Legacy Biomarker Qualification Project Status Update[1]

Administrative Information

Requesting Organization

Name: Critical Path Institute Predictive Safety Testing Consortium

Address: 1730 E. River Rd., Suite 200 / Tucson, AZ 85718

Phone: 520-547-3440

Email:

Website: www.c-path.org

Primary Contact

Name: John-Michael Sauer

Address: 1730 E. River Rd., Suite 200 / Tucson, AZ 85718

Phone: 520-382-1396

Email: jsauer@c-path.org

Alternate Contact

Name: Nicholas King

Address: 1730 E. River Rd., Suite 200 / Tucson, AZ 85718

Phone: 520-777-2881

Email: nking@c-path.org

Submission Date (MM/DD/YYYY): 05/22/2018

[1] The content you provide in this completed Status Update will be publicly posted as part of the section 507 transparency provisions.

Table of Contents

I. Context of Use

A. Biomarker Category

Safety

B. Intended Use in Drug Development

General Area: Clinical drug safety biomarker

Tissue injury of interest: Liver

Target Population for Use: Normal healthy volunteers and patients with increases in alanine aminotransferase (ALT) from suspected extrahepatic sources such as muscle.

Stage of Drug Development for Use: First-in-Human and other Phase I Single Ascending Dose or Multiple Ascending Doses studies, as well as Phase II and III studies in patients.

Conditions for Qualified Use:

- A change of 2.5x and 5x upper limit of normal (ULN) for glutamate dehydrogenase (GLDH) activity levels corresponds to 3x and 5x ULN ALT. These fold changes of GLDH can be utilized along with the standard hepatic injury monitoring panel for the assessment of drug-induced liver injury (DILI), in the same manner as 3x and 5x ULN ALT. Although, like ALT, the absolute values of the ULN for GLDH will vary from region to region, the 2.5x and 5x fold change from ULN should not change.
- GLDH activity can be used to ensure patient liver safety in drug development studies in which unexplained elevations of ALT are evidenced, and/or in subjects with known muscle impairment (e.g., due to strenuous exercise or muscular dystrophy). If GLDH activity increases to 2.5x ULN or 5x ULN, the same precautions should be taken as if 3x or 5x ULN ALT increases are observed as described in the FDA Guidance for Industry Drug-Induced Liver Injury: Premarketing Clinical Evaluation (2009).
- If GLDH is not elevated in the previously defined subjects, liver can be ruled out as a target tissue of toxicity and diagnosis of alternative sources of ALT increases should be sought, (i.e., via biomarkers of muscle injury such as creatinine kinase activity).

C. Context of Use Statement

Serum enzymatic activity of glutamate dehydrogenase (GLDH) is a safety biomarker for monitoring specifically for hepatocellular injury in healthy subjects and patients in conjunction with a panel of standard DILI biomarkers in all stages of drug development trials.

II. Drug Development Need

Drug-induced liver injury (DILI) remains the single greatest cause for termination of development of drug candidates and withdrawal of approved drugs from the market (Yuan and Kaplowitz, 2013; Kaplowitz, 2005). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum activity measurements are used as gold standard biomarkers for the identification of liver injury in clinical practice, and ALT is commonly used to assess risk of liver injury during drug development. However, increases in ALT do not always signal hepatocellular injury that will progress to severe DILI. Hy's Law and a recent definition by an international DILI Expert Working Group (EWG) provide guidance to help differentiate mild or transient hepatocellular injury from more severe DILI and functional hepatocellular damage (Temple, 2006; FDA, 2009; Aithal et al., 2011). Three-fold and five-fold ALT elevations from the upper limit of normal (ULN) are commonly used thresholds of concern in clinical trials, triggering confirmatory testing and close observation of an individual as suggested in the Food and Drug Administration (FDA) Guidance for Industry: Drug-Induced Liver Injury: Premarketing Clinical Evaluation (FDA, 2009). Despite these useful algorithms, unexplained elevations in ALT continue to slow or halt clinical trials, and ALT is insufficient to aid in the diagnosis of DILI in multiple scenarios.

One reason for high ALT and AST in the absence of liver injury is that these enzymes are also found in tissues other than the liver, namely in muscle. This severely limits the utility of ALT/AST as specific markers of liver damage in subjects with underlying muscle impairments, such as those with Duchenne muscular dystrophy (DMD) or other neuromuscular disease, those engaging in strenuous exercise, or subjects with simultaneous drug-induced liver and muscle injury, and creates a diagnostic challenge for clinicians. Therefore, the development of additional biomarkers of DILI is essential.

III. Biomarker Information

A. Biomarker Name, Source, Type and Description

Glutamate dehydrogenase (GLDH)

	Type of Biomarker (Check relevant type(s))		
X	Molecular		Radiologic/Imaging
	Histologic		Physiologic Characteristic
	Other (please describe):		

B. For molecular biomarkers, please provide a unique ID.

Scheme: UniPRot

ID: UniPRot ID P00367

Matrix: serum

C. Rationale for Biomarker

Glutamate dehydrogenase (GLDH), is a mitochondrial enzyme that plays a role in amino acid oxidation and urea production. GLDH is primarily found in the liver and to a lesser degree in the kidney, with only a trace amount in skeletal muscle (Mastorodemos et al., 2005). In humans, serum GLDH activity is elevated in patients with hepatic ischemia (Kretzschmar et al., 2003), progressively increasing with increased severity of disease (Schmidt and Schmidt, 1988). Furthermore, serum GLDH activity has recently been shown to be a sensitive marker of a mild hepatocyte necrosis in patients treated with heparin (Harrill et al., 2012). Increases in GLDH within 8 hours of acetaminophen (APAP) overdose predicted patients that proceeded to acute liver injury (Antoine et al., 2013).

As part of the Critical Path Institute (C-Path) Predictive Safety Testing Consortium's (PSTC) ongoing efforts to augment translational biomarker tools for DILI, the Hepatotoxicity Working Group (HWG) is proposing to qualify GLDH activity as a marker of liver injury in human subjects with ALT elevations from suspected extrahepatic sources such as muscle, ie. as a biomarker to confer tissue specificity to the liver. **GLDH activity is proposed to be utilized as a complement to the existing guidance and standard methods for assessing DILI.**

V. Biomarker Measurement Information

A. General Description of Biomarker Measurement

GLDH can be reliably measured in serum. The Randox GLDH assay utilizes the conversion of α-oxoglutarate to glutamate for detection of GLDH enzymatic activity as recommended by the Deutsche Gesellschaft für Klinische Chemie (DGKC).

$$\alpha - \text{oxoglutarate} + \text{NADH} + \text{NH}_4^+ \xrightarrow{\text{GLDH}} \text{glutamate} + \text{NAD}^+ + \text{H}_2\text{O}$$

In this reaction, the kinetics of nicotinamide adenine dinucleotide (NADH) oxidization are proportional to the GLDH activity and measured spectrophotometrically as a decrease in absorbance per minute at 340 nm.

The Randox GLDH assay kit was manufactured in the United Kingdom (UK) with ISO13485 certification as evidence of Good Manufacturing Practice (GMP). The assay is an approved *in vitro* diagnostics (IVD) assay in Europe, Health Canada, and China. The UK manufacturing facility was FDA inspected in 2012 and 2013.

B. Test/Assay Information

Indicate whether the biomarker test/assay is one or more of the following:

i. Laboratory Developed Test (LDT)

☒ Yes ☐ No

ii. Research Use Only (RUO)

☒ Yes ☐ No

iii. FDA Cleared/Approved.

☐ Yes ☒ No

If yes, provide 510(k)/PMA #: _____

iv. If the biomarker is qualified, will the test/assay be performed in a Clinical Laboratory Improvement Amendments (CLIA)–certified laboratory?

☒ Yes ☐ No

v. Is the biomarker test currently under review by the Center for Devices and Radiological Health or the Center for Biologics Evaluation and Research?

☐ Yes ☒ No

vi. Is there a standard operating procedure (SOP) for sample collection and storage?

☒ Yes ☐ No

vii. Is there a laboratory SOP for the test/assay methodology?

☒ Yes ☐ No

C. Biomarker Measurement

A complete validation report is available in Appendix II. During the course of this qualification project, two separate analyzers were utilized at one site (Siemens Advia 1800 and Advia 2400 Chemistry Analyzers). The current assay is being run on the Siemens Adva 1800 Chemistry Analyzer and assay validation parameters have been established using this instrument. Assay validation data using the Advia 2400 Chemistry Analyzer has been included to bridge the performance characteristics of both analyzers. Assay validation included measurement of the following parameters: precision, linearity, recovery, sample freeze/thaw stability, sample stability, reference interval, and interference. A short description of each validation acceptance criteria is listed in the following paragraphs and in Table 1.

Precision: The GLDH human serum assay demonstrated appropriate precision, as per the analyzed human serum samples and quality control material (<10% coefficient of variation (CV)). However, at the low end of the linear range, there were several points that did not meet the predefined acceptance criteria. Higher CV values obtained at low concentrations of GLDH (12.96%) and at Limit of Detection (LOD) (37.65%) are anticipated since CV is impacted, more substantially, by changes in small values. Close examination of the individual data points showed small differences. In both cases, standard deviation was less than 1.00 demonstrating that the variation is small and that assay precision is acceptable.

Linearity: The GLDH human serum assay demonstrated acceptable linearity. Samples had appropriate percent recovery values down to 3 U/L on the Siemens Advia 1800 Chemistry Analyzer and assay linearity has been established from 3 to 500 U/L on this instrument. In a previous validation of the GLDH assay on the Siemens Advia 2400 Chemistry Analyzer, assay linearity was preliminarily determined to be 1 to 200 U/L. The reference range of 1 to 10 U/L for GLDH was therefore reported in Schomaker *et al* (2013) based on the previous validation. As described above, the current assay on the Siemens Advia

1800 Chemistry Analyzer has an established assay linearity of 3 to 500 U/L, thereby resulting in a reference range for GLDH in human serum of <3 to 10 U/L.

Recovery: The GLDH human serum assay demonstrated acceptable recovery. All samples had appropriate percent recovery values (80 - 120%) based upon the results of this testing and the acceptance criteria.

Reference Interval: Historical data for GLDH was generated on the Siemens Advia 2400 Chemistry Analyzer using 552 human samples and a reference interval was generated from 274 males and 278 females. Based on these data, a reference interval for GLDH in human serum of 1 to 10 U/L was initially established. However, following the more stringent assessment of linearity (requirement 4, as described in Appendix II) in the validation of GLDH on the Siemens Advia 1800 Chemistry Analyzer, the reference range interval for GLDH in human serum was updated to <3 to 10 U/L.

Sample Freeze/Thaw Stability: All samples had appropriate percent recovery (80 - 120% of initial thaw). Based upon the results of this testing and the acceptance criteria, GLDH in human serum demonstrates acceptable stability for 4 freeze thaw cycles.

Sample Stability: Most samples had appropriate percent recovery (80 – 120%). Based upon the results of this testing and the acceptance criteria, GLDH in human serum demonstrates acceptable stability at room temperature up to 48 hours, refrigerated up to 14 days, and frozen at -80°C up to 18 months.

Interference: All samples met the acceptance criteria with the exception of lipemia. At high concentrations of triglycerides there is interference with GLDH testing. This agrees with the manufacturer's reagent package insert. Any sample positive for high triglycerides was omitted from the analysis and had no impact on the results. Hemolysis (hemoglobin) and icterus (bilirubin) did not interfere with the measurement of GLDH.

Table 1. Summary of GLDH assay performance characteristics for human serum

Platform	Siemens Advia 1800 Chemistry Analyzer
Assay Vendor	Randox
Detection	Kinetic Enzymatic Assay (NADH 340 nm)
Units	U/L
	Mean ± SD (%CV)
Precision – LOD	2 ± 0.89 (37.7) U/L
Precision – Low	7 ± 0.89 (13.0) U/L
Precision – Quality Control (QC) Level 2	16 ± 1.36 (8.0) U/L
Precision – QC Level 3	29 ± 1.36 (4.7) U/L
Precision – Mid	53 ± 2.43 (4.6) U/L
Precision – Conc. Calibrator 3	59 ± 3.27 (5.6) U/L
Precision – High	623 ± 15.37 (2.5) U/L
Lower Limit of Quantification (LLOQ)	3 U/L
Upper Limit of Quantification (ULOQ)	500 U/L
Upper reportable limit	125,000 U/L
Recovery range	85 –118%
Reference interval	<3 – 10 U/L
Dilutional range	1:250
Interferences – No Effect	Hemolysis (Hemoglobin) Icterus (Bilirubin)
Interferences – False Negative	Lipemia (Triglyceride)
Interferences – False Positive	None Determined

D. Additional Considerations for Radiographic Biomarkers

Not applicable

VI. Assessment of Benefits and Risks

A. Anticipated Benefits

Elevated serum enzymatic activity of GLDH is a specific measure of hepatocellular injury and can be used in healthy subjects and patients as an adjunct to ALT in all stages of drug development trials. When ALT increases are observed from suspected extrahepatic sources such as muscle, GLDH can lend weight of evidence to confirm or rule out hepatocellular injury. The specificity of GLDH will allow clinical drug developers to better define liver injury in both normal subjects and patients with disease that confound the ability of ALT to accurately identify hepatoxicity.

B. Anticipated Risks and Risk Mitigation Strategy

Potential risks for using GLDH include false positive or false negative for detecting drug-induced liver injury. As GLDH will be used to indicate that increases in ALT are hepatic or extrahepatic in origin, a false negative result would have potential liability. However, since GLDH will be used in conjunction with the current standard panel of DILI biomarkers, there is a low risk for misdiagnosing patients. Potential false negatives due to GLDH will be addressed by utilizing the Expert Working Group's (EWG) definition of liver injury. The EWG definition of hepatotoxicity is met if one of the following statements is true: ≥3 times the upper limit of normal (ULN) for ALT and ≥2 times the ULN for total bilirubin (Tbil), OR ≥5 times the ULN for ALT, OR ≥2 times the ULN for alkaline phosphatase (ALP).

When GLDH is used, in place of ALT, the EWG definition of hepatotoxicity is met if one of the following statements is true: ≥2.5 times the upper limit of normal (ULN) for GLDH and ≥2 times the ULN for Tbil, OR ≥5 times the ULN for GLDH, OR ≥2 times the ULN for ALP. Therefore, if GLDH does not change, a significant change in ALP would indicate hepatotoxicity. Therefore, the risk of missing DILI is mitigated using several indicators of hepatotoxicity.

C. Conclusions

The data summarized herein strongly support our proposed context of use and the utility of GLDH as a complement to the existing standard methods for assessing potential DILI in clinical trials. The proposed confirmatory studies should yield evidence sufficient to qualify GLDH as a sensitive and specific measure of hepatocellular injury that provides valuable information when extrahepatic sources of ALT are suspected or may be anticipated due to underlying conditions.

To provide further confirmation of how GLDH should be interpreted in the context of monitoring for DILI, we propose the following targeted assessments be conducted: confirmation of the linear relationship

of ALT and GLDH; confirmation that GLDH does not increase with muscle injury; confirmation of the specificity of GLDH for liver injury in humans; and further characterization of the elimination kinetics of GLDH with respect to ALT in humans.

For this qualification project, GLDH serum activity has already been measured in several studies and across multiple populations. The reference range for GLDH has been established in healthy subjects in which the effect of age, gender, and ethnicity and inter- and intra-subject variability were evaluated. GLDH has been established as a marker of hepatotoxicity that strongly correlates with ALT in both humans and rats. The GLDH serum activity cutoffs of concern have been established in a population with a variety of liver diseases, which correspond to 3x and 5x ALT increases,. We have also assessed the capability of GLDH to differentiate liver and muscle injury in human subjects both with DMD and a variety of myopathies and confirmed this in rats.

VII. Evaluation of Existing Biomarker Information: Summaries

A. Pre-Clinical Information, as appropriate

PSTC's Hepatotoxicity Working Group members conducted studies in rodents demonstrating GLDH's sensitivity and specificity as a biomarker of hepatotoxicity. Additionally, studies have demonstrated that GLDH is a sensitive liver specific biomarker capable of differentiating liver and other organ injuries in rats. Twenty-seven different toxicants were evaluated that injured liver, heart, skeletal muscle, and the pancreas. A common histopathology lexicon was developed to assess liver injury and applied across all studies to define if a toxicity occurred in an individual animal.

B. Completed Clinical Information, as appropriate

The completed clinical studies are described in Table 2 below. GLDH serum activity was measured in several populations with the following goals:

1. Establish a reference range for GLDH in healthy subjects; evaluate the effect of age, gender, and ethnicity; and characterize inter- and intra-subject variability (Study Numbers 1, 2, and 3).
2. Establish GLDH as marker of hepatotoxicity that strongly correlates with ALT.
    a. Human studies examining a diverse and large population with hepatic injury (Study Number 4).
    b. Rat studies utilizing histopathology as a gold standard (Study Numbers 8 and 9).
3. Establish GLDH serum activity cutoffs of concern that correspond to 3x and 5x ALT increases in a population with DILI and populations with a variety of liver diseases (Study Number 4).

4. Assess the capability of GLDH to differentiate liver and muscle injury in human subjects with DMD and a variety of myopathies (Studies Number 5, 6, and 7).

C. Summary of Ongoing Information Collection/Analysis Efforts

Four additional studies will (1) confirm the relationship between ALT and GLDH; (2) confirm that GLDH does not increase with muscle injury in humans; (3) confirm the specificity of GLDH for liver injury in humans; and (4) characterize the elimination kinetics of GLDH and ALT in humans. Additionally, the specificity of serum GLDH for detection of liver injury will be further assessed by evaluating the performance of GLDH following drug-induced hepatocellular injury in a genetic mouse model of muscular dystrophy and a rat model of pancreatic injury, for a total of five studies. It is expected that all samples for these studies will be collected by year end 2018 and analysis will be completed in early 2019. Summaries of these studies are shown below.

1) Confirmation of the relationship of ALT and GLDH in humans / confirmation of the performance of serum glutamate dehydrogenase (GLDH) as biomarker of liver injury in humans

The goal of this study is to confirm the performance of GLDH as a biomarker of liver injury, as well as to confirm the performance of GLDH thresholds of concern to identify subjects with liver injuries. In this study, the serum levels of GLDH and ALT will be evaluated in healthy subjects and subjects with liver injury featuring a wide variety of etiologies. Approximately 200 samples from heathy subjects and 200 samples from subjects with liver injury, including subjects with acetaminophen overdose and two additional investigational drugs, will be collected for the study. The ability of GLDH to substitute for ALT in the determination of liver injury will be confirmed across the entire data set, as well as individually for each liver injury etiology.

2) Confirmation that GLDH does not increase with muscle injury in humans / confirmation that serum glutamate dehydrogenase (GLDH) is unaffected by muscle injury in humans

The goals of this study are to confirm serum GLDH levels are unaffected by muscle injury in humans, and that GLDH outperforms ALT with regard to specificity for liver injury. The study will compare levels of GLDH and ALT in healthy subjects and subjects with muscle impairments featuring a wide variety of etiologies. Serum samples from approximately 120 subjects with muscle injury will be selected. Muscle injury will be defined by creatine kinase (CK) levels greater than two times normal healthy levels or based on a diagnosed muscle injury. The serum concentration of GLDH from subjects with muscle injury will be compared to healthy volunteer samples to confirm that muscle injury does not affect serum GLDH levels, and thereby does not interfere with the ability of GLDH to detect liver injury.

3) Confirmation of the specificity of GLDH for liver injury in humans / confirmation that serum glutamate dehydrogenase (GLDH) is unaffected by pancreatic, gastrointestinal and kidney injuries in humans

The goal of this study is to confirm that GLDH does not increase with pancreatic, gastrointestinal, or kidney injury. The study will compare GLDH levels from subjects with pancreatic, gastrointestinal, or kidney injury to healthy volunteers. Approximately 200 serum samples from subjects with pancreatic, gastrointestinal, and kidney injury will be collected for this study. The serum concentration of GLDH from subjects with pancreatic, gastrointestinal, or kidney injury will be compared to healthy volunteer samples to confirm that these injuries do not affect serum GLDH levels, and thereby do not interfere with the ability of GLDH to detect liver injury.

4) Further characterization of the elimination kinetics of GLDH and ALT in humans / confirmation that serum glutamate dehydrogenase (GLDH) detects the onset of liver injury by acetaminophen

The goal of this study is to confirm that GLDH detects liver injury caused by acetaminophen (APAP) overdose and that the half-life of GLDH, which is shorter than the half-life of ALT in human serum, reflects the time course of hepatocellular injury. In this study, the time course of GLDH activity in human serum will be compared with ALT activity and the medically adjudicated clinical outcome of APAP poisoning. Approximately 15 cases of APAP overdose will be evaluated.

5) Additional nonclinical support for the use of GLDH to detect liver injury during concurrent muscle or pancreatic injury

The specificity of serum GLDH for detection of liver injury will be further assessed by evaluating the performance of GLDH following drug-induced hepatocellular injury in a genetic mouse model of muscular dystrophy. This animal model of DILI on a background of increased ALT due to muscle injury/disease, mimics the proposed COU for GLDH use. Liver and muscle histopathology and serum chemistry analysis including ALT, AST, CK, ALP, Tbil, and GLDH will be completed to demonstrate that, in contrast to serum ALT, serum GLDH is a liver-specific biomarker of liver injury and is not affected by muscle damage.

The potential interference of underlying pancreatic injury on GLDH performance to detect liver injury will be assessed in a rat model of pancreatic injury. The objective of this study will be to investigate the effects of a single dose of a model exocrine pancreas toxicant, cyanohydroxybutene (CHB), on levels of amylase, lipase, ALT, ALP, Tbil, and GLDH. CHB is known to cause selective damage to the exocrine pancreas, possibly through alteration of glutathione metabolism, resulting in acinar cell apoptosis and

atrophy (Maher et al. 1991). Male Wistar-Han rats will be administered a single subcutaneous injection of CHB and a time course of the response of the biomarkers will be evaluated and compared to histopathology.

Table 2. List of Studies

Briefing Package Section	Study number	Study	Description	Objective(s)
Reference range	1	Pfizer healthy volunteers; Pfizer Clinical Research Unit (CRU)	Analysis of serum from healthy volunteers meeting recruitment criteria for Phase I trials (n = 186)	Establish GLDH reference range for healthy subjects and evaluate influence of sex, ethnicity, and age.
	2	UM healthy subjects; University of Michigan health care system (UM)	Analysis of serum from healthy subjects with normal liver function (n = 364).	Establish GLDH reference range for healthy subjects and evaluate influence of sex, ethnicity, and age.
	3	PSTC healthy volunteers; Jasper Clinic, Kalamazoo, MI	Analysis of serum from healthy volunteers meeting recruitment criteria for PSTC biomarker study (n = 81)	Establish GLDH reference range and evaluate influence of sex, ethnicity, and age, as well as intra- and inter-subject variability.
Correlation of GLDH and ALT	4	Hepatic injury subjects; University of Michigan health care system	Serum samples from subjects with AST and ALT > 2xULN and with diagnosed disease or injury resulting in increased liver enzymes (total n = 479). All healthy subjects used in reference range study were included in analysis.	1) Confirm correlation between GLDH and ALT. 2) Establish specificity and sensitivity of GLDH for liver injury using Expert Working Group definition (Aithal et al., 2011): ≥ 5x ALT or ≥ 2x ALP or [≥ 3x ALT and ≥ 2x TBil]. 3) Plot ALT and GLDH values in APAP overdose patients over a time course to observe kinetics of biomarker change. 4) Establish GLDH threshold values for liver injury through linear regression model with log GLDH and log ALT, then calculate GLDH levels that correspond to 3x and 5xULN ALT.
Specificity of GLDH for liver	5	Muscle injury subjects; University of Michigan health care system	Serum samples from (a) healthy subjects (n = 125; 3-64 years of age, and (b) subjects with muscle injury (n = 131; 2-78 years of age).	1) Examine specificity of GLDH for liver injury in human subjects with muscle impairment. 2) Determine if GLDH can detect liver injury onset in rhabdomyolysis patients (case study).
	6	University of Florida DMD	Serum samples from DMD patients (n=40; 5-14 years of age).	Determine GLDH specificity for liver and characterize levels in DMD subjects.
	7	Exercise study (Thulin et al., 2014)	Observational study in subjects (n=12) participating in extreme adventure race. Samples taken pre- and post-race.	Determine whether GLDH changes with exercise.
Correlation of ALT and GLDH	8	Rat analysis data set	Histopathology and biomarker data for rat toxicology studies with multiple toxicants (n=30), including those targeting liver, kidney, heart, and pancreas.	Confirm correlation of ALT and GLDH for hepatocellular injury, and specificity of GLDH for hepatocellular injury when other organ toxicities present.

Specificity of GLDH for liver	9	Rat study with liver and skeletal muscle toxicants	Histopathology and biomarker data (ALT, AST, and GLDH) collected from rats treated with acetaminophen (APAP) and of 2,3,5,6-Tetramethyl-p-phenylenediamine (TMPD)	Examine specificity of GLDH for liver injury.

VIII. Knowledge Gaps in Biomarker Development

A. List and describe any knowledge gaps, including any assumptions, that exist in the application of the biomarker for the proposed COU

The studies described in Section VI.C. address any remaining gaps pertaining to the proposed context of use. Specifically, we will address the gaps in measuring the elimination kinetics of GLDH and confirming the specificity of GLDH to liver injury and not pancreatic, kidney, nor gastrointestinal injury. The following studies will address these gaps:

- Confirmation that serum glutamate dehydrogenase (GLDH) is unaffected by pancreatic, gastrointestinal and kidney injuries in humans
- Confirmation that serum glutamate dehydrogenase (GLDH) detects the onset of liver injury by acetaminophen

B. List and describe the approach/tools you propose to use to fill in the above-named gaps when evidence is unknown or uncertain, (i.e., statistical measures and models, meta-analysis from other clinical trials).

The studies described in Section VI.C. address the remaining gaps pertaining to the proposed context of use. The studies are listed below with the planned statistical analysis.

- To confirm the performance of serum glutamate dehydrogenase (GLDH) as biomarker of liver injury in humans, the following analyses will be used:
    - Primary Analysis
        - Construct 2x2 contingency tables of the EWG definition with ALT compared to the EWG definition with GLDH. Compute measures of concordance, sensitivity, and specificity of the GLDH-based EWG definition of liver injury, defined in Section 2, to predict ALT-based EWG definition of liver injury, using proposed GLDH thresholds and computed ALT thresholds determined in the exploratory studies. The target success for each measure is ≥ 0.90, 95% Lower Confidence Bound ≥ 0.85.
    - Additional Analysis: Assess the performance of GLDH thresholds of concern across each disease state and drug treatment
        - The same analysis as described above will be conducted for each individual disease state and drug treatment. The target success for each measure is ≥ 0.90.

- To confirm that serum glutamate dehydrogenase (GLDH) is unaffected by muscle injury in humans, the following analyses will be used:
    - Primary Analysis
        - The primary endpoint is the false positive rate (FPR), i.e., the % of subjects exceeding a prespecified threshold, who do not have liver toxicity. In the first analysis, the percentage of subjects with GLDH values exceeding ULN (10), 2.5X ULN (25) and 5X ULN (50) will be computed. The target success criteria are:
            - ULN: FPR $\leq$ 10%
            - 2.5X ULN: FPR $\leq$ 5%
            - 5X ULN: FPR $\leq$ 1%
        - These target percentages reflect a balance between how GLDH will be utilized in drug development trials, and a recognition that (1) there will be sampling variability (although we anticipate the % of subjects < ULN to be around 2.5% by definition, the observed percentage from a given sample will vary, especially with a limited sample size); and (2) even with the robust inclusion/exclusion criteria, subjects with comorbidities that impact GLDH values may be enrolled into this study.
    - Secondary Analysis
        - In the second analysis, subjects will be classified as to whether they exceed the ULN for GLDH and whether they exceed the ULN for ALT. McNemar's Test for Correlated Proportions will be used to test for a difference in the proportions. The target success criterion is:
            - [% of subjects > ULN GLDH] significantly ($p<0.05$) lower than [% of subjects > ALT GLDH]
            - Note that because both GLDH and ALT are measured for each subject, McNemar's Test is more appropriate than the usual tests (e.g., Chi-Squared Test) to compare two proportions, which assume independence of the samples.
    - Additional Analysis
        - Qualitative Analysis: Scatterplots of GLDH vs. log(CK), and log(ALT) vs log(CK), will be produced. The Pearson correlation coefficient between GLDH and log(CK), and between log(ALT) and log(CK), will be computed and compared.

- To confirm that serum glutamate dehydrogenase (GLDH) is unaffected by pancreatic, gastrointestinal and kidney injuries in humans, the following analysis will be used:
    - Primary Analysis
        - The primary endpoint is the false positive rate (FPR). More specifically, the percentage of subjects with GLDH values exceeding 2.5X ULN (25) and 5X ULN (50) will be computed. The target success criteria are:
            - 2.5X ULN: FPR ≤ 5%
            - 5.X ULN: FPR ≤ 1%
- Confirmation that serum glutamate dehydrogenase (GLDH) detects the onset of liver injury by acetaminophen will have the following primary analysis:
    - Primary Analysis
        - Compare each subject's GLDH level at admission to the hospital to the ULN.
        - Target success criteria: all subjects have GLDH greater than ULN.

C. Describe the status of other work currently underway and planned for the future toward qualification of this biomarker for the proposed context of use.

The studies described in Section VI.C. address any remaining gaps pertaining to the proposed context of use. The proposed studies are the following:

1) Confirmation of the relationship of ALT and GLDH in humans / confirmation of the performance of serum glutamate dehydrogenase (GLDH) as biomarker of liver injury in humans
2) Confirmation that GLDH does not increase with muscle injury in humans / confirmation that serum glutamate dehydrogenase (GLDH) is unaffected by muscle injury in humans
3) Confirmation of the specificity of GLDH for liver injury in humans / confirmation that serum glutamate dehydrogenase (GLDH) is unaffected by pancreatic, gastrointestinal and kidney injuries in humans
4) Further characterization of the elimination kinetics of GLDH and ALT in humans / confirmation that serum glutamate dehydrogenase (GLDH) detects the onset of liver injury by acetaminophen
5) Additional nonclinical support for the use of GLDH to detect liver injury during concurrent muscle or pancreatic injury

IX. Attachments

See the following Appendices

*Optional information will not be posted publicly.

X. APPENDIX I. References

Aithal GP, Watkins PB, Andrade RJ, Larrey D, Molokhia M, Takikawa H, Hunt CM, Wilke RA, Avigan M, Kaplowitz N, Bjornsson E, Daly AK. Case Definition and Phenotype Standardization in Drug-Induced Liver Injury. Clinical Pharmacology and Therapeutics. 2011 Jun;89(6):806–15.

Antoine DJ, Dear JW, Lewis PS, Platt V, Coyle J, Masson M, Thanacoody RH, Gray AJ, Webb DJ, Moggs JG, Bateman DN, Goldring CE, Park BK. Mechanistic Biomarkers Provide Early and Sensitive Detection of Acetaminophen-Induced Acute Liver Injury at First Presentation to Hospital. Hepatology. 2013 Aug 1;58(2):777–87.

Begum T, Oliver MR, Kornberg AJ, Dennett X. Elevated Aminotransferase as a Presenting Finding in a Patient with Occult Muscle Disease. Journal of Paediatrics and Child Health. 2000 Apr;36(2):189–90.

Braakman, I., J. Keij, M. J. Hardonk, D. K. Meijer, and G. M. Groothuis. 1991. “Separation of Periportal and Perivenous Rat Hepatocytes by Fluorescence-Activated Cell Sorting: Confirmation with Colloidal Gold as an Exogenous Marker.” Hepatology (Baltimore, Md.) 13 (1):73–82.

Coco TJ, Klasner AE. Drug-Induced Rhabdomyolysis. Current Opinion in Pediatrics. 2004 Apr;16(2):206–10.

Cornell University College of Veterinary Medicine. 2013. “GLDH.” EClinpath. 2013. http://www.eclinpath.com/chemistry/liver/liver-injury/glutamate-dehydrogenase/.

Edge K, Chinoy H, Cooper RG. Serum Alanine Aminotransferase Elevations Correlate with Serum Creatine Phosphokinase Levels in Myositis. Rheumatology. 2006 Apr 1;45(4):487–8.

FDA. Guidance for Industry. Drug-Induced Liver Injury: Premarketing Clinical Evaluation. U.S. Food and Drug Administration. 2009. Available at: https://www.fda.gov/downloads/Guidances/UCM174090.pdf. Accessed on 1/31/18.

FNIH and FDA. FNIH Biomarkers Consortium Evidentiary Standards Writing Group. Framework for Defining Evidentiary Criteria for Biomarker Qualification. 2016. Available at: http://fnih.org/sites/default/files/final/pdf/Evidentiary%20Criteria%20Framework%20Final%20Version%20Oct%2020%202016.pdf Accessed on 12/2/17.

Fedoravicius A, Charlton M. Abnormal Liver Tests after Liver Transplantation. Clinical Liver Disease. 2016 Apr 1;7(4):73–9.

Giannini EG, Testa R, Savarino V. Liver Enzyme Alteration: A Guide for Clinicians. CMAJ: Canadian Medical Association journal = Journal de l'Association Medicale Canadienne. 2005 Feb 1;172(3):367–79.

Giffen PS, Pick CR, Price MA, Williams A, York MJ. Alpha-Glutathione S-Transferase in the Assessment of Hepatotoxicity—Its Diagnostic Utility in Comparison with Other Recognized Markers in the Wistar Han Rat. Toxicologic Pathology. 2002 Apr 1;30(3):365–72.

Harrill AH, Roach J, Fier I, Eaddy JS, Kurtz CL, Antoine DJ, Spencer DM, Kishimoto TK, Pisetsky DS, Park BK, Watkins PB. The Effects of Heparins on the Liver: Application of Mechanistic Serum Biomarkers in a Randomized Study in Healthy Volunteers. Clinical Pharmacology and Therapeutics. 2012 Aug 1;92(2):214–20.

Hickman PE, Potter JM, Pesce AJ. Clinical Chemistry and Post-Liver-Transplant Monitoring. Clinical Chemistry. 1997 Aug 1;43(8):1546–54.

Jackson ER, Kilroy C, Joslin DL, Schomaker SJ, Pruimboom-Brees I, Amacher DE. The Early Effects of Short-Term Dexamethasone Administration on Hepatic and Serum Alanine Aminotransferase in the Rat. Drug and Chemical Toxicology. 2008;31(4):427–45.

Jaeschke H, McGill MR. Serum Glutamate Dehydrogenase—Biomarker for Liver Cell Death or Mitochondrial Dysfunction? Toxicological Sciences. 2013 Jul 1;134(1):221–2.

Kaplowitz N. Idiosyncratic Drug Hepatotoxicity. Nature Reviews Drug Discovery. 2005 Jun;4(6):489–99.

Kasarala, George, and Hans L. Tillmann. 2016. Standard Liver Tests. Clinical Liver Disease 8 (1):13–18. https://doi.org/10.1002/cld.562.

Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer HC. Serum Activity of Alanine Aminotransferase (ALT) as an Indicator of Health and Disease. Hepatology. 2008 Apr 1;47(4):1363–70.

Kretzschmar M, Krüger A, Schirrmeister W. Hepatic Ischemia-Reperfusion Syndrome after Partial Liver Resection (LR): Hepatic Venous Oxygen Saturation, Enzyme Pattern, Reduced and Oxidized Glutathione, Procalcitonin and Interleukin-6. Experimental and Toxicologic Pathology: Official Journal of the Gesellschaft Für Toxikologische Pathologie. 2003 Jun;54(5–6):423–31.

Lackman, G.M. Clinical Biochemistry, Vol 29 No. 6 pp 599-602, 1996

Lumeij JT, Westerhof I. Blood Chemistry for the Diagnosis of Hepatobiliary Disease in Birds. A Review. The Veterinary Quarterly. 1987 Jul;9(3):255–61.

Luo L, Schomaker S, Houle C, Aubrecht J, Colangelo JL. Evaluation of Serum Bile Acid Profiles as Biomarkers of Liver Injury in Rodents. Toxicological Sciences: An Official Journal of the Society of Toxicology. 2014 Jan 1;137(1):12–25.

Mastorodemos V, Zaganas I, Spanaki C, Bessa M, Plaitakis A. Molecular Basis of Human Glutamate Dehydrogenase Regulation under Changing Energy Demands. Journal of Neuroscience Research. 2005 Jan 1;79(1–2):65–73.

Mathur T, Manadan AM, Thiagarajan S, Hota B, Block JA. Serum Transaminases Are Frequently Elevated at Time of Diagnosis of Idiopathic Inflammatory Myopathy and Normalize with Creatine Kinase. Journal of Clinical Rheumatology: Practical Reports on Rheumatic & Musculoskeletal Diseases. 2014 Apr;20(3):130–2.

Maher, M., Chernenko, G., Barrowman, A. J. The acute pancreatotoxic effects of the plant nitrile 1-cyano-2-hydroxy-3-butene. Pancreas 1991 6:168-74.

McGill MR, Sharpe MR, Williams CD, Taha M, Curry SC, Jaeschke H. The Mechanism Underlying Acetaminophen-Induced Hepatotoxicity in Humans and Mice Involves Mitochondrial Damage and Nuclear DNA Fragmentation. The Journal of Clinical Investigation. 2012 Apr;122(4):1574–83.

McGovern AJ, Vitkovitsky IV, Jones DL, Mullins ME. Can AST/ALT Ratio Indicate Recovery after Acute Paracetamol Poisoning? Clinical Toxicology (Philadelphia, Pa). 2015 Mar;53(3):164–7.

Nathwani RA, Pais S, Reynolds TB, Kaplowitz N. Serum Alanine Aminotransferase in Skeletal Muscle Diseases. Hepatology (Baltimore, MD). 2005 Feb;41(2):380–2.

O'Brien PJ, Slaughter MR, Swain A, Birmingham JM, Greenhill RW, Elcock F, Bugelski PJ. Repeated Acetaminophen Dosing in Rats: Adaptation of Hepatic Antioxidant System. Human & Experimental Toxicology. 2000 May;19(5):277–83.

O'Brien PJ, Slaughter MR, Polley SR, Kramer K. Advantages of Glutamate Dehydrogenase as a Blood Biomarker of Acute Hepatic Injury in Rats. Laboratory Animals. 2002 Jul 1;36(3):313–21.

Rosales XQ, Chu ML, Shilling C, Wall C, Pastores GM, Mendell JR. Fidelity of Gamma-Glutamyl Transferase (GGT) in Differentiating Skeletal Muscle from Liver Damage. Journal of Child Neurology. 2008 Jul;23(7):748–51.

Rutledge J, Andersen J, Fink CW, Cook J, Strickland A. Persistent Hypertransaminasemia as the Presenting Finding of Childhood Muscle Disease. Clinical Pediatrics. 1985 Sep;24(9):500–3.

Schmidt E, Schmidt W, Otto P. Isoenzymes of Malic Dehydrogenase, Glutamic Oxaloacetic Transaminase and Lactic Dehydrogenase in Serum in Diseases of the Liver. Clinica Chimica Acta. 1967;15(2):283–289.

Schmidt ES, Schmidt FW. Glutamate Dehydrogenase: Biochemical and Clinical Aspects of an Interesting Enzyme. Clinica Chimica Acta; International Journal of Clinical Chemistry. 1988 Mar 31;173(1):43–55.

Schomaker S, Warner R, Bock J, Johnson K, Potter D, Van Winkle J, Aubrecht J. Assessment of Emerging Biomarkers of Liver Injury in Human Subjects. Toxicological sciences: an official journal of the Society of Toxicology. 2013 Apr;132(2):276–83.

Singhal R, Harrill AH, Menguy-Vacheron F, Jayyosi Z, Benzerdjeb H, Watkins PB. Benign Elevations in Serum Aminotransferases and Biomarkers of Hepatotoxicity in Healthy Volunteers Treated with Cholestyramine. BMC pharmacology & toxicology. 2014 Aug 3;15:42.

Sutherland RJ. Biochemical Evaluation of the Hepatobiliary System in Dogs and Cats. The Veterinary Clinics of North America Small Animal Practice. 1989 Sep;19(5):899–927.

Temple R. Hy's Law: Predicting Serious Hepatotoxicity. Pharmacoepidemiology and Drug Safety. 2006 Apr;15(4):241–3.

Thulin P, Nordahl G, Gry M, Yimer G, Aklillu E, Makonnen E, Aderaye G, Lindquist L, Mattsson CM, Ekblom B, Antoine DJ, Park BK, Linder S, Harrill AH, Watkins PB, Glinghammar B, Schuppe-Koistinen I. Keratin-18 and microRNA-122 Complement Alanine Aminotransferase as Novel Safety Biomarkers for Drug-Induced Liver Injury in Two Human Cohorts. Liver International. 2014 Mar;34(3):367–78.

Van Waes L, Lieber CS. Glutamate Dehydrogenase: A Reliable Marker of Liver Cell Necrosis in the Alcoholic. British Medical Journal. 1977 Dec 10;2(6101):1508–10.

Yuan L, Kaplowitz N. Mechanisms of Drug Induced Liver Injury. Clinics in liver disease. 2013 Nov;17(4):507–18.

XI. APPENDIX II. Validation Report

Amended Validation Memo for Glutamate Dehydrogenase (GLDH or GDH) in Human Serum on the Advia 1800 Chemistry Analyzer (Serial Number CA1291000770077)

TESTING FACILITY

Pfizer Worldwide Research & Development
Drug Safety Research and Development
445 Eastern Point Road, Groton, CT 06340

APPROVAL SIGNATURES

I prepared the content of the amended Validation Memo for Glutamate Dehydrogenase (GLDH or GDH) in human serum on the Advia 1800 Chemistry Analyzer:

Johanna Wisniewski 12 Jul 2017

Johanna Wisniewski, MT (ASCP), MS — Date
Associate Director, Biomarker Operations
Drug Safety Research & Development – Groton, CT
Pfizer, Inc

I contributed to the content of amended Validation Memo for Glutamate Dehydrogenase (GLDH or GDH) in human serum on the Advia 1800 Chemistry Analyzer:

12 JUL 2017

Richard Giovanelli BS, MT (ASCP) — Date
Director, Clinical Pathology Laboratory
Drug Safety Research & Development - Groton, CT
Pfizer, Inc

on behalf of

Jeffrey Bock, MS, MT (ASCP)
Scientist, Clinical Pathology
Drug Safety Research & Development – Groton, CT
Pfizer, Inc

Ryan Criswell 12 Jul 2017

Ryan Criswell, BS, HT (ASCP) — Date
Senior Associate Scientist, Clinical Pathology
Drug Safety Research & Development – Groton, CT
Pfizer Inc

Gretchen Volberg 12 Jul 2017

Gretchen Volberg, BS, MLS (ASCP)^{CM} — Date
Associate Scientist, Clinical Pathology
Drug Safety Research & Development – Groton, CT
Pfizer Inc

APPROVAL SIGNATURES

I have reviewed the content of the amended Validation Memo for Glutamate Dehydrogenase (GLDH or GDH) in human serum on the Advia 1800 Chemistry Analyzer prior to initiation of testing and found it to be acceptable:

12 Jun 2017

Richard Giovanelli BS, MT (ASCP) Date
Director, Clinical Pathology Laboratory
Drug Safety Research & Development - Groton, CT
Pfizer, Inc

Shelli Schomaker 12 June 2017

Shelli Schomaker, BA Date
Senior Principal Scientist, Clinical Pathology and Biomarker Strategy Group
Drug Safety Research & Development – Groton, CT
Pfizer, Inc

12 July 2017

Jiri Aubrecht, PhD, Pharm.D Date
Senior Director, Biomarker Clinical and Translational Lead
Drug Safety Research & Development – Groton, CT
Pfizer, Inc

Kay Criswell 12-Jul 2017

Kay Criswell, MT (ASCP) SBB, MS, PhD, DABT Date
CAP/CLIA Clinical Pathology Laboratory Director
Drug Safety Research & Development – Groton, CT
Pfizer, Inc

TABLE OF CONTENTS

1. BACKGROUND

1.1. Introduction

This purpose of this memo is to provide documentation of the validation of Glutamate Dehydrogenase (GLDH or GDH) in Human Serum on the Siemens Advia 1800 automated chemistry platform as a laboratory-developed test.

1.2. Background

Laboratory-developed tests are considered to be tests used for patient management but have been developed within CLIA certified laboratories for use within those laboratories. Laboratory-developed tests are accepted as being scientifically valid and are relied on routinely in the delivery of health care in the United States. Laboratory developed tests are extensively regulated by Centers of Medicare and Medicaid Services (CMS) under the Clinical Laboratory Improvement Amendments (CLIA). Clinical laboratories must determine performance specifications for all laboratory-developed tests as required by CLIA and are responsible for both the quality and interpretation of results generated from those tests. Performance specifications for laboratory-developed tests must be established for the following characteristics: accuracy, precision, analytical sensitivity (LOB), analytical specificity to include interfering substances, reportable range, and reference interval. The validation of GLDH as a laboratory-developed test included these specific criteria as well as long term stability and freeze/thaw stability.

1.3. Clinical Significance

Glutamate Dehydrogenase, a mitochondrial enzyme located primarily in the centrilobular region of the liver, plays a role in amino acid oxidation and urea production. GLDH is primarily found in the liver and to a lesser degree in the kidney with trace amounts in skeletal muscle (Mastorodemos et al. (2005) 79(1-2):65-73). GLDH activity is a sensitive enzymatic serum marker of liver toxicity increasing with hepatocelluar damage in preclinical species (O'Brien, et al. *Laboratory Animals* (2002) 36, 313–321; Giffen, et al. Toxicol. Pathol. (2003) 30, 365-372). GLDH is elevated in humans with hepatic ischemia (Exp Toxic Path (2003) 54, 423-431) and progressively increased in patients with increased severity of disease (Schmidt et al. Clinica Chimica Acta (1988) 173 (1):43-55). More recently GLDH has been shown to correlate with ALT in patients with a broad range of clinically demonstrated liver injuries including acetaminophen-induced liver injury (Schomaker et al Tox. Sci. (2013) 132(2):276-83) and to detect mild hepatocyte necrosis in patients treated with heparin (Harrill et al Clin. Pharm. Ther. (2012) 92, 214-220).

1.4. Methodology Description

The Randox GLDH method measures GLDH using an optimized standard method according to the recommendations of the Deutsche Gesellschaft für Klinische Chemie (DGKC). The reaction below is used for measurement of GLDH.

$$\alpha\text{-oxoglutarate} + NADH + NH_4^+ \xrightarrow{GLDH} \text{glutamate} + NAD^+ + H_2O$$

As NADH is oxidized, the decrease in the absorbance per minute is measured spectrophotometrically at 340 nm and is proportional to the GLDH activity.

This kit was manufactured in the United Kingdom (UK) with ISO13485 certification as evidence of GMP. The assay is an approved IVD assay as in Europe, Health Canada and China. The UK manufacturing facility was FDA inspected in 2012 and 2013.

1.5. Reagents Selected for Validation

- Analyte: Glutamate Dehydrogenase (GLDH or GDH)
- Reagent: Randox GLDH reagent (GL441)
- Manufacturer: Randox
- Platform/Instrument: Siemens Advia 1800
- Specimen: Human Serum
- Randox Acusera Human Assay Control 2 Reference number HN1530
- Randox Acusera Human Assay Control 3 Reference number HE1532
- Randox Acusera Calibration 2 Reference number CAL2350
- Randox Acusera Calibration 3 Reference number CAL2351

1.6. Reagent Preparation

Procedures listed in the package insert for GLDH were followed for reagent preparation, sample collection/preparation and storage and stability of the reagent.

1.7. Sample Generation

Pfizer has a Biofluid agreement with University of Michigan to collect samples (blood or tissues) retrospectively from humans entering the clinical hospital. This activity is covered by a pre-existing Institutional Review Board (IRB) that allows Pfizer access to samples which can be used for biomarker and investigational work. To validate/qualify various hepatic injury biomarkers in humans, including GLDH, Pfizer requested blood samples from healthy subjects and patients with evidence of liver injury. Samples were obtained from samples that would have been discarded and were not prospectively ordered for biomarker development. Selected serum samples were shipped to the Safety Biomarkers Group, Groton DSRD for storage and utilized in this GLDH qualification and translation initiative. Additional samples may be obtained as required from Pfizer Occupational Health and/ or from commercial vendors.

2. QUALIFICATION PARAMETERS

Validation parameters for GLDH on the Advia 1800 platform are shown below.

Table 1 shows assay validation parameters, testing strategy, and acceptance criteria.

Requirement Number	Requirement	Testing strategy	Acceptance Criteria	Pass/Fail
1.	Limit of Blank (LOB) – the manufacturer's claim of LOB must be verified. Limit of Detection (LOD) – the lowest analyte concentration that can be reliably distinguished from the LOB must be determined.	At least 20 replicates of a blank and 10 replicates of the low concentration sample will be analyzed in a single run. The mean and standard deviation (SD) of the blank will be used to calculate the LOB. The LOB and the SD of the low concentration sample will be used to calculate the LOD utilizing CLSI guidance EP17A.	The LOB will be calculated according to the following formula: $LOB = mean_{blank} + 1.645(SD_{blank})$ The LOD will be calculated according to the following formula: $LOD = LOB + 1.645(SD_{low\ concentration\ sample})$	Pass
2.	Precision – the assay must be acceptably precise within run, between runs and day to day over a time course.	Minimum of 3 concentrations (Low, High and Near Detection Level (NDL)) in addition to both levels of Quality Control in duplicate 1- 2 times per day over 20 days. Calculate the SD and/or CV within run, between run, day to day and total variation	Values < 10% will be considered acceptable and/or should be consistent with information in the manufacturers' package insert. Values <15% are acceptable for the samples near the detection level of the assay.	Pass
3.	Method to Method Comparison	At least 40 human serum samples will be analyzed in duplicate over 5 operating days by both methodologies. A linear regression analysis will be performed and a correlation coefficient (R), slope and %bias will be calculated. Method to Method comparison data will be used to characterize the %bias between the Advia 1800 and a similar platform utilizing the same Randox GLDH reagent system at a second site. EP Evaluator version 7.0 will be used for the regression analysis.	Negative and positive biases are expected due to differences in methodology and will be used to assess impact on correlation. An R value of ≥ 0.90 is expected in most analytes in which the dynamic range is adequately tested.	Pass

Requirement Number	Requirement	Testing strategy	Acceptance Criteria	Pass/Fail
4.	Reportable range/ Linearity – the instrument's analytic measurement range must be established for each analyte tested	7-9 concentrations in duplicate or triplicate across anticipated measuring range (or 20-30% beyond the anticipated measurment range to ascertain widest possible range) or a series of dilutions of a highly elevated patient sample with concentrations across the anticipated measuring range. Linear regression analysis of the data will be performed using Microsoft Excel.	Acceptable performance will be based on percent recovery at each dilution (80% to 120%) and visual assessment of the linearity using the slope and correlation coefficient as guides.	Pass
5.	Accuracy (trueness)/ Recovery – spiked sample recovery studies must be evaluated using a species specific matrix. Investigations are run primarily as an indication of sample matrix effects and to test the ability of the assay to measure the "true" known concentration of the analyte in the sample.	Recovery evaluations will be made by utilizing serum samples (working dilution) with low analyte concentrations. The working diluted sample is 'spiked' with different concentrations of kit calibrator. Recovery should be evaluated at several concentrations over the assays dynamic range.	Acceptable performance will be based on percent recovery at each spiked concentration dilution (80% to 120%) and/or visual assessment of the linearity using the slope and correlation coefficient as guides.	Pass
6.	Reference Interval Establishment	At least 60 specimens representative of the population.	Historical data was generated using 552 human samples. See Reference 10.	Pass

Requirement Number	Requirement	Testing strategy	Acceptance Criteria	Pass/Fail
7.	Sample Freeze/ Thaw stability; Evaluate the effect of three freeze-thaw cycles on measurable analyte concentrations using two sample sets, ideally with analyte concentrations in low or mid/high range.	Freeze/ Thaw stability will be evaluated as multiple freeze/thaw cycles. Multiple freeze/thaw cycles: Four aliquots of undiluted sample/ sample pool are reserved for freeze/thaw stability studies. The samples are allowed to undergo 1, 2, 3 or 4 freeze/thaw cycles before being working dilutions are prepared and all samples are assayed in a single assay run. Freeze-thaw stability will be evaluated as recovered analyte concentration relative to the sample undergoing one (i.e. initial) freeze/thaw cycle	Acceptable freeze thaw stability would be within the inter-assay precision for the assay and/or 80-120% recovery of the baseline (initial thaw sample) concentration.	Pass

Requirement Number	Requirement	Testing strategy	Acceptance Criteria	Pass/Fail
8.	Sample Stability – long-term room temperature, refrigerated and frozen sample stability must be defined.	Sample Storage Stability will be evaluated using freshly collected samples/ sample pools that are aliquoted as soon as possible after collection and stored at room temperature (20-25°C), refrigerated temperature (1-8°C) and frozen at (-70 to -80°C). Ideally, three sample sets with low, mid, or high analyte concentrations are assayed at 4, 24, 48, 72 and 96 hours for both room and refrigerated temperatures plus 1, 2, 4, 8, 10, 14, 21, and 28 day refrigerated. Three sample sets with low, mid, or high analyte concentrations are assayed within the first week of collection and after storage of approximately: 1 week, 2 weeks, 1, 3, 6, 12, and 18 months frozen. The percent recovery for the each storage timepoint will be calculated relative to the baseline value.	Acceptable long-term storage stability: Measured concentration would be within the inter-assay precision for the assay and/or 80%- 120% recovery of the baseline (initial thaw) concentration.	Pass
9.	Analytical Specificity/Interference	Matrix components can potentially interfere with assay performance. Therefore, the potential for variable matrix-related interferences will be evaluated in at least 1 validation run in a patient pool with independent sources (n≥5) of icterus, hemolysis, and lipemia, spiked at different concentrations (n≥1).	Results are acceptable if ≥80% of the matrix lots tested meet the following criteria: • With-in lot precision (%CV) of ≤30% • With-in Lot accuracy (%RE) within ±30% of the respective nominal concentrations.	Pass

Requirement Number	Requirement	Testing strategy	Acceptance Criteria	Pass/Fail
10.	Proficiency Testing	GLDH proficiency testing samples were established using Randox Serum Level 2 (Calibrator 2) catalog number CAL 2350 prepared at least 2 and/or 3 known concentrations for blinded testing.	Acceptable performance will be based on obtaining values within the defined acceptance range established by the Laboratory Director	Pass

3. RESULTS

3.1. Requirement 1 - Limit of Blank (LOB) and Limit of Detection (LOD)

Limit of Blank (LOB) was performed using a blank, deionized water. Twenty replicates of the blank were analyzed in a single run to verify the LOB. The mean and SD of the blank was calculated and the LOB established according to the following formula:

$$LOB = mean_{blank} + 1.645(SD_{blank}) = 0.1 + 1.645\ (0.31) = 0.610 = 1$$

Limit of Detection (LOD) was performed using the blank, deionized water, and a low concentration sample. The low concentration sample was created using a 10% solution of the lowest available calibrator (Calibrator 1 = 28 U/L). The mean and SD of the blank was calculated and the LOD established according to the following formula:

$$LOD = LOB + 1.645(SD_{low\ concentration\ sample}) = 0.1 + 1.645\ (0.92) = 1.61 = 2$$

Conclusion: The LOB was demonstrated as 1 U/L for GLDH. The LOD was demonstrated as 2 U/L for GLDH. Results are shown in Table 2 below.

Table 2. Summary of LOB for GLDH (U/L) in Human Serum

Replicate Samples	Deionized Water (Blank)	Low Concentration Sample (10% Calibrator 1 in Deionized Water)
1	0	1
2	0	3
3	1	0
4	0	2
5	0	1
6	0	1
7	0	1
8	0	2
9	0	0
10	0	0
11	0	0
12	0	2
13	0	0
14	0	1
15	0	2
16	0	2
17	0	0
18	0	1
19	0	1
20	0	0
Mean	0.1	1.0
SD	0.31	0.92

3.2. Requirement 2 – Precision

Three concentrations (Near Detection Level (NDL), Low, and High) in addition to both levels of Quality Control and Concentrated (Conc.) Calibrator 3 in duplicate 2 times per day over 20 days.

Precision testing was performed using three human serum concentrations, 2 U/L (NDL), 7 U/L (low), and 623 U/L (high), two levels of quality control material from the kit manufacturer (Randox) Assay Control Level 2 (16 U/L) and Assay Control Level 3 (29 U/L), and concentrated Randox Acusera Calibration 3 (CAL2351) (prepared in a smaller volume of diluent than prescribed by the reagent preparation procedure) run in duplicate 2 times per day over 20 days. Total variation was calculated and is shown in Table 3 below. SD and CV were calculated within run, between run, and day to day which is shown in Tables 4-8. Data for the fourth replicate of day 20 for Table 8 was not included as part of the calculation shown. The sample had insufficient volume and the instrument gave an erroneous result.

Conclusion: Analyzed human serum samples, both quality control materials and Conc. Calibrator 3 demonstrated appropriate assay precision with a CV <10%. The Near Detection Level (NDL) sample (2 U/L) was below the LLOQ and would not be expected to meet a CV limit of <10%. Higher CV values obtained at low concentrations of GLDH (12.96% CV for the Low sample with a GLDH value of 7 U/L) are anticipated since CV is more substantially impacted by changes in small values. However, close examination of individual data points showed small differences. SD is a more reliable parameter for assessing precision for low values; and for the Low sample (7 U/L), the SD was less than 1.00, specifically 0.89, demonstrating that variation was small. Since liver injury will be assessed with increasing levels of GLDH and the normal reference range for GLDH has been determined to be <3 to 10 U/L, there is little biological relevance to a SD of 0.89 for a mean value of 7.0 U/L. Based upon the results of this testing and the acceptance criteria, GLDH in human serum is considered to have acceptable precision.

Table 3. Summary of Precision for GLDH in Serum and Quality Control

	NDL	Low	Mid	High	QC Level 2	QC Level 3	Conc. Cal 3
Mean	2	7	53	623	16	29	59
SD	0.89	0.89	2.43	15.37	1.36	1.36	3.27
%CV	37.7	13.0	4.6	2.5	8.0	4.7	5.6

Table 4. Summary of Precision for GLDH NDL in Human Serum

Day	Rep	GLDH (U/L)	Within Run SD	Within Run CV	Between Runs SD	Between Runs CV	Day to Day SD	Day to Day SD	Day to Day CV	Day to Day CV
Day 1	1a	2	0.00	0.0	0.00	0.0	0.35		16.6	
	1b	2								
	2a	2	0.00	0.0						
	2b	2								
Day 2	1a	3	0.71	28.3	0.50	22.2		0.71		31.4
	1b	2								
	2a	2	0.00	0.0						
	2b	2								
Day 3	1a	1	1.41	70.7	0.96	42.6	0.71		31.4	
	1b	3								
	2a	2	0.71	0.0						
	2b	3								
Day 4	1a	3	0.71	28.3	0.50	22.2		0.74		31.3
	1b	2								
	2a	2	0.00	0.0						
	2b	2								
Day 5	1a	3	0.00	0.0	1.00	40.0	0.83		39.3	
	1b	3								
	2a	3	1.41	0.0						
	2b	1								
Day 6	1a	1	0.71	47.1	0.50	28.6		0.53		26.7
	1b	2								
	2a	2	0.00	0.0						
	2b	2								
Day 7	1a	2	0.00	0.0	0.50	22.2	0.46		20.6	
	1b	2								
	2a	2	0.71	0.0						
	2b	3								
Day 8	1a	3	0.71	28.3	0.50	22.2		0.76		30.2
	1b	2								
	2a	2	0.00	0.0						
	2b	2								
Day 9	1a	3	0.71	20.2	0.96	34.8	0.89		32.2	
	1b	4								
	2a	2	0.00	0.0						
	2b	2								
Day 10	1a	2	0.00	0.0	0.96	34.8		0.83		26.7
	1b	2								
	2a	4	0.71	0.0						
	2b	3								
Day 11	1a	4	0.71	20.2	0.58	16.5	1.30		49.6	
	1b	3								
	2a	4	0.71	0.0						
	2b	3								
Day 12	1a	0	1.41	141.4	1.26	71.9		1.41		53.6
	1b	2								
	2a	2	0.71	0.0						
	2b	3								
Day 13	1a	3	0.00	0.0	1.00	28.6	1.04		37.6	
	1b	3								
	2a	3	1.41	0.0						
	2b	5								
Day 14	1a	2	0.00	0.0	0.00	0.0		0.53		21.4
	1b	2								
	2a	2	0.00	0.0						
	2b	2								
Day 15	1a	3	0.00	0.0	0.00	0.0	1.06		44.7	
	1b	3								
	2a	3	0.00	0.0						
	2b	3								
Day 16	1a	3	0.71	28.3	1.26	71.9		1.26		65.3
	1b	2								
	2a	2	1.41	0.0						
	2b	0								
Day 17	1a	2	0.00	0.0	1.00	66.7	0.71		40.4	
	1b	2								
	2a	2	1.41	0.0						
	2b	0								
Day 18	1a	2	0.00	0.0	0.00	0.0		0.53		26.7
	1b	2								
	2a	2	0.00	0.0						
	2b	2								
Day 19	1a	3	0.71	28.3	0.82	40.8	1.06		40.4	
	1b	2								
	2a	1	0.71	0.0						
	2b	2								
Day 20	1a	2	0.71	28.3	0.96	29.5				
	1b	3								
	2a	4	0.00	0.0						
	2b	4								

Table 5. Summary of Precision for Low GLDH in Human Serum

Day	Rep	GLDH (U/L)	Within Run SD	Within Run CV	Between Runs SD	Between Runs CV	Day to Day SD	Day to Day SD	Day to Day CV	Day to Day CV
Day 1	1a	7	0.71	10.9	0.50	8.0	0.74		11.2	
	1b	6								
	2a	6	0.00	0						
	2b	6								
Day 2	1a	7	0.00	0	0.82	11.7		0.92		14.4
	1b	7								
	2a	6	1.41	20.2						
	2b	8								
Day 3	1a	6	0.00	0	0.50	8.7	0.64		10.5	
	1b	6								
	2a	6	0.71	12.9						
	2b	5								
Day 4	1a	6	0.00	0	0.58	8.9		0.74		11.2
	1b	6								
	2a	7	0.00	0						
	2b	7								
Day 5	1a	8	0.71	9.4	0.96	14.2	0.76		11.6	
	1b	7								
	2a	6	0.00	0						
	2b	6								
Day 6	1a	7	0.71	10.9	0.50	8.0		0.83		12.1
	1b	6								
	2a	6	0.00	0						
	2b	6								
Day 7	1a	8	0.00	0	0.58	7.7	0.76		10.8	
	1b	8								
	2a	7	0.00	0						
	2b	7								
Day 8	1a	7	0.00	0	0.58	8.9		0.52		8.1
	1b	7								
	2a	6	0.00	0						
	2b	6								
Day 9	1a	7	0.71	10.9	0.50	8.0	0.83		12.1	
	1b	6								
	2a	6	0.00	0						
	2b	6								
Day 10	1a	8	0.71	9.4	0.58	7.7		0.46		6.4
	1b	7								
	2a	8	0.71	9.4						
	2b	7								
Day 11	1a	7	0.00	0	0.00	0.0	0.46		6.9	
	1b	7								
	2a	7	0.00	0						
	2b	7								
Day 12	1a	6	0.00	0	0.58	8.9		0.99		13.9
	1b	6								
	2a	7	0.00	0						
	2b	7								
Day 13	1a	8	0.71	8.3	0.96	12.4	0.92		12.4	
	1b	9								
	2a	7	0.00	0.0						
	2b	7								
Day 14	1a	6	0.71	10.9	0.82	11.7		0.83		11.7
	1b	7								
	2a	8	0.71	9.43						
	2b	7								
Day 15	1a	7	0.71	9.43	0.96	13.2	0.76		10.8	
	1b	8								
	2a	6	1.41	20.2						
	2b	8								
Day 16	1a	7	0.71	10.9	0.50	7.4		0.50		7.4
	1b	6								
	2a	7	0.00	0.0						
	2b	7								
Day 17	1a	6	0.00	0.0	0.50	8.0	1.13		16.4	
	1b	6								
	2a	7	0.71	10.9						
	2b	6								
Day 18	1a	6	0.71	10.9	1.29	17.2		1.20		15.9
	1b	7								
	2a	8	0.71	8.3						
	2b	9								
Day 19	1a	9	1.41	17.7	1.29	17.2	0.99		12.6	
	1b	7								
	2a	6	1.41	20.2						
	2b	8								
Day 20	1a	8	0.00	0.0	0.50	6.1				
	1b	8								
	2a	9	0.71	8.3						
	2b	8								

Table 6. Summary of Precision for Mid GLDH in Human Serum

Day	Rep	GLDH (U/L)	Within Run SD	Within Run CV	Between Runs SD	Between Runs CV	Day to Day SD	Day to Day SD	Day to Day CV	Day to Day CV
Day 1	1a	52.4								
	1b	52.9	0.35	0.7						
	2a	48.7					2.29		4.3	
	2b	52.1	2.40	4.8	1.91	3.7				
Day 2	1a	53.4	2.26	4.1						
	1b	56.6						2.88		5.5
	2a	54.9	1.77	3.3						
	2b	52.4			1.83	3.4				
Day 3	1a	49.8	0.21	0.4						
	1b	50.1							4.9	
	2a	47.8	2.62	5.3			2.54			
	2b	51.5			1.53	3.1				
Day 4	1a	54.4	0.57	1.0						
	1b	53.6						2.86		5.5
	2a	54.1	0.28	0.5						
	2b	54.5			0.40	0.7				
Day 5	1a	48.1	1.06	2.2						
	1b	49.6							7.1	
	2a	47.8	2.47	5.0			3.72			
	2b	51.3			1.61	3.3				
Day 6	1a	55.6	0.49	0.9						
	1b	54.9						2.49		4.6
	2a	57.4	1.70	3.0						
	2b	55.0			1.16	2.1				
Day 7	1a	51.0	1.41	2.7						
	1b	53.0							3.2	
	2a	51.5	0.71	1.4			1.68			
	2b	50.5			1.08	2.1				
Day 8	1a	54.4	0.49	0.9						
	1b	53.7						1.66		3.2
	2a	49.5	1.27	2.5						
	2b	51.3			2.25	4.3				
Day 9	1a	54.1	0.71	1.3						
	1b	53.1							4.0	
	2a	52.5	0.92	1.7			2.08			
	2b	53.8			0.72	1.3				
Day 10	1a	49.9	0.64	1.3						
	1b	50.8						3.38		6.4
	2a	48.9	0.28	0.6						
	2b	49.3			0.83	1.7				
Day 11	1a	55.6	0.35	0.6						
	1b	56.1							6.2	
	2a	57.0	1.63	2.9			3.30			
	2b	54.7			0.96	1.7				
Day 12	1a	49.7	0.85	1.7						
	1b	48.5						1.80		3.5
	2a	49.9	1.91	3.7						
	2b	52.6			1.73	3.4				
Day 13	1a	51.1	1.70	3.2						
	1b	53.5							3.0	
	2a	53.0	1.98	3.8			1.55			
	2b	50.2			1.56	3.0				
Day 14	1a	52.2	1.56	3.0						
	1b	50.0						2.14		4.1
	2a	49.1	2.12	4.2						
	2b	52.1			1.55	3.0				
Day 15	1a	54.6	1.20	2.2						
	1b	52.9							3.7	
	2a	55.6	2.05	3.8			2.03			
	2b	52.7			1.39	2.6				
Day 16	1a	59.1	1.98	3.4						
	1b	56.3						2.84		5.2
	2a	54.9	0.64	1.1						
	2b	55.8			1.81	3.2				
Day 17	1a	55.0	2.12	4.0						
	1b	52.0							3.4	
	2a	50.1	1.63	3.2			1.78			
	2b	52.4			2.02	3.9				
Day 18	1a	53.2	0.78	1.5						
	1b	52.1						1.26		2.4
	2a	55.7	2.33	4.3						
	2b	52.4			1.63	3.1				
Day 19	1a	52.6	0.07	0.1						
	1b	52.5							2.0	
	2a	54.0	1.48	2.8			1.07			
	2b	51.9			0.89	1.7				
Day 20	1a	54.3	2.05	3.9						
	1b	51.4								
	2a	51.5	0.99	1.9						
	2b	52.9			1.37	2.6				

Table 7. Summary of Precision for High GLDH in Human Serum

Day	Rep	GLDH (U/L)	Within Run SD	Within Run CV	Between Runs SD	Between Runs CV	Day to Day SD	Day to Day SD	Day to Day CV	Day to Day CV
Day 1	1a	658	9.90	1.5	18.26	2.9	14.83		2.4	
	1b	644								
	2a	622	1.41	0.2						
	2b	620								
Day 2	1a	634	7.07	1.1	8.21	1.3		7.37		1.2
	1b	624								
	2a	619	2.83	0.5						
	2b	615								
Day 3	1a	618	2.12	0.3	2.87	0.5	5.38		0.9	
	1b	615								
	2a	612	0.00	0.0						
	2b	612								
Day 4	1a	603	0.00	0.0	2.89	0.5		15.96		2.6
	1b	603								
	2a	608	0.00	0.0						
	2b	608								
Day 5	1a	633	0.71	0.1	5.07	0.8	21.23		3.5	
	1b	632								
	2a	631	7.78	1.2						
	2b	642								
Day 6	1a	588	8.49	1.4	7.14	1.2		12.04		2.0
	1b	600								
	2a	604	7.78	1.3						
	2b	593								
Day 7	1a	610	4.95	0.8	4.57	0.7	8.64		1.4	
	1b	617								
	2a	621	2.83	0.5						
	2b	617								
Day 8	1a	630	2.83	0.4	3.59	0.6		14.70		2.4
	1b	634								
	2a	633	4.95	0.8						
	2b	626								
Day 9	1a	610	0.00	0.0	6.40	1.1	14.87		2.5	
	1b	610								
	2a	602	3.54	0.6						
	2b	597								
Day 10	1a	624	9.19	1.5	21.67	3.6		20.00		3.2
	1b	611								
	2a	619	30.41	5.1						
	2b	576								
Day 11	1a	635	2.83	0.4	4.65	0.7	11.80		1.8	
	1b	639								
	2a	629	0.71	0.1						
	2b	630								
Day 12	1a	648	3.54	0.5	5.45	0.8		22.51		3.6
	1b	653								
	2a	652	6.36	1.0						
	2b	661								
Day 13	1a	617	1.41	0.2	6.75	1.1	9.80		1.6	
	1b	619								
	2a	604	4.95	0.8						
	2b	611								
Day 14	1a	622	11.31	1.8	8.39	1.3		8.29		1.3
	1b	638								
	2a	622	1.41	0.2						
	2b	620								
Day 15	1a	636	7.07	1.1	6.24	1.0	9.09		1.4	
	1b	626								
	2a	634	4.95	0.8						
	2b	641								
Day 16	1a	630	7.78	1.2	6.06	1.0		6.06		1.0
	1b	619								
	2a	618	7.78	0.1						
	2b	617								
Day 17	1a	639	2.83	0.4	8.77	1.4	9.59		1.5	
	1b	643								
	2a	638	10.61	1.7						
	2b	623								
Day 18	1a	622	4.24	0.7	4.03	0.6		4.90		0.8
	1b	628								
	2a	620	0.71	0.1						
	2b	619								
Day 19	1a	613	3.54	0.6	2.16	0.4	8.22		1.3	
	1b	618								
	2a	615	0.71	0.1						
	2b	614								
Day 20	1a	629	2.83	0.5	4.19	0.7				
	1b	625								
	2a	635	4.95	0.8						

Table 8. Summary of Precision for GLDH in Control 2

Day	Rep	GLDH (U/L)	Within Run SD	Within Run CV	Between Runs SD	Between Runs CV	Day to Day SD	Day to Day SD	Day to Day CV	Day to Day CV
Day 1	1a	12	3.54	24.4	2.22	15.0	1.55		10.3	
	1b	17								
	2a	16	1.41	9.4						
	2b	14								
Day 2	1a	15	0.71	4.6	0.58	3.7		1.04		6.6
	1b	16								
	2a	16	0.71	4.6						
	2b	15								
Day 3	1a	17	2.12	13.7	1.41	8.8	1.16		7.4	
	1b	14								
	2a	17	0.71	4.3						
	2b	16								
Day 4	1a	16	0.00	0.0	1.00	6.5		0.76		4.9
	1b	16								
	2a	16	1.41	9.4						
	2b	14								
Day 5	1a	16	0.00	0.0	0.58	3.7	1.04		7.0	
	1b	16								
	2a	15	0.00	0.0						
	2b	15								
Day 6	1a	14	0.71	4.9	0.82	5.8		1.19		8.1
	1b	15								
	2a	14	0.71	5.2						
	2b	13								
Day 7	1a	15	1.41	8.8	1.26	8.3	1.46		9.0	
	1b	17								
	2a	15	0.71	4.9						
	2b	14								
Day 8	1a	16	0.00	0.0	1.15	6.8		1.55		9.6
	1b	16								
	2a	18	0.00	0.0						
	2b	18								
Day 9	1a	17	2.12	13.7	1.50	9.8	2.14		13.4	
	1b	14								
	2a	14	1.41	9.4						
	2b	16								
Day 10	1a	18	3.54	22.8	2.63	15.7		1.98		12.2
	1b	13								
	2a	19	1.41	7.9						
	2b	17								
Day 11	1a	17	0.71	4.3	1.26	8.0	1.51		9.2	
	1b	16								
	2a	14	1.41	9.4						
	2b	16								
Day 12	1a	17	1.41	8.8	1.63	9.6		1.73		10.7
	1b	15								
	2a	19	1.41	7.9						
	2b	17								
Day 13	1a	17	2.12	13.7	1.50	9.8	1.19		7.6	
	1b	14								
	2a	14	1.41	9.4						
	2b	16								
Day 14	1a	17	0.71	4.3	0.82	5.1		1.20		7.2
	1b	16								
	2a	16	0.71	4.6						
	2b	15								
Day 15	1a	18	2.12	12.9	1.41	8.3	1.30		8.0	
	1b	15								
	2a	18	0.71	4.0						
	2b	17								
Day 16	1a	16	0.71	4.3	0.96	6.1		0.96		6.1
	1b	17								
	2a	15	0.00	0.0						
	2b	15								
Day 17	1a	16	0.00	0.0	0.50	3.2	0.52		3.3	
	1b	16								
	2a	15	0.71	4.6						
	2b	16								
Day 18	1a	15	0.71	4.6	0.58	3.7		0.74		4.8
	1b	16								
	2a	15	0.71	4.6						
	2b	16								
Day 19	1a	15	1.41	8.8	0.96	6.1	0.90		5.7	
	1b	17								
	2a	16	0.71	4.6						
	2b	15								
Day 20	1a	15	1.41	8.8	1.00	6.3				
	1b	17								
	2a	16	0.00	0.00						
	2b	-								

Table 9. Summary of Precision for GLDH Control 3

Day	Rep	GLDH (U/L)	Within Run SD	Within Run CV	Between Runs SD	Between Runs CV	Day to Day SD	Day to Day SD	Day to Day CV	Day to Day CV
Day 1	1a	28	2.12	7.2	1.71	5.6	1.46		4.9	
	1b	31								
	2a	32	1.41	4.6						
	2b	30								
Day 2	1a	30	0.71	2.4	1.29	4.4		1.04		3.5
	1b	29								
	2a	31	2.12	7.2						
	2b	28								
Day 3	1a	30	0.71	2.4	0.82	2.8	1.19		4.1	
	1b	29								
	2a	28	0.71	2.5						
	2b	29								
Day 4	1a	30	2.12	7.4	1.5	5.3		1.30		4.6
	1b	27								
	2a	27	1.41	5.1						
	2b	29								
Day 5	1a	30	1.41	4.9	1.15	4.0	1.41		5.1	
	1b	28								
	2a	30	1.41	4.9						
	2b	28								
Day 6	1a	27	0.00	0.0	0.82	3.0		1.36		4.8
	1b	27								
	2a	26	1.41	5.2						
	2b	28								
Day 7	1a	29	0.00	0.0	0.50	1.7	0.71		2.4	
	1b	29								
	2a	29	0.71	2.4						
	2b	30								
Day 8	1a	30	0.71	2.4	0.96	3.3		1.13		3.9
	1b	29								
	2a	30	1.41	4.9						
	2b	28								
Day 9	1a	29	1.41	5.1	1.41	4.9	1.07		3.6	
	1b	27								
	2a	30	0.00	0.0						
	2b	30								
Day 10	1a	30	0.00	0.0	0.00	0.0		1.67		5.6
	1b	30								
	2a	30	0.00	0.0						
	2b	30								
Day 11	1a	26	4.24	14.6	2.52	8.5	1.69		5.7	
	1b	32								
	2a	30	0.00	0.0						
	2b	30								
Day 12	1a	29	0.71	2.4	0.50	1.7		0.74		2.5
	1b	30								
	2a	30	0.00	0.0						
	2b	30								
Day 13	1a	28	1.41	4.9	1.00	3.4	1.41		4.8	
	1b	30								
	2a	30	0.00	0.0						
	2b	30								
Day 14	1a	29	1.41	4.7	1.91	6.5		1.58		5.5
	1b	31								
	2a	31	2.83	9.8						
	2b	27								
Day 15	1a	28	0.71	2.5	0.82	2.9	0.92		3.2	
	1b	29								
	2a	27	0.71	2.6						
	2b	28								
Day 16	1a	28	1.41	4.9	0.96	3.3		0.96		3.3
	1b	30								
	2a	29	0.71	2.5						
	2b	28								
Day 17	1a	28	2.12	7.2	1.29	4.4	1.31		4.5	
	1b	31								
	2a	30	0.71	2.4						
	2b	29								
Day 18	1a	28	1.41	4.9	1.29	4.5		1.20		4.3
	1b	30								
	2a	29	1.41	5.1						
	2b	27								
Day 19	1a	28	0.00	0.0	1.00	3.6	1.51		5.3	
	1b	28								
	2a	26	1.41	5.2						
	2b	28								
Day 20	1a	30	1.41	4.9	1.29	4.4				
	1b	28								
	2a	29	1.41	4.7						
	2b	31								

Table 10. Summary of Precision for GLDH in Conc. Calibrator 3

Day	Rep	GLDH (U/L)	Within Run SD	Within Run CV	Between Runs SD	Between Runs CV	Day to Day SD	Day to Day SD	Day to Day CV	Day to Day CV
Day 1	1a	63.4	5.44	9.1	3.38	5.6	2.26		3.7	
	1b	55.7								
	2a	61.9	0.35	0.6						
	2b	61.4								
Day 2	1a	60.3	0.28	0.5	0.57	0.9		2.73		4.7
	1b	60.7								
	2a	59.8	0.28	0.5						
	2b	59.4								
Day 3	1a	53.4	1.98	3.6	2.05	3.7	3.45		5.9	
	1b	56.2								
	2a	54.8	2.40	4.3						
	2b	58.2								
Day 4	1a	63.5	2.97	4.8	1.81	3.0		3.77		6.5
	1b	59.3								
	2a	61.6	0.92	1.5						
	2b	60.3								
Day 5	1a	58.7	3.89	7.0	3.08	5.5	3.68		6.3	
	1b	53.2								
	2a	57.9	3.61	6.5						
	2b	52.8								
Day 6	1a	61.9	0.71	1.2	2.67	4.4		2.45		4.1
	1b	60.9								
	2a	56.5	4.10	6.9						
	2b	62.3								
Day 7	1a	61.9	1.84	3.0	2.34	4.0	2.19		3.7	
	1b	59.3								
	2a	56.3	1.34	2.3						
	2b	58.2								
Day 8	1a	62.3	3.39	5.7	2.05	3.4		2.57		4.4
	1b	57.5								
	2a	60.9	0.07	0.1						
	2b	61.0								
Day 9	1a	60.3	3.75	6.5	2.31	4.0	2.89		5.2	
	1b	55.0								
	2a	56.3	1.34	2.3						
	2b	58.2								
Day 10	1a	54.4	1.63	2.9	2.95	5.4		4.38		7.5
	1b	56.7								
	2a	50.5	4.45	8.3						
	2b	56.8								
Day 11	1a	62.4	0.85	1.4	0.59	0.9	3.82		6.5	
	1b	61.2								
	2a	62.4	0.49	0.8						
	2b	61.7								
Day 12	1a	53.1	0.42	0.8	2.83	5.1		2.53		4.4
	1b	53.7								
	2a	59.1	1.48	2.6						
	2b	57.0								
Day 13	1a	59.0	1.06	1.8	0.96	1.6	2.23		3.9	
	1b	57.5								
	2a	58.6	0.85	1.4						
	2b	59.8								
Day 14	1a	60.0	3.32	5.8	2.81	5.0		3.10		5.4
	1b	55.3								
	2a	53.6	2.97	5.3						
	2b	57.8								
Day 15	1a	53.6	2.90	5.2	3.68	6.4	4.83		7.8	
	1b	57.7								
	2a	62.6	3.39	5.6						
	2b	57.8								
Day 16	1a	63.8	3.25	4.9	2.36	3.6		4.15		6.7
	1b	68.4								
	2a	63.1	1.70	2.6						
	2b	65.5								
Day 17	1a	61.7	4.67	8.0	2.74	4.7	2.51		4.2	
	1b	55.1								
	2a	59.4	0.21	0.4						
	2b	59.1								
Day 18	1a	59.9	2.26	3.9	2.48	4.1		2.47		4.2
	1b	56.7								
	2a	62.3	0.92	1.6						
	2b	61.5								
Day 19	1a	62.2	2.47	4.1	2.58	4.4	2.07		3.5	
	1b	58.7								
	2a	56.2	0.92	1.6						
	2b	57.5								
Day 20	1a	56.7	3.04	5.2	1.82	3.1				
	1b	61.0								
	2a	58.6	0.57	1.0						
	2b	57.8								

3.3. Requirement 3 - Method to Method Comparison

In the absence of a FDA cleared assay, this study was utilized as a method comparison study to evaluate “accuracy” as a measure of the closeness of agreement between a split-sample experiment performed at 2 separate CLIA certified laboratories using the same assay. Forty human serum samples were split and analyzed in duplicate over 5 operating days at Pfizer on the Siemens Advia 1800 platform and compared to the Siemens Advia 1800 platform at Huntingdon Life Sciences (Princeton Research Center, P.O. Box 2360, Mettlers Road, East Millstone, NJ 08875-2360) utilizing the Randox GLDH method. A linear regression analysis was performed and correlation coefficient (R), slope and %bias calculated as shown in Figure 1 below. Table 9 shows a summary of method comparison data for GLDH (U/L) in human serum. The original run of human samples 40-47 produced erroneous results at Pfizer, due to inadequate sample volume for testing on the instrument. The identical, split sample was transferred back from Huntingdon Life Sciences to the Pfizer lab for reanalysis.

Conclusion: The slope, R value and average bias were calculated using EP Evaluator, release 7 and can be seen in Figure 1. Based upon the acceptance criteria stated in Table 1, the dynamic range of GLDH in human serum was adequately tested and all criteria were met.

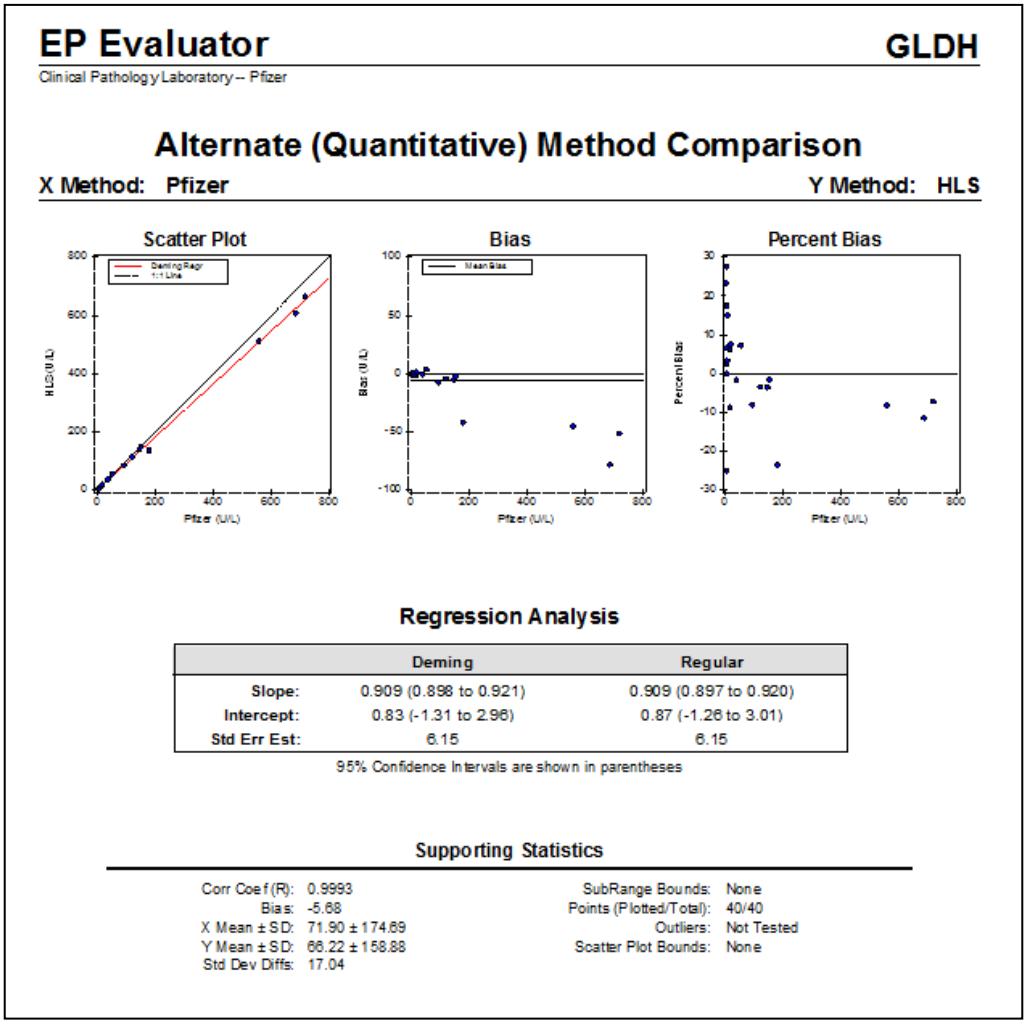

Figure 1. Summary of Method Comparison for GLDH in Human Serum in EP Evaluator

Table 11. Summary of Method Comparison Data for GLDH (U/L) in Human Serum

Cerner Subject ID	Pfizer	HLS		Cerner Subject ID	Pfizer	HLS
9	<3	<3		29	3	<3
10	3	<3		30	3	<3
11	4	3		31	<3	<3
12	4	4.7		32	<3	<3
13	3	<3		33	6	6.2
14	<3	3.2		34	<3	<3
15	6	6		35	15	13.7
16	<3	<3		36	16	17
17	<3	<3		37	17	18.3
18	3	3.1		38	3	<3
19	4	4.7		39	37	36.4
20	3	3.7		40	52	55.8
21	<3	<3		41	151	148.6
22	<3	<3		42	92	84.6
23	<3	<3		43	178	136.1
24	<3	<3		44	119	114.9
25	6	6.9		45	145	140
26	4	4.1		46	557	511.8
27	4	5.1		47	717	665.6
28	<3	<3		48	685	607.2

3.4. Requirement 4 – Linearity

Several concentrations of GLDH were analyzed in triplicate across the anticipated measuring range (or 20-30% beyond the anticipated measurable range to ascertain widest possible range). Two separate linearity studies were performed. The first used a human sample that had elevated GLDH concentration but still within the measurable range of the assay. The second used a highly elevated human sample that started above the measurable range of the assay. Dilutions were made of both the moderately and highly elevated human samples with concentrations across the anticipated measuring range. Data is shown in Figures 2 and 3 below.

Conclusion: Samples had appropriate percent recovery values for both experiments down to 3 U/L. Assay linearity have been established from 3 to 500 U/L. Based upon the results of this testing and the acceptance criteria stated in Table 1, GLDH in human serum demonstrated acceptable linearity.

EP Evaluator

GLDH-Mid

Clinical Pathology Laboratory -- Pfizer

Instrument: 1800-1

Linearity

	Assigned	N	Est	Mean	Residual	Percent Recovery
1:256	0.3	3	0.90	0.37	-0.53	122.2
1:128	0.7	3	1.30	0.23	-1.07	33.3
1:64	1.4	3	2.00	2.17	0.16	154.8
1:32	2.8	3	3.41	3.27	-0.14	116.7
1:16	5.6	3	6.21	6.13	-0.08	109.5
1:8	11.1	3	11.72	12.00	0.28	108.1
1:4	22.3	3	22.94	24.20	1.26	108.5
1:2	44.6	3	45.27	46.17	0.89	103.5
1	89.2	3	89.95	89.17	-0.78	100.0

Linearity Summary

	Reg. Regression
Slope	1.002 ± 0.010
Intercept	0.60 ± 0.33
SEE	0.81
N	9

Experimental Results

1:256	1.1	0	0
1:128	0.2	0.1	0.4
1:64	1.8	1.9	2.8
1:32	3.9	3.7	2.2
1:16	5.1	7.0	6.3
1:8	11.6	12.9	11.5
1:4	24.2	24.3	24.1
1:2	46.8	46.7	45.0
1	87.6	89.4	90.5

X: Excluded from calculations

User's Specifications

Allowable Total Error: --
Systematic Error Budget: --
Allowable Systematic Error: --

Supporting Data

Analyst: AB
Date: 14 Jan 2015
Value Mode: Preassigned
Units: U/L
Lot Number:
Comment

Analytical Claim

The Linearity of GLDH-Mid was analyzed on 1800-1 over a measured range of 0.23 to 89.17 U/L.

Scatter Plot

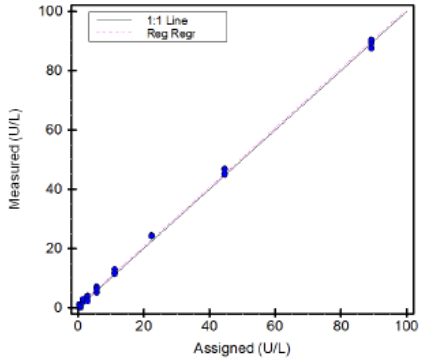

Figure 2. Summary of Linearity for GLDH in Human Serum Mid-Range

EP Evaluator

GLDH-High

Clinical Pathology Laboratory -- Pfizer

Instrument: 1800-1

Linearity

	Assigned	N	Est	Mean	Residual	Percent Recovery
1:1024	0.5	3	0.75	0.80	0.05	160.0
1:512	1.1	3	1.35	1.87	0.52	169.7
1:256	2.2	3	2.45	1.77	-0.68	80.3
1:128	4.3	3	4.54	4.27	-0.27	99.2
1:64	8.6	3	8.82	9.27	0.45	107.8
1:32	17.3	3	17.48	18.67	1.19	107.9
1:16	34.5	3	34.60	35.03	0.43	101.5
1:8	69.0	3	68.95	69.90	0.95	101.3
1:4	138.1	3	137.74	137.63	-0.11	99.7
1:2	276.2	3	275.23	270.50	-4.73	97.9
1	552.3	3	550.10	552.30	2.20	100.0

Linearity Summary

	Reg. Regression
Slope	0.996 ± 0.003
Intercept	0.26 ± 0.65
SEE	1.85
N	11

Experimental Results

1:1024	0.2	0.9	1.3
1:512	1.8	1.0	2.8
1:256	1.1	2.6	1.6
1:128	4.8	3.2	4.8
1:64	8.2	9.6	10.0
1:32	18.9	19.2	17.9
1:16	34.6	35.3	35.2
1:8	70.4	69.8	69.5
1:4	138.1	137.5	137.3
1:2	271.4	270.2	269.9
1	545.7	557.4	553.8

X: Excluded from calculations

User's Specifications

Allowable Total Error: --
Systematic Error Budget: --
Allowable Systematic Error: --

Supporting Data

Analyst: AB
Date: 14 Jan 2015
Value Mode: Preassigned
Units: U/L
Lot Number:
Comment

Analytical Claim

The Linearity of GLDH-High was analyzed on 1800-1 over a measured range of 0.80 to 552.30 U/L.

Scatter Plot

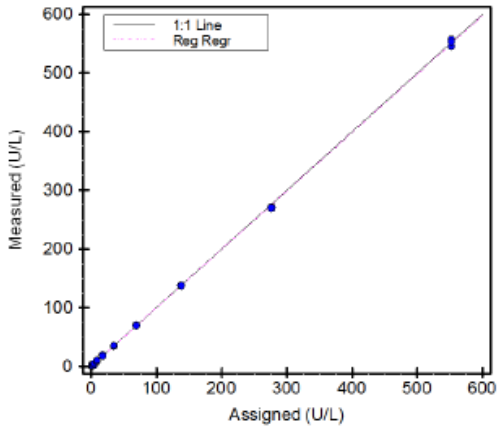

Figure 3. Summary of Linearity for GLDH in Human High-Range

3.5. Requirement 5 – Accuracy/Recovery

In the absence of commercially available standard material with GLDH values >30 U/L, the Randox Acusera Calibration Reference number CAL2351 was concentrated (prepared in a smaller volume of diluent than prescribed by the reagent preparation procedure) and obtained a GLDH value of ~110 U/L. The concentration of this sample (Conc. Calibrator 3) and a patient sample with a high GLDH value (Patient LT-1041) was confirmed by measuring each in triplicate. The mean value of the triplicate measures was then assigned to that sample for the recovery experiment. Recovery was performed by spiking Conc. Calibrator 3 or Patient LT-1041 into serum samples with low analyte concentration, <3 U/L GLDH. Human samples 049, 050, and 051 were spiked with Conc. Calibrator 3 (110 U/L). Human sample 052 and human sample 053 were spiked with serum from Patient LT-1041 (854 U/L).

In a second experiment, Randox Acusera Calibration Reference number CAL2351 was concentrated (prepared in a smaller volume of diluent than prescribed by the reagent preparation procedure) and obtained a GLDH value of ~93 U/L. Recovery was performed by spiking Conc. Calibrator 3 in a Human Pool (<3 U/L). The mean value of the triplicate measures was then assigned to that sample for the recovery experiment. Results are shown in Table 12 below.

Conclusion: All samples had appropriate percent recovery values (80 - 120%) based upon the results of this testing and the acceptance criteria stated in Table 1, GLDH in human serum demonstrated acceptable recovery and accuracy.

Table 12. Summary of Recovery for GLDH (U/L) in Human Serum

Concentration (U/L)					
Human 049 (5%) spiked with Conc. Calibrator 3	GLDH	GLDH Mean	Conc. Calibrator 3 (Experiment 1)	Expected Conc.	% Recovery
Replicate 1	8.5	7.6	110	6.8	112
Replicate 2	6.7				
Human 050 (11%) spiked with Conc. Calibrator 3	GLDH	GLDH Mean	Conc. Calibrator 3 (Experiment 1)	Expected Conc.	% Recovery
Replicate 1	12.5	13.35	110	13.3	100
Replicate 2	14.2				
Human 051 (21%) spiked with Conc. Calibrator 3	GLDH	GLDH Mean	Conc. Calibrator 3 (Experiment 1)	Expected Conc.	% Recovery
Replicate 1	21.5	20.65	110	24.2	85
Replicate 2	19.8				
Human 052 (16%) spiked with Patient LT-1041 seurm	GLDH	GLDH Mean	Patient LT-1041	Expected Conc.	% Recovery
Replicate 1	124.5	124.1	110	137.8	90
Replicate 2	123.7				
Human 053 (39%) spiked with Patient LT-1041 seurm		Mean	Patient LT-1041	Expected Conc.	% Recovery
Replicate 1	282.8	282.45	110	333.9	85
Replicate 2	282.1				
Human Pool (50%) spiked with Conc. Calibrator 3	GLDH	GLDH Mean	Conc. Calibrator 3 (Experiment 2)	Expected Conc.	% Recovery
Replicate 1	55.4	54.75	93	46.5	118
Replicate 2	54.1				

3.6. Requirement 6 – Reference Interval

Historical data was generated using 552 human samples and a reference interval was generated from 274 males and 278 females (see reference 10). The reference interval for GLDH in human serum is 1 – 10 U/L. Due to the linearity assessment (requirement 4) the reference range interval for GLDH in human serum will be updated to <3 – 10 U/L.

3.7. Requirement 7 – Freeze/Thaw Stability

Sample freeze/thaw stability was performed with 3 human serum samples with varied GLDH concentrations. Human sample 054 had an initial concentration of 4 U/L and human sample 055 and 056, <3 U/L. Human sample 055 and 056 were spiked with sample LT-1040 and LT-1044

respectively which had known, high concentration of GLDH to establish a mid and high range sample for this analysis. The samples were assayed after 1, 2, 3, and 4 freeze/thaw cycles from -80°C and the data is shown in Tables 12-14 below. Freeze-thaw stability was evaluated as recovered analyte concentration relative to the sample undergoing one (i.e. initial) freeze/thaw cycle.

Conclusion: All samples had appropriate percent recovery (80 - 120% of initial thaw) based upon the results of this testing and the acceptance criteria stated in Table 1, GLDH in human serum demonstrates acceptable stability for 4 freeze thaw cycles.

Table 13. Summary of Freeze/Thaw Stability for GLDH in Human 054 Serum

Human 054	Result (U/L) Rep 1	Result (U/L) Rep 2	Mean	% Recovery
Baseline	4	<3	3.5	100
1 Thaw Cycle	<3	4	3.5	100
2 Thaw Cycles	3	3	3	86
3 Thaw Cycles	3	4	3.5	100
4 Thaw Cycles	<3	3	3	86

Table 14. Summary of Freeze/Thaw Stability for GLDH in Human 055 Spiked with Human LT-1040 Serum

Human 055	Result (U/L) Rep 1	Result (U/L) Rep 2	Mean	% Recovery
Baseline	68	67	67.5	100
1 Thaw Cycle	69	70	69.5	103
2 Thaw Cycles	69	70	69.5	103
3 Thaw Cycles	66	69	67.5	100
4 Thaw Cycles	69	67	68.0	101

Table 15. Summary of Freeze/Thaw Stability for GLDH in Human 056 Spiked with Human LT-1044 Serum

Human 056	Result (U/L) Rep 1	Result (U/L) Rep 2	Mean	% Recovery
Baseline	274	272	273.0	100
1 Thaw Cycle	285	284	284.5	104
2 Thaw Cycles	281	284	282.5	103
3 Thaw Cycles	282	280	281.0	103
4 Thaw Cycles	281	284	282.5	103

3.8. Requirement 8 – Sample Stability

Sample stability was performed with 3 human serum samples with low, mid, or high GLDH concentrations. The samples were assayed at baseline, 4, 24, 48, 72 and 96 hours for both room and refrigerated temperatures plus 1 day, 2, day, 4 day, 8 day, 10 day, 14 day, 21 day and 28 day refrigerated. Three sample sets with low, mid, or high analyte concentrations were assayed at baseline and after storage of approximately: 1 week, 2 weeks, 1 month, 3, 6, 12 and 18 months frozen. The percent recovery for each storage timepoint was calculated relative to the baseline value. Stability data is shown in Tables 15 -17 below.

The low sample was a fresh human specimen with a concentration of ~4 U/L. In order to establish a mid and high range sample for this analysis, 2 separate frozen human samples with a known high GLDH concentration were spiked into a fresh, normal human serum sample

An additional serum sample set was used to assess refrigerated stability (2-8°C) up to 28 days. In order to establish a low sample above baseline (>3 U/L), a mid and high sample, samples with known GLDH concentrations were spiked into a fresh, normal human serum sample.

Conclusion: Most samples had appropriate percent recovery (80 – 120%); based upon the results of this testing and the acceptance criteria stated in Table 1, GLDH in human serum demonstrates acceptable stability for room temperature up to 48 hours, refrigerated up to 14 days, and frozen up to 18 months. The variability of Human 057, 058, and 059 is due to the very low concentration of GLDH, the actual variation between points is relatively minor and likely to be of little biologic relevance. Human 059 had a percent recovery of 157% at the 1 month stability timepoint. Close examination of individual data points showed only small differences (≤ 2 U/L) as detailed in Tables 16 -18 below.

Table 16 . Summary of Room Temperature Stability for GLDH in Human Serum

Room Temperature	Human 057 (U/L)	Mean	% Recovery	Human 060 (U/L)	Mean	% Recovery	Human 063 (U/L)	Mean	% Recovery
Neat	4	3	100	68	67.5	100	274	273	100
	2			67			272		
4 Hour	4	3.5	117	66	66	98	271	271	99
	3			66			271		
24 Hour	4	4.5	150	62	62	92	262	262	96
	5			62			262		
48 Hour	2	2.5	83	60	60.5	90	259	261.5	96
	3			61			264		
72 Hour	5	5	167	53	53	79	228	229.5	84
	5			53			231		
96 Hour	3	3.5	117	50	50	74	216	217.5	80
	4			50			219		

Table 17. Summary of Refrigerated Stability for GLDH in Human Serum

Refrigerated	Human 058 (U/L)	Mean	% Recovery	Human 061 (U/L)	Mean	% Recovery	Human 064 (U/L)	Mean	% Recovery
Baseline	4	3	100	68	67.5	100	274	273	100
	2			67			272		
4 Hour	2	3	100	65	66.5	99	271	270.5	99
	4			68			270		
24 Hour	4	4.5	150	67	68	101	274	273.5	100
	5			69			273		
48 Hour	3	3	100	69	68	101	281	281.5	103
	3			67			282		
72 Hour	5	4.5	150	64	63	93	262	261.5	96
	4			62			261		
96 Hour	4	3.5	117	64	64	95	264	264	97
	3			64			264		
Refrigerated	Human 001 (U/L)	Mean	% Recovery	Human 002 (U/L)	Mean	% Recovery	Human 003 (U/L)	Mean	% Recovery
Baseline	10	10.0	100	75	74.0	100	259	259.0	100
	10			73			259		
1 Day	12	12.0	120	70	70.5	95	244	246.0	95
	12			71			248		
2 Day	12	12.5	125	75	75.5	102	261	261.5	101
	13			76			262		
4 Day	13	12.0	120	76	76.0	103	261	260.5	101
	11			76			260		
8 Day	10	10.5	105	65	66.5	90	234	232.0	90
	11			68			230		
10 Day	10	10.0	100	70	69.0	93	237	235.5	91
	10			68			234		
14 Day	10	9.5	95	66	66.5	90	241	238.0	92
	9			67			235		
21 Day	11	10.5	105	62	62.0	84	241	238.0	92
	10			62			235		
28 Day	11	9.5	95	60	58.5	79	205	200.5	77
	8			57			196		

Table 18. Summary of Frozen Stability for GLDH in Human Serum

Frozen	Human 059 (U/L)	Mean	% Recovery	Human 062 (U/L)	Mean	% Recovery	Human 065 (U/L)	Mean	% Recovery
Baseline	4	3.5	100	68	67.5	100	274	258	100
	3			67			242		
7 Days	3	3.5	100	68	68.5	101	273	272	105
	4			69			271		
14 Days	4	4	114	67	66.5	99	267	270	105
	4			66			273		
1 Month	6	5.5	157	64	64.5	96	261	262	102
	5			65			263		
3 Months	3	3	86	66	64.5	96	266	264.5	103
	3			63			263		
6 Months	<3	<3	NA*	66	66	98	271	271	105
	<3			66			271		
12 Months	4	3.5	100	64	64.5	94	255	256	94
	3			65			257		
18 Months	4	3.5	100	64	64	96	263	263	97
	3			64			262		

*****not calculated due to all samples below the lower assay linearity of 3

3.9. Requirement 9 – Interference

Matrix components can potentially interfere with assay performance. Variable matrix-related interferences were evaluated including hemolysis, lipemia, and icterus. This was achieved by spiking a high GLDH pooled human sample with interferent.

A pair of test interference samples was prepared at 6 different interferent concentrations. A serum sample with a high GLDH value was used to prepare the test interference samples. The pooled serum sample spiked with interferent at 1 of 6 different concentrations was run in parallel with the same high GLDH pooled serum sample spiked with a serum sample with a GLDH value <3 U/L at the same volume as the interferent. Both samples were analyzed and compared to each other. Results were deemed acceptable if ≥80% of the samples tested resulted in %CV of ≤30% and were within ±30% of the respective nominal concentrations.

For evaluation of hemolysis interference, human EDTA whole blood was collected and washed twice with saline. The blood was lysed by adding deionized water. This was then spun down to pellet white blood cell (WBC) and red blood cell (RBC) debris and the supernatant was collected. The supernatant was analyzed on an Advia 120 hematology instrument in order to determine the hemoglobin value, which was measured at 11000 mg/dL. To achieve icterus, a Maine Standards GC4 level 5 linearity material (cat # 1400sa, lot # AL2881305) containing a known concentration of total bilirubin (36 mg/dL), was spiked into the pooled patient sample. A commercial source of lipemic material, Intralipid (Sigma # I141-100ml) a 20% emulsion of phospholipid stabilized soybean oil, was used in order to evaluate lipemia interference. The triglyceride value, of the Intralipid, measured at 44210 mg/dL, was determined by analyzing various dilutions of the solution on an Advia 1800 chemistry analyzer.

A pair of test interference samples was prepared for each level. The first sample contained the interfering material. The second sample contained an equal volume of normal human serum. Both samples were analyzed and compared to each other.

Conclusion: All samples met the acceptance criteria with the exception of lipemia. There is interference with GLDH testing in samples with high concentrations of lipemia tested on the Advia 1800 analyzer (++++; marked lipema). This is in agreement with the manufacturer's reagent package insert. For the Lipemia interference, the 1288 Spiked Triglyceride (mg/dL) level percent CV and RE were within acceptability criteria however the results were not accepted due to the presence of a system generated "K" flag.

The Siemens Advia 1800 analyzer has flagging capabilities to detect when maximum absorbance limits are exceeded. This "K" flag is linked to the maximum absorbance limit field for the GLDH reaction. The Advia 1800 chemistry analyzer will automatically flag GLDH results with the letter "K" when high levels of lipemia are present and/or if maximum absorbance limits of 2.5 optical density are exceeded. Any GLDH result with a "K" flag is not reportable and these results will be rejected.

Table 19. Summary of Hemolysis Interference Study for GLDH in Human Serum

Spiked Hemoglobin (mg/dL)	System Flags	Comment	%CV	%RE
27	+	Slight	2.6	3.7
109	+	Slight	1.0	1.4
320	+++	Marked	0.7	0.9
524	++++	Marked	2.4	3.3
1000	++++	Marked	5.8	7.9
2538	++++	Marked	12.0	15.7

Table 20. Summary of Lipemia Interference Study for GLDH in Human Serum

Spiked Triglyceride (mg/dL)	System Flags	Comment	%CV	%RE
55	++	Moderate	0.5	0.7
110	++	Moderate	0.4	0.5
220	++	Moderate	1.0	1.4
438	+++	Marked	1.6	2.3
1288	++++*	Marked	10.1	13.4
2105	++++*	Marked	48.3	50.9

*with a "K" flag

Table 21. Summary of Icteric Interference Study for GLDH in Human Serum

Spiked Total Bilirubin (mg/dL)	System Flags	Comment	%CV	%RE
1.1	+	Slight	0.3	0.4
1.7	+	Slight	0.4	0.5
3.3	++	Moderate	1.1	1.5
8.3	++	Moderate	1.6	2.2
10.3	++++	Marked	4.1	5.7
18	++++	Marked	4.7	6.4

3.10. Proficiency Testing (PT)

Currently, there is no external PT program for GLDH, so an alternative proficiency testing was performed. Alternative PT methods may include: replication of results by reanalysis on a stored sample, technologist-to-technologist comparison, instrument-to-instrument comparison, split sample analysis with reference or other laboratories, split samples with an established in-house method, assayed materials, clinical validation by chart review, or other suitable and documented means. The laboratory director and clinical pathology manager defined the alternative assessment procedure and the acceptability criteria for successful performance in accordance with good clinical and scientific laboratory practice. Randox calibration material was reconstituted to yield two to three differing concentrations of GLDH. Acceptablility ranges for GLDH proficiency testing samples were established by using similar ranges as those utilized for proficiency testing samples of standard liver enzymes by the College of American Pathologist.

Conclusion: Proficiency testing for 2016 and 2017 was performed on the Siemens Advia 1800 clinical chemistry analyzer. Samples were blinded, processed and analyzed in the same manner patient samples are analyzed. All proficency samples were within the acceptable range for all concentrations.

Table 22. Summary of GLDH Proficiency Testing

Date	GLDH Proficiency Sample	GLDH Result (U/L)	Acceptable Range (U/L)	Pass/Fail
4-May-16	Sample A	27.8	24.0 - 36.0	Pass
4-May-16	Sample B	16.8	12.0 - 18.0	Pass
20-Feb-17	Sample A	9.0	6.0 - 12.0	Pass
20-Feb-17	Sample B	19.0	15.0 - 21.0	Pass
20-Feb-17	Sample C	35.0	31.0 - 41.0	Pass

4. USER ACCEPTANCE

This memo fully documents that testing is complete and meets the acceptance criteria specified in section 2, and that GLDH assay in human serum is released for use in the Groton Clinical Pathology Laboratory on the Advia 1800.

5. END USER TRAINING

Scientists performing these assays will require documented training prior to analysis of GLP/GCLP samples.

All general users of the Advia 1800 instrument have been trained by a key operator, in accordance with the Groton Clinical Pathology Laboratory Quality Manual and global and SOP titled "*PERS-001 Training and Education Program"*. Training has been documented in the colleague's training file. Scientists will not require re-training to perform this procedure.

6. ARCHIVING

All supporting scientific (hard copy) data and a hard copy of the final report will be archived in the DSRD Document Archive. Electronic records of some aspects of the requalification may be retained in the laboratory as an easily accessible resource for scientists.

7. REFERENCES

1 Assay Validation Memo for Glutamate Dehydrogenase on the Advia 2400 Chemistry Analyzer (Serial Number CA12420078), dated 20-August-2009, located at: http://insight.pfizer.com/livelink/llisapi.dll?func=ll&objId=73917790&objAction=browse&sort=name&viewType=1

2 Biomarker Requalification Guidelines, version 1.0, located at: http://ecf.pfizer.com/sites/DSRDBiomarkers/LAB-USE/Requalification%20Templates/Forms/AllItems.aspx

3 GLDH Package Insert, revised 14-Nov-08, Randox Laboratories Ltd., Co. Antrim, UK.

4 Validation of Laboratory-Developed Molecular Assays For Infectious Diseases; Burd, Eileen M., Clinical Microbiology review, July 2010, Vol.23, No.3. , Emory University Hospital, Atlanta Georgia, p. 550-576.

5 Mastorodemos, V., *et. al.* (2005).Molecular basis of human glutamate dehydrogenase regulation under changing energy demands. *J. Neurosci. Res*. 79(1-2):65-73.

6 O'Brien, P.J., *et. al*. (2002). Advantages of glutamate dehydrogenase as a blood biomarker of acute hepatic injury in rats. *Lab. Anim.* 36, 313–321.

7 Giffen, P.S. *et. al*.(2003). Alpha-gluthatione-S-transferase in the assessment of hepatotoxicity: Its diagnostic utility in comparison with other recognized markers in the Wistar Han Rat.- *Toxicol. Pathol*. 30, 365-372.

8 Kretzshmar, M. *et. al.* (2003). Hepatic ischemia-reperfusion syndrome after partial liver resection (LR): Hepatic venous oxygen saturation, enzyme pattern, reduced and oxidized gluathione, procalcitonin, and interleukin-6. *Exp. Toxic. Pathol*. 54, 423-431.

9 Schmidt, E.S., *et. al*. (1988). Glutamate dehydrogenase: Biochemical and clinical aspects of an interesting enzyme. *Clin. Chim. Acta*. 173 (1):43-55.

10 Schomaker, S. *et. al*. (2013). Assessment of emerging biomarkers of liver injury in human subjects. *Tox. Sci*. 132(2):276-83.

11 Harrill, A.H. *et. al* (2012).The effects of heparins ont he liver: Application of mechanistic serum biomarkers in a randominzed study in healthy volunteers *Clin. Pharmacol. Ther*. 92, 214-220.

12 Customer Bulletin, 2008-06. New serum indices normalized for Advia chemistry systems

13 Westgard et al. Basic method validation; Interference and recovery experiments; lesson 27. Retreived at https://www.westgard.com/lessons.htm.

14 Dimiski, G. (2008) Interference Testing; *Clin. Biochem. Rev*, Vol 29, Suppl (1) S43-48.

15 Advia Chemistry; Customer Bulletin 2012-07; Manually rerunning sample results marked with a K flag.

16 Armbruster, D.A., and Pry, T. (2008) Limit of blank, limit of detection, and limit of quantitation. *Clin. Biochem. Rev*. Vol. 29, Suppl.(1) p. S49-52.

17 Burd, E.M. (2010). Validation of laboratory-developed molecular assasy for infectious diseases. *Clinical Microbiology Reviews*. Vol. 23, No. 3, p. 550-576.

18 Clinical and Laboratory Standards Institute. Protocols for Determination of Limits of Detection and Limits of Quantitation, Approved Guideline. CLSI document EP17. Wayne, PA USA: CLSI; 2004.

8. CHANGE LOG

Version	Author	Change Description	Reason / Comment
2.0	Abigail Bull	Updated to include testing and results for additional work performed to extend the frozen serum stability to 3 and 6 months. Also included extended frozen stability timepoint of 18 months.	Updated frozen stability
3.0	Johanna Wisniewski	Additional 12 and 18 month frozen stability data added Updated to included extended refrigerated stability to 14 days Original Stability	Additional stability studies have been completed and summarized. Stability tables 15 - 17 were corrected, amended, and inserted. There is no impact

		tables were calculated from a single baseline value, not the mean. Tables were corrected and replaced in this validation memo amendment.	as a result of this change.
4.0	Johanna Wisniewski	Updated Table 1. Requirement 1: Values for LOB and added LOD Requirement 3: Removed accuracy (trueness) from the description Requirement 5: Added accuracy (trueness) to the description Requirement 9: Analytical Specificity/Interference Added Requirement 10 Section 3.2 Precision section updated	 Requirement 1: LOB was incorrectly defined as 2.8 U/L, the correct value was 1.0 U/L according to the definitions established in Table 1. Requirement 3: Method to Method Comparison was the expected and tested strategy for requirement 3. Requirement 5: Recovery experiments are performed to also test the ability of the assay to measure the “true” known concentration of the analyte in the sample. Added clarity around patient pools in the testing strategy Proficiency testing requirement added for amendment 3.0 Limit of Detection (LOD) was replaced by Near Detection

			Level (NDL) to accurately define the sample concentration Added a mid and Cal 3 sample concentration for precision
		Section 3.5 Accuracy/Recovery updated	Added Human 054 spiked with Conc. Calibrator 3
		Section 3.9 Interference updated	Clarified the K flag verbiage for lipemia interference in the conclusion
		Added Section 3.10	New section for Proficiency testing
		Updated references	Added reference titles and standardized formatting

XII. APPENDIX III. Study Protocol Synopses

PROTOCOL SYNOPSIS

CONFIRMATION OF THE PERFORMANCE OF SERUM GLUTAMATE DEHYDROGENASE (GLDH) AS BIOMARKER OF LIVER INJURY IN HUMANS

Table of Contents

1 OBJECTIVES AND ENDPOINTS

The goal of this study is to confirm the performance of GLDH as a biomarker of liver injury, as well as to confirm the performance of GLDH thresholds of concern to identify subjects with liver injuries. In this study, the serum levels of GLDH and ALT will be evaluated in healthy subjects and subjects with liver injury featuring a wide variety of etiologies. Blood samples will be collected at the University of Michigan health care system (UM) under an approved IRB (HUM-44422), with the exception of samples taken from subjects with medically adjudicated liver injury in two investigational trials. The specific objectives and endpoints are outlined below in Table 1.

Table 1 Objectives and Corresponding Endpoints

	Corresponding Endpoints
Primary Objective:	
Confirm the performance of GLDH thresholds of concern to identify subjects with liver injuries.	• Serum ALT • Serum GLDH
Additional Analysis:	
Assess the performance of GLDH thresholds of concern across each disease state and drug treatment.	• Serum ALT • Serum GLDH

2 STUDY DESIGN

2.1 Description of the Study

Serum samples from healthy subjects and subjects with liver injury will be selected as described in detail below. Briefly, subjects will be defined as healthy based on normal levels of ALT, AST, ALP, total bilirubin, glucose, blood urea nitrogen, serum creatinine and CK. Hepatic injury subjects will be selected based on elevated levels of ALT, AST, ALP, or total bilirubin. Subjects will be further classified as having liver injury if they meet the clinical chemistry criteria of DILI. The criteria include the manifestation of one or more of the following: ≥5x ALT, or ≥2x ALP, or ≥3x ALT and ≥2x total bilirubin. The ability of GLDH to substitute for ALT in the determination of liver injury will be confirmed.

2.2 Number of Patients

Approximately 200 samples from heathy subjects and 200 samples from subjects with liver injury, including subjects with acetaminophen overdose and two additional investigational drugs, will be collected for the study. 200 subjects in each group should provide adequate power to achieve the target success criteria for sensitivity and specificity described in the Statistical Methods section. Specifically, assuming a Beta (5,6) distribution of sensitivity and specificity values over the range of 87.5% to 100%, reflecting our belief in the true values of these performance measures, we computed the average power to meet our primary objectives.

200 subjects per group yields approximately 90% power to achieve the success criteria for each measure (sensitivity, specificity).

2.3 Target Population

Heathy subjects and subjects with clinically demonstrable liver injuries could include, but may not limited to, those with hepatic carcinoma (diagnosed by biopsy or histopathology after resection), cirrhosis, liver impairment (Hepatitis B or C, hepatic graft vs host disease, ethanol cirrhosis, drug abuse, transaminitis/hepatic congestion, accidental acetaminophen overdose), and 2 investigational drugs with cases of medically adjudicated liver injury in clinical trials. It should be noted that additional co-morbidities might be present and will be documented.

2.3.1 Inclusion Criteria

Subjects must meet the following criteria to be eligible for study entry:

- Age at least 2 years.
- Blood samples will be collected at the University of Michigan health care system (UM) under an approved IRB (HUM-44422), with the exception of samples taken from subjects with medically adjudicated liver injury in two investigational trials.
- Healthy subjects will be selected using the following criteria:
    - Normal levels of ALT, AST, ALP, total bilirubin, glucose, blood urea nitrogen, serum creatinine and creatine kinase. In addition, subjects will be classified as healthy by medical adjudication.
- Subjects with liver injury will be selected using the following criteria:
    - Subjects classified as having liver injury by meeting the clinical chemistry criteria of DILI (≥5x ALT, or ≥2x ALP, or ≥3x ALT and ≥2x total bilirubin). These criteria have been derived from the Expert Liver Working Group (EWG) definition of liver injury. Subjects with clinically demonstrable liver injuries could include, but may not limited to, those with hepatic carcinoma (diagnosed by biopsy or histopathology after resection), cirrhosis, and liver impairment (Hepatitis B or C, hepatic graft vs host disease, ethanol cirrhosis, drug abuse, transaminitis/hepatic congestion, accidental acetaminophen overdose). Additional co-morbidities might be present. The status of liver injury will be determined by medical adjudication.

2.3.2 Exclusion Criteria

Samples from subjects who meet any of the following criteria will be excluded from study entry:

- Subjects with ongoing health problems or immunological flares that could influence liver health as determined by medical adjudication.
- Subjects with muscle injury as determined by medical adjudication.

- Subjects with pancreatic, kidney, or gastrointestinal injury as determined by medical adjudication may be included based on the outcome of the pancreatic, kidney, or gastrointestinal injury specificity study (STUDY 3: CONFIRMATION THAT SERUM GLUTAMATE DEHYDROGENASE (GLDH) IS UNAFFECTED BY PANCREATIC, GASTROINTESTINAL AND KIDNEY INJURIES IN HUMANS).

3 Statistical Methods

3.1 Primary Analysis

Construct 2x2 contingency tables of the EWG definition with ALT compared to the EWG definition with GLDH. Compute measures of concordance, sensitivity, and specificity of the GLDH-based EWG definition of liver injury, defined in Section 2, to predict ALT-based EWG definition of liver injury, using proposed GLDH thresholds and computed ALT thresholds determined in the exploratory studies. The target success for each measure is ≥ 0.90, 95% Lower Confidence Bound ≥ 0.85.

3.2 Additional Analysis: Assess the performance of GLDH thresholds of concern across each disease state and drug treatment

Same analysis as described above (Section 3.1) will be conducted for each individual disease state and drug treatment. The target success for each measure is ≥ 0.90.

CHANGE LOG for Version Dated 11Dec2107

Section	Change Description	Reason/Comment
2.2 Number of Patients	Number of Patients changed from 400 healthy subjects and 400 subjects with liver injury to 200 per group	Updated based on statistical considerations.
2.3.1 Inclusion Criteria	Age changed from 15-70 years of age to age at least 2 years. Added wording to include the inclusion/exclusion of subjects based on medical adjudication.	Updated based on relevance of ages. Updated for accuracy and clarity.
2.3.2 Exclusion Criteria	Added wording regarding the inclusion of subjects with pancreatic, kidney, and gastrointestinal injury.	Updated so that subjects with pancreatic, kidney, and gastrointestinal injury could potentially be included based on the outcome of Study 3.
3.1 Primary Analysis	Modified statistical method.	Updated so that EWG-based definitions of liver injury use ULN values for GLDH and ALT previously established for learning phase, rather than based on confirmatory study data.

PROTOCOL SYNOPSIS

CONFIRMATION THAT SERUM GLUTAMATE DEHYDROGENASE (GLDH) IS UNAFFECTED BY MUSCLE INJURY IN HUMANS

Table of Contents

1 OBJECTIVES AND ENDPOINTS

The goals of this study are to confirm serum GLDH levels are unaffected by muscle injury in humans, and that GLDH outperforms ALT with regard to the specificity for liver injury. The study will compare levels of GLDH and ALT in healthy subjects and subjects with muscle impairments featuring a wide variety of etiologies. Blood samples will be collected at the University of Michigan health care system (UM) under an approved IRB (HUM-44422). The specific objectives and endpoints are outlined below in Table 1.

Table 1 Objectives and Corresponding Endpoints

	Corresponding Endpoints
Primary Objective:	
Confirm that serum GLDH does not increase in subjects with muscle injury, and that GLDH exhibits improved liver injury specificity, relative to ALT.	• Serum GLDH • Serum ALT
Additional Analysis:	
Qualitative analysis: Compare relationship between GLDH and CK to the relationship between ALT and CK.	• Serum GLDH • Serum ALT • Serum CK

2 STUDY DESIGN

2.1 Description of the Study

Serum samples from subjects with muscle injury will be selected based on creatine kinase (CK) levels greater than two times normal healthy levels or based on a diagnosed muscle injury. The serum concentration of GLDH from subjects with muscle injury will be compared to healthy volunteer samples in order to confirm that muscle injury does not affect serum GLDH levels, and thereby does not interfere with the ability of GLDH to detect liver injury.

2.2 Number of Patients

Approximately 120 samples from subjects with muscle injury will be enrolled in the study. 120 subjects should provide adequate power to achieve the target success criteria for the Primary Analysis false positive rate described in the Statistical Methods section. Specifically, assuming a Beta (5,10) distribution of false positive rates over the range of 0% to 10%, reflecting our belief in the true values of this performance measure, we computed the average power to meet our primary objective. 120 subjects yield approximately 85% power. Note that this sample size calculation was based on expected false positive rates relative to the 2.5X ULN cutoff.

2.3 Target Population

Subjects with muscle impairments resulting from a wide variety of etiologies determined by medical adjudication.

2.3.1 Inclusion Criteria

Subjects must meet the following criteria to be eligible for study entry:

- Age at least 2 years.
- Blood samples will be collected at the University of Michigan health care system (UM) under an approved IRB (HUM-44422).
- Healthy subjects will be selected using following criteria:
    - Normal levels of ALT, AST, ALP, total bilirubin, glucose, blood urea nitrogen, serum creatinine and creatine kinase (CK). In addition, subjects will be classified as healthy by medical adjudication.
- Subjects with muscle impairments will be selected using the following criteria:
    - Diagnosed by either i) medical adjudication, ii) a muscle biopsy, iii) genetic testing or iv) clinically determined injuries, which may include, but are not limited to, Primary Disorders of Muscle (Dystrophies, Myotonic Disorders, Congenital Myopathies and Mitochondrial Myopathies) and Toxic Myopathies (Drug, Alcohol and Toxicants), as exhibited by, myositis (inflammatory muscle injury), neurogenic atrophy, necrotizing inflammatory muscle injury, chronic severe atrophy, AAF, type II fiber atrophy, nuclear myobags, denervation atrophy, and increased lipids in myofibers.

2.3.2 Exclusion Criteria

Samples from subjects who meet any of the following criteria will be excluded from study entry:

- Subjects with ongoing health problems or immunological flares that could influence liver health as determined by medical adjudication.
- Subjects classified as having clinically demonstrable liver injury.
- Subjects with total bilirubin, ALP or GGT above the normal healthy range and/or evidence of liver injury in the medical records. Thiscriteria is more stringent than that used for the learning phase and is based on the outcome of the learning phase data and input from the regulators.
- Subjects with pancreatic, kidney, or gastrointestinal injury as determined by medical adjudication may be included based on the outcome of the pancreatic, kidney, or gastrointestinal injury specificity study (STUDY 3: CONFIRMATION THAT SERUM GLUTAMATE DEHYDROGENASE (GLDH) IS UNAFFECTED BY PANCREATIC, GASTROINTESTINAL AND KIDNEY INJURIES IN HUMANS).
- Subjects with elevations in ALT and AST without evidence of liver injury **will not be excluded**.

3 STATISTICAL METHODS

3.1 Primary Analysis

The primary endpoint is the false positive rate (FPR), i.e., the % of subjects exceeding a prespecified threshold. In the first analysis, the percentage of subjects with GLDH values exceeding ULN (10), 2.5X ULN (25) and 5X ULN (50) will be computed. The target success criteria are:

- ULN: FPR ≤ 10%
- 2.5X ULN: FPR ≤ 5%
- 5X ULN: FPR ≤ 1%

These target percentages reflect a balance between how GLDH will be utilized in drug development trials, and a recognition that (1) there will be sampling variability (i.e., though we anticipate the % of subjects < ULN to be around 2.5% by definition, the observed percentage from a given sample will vary, especially with a limited sample size); and (2) even with the robust inclusion/exclusion criteria, subjects with comorbidities that impact GLDH values may be enrolled into this study.

In the second analysis, subjects will be classified as to whether they exceed the ULN for GLDH and whether they exceed the ULN for ALT. McNemar's Test for Correlated Proportions will be used to test for a difference in the proportions. The target success criterion is:

- [% of subjects > ULN GLDH] significantly ($p<0.05$) lower than [% of subjects > ALT GLDH]

Note that because both GLDH and ALT are measured for each subject, McNemar's Test is more appropriate than the usual tests (e.g., Chi-Squared Test) to compare two proportions, which assume independence of the samples.

3.2 Additional Analysis

Qualitative Analysis: Scatterplots of GLDH vs. log(CK), and log(ALT) vs log(CK), will be produced. The Pearson correlation coefficient between GLDH and log(CK), and between log(ALT) and log(CK), will be computed and compared.

CHANGE LOG for Version Dated 11Dec2107

Section	Change Description	Reason/Comment
1 Objectives and Endpoints	Added wording to objective.	Updated for clarity.
2.1 Description of the Study	Deleted parts of the description of subjects	Detailed description is provided in Section 2.3.1 Inclusion Criteria.
2.2 Number of Patients	Number of Patients changed from 200 with muscle injury to 120.	Updated based on statistical considerations.
2.3.1 Inclusion Criteria	Age changed from 15-70 years of age to age at least 2 years. Added wording to include the inclusion/exclusion of subjects based on medical adjudication.	Updated based on relevance of ages. Updated for accuracy and clarity.
2.3.2 Exclusion Criteria	Added wording regarding the inclusion of subjects with pancreatic, kidney, and gastrointestinal injury.	Updated so that subjects with pancreatic, kidney, and gastrointestinal injury could potentially be included based on the outcome of Study 3.
3.1 Primary Analysis	Modified statistical method.	Updated to include a direct statistical comparison of GLDH and ALT.

PROTOCOL SYNOPSIS

CONFIRMATION THAT SERUM GLUTAMATE DEHYDROGENASE (GLDH) IS UNAFFECTED BY PANCREATIC, GASTROINTESTINAL AND KIDNEY INJURIES IN HUMANS

Table of Contents

1 OBJECTIVES AND ENDPOINTS

The goal of this study is to confirm that GLDH does not increase with pancreatic, gastrointestinal, or kidney injury. The study will compare GLDH levels from subjects with pancreatic, gastrointestinal, or kidney injury to healthy volunteers. Blood samples will be collected at the University of Michigan health care system (UM) under an approved IRB (HUM-44422). The specific objectives and endpoints are outlined below in Table 1.

Table 1 Objectives and Corresponding Endpoints

	Corresponding Endpoints
Primary Objective:	
Confirm that pancreatic, gastrointestinal, and kidney injury does not increase serum GLDH.	• Serum GLDH

2 STUDY DESIGN

2.1 Description of the Study

Serum samples from subjects with pancreatic, gastrointestinal or kidney injury will be selected as described in detail below. Briefly, subjects with acute and chronic pancreatitis, gastrointestinal abnormalities, and Chronic Kidney Disease (CKD) will be included in the study. The serum concentration of GLDH from subjects with pancreatic, gastrointestinal, or kidney injury will be compared to healthy volunteer samples in order to confirm that pancreatic, gastrointestinal, or kidney injury does not affect serum GLDH levels, and thereby does not interfere with the ability of GLDH to detect liver injury.

2.2 Number of Patients

Approximately 200 samples from subjects with pancreatic, gastrointestinal, and kidney injury be enrolled in the study. A sample size as low as 50 subjects in any of the three disease groups should provide modest power to achieve the target success criteria for the Primary Analysis false positive rate described in the Statistical Methods section. Specifically, assuming a Beta(5,10) distribution of false positive rates over the range of 0% to 10%, reflecting our belief in the true values of this performance measure, we computed the average power to meet our primary objective. 50 subjects yield approximately 75% power. Note that this sample size calculation was based on expected false positive rates relative to the 2.5X ULN cutoff.

2.3 Target Population

Subjects with pancreatic, gastrointestinal, and kidney impairments resulting from a wide variety of etiologies determined by medical adjudication.

2.3.1 Inclusion Criteria

Subjects must meet the following criteria to be eligible for study entry:

- Age at least 2 years.

- Blood samples will be collected at the University of Michigan health care system (UM) under an approved IRB (HUM-44422).

  Subjects with pancreatic Injury will be medically adjudicated as having pancreatitis (Acute, Chronic, Hereditary) that is diagnosed by either i) Persistent Severe Epigastric Pain, ii) Diagnostic Armamentarium [Endoscopic Ultrasound (ES), Magnetic Resonance Cholangiopancreatography (MRCP), Computerized Tomography (CT) or Transabdominal ultrasound] iii) Clinically Demonstrable Deficiencies or iv) Amylase or Lipase 3X ULN.

- Subjects will be medically adjudicated as having gastrointestinal abnormalities diagnosed by either i) Endoscopy, ii) Sigmoidoscopy or iii) Colonoscopy, or iv) Clinically Demonstrable Deficiencies, which could include, but is not limited to, Gastroesophageal Reflux Disease (GERD), Esophagitis, Irritable Bowel Syndrome (IBS), Celiac Disease, Crohn's Disease, Ulcerative Colitis, Ulcerative Pancolitis, Ulcerative Proctosigmoiditis and Appendicitis.
- Subjects will be medically adjudicated as having Chronic Kidney Disease (CKD) diagnosed by either i) Biopsy-Proven or ii) Clinically Demonstrable Deficiencies, which could include, but are not limited to, Diabetes, High Blood Pressure, Glomerulonephritis, Interstitial Nephritis, Polycystic Kidney Disease and Malformations, as exhibited by, CKD stage II – V, End Stage Renal Disease (ESRD) and patients on Dialysis.

2.3.2 Exclusion Criteria

Samples from subjects who meet any of the following criteria will be excluded from study entry:

- Subjects classified as having clinically demonstrable liver injury.
- Subjects with ALT, AST, total bilirubin, ALP or GGT above the normal healthy range or evidence of liver injury in the medical records. This criteria is more stringent than that used for the learning phase and is based on the outcome of the learning phase data and input from the regulators.
-

3 STATISTICAL METHODS

3.1 Primary Analysis

The primary endpoint is the false positive rate (FPR). More specifically, the percentage of subjects with GLDH values exceeding 2.5X ULN (25) and 5X ULN (50) will be computed. The target success criteria are:

2.5X ULN: FPR ≤ 5%

5.X ULN: FPR ≤ 1%

CHANGE LOG for Version Dated 11Dec2107

Section	Change Description	Reason/Comment
2.1 Description of the Study	Deleted parts of the description of subjects	Detailed description is provided in Section 2.3.1 Inclusion Criteria.
2.2 Number of Patients	Additional detail added.	Updated based on statistical considerations.
2.3.1 Inclusion Criteria	Age changed from 15-70 years of age to age at least 2 years. Added wording to include the inclusion/exclusion of subjects based on medical adjudication.	Updated based on relevance of ages. Updated for accuracy and clarity.
3.1 Primary Analysis	Modified statistical method.	

PROTOCOL SYNOPSIS

CONFIRMATION THAT SERUM GLUTAMATE DEHYDROGENASE (GLDH) DETECTS THE ONSET OF LIVER INJURY BY ACETAMINOPHEN

Table of Contents

1 OBJECTIVES AND ENDPOINTS

The goal of this study is to confirm that GLDH detects liver injury caused by acetaminophen (APAP) overdose and that the half-life of GLDH, which is shorter than the half-life of ALT, in human serum reflects the time course of hepatocellular injury. In this study, the time course of GLDH activity in human serum will be compared with ALT activity and the medically adjudicated clinical outcome of APAP poisoning. Blood samples will be collected at the University of Michigan health care system (UM) under an approved IRB (HUM-44422). The specific objectives and endpoints are outlined below in Table 1.

Table 1 Objectives and Corresponding Endpoints

	Corresponding Endpoints
Primary Objective:	
Confirm that GLDH detects the onset of liver injury in subjects with APAP overdose.	• Serum GLDH • Serum ALT

2 STUDY DESIGN

2.1 Description of the Study

Serum samples spanning the time course of APAP overdose will be collected from subjects hospitalized for APAP intoxication at University of Michigan. The time course of GLDH activity in human serum will be compared with ALT activity and the medically adjudicated clinical outcome of APAP poisoning.

2.2 Number of Patients

Approximately 15 cases of APAP overdose will be evaluated.

2.3 Target Population

Subjects with accidental APAP overdose admitted and treated at University of Michigan.

2.3.1 Inclusion Criteria

Subjects must meet the following criteria to be eligible for study entry:

- Age at least 2 years.
- Subjects with medically adjudicated signs of APAP overdose that include elevated levels of ALT greater than the ULN.
- Blood samples will be collected at the University of Michigan health care system (UM) under an approved IRB (HUM-44422).

2.3.2 Exclusion Criteria

Samples from subjects who meet any of the following criteria will be excluded from study entry:

- Subjects with ongoing health problems or immunological flares that could influence liver health as determined by medical adjudication.
- Subjects with muscle injury as determined by medical adjudication.
- Subjects with pancreatic, kidney, or gastrointestinal injury, as determined by medical adjudication, may be included based on the outcome of the pancreatic, kidney, or gastrointestinal injury specificity study (STUDY 3: CONFIRMATION THAT SERUM GLUTAMATE DEHYDROGENASE (GLDH) IS UNAFFECTED BY PANCREATIC, GASTROINTESTINAL AND KIDNEY INJURIES IN HUMANS).

3 STATISTICAL METHODS

3.1 Primary Analysis

Compare each subject's GLDH level at admission to the hospital to the ULN.

Target success criteria: all subjects have GLDH greater than ULN.

CHANGE LOG for Version Dated 11Dec2107

Section	Change Description	Reason/Comment
2.3.1 Inclusion Criteria	Age changed from 15-70 years of age to age at least 2 years.	Updated based on relevance of ages.
2.3.2 Exclusion Criteria	Added wording regarding the inclusion of subjects with pancreatic, kidney, and gastrointestinal injury.	Updated so that subjects with pancreatic, kidney, and gastrointestinal injury could potentially be included based on the outcome of Study 3.

CRITICAL PATH INSTITUTE
collaborate · innovate · accelerate

May 22, 2018

Ms. Beth Walton
Regulatory Project Manager
Office of New Drugs (OND)
Biomarker Development and Regulatory Science

Dear Ms. Walton,

On behalf of the Hepatotoxicity Working Group (HWG) of the Critical Path Institute's Predictive Safety Testing Consortium (C-Path PSTC), we are pleased to submit this Legacy Biomarker Qualification Project Transition Summary to the FDA for DDT #DDTBMQ000050. As part of our ongoing efforts to augment translational biomarker tools for drug-induced liver injury (DILI), the HWG is proposing to qualify serum Glutamate Dehydrogenase (GLDH) activity as a marker of liver injury in human subjects with ALT elevations from suspected extrahepatic sources such as muscle. GLDH activity is proposed to be utilized as a complement to the existing guidance for assessing DILI.

As summarized in the Transition Summary, several clinical studies have been conducted and analyzed that support our proposed context of use. GLDH is not a novel biomarker, is commonly utilized by some drug developers, and is highly valuable in specific drug-development scenarios for interpreting ALT elevations. Through regulatory qualification, our hope is that GLDH will be routinely incorporated into the toolbox for assessing risk of severe DILI.

We look forward to any questions or comments FDA. Please feel free to contact me with any questions regarding this submission.

Most sincerely,

Nicholas M.P. King, MS
Senior Project Manager, Predictive Safety Testing Consortium (PSTC), Critical Path Institute

John-Michael Sauer, PhD
Executive Director, Predictive Safety Testing Consortium (PSTC), Critical Path Institute

1730 E River Rd, Suite 200
Tucson, AZ 85718
T 520.547.3440 F 520.547.3456
c-path.org