Total Galactose Screening Assay

An Enzymatic Colorimetric Assay for Quantitative Determination of total Galactose (Galactose and Galactose-1-phosphate) Levels in Neonates.

A SCREENING KIT FOR DRIED BLOOD SPOTS

PISHTAZ TEB DIAGNOSTICS

Introduction

The Total Galactose Screening Kit is an Enzyme Assay (EA) for the quantitative measurement of total Galactose (Galactose and Galactose-1-phosphate) concentrations in dried blood spot samples that have been collected onto Schleicher & Schuell (S&S®) 903™ specimen collection paper.

Clinical Utility

Galactosemia is a disorder caused by an inborn error of Galactose metabolism and characterized by elevated concentrations of Galactose in the blood resulting from the absence or dysfunction of any of the three enzymes responsible for the transformation of Galactose to glucose, i.e., D-Galactose-1-phosphotransferase, -D-Galactose-1-phosphate-uridyltransferase or UDP-glucose-4-epimerase. The rate of incidence is approximately 1 in 50,000 newborns worldwide. The symptoms associated with Galactosemia in the newborn period can include vomiting, diarrhea, dehydration, jaundice, hepatic failure, hypoglycemia, cataracts, and developmental retardation. The increased circulating concentrations of Galactose, if left untreated, can result in a variety of symptoms including metabolic cirrhosis of the liver, mental retardation, cataract formation, and kidney damage. Screening is now a fundamental component of health care programs which is recommended for all newborn infants in most countries.

Principle of the Assay

After extraction the eluted sample is combined with an Enzyme-Coenzyme solution containing Galactose dehydrogenase, alkaline phosphatase and nicotinamide adenine dinucleotide (NAD). Galactose-1-phosphate is converted to Galactose by the enzymatic action of alkaline phosphatase. Galactose is oxidized by Galactose dehydrogenase in the presence of NAD and results in the production of galactono-lactone and NADH. The amount of Galactose in the dried blood spot sample can be quantitated by measuring the reduction of NAD to NADH. The amount of NADH produced is measured colorimetrically by the conversion of the colourless indicator into a pink to red color. The intensity of this reaction product can be measured at a 490 nm and is directly proportional to the concentration of Galactose present in the sample. (Fig. 1)

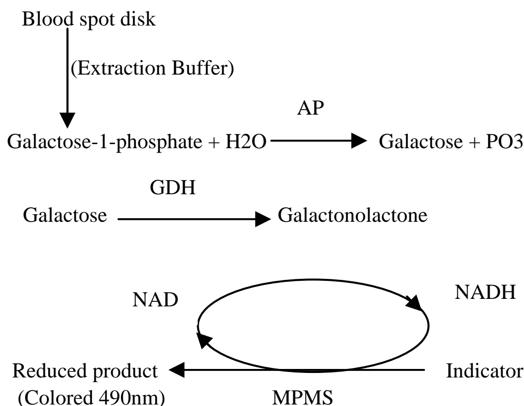

Fig1. Assay principle

Sample Preparation & Storage

Dried blood spot samples that have been collected onto Schleicher & Schuell (S&S®) 903™ specimen collection paper are used for Galactose determination. Samples should be collected 24-72 hours after birth from infant plantar heel surface. (Fig. 2)

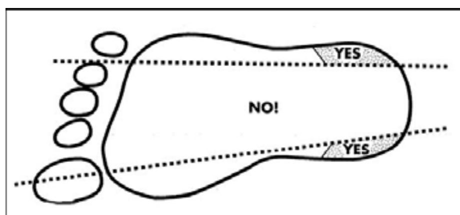

Fig 2. "Yes" in grey areas showed proper infant blood collection site

Sample collection should be based on standard protocol of National Committee for Clinical Laboratory Standards, now known as Clinical Laboratory Standards Institute (CLSI): LA4-A4 "Blood Collection on Filter Paper for Newborn Screening Programs; Approved Standard - Fourth Edition which is briefly stated here:

1. Cleanse infant's heel with 70% isopropyl alcohol (use only rubbing alcohol). *Note:* Warming the skin-puncture site with a warm moist cloth, or a heel warming device, for 3 minutes can increase blood flow through the site.

2. Allow heel to air dry.

3. Using a lancet, or heel incision device, and wearing gloves, performs the puncture on the plantar surface of the heel (as indicated in the drawing). The puncture should be made to a depth of less than 2.0 mm with a sterile lancet or incision device.

4. Gently wipe off first drop of blood with sterile gauze or cotton ball. The initial drop contains tissue fluids that may dilute sample.

5. Wait for formation of large blood droplet; apply gentle pressure with thumb and ease intermittently as drops of blood form.

6. Gently touch the printed side of the filter paper card to the blood drop and in one step, allow a sufficient quantity of blood to soak through and completely fill a pre-printed circle. Do not press the filter paper against the puncture site on the heel. Fill each printed circle with a SINGLE application of blood. Observe both sides of the filter paper card to assure that blood uniformly penetrated and saturated the card. Spotting should be done *only* on the printed side. The filter paper must not touch the skin puncture site.

7. Fill the required number of blood spots for mandated tests.

8. All used items should be disposed of in an appropriate biohazard container.

9. Elevate infant's foot above the body and apply pressure using sterile gauze. Do not apply adhesive bandages.

10. Allow blood specimen to air dry thoroughly, on a horizontally level—non-absorbent open surface, such as a plastic-coated test tube rack—for a minimum of 3 hours at ambient

temperature and away from direct sunlight. Do not stack, heat, or allow touching other surfaces during the drying process.

11. Each dried sample card should be placed into a separate envelope with all required information for shipment. Humidity and moisture are detrimental to stability of dried blood spot specimens and can affect results.

12. Sample cards keep in humidity resistant envelope can be stored for 1 week and those which keep in silica gel or plastic bags at 4-8 °C can store for 4 months. Keeping the samples in lower temperature (-20 °C) increase the storage duration. All removed kit controls or standards should returned back to its original aluminum bag to maintain their function during test.

Kit Contents

1 - Extraction micro titer plate : (1 plate /96 wells)
Polystyrene well
2 - Reader micro titer plate: (1 plate /96 wells)
Polystyrene well
3 - Chromogen Solution (1 vial / 6 ml)
0.1 M Tris buffer solution contain Tetrazolium salt and Sodium Azide as preservative
4- Substrate Solution (1 vial / 6 ml)
0.1 M Tris buffer solution contain NAD and Sodium Azide preservative
5 - Extraction Solution: (1 vial / 20 ml)
0.1 M Tris buffer solution contain 0.05 % sodium azide
6 - Enzyme solution (1 vial / 6 ml)
0.1 M Tris buffer solution contain alkaline phosphatase and Galactose Dehydrogenase and Sodium Azide as preservative
7 - Standard set (6 x 1 papers)
Filter paper Contain Whole blood Dried (ss2992) with exact amount of Galactose
8 - Controls (2x 1 paper)
Filter paper Contain Whole blood Dried (ss2992) and certain amount of Galactose

Assay Procedure

1. Take a clean 96-well microplate (elution microplate).
2. Equilibrate all reagents to room temperature.
3. Add related collection paper of controls, standards and specimen dried blood spots, each cut in 5 mm diameter into appropriate well. Run tests in duplicate for reliable results.
4. Incubate the microplate in a 90-95 °C water bath on a horizontally level, such as a test tube rack, for 10 minutes.
5. Add 150 µl of Reagent 1 (extraction buffer) into each well to release Galactose from collection paper disks. Note to fully covered disks and avoid air bubble formation.
6. Cover the plate with cardboard sealer and shake for 60 minutes.
7. Transfer 100 µl of extracted solutions into the equivalent transparent microplate wells which appropriately marked.
8. Depend on number of wells required, mixed Reagent 2, 3 and 4 in equal volumes. This mixture remains stable for 5 minutes.
9. Add 150 µl of mixture prepared in previous step into each well. Cover the plate with cardboard sealer and shake for 60 minutes to complete the reaction.
10. Read the microplate at 490 nm endpoint mode, single measurement within 10 minutes.

Results calculation

Construct a point to point standard curve by plotting the mean absorbance obtained for each standard against its concentration in mg/dl on linear graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis. Fig. 3

Limitations

The Kimia Pajouhan Total Galactose Screening Assay is intended for use as a tool to screen neonates for elevated levels of total Galactose (Galactose and Galactose-1-phosphate). This Kit is not to be used for confirmatory testing or to monitor therapy. A definitive clinical diagnosis should not be based on the results of a single test but should be made by the physician only after all clinical and laboratory findings have been evaluated. Another diagnostic procedure performed on a serum sample should be used to confirm the diagnosis of Galactosemia.

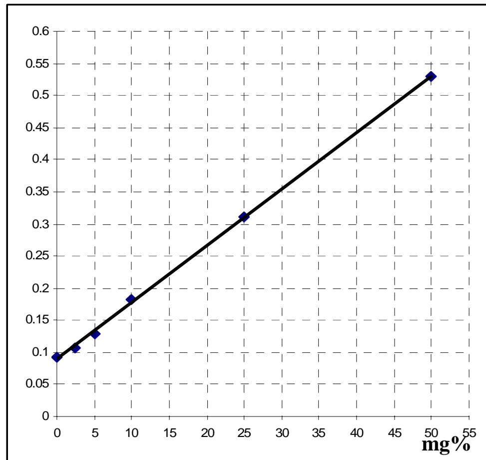

Fig. 3 Standard curve

Expected Values

To determine normal range for Galactose, 250 blood samples from 1-4 days neonates were collected and their Galactose level measured by this kit. (Table 1)

Table 1. Expected value

1-4 days Newborn infants	Galactose Conc.
NORMAL	0 – 5 mg/dL
SUSPECT	5 – 18 mg/dL
ABNORMAL	>18 mg/dL

The recommended cut off point for neonate Galactose level is 5 mg/dl. Neonates with higher Galactose levels should be re-tested.

Note: New standard curve should be plotted for each run.

Performance Characteristics
Precision test
Tree dried blood spot samples with different concentration of Galactose were repeatedly tested. Results are shown in table 1 and 2:

Intra assay

No.	No. of tests performed	Means mg / dl	SD mg / dl	CV %
1	24	1.72	0.15	8.7
2	24	5.62	0.29	5.1
3	24	11.37	0.74	6.5

Inter assay

No.	No. of tests* performed	Means mg / dl	SD mg / dl	CV %
1	10	1.58	0.18	11.3
2	10	5.35	0.43	7.9
3	10	11.4	0.77	6.8

*Each test has been run in duplicate

Accuracy

Recovery
The certain amount of Galactos was added into one sample with known concentration of Galactos and then their recovery were determined. The results shown below:

Added mg/dl	Measured mg/dl	% Recovery
4	**3.4**	**85**
8	**7.7**	**96**
12	**12.8**	**106**
16	**17**	**106**
20	**21.6**	**108**

Linearity

To verify test linearity, one sample with known Galactos concentration was diluted sequentially by zero standard. Then the sample was tested by kit. The results and serum recovery were determined considering dilution factor.

Sample	% Recovery
1 / 2	108
1 / 4	106
1 / 8	93
1 / 16	98

References

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