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PROTOCOL

Assessment of galactose-1-phosphate uridyltransferase activity in cells and tissues

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Abbreviations used: CG, classic galactosemia; ER, endoplasmic reticulum; HPLC, high-performance liquid chromatography; GALT, galacose-1-phosphate uridyltransferase

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ABSTRACT

Galactosemias are a family of autosomal recessive genetic disorders resulting from impaired enzymes of the Leloir pathway of galactose metabolism including galactokinase, galactose uridyltransferase, and UDP-galactose 4-epimerase that are critical for conversion of galactose into glucose-6-phosphate. To better understand pathophysiological mechanisms involved in galactosemia and develop novel therapies to address the unmet need in patients, it is important to develop reliable assays to measure the activity of the Leloir pathway enzymes. Here we describe in-depth methods for indirectly measuring galacose-1-phosphate uridyltransferase activity in cell culture and animal tissues.

Keywords: classic galactosemia, GALT, HPLC

BACKGROUND

Classic galactosemia (CG), or type I galactosemia, is a rare disorder of autosomal recessive inheritance caused by point mutations in the gene encoding the galacose-1-phosphate uridyltransferase (GALT) enzyme. This enzyme functions in the Leloir pathway of galactose metabolism and transfers a uridyl group from UDP-glucose to galactose-1-phosphate to generate UDP-galactose and glucose-1-phosphate which is further metabolized in the glycolytic pathway. Of the more than 350 mutations identified, the vast majority result in reduced activity of the GALT enzyme [1]. Biochemically, this results in buildup of galactose, galactitol, and galactose-1-phosphate. While the pathophysiology of CG is not fully elucidated it has been hypothesized that these metabolites reach toxic levels that result in oxidative stress, endoplasmic reticulum (ER) stress, and inflammation [2].

In untreated infants, CG may result in failure to thrive, liver failure, kidney failure, sepsis, cataracts, and death within the first two weeks of life. Even with dietary treatment, patients may exhibit long term phenotypes such as cognitive, language, and motor impairments as well as subfertility [3,4]. Standard newborn screening in the USA includes a test for CG which involves determining the activity of GALT in red blood cells from blood spots. This assay is easily adapted for preclinical laboratory use [5]. However, most laboratory protocols for determining the activity of GALT in *in vitro* systems, such as patient-derived cell lines or cells transfected with plasmids expressing GALT, involve the use of high-performance liquid chromatography or mass-spectrometry [6,7]. Some research laboratories may not have access to equipment or training necessary for these techniques. Furthermore, these techniques involve feeding cells C^{14} radiolabeled galactose and require additional laboratory safety regulations, training, and engineering controls to be established (**Table 1**) [7-9].

We report an improved and rapid protocol adapted from the GALT blood spot assay [5] for the analysis of GALT activity in cell and tissue lysates (**Fig. 1**). We describe a simple step-by-step protocol describing cell culture conditions, cell lysate preparation, an indirect assay for GALT enzyme activity, and data analysis. We believe this protocol will benefit researchers interested in studying the effects of different mutations in the GALT gene and potential therapies to restore GALT activity in CG as well as potential small molecule activators and inhibitors.

MATERIALS

Reagents

- ✓ α-D-galactose 1-phosphate dipotassium salt pentahydrate (Sigma, Cat. # G0380)
- ✓ UDP-α-D-glucose, disodium salt (Sigma, Cat. # 670120)
- ✓ Nicotinamide adenine dinucleotide phosphate (NADP), monosodium salt (Sigma, Cat. # 481971-M)
- ✓ Tris base (Fisher, Cat. # BP152)

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- ✓ Glacial acetic acid (Sigma, Cat. # 695092)
- ✓ Ethylenediaminetetraacetic acid (EDTA) (Fisher, Cat. # S311)
- ✓ Saponin (Sigma, Cat. # 47036)
- ✓ Acetone (Sigma, Cat. # 650501)
- ✓ Methanol (Sigma, Cat. # 34860)
- ✓ cOmplete Mini, EDTA-free protease inhibitor cocktail tablets, Roche, REF 11836170001
- ✓ HEPES (Sigma, Cat. # RES6003H-B7)
- ✓ Potassium hydroxide (KOH) pellets (Sigma, Cat. # 221473)
- ✓ Dithiothreitol (DTT) (Sigma, Cat. # D9779)
- ✓ Bovine serum albumin (BSA) (Fisher, Cat. # 52-171-00G)
- ✓ Gibco™ DMEM, high glucose, pyruvate (Fisher, Cat. # 11-995)
- ✓ Gibco™ DMEM, low glucose, pyruvate (Fisher, Cat. # 11-885)
- ✓ Gibco™ DMEM, no glucose (Fisher, Cat. # 11-966-025)
- ✓ Gibco™ PBS, pH 7.4 (Fisher, Cat. # 10-010-023)
- ✓ Gibco™ Fetal bovine serum (Fisher, Cat. # 26-140)
- ✓ Gibco™ Penicillin-streptomycin (10000 U/ml) (Fisher, Cat. # 15-140)
- ✓ Gibco™ MEM non-essential amino acids solution (100X) (Fisher, Cat. # 11-140-050)
- ✓ Gibco™ Sodium pyruvate, 100 mM (Fisher, Cat. # 11-360-070)
- ✓ Pierce™ BCA protein assay kit (Fisher, Cat. # PI23227)
- ✓ The following cell lines were obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository at the Coriell Institute for Medical Research: GM05757, GM01703, and GM00422
- ✓ ddH_2O (double distilled water)

Recipes

- ✓ Human fibroblast complete culture media: DMEM containing 10% FBS, 1% Penicillin-Streptomycin, and 1% MEM non-essential amino acids
- ✓ HEPES/KOH lysis buffer: Dissolve 2.383 g HEPES in 400 ml ultrapure water, pH to 7.5 with KOH pellets, dissolve 0.077 g DTT and 150 mg BSA, bring volume to 500 ml (20 mM HEPES/KOH pH 7.5, 1 mM DTT, 0.3 mg/ml BSA). At time of use, add 1 protease inhibitor cocktail tablet to 10 ml of HEPES/KOH lysis buffer and keep on ice.
- ✓ Acetone-methanol solution: Combine equal volumes of acetone and methanol (1:1). Fifty ml of each for a total volume of 100 ml is suitable for several 96-well plates.
- ✓ GALT activity assay buffer: Dissolve 15.14 g Tris Base in 450 ml of ultrapure water, and pH to 8.0 with Acetic Acid. Dissolve the following: 0.098 g UDP-glucose, 0.253 g NADP, and 0.025 g EDTA. Bring the volume to 500 ml. Store at 4°C. (0.25 mol/L Tris-acetate pH 8.0, 0.32 mmol/L UDP-glucose, 0.66 mmol/L NADP, 0.135 mmol/L EDTA)
- ✓ GALT activity assay reaction buffer: Divide the GALT Activity Assay into two for 2 aliquots of 250 ml each. Dissolve 0.192 g Galactose 1-phosphate. Store at 4°C. At time of use, add 0.013 g of Saponin to 10 ml of GALT activity assay reaction buffer. (0.25 mol/L Tris-acetate pH 8.0, 0.32 mmol/L UDP-glucose, 0.66 mmol/L NADP, 0.135 mmol/L EDTA, 1.8 mmol/L Galactose 1-phosphate, 1.3 g/L saponin)
- ✓ GALT activity assay control buffer: 250 ml of GALT Activity Assay Buffer. Store at 4°C. At time of use, add 0.013 g of Saponin to 10 ml of GALT Activity Assay Control Buffer. (0.25 mol/L Tris-acetate pH 8.0, 0.32 mmol/L UDP-glucose, 0.66 mmol/L NADP, 0.135 mmol/L EDTA, 1.3 g/L saponin)

Equipment

- ✓ 96-well conical bottom plate (Thermo, Cat. # 249946)
- ✓ 96-well optical bottom plate (Thermo, Cat. # 165305)
- ✓ Orbital plate shaker
- ✓ 37°C incubator
- ✓ Centrifuge with plate adaptor
- ✓ Centrifuge at 4°C
- ✓ Plate reader capable of excitation of 350–355 nm and emission of 460 nm

Table 1. Comparison of GALT activity assay methods.

	HPLC and GC-MS	Modified blood spot assay
Pros	• Very accurate • Very sensitive	• Rapid • Requires more common equipment
Cons	• Time consuming • Requires specialized equipment • Requires use of radiolabeled glucose	• Less accurate • Less sensitive

PROCEDURE

1. Sample preparation
   - 1.1. Fibroblasts were plated at a minimum of 1.0×10^5 cells per well in 6 well plates in high glucose media.
   - 1.2. Allow cells to reach 70% confluency or higher for 24–72 h.
   - 1.3. For assay conditions involving media containing galactose: 24–48 h post plating cells, incubate cells in low glucose media for 24 h followed by galactose-containing media for 24 h.

NOTES: Plating 1.0×10^5 cells per well in 6 well plates in low glucose media for 72 h were the minimum plating

conditions used to achieve a lysate with sufficient total protein concentration to plate 10 µg total protein per sample in duplicate. 10 cm dishes at 70% confluency or higher provided more optimal lysates. Incubating cells in low glucose media between high glucose media and galactose-containing media prevents excessive ER stress in the cells [10].

2. Harvesting cell and tissue samples.
   - 2.1. Place samples on ice.
   - 2.2. Lyse samples on ice with cold HEPES/KOH lysis buffer [1,11]. Use a volume that will obtain > 1 µg/ml of total protein content in the lysate.
   - 2.3. Transfer lysate to 1.5 ml tube, vortex briefly, incubate for 5 min on ice, vortex, incubate for 5 min on ice, vortex briefly, and centrifuge at 17000 to 20000 RCF for 10 min at 4°C.
   - 2.4. Transfer supernatant to a new 1.5 ml tube.
   - 2.5. Determine total protein content using Pierce BCA Protein Assay Kit.

NOTES: Appropriate positive controls include cells or blood spots from a normal individual or non-classic galactosemia patient, HEK239T cells transfected with a plasmid expressing WT GALT, as well as cells, tissues, or blood spots from wild-type mice (*e.g.*, C57Bl6 strain). It is best to use a positive control of the same sample matrix type (such as liver lysate, cells in culture or serum sample) to enable comparison between experimental samples. If cell culture plates are used: plates should be placed on ice and washed with cold PBS 3 times; all remaining liquid should be gently aspirated prior to lysis. We have found 12 well plates, with lysis directly in the well, to be the smallest size that still allows for adequate total protein concentration. For data shown, cells were lysed directly in the 10 mm dishes used for cell culture. For tissue, we have achieved optimal results with approximately 10 mg of pulverized liver tissue powder. Optimization may be required depending on tissue. The volume of lysis buffer used to number of cells or tissue weight will depend on sample matrix and may need to be further optimized. A total protein concentration that allows for 15 ml or less sample volume per well of the 96 well assay plate is optimal. Larger volumes seem to result in higher variability between technical replicates (data not shown). While results can be obtained with low glucose media (1 g/L), we found growing cells in high glucose media (4.5 g/L) is optimal in differentiating GALT activity between wild-type and CG patient fibroblasts (see **Fig. 2B**). Other lysis buffers may also be used; however, they should be non-denaturing to maintain the functional GALT enzyme and be EDTA-free, which may interfere with the assay.

3. GALT activity assay
   - 3.1. Determine volume of each sample for 10–20 µg of total protein.
   - 3.2. Add a minimum of 10 µg total protein of each sample to 2 wells of a 96-well plate with conical bottom.
   - 3.3. Add HEPES/KOH lysis buffer to 2 wells of the plate as a control. Use a volume equivalent to the volume of the samples. Note, a range of volumes may need to be used depending on experimental design and sample types.
   - 3.4. Add 50 µl of GALT Activity Assay Reaction Buffer to 1 duplicate of each sample and control.
   - 3.5. Add 50 ul of GALT Activity Assay Control Buffer to the other duplicate of each sample and control [12].
   - 3.6. Shake on a plate shaker for 1 min at room temperature for 200–400 rotations per minute.
   - 3.7. Incubate the plate at 37°C for 1.5 h.
   - 3.8. Shake the plate on a plate shaker for 1 min at room temperature for 200–400 rotations per minute.
   - 3.9. Add 100 µl of Acetone-Methanol solution to each well.
   - 3.10. Shake on plate shaker for 1 min at room temperature for 200–400 rotations per minute.
   - 3.11. Centrifuge the plate at 2010 *g* for 15 min.
   - 3.12. Add 160 µl of ddH_2O to the wells of a 96-well optical bottom plate.
   - 3.13. Transfer 70 µl of solution in each well of the 96-well conical bottom plate to the wells of the optical bottom plate containing ddH_2O.
   - 3.14. Shake on plate shaker for 1 min at room temperature for 200–400 rotations per minute.
   - 3.15. Read the plate at an excitation of 355 nm and emission of 460 nm on a plate reader.

NOTES: Ten micrograms of total protein per sample is the minimum to produce reliable results. Each sample

should be loaded onto the 96-well plate in duplicate or quadruplicate if enough sample is available. One and a half (1.5) hours was chosen for the GALT activity assay incubation at 37°C based on results from Fujimoto *et al*. 2000 [5]; this length of time appeared to be sufficient to differentiate fibroblasts from heterozygotes and fibroblasts from both normal individuals and CG patients. While longer incubation times may increase the ability to differentiate fibroblasts from healthy controls and fibroblasts for CG patients, 1.5 h is sufficient (**Fig. 3**).

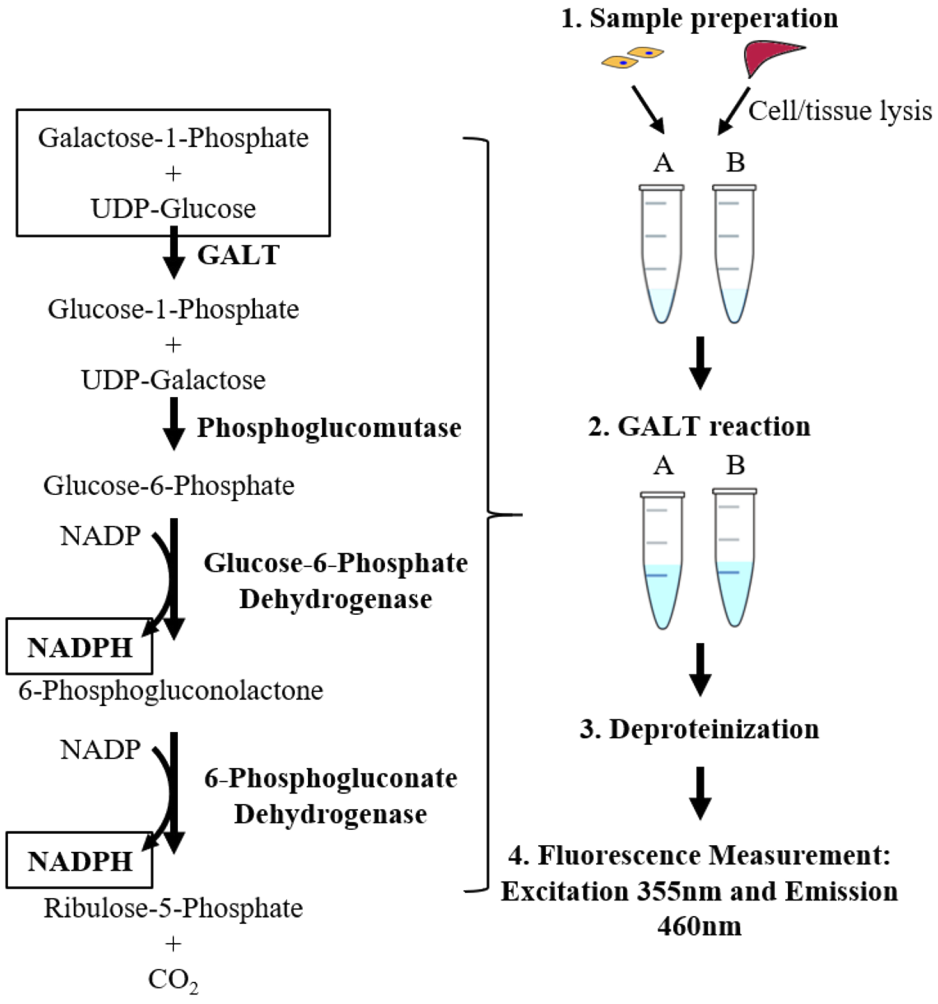

Figure 1. GALT activity assay and reaction pathway. The GALT activity assay is an indirect measure of GALT activity. It measures the conversion of NADP to NADPH in subsequent enzymatic reactions for which the GALT enzyme is the rate limiting step. In Step 1, cell or tissue lysates are prepared and loaded onto a 96 well plate in duplicate (A and B) or quadruplicate. Step 2, Reaction Buffer is added to tube A and Control buffer is added to tube **B.** The Control Buffer lacks galactose-1-phosphate, a substrate of GALT. Step 3, acetone:methanol solution is added to deproteinize the contents of each well followed by centrifugation and transfer to a optical 96 well plate. Step 4, NADH is measured.

4. Analysis of GALT activity assay data

4.1. Subtract the reading of the lysis buffer control with reaction buffer (r_c) from the reading of all samples with reaction buffer (r_{sample}) to obtain a corrected reading with reaction buffer for each sample (R_{sample}).

$$r_{sample} - r_c = R_{sample} \text{ (Eqn. 1)}$$

4.2. Subtract the reading of the lysis buffer control with control buffer (n_c) from the reading of all samples with control buffer (n_{sample}) to obtain a corrected reading with control buffer for each sample (N_{sample}).

$$n_{sample} - n_c = N_{sample} \text{ (Eqn. 2)}$$

4.3. Subtract the correcting reading with control buffer for each sample from its corresponding corrected reading with reaction buffer to obtain a Δ value for each sample.

$$R_{sample} - N_{sample} = \Delta_{sample} \quad \text{(Eqn. 3)}$$

4.4. If multiple WT cell lines or controls conditions were analyzed, average the Δ values ($\Delta_{average}$).

4.5. Divide all of the Δ values by the average WT or control Δ value to obtain a fold change for each sample (F_{sample}).

4.6.
$$\frac{\Delta_{sample}}{\Delta_{average}} = F_{sample} \quad \text{(Eqn. 4)}$$

4.7. Multiply the fold-change for each sample by 100 to obtain a percentage.

NOTES: For certain statistical analysis purposes it may be optimal to use the raw Δ_{sample} data value.

ANTICIPATED RESULTS

This manuscript describes a step-by-step protocol for determining the activity of the GALT enzyme in cells and/or tissue lysate. It relies on measuring the intrinsic fluorescence in NADPH after it is converted from NADP (**Fig. 1**). This test also utilizes phosphoglucomutase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase present endogenously in the sample. The enzyme reaction rates of these other enzymes are 7- to 20-fold higher than GALT [5]. Thus, GALT is the rate limiting enzyme in the conversion of NADP to NADPH.

The examples provided here use fibroblast cells both from normal individuals and several CG patients with a variety of mutations (**Table 1**). Different media conditions were also used to demonstrate the effect of the media on the activity levels obtained using this assay (**Table 2**). Different media conditions were also used to demonstrate the effect of the media on the activity levels obtained using this assay (**Table 3**).

The exact raw data values from the plate reader may vary depending on the machine or software used. In this case, a SpectraMax M5 with SoftMax Pro 7.0.2 software was used. Despite variations in the raw data, the percentage obtained should be similar regardless of machine or software used. If samples are plated in quadruplicate, duplicate readings from the plate reader would be averaged together. If samples are only plated in duplicate, with 1 well receiving the GALT Assay Reaction Buffer and the other receiving the GALT Assay Control Buffer, readings would not be averaged together. Different total protein concentrations and different media concentrations were assessed (**Fig. 2**). Ten micrograms of total protein was determined to be the minimum amount of total protein needed per well to obtain consistent and reproducible results. Culturing fibroblasts in high glucose media provided the most consistent and reproducible results as well. Low glucose media also provides consistent and reproducible results but with more variability than high glucose media.

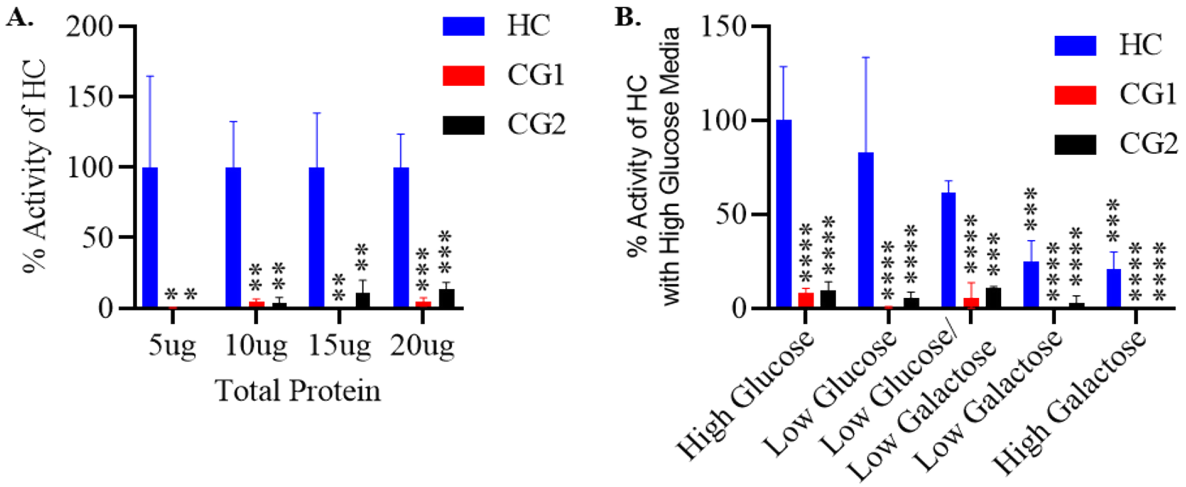

Figure 2. GALT activity with different total protein and different media conditions. A. 5, 10, 15, and 20 μg of total protein of healthy control cells (HC), CG patient cells 1 (CG1), and CG patient cells 2 (CG2) with high glucose media were assessed to determine GALT activity. *n* = 3 independent cultures. One-way ANOVA with Tukey's multiple comparisons test was used. **B.** 10 μg of total protein of HC, CG1, and CG2 in the indicated media conditions were assessed to determine GALT activity. Each condition in all both CG cells is compared to HC cells with high glucose media. For HC and CG1, *n* = 3 independent cultures tested in duplicate. For media conditions containing galactose, CG2 *n* = 2 independent cultures tested in duplicate. For high glucose and low glucose media conditions, CG2 *n* = 3 independent cultures tested in duplicate. One-way ANOVA with Tukey's multiple comparisons test was used. *P < 0.05. **P < 0.01. ***P < 0.001. ****P < 0.0001.

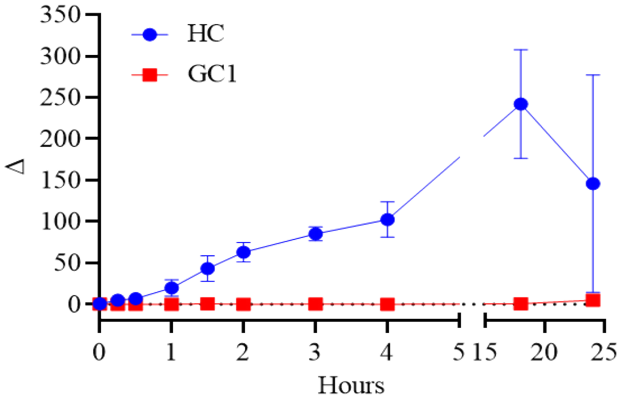

Figure 3. GALT activity time course. 10 μg of total protein from healthy control cells (HC) and CG patient cells 1 (CG1) with high glucose media were assessed at the indicated incubation times with GALT activity assay reaction buffer and GALT activity assay control buffer. The average Δ value for three independent cultures are shown for each time point (0 min, 15 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 18 h, and 24 h).

Table 2. Fibroblast cells.

	Cell line	Allele 1	Allele 2	Age	Gender
Healthy control	GM05757	WT	WT	7 yr	M
CG Patient 1	GM01703	Q188R	Q188R	9 mo	M
CG Patient 2	GM00422	Q188R	Q344K	9 yr	F

Table 3. Media conditions.

Media	Glucose (g/L)	Galactose (g/L)
High glucose	4.5	0
Low glucose	1	1
Low glucose/Low galactose	1	1
Low glucose	0	1
High glucose	0	4.5

TROUBLESHOOTING

Possible problems and their troubleshooting solutions are listed in **Table 4**.

Table 4. Troubleshooting.

Step	Problems	Causes	Suggestions
2.1	Low sample protein concentration	• Insufficient cells lysed • Large volume used to lyse cells • Insufficient collection of lysed sample	• Increase the number of cells used • Decrease the volume of lysis buffer used • Ensure sufficient coverage of lysis buffer (spread across plate with cell scraper) and sufficient scraping • Ensure all PBS is aspirated from the plate prior to adding lysis buffer
3.3	**Δ** values for WT samples are very low or negative	• Poor sample quality • Volume of sample used exceeds 15 ul • GALT Activity Assay buffer quality is poor • Media conditions • Lysis buffer is not suitable	• Use fresh samples or samples that have limited (< 2) freeze thaw cycles • Adjust plate size, cell density, or lysis buffer volume to increase total protein concentration • A reagent within the buffer may have degraded, such as NAD • Ensure that media conditions are optimal • Ensure that a non-denaturing lysis buffer lacking EDTA is used

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