

**Automated Newborn Screening Method for the
Determination of Galactose-1-Phosphate Uridyl-
transferase (TRA) and Biotinidase (BIO) Activities**

**Tracking Number CN005
Version 1.7**

Appendix 5H

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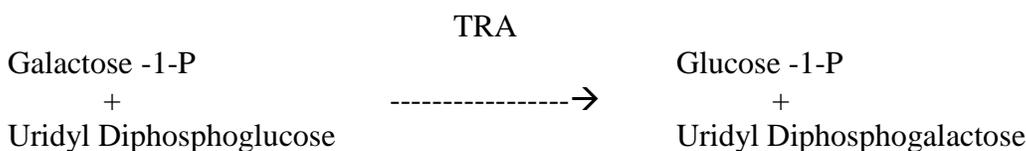
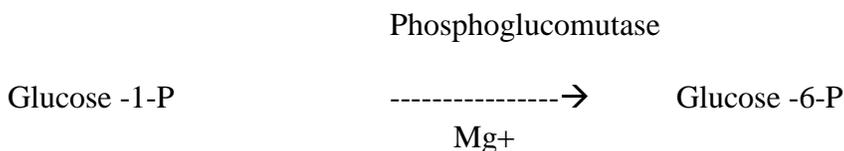
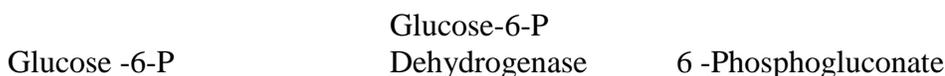
I. Title

The Automated Newborn Screening Method for the Determination of Galactose-1-Phosphate Uridyltransferase (TRA) and Biotinidase (BIO) Activities

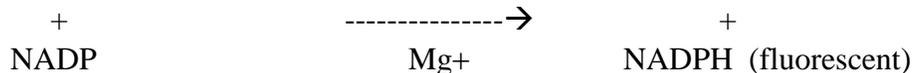
II. PrincipleTRA

The screening method measures the activity of the enzyme, galactose-1-phosphate uridyltransferase (TRA), in blood dried on filter paper collected from newborns. The deficiency of the enzyme leads to a carbohydrate metabolism disorder, Galactosemia. Early detection of the TRA deficiency identifies affected newborns for follow-up and treatment to prevent mental retardation, liver damage, cataracts, and other adverse effects related to Galactosemia.

The method is based on the principle of Beutler and Baluda's 1966 Screening Method for Galactosemia. Two (or one) 6-mm diameter disks punched from blood dried on filter paper are eluted with 850 μ l (or 425 μ L) of distilled water. The eluant is distributed into microtiter plates for TRA, BIO, and Hemoglobin testing. The TRA microtiter plate is loaded onto a continuous flow analyzer, API 300, where the eluant is aspirated and the reagents are added on line. Incubation of each sample is performed on-line at 37 $^{\circ}$ C for approximately one hour. During incubation, TRA, present in the blood eluant, catalyzes the reaction in the following sequential order.

Step 1Step 2Step 3

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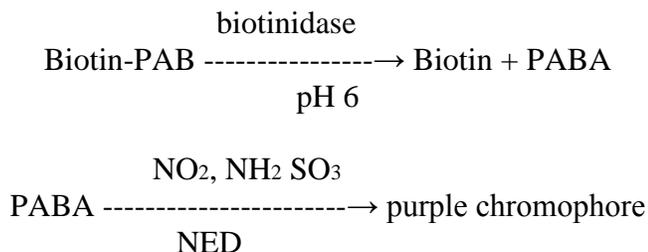


At the end of one hour, the sample is dialyzed and the NADPH reaction product is measured for its fluorescence. The amount of fluorescence produced in one hour is proportional to the TRA activity in the blood spots. A reagent blank is employed to subtract the background fluorescence.

BIO

Biotinidase deficiency is an inherited metabolic disorder in which the enzyme, biotinidase, cannot 1) liberate biotin from ingested protein and cannot 2) liberate covalently bound biotin from degraded carboxylases to recycle biotin.

The screening method measures the activity of the enzyme, biotinidase. The TRA and BIO assays require only one punch, one elution, and one distribution plate. The eluate stream on the API continuous flow system (for TRA and BIO) is split and directed to the TRA and BIO channels where reagents are added on line. Incubation for BIO is performed on-line at 40° C for approximately 110 minutes. After incubation, the p-aminobenzoic acid (PABA) released from biotinyl-p-aminobenzoate (Biotin-PAB) is separated from proteins in the sample by dialysis. The PABA is diazotized and coupled to a naphthol derivative to form an azo dye (purple) by the successive addition of sodium nitrite, acidic ammonium sulfamate, and N-1-naphthylethylenediamine dihydrochloride (NED). The color is measured colorimetrically at 550 nm.



The color developed is proportional to the biotinidase activity in the specimen. A standard curve prepared from a stock PABA solution is used to quantitate the results.

A copy of this protocol is in the Supervisor's PC. To access, login, click **NBS Assays SOP** and select the TRA/BIO assay.

III. Specimen Collection and Type

Blood spots are collected from a heel stick puncture and air-dried on to filter paper. The collection procedure is performed on the newborn at the time of discharge from the

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hospital but no later than 6 days of life. The dried specimen is transported to one of the State's contract laboratories immediately after collection.

IV. Equipment and Supplies

A. Equipment

Supplied by Wallac

1. API 300 Analyzer for TRA/BIO assay, 2 systems, each equipped with:
 - a. 311 Sampler with XYZ sampling format and a sampler cooling unit
 - b. 302A Micropump, one
 - c. 303A Cartridge base, one
 - d. TRA Active Channel, one
 - e. TRA Blank Channel, one
 - f. BIO channel, one
 - g. Fluorometer, two
 - h. Colorimeter, one
 - i. Incubator with Temperature Regulator for TRA
 - j. Incubator with Temperature Regulator for BIO
 - k. 350 Interface unit.
 - l. PC with FASpac software for API 300.
2. NeoGo workstation PC, shared.

B. Supplies

1. Supplied by Wallac
 - a. Autosampler with a cooling unit
 - 1) Sample cups, 4 ml
 - 2) 96 well microtiter plates
 - 3) Sample probe
 - 4) Sampler cover
 - b. Micropump
 - 1) Pump tubing, see TRA/BIO Pumptube Diagram, Attachment A.1
 - 2) Pump platen

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c. Analytical Cartridge

- 1) Coils and transmission tubing, see flow diagram, Attachment A.2 and A.3.
- 2) Dialyzer Plates, Upper
- 3) Dialyzer Plates, Lower
- 4) Dialysis Membrane, Type H
- 5) Torque Wrench
- 6) U- groove screws
- 7) Washers
- 8) O-rings
- 9) Helium pillow (for BIO)

d. Fluorometer, detector for TRA

- 1) Excitation filter, wavelength 365 nm
- 2) Emission filter, wavelength 500 nm
- 3) Source lamp, Quartz Halogen
- 4) Flowcell, size 9 ul

e. Colorimeter, detector for BIO

- 1) Tungsten lamp
- 2) Flowcell

f. General Supplies

- 1) Carboy Polyethylene Container, 1 liter, 10 liter, 3 each
- 2) Reagent Bottle, Polyethylene, 50 mL, 100 mL, 250 mL, 500 mL, and 1000 mL
- 3) Graduated glass cylinders, 50, 100 mL, 250 mL, 500, 1000 mL, each is dedicated to specific reagent preparation
- 4) Volumetric flask, glass, Class A, 50 mL, 100 mL
- 5) Volumetric Pipette, glass, Class A, TD, 1, 2, 3, 5, 7, 50 mL
- 6) Vacuum flask, 4, each is dedicated to specific reagent filtration
- 7) Vacuum pump
- 8) Syringe, polyethylene, 60 mL
- 9) 0.45 micron filter
- 10) Kryoraks for active and blank substrate reagents
- 9) Reagent rack

2. Supplied by NAPS Laboratories

a. Cotton swab

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- b. Parafilm
- c. Kimwipes
- d. Soft cloth
- e. Pipette, disposable.
- f. Pipettor, Eppendorf, 25 - 1000 uL
- g. Pipette tips
- h. Forceps for handling punched blood spot disks

V. ReagentsA. Supplied by Wallace**For TRA**

- 1. TRA Reagent Kit
Each kit contains:

| | | | |
|----|-------------------|-----------------------|-----------|
| a. | TRA Buffer Part A | 150 mL / bottle, | 5 bottles |
| | - Consists of | TRIS Buffer 50 mM | |
| | | NADP 1.3 mM | |
| | | Dithiothreitol 2.0 mM | |

| | | | |
|----|-------------------|-----------------------|-----------|
| b. | TRA Buffer Part B | 150 mL / bottle, | 5 bottles |
| | - Consists of | Magnesium 0.6 mM | |
| | | EDTA 0.1 mM | |
| | | Triton X-100 0.5 ml/L | |

| | | | |
|----|-----------------------|----------------------------|-----------|
| c. | TRA Substrate Reagent | 60 mL / bottle, | 5 bottles |
| | - Consists of | Gal-1-Phosphate 4.3 mM | |
| | | UDP-Glucose 0.5 mM | |
| | | G6PDH from Yeast 1.6 KU/L | |
| | | PgluM from Rabbit 0.8 KU/L | |

- 2. Lyophilized NADH Stock
Standard 50 mL / bottle

- 3. Lyophilized TRA System Control,
Stock Standard 50 mL / bottle

For BIO

- 4. BIO Reagent Kit
Each kit contains

- a. Sodium Nitrite, 1 bottle
- b. Hydrochloric Acid, 5N, 1 bottle, 30 mL
- c. Ammonium Sulfamate, 1 bottle
- d. N-1-Naphthylethylenediamine Dihydrochloride, (NED), 1 bottle,
300 mg

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- e. Biotinidase Substrate, Biotin-PAB, 1 bottle, 18 mg
 - f. Substrate Diluent, 1 bottle, 300 mL
 - g. Substrate Buffer, 1 bottle
- 5. BIO Stock Standard, is p-Aminobenzoic Acid (PABA), ready for use, 50 mL
 - 6. BIO System Control, PABA, ready for use, 50 mL
 - 7. Helium, tank, one tank, call for replacement tank at 500 psi
 - 8. FASCleaner, ready for use, 250 mL, use until the expiration date

NOTE: All unopened kit reagents are stored refrigerated at 2 - 8C. Use until the expiration date.

Reagents for TRA and BIO

- 9. Tris buffer, pH 7.8, 73gm/bottle and 7.3gm/vial.
- 10. Detergent TX - 10 (Triton), 50 mL/bottle.
- 11. Cleaning Solution (Chem Wash).

B. Supplied by GDL

- 1. Tray control, 1 level, prebarcoded, whole blood spotted on filter paper.
- 2. Special proficiency control, prebarcoded, whole blood spotted on filter paper.

C. Supplied by NAPS laboratories

- 1. Distilled water or equivalent to NCCLS Type 1 water.
- 2. Household bleach.
- 3. Isopropanol or Ethanol.

D. Preparation of Reagents

- 1. Warning and Precautions
 - a. Tray controls are human blood components. The source material has been tested for hepatitis B surface antigen, anti-hepatitis C, and anti-HIV antibodies and found to be negative. Nevertheless, all blood derivatives should be considered potentially infectious.
 - b. The reagents may cause irritation or sensitization by skin contact. Wear lab coat, disposable gloves and safety glasses while handling reagents and blood specimens. Refer to the U.S. Department of

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Health and Human Services publication of No. (CDC) 88-8395 on laboratory safety procedures.

2. Stock Solution Preparation

a. Triton X-10 Solution, use as start up/shut down solution for API 300 System

- 1) Rinse and fill the 10 liters carboy labeled Triton carefully to the mark with distilled water.
- 2) Add one bottle Detergent, TX - 10. Cap tightly.
- 3) Invert gently 5 times.
- 4) Store at room temperature. Discard after one week.

b. Tris buffer + Triton, use as sampler wash

- 1) Rinse and fill the 10 liter carboy labeled Tris Buffer with Triton carefully to the mark with distilled water.
- 2) Add one bottle, 73g of the Tris Buffer, pH 7.8, and cap tightly.
- 3) Mix vigorously for 2-3 minutes.
- 4) Add one bottle of the detergent, TX-10 and cap tightly.
- 5) Invert gently 5 times. Store at room temperature. Discard after one week.

c. 10% Bleach Solution (1:10 dilution), to wash reagent bottles and carboys

- 1) Fill one liter polyethylene bottle with approximately 900 ml distilled water.
- 2) Pour 100 ml of commercial bleach into the polyethylene bottle.
- 3) Mix before use.
- 4) Prepare fresh whenever needed.

d. Tris Buffer (no Triton) use to dilute TRA calibrators and system controls

- 1) Rinse the 1000 mL Tris Buffer (no Triton) graduated cylinder thoroughly with distilled water.
- 2) Fill the cylinder with approximately 500 mL of distilled water.

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- 3) Open 1 vial, 7.3g of Tris Buffer, pH 7.8, and pour it into the cylinder. Mix.
 - 4) Bring volume to 1000 mL and mix well.
 - 5) Store at room temperature. Discard after one week.
- e. TRA Stock Calibrator
- 1) Open a new vial of NADH Stock Standard.
 - 2) Use a 50 mL volumetric pipette to add 50mL of the distilled water directly into the vial.
 - 3) Mix thoroughly for 2-3 minutes until dissolved.
 - 4) Store refrigerated (2-8C) when not in use. Discard after 2 weeks.
- f. TRA Stock System Controls
- 1) Open a new vial of TRA Stock System Control.
 - 2) Use a 50 mL volumetric pipette to add 50 mL of the distilled water directly into the vial.
 - 3) Mix thoroughly for 2-3 minutes until dissolved.
 - 4) Store refrigerated (2-8C) when not in use. Discard after 4 weeks.
3. Working Solution Preparation
- a. TRA Blank Substrate Reagent
- 1) Rinse the 250 mL graduated glass cylinder labeled TRA blank thoroughly with distilled water.
 - 2) Fill the cylinder to about 75 mL with distilled water.
 - 3) Open 1 vial of TRA Buffer Part A (blue dot on lid) and pour it into the cylinder. Rinse the bottle with distilled water and add to the cylinder. This will have a sulfur smell.
 - 4) Open 1 vial of TRA Buffer Part B (blue crimp seal) and pour into the cylinder. Rinse the vial with distilled water and add to the cylinder.
 - 5) Bring volume to 150 mL with distilled water.
 - 6) Cover with parafilm and mix gently until dissolved.

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- 7) Pour 60 mL of the above solution into the labeled 100 mL TRA Active graduated cylinder.
- 8) Pour the remaining 90 mL into the labeled Blank reagent bottle. Label with the date of preparation.
- 9) Seal with septum and refrigerate (2-8° C) until use. Use for 2 days.

b. TRA Active Substrate Reagent

- 1) Take the TRA Active graduated cylinder with the 60 mL of Blank Substrate
- 2) Open 1 vial of TRA Substrate (red crimp seal) and pour into the cylinder.
- 3) Take 1-2 mL out of the cylinder using a new transfer pipette to rinse the vial and septum and add back into the cylinder.
- 4) Repeat step 3) two more times.
- 5) Cover with parafilm and mix gently to dissolve.
- 6) Remove the plunger from the TRA Substrate syringe, place the filter on the end, fill the syringe with the substrate reagent, and filter it directly into the labeled Active reagent bottle.
- 7) Seal with septum and refrigerate (2-8C) until use. Use for 2 days.
- 8) Remove the filter and discard it. Rinse the syringe with distilled water.

c. Sodium Nitrite, 14 mM

- 1) Add ~200 mL distilled water to the labeled 500 mL graduated cylinder.
- 2) Open the Sodium Nitrite bottle and pour content into the graduated cylinder.
- 3) Rinse the bottle with distilled water and add to the cylinder.
- 4) Repeat step 3) two more times.
- 5) Bring volume to 300 mL.
- 6) Cover with parafilm and mix 2-3 minutes until dissolved.
- 7) Filter reagent with a 0.45 micron filter directly into the labeled vacuum flask. Pour into the labeled reagent bottle and cap. Remove filter and discard. Rinse the vacuum flask with distilled water.

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- 8) Label with expiration date, store at room temperature. Discard after 2 weeks or when turbid or cloudy.
- d. Ammonium Sulfamate in Hydrochloric Acid, 44 mM in .5M HCL
- 1) Add ~200 mL of distilled water to the labeled 500 mL graduated cylinder.
 - 2) Open a bottle of Ammonium Sulfamate and add to the graduated cylinder. Rinse the bottle with distilled water and add to cylinder.
 - 3) Open a bottle of 5N HCL and add cylinder.
 - 4) Bring volume to 300 mL.
 - 5) Cover with parafilm and mix 2-3 minutes until dissolved.
 - 6) Filter reagent with a 0.45 micron filter directly into the labeled vacuum flask. Pour into the labeled reagent bottle and cap. Remove filter and discard. Rinse the vacuum flask with distilled water.
 - 7) Label with expiration date, store at room temperature. Discard after 2 weeks or when turbid or cloudy.
- e. N-1-Naphthylethylenediamine Dihydrochloride (NED), 3.8 mM
- 1) Add ~200 mL of distilled water to the labeled 500 mL graduated cylinder.
 - 2) Open the bottle of NED reagent and add to the cylinder. Most of the powder may remain in the bottle.
 - 3) Rinse the bottle with distilled water and add to the cylinder.
 - 4) Repeat step 3) two more times.
 - 5) Bring volume to 300 mL.
 - 6) Cover with parafilm and mix on a stirrer for 5 – 10 minutes or until dissolved.
 - 7) Filter reagent with a 0.45 micron filter directly into the labeled vacuum flask. Pour into the labeled reagent bottle and cap. Remove filter and discard. Rinse the vacuum flask with distilled water.
 - 8) Label with expiration date, store at room temperature protected from light. Discard after 2 weeks or when turbid or cloudy.
- f. BIO Substrate
- 1) Open a bottle of Substrate Diluent. Allow it to warm to room temperature. It can be removed from the refrigerator the night before.
 - 2) Open a vial of Biotin-PAB Substrate and pour the contents into the diluent bottle. Rinse the vial with the diluent and add back to the diluent bottle.

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- 3) Repeat step 2) two more times.
- 4) Cap and mix for 5 – 10 minutes until completely dissolved. The substrate MUST be completely dissolved before adding the buffer.
- 5) Open a vial of Substrate Buffer and pour contents into the diluent bottle. Rinse the vial with the diluent/substrate mixture and add back to the diluent bottle.
- 6) Repeat step 5) two more times.
- 7) Cap and mix for 2 – 3 minutes until dissolved.
- 8) Filter reagent with a 0.45 micron filter directly into the labeled vacuum flask. Pour into the labeled reagent bottle and cap. Remove filter and discard. Rinse the vacuum flask with distilled water.
- 9) Label with expiration date and store in refrigerator. Discard after 2 weeks or when turbid or cloudy.

g. TRA and BIO Calibrators

Each calibrator contains both NADH and PABA at the correct concentration to construct a standard curve for their specific assay.

- 1) Use the 5 appropriately labeled 100 mL volumetric flasks.
- 2) Remove the reconstituted TRA stock STD solution and the ready-to-use BIO stock STD from the refrigerator and allow it to warm to room temperature.
- 3) Use volumetric pipets or the 1000 ul Eppendorf pipettor to prepare dilutions as indicated in the table below using the Stock Tris Buffer (no Triton) to dilute to volume.

| Volume of TRA Stock STD Solution | Volume of BIO Stock STD Solution | Final Volume (mL) | Conc of TRA STD in μM | Conc. of BIO STD in ERU |
|----------------------------------|----------------------------------|-------------------|----------------------------------|-------------------------|
| 0 mL | 50 μL | 100 | 0 | 5 |
| 1.25 mL | 250 μL | 100 | 25 | 25 |
| 2.50 mL | 500 μL | 100 | 50 | 50 |
| 5.00 mL | 1000 μL | 100 | 100 | 100 |
| 7.50 mL | 2000 μL | 100 | 150 | 200 |

- 4) Label and store the calibrators in the refrigerator (2-8C) and protected from light. Use for one week.
- 5) Use the open PABA Stock Standard for 30 days when stored refrigerated and protected from light.

h. TRA and BIO System Controls

- 1) Use the 3 appropriately labeled 100 mL volumetric flasks.

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- 2) Remove the reconstituted TRA stock System Control solution and the ready-to-use BIO stock System Control from the refrigerator and allow it to warm to room temperature.
- 3) Use volumetric pipets or the 1000 ul Eppendorf pipettor to prepare dilutions as indicated in the table below using the Stock Tris Buffer (no Triton) to dilute to volume.

| Volume of TRA Stock System Control | Volume of BIO Stock System Control | Final volume (mL) | System Control |
|------------------------------------|------------------------------------|-------------------|----------------|
| 0.625 mL | 50 μ L | 100 | CL |
| 1.25 mL | 100 μ L | 100 | CM |
| 2.50 mL | 250 μ L | 100 | CH |

- 4) Label and store the controls in the refrigerator when not in use. Discard after one week.
- 5) Use the open PABA Stock System Control for 30 days when stored refrigerated.

VI. Calibration and Quality Control

A. Calibration

The calibration for the TRA assay is performed using five levels of TRA calibrators 0, 25, 50, 100, and 150 μ M/L prepared in aqueous solution. The calibration for the BIO assay is performed using five levels of BIO calibrators, 5, 25, 50, 100, 200 ERU prepared in the same solution with NADH. The calibrators are run in single replicate each time the assay is performed.

The calibration curve is constructed automatically by the software as a spline smoothed line using log concentration on the x axis and log response on the y-axis.

B. System Controls

The system controls (SQC) consist of three levels of NADH and three levels of PABA, low, medium, and high SQC, prepared in the same aqueous solution. The SQCs are run in single replicate each time the assay is performed.

C. Tray Controls

The tray control material is dried human blood on filter paper prepared at the Genetic Disease Laboratory.

Two types of tray controls, CT and CP, are used for each tray. The CT is tested at the beginning and at the end of each tray whereas the CP is tested at position 49

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for each tray. The CP specimen is tested only if the partial tray has sufficient specimen to reach that position.

VII. Procedures

A. Barcoding

Refer to NBS Accession and Reporting Protocol for details.

B. Specimen Handling

Refer to Specimen Handling Protocol for details.

C. Preparation for analysis

1. Daily Preparation of API 300

NOTE: This is done before the power is turned on for the API 300 system.

- a. Dump, rinse with distilled water, and refill with distilled water in the water bottle. Place all reagent lines in the rinse water bottle.
- b. Dump, rinse thoroughly with distilled water, and refill with startup/shutdown and the sampler wash solution. Move all reagent lines from the rinse water bottle to the startup/shutdown solution.
- c. Check that the non-daily reagents, standards and controls have not expired. Prepare if necessary.
- d. Inspect the cartridge connections for looseness, gaps and kinks. Make sure there are no loose fittings and connections. Check the drain tubings for wear and tear. Drain tubing must be placed properly in the drainage system.
- e. Inspect the pump tubings for wear. Replace pump tubing if necessary.
- f. Remove loose dust on the exterior surfaces of all equipment with soft cloth. Dirt which remains can be removed with a soft cloth dampened in a mild detergent and water solution. Abrasive cleaners should not be used. The exterior surfaces of all equipment are coated with protective finish.

D. Set up and analysis on API 300

1. Turn on the API 300 Analyzer by pushing the universal power switch on. Check that the sampler, pumps, fluorometers, colorimeter, incubators and 350 interface unit are on and plugged into the power box. Check that the

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range indicator switch for the TRA blank channel is ~ **0.2** RFU and for the TRA active channel is ~ **0.5** RFU. Adjust the range if needed. Range setting for the BIO colorimeter is behind the removable panel and is set at ~0.02. Once set it is not necessary to remove the panel to check each day.

NOTE: If the panel is removed, when putting the panel back on the colorimeter, be sure tubings are in the cut out sections and are not pinched.

2. Enter your **user name** and the **password** using the FASPac PC. The screen shows three icons.
3. Double click on the **Data Logger** icon. A brief handshake will appear.
4. Double click on the **API NeoGo** icon. The software takes you to the “Welcome to Wallac NeoGo” screen.
5. Follow the directions on the NeoGo screen. Click next when task is completed.
6. Click **Next**.
7. Remove Kryoraks from freezer. Run under warm water for 5 minutes before placing reagents in them.
8. Place all lines in the start-up solution, Triton X-100. See Attachment A and A1 for flow diagrams for the TRA and BIO channels.
9. Start the pump and wipe the platen bottom surface using a tissue moistened with isopropanol or ethanol while the pump is running. Then latch the platens. Check that the pump speed is 42 for all channels. Use push buttons located on the pump to adjust the required pump speed.
10. Check the level of water in each of the two incubators. Add more if necessary to cover the coils. Check that the temperature regulator display switch is on.

NOTE: Unlike the glass TRA coil, the BIO coil in the incubator is plastic.

11. Click **Next**. **Set the timer for 10 minutes**. Pump the startup solution for 10 minutes. When 10 minutes timer goes off, pour approximately 40 ml of cleaning solution into a 100 ml beaker. Place all lines, **except the sampler_wash line**, in the Cleaning Solution (Chem Wash) for 3 minutes. During this 3 minute period perform the probe cleaning procedure described in step 12.
12. Place Cleaning Solution cup at position 20 (of the Standard Block) and click **Next**. The sample probe cleaning process begins. A FasPac message, “Running cleaning protocol”, is on the screen.
13. Rinse the reagent lines briefly in distilled water at the end of 3 minutes and return to the startup solution. **Set timer for 20 minutes**.

NOTE: Do not return the excess cleaning solution to the original bottle to avoid contamination.

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14. Attach the helium pillow onto the BIO channel. Every third day deflate and refill the pillow.
- a. Open the He pillow valve by turning it counterclockwise.
 - b. Attach the short piece of tubing labeled ‘pillow deflater’ to the neck of the flask (used for filtering reagents).
 - c. Connect the valve of the He pillow to the ‘pillow deflater’ tubing.
 - d. Turn on pump to remove air/helium from the pillow. When the pillow is flat, turn off pump.
 - e. Turn on the helium tank by turning the handle counterclockwise.
 - f. Connect the tubing from the tank to the valve on the pillow.
 - g. Push the outlet valve on the tank sideways. You can hear the flow of helium.
 - h. Fill the bag. The bag does not have to be completely filled.
 - i. Remove the pillow and close the valve. Close the tank by turning the handle clockwise.
 - j. Attach the pillow valve to the Helium pump tubing.
15. Prepare the following reagents as described in Section VII.D., Preparation of Reagents:
- a. Blank and active substrate for TRA, if needed
 1. Take the stored blank and active substrate from refrigerator.
 2. Determine if the volume of stored blank and active substrate is sufficient for today’s run. If sufficient, use the substrate on Day 2 and dump at the end of the run. If insufficient, prepare new substrate and add a volume of the newly prepared substrate to the stored substrate to enable completion of the day’s run. Mix with gentle swirling. Use and dump the combined substrate at the end of the run. Cover the bottle and store the newly prepared substrate in the refrigerator for the next day, Day 2, and dump after use.
 3. Use the table below to help determine if there is sufficient volume. The numbers are the same for blank and active substrate. Please note that dead volume is a very conservative number and should be increased based on how much time the substrate is pumped before and after sampling.

| # of Trays | Dead Volume in | Volume Pumped in | Total Volume in mL |
|------------|----------------|------------------|--------------------|
|------------|----------------|------------------|--------------------|

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| | | | |
|---|----|----|----|
| | mL | mL | |
| 1 | 4 | 13 | 17 |
| 2 | 4 | 23 | 27 |
| 3 | 4 | 32 | 36 |
| 4 | 4 | 42 | 46 |

- b. Sodium Nitrite, if needed
 - c. Ammonium Sulfamate in Hydrochloride Acid, if needed
 - d. NED Reagent, if needed
 - e. Biotinidase Substrate, if needed
16. Check that the incubator temperature is 37° C +/- 1° for the TRA channel and is 40° C +/- 1° for the BIO channel.
 17. Verify that there is sufficient volume of reagents for the sample load. See table below.

NOTE: Minimize potential waste of prepared BIO reagents. Do not dump remaining reagents if within 14 days of preparation. If there is insufficient reagent volume for today's run, open a new kit and prepare reagents. Add the newly prepared reagents to the remaining reagents to enable completion of the day's run. Mix reagents completely with gentle swirling. Combine reagents only if the lot numbers are the same. Dump combined reagents at the end of the day's run and use newly prepared reagents for the next days' runs until expiration in 14 days.

| Reagent | Tray 1 | Tray 2 | Tray 3 | Tray 4 |
|------------------|--------|--------|--------|--------|
| Tris + Triton | 200 mL | 300 mL | 400 mL | 500 mL |
| Triton | 200 mL | 300 mL | 400 mL | 500 mL |
| Sodium Nitrite | 14 mL | 20 mL | 30 mL | 40 mL |
| NH SO in HCL | 14 mL | 20 mL | 30 mL | 40 mL |
| NED | 14 mL | 20 mL | 30 mL | 40 mL |
| BIO Substrate | 14 mL | 20 mL | 30 mL | 40 mL |

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18. Click **Next**. Remove working calibrators and system controls from the refrigerator. Pour into 4 ml cups (fill to about $\frac{3}{4}$ full) and place on the block (designed for TRA calibrators and controls) in the following order (see Table A).

Table A

| Cup # | Calibrator |
|-------|-----------------------|
| 1 | 150 M+200 ERU |
| 2 | Tris Buffer |
| 3 | Tris Buffer |
| 4 | 0 + 5 ERU |
| 5 | 25 μ M + 25 ERU |
| 6 | 50 μ M + 50 ERU |
| 7 | 100 μ M + 100 ERU |
| 8 | 150 μ M + 200 ERU |
| 9 | Tris Buffer |
| 10 | Tris Buffer |

| Cup # | Control |
|-------|-------------|
| 11 | CL |
| 12 | CM |
| 13 | CH |
| 14 | Tris Buffer |

19. Take the block to the sampler and place cups into positions labeled as Standards, Tris and System Controls.
20. Return the working calibrators and system controls to the refrigerator (2-8° C) immediately. **DO NOT LEAVE THE CALIBRATORS AT ROOM TEMPERATURE.**
21. Turn on sampler cooling tray at the power strip approximately 10 minutes before the start of the run.
NOTE: After **Table A** is shown on the screen, do not Click **Next** until the worklist has been downloaded from the NeoGo workstation i.e., the worklist has been printed. You can use this time to fill out the Checklist for NBS, see Attachment B.
22. Visually inspect the flow and air bubble patterns when the 20 minute times goes off. Pay special attention to both streams above and below the

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dialyzers and the tubing leaving the last coil before the debubbler. Bubble pattern must be uniform and consistent.

23. Click **Next**. Place all the reagent lines carefully in the correct reagent bottles working from the front of the pump towards the back, one line at a time. **Set the timer for 15 minutes.**

NOTE: For the BIO channel, there is a way to determine if there are air bubbles in the flowcell. Pinch the “fat” portion of the tubing taped to the 303 unit and watch the display on the colorimeter. If the display does not change, there are no air bubbles in the flowcell. If the display changes and shows a negative number, there are air bubbles in the flowcell. To remove air bubbles, pinch the debubbler line just before the pump for about 15 – 20 seconds and watch the display go to a high number, e.g., 1.000 and release the line.

24. Click **Next**. **Next** again. The screen shows the worklist or the plate map with the location of the daughter plate barcode ID and its position number.
- a. If a plate map is on the screen
 - 1) Place the first daughter plate in the correct position on the sampler. Make sure that the plate is placed in the position properly. Scan the daughter plate ID using the hand held barcode reader. Hold the barcode reader as close as possible to the barcode and press the button. If the barcode does not scan, type in the number. Once the barcode is read, the ID will start blinking on the screen. Click **Close**.
 - 2) Continue to scan and click **Close** for the remaining plates. All daughter plates must be scanned to start the run.
 - 3) Check the filename and sample IDs in the worklist as soon as it appears on the screen after reading the daughter plate IDs. Proceed to step 24.
 - b. If the correct worklist is displayed on the screen
 - 1) Check the filename highlighted on the top of the screen. If the filename and the sample IDs on the worklist match the one generated by the NeoGo Workstation, proceed to step 24.a.1) to scan the barcode when the first daughter plate appears on the screen. Then, proceed to step 25.
 - c. If an incorrect worklist is displayed on the screen

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- 1) Click **File/Exit**. A message appears “file name. ACF has changed. Save it?” Click **No**.
 - 2) Another message appears “Do you want to send to database?” Click **No**.
 - 3) Click **Exit**.
 - 4) Message appears “Do you want to restart the program to set up another assay?” Click **Yes**.
 - 5) Remove the cleaning solution from position 20 and replace with the startup solution.
 - 6) Restart API NeoGo. Continue to Click **Next** until the Cleaning Protocol is completed and the correct worklist appears on the screen. There is no need to pump startup solution for 20 minutes after the cleaning protocol is completed.
 - 7) Proceed to step 24.a.1) to read the barcode when the daughter plate is on the screen.
 - 8) Call Proxy if all attempts to download the correct worklist fail.
- d. If more than one worklist is imported from the NeoGO workstation, a window with all the worklists will appear. Select the correct worklist. Click **OK**. Proceed to step 24.a.1).
- e. If you get an error message, “No worklists are waiting, you need to create one with the NeoGo workstation.
- 1) Wait for the worklist to be printed by NeoGo workstation. Click **Retry**.
 - 2) Proceed to step 24.a.1) if you have the correct worklist and there are no more error messages on the screen.
 - 3) If you press **Retry** again before the worklist is ready, another error message appears “No worklist found.”
 - a) Click **OK**.
 - b) Another message appears “Do you want to restart the program to setup another assay? Click **Yes**.
 - c) Remove the Cleaning Solution from position 20 on the sampler and replace it with the startup solution.
 - d) Restart API neo. Continue to click **Next** until the cleaning protocol is completed. Continue to click **Next** until the correct plate map or the worklist appears. There is no need to pump startup solution for 20 minutes

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- e) after the cleaning protocol is completed.
Call Proxy if all attempts to download the correct worklist fail.
25. Click on the icon **S1** if the FASpac screen does not display the signal for the **blank** and the **active** channels. Click on **Options**; click on **Autotracking** and click on **Display Signal** for both channels. **Verify a smooth and stable baseline for each channel.** If the baseline is unacceptable (baseline response must be less than 10% of the lowest calibrator response), perform troubleshooting as described in the troubleshooting Section VII.J. before proceeding to the next step.
26. When 15 minute timer goes off, press **Autozero** on both TRA detectors and **Zero** on the BIO 315 detector. Baseline must read near 0.5 volts on both TRA channels and 0.5 volts on the BIO channel.
27. Click on the **green flask** icon, click **run**, click **begin**. The computer will ask “Start run for system 1”. Click **yes**. **Set timer for 70 minutes.**
- NOTE:** It is critical that the plates are processed **immediately** after elution and distribution into daughter plates. When distributed, place the daughter plates **immediately** on the cold API cooling plate. If for some reason there are delays, e.g., troubleshooting, the microplates must be stored in the refrigerator and the time from the completion of elution to the completion of sampling on the API system must not exceed **six hours**. In exceptional cases, consult with GDL before releasing trays.
28. Click on **S1** to return to the chart tracing.
29. Check for Instrument Sensitivity for **TRA Blank Channel**. View the peak height of the sync peak (highest standard). See Attachment C. Voltage number must be 3.0 - 4.0 after subtraction of baseline. If the instrument does not show the desired sensitivity, see Section XII.
30. Check for **Dwell Time** of the **TRA Blank Channel SYNC peak**. The dwell time is the time the SYNC peak sample takes from the point of its aspiration to the appearance of peak maxima. The dwell time must be 4.5 – 6.5 minutes for the Blank Channel.
31. Press Autozero on the **TRA Active Channel only** when the 70 minute timer goes off. Set the timer for 25 minutes.
32. Check for Instrument Sensitivity for **TRA Active Channel**. View the peak height of the sync peak (highest standard). See Attachment C. Voltage number must be 1.5 – 2.5 after subtraction of baseline. Once the baseline is set, about 2 minutes after the emergence of the SYNC peak, the voltage # will change to include the subtraction of baseline. If the instrument does not show the desired sensitivity, see Section XII.
33. Check for **Dwell Time** of the **TRA Active Channel SYNC peak**. The dwell time is the time the SYNC peak sample takes from the point of its

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aspiration to the appearance of peak maxima. The dwell time must be 78 – 82 minutes for the active channel.

34. Check the peaks of the TRA calibrators and system controls on the Blank Channel to detect gross errors while the sampler is still sampling the water cups in tray 01 (Positions A1 to B3). If gross errors are detected:
 - a. Click **Run/End**.
 - b. Correct the problem. See General Guidance for Troubleshooting.
 - c. Click **Run /Begin** again after the problem is resolved.
 - d. Click **S1** to display the blank and the active channels.
 - e. Allow the peaks in the system from the first run to emerge from the detector. When the new SYNC peak appears, check that the peak maximum is marked correctly and move the marker if needed.

NOTE: Do not walk away until the probe has finished sampling the first two wells of the first microtiter plate to ensure that the probe is sampling properly.
35. Press **Zero** on the BIO 315 Detector when the 25 minute timer goes off.
36. Check for Instrument Sensitivity for **BIO Channel**. View the peak height of the sync peak (highest standard). See Attachment C1. Voltage number must be 3.0 – 4.0 after subtraction of baseline. Once the baseline is set, about 2 minutes after the emergence of the SYNC peak, the voltage # will change to include the subtraction of baseline. If the instrument does not show the desired sensitivity, see Section XII.
37. Check for **Dwell Time** of the **BIO Channel SYNC peak**. The dwell time is the time the SYNC peak sample takes from the point of its aspiration to the appearance of peak maxima. The dwell time must be 105 -115 minutes for the BIO channel.
38. **Wait** for the sampler to finish sampling all wells (sampler will move to the home position after the sampling is done). After the sampler has finished sampling, set the timer for 10 minutes. When the 10 minute timer goes off, move the TRA blank and active substrate lines, the sampler wash line, and BIO substrate line to the startup/shutdown solution. Cover the TRA blank and active substrate bottles and store in refrigerator for next day's testing if the substrate was prepared today. Cover the BIO substrate bottle and store refrigerator if prepared within 14 days.

NOTE: Do not remove the two Tris+Triton lines and the 4 lines for BIO, the 3 color reagent lines and the Triton line.
39. Pour FASCleaner into a beaker. Move the BIO substrate line from the startup/shutdown solution to the FASCleaner after pumping 10 minutes.

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Pump FASCleaner for 5 minutes, rinse in distilled water, and return line to the startup/shutdown solution.

40. Move all the reagent lines to the startup/ shutdown solution when the signal screen shows the last peak. Confirm by clicking the green flask that the run has ended (click on the green flask to worklist to see Done). Set the timer for 45 minutes.
41. Disconnect the He pillow and close the valve.

E. Peak Review

After the run has ended, review the peaks for the three channels before sending the data to the Supervisor's PC.

1. Click **Options**. Click **Autotracking** to turn it off. Review the peaks.
2. Inspect the first tray control peak. The peaks are marked with the yellow marker when the FASPac Software finds the apex of the peaks based on the rise and fall of the slope. When the apex is not found, the peaks are marked with the red marker as in the case of the 15 consecutive water cups on the first tray. FASPac marks the peaks based on the timing window. This is based on the total time needed to complete the sampling and washing for each sample, which is 43 seconds.
3. If the first tray control peak is not marked properly at the apex, click on the red marker and drag the marker to the apex of the peak and release. A message appears "Search for Peaks to the right of this peak?"
4. Click **Yes**, if the peaks immediately to the right are not marked correctly at peak maximum, otherwise click **No**.
5. Scan the rest of the peaks and reset marker if needed. Data will now be saved in a different filename.

F. Data transfer to supervisor's PC

After the run is done and the peaks have been reviewed, the data must be transferred to the supervisor's PC.

1. Click on the **Green Flask** to see DONE.
2. Click on **File**. Click **Exit**.
3. View message on the screen, "**do you want to send the results to the data base?**" Click **Yes**.
4. Click **Next** until option **Next** is not highlighted.
5. Click **Exit**.
6. See message on the screen, "do you want to restart the program to set up another run?"
7. Click **No**, if this is the last run of the day and click **Yes** if you want to start another run.

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8. If you click **No** in step 7, click on **Result Transfer** button on the FASPC PC. View message on the screen, “**No Input filename given! Do you want to specify manually?**” Click **Yes**.
 9. Select the FASPC result file and press “**Ok**”.
 10. Click the **start** button located in the lower left corner of the FASPC PC.
 11. Click **Shut down**.
 12. Select “Close all programs and log on to a different user”. Click **Yes**.
 13. If you click **Yes** in step 7, “welcome to the Wallac neo” screen will appear and you can start another run by following the run startup procedure.
- G. Daily Shutdown
1. Rinse the blank and active substrate reagent bottles for the next day if this is Day 2 (prepared yesterday) for the substrate.
 2. Put the Kryoraks into the freezer.
 3. Cap BIO reagents and store room temperature if within 14 days of preparation.
 4. Empty the waste container. Discard this liquid waste into the sink with copious amount of water.
 5. Discard the sample trays and the sample cups in the biohazard container.
 6. Clean up spill.
 7. When the 45 minutes timer goes off, release the platens and tilt them back. Turn off the universal POWER SUPPLY.
- H. Repeat Runs
1. Repeat testing if a run (e.g., low system control >3SD), tray (e.g., 2 TQC >2SD), or specimen (e.g., unresolved peak) was scored red and prevented from release.
 2. Repeat testing a potential TRA presumptive positive the same day. Repeat all others no later than the next day.
 3. Set up a run for TRA or BIO separately or include specimen(s) with new specimens for TRA/BIO run the next day.
NOTE: A requirement for FASPC to complete a run is to mark the sync peak in all 3 channels. To run BIO only, pump startup solution through the TRA active and blank channels. The TRA standard of the TRA/BIO standards is the end product and sync peak appears on the TRA active and blank channels. To run TRA only, you must pump the color developing

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reagents, sodium nitrite, ammonium sulfamate, and N-1-naphthylethylenediamine, through the system for the BIO standard which is p-aminobenzoic acid to develop color to produce the sync peak. If there is no sync peak for BIO, FASpac will never complete this run. Multicalc needs to see 3 standard curves, TRA, TRB, and BIO. Run the color reagents until there is a standard curve for BIO.

I. Preventive Maintenance

1. Daily

Refer to daily preparation of analysis and shutdown.

2. Weekly

a. Clean the sample probe and the sample line before the sampler is turned on. Move the probe arm to the center. Move the probe to the front and tilt the protective box to the back. If the protective box has an opening at the top, access the probe through this opening.

- 1) Remove the sample probe from the sample line. Hold the probe while loosening the white screw and lift the probe from the wash reservoir.
- 2) Fill a syringe with 20 ml distilled water and flush the sample probe.
- 3) Connect the sample line to the probe.
- 4) Insert and align the sample probe into the wash reservoir.
- 5) Tighten the white screw halfway.
- 6) Adjust the probe height so that the probe is centered between the inlet outlet ports of the wash reservoir. The probe will be approximately $\frac{1}{4}$ inch from the bottom of the wash reservoir. See Attachment D.
- 7) Lock the sample probe into position by securely tightening the white screw on the probe holder.

b. Turn on the pump. Wipe platens and rollers using a tissue moistened with 70 % isopropanol.

3. BiWeekly

Perform the biweekly maintenance even if the system is not used on a daily basis.

a. Clean the system prior to changing the tubing and the dialysis membrane.

- 1) Disconnect the backpressure tubing at the point where the

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- tubing from the detector is connected to the backpressure tubing.
- 2) Place all the reagent lines in the startup solution. Set the timer and pump startup solution for 10 minutes.
 - 3) Place all reagent lines including the sampler wash in the cleaning solution when the 10 minute timer goes off and perform the cleaning protocol for 3 minutes followed by startup solution for 15 minutes.
 - 4) Place all reagent lines including the sampler wash in 1N HCL acid solution when the 15 minute timer goes off. Pump acid solution for 10 minutes. Rinse briefly all reagent lines with distilled water, then return to the startup solution.
 - 5) Set **pump speed of 99** and pump the startup solution for 40 minutes.
 - 6) Reconnect the backpressure tubing.
- b. Change all pump tubing. See VII.H.5.a.
 - c. Change the dialysis membranes. See VII.H.5.b.
 - d. After changing pump tubings and dialyzer membranes, pump the startup solution for 40 minutes at **pump speed 42** before setting up the system for analysis.
 - e. Inspect the temperature of water for both incubators. Check that the temperature regulator display switch is on. Put the thermometer inside the water and make sure that the temperature regulator display shows the correct temperature.
 - f. Empty the reagent bottles and the two 10 L carboy for the Triton solutions. Add 10% bleach solution, swirl the container, and scrub the inside area with a brush. Discard the bleach, rinse the carboy with copious amount of tap water and rinse with distilled water.
4. Quarterly
- Volumes tested at your laboratory may be different based on how volumes are measured when making reagents, standards, and system controls. Your laboratory is required to verify each quantitative volume measured. Criteria used are +/- 2% of target volume and CV is 1.5% for volumes $\leq 50 \mu\text{L}$ and 1% for all others.
- a. Perform accuracy and precision determination for pipettes:

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- 1) Weigh 50 4mL cups.
- 2) Set 1000 ul adjustable Eppendorf pipette to 50 uL and dispense 50 uL distilled water into the first 10 cups.
- 3) Set the adjustable pipette to 250 uL and dispense 250 uL distilled water into the next 10 cups.
- 4) Set the adjustable pipette to 500 uL and dispense 500 uL distilled water into the next 10 cups.
- 5) Set the adjustable pipette to 625 uL and dispense 625 uL distilled water into the next 10 cups.
- 6) Set the adjustable pipette to 1000 uL and dispense 1000 uL distilled water into the next 10 cups
- 8) Reweigh the 50 cups.
- 9) Calculate the volume dispensed.
- 10) Determine the average volume and CV for each set. The acceptable results must meet the following criteria:

| Volume | Average (+/-2%) | CV |
|---------|-----------------|------|
| 50 µL | 49 – 51 | 1.5% |
| 250 µL | 245 - 255 | 1.0% |
| 500 µL | 490 - 510 | 1.0% |
| 625 µL | 612.5 - 637.5 | 1.0% |
| 1000 uL | 980 - 1020 | 1.0% |

5. Replacement procedures

a. Pump tubing

Normal life expectancy is 160 to 200 hours of operation. Actual life expectancy depends on many factors including platen wear, pump speed, pump condition, start up and shut down and the chemical composition of the solutions pumped.

NOTE: Pump tubing should not be left compressed under the platens without the pump on.

- 1) Replace all pump tubing on a cartridge when changing any of the tubing.
- 2) Remove the tubing from the front of the pump towards the back, one tubing at a time.
- 3) Cut the new tubing to match the length of the old one.
- 4) Attach the new tubing to the inlet connector block or cartridge fitting and also to the reagent line or sample line connection as required. Wet the end of the tubing with Brij to facilitate the connection.
- 5) Inspect each connection to ensure there are no gaps.

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- 6) Place the pump tubing on the 302 A Micropump using the position map.
 - 7) Pump tubing must be stretched straight across the rollers from one side to the other. Caution: do not twist the tubing.
 - a) Press one side of the pump tubing into the shoulder grip on the 302A micropump so that the colored shoulder is held firmly in place. **Put all the orange colored shoulder at the left side of the platen.**
 - b) Stretch the tubing straight across the rollers to the shoulder grip exactly across from the other end.
 - c) Press the pump tubing into the opposite shoulder grip as before.
 - d) For a neater appearance, excess tubing between the pump and the cartridge may be cut away. Be careful to make the connections exactly the same. Modify one at a time.
- b. Dialyzer Membranes
- A loss of sensitivity may indicate the need to change dialyzer membranes. Normal life expectancy is two weeks. Dialyzer membranes are fragile in nature.
- 1) Common problems encountered include:
 - a) Leakage around the dialyzer block
 - b) Rupture of the membranes
 - c) Degraded performance
 - 2) Root causes of these problems are:
 - a) Warped dialyzer blocks
 - b) Mechanical or chemical damage to the membrane
 - c) Backpressure in the cartridge due to partial blockages
 - d) Insufficient surfactant
 - 3) Steps in changing the dialyzer membrane:
 - a) Examine the top and the bottom plate of the dialyzer block for dust, lint or foreign material. Wipe and rinse with distilled water before proceeding. Pay special attention to the liquid tracts. Use a soft toothbrush to clean them if necessary. Check for debris in the alignment holes on the bottom part of the dialyzer. This would prevent the pins on the top part of the dialyzer from seating properly and may cause leakage. Use thin

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wire to clean debris. **Make sure that there are no gaps between the connections.**

- b) Remove the dialyzer membrane from its seated pouch and inspect carefully for tears.
- c) Use “H” type membrane only. Do not wipe or rinse the coating from the dialyzer membrane. Avoid getting any dust, lint or foreign material on the dialyzer membrane because this may cause a poor seal and leakage on the dialyzer.
- d) Lay the dialyzer membrane on the bottom part of the dialyzer.
- e) Place the top part of the dialyzer block on the membrane. Carefully align the pins protruding at the back corner of the top dialyzer so that it is placed straight down without twisting or turning into the alignment holes in the bottom of the dialyzer.
- f) Use a sharp tool to make holes to facilitate the entry of the dialyzer screws through the dialyzer membrane.
- g) Slowly slide the dialyzer screws through the dialyzer membrane into the opening to the bottom of the dialyzer and finger tight the screws.
- h) Turn the screws slowly using the torque wrench. Alternate the wrench from one screw to the other turning each slightly until the wrench clicks on each of them. Tighten the screws again until you feel that the wrench is ready to click.

b. Dialyzer Assembly

Steps for changing the dialyzer assembly

- 1) Loosen the dialyzer screws using a torch wrench. **Do not remove the screws.**
- 2) Remove the analytical cartridge from the cartridge module and the pump.
- 3) Remove carefully all the tubing connected to the dialyzer. **Label** tubing to be sure it is replaced in the correct locations.
- 4) Remove the two mounting screws on the bottom of the cartridge tray. The dialyzer assembly will fall free.
- 5) Remove the dialyzer screw and the dialyzer mount from the old dialyzer.
- 6) Orient the new dialyzer in place.
- 7) Replace the dialyzer mount and the two mounting screws.

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- 8) Reconnect the dialyzer tubing. Be sure there are no gaps or spaces.
- 9) Replace the cartridge on the cartridge module.
- 10) Install a new dialyzer membrane.

6. Maintenance Charts

Refer to Automated Dissociated Enhanced Lanthanide Fluoro Immunoassay System (AutoDELFIA) for the determination of Thyrotropin (TSH) and 17-hydroxyprogesterone (17OHP), Version 1.2. Select API300-TRA from equipment list.

Follow the instructions in the TSH protocol and access the Daily Operator Maintenance Checklist.

- a. Check the box when each step is completed.
- b. Record manually the temperature of the incubator, sync peak volts, and sync peak dwell time in a maintenance chart.

J. Inventory

Refer to TSH/17OHP protocol, Version 1.2.

K. Troubleshooting**1. General guidance for troubleshooting**

- a. Do not overlook the obvious: Look for the simplest and most obvious cause for a particular problem or solution.
- b. Rule out operator induced errors: Review test methodologies and flow diagram to verify operating parameters.
- c. Isolate and define the problem: The following three categories of problems can occur in the API system.
 - 1) **CHEMISTRY:** Reagents, standards, controls, and temperature. Prepare fresh reagents, and discard the expired ones. Adjust the incubator temperature. Pump distilled water with surfactant through the cartridge and observe the signal. If the problem persists, it is most likely hydraulic.
 - 2) **HYDRAULIC:** Pump, pump tubing, surfactant, flowcell, and blockage in the cartridge. Call Proxy if the hydraulic problem is due to the flow cell or pump.

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- 3) ELECTRONIC: PC Components, optics, detector, cable and lamp. Call Proxy.
- d. Eliminate one variable at a time. Use good experimental technique and exchange one component at a time.
- f. Document the solution. Maintain a log of troubleshooting activity. Describe the symptom, cause and the solution for future reference.
- g. Also see Attachment E, Troubleshooting Common assay Problems for the API Systems.

VIII. Calculations

- A. A set of calibrators is tested for each run to construct a calibration curve (STD curve). The calibration curve is a spline smoothed line constructed automatically by the software using log concentration on the x axis and log response on the y-axis for each calibrator at the supervisor's PC.
- B. The FASpac software allows viewing of the chart tracing and instrumental response in real time. At the completion of the run, the file is downloaded to the supervisor's PC where quantitation of the responses against the STD curve occurs using Multicalc. A complete worksheet is generated for review and release by the supervisor.

VIII. Reporting Results

- A. Quality Control Program

The quality control program is used to validate results before reporting.

The quality control program is designed to 1) monitor the day to day performance of the API 300 system and 2) monitor the day to day performance of the method including the punching and elution steps and 3) confirm that sample trays were processed in the correct sequence.

1. Monitor Performance of the API 300
 - a. System Controls (SQC)
 - 1) Three system controls are prepared from a single stock control material for TRA and BIO provided by Perkin Elmer to monitor the performance of the API300 system. The system controls are formulated to contain NADH and

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PABA at a low, medium, and high concentration level. A single replicate of each of the system controls is run at the beginning of each run. The judgment of whether or not the results for the system controls are acceptable is made using the limits established by Genetic Disease Laboratory in conjunction with the application of the Westgard Rules.

- 2) The Westgard rules are applied to three results for the low, medium, and high SQC. By definition, the run is out of control if “3SD 1” (one is outside 3 SD limits), “2SD 2” (two are outside 2SD), or “R4s” (range of SD exceeds 4 SD). The system issues a warning if “2SD 1” (one is outside 2SD). These limits and the Westgard rules are the part of the quality control software.
- 3) The analytical run is scored automatically as being in control (green light), out of control (red light) or as a warning (yellow light). Each result which is outside the +/- 2SD or +/-3 SD limits is appropriately flagged. System control results can be used to aid in troubleshooting the entire method in the event the results are outside acceptable limits.
- 4) Specifically, the results of the system controls together with that of tray controls will assist in determining the source of error, such as degradation of standards and/or reagents, inaccurate reconstitution of reagents, instrument malfunctioning, operator error, sample dilution, or inaccurate sampling.
- 5) The quality control software will plot the system control results showing the mean and acceptable limits. Access the plots using the Supervisor’s PC.

2. Monitor Performance of the Method

a. Tray Control (TQC)

- 1) A blood spot tray control is provided by Genetic Disease Laboratory to monitor the performance of the method. The tray control is formulated to contain TRA activity, BIO activity, TSH, 17OHP, and Hb and is run exactly as newborn specimens. The tray control is run as the first and the last blood spot sample on each tray. The judgment of whether or not the results for the tray control are acceptable is made using the limits established by Genetic Disease Laboratory in conjunction with the application of the Westgard Rules.
- 2) The Westgard Rules are applied to the pair of tray controls at the beginning and end of each tray. By definition, the

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tray is out of control if “3SD 1” (one is outside 3 SD limits), “2SD 2” (two are outside 2SD), or “R4s” (range of SD exceeds 4 SD). The system issues a warning if “2SD 1” (one is outside 2SD). These limits and the Westgard rules are the part of the quality control software.

- 3) The tray is scored automatically as being in control, out of control, or as a warning. Each result that is outside the ± 2 SD or ± 3 SD limits is appropriately flagged. If tray control results fall outside the acceptable limits, the supervisor, in consultation with the Genetic Disease Laboratory will determine the source of error, interpret its meaning, and take corrective action.
- 4) The quality control software will plot the system control results showing the mean and acceptable limits. Access the plots using the supervisor’s PC.

b. Special proficiency control (CP)

A prebarcoded bloodspot proficiency control that has the same concentration as the tray control is provided by GDL. The CP is run on each tray at position E1. Multicalc does not score this control using Westgard Rules. The results of the CP in conjunction with the tray controls are used to further assist the Genetic Disease Laboratory to evaluate the validity of all data.

3. Confirm that sample trays were processed in correct sequence

- a. The software is programmed to skip the positional control well and move to the next sample well automatically without punching a filter paper disk.
- b. The well positions for the positional controls on a sample tray are designated in the following order:

| | |
|--------|--------|
| Tray 1 | Pos C1 |
| Tray 2 | Pos C2 |
| Tray 3 | Pos C3 |
| Tray 4 | Pos C4 |

- c. The tray is judged to be processed in the correct sequence if results of the positional control meet the limits set by the department. If the positional control results fall outside the acceptable limits, the supervisor in consultation with the Genetic Disease Laboratory, will determine the source of error, interpret its meaning and take corrective action.

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- B. Test must be repeated when results are prevented from release. Refer to the Repeat Testing for Newborn Specimens Protocol for details.
- C. Refer to NBS Accession and Reporting Protocol for details on reporting presumptive positives.

X. Procedure Notes

- A. TRA and BIO deficiencies are autosomal recessive disorders.
- B. The temperature variation has significant effect on the performance of the assays and the patient results. The analysis must be performed at recommended temperatures.
- D. The tray control values of the assay are measured without subtracting the background fluorescence, but the patient results are reported after subtracting the background fluorescence.

XI. Limitations of Procedure

- A. Samples incompletely dried, exposed to moisture, heat or sunlight after drying will reduce TRA and BIO activities.
- B. The procedure is not suitable for the detection of Kinase and Epimerase Galactosemia.
- E. There is approximately 5% carry over from sample to sample. The correction to the carry over cup (CO) is entered in the sample table of the FASpac software.

XII. Special Procedures

Sync Peak Optimization/Equation Setting on the API Systems

- A. Log onto the API PC as Level 1, analyst.
- B. Double click the **Data Logger** icon located on the computer's desktop.
- C. Click **API NeoGo**. Prepare the API system for testing per protocol. Pump reagents for at least 20 minutes. Stop the run and go to the log on screen. Log on as Level 2.
NOTE: If you are making a run and are running the standards from the standard rack when you determine that the sync peak must be adjusted, you must stop the run and go to the log on screen.
Bypass the incubation bath when performing this procedure on the TRA/TRB or BIO channel. Disconnect the line from the coil leading to the incubation bath. Disconnect the line from the incubation bath leading to the dialyzer. Connect the two lines.
- D. Double click on the **Data Logger**.

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- E. Double click on the **EQUChem** icon.
“C:\FASPAC\ACF\noname00.ACF already exists. Do you wish to use this name?” appears. Click **Yes**.
- F. Click the green flask. The EQUChem sample table will appear. Autozero the detector for each channel you are adjusting the sync peak. You can adjust both the TRA and TRB channels at the same time or the BIO channel.
- G. Click S1. Go to the channel window and select **Options/Display Signal** for each channel. Verify that the baseline is acceptable.
- H. Select **Settings/Equations** for each channel. This opens the **Equations** window. **NOTE:** The equation in the **Raw signal modification** box. The number after **)*** and before **+** is the amplification factor. **THE AMPLIFICATION FACTOR IS THE ONLY PART OF THE EQUATION YOUR LABORATORY IS ALLOWED TO CHANGE.**
- I. Use the labeled **chart divisions** to optimize the sync peak height. Volts will not be on the screen. The table below lists the acceptable ranges for each assay. When optimizing the peak height aim toward the middle of the range. The ranges and targets are without baseline subtraction.

| Assay | Sync Peak Height Range | Target, chart divisions |
|--------|------------------------|-------------------------|
| UT – A | 2.0 – 3.0 | 2.5 |
| UT - B | 3.5 – 4.5 | 4.0 |
| BIO | 3.5 – 4.5 | 4.0 |

- J. Reduce the amplification factor if the sync peak is too tall. Increase the factor if the sync peak is too short. Amplification factor must fall between 2 and 10. Change the range setting on the fluorometer or colorimer if the amplification factor cannot fall between 2 and 10 to get the correct sync peak size.
- F. Click the green flask to return to the EQUChem sample table window. Fill the standard rack per EQUChem sample table, i.e., alternate five sync peak (Std E) with five water cup.
- G. Select **Run/Begin**. “Start the run for system?” appears. Click **Yes**. Go to the channel window by clicking the S1 button.
- H. Highlight the amplification factor so that you are ready to change it as required.
- I. Use the formula below to help determine the new amplification factor.

$$\frac{(\text{New amplification factor}) = (\text{Desired peak height}) * (\text{Current amplification factor})}{(\text{Current peak height})}$$

Once the sync peak resolves, use the formula above to determine how the amplification factor should be changed. Enter the newly calculated amplification factor. Click **OK**.

- Q. Wait for the next sync peak to resolve and determine if another change is required.

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- Repeat this process until the sync peak is close to the target value.
- R. Allow any remaining sync peaks to resolve to verify the new setting. Click on the green flask to go to the EQUChem sample table window.
 - S. Select **File/Save** from the menu. “EQU1.ACF has changed. Save it?” appears. Click **Yes**. This will save the new equation setting to your FASpac configuration.

References

- A. Cantarow and Schepartz, “Carbohydrate Metabolism”, Biochemistry, Second Edition. W.B. Saunders Co., Philadelphia, 1975, Chapter 17, pp. 389-392.
- B. Astoria - Pacific SPOTCHECK Uridyltransferase 50 - Hour Reagent Kit for the qualitative determination of galactose-1- phosphate uridyltransferase, EC 2.7.7.12 (GALT).
- C. Beutler, E., Baluda, M: “A Simple Spot Screening Test for Galactosemia”, J. Lab. Clin. Med., 68 (137), 1996.
- D. Approved Standard From National Committee For Clinical Laboratory Standards, LA-4-A, “Blood Collection on Filter Paper For Neonatal Screening Programs”, Approved Standard, 1987.
- E. Astoria Pacific SPOTCHECK Biotinidase 50 Hour Reagent Kit for qualitative determination of Biotinidase activity.
- F. Heard, G., McVoy, J., Wolf, B., A Scefening Method for Biotinidase Deficiency in Newborns, Clin Chem, 30, 125-127, 1984.

Appendix 5H

Attachment 1

TRA/BIO Pumptube Diagram

| | | | |
|------------|-----------|------------------|---------------------------------|
| Grn | T1 | Grn | From Sampler |
| Yel | | Blu | To Sampler (Tris Buffer) |
| Wht | B6 | Wht | Detergent TX-10 |
| | | | |
| Orn | T4 | Wht | Helium |
| Blk | B2 | Blk | Helper |
| | | | |
| Orn | B1 | Orn | Debubble |
| | | | |
| Orn | T2 | Yel ----- | Air |
| | | | |

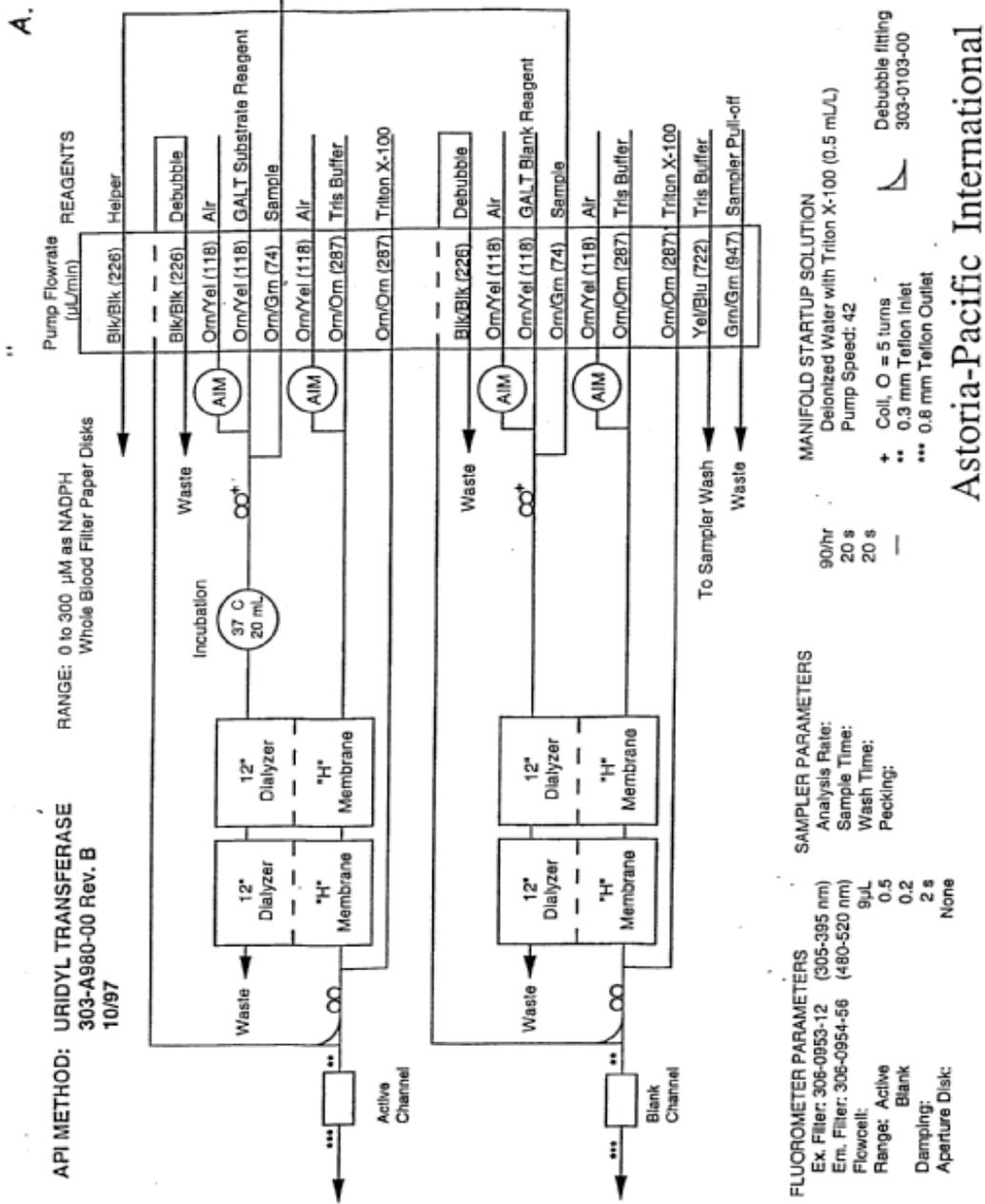
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| | | | |
|------------|-----------|------------------|-------------------------|
| Orn | | Grn ----- | Sample |
| Orn | B3 | Grn ----- | Bio Substrate |
| | | | |
| Orn | T5 | Grn | Nitrite |
| Orn | T7 | Grn | Sulfamate |
| Orn | T8 | Grn | NED |
| | | | |
| Orn | B4 | Orn | Tris Buffer |
| Orn | T8 | Orn | Detergent TX-10 |
| Blk | B1 | Blk | Debubble |
| | | | |
| Orn | T5 | Yel | Air |
| Orn | B6 | Yel ----- | Active Substrate |
| Orn | T7 | Yel ----- | Air |
| Orn | | Grn ----- | Sample |
| | | | |
| Orn | | Grn | Sample |
| Orn | T5 | Yel | Air |
| Orn | B6 | Yel | Blank Substrate |
| Orn | T7 | Yel | Air |
| | | | |
| Blk | B1 | Blk | Debubble |
| Orn | T8 | Orn | Detergent TX-10 |
| Orn | B4 | Orn | Tris Buffer |

T = Top
B = Bottom
 ----- = black mark on white nib of colored collar

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Appendix 5H



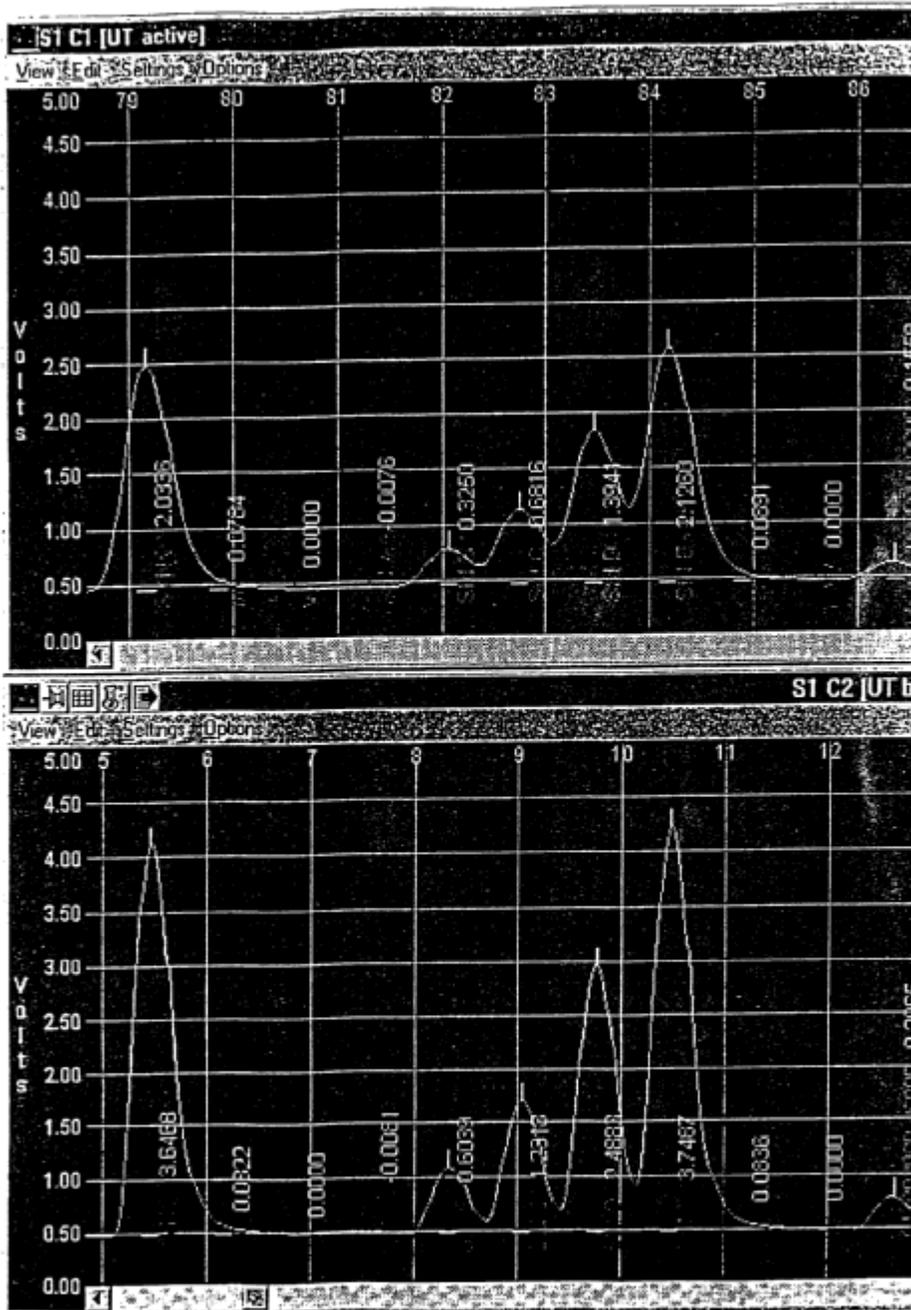
NBS CHECKLIST

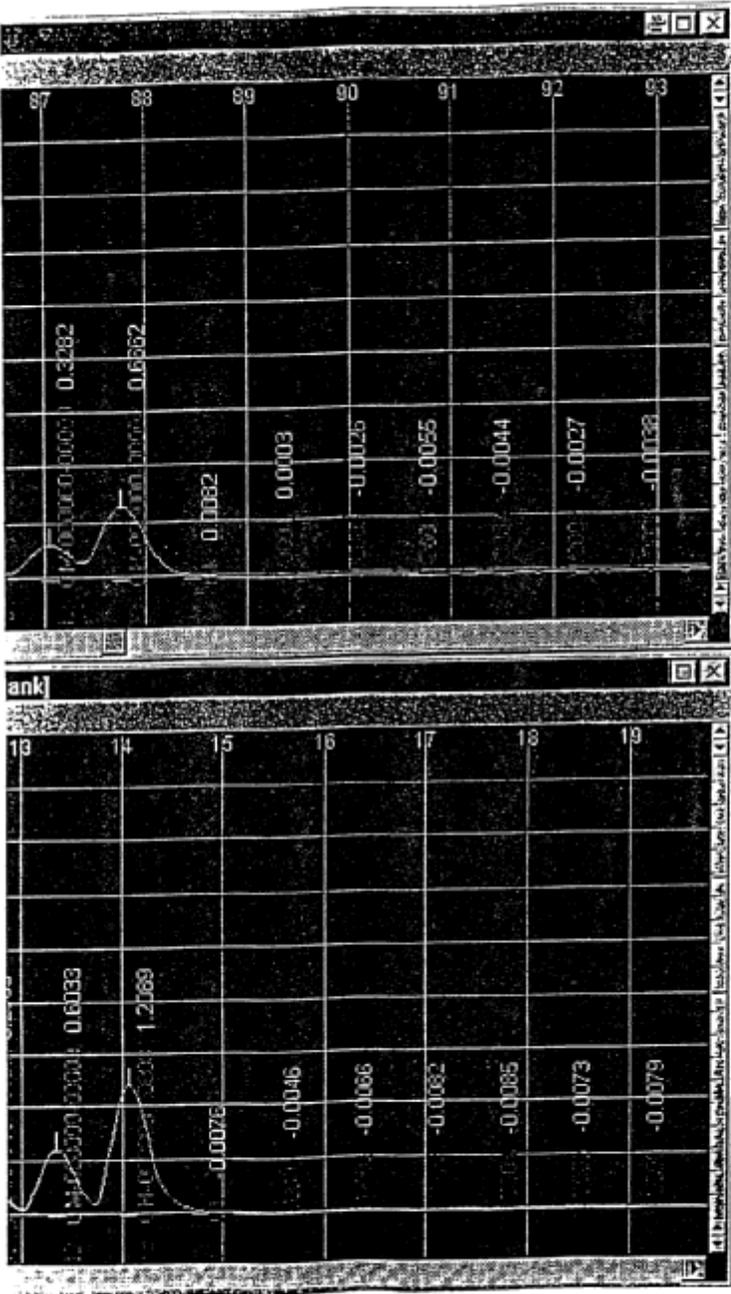
LABORATORY SITE: _____

Please FAX checklist to GDLB Daily

| NBS LABORATORY | | | | | | | | | | GDLB NBS QA | | | | | | | | | |
|--|----------------|--------------|------------|----------------|-------|-----|-----|-------|-------------------------|---------------------------|-----|-----|-------|----------------------|-----|-----|-------|--|--|
| Run Date | Assay Run ID # | Instrument # | Batch ID # | Accession Date | Assay | | | | Releaser Initial & Date | Reviewer Name & Date | | | | Releaser Name & Date | | | | | |
| | | | | | APSC | IRT | TSH | 17OHP | | APSC | IRT | TSH | 17OHP | APSC | IRT | TSH | 17OHP | | |
| | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | |
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| | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | |
| Are any specimens delayed more than 2 days? Yes _____ No _____ If yes, accession #s: | | | | | | | | | | Request to Transfer Data: | | | | | | | | | |
| Are any instruments down today? Yes _____ No _____ If yes, describe: | | | | | | | | | | Date: _____ | | | | | | | | | |
| | | | | | | | | | | Time: _____ | | | | | | | | | |
| | | | | | | | | | | NAPS Contact: _____ | | | | | | | | | |

C.





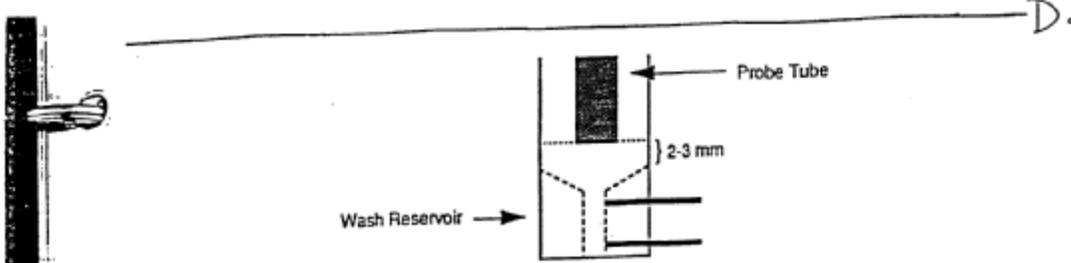


Diagram 6-13

CAUTION: Always lock the probe tube collar with the lock screw on the smooth side of the probe tube. Tightening the lock screw into the gear teeth on the probe tube can damage the teeth and prevent smooth operation beyond this point if the collar is re-adjusted upwards.

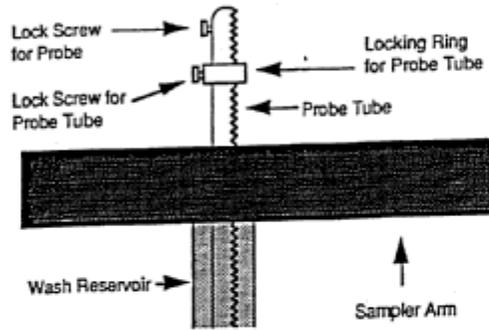


Diagram 6-14

Gently pull the probe tube up as far as it will go. Be sure that the probe tube's movement is smooth. Insert the black Teflon-coated end of the probe into the probe tube and down through the wash end of the probe should be clearly visible. Position the end of the probe between the IN and OUT bottom of the wash reservoir. Refer to diagram 6-15. Tighten the nylon lock screw to hold the probe in place.

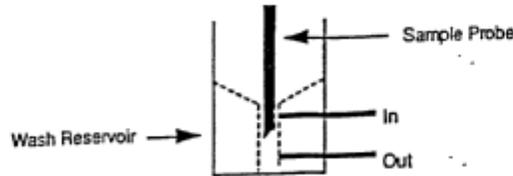


Diagram 6-15

Attachment E**Troubleshooting Common Assay Problems for the API Systems**

| Event | Cause | Action |
|---|-----------------------------------|---|
| I. Curve does not match prior, flat or max/min incorrect | Prepare Stds incorrectly | Use Type 1 water, use accurate + precise pipets, use clean volumetric flask, follow protocol |
| | Place on system in wrong order | Label a cup and pour Std one at a time, recheck order after placement into Std block |
| | Insufficient liquid in cup | Fill all cups to at least $\frac{3}{4}$ full |
| | Contaminated cup/pipet used | Store cups in clean space, clean the ends of pipets frequently |
| | Bad bottle of stock Std | Call GDL |
| | Brij solution contaminated | Prepare new solution, cap immediately after preparation, store and use properly, keep spigot clean |
| | Use water that is not Type 1 | Test water periodically to determine that it meets Type 1 specifications |
| | II. Water cups > limits | Dirty system-coil, probe |
| Contaminated cups on Std block | | Store cups in clean space, do not touch cups with dirty gloves |
| Carryover due to poor washout | | Check system for blockage, clean with cleaning solution/HCL for TRA/BIO system, check tubing/nipple connections, prepare brij/triton solution incorrectly, pump brij/triton solution for the required times |
| Contaminated wash reservoir for sample probe | | Clean wash reservoir with cleaning solution/HCL for TRA/BIO systems |

| | | |
|--------------------------------------|--|---|
| III. Dwell time is short/long | Platen is uneven or damage | Call Proxy, refer to TSH/17OHP Assay Protocol |
| | Wrong pump speed | Follow protocol and set speed correctly |
| | Wrong tubing | Follow protocol and use correct tubing |
| | Plug in system | Find plug by observing bubble pattern, find by visual inspection, remove plug, change tubings/nipples |
| | Did not pump brij/triton sufficiently | Follow times listed in protocol |
| | Did not filter reagents causing back pressure | Follow protocol and filter reagents |
| | Dirty coil in incubator for TRA | Clean with cleaning solution/HCL, call Proxy to replace if needed |
| | Excess tubing for TRA | Remove some of the tubing |
| IV. Sync peak is high/low | Equation is incorrect | Change equation for fine adjustments |
| | Plug in system | Find plug by observing bubble pattern, find by visual inspection, remove plug, change tubings/nipples |
| | Leak in system | Find leak by observing bubble pattern, find excess water on the system, fix, call Proxy if needed |
| | Did not turn heater on for Phe/Tyr | Follow protocol and turn on heater |
| | Pour wrong Std into cup for sync peak | Read label on volumetric flask before pouring and label cup |
| | Contaminated Std | Replace stopper immediately after use, use clean pipets and glassware, store properly |
| | Incorrect Std preparation | Use accurate and precise pipets, clean volumetric flask, Type 1 water, |
| | Incorrect reagent preparation for Phe/Tyr, e.g., ninhydrin, nitrous acid | Follow protocol, use accurate and precise pipets, clean glassware, use Type 1 water, |
| | Dirty flowcell | Clean with cleaning |

| | | |
|--|--|---|
| | | solution/HCL for the TRA/BIO system, call Proxy is needed |
| | Lamp not working | Call Proxy |
| | System has wrong sample tubing | Follow protocol flow chart and use the right tubing |
| V. Control results outside limits | | |
| A. SQC | Incorrect preparation | Follow protocol, use clean pipets and glassware, use accurate and precise pipets |
| | Contaminated with high Std or stock | Use clean pipets and glassware, do not touch vials/tubes with dirty gloves |
| | Air spike | Check for leaks by observing bubble pattern, find excess water on the system, fix, call Proxy if needed |
| B. TQC | Bad spot | Check inventory for affected spots, call to inform GDL |
| | Bad punch | Have entire spot under punch head |
| | Bad tip on Apricot when adding water for elution | Call Proxy to replace O ring for better grip |
| | Did not elute completely, spot is not in liquid | Before putting on shaker, check for spots above the liquid level |
| | Crack in well | Visually inspect all trays, check inventory for affected plates, call GDL if found |
| | Incorrect vortexer speed | Call Proxy |
| | Incorrect storage of TQC spots | Follow protocol for correct storage |
| | Air spike | Check for leaks by observing bubble pattern, find excess water on the system, fix, call Proxy if needed |
| VI. Run/Tray has low/high bias | | |
| TRA/BIO | Deactivated enzyme in specimens by UV/heat | Call AGN to correct collection errors, refrigerate |

| | | |
|---|---|--|
| | | specimens until testing in labs |
| | Incorrect substrate preparation | Follow protocol, use clean glassware |
| | System is dirty | Clean with cleaning solution/HCL, change tubings/nipples |
| | Incorrect elution - volume, time | Call proxy for equipment service, follow protocol and use correct Apricot program |
| | Incorrect vortexer speed | Call proxy |
| | Improper Std curve | Follow protocol, use accurate and precise pipets, use clean glassware |
| | Blockage in flowcell | Clean with cleaning solution/HCL, call Proxy if needed |
| | Incubator temp is high/low | Call Proxy |
| VII. Repeats are inconsistent with initial | Difference is spots | Call AGN to correct collection procedures |
| | Elution problem with time, volume, or speed | Follow protocol, use correct Apricot program, call Proxy for equipment service |
| | Bad tip on Apricot | Call Proxy to replace O ring |
| | Jumping chad | Use Zerostat anti-static device |
| VIII. TRB results are high | Contaminated/dirty blk channel | Clean system with cleaning solution/HCL, change tubings/nipples |
| | Contaminated specimen at collection site e.g., splash | Call AGN to correct collection procedures |
| | Pharmacologic effects from patient | Call AGN |
| | Contaminated wells | Store all trays properly with plastic and in clean space, handle with clean gloves |
| IX. Assay/Tray median >limits | | |
| TRA/BIO | Incorrect elution time, volume | Follow protocol, use correct Apricot program, call Proxy for equipment service |
| | Incorrect incubator temperature | Call Proxy |
| | Partially denatured enzyme due to sun, hairdryer | Call AGN to correct collection procedures |

| | | |
|-----------------------------------|--|---|
| X. Poor resolution | Dirty flowcell | Clean with cleaning solution/HCL for TRA/BIO systems, call Proxy if needed |
| | Lines are partially plug | Find blockage by observing bubble patterns, find by visual inspection, remove plug, change tubings/nipples |
| | Did not pump brij/triton sufficiently | Follow times listed in protocol |
| | Leaking membrane | Change membrane |
| | Poor connections | Check/change tubings/nipples |
| | Air bubbles in lines | Check for leaks by observing bubble pattern, find excess water on the system, fix or call Proxy if needed |
| XI. Unstable baseline | Brij/triton solution is not lubricating the system | Dump and prepare new, do not use expired brij/triton solutions, prepare per protocol |
| | Noise in lamp | Call Proxy |
| | Temperature of heater/incubator drops steadily or suddenly | Call Proxy |
| | Dirty flowcell | Clean with cleaning solution/HCL for TRA/BIO systems, call Proxy if needed |
| | Lines are partially plug | Find blockage by observing bubble patterns, find by visual inspection of system, remove plug, change tubings/nipples |
| | Worn pump tubings | Replace tubing, follow protocol for maintenance schedule |
| XII. Unusually low results | One spot in well | Use Zerostat anti-static device |
| XIII. PQC outside limits | Jumping chat in PQC well | Use Zerostat anti-static device |
| | Turn tray 180° | Always check for the A1 position |
| | Carryover | Clear any blockage in system, use properly prepared brij/triton solutions, pump brij/triton solutions for the required times, check concentration of prior sample |

| | | |
|--|-------------------|--|
| | Tray out of order | Follow protocol to maintain sets of grandmother, mother, daughter plates |
|--|-------------------|--|

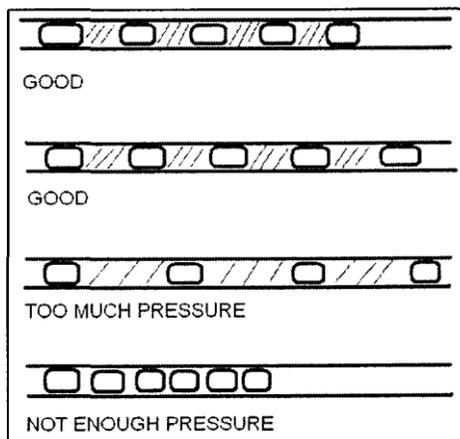
Attachment F

Troubleshoot Backpressure Problems for BIO

The most direct diagnostic tool for examining the backpressure in the Bio cartridge is the bubble spacing in the debubble line coming from the 315 detector. You do not have to take the cover off. Just look at the debubbler tube to the right of the detector as it heads toward the orn/orn debubble pump tube.

“GOOD” = Liquid and bubble segments are about the same size, or up to about 1.5 to 2 times more liquid than bubble.

First be certain that there is no plug or partial plug at the inlet of the small backpressure tubing. Then compare the observed flow with the diagram above. Allow enough time for flow to equilibrate before judging. 5 - 10 minutes should be enough. Add (to increase pressure) or subtract (to decrease pressure) lengths of backpressure tubing to achieve a “GOOD” pattern.

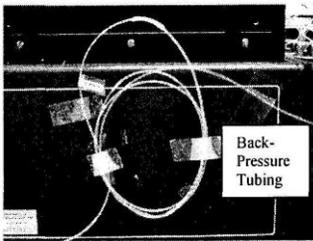
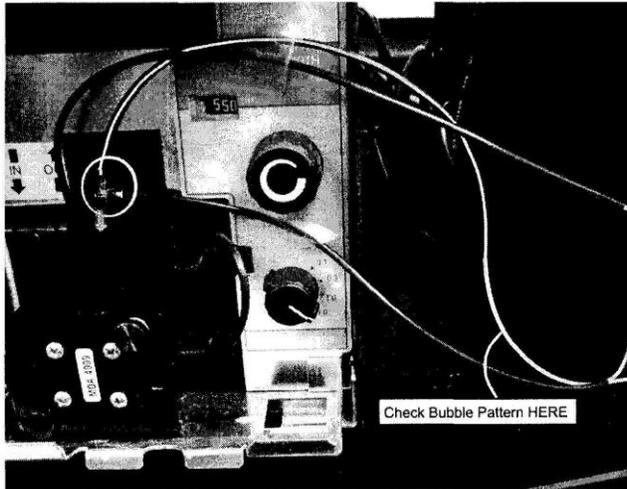


The usual cause of nearly any excessive pressure problem is a plug or a crimp in the backpressure tube. This is true no matter what the symptom may be or where a leak may appear on the cartridge or flowcell. Remove the plug using one or more of the following:

- 1) Disconnect and reconnect the backpressure tubing at its inlet.
- 2) Carefully cut 1/8" to 1/4" from the inlet of the backpressure tubing, being careful not to crimp it.
- 3) With the backpressure tubing disconnected, back flush it with a syringe and TX10. **OR** Replace the tubing.

If something was plugging it, it had to come from somewhere and that is the root of the problem. The 5 choices are: NED, Nitrite, Sulfamate, TX10, and Chemwash. These bottles and solutions should be carefully examined. Clean the bottles and filter the solutions as needed. You do not need to discard the solutions if they have not passed their expiration date.

The blood sample extracts, substrate and incubation coil cannot cause backpressure in this part of the cartridge; they are on the top side of the dialyzer membrane.



Prepared by _____ Date _____
Stephen Singh

Reviewed by _____ Date _____
Faezeh Samimi

Approved by _____ Date _____
Dr. George Helmer

