Joe:

I'm sending this again.   This time in Unicode format.   Hopefully, one will
work.

1. Phase II Specific Aims

a. Background and Introduction

This proposal addresses the development of a bioluminescence biosensor
for both "total " blood galactose and urine galactose. The biosensor would
be used to screen newborns for galactosemia, for disease management and for
use by researchers working on new approaches to improve disease management.
The biochemistry of galactose metabolism is summarized in Figure 1. The
current literature addressing the diagnosis and clinical management of
galactosemia [1,2,6-13] and our recent discussions with several clinicians
and experts [3-6] (see consultants) suggests that the major immediate need
is for a sensor for both erythrocyte galactose-1-phosphate (Gal-1-P) and
"free" or plasma galactose AND a sensor for urine galactose. There is likely
to be a need for a second generation sensor which also measures urine
galactitol and galactonate [1,2,15-16]. These two metabolites are produced
when blood galactose and/or Gal-1-P are elevated and, together or
individually, they may be responsible for some of the chronic effects of
galactosemia (1-2, 12-16). Although we are confident that our
bioluminescence-based sensing technology can also be applied to the
measurement of urine galactitol and galactonate, it is, at this point, not
yet clear if such measurement will indeed be necessary for the chronic
management of galactosemia. Thus, we have chosen to focus our efforts in
this Phase II application on first generation sensors for blood galactose
and Gal-1-P and urine galactose. The biochemistry and associated disposable
biosensor platform are the subject of this effort. The luminescence
detector component of the device is currently under development and is being
funded by another NIH SBIR [36].

Figures 2 and 3 depict the necessary functions and preliminary design

for the first generation blood sensor, respectively. The urine biosensor (not shown) is also a two channel device having a galactose channel and a creatinine channel, as urine measurements are normally reported relative to creatinine, to correct for dilution and volume effects. Although it is normally felt that urine galactose is not as useful as blood values for screening and early management, there is very little data with which to assess the expected correlation between urine and plasma galactose values at this time. There is growing sentiment that knowledge of urine galactose, particularly if provided together with urine galactitol and galactonate, may be suitable for the management of galactosemia [6]. This may make blood measurements unnecessary. Urine measurement was a key focus of the Phase I work. We have also made considerable progress on the analysis of blood galactose and Gal-1-P. Consequently, we have chosen to focus this Phase II project on the development of first generation sensors for both urine galactose AND blood galactose and Gal-1-P. The availability of such sensors would permit the study of the correlation between urine and blood galactose and also facilitate the eventual development of a second generation urine sensor. The blood galactose/Gal-1-P sensor would be immediately applicable to the management of galactosemia and to clinical research addressing this disease.

The Phase I research established the feasibility of using a homogeneous, multi-enzyme bioluminescence assay for galactose via galactokinase and the ATP-firefly luciferase-luciferin bioluminescence reaction to produce light that is inversely proportional to the concentration of galactose [27,30]. In the phase II effort, we will develop and optimize the biosensor for both the management of galactosemia and as a research tool for better understanding of the disease.

The blood galactose biosensor design depicted in Figures 2 and 3 is comprised of two channels, one for free plasma galactose and one for “total” galactose (plasma galactose plus galactose-1-phosphate in erythrocytes). The galactose existing as galactose-1-phosphate (Gal-1-P) can be then determined

by subtracting plasma galactose from the total galactose. In the free plasma galactose channel (Channel 1) plasma is separated from the formed elements of the blood sample and the free plasma galactose is determined using galactokinase and the firefly bioluminescence reaction. In the "total" galactose channel (Channel 2), erythrocyte Gal-1-P is released via cell lysis and then converted to galactose using a phosphatase, followed by galactose analysis as in the Channel 1 analysis.

The urine biosensor for galactose incorporates the free galactose channel approach of Figure 1 with urine as the sample rather than blood. Consequently, a blood separation means is not necessary. The basic biochemistry and subsequent bioluminescence approach are the same. The urine biosensor will also have a second channel for measuring blood creatinine for reference purposes. At this time, we have an NIH Phase I SBIR underway to develop a creatine-based bioluminescence sensor for blood and urine creatinine (1R43 DK55426-01, "Creatinine Biosensor for Renal Transplant Monitoring")

Both the urine and blood galactose biosensors will utilize a small, hand held CCD-based luminometer [36] for signal detection, processing, and recording. This device (Bio-light⍰) is currently being developed in house and is funded by another NIH SBIR ("CCD-based Analyzer for Multi-Channel Biosensor" 1R43 RR13087-1). This detector will also be applicable to the analysis of other metabolites using other specific biosensors. Figure 4 summarizes our experience with the detection of bioluminescence by various methods.

b. The Phase II Specific Aims are:

1. Optimize the individual biochemical reactions and basic functions for each of the three zones in the blood galactose biosensor. Refer to Figures 2 and 3. It is anticipated that each channel of the galactose/Gal-1-P biosensor will require about 50 μL of blood. For reference, a normal lancet derived finger tip blood drop used for glucose monitoring by Type I diabetics is 50-100 μL.

(a) Zone 1 - Blood Processing/Pre-treatment: The first step is to

lyse
erythrocytes and release galactose-1-P. Several erythrocyte lysing approaches will be evaluated, i.e., several surfactants, enzymes and commercial red cell lysing reagents will be evaluated for this purpose. Of
course, hemoglobin, ATP, ATPases and other enzymes will also be released
during lysis. The freed cellular ATP will be consumed by a variety of ATPases in the cell lysate. The galactose-1-phosphate released must be converted to galactose. Alkaline phosphatase (EC.3.1.3.1), or another suitable phosphatase, will be employed to hydrolyze the phosphoric monoester
bond of galactose-1-phosphate to produce galactose. This enzyme will also
consume any residual ATP in the lysate. We will evaluate the performance of
alkaline phosphatase from several sources for this purpose. These processes are depicted in Reactions 1 and 2 of Figure 2. Note that this
step is only required for the Gal-1-P channel.

(b) Zone 2 - Protein Removal In this region, residual cells, cell membranes
and proteins (particularly the hemoglobin and any residual ATPases) will be
largely removed by a combination of filtration and non-specific adsorption.
A variety of commercial blood cell-plasma separation materials and wicking
materials currently used for immunoassays will be evaluated for optimal
separation of components [23]. This zone may also include pre-deposited
buffers and/or precipitants to facilitate adsorption or aggregation of most
of the protein in the lysate [23].

(c) Zone 3 - Bioluminescence Signal Generation When the clarified, largely
protein free, plasma reaches this zone the galactose originating from both
Gal-1-P and free plasma galactose will be acted upon by galactokinase with
the subsequent depletion of pre-deposited ATP. The residual ATP left after
the reaction is quantified by the firefly reaction employing luciferin oxidation catalyzed by luciferase and utilizing ATP. Luciferase is a much
slower enzyme than galactokinase, thus the luciferase responds to the depleted ATP level with a bioluminescence output which is proportional to
the ATP concentration and inversely proportional to the galactose

concentration [27,30]. These processes are depicted in Reactions 3 and 4 of Figure 2. Activity here will focus upon optimizing the reactants for good sensitivity and accuracy in the analysis range.

2. Device Construction and Performance Optimization. After the various parameters of the three zones are thoroughly understood and optimized, the three zones will be combined together into a two channel biosensor device. The current design prototype is illustrated in Figure 3. Evaluation and optimization of this prototype will then proceed. Of primary concern is the appropriate generation of galactose from Gal-1-P and filtration of most of the proteins and enzymes from the lysate. Of special importance is the removal of much of the hemoglobin (it can absorb the bioluminescence) and the ATPases which could consume some of the analyte ATP in Zone 3.

3. Development of urine biosensor for galactose. The optimization activities described in Aims 1 and 2 above will also be applied to the urine biosensor. A galactose channel very similar to that described for plasma will be developed and evaluated. Blood cell separation and protein removal functions will, however, not be necessary. This will make the actual sensor much easier to develop. The creatinine channel needed for optimal, quantitative urine analysis will be available from a parallel project [36]. The primary effort will be focused on adjusting the concentration of reagents in the bioluminescence Zone 3 to accommodate the lower galactose concentration range of urine.

4. Preservation of Biosensor Analytical Reagents. Dry reagents are a virtual necessity to enhance the stability and shelf-life of the biosensor. Our experience with a variety of preservation techniques suggests that lyophilization yields the best result for long term storage. We will study the optimum conditions (combination and concentration of preservatives, temperature and vacuum control) for the lyophilization process. Various lyophilization approaches will be evaluated in short term (120 day) stability tests. The most promising conditions and formulations will

then
be evaluated in longer term (one year) stability studies. The effect of
various storage conditions on reagent preservation will also be evaluated.
Preservation success will be evaluated by comparing the stored samples with
a fresh standard multi-enzyme bioluminescence reference assay.
5. Preparation of Recombinant Enzymes. Both luciferase and galactokinase
(EC.2.7.1.6) are very expensive. Galactokinase is only available from a few
commercial sources. Both enzymes account for a considerable fraction of
the biosensor cost, so less expensive and reliable sources are highly
desirable. Recombinant protein engineering, expression, purification and
characterization are proposed to generate both firefly luciferase and
galactokinase in house. Through our University of Utah collaborator and
consultant, Dr. R. Stewart, we have already expressed and regularly produce
an engineered firefly luciferase which can be immobilized and purified in
one step using small poly-histidine and biotin segments [29]. Preliminary
studies in Dr. Stewart's lab indicate that we can also express engineered
galactokinases with similar properties [37]. We plan to express, produce,
purify, and characterize galactokinase and galactokinase-luciferase hybrids
[25]. The availability of such hybrids will permit less expensive, more
reproducible, reliable and probably higher activity sources of our key
biochemical reagents. We also plan to produce modified galactokinases with
altered enzyme activities, thus facilitating preparation of biosensors with
higher or lower analytical sensitivity for galactose and
galactose-1-phosphate. We also expect to utilize galactokinase sequences
from thermophilic organisms for enhanced enzyme stability.
6. Performance Validation of the Biosensors. The blood and urine galactose
biosensors will be evaluated for accuracy, precision and linearity for the
ranges of galactose encountered in blood and urine. The reference standard
assay for galactose will be the galactose dehydrogenase based

spectrophotometric assay [27, Appendix 1].

2. Significance

A need exists to screen all newborns for galactosemia [1,2,7,8]. Even though this metabolic disease is quite rare (about 1 in 60,000 live births), the consequences of missing the rare case are traumatic for both the patient and family and costly for the healthcare system. Newborns in the United States are regularly screened for several inborn errors of metabolism during the first days of life, e.g., galactosemia, phenylketonuria, maple syrup urine disease, homocystinuria, etc. Currently, 46 states screen for galactosemia. A typical screening requires that a blood sample be drawn and sent to a central clinical lab as a dried spot on a sample card. The test costs between $10 and $40 with a mean of about $20. Most importantly, the results are often not available for several days. Infants with "classic" or transferase deficiency galactosemia develop jaundice, fail to gain weight and grow, act lethargic and exhibit liver and spleen enlargement [1,7,8]. Without dietary intervention they often die of sepsis within two weeks of birth. Early detection of galactosemia and prompt implementation of a galactose-free diet can result in elimination of the acute mental and physical effects of the disease, however, such intervention must be in the first few days of life and is, quite literally, a matter of life and death. If dietary control is instituted late or dietary compliance is poor, cataracts and mental retardation occur. The current trend toward early discharge of mother and child following birth often results in hospital stays of less than 24 hours. This works against early detection and treatment of galactosemia. Point of care newborn testing for galactosemia at birth has the potential to reduce delays in diagnosis and treatment thus lowering the impact to the national healthcare system.

Although galactosemia is routinely screened for in American newborn screening programs, it is not screened in several European nations.

There
are several methods used in the USA for the screening of galactosemia in
newborns, but they require specialized laboratory instrumentation and trained personnel and taking a blood sample followed by the time needed for
transport to a central lab for analysis. No simple quantitative "dipstick"
assay is currently available. There is a need for a fast and accurate screening technology. A low cost, instrument based biosensor or "dipstick"
seems most appropriate for these purposes. In fact, a point of care blood
glucose test is regularly performed on newborns only 4-6 hours after birth
as a screen for diabetes. A point of care test for blood galactose and
galactose-1-phosphate could just as easily be performed as an early screen
for galactosemia. If the screen is positive a lactose (galactose) free diet
could be initiated immediately, three or more days earlier than would happen while confirmation is pending from the typical clinical laboratory.

Significantly elevated levels of galactose or Gal-1-P in blood are
indicative of possible galactosemia. Positive screening tests are often
followed by specific galactose enzyme assays, which indicate the type of
galactosemia or the presence of a benign variant, such as the Duarte variant. Because the acute consequences of elevated blood galactose are so
severe (cataracts, liver problems, mental retardation, and often death via
sepsis), nearly all patients with suspected galactosemia are placed on galactose restricted diets. The Duarte variant patients are often kept on
galactose restricted diets until their blood galactose reaches normal levels
6 to 12 months after birth [1-11,34].

Galactosemia is considered to be an "orphan" disease, because of its
low incidence. The relatively large firms that routinely supply diagnostic
kits and equipment have tended to ignore the need for inexpensive, easy-to-use, assays for the screening and dietary management of galactosemia. Even for a small, niche company like ours the galactosemia
"market" does not appear profitable. However, there is growing

recognition
of the need to better monitor and manage inborn metabolic diseases, and it
is likely that appropriate economic incentives for "orphan" devices will
materialize in the near future. Studies of tightly monitored and regulated
Type I Diabetes have demonstrated both the health and economic benefits of
frequent monitoring and aggressive disease management. The phenylketonuria
(PKU) community is also beginning to recognize such benefits [20,21].
Regular monitoring of galactosemics is likely to result in better management
and improved outcomes [4]. Unfortunately, galactosemia is more complex and
less understood than PKU. Although early and rigorous dietary management
does indeed prevent the severe, acute consequences of the disease, long term
problems often result, even with aggressive management [1,2,15-19].

Figure 1 illustrated the simplified, normal metabolism of galactose. The
most common form of galactosemia ("classic" or transferase defficiency)
results from a deficiency of galactose-1-phosphate uridyltransferase (GALT).
The absence of GALT results in highly elevated levels of Gal-1-P, which, in
turn, causes elevated levels of galactose. Even if galactose is rigidly
controlled via dietary lactose restriction it still accumulates in the blood
and tissues due to the presence of galactose in many foods, including fruits
and vegetables (9-12). Also, galactose is an endogenous component of the
body as it is a key component of galacto-proteins and galacto-lipids.
Galactose can apparently be incorporated in these compounds via the
glucose-1-P and UDP-galactose pathways. See Figure 1. The natural turnover
and recycling of galacto-proteins and related compounds results in the
endogenous production of galactose. The resultant elevated galactose and
Gal-1-P levels promote the production of galactitol and galactonate,
resulting in their abnormally high levels. Galactose can be reduced to
galactitol via aldose reductase as in Reaction 7 of Figure 2. Galactitol is
then excreted in the urine. Galactose may also be oxidized to galactonate

by galactose oxidase in Reaction 6 of Figure 2. The galactonate can then be
metabolized to xylulose and carbon dioxide. It is possible that galactitol
and/or galactonate are responsible for the chronic, insidious problems of
galactosemics; it is also possible that their problems may be due to
abnormal production and/or synthesis of galacto-proteins or galacto-lipids.
The issue is far from clear [1,2,4, 12-19,34].

It is difficult for the biochemical and clinical research communities
to make rapid progress due to the relative difficulty in accessing suitable
numbers of patients. Both funding and patients are greatly limited, typical
problems with rare, orphan diseases. The patient issue could be partially
alleviated by the availability of a simple, minimally invasive, inexpensive
assay which patients and their care-givers could routinely use to monitor
and manage their disease AND provide a substantive increase in the amount of
clinical biochemical data for the research community. The availability of
the internet has allowed much of the galactosemia community to communicate,
organize and interact [22]. This will enhance the organization,
involvement, and empowerment of this community in better disease management
and participation in research that will hopefully lead to better
understanding, treatment and outcome for this insidious, orphan disease.

A major first step in meeting the analytical needs is this Phase II
application. The development and availability of a dipstick-like device to
measure blood galactose, Gal-1-P and urine galactose (the first generation
of our Galactose Direct! sensor) will provide improved disease management,
patient/provider empowerment AND the much needed data for research. We hope
to follow this work with the development of a second generation sensor
(Galactose Direct II) which will include the analysis of urine galactitol
and galactonate, providing far more biochemical information for research and
hopefully treatment. The metabolites galactose, galactose-1-

phosphate, glucose-1-phosphate, galactitol and galactonate, individually or in some combination, may be responsible for the negative outcomes observed in most surviving galactosemics.

Patients with the more common Duarte variant (1 in 5,000 births) also should be on a galactose restricted diet and should be monitored for their first 6 to 12 months of life. This population substantially increases the potential market, making it more comparable to PKU. There is also a market opportunity in those regions which do not yet have screening programs in place for galactosemia, such as England, Wales and France [4]. The total market would still be small, but one appropriate to a small firm such as ours. We should at least break even, while developing a set of technologies which we can, hopefully, apply to other, more profitable markets. We have been greatly inspired by the dedication, motivation, and commitment of the various communities involved with galactosemia and PKU, and we believe we can help.

3. Phase I Final Report

SBIR Phase I Grant No. 1 R43 MH57591-01

Project period and Dates of Service for Key Individuals: 10/01/97 through 04/30/98

Key Individuals and Level of Effort:

Name	Project Role	% FTE	% Effort
C.-Y. Wang, Ph.D.	Principal Investigator	100	50
Rob Scheer, Ph.D.	Research Scientist	33	50
R. Van Wagenen, Ph.D.	Research Scientist	100	20
Mara Hammer	Lab Technician	50	50
J.D. Andrade, Ph.D.	Technical Advisor	25	10

This report summarizes two periods of activity: (1) the work done on the Phase I project itself, which concluded on 04/30/98 and (2) additional work done using internal funds through 04/13/99. The latter work was, in part, based on the critique to our Phase II proposal. The present proposal is a highly augmented and revised submission of the original Phase II

proposal.

The critical objective of the phase I research was to assess the feasibility of a direct reading quantitative, dipstick type sensor for the measurement of galactose in urine (an indicator of galactosemia). The specific aims of this Phase I Research were to:

1. Characterize the available galactokinases with respect to cost, specificity, pH and temperature stability, and sensitivity.
2. Study galactose in water solution using the luciferase based ATP detection system.
3. Study the singular effect of individual urine components on the luciferase-based ATP detection system, including: glucose, lactose, fructose, maltose, pentose, chlorine, sodium, urea, creatinine, uric acid, and ketone bodies.
4. Quantitate galactose in commercial urine standards using the luciferase based ATP detection system.
5. Study device stability and reliability with emphasis on the preservation of galactokinase and ATP.

Phase I Results by Specific Aim

1. Characterize the available galactokinases with respect to availability, cost, specificity, pH and temperature stability, and sensitivity.

Although there are several sources for galactokinase, the only commercially available source was from galactose adapted yeast. The literature summary of galactokinase enzymes is summarized in Table I. The kinetic characterization of the E. coli enyzme has been reported [33].

Galactokinase was purchased from Sigma (G-0130). Recombinant luciferase was made and purified in the laboratory of R. Stewart [29], one of our consultants. D-Luciferin was purchased from Molecular Probes. Mg2O4?7H2O was purchased from Mallinckrodt. The urine standard was from Scantibodies Laboratory, Inc. (level 2, Lot No. C882B). All other chemicals were obtained from Sigma. All the reagents were prepared in 0.45 M glycyl glycine (gly-gly) buffer, pH 7.8, unless specified otherwise.

The specific activity of recombinant luciferase was determined by our standard in house protocol [27]. 50 ?l of 10 mM ATP in 0.45 M gly-gly buffer, 40 ?l of 1mg/ml BSA in 0.45 M gly-gly buffer, and 10 ?l of 5

μg/ml luciferase in 0.45 M gly-gly buffer were mixed in a 12x50 mm disposable polypropylene test tube. 50 μl of 0.1 mM luciferin/10 mM $MgSO_4 \cdot 7H_2O$ in 0.025 M gly-gly buffer was added to the tube by an auto injector. Bioluminescence was recorded by a Turner TD 20/20 luminometer for 10 s. The flash peak intensity divided by total amount of luciferase in the assay is used to represent the specific activity of the enzyme. The whole procedure was repeated three times with a fixed luciferase concentration. The averaged specific activity from three runs is 7.1±0.21 x108 Relative Light Units (RLU)/mg based on a Turner TD 20/20 luminometer.

The specific activity of galactokinase [27] was determined using 100 mM of D-galactose, 5.9 mM ATP, 16.2 mM phospho (enol) pyruvate (PEP), 800 mM KCl, 100 mM $MgCl_2$, and 20 mM ethylenediaminetetraacetic acid (EDTA) prepared in reagent grade water. 3.76 mM β-Nicotinamide adenine dinucleotide (β-NADH) was prepared in 160 mM potassium phosphate buffer. A pyruvate kinase/ lactate dehydrogenase mixture was prepared in 1:1 volume ratio of glycerol and 0.45 M gly-gly buffer. The total activity of pyruvate kinase and lactate dehyhrogenase was 500 U/ml and 200 U/ml, respectively. 5.75 ml of 160 mM potassium phosphate buffer, 1.5 ml of ATP, 1.5 ml of PEP, 1.5 ml of KCl, 1.5 ml of $MgCl_2$, 1.5 ml of EDTA, and 0.5 ml of β-NADH were mixed together as a reaction cocktail. The pH of reaction cocktail was adjusted to 7 at room temperature with 1N HCl or 1N NaOH if necessary. 917 μl of reaction cocktail was then added to a quartz cuvette, followed by 17 μl of pyruvate kinase/lactate dehydrogenase mixture, then 33 μl of galactokinase solution. The reaction was started by adding 33 μl of 100 mM galactose. Absorbance was measured with a Beckman-35 UV-Vis spectrophotometer at 340 nm. Absorbance was recorded immediately after the addition of galactose and every minute afterwards up to 5 min. Background absorbance change can be measured by

replacing galactokinase solution with buffer. The activity of galactose was
calculated from the change of absorbance according to the following equation:

$$? \quad \text{Units/mg} = [?A340nm / min\ test - ?A340\ nm / min\ blank] / [(6.22)(xmg / mlRM)]$$

Where 6.22 is the millimolar extinction coefficient of ?-NADH at 340 nm, RM
refers to reaction mixture; x is the total amount of galactokinase in 1 ml
reaction mixture.

The specificity of the galactokinase was studied using the ATP-firefly
bioluminescence assay [27]. Because the turnover rate of galactokinase is
2000 times faster than the luciferase, a lower light intensity can be
interpreted as the consumption of ATP by the galactokinase catalyzed
phosphorylation of galactose. Using a set of standard assay conditions, a
decrease in light intensity of 98% was observed after 30 minutes in the
presence of galactose while the
other sugars resulted in a decrease in light intensity of only 5.1-5.4% and
much of that arises from the intrinsic ATP consumption of luciferase itself.
See TABLE II. These results suggest that galactokinase is very specific to
galactose.

The pH sensitivity of galactokinase was also studied using the
bioluminescence assay. Galactokinase showed variable activity in the pH
range from 6.2 to 8.6 (Table II). In acidic solution (pH 6.2) the
galactokinase catalyzed phosphorylation reaction was slow and only about 77%
of the ATP was consumed after a 10 min reaction. In basic (pH 8.6) and
neutral solution (pH 7.0-7.8) the reaction was faster and up to 99% of the
ATP was consumed after 10 min reaction at pH 8.6 However, for all pH
conditions tested (pH 6.2 - 8.6), a 30 min incubation was sufficient for
galactokinase to reach the equilibrium of the phosphorylation reaction.
Consequently, we elected to conducted our work at the pH value where
luciferase catalyzed bioluminescence is optimal, i.e., 7.8

Recombinant luciferase in solution retained about 86% activity after

being stored at 4 oC for four weeks. Galactokinase in solution was stored at -20 oC, 4 oC, room temperature and 37 oC for four days. Our results indicate that the best galactokinase activity retention (58%) was at 4 oC. Galactokinase activity dropped to zero when stored at 37 oC for several hours. It is obvious that galactokinase is a labile enzyme and cannot be stored in solution at room temperatures for long times, thus stabilization and preservation processes must be developed. This is one of the specific aims of Phase II. Consideration should also be given to recombinant protein engineering of enzyme reagents from thermophilic organisms which typically have more thermally robust biochemistries. This is considered in Specific Aim 5.

2. Study galactose in water solution using the luciferase based ATP detection system.

A semi-quantitative assay for galactose was developed using a substrate controlled approach. 45 ?l of Solution A [two volume units of galactokinase (4 Units/ml) plus 2.5 volume units of bovine serum albumin (0.8 mg/ml) and 8 mM dithiothritol (DTT)] was mixed with 50 ?l trehalose (500 mg/ml) and 30 ?l Solution B [two volume units of 2 mM luciferin plus one volume unit of recominant luciferase (1.62 x108 RLU/ml]. Different ATP concentrations (0, 0.5, 1, 3, 5 and 10 mM) were prepared in 0.45 M glycylglycine buffer, pH 7.8. Sample solutions containing different amount of galactose (0, 0.5, 1, 3, 5 and 10 mM) were prepared in 0.45 M glycylglycine buffer, pH 7.8. Fifty microliters of sample solution and 50 microliters of ATP solution were then added to the enzyme assay. The bioluminescent intensity generated was recorded with both a CCD camera (Santa Barbara Instrument Group, ST-6 opto-head) and photographically using a Tektronix C-4 CRT camera with Polaroid 667 film (3000 ISO).

In this ATP range, the bioluminescent intensity of the luciferase reaction is not linearly related to the ATP concentration. However, this semi-quantitative assay can measure a range of galactose

concentrations between 0-0.5, 0.5-1, 1-3, 3-5, or 5-10 mM. This is illustrated in Figure 5 where a prototype biosensor for galactose is composed of six vertical wells creating a gradient of ATP concentration. Each horizontal row of wells was loaded with ATP (0, 0.5, 1, 3, 5 and 10 mM ATP from bottom to top). The bright spots on each horizontal sensor strip decreased as the galactose concentration increased. Figure 5 is a photographic record of such a sensor array responding to various concentrations of galactose. This semi-quantitative sensor can be described as a spatial array of light intensity detectable either visually, photographically or with a CCD camera.

3. Study the singular effect of individual urine components on the luciferase-based ATP detection system, including: glucose, lactose, fructose, maltose, pentose, chlorine, sodium, urea, creatinine, uric acid, and ketone bodies.

Sugar solutions containing 10 mM glucose, fructose, lactose, maltose, sucrose, trehalose, D-ribose, 0.4 M NaCl, 0.5 M urea, 5 mM uric acid, 7.4 mM acetoacetic acid, 2.84 mM ascorbic acid, and 0.58 mM hydroxybutyrate were prepared in reagent grade water. In micro-titer wells, 20 μl of 8 mg/ml BSA, 8 mM DTT, 20 μl of 2 U/ml galactokinase, 25 μl of 10 mM galactose in 0.025 M gly-gly buffer were mixed. The galactokinase reaction was initiated by adding 25 μl of 50 mM $MgSO_4 \cdot 7H_2O$ and 50 μl of 1 mM ATP in 0.45 M gly-gly buffer. 2 min later, the BAR was added to the microtiter well. Bioluminescence was recorded by a SBIG ST-6 CCD camera at 1 min, 8 min, and 28 min after the addition of the BAR. The integration time of the CCD camera was 20 s. Controls were performed by replacing the interfering components with reagent grade water. The whole procedure was repeated to study the interfering effects on luciferase alone by replacing the galactokinase solution with 0.45 M gly-gly buffer (pH 7.8).

We know that the bioluminescence assay is inhibited by urine [31]. At the concentrations indicated most of the compounds added did not

inhibit the galactokinase assay. Sodium chloride was the only chemical that showed significant inhibition. Our studies with a urine standard showed a decrease of about 20% in bioluminescent intensity. This is caused primarily by the presence of the chloride ion in the urine. The results of these studies are shown in TABLE II [33]. The specificity of galactokinase for galactose is clearly evident. The only major interference is from sodium chloride. This can be addressed by appropriate calibration of the final sensor.

4. Quantify galactose in commercial urine standards using the luciferase based ATP detection system.

To simulate clinical cases, a urine standard (Scantibodies Laboratory, Santee, CA, Lot # C8102C) termed Syscon was used in this study. This urine standard was sent to Children's Hospital, Los Angeles, CA for determination of the baseline galactose. The test showed only 4.6 µM galactose in the urine standard. Galactose standards (1, 2, 4, 6, 8, 10, 15, and 20 mM) were prepared in Syscon standard urine. Ten µl of 40 µM ATP in 0.45 M gly-gly buffer, 20 µl of 4.5 µg/ml firefly luciferase in 0.45 M gly-gly buffer, 2 µl of 1 U/ml galactokinase in 1 mg/ml BSA, and 28 µl of 1 mg/ml BSA were mixed in a 1.7 ml microcentrifuge tube. Ten µl of galactose standard was added followed by the addition of 50 µl 0.1 mM luciferin/ 10 mM $MgSO_4 \cdot 7H_2O$ mixture in 0.025 M gly-gly buffer with an auto injector. Bioluminescence was recorded by a Turner TD 20/20 luminometer for 300 s. The results of this study are discussed in the final section of this progress report and in the Appendix.

5. Study device stability and reliability with emphasis on the preservation of galactokinase and ATP.

A lyophilization protocol was developed to enhance the storage stability of the complete assay. The galactose assay was prepared in two buffer systems: 10 mM Tris and 100 mM Tris, pH 7.5. The other components in

the buffer include 0.3 mg/ml BSA, 3.1 mM DTT, 57.7 mg/ml PEG 8000, 123 mg/ml
trehalose, 19.2 mM $MgSO_4$, 0.6 mM luciferin, 0.15 unit/ml luciferase, and 1.2
unit/ml galactokinase. 65 ?l of the assay was delivered to each well of a
multi-well strip and tested with bioluminescent assay. The other assays
were frozen at -70 oC overnight. The frozen assays were then placed in a
freeze-dryer and dried at below -20 oC for 24 hr. The lyophilized assays
were rehydrated with 65 ?l de-ionized water for 10 min and then the assay
performance was tested in the same way as the fresh assay.

There is very little deterioration of performance, about 3%, for assays
preserved and lyophilized in the lower concentration buffer and stored at
room temperature for one month (Figure 6). For the assay preserved with the
higher concentration buffer (100 mM Tris not shown) a 54% lower performance
was observed. The preservatives and the lower concentration buffer
successfully preserve the assay performance. A lower ionic strength and pH
resulted in improved stability. This was probably due to preventing
luciferin oxidation.

Additional Phase I Progress Relevant to Phase II Proposal

1. Homogeneous Bioluminescent Assay for Galactose

Much of Mr. J.-Y. Eu's extensive work [27,37] was completed after the
formal end of the Phase I grant period. Limited space allows a discussion
of the following results.

a. Cross Interference between the Two Reactions

One of the most important considerations in coupled reactions is the
possible cross interference between the two reactions. Cross interference
must be minimized. The activity of firefly luciferase was determined in the
presence of the substrates from the galactokinase reaction, and vice versa.
The results are summarized in Table III.

For the firefly luciferase reaction, the alteration in enzyme activity
was represented by normalizing the peak intensity to the control (no
interfering components). For the galactokinase reaction, the effect was

calculated by normalizing the absorbance change at 340 nm to the control, since the activity was determined using a spectrophotometer. All the results show less than 7.6% change in enzyme activities (Table III). This suggests that cross interference is small when the two reactions are coupled. Besides the components studied in Table II, the adenosine-5'-diphosphate (ADP), product from the galactokinase reaction has been known to be a competitive inhibitor for ATP in the firefly luciferase reaction (Ki = 2.5 mM). Since the initial ATP concentration in the assay was 3.3 ?M, ATP was the limiting substrate for both galactokinase and firefly luciferase. The final concentration of ADP in the assay should be less than 3.3 ?M, far below its inhibition constant. ADP formed during the reaction should not significantly influence the activity of firefly luciferase. The results suggest that the reaction of galactokinase and firefly luciferase do not interfere with each other, assuring that a homogeneous assay for galactose is practical.

b. Galactose Concentration Determination with Bioluminescence Assay

Figure 7 shows the light intensity profile for a homogeneous galactose assay from 0 to 300 s. The control experiment (Figure 7, curve A, no added galactose) showed the typical pseudo steady state kinetics of firefly luciferase with the expected slow decrease in light intensity. All the intensity time profiles reached a similar maximum intensity at about 3 s, suggesting that the delay in light emission is probably due to the mixing of the reactants. The decrease following the maximal light intensity agrees with exponential decay (fitted lines not shown, but R2 for all the fitted curves are greater than 0.95, except for the control which ideally should be constant light emission). For higher concentrations of galactose in Figure 7 (10, 15, and 20 mM), the decays are very similar due to the saturation of galactose. The kinetics of the reactions have been thoroughly modeled (See Reference 27 and the Appendix). A simplified expression is:

Equations (1) – (5) are defined and presented in Reference 27 and the Appendix.

By taking the reciprocal of both sides of equation (6), a linear relation between $1/\ln(I_0/I_{t1})$ and $1/[gal]_0$ is derived:

where the slope is K_aK_b/V_1t_1 and the intercept is K_b/V_1t_1. The plot of $1/(\ln(I_0/I_{t1})$ against $1/[gal]_0$ should produce a straight line (Figure 8).

Ideally, curve A in Fig. 7 (0 mM galactose) should remain a constant light
output since there is no galactose present. However, there is about 10%
light intensity decrease due to the firefly luciferase reaction for the
first 60 s. This intensity decrease is compensated in Figure 8 by adding the
difference between I_{max} (0 mM galactose) and I_{60} (0 mM galactose) to the
intensities at 60 s for other galactose-containing samples. It is reasonable
to use the maximal light intensity (I_{max}) to approximate the initial light
intensity (I_0) used in the mathematical analysis since different initial
concentrations of galactose all reached similar maximal light intensity at
roughly the same time due to mixing. In Figure 8, the observation time was
chosen to be 60 s (t_1= 60 s) since most of the assumptions made in the derivation of Equation (7) are valid at the beginning of the reaction.

Figure 8 shows a good linear fit (R^2=0.99) for urine containing 1 to 20
mM galactose. For normal newborns during the second to sixth day after birth, the galactose concentration in urine ranges from 0.02 to 6.27 mM with
a mean of 1.13 mM, which is mostly covered by the linear range of the assay.
For adults, the galactose concentration in urine ranges from 0 to 0.57 mM.
To determine galactose concentration beyond the linear range using the current assay, the sample needs to be diluted or concentrated. The lower
detection limit (1mM galactose) results from the slow consumption of ATP by
galactokinase. At galactose concentration lower than 1 mM, the background
ATP consumption by the firefly luciferase reaction becomes predominant. The

higher detection limit (20 mM galactose) results from galactose saturation as described before. These approximate limits on the concentration range for galactose and galactose-1-P can be changed by using galactokinases with different reactions characteristics. See Specific Aim 5.

Standard urine was tested for ATPase activity and residual ATP concentration to assure the accuracy of the calibration curve in Figure 7
Standard urine is essentially free of ATPase activity by measuring ATP concentration using firefly luciferase bioluminescence. Residual ATP concentration in the urine was 1.8 nano mol/L determined by firefly luciferase bioluminescence using internal ATP standards. Residual galactose in standard urine was 4.6 ?mol/L, determined by Biochemical Genetics Laboratory of Children’s Hospital, Los Angeles, USA. Since the galactose concentration in standard urine was spiked into the millimolar range, the residual galactose in standard urine is insignificant. The pH change due to the addition of standard urine is negligible (from 7.8 to 7.67) since the assay has enough buffer capacity to maintain the pH.

The homogeneous bioluminescence assay was compared with the galactose dehydrogenase-based spectrophotometric assay. See Figure 9. The bioluminescence assay has a good agreement with the spectrophotometric assay using standards (1 to 8 mmol/L galactose) prepared in Syscon standard urine.

The galactose dehydrogenase-based galactose assay is an end point assay which requires an incubation period to convert the galactose present to NADH. The length of the incubation period also depends on the concentration of galactose present in the sample. For a high galactose sample, the assay generally needs at least 30 mins to bring the reaction close to equilibrium.
The kinetic bioluminescence assay requires a relatively short reaction time and provides similar accuracy over the physiological relevant concentration range.

c. Effect of Galactitol and Galactonate

In urine, galactonate ranges from 0.18 to 10.6 mM and galactitol ranges

from 0.51 to 118.7 mM. All the values are converted from mmole/mole creatinine by using an approximate urinary creatinine concentration of 9 mM.

The effect of galactonate and galactitol on the bioluminescence assay was studied with galactose containing standard urine spiked with galactonate and galactitol up to 25 mM and 125 mM, respectively. Galactose standards (5 and 10 mM) were prepared in Syscon standard urine (lot# C8102C) as previously characterized. Each galactose standard was spiked with 2.5 mM galactonate/12.5 mM galactitol and 25 mM galactonate/125 mM galactitol. Galactose concentration was determined as described previously. Bioluminescence was recorded for 180 s.

Figure 10 shows the intensity time course in the presence of galactonate and galactitol; all samples contained 10 mM galactose. All the curves in Figure 10 are almost identical, which suggests that galactonate and galactitol have minimal effect on the assay up to 25 mM and 125 mM, respectively.

The galactose concentrations determined by using the standard curve in Figure 7 are: 9.28 � 0.48 (curve A, 0 mM galactonate/ 0 mM galactitol), 10.37 � 0.81 (curve B, 2.5 mM galactonate/ 12.5 mM galactitol), and 9.8 � 0.73 mM (curve C, 25 mM galactonate/ 125 mM galactitol). All the values are the average of triplicates. The result demonstrates that the presence of galactonate and galactitol have minimal effect on the accuracy of the assay. Identical experiments were performed for standard urine with 5 mM galactose. The same conclusion was reached.

2. Preliminary Blood and Plasma Studies

A number of studies were conducted related to the development of a sensor for plasma galactose and erythrocyte Gal-1-Phosphate. The background ATP level in plasma is about two orders of magnitude lower than the initial ATP level used in an ATP consumption assay, including our galactose assay. Blood contains various ATPases which will compete with galactokinase and

luciferase for ATP. These endogenous ATPases result in a decrease in added ATP level from 100 micromolar to 1 micromolar or so in 20 minutes. Other preliminary work showed that the ATPase activity can be eliminated by thermal denaturation of the plasma, by non-specific adsorption and by 2 molar ammonium sulfate precipitation. Depending upon the specific design and kinetics of the biosensor, endogenous blood ATP must be considered. See Specific Aim #1 of this Phase II application.

Analysis of Gal-1-P requires that it be released from erythrocytes. This is addressed in Zone 1 of Specific Aim 1. Our preliminary work has shown that the commonly used erythrocyte lysing reagent saponin (Sigma R1129, 0.2% saponin) was very effective in lysing erythrocytes at a concentration of 0.1% saponin. In addition, we found that these small amounts of saponin detergent did not inhibit the luciferase bioluminescent ATP assay. Indeed, the presence of the saponin actually appeared to enhance the bioluminescence intensity (the effect of "detergent enhancement" has been observed by others).

Preliminary work with the alkaline phosphatase in buffered solutions has shown that it is indeed suitable for the conversion of Gal-1-P to galactose. We were able to convert 90 percent of 2.5 mM galactose-1-phosphate to galactose in less than one hour using 0.9 Units of calf intestine mucosa alkaline phosphatase in a volume of 200 ?L. Alkaline phosphatase can also be used to remove endogenous ATP since nucleoside triphosphotates are natural substrates for the enzyme.

During the development of the ATP bioluminescence platform we evaluated several approaches to the detection of emitted bioluminescent light. The results of this work were summarized in Figure 4. The original work addressed an ATP biosensor with an ATP consumase gradient that produced a spatial distribution of bioluminescent light when observed in the dark, much like a glowing thermometer. See Figure 5. The concept does indeed work, however, there are a variety of problems with human visual detection; foremost among these being that: (1) there is a wide range in visual

response and dark adaptation in the general population, (2) there is no verifiable, permanent record for documentation and quality control, (3) visual sensitivity is limited compared to the electro-optic approaches, and (4) examination in the dark is inconvenient. Luminometers which employ photomultiplier tubes (PMTs) are the quantitative monitoring standard and are extremely sensitive being able to detect pico-molar (sub femto-moles) of ATP, however, these devices are quite expensive (even the small hand-held instruments cost several thousand dollars). We are developing small, hand held luminometers (Bio-Light�) for the analysis of inexpensive, disposable biosensors which employ bioluminescence. These instruments utilize inexpensive, silicon based charge coupled devices (CCD) arrays and PIN photodiodes. These detectors are more than adequate to detect the luciferase catalyzed, ATP-luciferin bioluminescence originating from our galactose biosensors (ATP analyte is in the range of 10-5 to 10-7 molar) as shown in Figure 4. Figures 2 and 3 summarize the current design of these galactose biosensors. These sensors are envisioned to be 5-10 mm wide, about 20 mm long and about 1 mm thick. This Phase II proposal addresses the development of these galactose biosensors.

Conclusion: The Phase I studies and subsequent work have demonstrated the feasibility of a homogeneous, multi-enzyme, ATP depletion type bioluminescence assay for galactose in urine [27]. Preliminary results conducted with blood are encouraging. The original concept of a spatial distribution of light with a pattern or linear distribution inversely proportional to the concentration of galactose was verified. However, the variability in human visual response and the lack of a rigorous quantitative capability via visual detection argues against this approach. Instead, we have shown that a electro-optic approach using an inexpensive CCD camera provides the necessary quantitative capability necessary for the galactose

biosensor.

4. Experimental design and methods

1.Sensor Design and Functional Zones

The design of the sensor has undergone several iterations and improvements.
Although the basic approach is similar to that in the previous Phase II
application, the design has been greatly simplified and improved (Figures 1,
2). In the Phase I proposal we emphasized direct visual detection and
demonstrated its feasibility in the Phase I work (Figure 5). Our emphasis on
visual detection has changed as a result of our discussion with clinicians
and caregivers [3-6] and the relatively recent insight gained from the
galactosemia community that the optimum management of galactosemia is likely
to require the measurement of several analytes and analysis of their ratios
as well as absolute concentrations. Also, the information needs of
researchers would best be met by analytical means which can record, store
and transmit data. As a result, we initiated the development of a
relatively inexpensive, small, hand held analyzer capable of detecting and
quantifying the biosensor bioluminescence. The device called Bio-Light? is
based on an inexpensive charge coupled device (CCD) camera and imaging
optics. Various CCD cameras have been evaluated and a functional prototype
device has been constructed. Evaluations conducted to date indicate that
the prototype signal levels are more than adequate to quantify galactose and
galactose occurring as Gal-1-P in blood and urine galactose (Figure 4).

The focus of this work will be on the disposable, multi-channel
biosensor for galactose (Galactose Direct?). Both Galactose Direct? and
Bio-Light? will be designed to be compatible. The blood sample for the
sensor will be obtained via a standard lancet typical of that used by
diabetics for glucometer samples. The amount of blood necessary will be a
typical droplet of 50-100 ?L volume. The droplet will be deposited in the
receiving depression of the biosensor (Figure 3) which will contain a fill
indicator line to indicate that sufficient blood plasma has been

collected
to complete the reactions in Zones 1-3 (Figures 2 and 3).
Blood Preparation Zone 1 - Blood erythrocytes will be lysed in Zone 1 of
Channel 2 (Figure 3). A variety of cell lysing agents will be evaluated for
this purpose, i.e., detergents, lipases, etc. Red cell lysing reagent (0.2%
saponin) will be obtained from Sigma. Whole blood will be purchased from
Vital Products, Inc., St. Louis, MO. Ten ml of whole blood will be mixed and
incubated with different amounts of lysing reagent at room temperature. All
work with biological materials will be performed in a Level 2 containment
hood. All blood and urine samples will be commercially obtained and
certified to be free of pathogens such as HIV and hepatitis antigen. An
aliquot of 100 ?l cell lysate will be withdrawn and observed with a
microscope every minute. Hematocrit will also be determined. The ATP in the
cell lysate will be determined by a bioluminescent assay. The hematocrit,
cell number, and amount of ATP will be used as indices to evaluate the
completeness of the lysing reaction. The ATP amount increases as the cell
contents empty followed by a sharp decrease as intracellular ATPases consume
the ATP, as demonstrated in our preliminary work. We will also evaluate
various phospholipases as lysis agents if we run into problems with saponin
and related detergents. Our preliminary work with saponin suggests that it
is very satisfactory, although we have not yet examined its long term
preservation via drying and reconstitution - that work is also part of this
specific aim.

Alkaline phosphatase will be more thoroughly evaluated for removal of
endogenous ATP (already demonstrated). Alkaline phosphatase is a robust
enzyme widely used in immunoassays. The conversion of galactose-1-phosphate
to galactose will also take place in Zone 1 of channel 2 (Figure 3).
Alkaline phosphatase catalyses the hydrolysis of almost any phosphomonoester
to release inorganic phosphate (Pi) and the corresponding nucleotide,
alcohol or sugar at alkaline pH. The activity of alkaline phosphatase

depends on the presence of divalent metal ions such as Mg++ and Zn++. Steady state kinetic analysis indicates that alkaline phosphatase obeys Michaelis-Menton kinetics [42]. The turnover rate of alkaline phosphatase from different sources ranges from 1,450 - 6550 s-1. Alkaline phosphatases are generally very stable enzymes at room temperature and they have been used to convert gal-1-P to galactose [43,44]. The reference range of galactose-1-phosphate in red blood cells is 0-0.04 mM for a normal person and above 1 mM in galactosemics. We will evaluate the performance of the alkaline phosphatases in glycyl glycine buffer for 0-1 mM galactose-1-phosphate. The galactose produced will be measured with the quantitative method developed in the Phase I work. The standard reference assay for galactose in all of our studies will be the galactose dehydrogenase based spectrophotometric assay [27, Appendix 1]. The optimal pH, divalent ion concentration, and enzyme concentration will be determined.
A wide range of these enzymes can be obtained from Sigma Chemical Co.

The region into which the blood sample is applied in channel 2 will include pre-deposited lysis agent and pre-deposited alkaline phosphatase. Preservation of these agents is addressed in Specific Aim 4. The fluid leaving Zone 1 is therefore a blood "lysate" with its gal-1-phosphate converted to galactose and endogenous ATP removed. Channel 1, the free plasma galactose channel, is different. Here the erythrocytes are trapped but not lysed. The function of this zone is to obtain free plasma and determine its galactose level. We will study various surface passivation agents to minimize cell lysis or hemolysis in this channel. The cell-free plasma leaves Zone 1 and enters Zone 2.

We will evaluate a wide range of materials for the filtration and reagent support functions, including surfactant coated polyester wicks manufactured by Filtronia Richamond, cellulose nitrate membranes of various pore sizes (0.2 ?m to 12 ?m) manufactured by several companies (Millipore, Paul-Gelman Sciences, Whatman, Micron Separations, Sartorius, Schleicher & Schuell, etc.), and both lateral and vertical flow blood separation

membranes manufactured by Spectral Diagnostics-Prime Care, Whatman (PlasmaSep), Pall Gelman (Hemasep V and H) and others that may become available. Prior to application of the enzyme the membranes and wicking materials will be treated with a 1 mg/ml solution of bovine serum albumin to decrease the detrimental effects of surface-enzyme reactions. The membranes will be evaluated in terms of effective separation of blood cells from plasma, minimizing the amount of damage to cellular elements and minimizing the amount of plasma volume necessary to fill the pores of the material.

Filtration Region - The Zone 1-Zone 2 boundary may require a filter for cells and cell debris. Its purpose in channel 1 is to permit only plasma to move through; its purpose in channel 2 is to permit the lysate, filtered of unlysed cells and cell fragments, to move through. A range of filters will be evaluated. These materials are relatively standard and widely used in the invitro diagnostics (IVD) industry (23). They are essentially the kind of materials noted above for Zone 1. It is quite possible that this filtration function can be completely accomplished using the materials evaluated for Zone 1.

Adsorption Region (Zone 2) - This region is continuous with the macrofiltration region noted above. This is a relatively high capacity protein adsorption zone whose main purpose is to remove endogenous ATPases, including any alkaline phosphatase arising from Zone 1. The materials evaluated for this zone are those previously described. A primary function of this zone is to remove as much hemoglobin as possible although all of the hemoglobin need not be removed. Our preliminary work on galactose and ATP analysis in lysed blood suggests however, that hemoglobin is not a significant interferent to either the galactokinase or luciferase reactions. Nevertheless, we can remove much of the hemoglobin in Zone 2 thereby even further minimizing possible optical and chemical interferences. There are several commercial sources of high surface area membranes designed to remove large quantities of protein by passive adsorption and also by covalent bonding to pre-activated surfaces (Pall Gelman, Millipore, etc.) we

will
thoroughly characterize the adsorption capacity of such candidate membranes
particularly for hemoglobin, albumin and endogenous ATPases.

Signal Generation Region (Zone 3) - We have already established that a
homogeneous (galactokinase and luciferase together with other bioluminescent
reagent) assay for galactose based on ATP depletion and bioluminescent
detection works very well in solution (27). We must now demonstrate that
this is also the case for reagents that are immobilized on reagent support
membranes and subsequently freeze dried in place. We must identify the
appropriate support membrane substrate that provides sufficient surface area
and at the same time does not appreciably denature the reagent enzymes. We
are confident that this can be addressed with albumin passivation and one of
the commercial membranes previously mentioned. We believe that passive
trapping of the bioluminescent reagents in the membrane of Zone 3 is
practical, however as the moving front of advancing plasma wets Zone 3 it
will re-hydrate the reagents and possibly carry some of them out of the
zone. If too much reagent is lost in this fashion smaller pore size
membranes will be evaluated as well as covalent immobilization to retain
them in zone 3.

The bioluminescence arising from the reactions depicted in Reactions 4
and 5 of Figure 2 must escape from Zone 3 and be detected. Because these
reactants are dispersed throughout the reagent support membrane of Zone 3
bioluminescence will arise throughout this zone, thus the concern that the
presence of hemoglobin would absorb a significant fraction of the light.
Hemoglobin will be studied as an optical and chemical interferent in Zone
3. However, we have determined that the presence of the hemoglobin will not
pose much of an absorbance problem due to its removal and the very thin
layer of support material. Of most importance is the fact that even though

these membrane materials are white they are generally opaque so most of the bioluminescence we have observed to date actually comes from the surface and near surface of the materials. We are confident that even the emitted surface bioluminescence would be sufficient to provide adequate signal so the presence of a considerable amount of hemoglobin reaching Zone 3 is not considered to be too serious.

2. Device Construction and Performance Optimization

Based on the results of Aim #1, we will modify, adjust and optimize the relative dimensions and placements of the zones to optimize the kinetics and performance of the galactose biosensors. The goal is to have the test completed within 60 to 90 seconds after application of the blood sample and insertion of the strip into the reader. Here we will also consider the rigidity of the strip itself, the possible requirements for a air tight, foil cover to minimize photo-oxidation of the components such as luciferin, the implementation of a "proper fill" indicating region (see Figure 2) and related practical issues of a reliable, reproducible, quantitative, prototype biosensor. One of the most important issues in the overall design is to chose materials and device dimensions which do not consume large quantities of plasma fluid sample. This issue would tend to drive the pore size specification (and thus priming or fill volume) down for our membranes in all three zones. The purpose of this Specific Aim is to fully optimize and finalize the design of Figure 2 so that we have functional biosensors for galactose. It is anticipated that the housing of our biosensor will be a poly(vinyl chloride) or similar material which is common to the IVD industry. Obviously, such a material would need to be inexpensive and easily to manufacture.

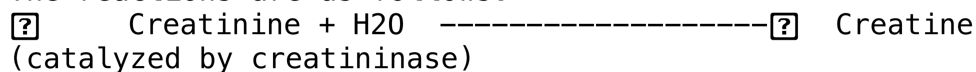
3. Urine Sensor for Galactose

The urine galactose channel is very similar to the free plasma galactose channel (Channel 1 shown in Figure 2). The design will be less challenging because no blood cells will have to be separated from the sample in Zone 1 and the amount of protein requiring removal in Zone 2 will be much

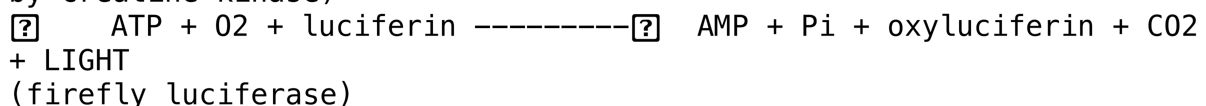
smaller than that of the blood plasma galactose channel. The primary focus will be the modification of the necessary bioluminescent reagents to produce an acceptable signal from the generally lower concentration of galactose that may be present in the urine sample. We can address lower bioluminescent signals by adding more reagent (particularly luciferase) to the reagent mix which will be placed in Zone 3.

The second channel in the urine galactose sensor is a creatinine sensor, as creatinine is the normal reference for all quantitative urine assays. We are currently developing a creatine/creatinine biosensor which is being funded by a Phase I NIH SBIR grant [36]. The approach we are using for this biosensor is also a homogeneous, multi-enzyme ATP depletion assay.

The reactions are as follows:


? Creatinine + H_2O --------------------? Creatine (catalyzed by creatininase)

? Creatine + ATP -----------? creatine phosphate + ADP (catalyzed by creatine kinase)


? ATP + O_2 + luciferin ---------? AMP + Pi + oxyluciferin + CO_2 + LIGHT (firefly luciferase)

The bioluminescence produced in this fashion is again quantified with our Bio-Light? CCD based analyzer. Our preliminary studies already indicate the feasibility of this approach, including a rather extensive kinetic simulation and model (38).

4. Preservation of Biosensor Analytical Reagents

A primary concern regarding the practicality of our biosensor design is the long term stability of the governing enzymes and substrates. In this case, we are most concerned with the storage stability of luciferase, luciferin, galactokinase, phosphatase, galactose, and ATP. We believe that storage periods of at least one year at room temperature are the minimum necessary specification and short periods of several days at 30 ?C may also be encountered. Preliminary studies using a lyophilization process and several preservatives have shown positive results (Phase I report). The

Phase I lyophilization process included several preservatives. Polyethylene glycol, PEG, (8,000 M.W.) was included as a freezing protectant (50 mg/ml) [39]. Trehalose was included as a dehydration protectant (120 mg/ml) [40]. Bovine serum albumin (0.3 mg/ml) was included as a denaturation protectant [41] and 3 mM dithiothreitol (DT) was employed as a sulfide bond linkage protectant. The Phase I work was done with a research grade lyophilizer that gave no quantitative indication of the process pressure or temperature of the materials during the freezing and lyophilization. We have subsequently purchased a production capacity lyophilizer (Virtis Model 12EL Genesis) which has full capability to provide temperature control of the lyophilization chamber shelves and condenser and provides measurements of the chamber pressure and shelf temperature throughout the freezing and lyophilization process. It now appears that our better controlled lyophilization process, as described below, will effectively preserve the viability of the governing enzymes and substrates for long term storage at room temperature in desiccated conditions in the absence of light and water vapor.

The lyophilization process closely follows protocols prescribed in the pharmaceutical literature [41]. After mixing the preservatives with the reagents necessary for the biosensor application, the solution is either pipetted into individual test wells or applied to BSA treated membranes or other wicking material for preservation. The sample is frozen to −70�C for at least one hour. The final step in the preservation process involves removing the water from the reagent solution at high vacuum and various shelf temperatures. The moisture removal begins with temperatures near −30�C and ends near room temperature or slightly higher to drive off additional bound water. The drying process lasts approximately 24 hours. Once the samples are dry, they can be stored in the dark at room

temperature
in dry air for extended periods without significant loss of activity.

We will investigate various freezing and lyophilization protectants. We have found dextran T40 to be a good alternative to PEG and sucrose to be good alternative to trehalose. It is important to minimize the presence of even trace amounts of heavy metals and to maintain rigorous control of the freezing rate and lyophilization shelf temperature. The primary variables to be evaluated in Phase II are: (1) preservation cocktail composition (the benefits of DTT, EDTA and bovine serum albumin are well established, but the optimal concentrations will have to be determined), (2) PEG's, dextrans and sugars such as sucrose will be evaluated in various concentrations. (3) Freezing rates will be optimized and the optimal shelf temperatures for both primary and secondary drying will be determined. (4) The amount of bound water in the final product will be determined using DSC. The primary guidance on this work will be from the many publications by Dr. John Carpenter's group [39,40]. The primary criteria of success will be to maximize the galactokinase and luciferase activity during freezing and lyophilization and to ensure the long term preservation of the assay for periods of at least one year. To some extent, unavoidable activity losses can be accounted for by adding excess luciferase, galactokinase, ATP and luciferin to the assay before the preservation process begins.

5. Preparation of Recombinant Proteins

Recombinant galactokinase can be genetically engineered to facilitate the fabrication of the sensor such as immobilization through an avidin-biotin interaction. The galactokinase gene (galK) from Escherichia coli will be isolated from genomic DNA using PCR technique. The galK gene purified from PCR will also carry two unique restriction endonuclease cutting sites using appropriate PCR primers (BamH I site at 5’ end and Eag I at 3’ end). We have been using recombinant firefly luciferase for several years. The recombinant firefly luciferase is fused with a histidine

tag and
a biotin carboxyl carrier protein (BCCP) domain at its amino terminus [29].
The most straightforward approach is to replace the firefly luciferase gene
(luc) on the vector we have been using with galK gene. See Figure 11 below.

Figure 11. Construction of pET 24a (+) BCCP-galK plasmid by replacing the
firefly luciferase gene (luc) with galactokinase gene (galK). ATG: start
codon; (His)6: six histidine tag; *: stop codon.

The luc gene-containing vector pET 24a (+) BCCP-luc will be digested
with restriction endonucleases BamH I and Eag I to remove luc gene. The galK
gene can be ligased into the vector using DNA ligase since the gene carries
the same restriction endonuclease cutting sites. The fused gene can be
transformed to a competent E. coli strain to express the protein.
The expressed recombinant galactokinase is fused with a histidine tag and
a BCCP domain on its amino terminus. Histidine tag binds specifically to
Ni2+ with a binding constant of K=1013M-1 at pH 8. A nitrilotriacetic
acid-agarose conjugate (NTA-agarose) contains four chelation sites for metal
ions, which can bind strongly with Ni2+. A Ni2+-NTA column can be used to
purify histidin-tagged galactokinase. The purification procedure for
histidine-tagged protein is much simpler than the traditional protein
purification procedure since the common precipitation and chromatography
steps can be avoided. It is sometimes necessary to immobilize enzymes in
biosensor applications. The BCCP domain can be used to immobilize
recombinant galactokinase through an avidin-biotin interaction. The
histidine tag can also be used in immobilization. The purity of the
recombinant galactokinase will be determined by SDS-PAGE. The basic kinetic
constants (Km, Vmax) will be measured spectrophotometically. We expect to
work with several recombinant galactokinases, including mutants with
enhanced thermal stability and altered kinetic constants. The latter should

facilitate the galactose and gal-1-P analysis at higher and lower analyte
concentrations. BCCP-galactokinase can also be fused with firefly luciferase to produce a bi-functional fusion protein. Fusion protein can
simplify the fabrication of the biosensor. Depending on the time and resources available, we will also develop a galactokinase-luciferase hybrid
enzymes [37].

We have requested equipment funds with which to establish a small recombinant enzyme development and characterization laboratory. Dr. Russell
Stewart, consultant and Scientific Advisory Board Member together with D.-J.
Min and a molecular biologist (to be hired), will establish and validate
this recombinant protein lab.

6. Performance Validation

The biosensors will first be tested in simple buffer solutions, followed by more comprehensive testing in commercially available blood and
urine samples which have been certified to be free of HIV and hepatitis
antigens. Internal standards (known amounts of galactose and/or galactose-1-phosphate) will be added for calibration purposes. The accuracy, linearity and precision of the assays will be determined. Variations between various sensors will also be determined. Our results
will be referenced against standard spectrofluorometric assays for galactose
and gal-1-P[27, Appendix 1]. Results will be compared to determine the
probability of false positives and false negatives. The clinical chemistry
service provided by an established and accredited analytical laboratory,
such as Associated Regional University Pathologists, Inc. (ARUP) in Salt
Lake City, UT and Children's Hospital Clinical Labs, Los Angels, CA, will
also be used to for confirmation of testing results.

A limited number of sensors will also be made available to our clinical
collaborators (Drs. Leonard and Berry) for testing and assessment in their
own facilities. These studies will be part of their ongoing clinical and
research activities purely for experimental testing purposes and as such
will require the approval of their own in house Institutional Review

Boards.

Phase III

We expect this Phase II research and development to result in a practical, functional, dry reagent, in vitro diagnostic biosensor prototype for the quantification of blood galactose and galactose-1-phosphate. A second functional biosensor prototype will also be developed for urine galactose. We refer to these devices as Galactose Direct!?. A subsequent generation biosensor would be developed for galactitol and galactonate if current ongoing biomedical research indicates a diagnostic need for analyzing these species in blood or urine. All of the biosensors under development will utilize the same electro-optic bioluminescence detecting instrument (Bio-light?) specifically designed and developed for our Direct? line of disease-specific analytical "dipstick" biosensors [35]. This is now under development in house.

One of us (J.D. Andrade) will be speaking with the FDA In Vitro Diagnostics Group in late May, 1999 to begin the process related to appropriate FDA oversight of our in vitro diagnostic device development. His recent discussion paper, "Personal Sensors in the Home", prepared for a recent NSF/FDA Home Healthcare Workshop, is available at www.hctr.be.cua.edu (click on HCT workshop then on Resource Materials).

As soon as we have a fully functional analytical device for quantifying the bioluminescence (Bio-light?) and functional prototype biosensors for galactose (Galactose Direct?) we will begin discussing possible collaborative relationships with interested, larger partners to generate additional follow on funding necessary for device refinements, clinical testing and validation, FDA approvals, and scale up production and marketing. Our goal is to find a partner with a well established marketing and distribution capability while our company provides the technical expertise and possibly the manufacturing and production of the analytical devices of disease-specific, clinical biosensors. The first such sensors will be for galactosemia management and later for PKU management and kidney

function monitoring [36].
5. Human Subjects No human subjects will be used in this research. All
human samples will be obtained from commercial sources which are certified
to be negative for HIV and hepatitis antigens.
6. Vertebrate Animals No work with vertebrate animals is planned for this
work.
7. Consultants
Three consultants are budgeted for this Phase II work. Their letters
agreeing to consult are included in this proposal with the exception of Dr.
Berry's letter which did not arrive in time. Dr. Russell Stewart is an
Associate Professor in the Department of Bioengineering at the University of
Utah. He has previously served as a consultant and Principal Investigator to
our company on a Phase II NSF STTR subcontract which addressed recombinant
firefly and bacterial luciferase. Dr. Stewart will meet with us monthly to
assist us in the areas of protein engineering and protein characterization.
He will assist us in transferring the recombinant luciferase know how over
to our company. This will also be particularly crucial as regards our
proposed work to develop the in house galactokinase protein engineering
capability in our own facility. Dr. Claire Leonard, M.D., is an Associate
Professor of Pediatrics and Director of the Metabolic Program in the
Department of Pediatrics at the University of Utah School of Medicine. She
has a thorough background in the genetics and biochemistry of inborn
metabolic erros of metabolism with special interest in PKU and galactosemia.
Dr. Leonard will be advising us in the area of clinical monitoring needs for
galactosemia patients. It is anticipated that he input will pave the way
for a more appropriate sensor design in follow on Phase III work. Dr.
Gerald Berry, M.D. is is a senior physician at The Children's Hospital of
Philadelphia. His areas of professional interest are pediatric
endocrinology, medical genetics, and clinical biochemical genetics. He has
published numerous articles addressing the chronic long term effects of

galactosemia and the biochemistry that may lead to these manifestations.
Dr. Berry will be advising us on the needs and methods of monitoring both
urine galactose as well as galactitol and galactonate assay in urine and
blood.

In addition to the consultants proposed for this research our strong
Scientific Advisory Board will continue to serve in an advisory and consulting capacity with reimbursement from other sources. The board members are as follows:

Dr. Woody Hastings - Professor of Biology at Harvard University. Dr. Hastings is internationally recognized for his basic research on bioluminescence.

Dr. Vladimir Hlady - Associate Professor of Bioengineering at the University
of Utah. Dr. Hlady is an expert on the study of proteins at surfaces using
interfacial fluorescence spectroscopy in conjunction with CCD detectors.

Dr. Henry Kopecek - Professor of Pharmaceutics and Bioengineering at the
University of Utah. Dr. Kopeck is an internationally recognized authority
on hydrogels and related polymers for drug delivery and biocompatibility.

Dr. Larry Kricka - Director of the General Chemistry Lab and Professor of
Pathology and Laboratory Medicine at the University of Pennsylvania. Dr.
Kricka is an internationally recognized for his work on applying both bioluminescence and chemiluminescence to clinical chemistry.

Dr. Russell Stewart - Assistant Professor of Bioengineering at the University of Utah. Dr. Stewart is an expert on recombinant techniques for
the synthesis and study of luciferases and motor proteins.

8. Consortium Arrangements No subcontracts are budgeted for this Phase II
work.

9. Literature Cited

1. S. Segal and G.T. Berry, "Disorders of Galactose Metabolism" in The Metabolic and Molecular Bases of Inherited Disease Vol. 1 7th ed., C.R.
Scriver, et al. eds., McGraw-Hill (1995) 967-1000.

2. S. Segal, "Galactosemia Today: The Enigma and the Challenge", J.Inher.
Metab. Dis. 21 (1998) 455-471.

3. N.R.M. Buist, M.D., written communication of March 26, 1998 and conversation of March 17, 1999.

4. J.H. Walters,M.D., Royal Manchester Children's Hospital, Manchester,
England, conversation of March 22, 1999 and J.H. Walters, et al., "Recommendations for the Management of Galactosemia", Arch. Dis. Child 80
(1999) 93-96.
5. Claire Leonard, M.D., Associate Professor of Pediatrics and Director of
the Metabolic Program, Department of Pediatrics, University of Utah School
of Medicine, Salt Lake City, Utah, conversation of March 16, 1999.
6. G. Berry, M.D., Children's Hospital of Philadelphia, conversation of
April 5, 1999.
7. E. Beutler, "Galactosemia: Screening and Diagnosis", Clin. Biochem. 24
(1991) 293-300.
8. T. Podskarbi and W.P. Bieger, "Neonatal Screening and Differential Diagnosis of
Galactosemia", Clin. Lab. 41 (1995) 843-847.
9. K. Widhalm, et al., "Diet Does Not Ensure Normal Development in Galactosemia", J. Amer.
Coll. Nut. 16 (1997) 204-208.
10. D.D. Waggoner, N.R.M.Buist, and G.N. Donnell, "Long-Term Prognosis in
Galactosemia:
Results of a Survey of 350 Cases", J. Inher. Metab. Dis. 13 (1990)
802-818.
11. F.R. Manis, et al., "Cognitive Functioning in Patients with Classical
Galactosemia, …..",
J. Inh. Met. Dis. 20 (1997) 549-555.
12. G.T. Berry, et al., "Quantitative Assessment of Whole Body Galactose
Metabolism in
Galactosemic Patients", Eur. J. Pediatr. 156-S1 (1997) S43-S49.
13. C. Jakobs, S. Schweitzer and B. Dorland, "Galactitol in Galactosemia",
Eur. J. Pediatr.,
154-S2 (1995) S50-S52.
14. R. Gitzelmann, R.G. Hansen, and B. Steinmann, "Biogenesis of Galactose:
A Possible
Mechanism of Self-Intoxication in Galactosemia" in F.A. Hommes and
C.J. Van den Berg,
eds., Normal and Pathological Development of Energy Metabolism, Academic Press
(1975) 25-38.

15. J.T. Allen, J.B. Holton, A.C. Lennox and I.C. Hodges, "Early Morning
Urine Galactitol Levels
in Relation to Galactose Intake: A Possible Method of Monitoring the
Diet in Galactokinase
Deficiency", J. Inher. Metab. Dis. 11-S2 (1988) 243-245.
16. G.T. Berry, et al., "The Effect of Dietary Fruits and Vegetables on
Urinary Galactitol
Excretion in Galactose-1-phosphate Uridyltransferase Deficiency", J.
Inher. Metab. Dis. 16
(1993) 91-100.
17. T.A. Tedesco and K.L. Miller, "Galactosemia: Alterations in Sulfate
Metabolism Secondary to
Galactose-1-Phosphate Uridyltransferase Deficiency", Science 205
(1979) 1395-1397.
18. P. Cuatrecasas and S. Segal, "Galactose Conversion of D-Xylulose: An
Alternative Route of
Galactose Metabolism" Science 153 (1966) 549-551.
19. R.J. Allen, "Window in Clouds Over Galactosemia", The Lancet 345 (1995)
128.
20. U. Wendel and U. Langenbeck, "Towards Self-Monitoring and Self-Treatment
of PKU:
A Way to Better Diet Compliance", Eur. J. Ped. 155-S1 (1996) S105-S107.
21. U. Langenbeck and U. Wendel, "Kinetic Analysis Phenylalanine Disposal….", Int. Ped. 12
12 (1997) 19-22.
22. Parents of Galactosemic Children, Inc., Newsletter (www.galactosemia.org).
23. K.D. Jones, "Troubleshooting Protein Binding in Nitrocellulose Membranes", IVD Tech. 5
(1999) 32-41.
24. J.D. Andrade, ed. Surface and Interfacial Aspects of Biomedical Polymers
Vol.2 - Protein
Adsorption, Plenum Press (1985).
25. H. Carlsson, et al., " Use of Genetically Prepared Enzyme Conjugates in
Lactose and
Galactose Analysis", Anal. Biochem. 218 (1994) 278-283.
26. P.G. Arthur, J.C. Kent and P.E. Hartmann, "Microanalysis of the Metabolic Intermediates of

Lactose Synthesis in Human Milk and Plasma Using Bioluminescent Methods", Anal. Biochem. 176 (1989) 449-456.
27. J.-Y. Eu, C.-Y. Wang, and J. Andrade, "Homogeneous Bioluminescence Assay for Galactosuria: Interference and Kinetic Analysis" Anal. Biochem. (1999) in press and Included as Appendix 1.
28. D.J. Min, J.D. Andrade, and R.J. Stewart, "Specific Immobilization of In Vivo Biotinylated Bacterial Luciferase and FMN:NAD(P)H Oxidoreductase", Anal. Biochem. (1999) in press.
29. C.-Y. Wang, S. Hitz, J.D. Andrade and R.J. Stewart, "Specific Immobilization of Firefly Luciferase Through a Biotin Carboxyl Carrier Protein Domain", Anal. Biochem. 246 (1997) 133-139.
30. S. Brolin and G. Wettermark, Bioluminescence Analysis, VCH (1992).
31. L.J. Kricka, "Chemiluminescence and Bioluminescence", Anal. Chem., 67 (1995) 499R.
32. A. Lundin, "Techniques Related to Accuracy of Luminometric ATP Measurements", in A Rapid Guide to Industrial Use of ATP-Luminescence in Rapid Microbiology, Cara Technology (1997) 55-62.
33. J.S. Gulbinsky, W.W. Cleland, "Kinetic Studies of E. Coli Galactokinase", Biochem., 7 (1968) 566.
34. K.G. Petry and J.K.V. Reichardt, "…Human Galactose Metabolism…", Trends in Genetics, 14 (1998) 98-102.
35. R. Van Wagenen, "CCD-Based Analyzer for Multi-Channel Biosensor" NIH Phase I SBIR, Grant#: 1R43RR13087-01.
36. R. Van Wagenen, "Creatinine Biosensor for Renal Transplant Monitoring", NIH Phase I SBIR, Grant#: 1R43DK55426-01.
37. J.-Y. Eu, Ph.D. Dissertation, University of Utah (1999) in preparation.
38. R. Davies, Ph.D. Dissertation, University of Utah (1999) in preparation.

39. J.F. Carpenter, S.J. Prestrelski, and T. Arakwa, "Separation of Freezing- and Drying-
Induced Denaturation of Lyophilized Proteins Using Stress-Specific
Stabilization, Arch.
Biochem. Biophys. 303 (1993) 456-464.
40. J.H. Crowe, L.M. Crowe, and J.F. Carpenter, "Preserving Dry Biomaterials: The Water
Replacement Hypothesis, Part 2", Biopharm. (May 1993) 40-43.
41. C.O. Fagain, "Lyophilization of Proteins" In Methods in Molecular Biology, Vol. 59, S.
Doonan, ed., Humana Press.
42. H.N. Fernley, "Mammalian Alkaline Phosphatases" in The Enzymes, 3rd
Edition, P.D. Boyer
ed., 4 Academic Press, p. 417.
43. F. Diepenbrock, et al., "Colorimetric Determination of Galactose and
Galactose-1-Phosphate
from Dried Blood", Clin. Biochem. 25 (1992) 37-39.
44. H. Misuma, et al., "Galactose and Galactose-1-Phosphate Spot Test for
Galactosemia
Screening", Clinica Chimica Acta 111 (1981) 27-32.