DEVELOPMENT OF NEWBORN GALACTOSEMIA SCREENING KITS

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ABSTRACT

DEVELOPMENT OF NEWBORN GALACTOSEMIA SCREENING KITS

Ünal, Sıdıka Yağmur

M.S., Department of Biotechnology

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Galactosemia is an autosomal recessive disorder, caused by an impaired galactose metabolism, resulting in acute complications with high morbidity and mortality as soon as the infant begins a milk-based diet. Mutations in GALT, GALE and GLAK genes that are in the Leloir Pathway are responsible for the phenotype. Removing galactose from the diet is a critical, lifesaving intervention; yet this treatment does not prevent multiple long-term complications. Long-term neurological outcomes, particularly movement disorders, have been reported but not well studied.

In this thesis, a kit prototype was constructed to measure the amount of galactose and galactose – 1 –phosphate in the collected dried blood samples using galactose dehydrogenase and alkaline phosphatase enzymes via fluorescence emission. In this prototype the first step is the conversion of galactose – 1- phosphate to galactose by alkaline phosphatase. Then the galactose formed in the first reaction and free galactose are converted to galactono – lactone by galactose dehydrogenase enzyme. Detection was done through the generation of NADH after this last step at 460 nm wavelength.

In Turkey, almost all galactosemia screening kits used in clinical laboratories are imported from various countries. Such an import brings extra costs both for families and hospitals. The galactosemia screening test kit prototype offers similar test parameters but to a lower a cost compared to imported screening kits. We have also

produced strips that detect galactosemia. In stips was based quantitative reading. It' color change white to pinky-red according to amount of galactose and galactose-1-phosphate amount. These strips does not need to fluorometric reading. This property became advantage in the absence of fluorimeters equipment and in rural areas.

Keywords: Galactose screening, galactosemia, metabolic diseases, newborn screening

ÖZ

YENİDOĞAN GALAKTOSEMİ TARAMA KİTLERİNİN GELİŞTİRİLMESİ

Ünal, Sıdıka Yağmur

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Galaktozemi, galactok metabolizmasındaki bozukluklardan kaynaklanan, yeni doğanların süt/süt bazlı diyete başladıkları zaman yüksek mortalite ve akut komplikasyonlara sebep olan otozomal ressesif bir hastalıktır. Leloir metabolik yolundakiGALT, GALE ve GALT genindeki mutasyonlar bu fenotipin sorumlusudu. Diyetten galaktoz çıkarmak bu durumda hayat kurtarıcı bir müdahaledir. Ama bu tedavi henüz uzun vadeli komplikasyonlara önlemek için yeterli değildir. Uzun vade de nörolojik sonuçlar, özellikle hareket bozuklukları, raporlanmış ama çok iyi çalışılmamış bir konudur.

Bu tezde, kandaki galaktoz ve galaktoz-1-fosfat miktarları, galaktoz dehidrogenaz ve alkalin fosfataz kullanılarak flurometrik olarak ölçülerek tayin edilir. Bu protokip kitte, ilk basamak galaktoz-1-fosfatın alkalin fosfataz kullanılarak galaktoza dönüşmesidir. Sonra, ikinci basamakta hem serbest galaktoz hem de ilk basamaktan oluşan galaktoz, galaktoz dehidrogenaz enzimi ile beraber galakto-laktona dönüşür. Bu son adımında NADH'nin oluşması ile 460 nm dalga boyunda okuma yapılır.

Türkiye' de, klinik laboratuvarlarda kullanılan galaktozemi görüntüleme kitleri bir çok ülkeden ithal edilir. Bu da hem hastaneler için hem de aileler için ekstra paha getirir. Bu tez de, klinik laboratuvalarda diyagnoz için prototip kit üretilmiştir. Bu kit dışardan ülkelerden alınan kitlerle benzer sonuçlar verirken daha düşük maliyetlidir.

Bu tez ile beraber stripler galaktozemi tanı için kullanılan stripler üretilmiştir. Bu stipler görsel okumaya dayalıdır. Kandaki galaktoz ve galaktoz-1-fosfat miktarına göre rengi beyazdan pembe-kırmızıya dönüşmektedir. Bu çalışmada florometrik okuma gerekli değildir. Bu özellik flurometre cihazının olmadığı yerlerde ve kırsal alanlarda bir avantaj haline gelmektedir.

Anahtar Kelimeler: Galaktoz taraması, galaktosemi, metabolik hastalıklar, yenidoğan tarama testi

To My Family Yasemin ÜNAL & Yelda EKİZ

&

Sinan YILDIRIM

For their support, encouragement & endless love…

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GADH	: Galactose dehydrogenase
AP	: Alkaline Phosphatase
Gal – 1 - P	: Galactose – 1 – Phosphate
GALT	: Galactose-Specific Kinase
GALE	: Uridine diphosphate galactose-4-epimerase
GALK	: Galactose-1-phosphate uridyl transferase
RFU	: Relative Fluorescence Unit
Gal	: Galactose
UDP-gal	: Uridine diphosphate galactose
UDP-glu	: Uridine diphosphate glucose
NAD	: Nicotinamide adenine dinucleotide

CHAPTER 1

INTRODUCTION

1.1 Sugars– Carbohydrates

Carbohydrates, lipids, proteins and nucleic acids are the four kinds of macromolecules found in living things. Most of these molecules are found in nature in polymer forms, mean that they are composed of smaller molecules, monomers. Both macromolecules and monomers have important job in living things. They participate in formation of cell membrane, DNA structure, formation of energy, conservation of organs, etc (Sadava et al., 2006).

Carbohydrates are source of usable energy for body tissues and these molecules can be arranged different forms that are essential for biological structures and functions (Voet & Voet, 2003).

General formula of carbohydrate is $C_x(H_2O)_y$, which shows proportion of carbon, hydrogen and oxygen in molecule. Carbohydrates are available in monosaccharide, disaccharide, oligosaccharide and polysaccharide forms. All living cells contain glucose molecule, which is a monosaccharide, uses as an energy source. Cells also contain other monosaccharide such as ribose, deoxyribose, mannose, galactose and fructose. These monosaccharides are linked to each other with glyosidic linkage and form disaccharides, oligosaccharides and polysaccharides. Figure 1-1 shows some of the monosaccharides which form disaccharides with glyosidic linkage. Starch, glycogen, cellulose are some polysaccharide molecules. (Michael & Farabee, 2006).

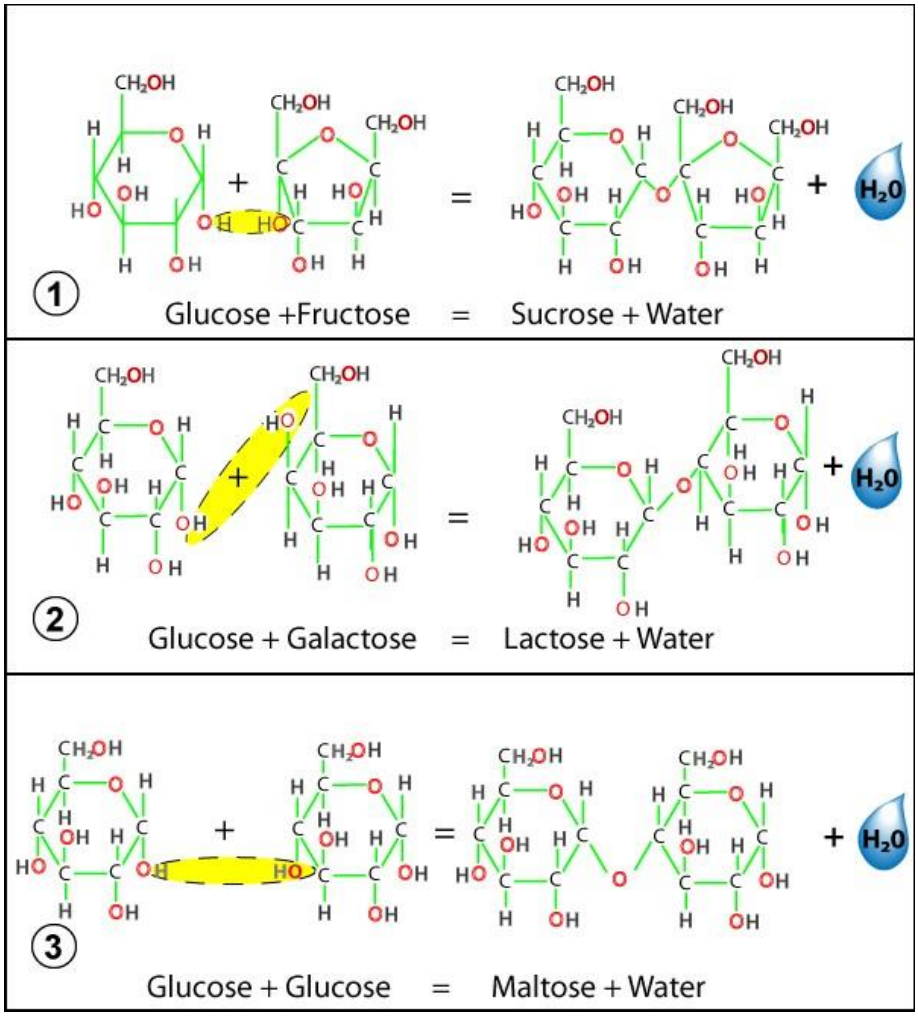


Figure 1-1 Formation of disaccharides from monosaccharides. Two monomers connected to each other with glyosidic linkage and form one disaccharide and one water molecule (Michael & Farabee, 2006).

Humans not only use dietary carbohydrate but also they modify carbohydrate for necessary reversible metabolism. When human take carbohydrate in to the body, they can digest them in to monomers for use in glycolysis, lipid metabolism or protein metabolism (Sadava et al., 2006).

1.2 Galactose metabolism & Leloir Pathway

Lactose is a disaccharide composed of galactose and glucose. It is generally known as milk sugar and occurs naturally only in milk that typically use in human diet. When it is hydrolyzed, it breaks down to its monomers, galactose and glucose. Galactose is not directly used in human body. It must be converted to glucose. Galactose is metabolized and utilized in human body via Leloir pathway (Frey, 1996). The metabolism of galactose was delineated in 1948 to 1951, mainly by L. F. Leloir and his associates. (Prey, 1996) Figure 1-2 shows galactose metabolism. (Holdent et al., 2003).

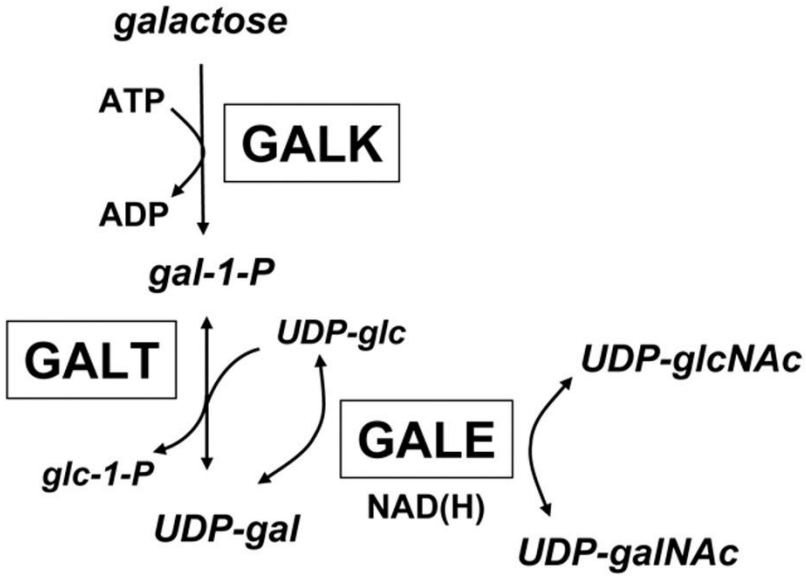


Figure 1-2 Galactose does not use directly in human body. It is metabolized via Leloir Pathway (Sanders et al., 2010).

From bacteria to human, galactose metabolized through Leloir Pathway (Sanders, et al., 2010). This pathway is only mechanism of utilization of galactose. (Grossiord et al., 2003; Kalckar et al., 1959; Slepak et al., 2005; Yarmolinsky, et al., 1959).

In 1959, Kalckar was examined three enzymes that have role on Leloir pathway. These were galactokinase (GALK), galactose-1-phosphate uridyl transferase (GALT), and galactose-4epimerase (GALE).

Each of these enzymes utilizes different metabolic intermediates. First enzyme of Leloir pathway, Galactokinase (GALK) plays role in formation galactose – 1 – phosphate from galactose in to (gal – 1 – p) (Frey, 1996). Galactose is first phosphorylated by galactokinase and produces one of the two substrate of GALT, galactose-1-phosphate (gal-1P) (Grossiord et al., 2003; Kalckar et al., 1959; Mollet & Pilloud, 1991).

In second step, galactose – one uridine diphosphoglactose (UDP-gal) and one glucose-1-phosphate (glu-1-P) are formed after one uridine diphosphoglucose (UDP-glu) and one gal-1P reacted with GALT (Frey, 1996).

UDP gal continue in the Leloir pathway to energy production also it can be used in synthesize number of glycoconjugates. Finally, third enzyme is UDP – galactose – 4' – epimerase (GALE). This enzyme catalyzes epimerization of UDP-galactose at 4-carbon into UDPglucose (Frey, 1996). UDP glucose can enter glycolysis or used as UDP to formation of necessary glucose derivative (Grossiord et al., 2003; Kalckar et al., 1959; Mollet & Pilloud, 1991).

1.3 Molecular Biology of GALT Gene

Human GALT gene, galactose 1 – phosphate, uridylytransferase, was completely identified and sequenced (Leslie, et. al 1992). GALT gene is located on the small arm of chromosome 9p13 (Figure 1-3). It has 4 kb gene length, comprises 11 exon, and encodes 397 amino acid of protein. The active protein molecular mass is approximately 86-88 kDa. Promoter of GALT gene contains two GC rich sites, a CCAAT and three AP-1 sequences. This gene thought to be housekeeping gene because of no consensus of TATA box. But regulatory domains of this gene play role in tissue specific and developmental tissue specific gene expression (Prasad et al., 2013).

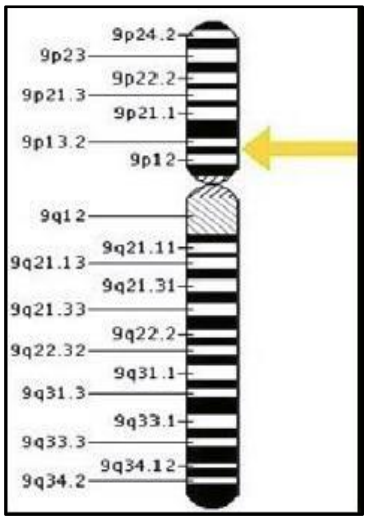


Figure 1-3 Galactose 1 – phosphate uridylytransferase gene, GALT gene is located on the small arm of chromosome 9p13 (Prasad et al., 2013).

Human GALT gene's His-Pro-His sequences are highly conserved. The site consists Histidine-Proline-Histidine-Cysteine-Glutamine at 184 to 188 sites at human GALT gene. This site is located on β-sheet at the core of catalytic structure. This site is critical because of most mutation is seen at the position of 188 (Prasad et al., 2013).

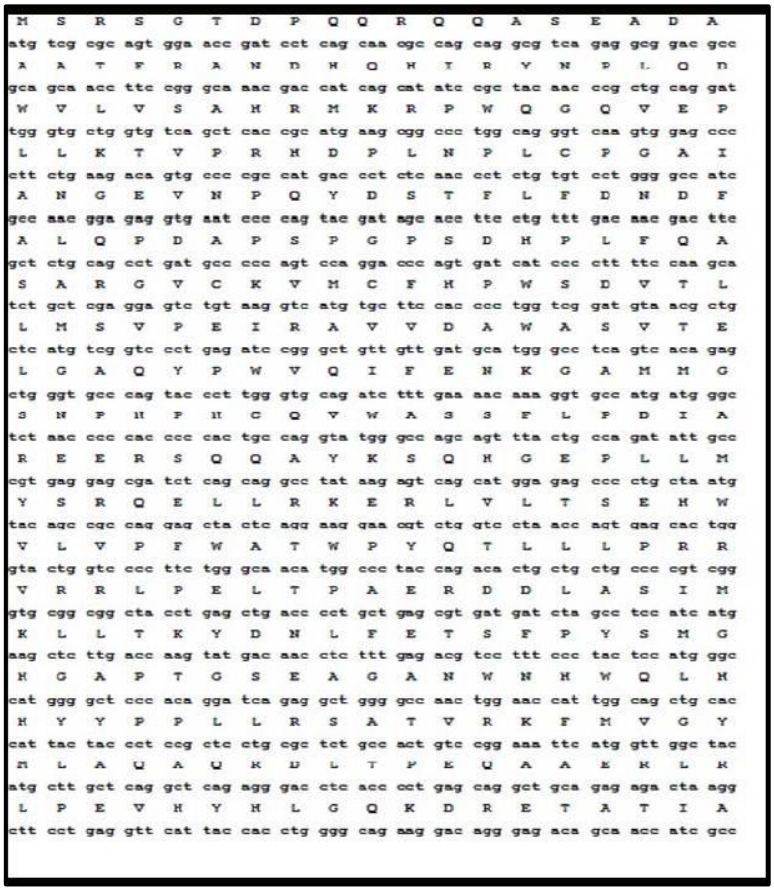


Figure 1-4 cDNA sequences of GALT gene (Prasad et al., 2013)

1.4 GALT Deficiency

If there is a homozygous mutation in this gene that can cause loss of function of GALT enzyme activity, then GALT deficiency also known as galactosemia can occur (Arn, 2003).

Until to date, more than 200 mutations occurred in GALT gene have been recorded. The most common mutations are Q188R, K825N, S135l and N314D (Prasad et al., 2013).

In Caucasian (white) Europeans and North Americans, conversion of glutamine residue to arginine residue at location 188 is the most common mutation (Tyfield et al., 1999). This mutation rate is around 60% to 70%. Glutamine is hydrophilic amino acid and arginine is basic, positively charged amino acid. Because of any

changes between these amino acids can result with conformational changes and leading to enzyme inactivity.

The S135L, serine to leucine replacement at location 135 mutations which is frequently observed in African Americans, is around 50% of the mutant alleles. Another mutation is K285N which is replacement of lysine to asparagine (Seyrantepe, et. al 1999). It is common in people of European descent and is around 25% to 40% of the alleles. 5% of general population of United States has another mutation, N314D. This mutation is replacement of asparagine by aspartate (Seyrantepe, et. al 1999).

Moreover GALT gene includes six missense mutations, are Y89H, Q103R, P166A, S181F, K825R, and R333L. And it also includes one nonsense mutation, is S307X and 3 silent mutations (Prasad et al., 2013)

1.5 Molecular Biology of GALK Gene

Galactokinase gene was characterized in 1995 (Stambolin et al., 1995) It is located on the chromosome 17q24 (Figure 1- 5). It is 1.35 kb long and encodes 392 amino acids (Prasad et al., 2013).

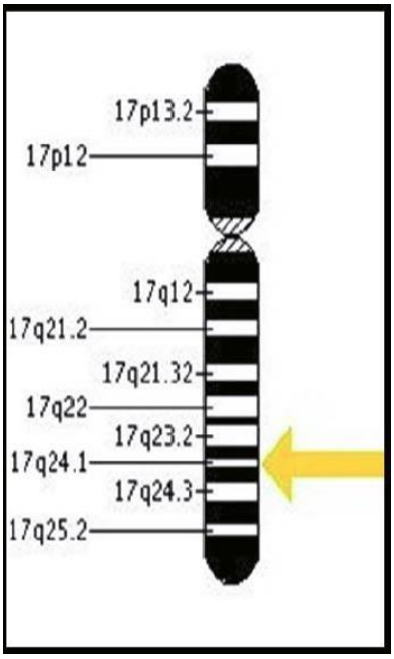


Figure 1-5 Galactokinase is located on the chromosome 17q24 (Prasad et al., 2013).

1.6 GALK Deficiency

Galactokinase deficiency is described in 1965 by Gizeltmann (Cook et al., 1971). It is an autosomal genetic disorder with an incidence of 1/1,000,000 in Caucasians (Segal and Berry, 1995), and 1/1,000,000 in Japan (Aoki and Wada, 1998). Till today, 25 different mutations were observed in GALK gene which results in Galactosemia II (Prasad et al., 2013).

The only consistent clinical finding of GALT deficiency is cataract. Cataract is the osmotic phenomena that result of accumulation of galactitol in the lens. This product is synthesized from reduction of galactose via aldose reductase. Complete or partial loss of activity in galactose metabolism, high consumption of lactose may result with cataract formation (Prasad et al., 2013).

1.6 Molecular Biology of GALE Gene

The human GALE, UDP-galactose-4-epimerase, gene is located in the short arm of chromosome 1 between position at 35 and 36 (Prasad et al., 2013). GALE gene has 1488 base pairs and a protein composed of 348 amino acids (Figure 1- 6).

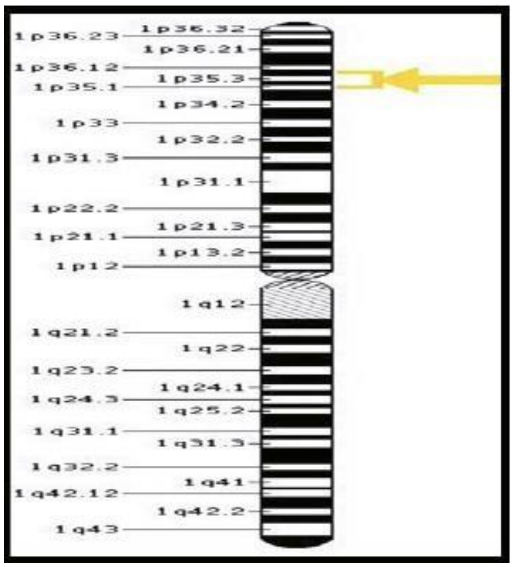


Figure 1-6 GALE gene is located in the short arm of chromosome 1 between position at 35 and 36 (Prasad et al., 2013).

1.7 GALE Deficiency

Up to date only 20 mutations have been recorded in GALE gene. Complete loss of function of enzyme is related with V94M and G908E mutations. On the other hand, L183P, D103G, L313M and N34S mutations have almost normal activity of enzyme (Prasad et al., 2013).

1.8 Galactosemia

Galactosemia is a genetic disorder which is caused by loss of GALT enzyme activity. It is an autosomal recessive disorder which means both gene comes from mother and father must be mutated (Waisbren et al., 2011).

The disease is typically seen after birth when infant start to be fed with breast milk, which contains lactose. Lactose is a disaccharide, when metabolized it can produce one mole of galactose and one mole of glucose. Glucose directly enters glycolysis but galactose does not. It should be converted into UDP – gal in order to enter glycolysis. (Charlwood et al 1998; Lai & Elsas, 2000; Lai et al., 2003; Liu et al., 2012; Sturiale et al., 2005).

There are three types of galactosemia: Galactosemia I, Galactosemia II, and Galactosemia III. Classic galactosemia, Galactosemia I, seen 1 in 30,000 to 60,000 newborns. Other types are seen less. Galactosemia II is seen fever than 1 in 100,000 and Galactosemia III is seen rare in newborns. Each type of galactosemia has different patterns of signs and symptoms (Fridovich et al., 2006)

Galactosemia I, the most common and most severe type, is occured when GALT enzyme is mutated. Infant with Galactosemia I have prone to feeding difficulties, no gain weight, liver damage and bleeding, skin with yellow color and jaundice. Sepsis and shock are severe complications. Also these children have increasing risk of cataract, speech difficulties and intellectual disability (Coman et al., 2010).

Galactosemia II occurs in deficiency of galactokinase enzyme. Galactosemia II has fewer medical problems than galactosemia I, classical type. These infants develops cataract. Other abnormalities of galactosemia II are muscular deposits, mental retardation and coincidental. (Thoden et al., 2005)

An increase in galactose or galactose-1-phospate and decrease in GALE activity cause galactosemia III. Galactosemia III was considered to exist in two forms- the severe or generalized, form and much milder peripheral form (Timson, 2006). GALE deficiency inhibits UDP glucose regeneration that results in accumulation of galactose-1-phosphate in the human body. Intellectual disability, liver diseases, hypotoina, splenomegaly, ovarian failure and kidney problems are complications of Galactosemia III (Prasad et al., 2013).

1.9 Long-term complications in classic galactosemia

Patients with galactosemia born with no clinical symptoms until beginning to nurse or drink milk or milk based. These infants shows symptoms of cataracts, vomiting, and diarrhea, to hepatomegaly, *E. coli* sepsis, and death. In order to prevent these acute results, galactose is removed from the diet. However, older people with galactosemia showed long-term complications of this disease. These complications were ovarian dysfunction of girl with 80% to 90%, cognitive, speech difficulties and variety of neurological outcomes (Testa, 1996).

1.10 Diagnosis and Screening

In the world, the incidence of galactosemia varies. In US, one baby in every 30,000 to 60,000 babies, birth with galactosemia. The screening is done via newborn screening or observation disease symptoms. ("Expanded Newborn Screening Using Tandem Mass Spectrometry Financial, Ethical, Legal and Social Issues (FELSI)," 2013). This mutation is also seen in Turkey. In Turkey, incidence of galactosemia is 1:23,775. In Europe, it is seen in 1:30-40,000 births (Aydın Özdemir, et.al 2014).

Diseases symptoms can start after infant is fed lactose containing products. In order prevent death or other complications, some screening methods were developed.

Galactose-1-phosphate urdiyltranferase catalyzes reaction of galactose-1-phosphate to UDPgalactose mechanism reversibly. The absent or deficiency of this enzyme partially blocks the galactose metabolism. Galactose-1-phosphate is accumulated in red blood cells. One of the galactosemia screening methods uses this accumulation. This technique detects galactosemia people with galactose tolerance or galactose-1-phosphate uridylytransferase level in red blood cell. In this method hemolyzates were as enzyme source. Incubation time with was 30 minutes. One sample was incubated with Gal-1-P alone and the second with UDPG alone. After deproteinization, the missing substrate was added. The third sample was incubated with both substrates. Specific enzymatic methods were used in order to determine of Gal-i-P, UDPG, and UDPGal. The indicator of this method is one of the pyridine nucleotides (TPN or DPN). Both nucleotides develop an increase in absorption at 340 mp. The Gal-i-P was determined by means of its liberation of G-1-P from UDPG, using purified PGal transferase and a TPN indicator system. UDPG was determined by means of a specific, purified UDPG dehydrogenase, using DPN as indicator. UDPGal was determined by the same principle. UDPGal was prepared by enzymatic techniques. It was confirmed and extended the observation of Schwartz et al. that galactose added to erythrocytes from galactosemic children gives rise to accumulation of Gal-i-P. The erythrocytes were incubated with galactose for three hours at 37° C and the Gal-i-P determined

enzymatically. Blood from normal children showed no accumulation of Gal-i-P under these conditions. However, in cases of galactosemia, about 0.1 MM of Gal-i-P accumulated per milliliter of red blood cells per hour. The presence of PGal transferase was studied by means of incubating Gal-i-P and UDPG together with hemolyzates and subsequently measuring by enzymatic techniques the conversion of UDPG to UDPGal. In order to insure that the hemolyzates were not fortuitously varying in activity with respect to this class of enzymes, another nucleotide transferase, which we call "PP transferase,"'0" was also measured by the same type of method and at the same time as the PGal transferase. The PP transferase catalyzes the following reaction, which involves inorganic pyrophosphate (PP) and uridine triphosphate (UTP): G-1-P + UTP = PP + UDPG. The PP transferase does not play a direct role in the metabolism of Gal-l-P. It brings about a conversion of G-1-P to UDPG, which by subsequent enzymatic dehydrogenation is converted to UDPglucuronic acid (Kalckar, 1956).

Another method of the galactosemia screening method is automated fluorometric techinique.This technique is used for determination of galactose from dried blood spots. The principle of this method based on the fluorometric determination of GADH-NAD^+/NADH reaction system. In this method, blood samples are collected on Schleicher and Schuell No. 903 C filter paper and punched. Blood punches are 7.94 mm diameter. Then, they are transferred to elute the blood from it by gentle shaking in 0.5 ml of the elution solution during 30 mm. Disc is discarded and samples are analyzed for with the Auto Analyzer. The Auto Analyzer system run the reagents for about 20 min to establish the baseline and then run a series of standards (20, 40,60, and 80 mg/100 ml). Each standard being separated from the next by a cup of Tris buffer. Transmittance should be at least 80% for the 100 mg/100 ml galactose standard if sensitivity and resolution are to be good when the samples will be run. In this method, although there is no detectable galactose in the blood of normal infant, each sample gave small peaks. Moreover, sometimes nonspecific fluorescence may be quite high in a given sample. This method was suitable for mass screening and it had very little false positive result. (Laberge & Grenier, 1973).

Fujimura and his colleagues were developed an enzymatic fluorometric assay for determination of galactose and galactose – 1- phosphate. The assay was based on GADH- NAD^+ / NADH reaction system combined with using alkaline phosphatase. This method needs fluorescence in order to read results. Samples of this reactions systems were dried blood spots. Each spots size of dried blood is 3 mm. This method needs to hemoglobin fixation. Fixation is done by methanol: acetone: de – ionized water (35:35:10 by vol). After fixation, each dried blood spots combine with enzyme solution, incubated and fluorescence reading was done. The sample concentration taken from regression curve with mean and SD values were calculated. In this method, galactosemia -positive results are automatically detected. This test' cutoff value is 80 mg/L (Fujimura et al., 1981).

Beutler Test which is, enzyme spot test, activity of GALT enzyme is monitored with the aim of phosphoglucomutase and glucose-6-phosphate dehydrogenase (G6PD) present naturally in RBCs as the enzyme reactions subsequent to the GALT enzyme. GALT activity in the Beutler enzyme spot test is determined by the fluorescence of NADPH converted from NADP+ in the G6PD reaction. On the basis of this principle, it is suspected that G6PD deficiency appears as a positive result. Yet, Beutler test may give false negative result after blood transfusion. Shih et al. identified an increased frequency of false positives in the Beutler spot tests. The deterioration of the GALT enzyme in dried blood spots was attributed to high humidity and high temperature, and was recognized as a weak point of Beutler enzyme spot test. Also it only depends on qualitative and relies on visual interpretation. With this situation DNA test should be done for K825N, Q188R, N314D and s135L (Fujimoto et al., 2000)

Another test for galactosemia is looking GALT enzyme and galactose in new – born baby's blood and urine sample. (Freer et al., 2010). In this method, galactose concentration and GALT enzyme activity is measured. According to this method, if level of galactosemia is high, GALT enzyme activity is low. These samples are further tested to detect other mutations of galactosemia (Freer et al., 2010). In normal way, the human GALT enzyme amount is 150 – 500 μmol/L but in

galactosemia condition with GALT enzyme deficiency, this enzyme is less than 32 μmol/L (Freer et al., 2010). However, if child is on intravenous fluids, there will be no galactose in urine. Moreover, galactosuria is found in people with liver disease and glucose and fructose give positive result also. This test should be supported by glucose dipstick test.

Benedict's test, described by Stanley Benedict in 1908, is a semi quantitative test for reducing sugar in urine. It is performed based on Benedict's reagent which is a chemical reagent that is used to detect presence of reducing sugar. Test consists of copper (II) sulfate, which is reduced in the presence of some compounds to give a copper (I) oxide precipitates, and also changes the color of the solution. If a result is positive, color change from a deep blue alkaline solution to red-brown depends on reducing agent concentration. Although, Benedict's tests have good correlation between quantitative analytical strategies, it is important to know Benedict's limitations. It has low specificity and sensitivity. For example; it gives positive result with other reducing substances. This includes all monosaccharides and many disaccharides, including lactose and maltose (Morell-Garcia et al., 2013).

1.11 Treatment / Management

The first step of management galactosemia is removal of galactose from diet. Other management method is using casein hydrolysates and dextrin maltose as carbohydrate source. According to complications, i.e. sepsis, liver, failure, alternative therapies may be applied. For abnormal clotting, Vitamin K and fresh-frozen plasma are used. Cow milk formula or breast milk must be replaced with soy milk formula (Prasad et al., 2013). Calcium supplements will be added in to diet if calcium intake does not meet recommended daily allowance. (Arn, 2003)

Also physical and speech therapy are applied to the patients. For galactosemia III, speech therapy may address speech delay or verbal dyspeaxia (Arn, 2003).

1.12 Aim of Study

In this study we aimed to a **neonatal galactose screening test kit prototype** to be used for screening of newborns for galactosemia. This kit has a cost advantage as it has been produced in Turkey using local sources and know-how. Currently, Galactosemia screening is not included in the Health Ministry's newborn screening program. With its low cost our kit will have the potential to be used in this program when ministry enlarges included test in the program. In this study we have also developed a strip test prototype which provides faster results and easier to use.

CHAPTER 2

MATERIALS AND METHOD

2.1 Materials

D - (+) - Galactose and Type II, ≥98% α-D-Galactose 1-phosphate dipotassium salt pentahydrate were used in this thesis. They were purchased SIGMA. For enzymatic reactions, buffered aqueous solution, 2,000-4,000 DEA units/mg protein alkaline phosphatase from bovine intestinal mucosa , recombinant, expressed in E. coli, ammonium sulfate suspension, ≥50 units/mg protein (biuret) beta-Galactose Dehydrogenase from *Pseudomonas fluorescens* and ≥96.5% (HPLC), ≥96.5% (spectrophotometric assay), ≥96.5% (enzymatic), from yeast βNicotinamide adenine dinucleotide hydrate were used. They were also purchased from SIGMA. For color formation reaction, 95% Iodonitrotetrazolium chloride and lyophilized powder, 3.0-20.0 units/mg protein (biuret) Diaphorase from *Clostridium kluyver* were used. Iodonitrotetrazolium chloride was purchased from SIGMA. Diaphorase from Clostridium kluyveri was purchased from ALDIRICH. For blood fixation filter paper 580 x 580 mm, grade 903 was used. It was purchased from GHEC-watman. For buffer preparation trizma base, magnesium chloride hexahydrate, potassium chloride and bovine serum albimum were used. Primary Standard and Buffer, ≥99.9% (titration), crystalline Trizma base, potassium chloride and bovine serum albimum were purchased from SIGMA. Magnesium chloride hexahydrate was purchased from MERCK.

2.2 Methods

Galactosemia diagnosis has different methods. One method is the measurement of activity of GALT enzyme. This measurement method is described in introduction part under galactosemia diagnosis header. Another pathway is, galactosemia may diagnose with using metabolites of Leloir Pathway.

In this thesis, galactose and galactose – 1 – phosphate concentrations were used for diagnosis of galactosemia. Amount of galactose and galactose – 1 –phosphate in the blood was detected using galactose dehydrogenase and alkaline phosphatase enzymes is done by measuring aid of emerging fluorimeter (Misuma & Wada, 1981).

This reaction has two consecutive steps. The first step galactose – 1- phosphate is converted to galactose by alkaline phosphatase. Second step both galactose that form in first reaction and free galactose are converted galactono – lactone by galactose dehydrogenase enzyme. In second step NAD^+ comprises. And that NAD^+ takes part in electron receiver and it became reduced NADH (Figure 2-1).

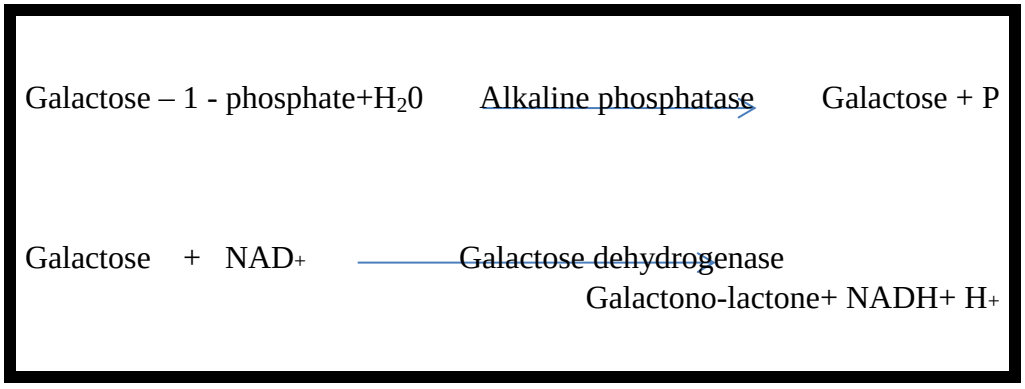


Figure 2-1 Reaction of galactose - 1 - phosphate to galactonolactone

NAD^+ and NADH have different fluorescence values in solutions. NAD^+ has no absorbance at 340 nm. On the other hand, NADH is radiated at 340 nm and read at 460 nm wavelength. And it gives half quantitative reading under UV-lamb (Misuma & Wada 1981). Alike, samples with known quantities of galactose and

galactose – 1 – phosphate are used to form standardized curve and then this curve is used for unknown quantities. Only this advantage of this method fluorimeter reader is expensive equipment (Misuma & Wada 1981).

There were some studies carried out that allow modifications on the determination to be made without requiring fluorescence measurements. Diepenbrock and his colleagues studied colorimetric reading (Diepenbrock et al. 1992). In the presence diaphoresis enzyme and Iodonitrotetrazolium, NADH is oxidized and formazan with red color is formed. This color is observed with naked eye. This method requires less laboratory costs and amount sufficient to scan gives the quantification (Diepenbrock et al. 1992) (Figure 2-2).

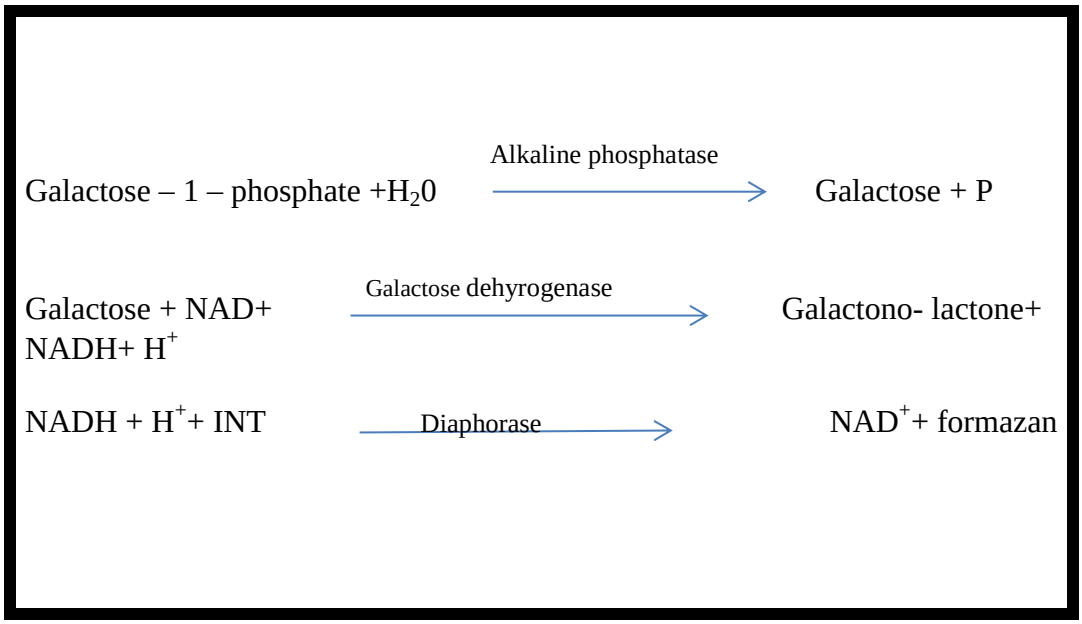
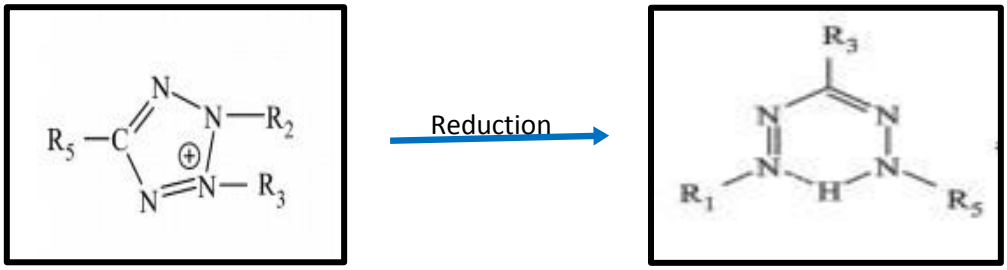


Figure 2-2 Formation of formazan. Formazan gave red color in to the solution. In this thesis, formazan was used as a colorimetric reading

Tetrazolium is an organic compound that has quaternized tetrazols. Its double bond can not strictly locate, it can be changed and isomers are formed. Tetrazolium salts are generally colorless although some of them are faintly yellow color. Tetrazolium salts are reduced to deeply colored compounds formazan (Figure 2-3). Formazan has color with ranging from cheery red to deep purplish black. Formazan molecules that contain Hydrogen Bridge are red color.



Tetrazolium Formazan

Figure 2-3 Tetrazolium to formazan formation. Tetrazolium salts are reduced to deeply colored compounds formazan

-

There were also some studies provide that to this method were studied with multiple well plates. In this method blood is saturated to special paper and then it is punched. Galactose and galactose-1-phosphate is revealed via chemical reagents. After this step, enzymatic reactions are done and whether microplate is read via fluorimeter or colorimetric reading is done (Fujimura & Ishii et al. 1981).

2.1.1 Reaction Solutions

In this thesis, one mainly enzyme solution was used. In order to prepare this solution, following chemicals were used.

- Tris –HCl Buffer with 0.2 M pH: 8.6
- 45 mM NAD^+
- 20 mM $MgCl_2$ x $6H_2O$
- Galactose dehydrogenase (Pseudomonas fluorescens), ≥50 units/mg protein
- Alkaline Phosphatase, 2,000-4,000 DEA units/mg protein

Enzyme solution was prepared before the experiment. And it must be kept at 4 °C in order to prevent degradation of enzymes.

For colorimetric reading, one mainly reaction solution was used. In order to prepare this solution, following chemicals were used:

- Diaphorase (*Clostridium kluyveri*), 31 U/mg
- 2.0 M Tris-HCl (pH: 8.6)
- KCl with 0.29 M,
- 250 mg/L BSA

Second solution was consisted of 3 mL INT with solution that described above. This solution was sensitive to light. Therefore it should be stored in dark at 4 °C.

Diepenbrock and his colleagues were got referenced to preparation of this enzyme solutions (Diepenbrock et al., 1992).

2.2.2 Galactose and Galactose – 1 – Phosphate Solutions

Galactose and Galactose – 1 – phosphate solutions were prepared according to Diepenbrock and colleagues paper. Different concentration of galactose concentrations were prepared 0.6 mM, 1.7 mM, 2.8 mM and 5.6 mM and galactose – 1 – phosphate were arranged 0.3 mM, 0.9 mM, 1.5 mM, and 3.0 mM. This mM were equal to 10, 30, 50 and 100 mg/dL.

When preparing galactose and galactose – 1 – phosphate solution, the important point was final concentration must be equal to concentration that to be mentioned above. Namely, when enzyme solution I was added, final concentration was equal to 10, 30, 50 and 100 mg/dL.

In the thesis, firstly galactose and galactose – 1- phosphate solutions were prepared in Tris-HCl solution with 2.0 M at pH: 8.6. 40 µl solutions were prepared. To prevent batch to batch preparation 100 mM stock solution was prepared. This stock was again prepared with Tris HCl solution with 2.0 M at pH: 8.6. 40 mL. Aim of using Tris-HCl solution range between pH 7- 9 coincides with the physiological pH typical of most living organisms.

In the first step only for galactose solutions which were 0.6mM, 1.7 mM, 2, 8 mM, 5.6 mM were arranged. Initial experiments were in order to providing optimization. Narrow substrate concentration was preferred. All substrate were arranged triplicate in order to ensure the accuracy of experiments.

After in the thesis, broad substrate concentrations were studied. Because of using broad spectrum, not to skip any concentration. Also it helped to increasing sensitivity of experiments.
With these studies level out values was determined.

2.2.3 Blood Galactose and Galactose – 1 – phosphate Solutions

In the blood studies, blood was taken only one person, in order to prevent batch variation. Blood was taken EDTA tubes and stored at 4 °C. Blood solutions were prepared as same as galactose solutions which described above. After blood solutions prepared, it dropped on to paper with 10 µl to 15 µl. At the beginning, normal filter paper was used and then blood fixation filter paper 580 x 580 mm, grade 903 was used.

Dropped blood on the paper dried 2 – 3 hours at room temperature, away from direct sun light and humidity. After desiccation, blood samples can be stored in refrigerator about 1 month.

For experiment, blood paper was punched. In Düzen Laboratories, experiments were done with 2 punches. Each of punch had 2.3 mm diameter. In this thesis, one punch was used which had approximately 4.8 mm. Therefore, one punch was enough to doing experiment in this thesis study.

In order to prevent, diffusion of color blood to solution, blood fixation was done. Blood fixation, firstly was done by using steam bath. Punched paper put down in to Eppendorf and then put on to the boiling water at 1 hour. But in this method, after adding enzyme solution, hemoglobin diffused in to solution and give cloudy –

reddish color. This color was negatively affecting the fluorometric reading. Moreover, colorimetric reading could not be done. After this unsuccessful experiment, ethanol precipitation was tried. In this method 80% ethanol was used. 20 µl ethanol was dropped each Eppendorf and dried at 37 °C at 1 hour. In this step, critical point was Eppendorf's cap must be open in order to provide ethanol evaporation. After fixation, enzyme solution was dropped. The amount of solution was 80 µl.

2.2.4 Strip Studies

Fluorometric reading was an expensive method of galactosemia diagnosis. This reader is expensive equipment and all clinics do not have this reader. Therefore, in this study, paper studies were done in order to minimize the necessity of equipment. Paper studies were tested firstly on filter paper. Filter papers were punched. Amount of solution that put on to the paper were chosen according to diameter of punch. Excessive amount of solutions was overflowed from paper. Also solution should be covering all the paper. According to these arrangements,

5 to 10 µl solutions were put on the papers. 10 µl enzyme solutions was added and dried.

After desiccation, 8 µl solution II was added and again dried. After adding solution II, paper must be dried at dark at 23°C. Dried paper put in to the different concentrations of galactose solution with Tris-HCl. They incubated at 37 °C 1 hour.

Another pathway of strip studies was only solution II was dropped on the paper. Enzyme solution was not dropped. Again, 8 µl solution II was dropped on the papers. The papers were put in to the tubes and dried at 23 ° C at 2 to 3 hours. The important point was tubes were covered with aluminum foil to protect from light. Simultaneously, 20 µl enzyme solutions were added to 40 µl different concentrations of galactose solutions and they were incubated at

37 °C 1 hour. After incubation, dried papers put in to the tubes and incubated at 23°C 10 minutes.

Other trials were also done with strips (Figure 2 - 4). Strips were bigger and thicker than filter paper strips. Some experiments were done with all strips some were divided in to two strips. Amount of solutions was bit increased. In the strips studies, firstly both of enzyme solution and solution II were dropped and dried at room temperature. After this step, strips were incubated at 37 °C 1 hour with different concentrations of galactose solutions which prepared with Tris-HCl.

Figure 2-4 Strips that were used in thesis study.

If enzyme solution and solution II were dropped simultaneously or one dried and other dropped, the solutions became inactive. Therefore, again in the paper studies solution II was dropped on the paper and dried. With this method, both fluorometric results were handled and both strips result were.

Because of thickness and size, solution II amount were increased to 10 µl. Strips were placed on to the heat block. Temperature was arranged to 23° C and dried 2 – 3 hours. Room must be kept dark. Galactose and enzyme solutions were added on the strips gave more accurate result because strips were thick and long and its all part did not contact with solution II. After dropping galactose-enzyme solution they incubated 10 min at 23° C at dark.

2.2.5 Shelf – Life Studies

In enzymatic reactions, stability enzyme was important point. In order to adjust enzyme stability, shelf life studies were done. In shelf-life experiments, 7 different galactose concentrations, 0.1 mM, 0.5 mM, 1.5 mM, 3.5 mM, 5.0 mM, 7.0 mM and 10.0 mM, were studied. Enzyme activity, seven weeks were measured.

2.2.6 Validation Studies

In the small value of galactose and galactose – 1- phosphate, enzyme activity gave near results to each other. In order to improve this validation studies were done. In validation studies, firstly, 4 different groups can be measured with different concentration of galactose – 1 – phosphate. These 4 different groups had different enzyme amount. Table 2 -1 was showed 4 different conditions of validation studies.

Table 2-1 Four different enzyme solution were tried in validation studies. Enzyme amounts were re-arranged.

Enzyme Solution	**Standard condition**	**1st Condition**	**2nd Condition**	**3rd Condition**
Tris - HCl	X	X	X	X
$MgCl_2$	X	X	X	X
NAD	X	X	X	X
GADH	X	2X	X	2X
AP	X	X	2X	2X

Another validation studies were done with 2 different conditions. One of these conditions was normal condition that used in the experiment; other condition was amount of enzymes in the solution was halved. Table 2-2 shows standard conditions and 4th condition.

Table 2-2 In 4th condition, both enzyme amount is halved.

Enzyme Solution	Standard Condition	4 th Condition
Tris - HCl	**X**	**X**
$MgCl_2$	X	X
NAD	X	X
GADH	X	X/2
AP	X	X/2

Other studies of validation were about on time. Time was important subject in the enzymatic activities. Half an hour of enzyme incubation was tested instead of incubating 1 hour.

2.2.7 Real Samples Experiment

In order to ensure whether galactose and galactose – 1 – phosphate results were true, real samples were studied. Real samples were taken Düzen Laboratory. The experiment assembly was established in the Düzen Laboratory. Enzyme solutions were prepared and send to the Düzen Laboratory. In laboratory, they were using enzyme solution and their substrates were real samples that taken from patients. They took results and gave feedback about enzymatic activity of solution.

2.2.8 Reaction Summary

General Experiment Procedure:

- Different concentrations of galactose or galactose -1-phosphate were prepared.
- Different concentrations of galactose or galactose-1-phosphate were prepared in Tris-HCL solution or in blood.
- In both solvent, stock solution was prepared.
- In the blood studies, blood was taken EDTA tubes and stored at 4 °C.
- After blood solutions prepared, it dropped on to filter paper 580 x 580 mm, Grade 903 with 10 µl to 15 µl.
- Dropped blood on the paper dried 2 – 3 hours at room temperature, away from direct sun light and humidity.
- Blood paper was punched. One punch was approximately 4.8 mm.
- 80% ethanol was used for blood fixation. 20 µl ethanol was dropped each Eppendorf and dried at 37 °C at 1 hour. Tube caps must be open.
- 80 µl enzyme solution was dropped into the each tubes and incubated at 37 °C at 1 hour.
- Fluorometric reading was done at 460 nm
- 10 µl solution II was added and incubated 23° C at 10 min.

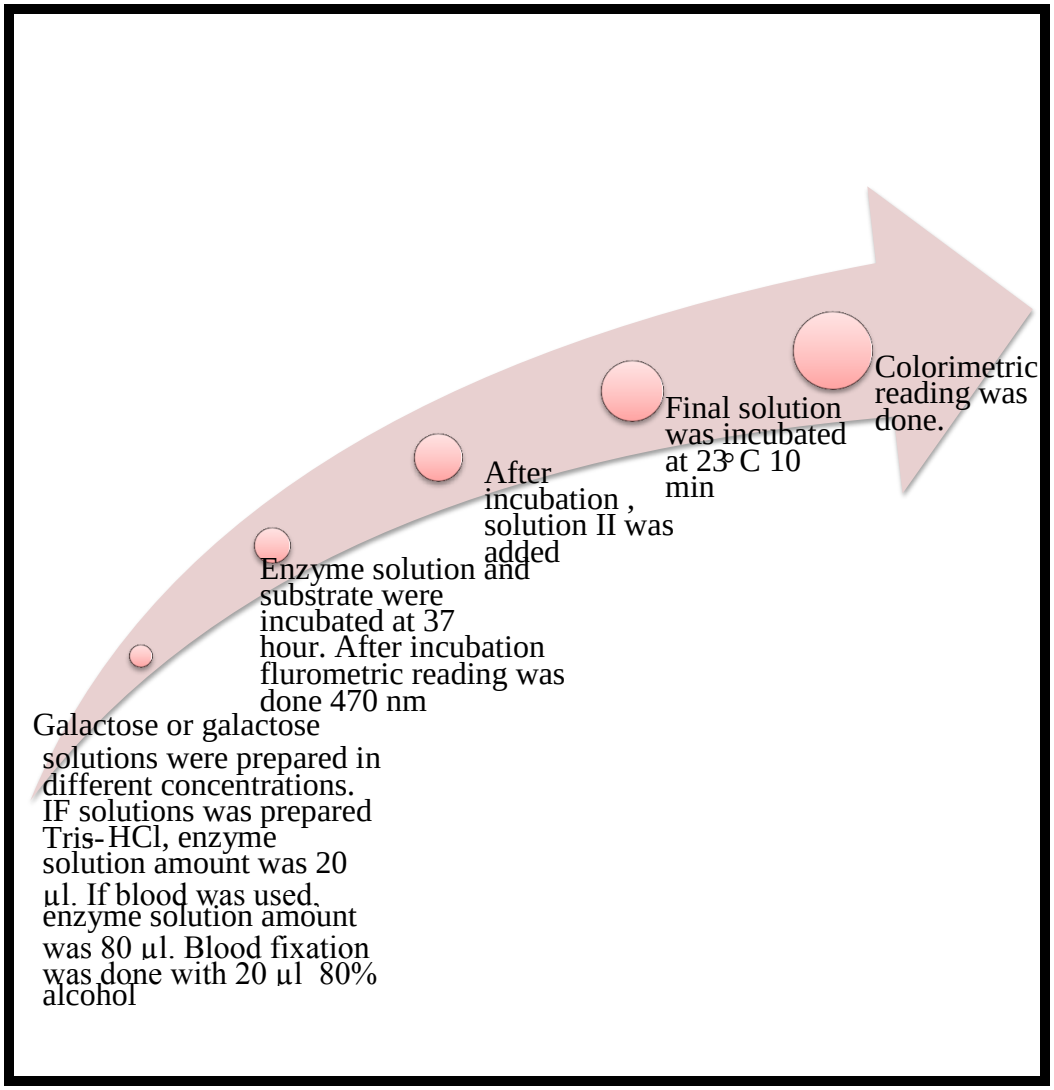


Figure 2-5 Summary of reaction

CHAPTER 3

RESULTS & DISCUSSION

3.1 Galactose and Galactose – 1 – Phosphate Results

Optimization experiments were done with galactose solution which had been prepared with Tris-HCl solution. Initial experiments were performed with the narrow concentration range. Each concentration was worked as triplicates. NADH was measured by NANO-drop and results are given as relative fluorescence values (RFU). The amount of NADH in solution was increased, the RFU value was also increase. The galactose and galactose-1-phosphate amount in the solution have correct proportions with RFU value.

According to studies performed at State University, Pathology department, in non galactosemic people's level is 1.0 mg/dL, people with galactosemic on galactose restricted diet level is 1.0-4.0 mg/dL and galactosemic on unrestricted diet people's level is higher than 4.0 mg/dL. Also according to State Newborn Screening programs, cut off value for galactose in blood is mg/dL. However if the value is upper than mg/dL, GALT enzyme is performed.

Concentration range that we have selected was from the studies of (Diepenbrock et al.1992). In order to understand level out values, concentration ranges were expanded. Seven different concentrations of galactose-Tris HCl solution were performed. These concentrations were 0.6 mM, 1.7 mM, 2.8 mM, 4.0 mM, 5.6 mM, 7.5 mM and 10 mM. Each concentration of galactose-Tris-HCl solution was studied in three batches.

Results of these studies were shown in the Table 3.1 and Figure 3.1. Each box composed of arithmetic value of three different batches and final result was obtained from values of 9 different batches. As can be seen in Figure 3.1, at 10 mM galactose concentration a sudden drop in RFU value was observed probably due to the negative effect of high galactose concentration on the enzyme. Table 3.1 and Figure 3.1 show results obtained before the addition of solution II. In order to see how values could be changed after adding solution II, RFU results were taken after incubating with solution II. These results were shown in Table 3-2 and Figure 3-2. RFU results of second measurement were coordinated with first measurement only different was they had smaller RFU values. The reason of these smaller values, free NADH molecules decreased in the solution of experiment. In the second step of experiment, free NADH molecule was formed and in the third step with Diaphorase and INT, these free NADH molecules again converted to NAD and formazan.

Quantitative result of experiment was shown in Figure 3- 3. The left column showed before adding solution II, right column was showed after adding solution II. In the presences of galactose tubes color were changed and turned to the pink-red color.

Table 3-1 RFU result of seven different concentrations of galactose and Tris- HCl solution. Each concentration was studied 3 different batches. Final results were shown arithmetic values.

Galactose Concentration [mM]	Experiment I (RFU)	Experiment II (RFU)	Experiment III (RFU)	Final results (RFU)
0,6mM	389	1503	1519	1137
1,7mM	519	1081	2160	1253
2,8mM	1604	2243	2341	2063
4,0mM	1433	3153	2379	2322
5,6mM	3420	3443	2393	3086
7,5mM	4139	1610	2608	3665

10mM	2605	2478	2934	2672

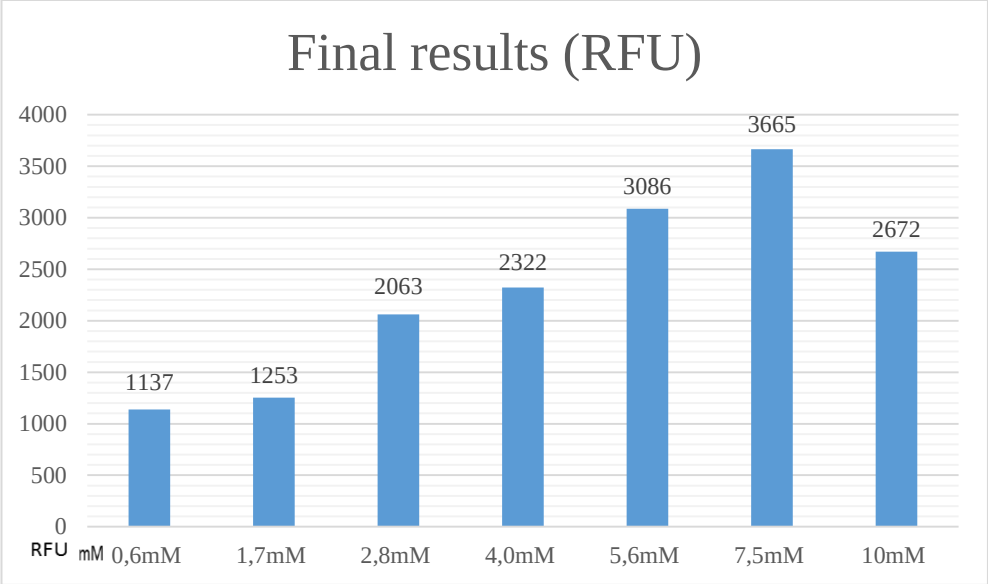


Figure 3-1 Graph of seven different concentrations of galactose. Graph was drawn according to final results. Each bar consisted 21 different values.

Table 3-2 RFU result of seven different concentrations of galactose and Tris-HCl solution after adding solution II. Each concentration were studied 3 different batches. Final results were shown arithmetic values.

Galactose Concentration [mM]	Optimization III (RFU)	Optimization IV (RFU)	Optimization (RFU)	Final results (RFU)
0,6mM	84	859	902	615
1,7mM	120	1081	1310	837
2,8mM	490	1687	1328	960
4,0mM	1064	1831	1427	1168
5,6mM	1800	2183	1551	1440
7,5mM	3111	2101	1736	2316
10mM	1420	1352	1541	1437

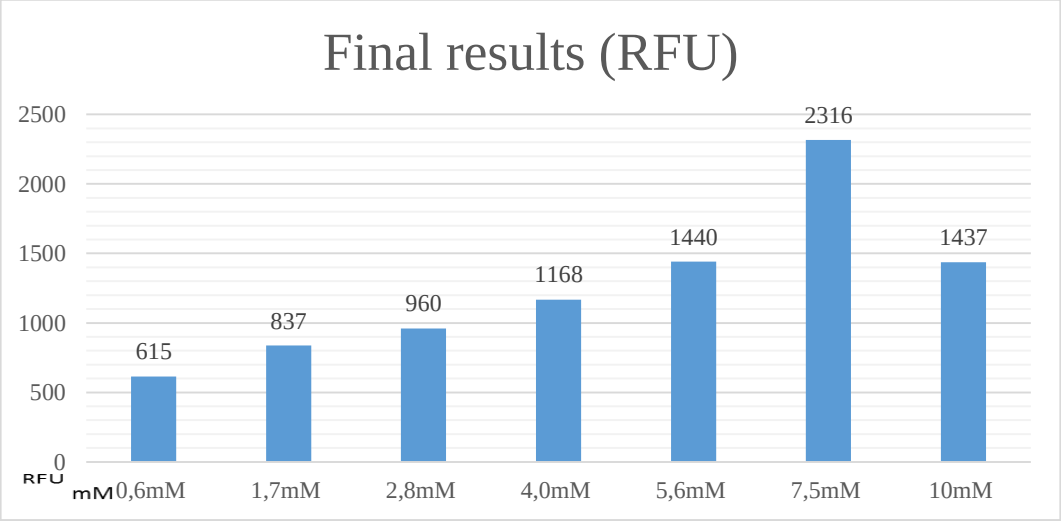


Figure 3-2 Graph of seven different concentrations of galactose after adding solution II. Graph was drawn according to final results. Each bar consisted 21 different values.

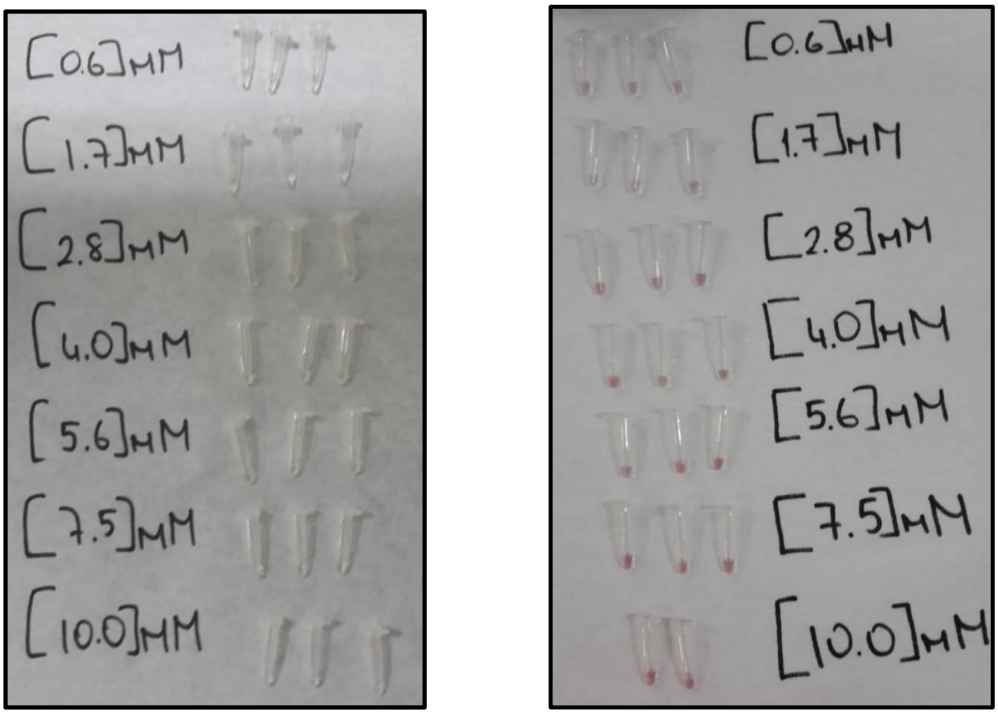


Figure 3-3 Colorimetric reading of above experiments. Left column showed before solution II addition. All tubes had transparent color. Right column showed after incubation with solution II. Tubes had pinky red color.

This work gave an idea about how to obtain a value at which concentration. But in order to improve this work, concentration range should be expanded. And according to another experiment sets were performed. In these studies, 6.5 mM and 8.5 mM concentration of galactose –Tris-HCl solutions were added. Again each concentration was studied three different tubes. Results of these studies were shown in the Table 3-3 and Figure 3-4. Seven different experiment sets were performed. In Table 3-3, under sets, each box was shown arithmetic value of 3 different RFU value. In final result section, each box was consisted 21 different RFU value.

Table 3-3 RFU result of nine different concentrations of galactose and Tris-HCl solution. Each concentration was studied 3 different batches. Final results shown arithmetic values.

Galactose Concentration [mM]	Set I	Set II	Set III	Set IV	Set V	Set VI	Set VII	Final Results (RFU)
0.6mM	745	489	517	1316	326	377	569	620
1.7mM	939	1007	567	1355	665	615	895	857
2.8mM	957	1075	1005	1519	614	721	1040	990
4.0mM	1135	1250	1208	2216	1007	913	1139	1267
5.6mM	1197	1834	1730	1764	1114	943	950	1361
6.5mM	1468	2164	1897	1837	1383	1318	1142	1601
7.5mM	1439	1142	1513	1859	1259	1306	933	1350
8.5mM	1449	1250	1777	1923	1055	1369	1272	1442
10 mM	1665	1265	1062	2055	1363	1452	1310	1453

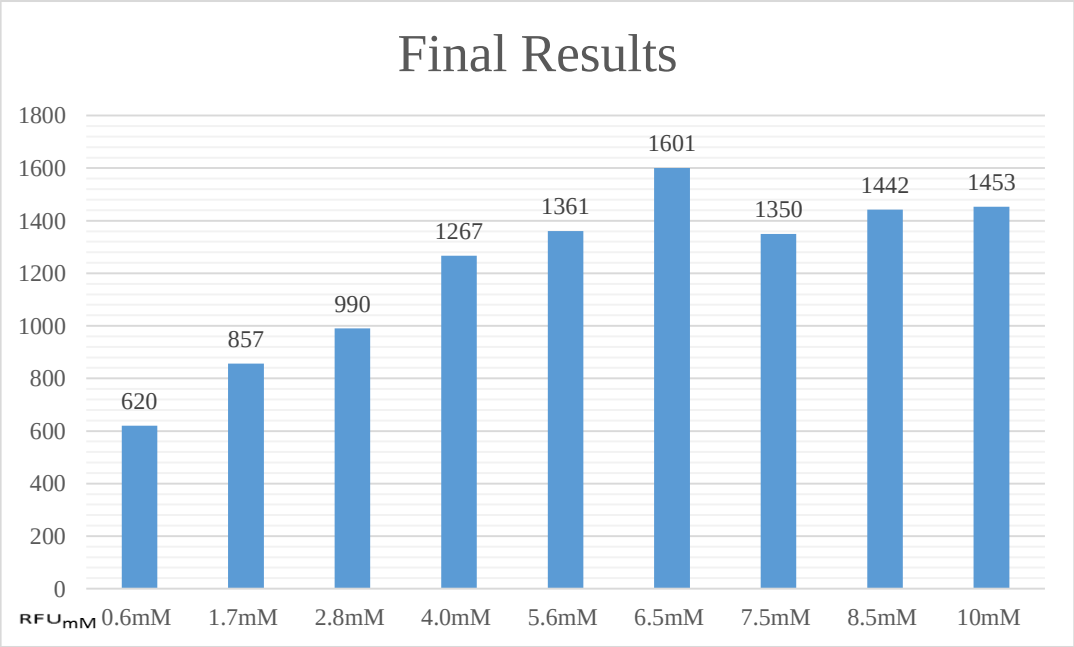


Figure 3-4 Graph of nine different concentrations of galactose. Graph was drawn according to final results. Each bar consisted 27 different values.

In order to see how values could be changed after adding solution II, RFU results were taken after incubating with solution II. These results were shown in Table 3-4 and Figure 3-5.

Table 3-4 RFU result of nine different concentrations of galactose and Tris-HCl solution after adding solution II. Each concentration was studied 3 different batches. Final results were shown arithmetic values.

Galactose Concentration [mM]	Set I	Set II	Set III	Set IV	Set V	Set VI	Set VII	Final Result (RFU)
0.6mM	386	203	200	821	151	134	332	318
1.7mM	490	362	472	853	412	422	386	485
2.8mM	498	486	820	887	304	576	338	541
4.0mM	616	615	858	1402	780	608	537	774
5.6mM	767	863	910	725	813	840	636	793
6.5mM	871	1303	940	1115	985	1144	889	1035
7.5mM	918	628	546	1154	978	1074	843	877
8.5mM	826	637	991	1172	1098	1104	906	962
10mM	1137	666	1243	734	1245	1173	1075	1201

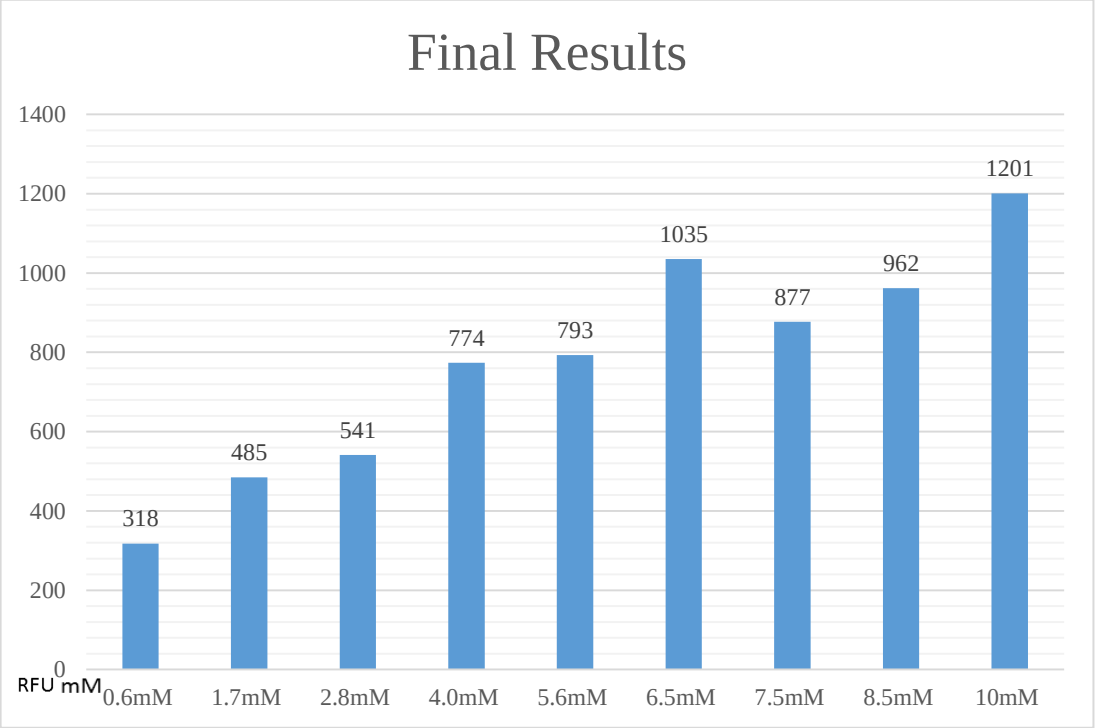


Figure 3--5 Graph of nine different concentrations of galactose after adding solution II. Graph was drawn according to final results. Each bar consisted 27 different values.

Experiment results color reading was shown in Figure 3-6. In this study, pinky-red color was observed in the tubes which comprised galactose in it.

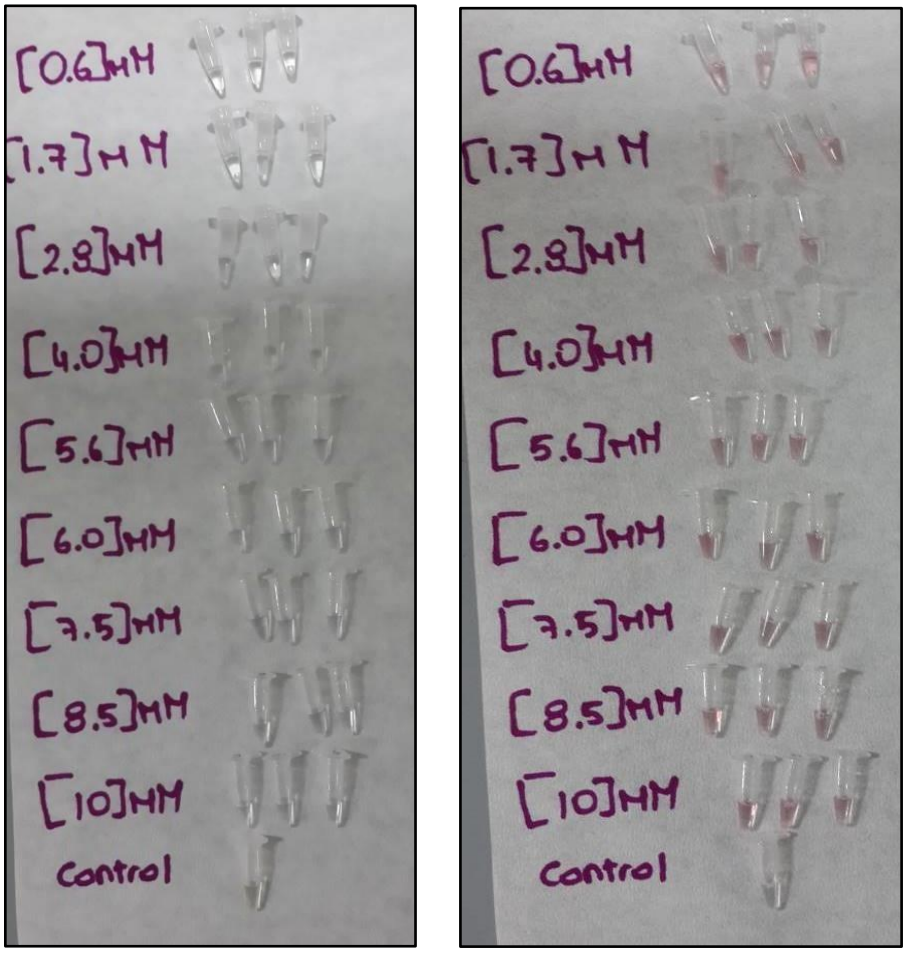


Figure 3-6 Colorimetric reading of above experiments. Left column showed before solution II addition. All tubes had transparent color. Right column showed after incubation with solution II. Tubes had pinky red color.

After using galactose solution, different galactose – 1 – phosphate concentrations were tested. In order to test galactose – 1 – phosphate, firstly five different concentrations were prepared. These concentrations were 0.3 mM, 0.9 mM, 1.5 mM, 3.0 mM and 5.0 mM. Difference between galactose experiments, alkaline phosphatase was added in to the enzyme solution. Alkaline phosphatase played role in conversion of galactose – 1 –phosphate to galactose. Each concentration of galactose – 1- phosphate had prepared in triplicate batch. Table 3-5 and Figure 3-7 gave result of galactose-1-phosphate experiment.

Table 3-5 RFU result of five different concentrations of galactose – 1 - phosphate and Tris-HCl solution. Final results shown arithmetic values.

Galactose- 1- Phosphate Concentration [mM]	Final Result (RFU)
0.3	868
0.9	1786
1.5	2351
3.0	2685
5.0	3174

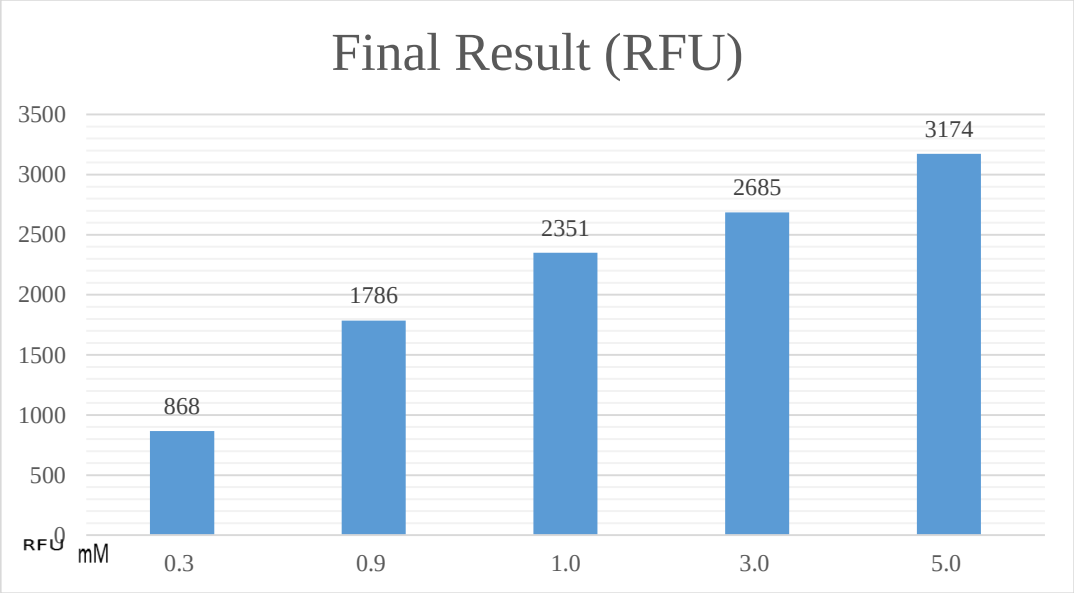


Figure 3-7 Graph of five different concentrations of galactose – 1 - phosphate. Graph was drawn according to final results. Each bar consisted 15 different values.

The 4.0 mM concentration of galactose – 1- phosphate was added to experiment. And experiments were redone. Figure 3-8 and Table 3-6 were showed results.

Table 3-6 RFU result of six different concentrations of galactose – 1 - phosphate and Tris-HCl solution. Final results shown arithmetic values.

Galactose- 1- Phosphate Concentration [mM]	Final Result (RFU)
0.3	260
0.9	400
1.5	575
3.0	712
4.0	775
5.0	970

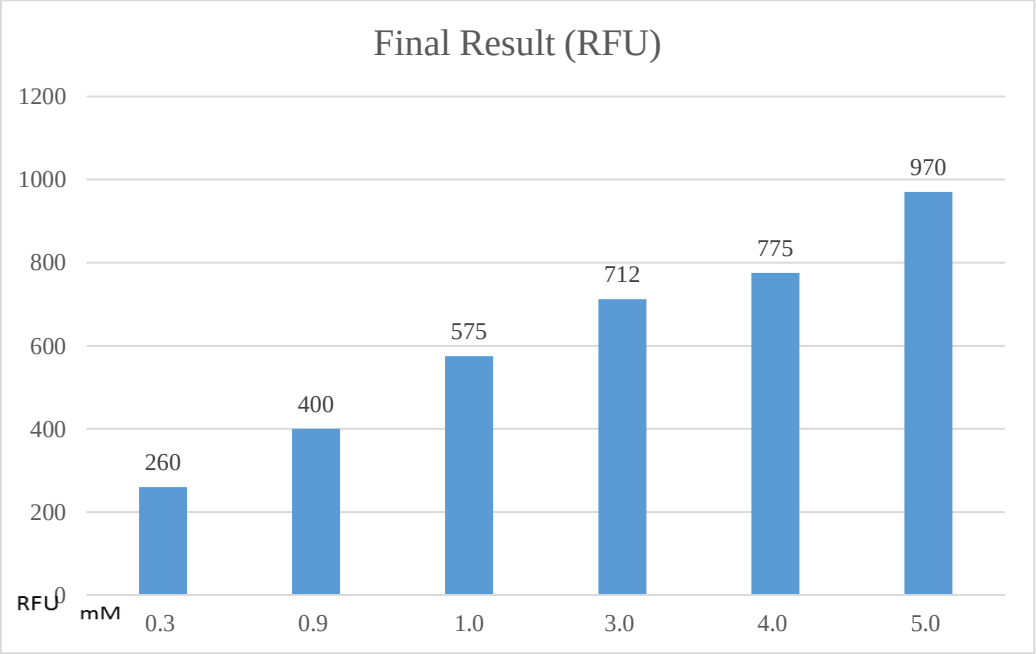


Figure 3-8 Graph of five different concentrations of galactose – 1 - phosphate. Graph was drawn according to final results. Each bar consisted 26 different values.

Both galactose and galactose-1-phosphate results were shown, kit works better with high concentrations of both galactose and galactose-1-phosphate. At lower concentrations, however; the discrimination power was lost.

3.2 Shelf - Life studies

In enzymatic reactions, enzyme stability is an important aspect. In shelf-life studies, seven different galactose concentrations were tried. These concentrations were 0.1 mM, 0.5 mM, 1.5 mM, 3.5 mM, 5.0 mM, 7.0 mM and 10.0 mM. Table 3-7 and Figure 3-9 gave results of changes in enzyme activity through weeks. A decreasing enzymatic activity has been observed through weeks. At Week IV and week V, enzymatic activity was dropped to half of initial values. To be used in clinical laboratories enzyme stability must be longer than seven weeks. Addition of bulking agents to protect enzymes or lyphilization of the enzyme itself is possible solutions to increase the shelf life.

Table 3-7 Shelf-life studies were done with seven different galactose concentrations. Shelf studies were taken seven weeks.

Galactose Concentration [mM]	Week I	Week II	Week III	Week IV	Week V	Week VI	Week VII
0.1 mM	624	621	666	698	623	503	563
0.5 mM	2670	1906	1452	1179	1282	1152	866
1.5 mM	4304	3323	2072	1727	2184	1859	1197
3.5 mM	5138	4711	2600	2111	2773	3114	1500
5.0 mM	5413	3571	2810	2024	2999	2226	1646
7.0 mM	5384	4233	2830	2210	3175	2191	1673
10.0 mM	5230	4634	3114	2778	3461	2619	1563

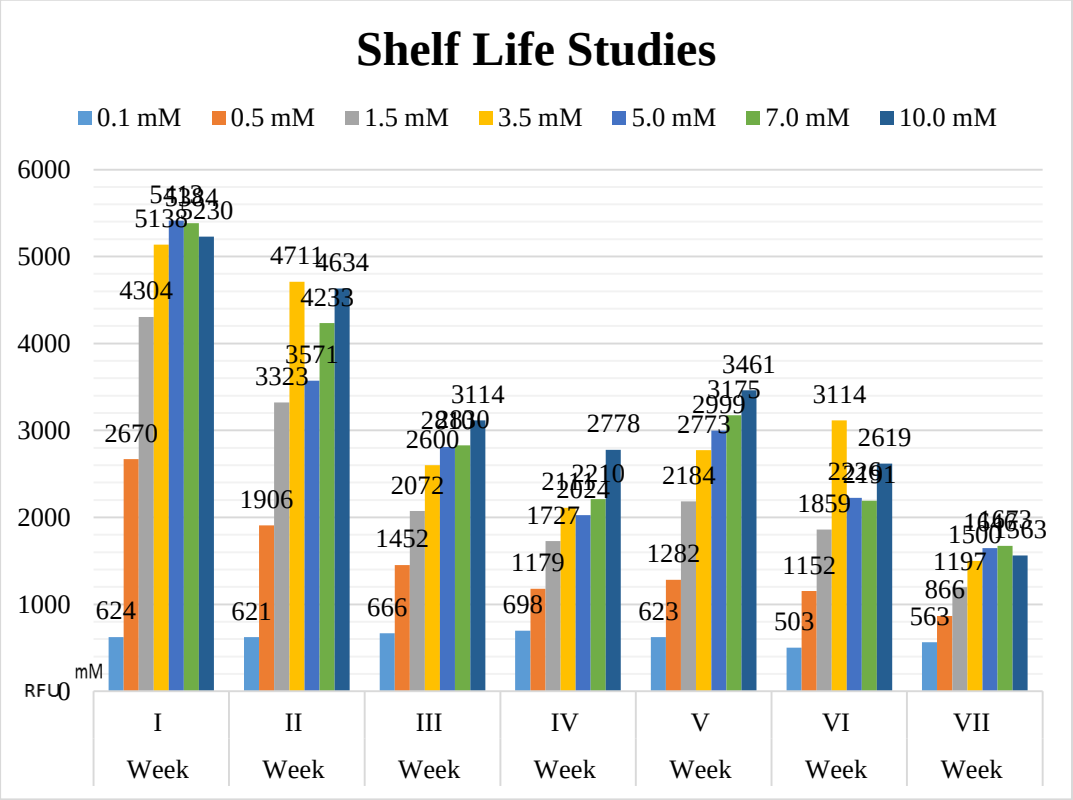


Figure 3-9 Graph of shelf-life studies. Enzyme activity decreased through weeks

3.3 Real Samples Experiments

In order to ensure its validity, prototype kit was used at Düzen Laboratories to measure galactose levels in control samples. In this experiment, nine anonymized patients' bloods were studied. Assay was done as in previous experiments. Patient's blood samples were fixed 20 µl with 80 % alcohol at 37 ° C at 1 hour. After fixation, 80 µl enzyme solutions was added and incubated at 37 ° C at 1 hour. After 1 hour fluorometric reading was done. Only difference between experiments, in Düzen studies two punches were used. Because these punches were 2.3 mm diameter. In the normal study, diameter of punch approximately was 4.8 mm. Results of this study was given Table 3-8.

Table 3-8 Prepared kit was tested in Düzen Laboratory with real samples. Ten samples were used. One of the samples was reference value and one sample was known as galactosemic. According to kit results, kit was caught reference and galactosemic value. But it gave missense result in smaller value.

Patients	Values	Düzen Laboratory Values
1	22	14.3
2	**28**	**27**
3 (galactosemia)	**60**	**55**
4(references value)	**31**	**34**
5	27	3.6
6	27	1.97
7	26	2.24
8	27	5.47
9(smallest value)	**20**	**1.9**
10	27	7.1

According to results, prototype kit detected galactosemic patients. In Düzen Laboratory result, galactosemic person result was 55, kit's result was 60. They were approximately same. Moreover, reference value results were near each other. When looking smallest value, kit read 20 however in Düzen laboratory, it read 1.9. Although 20 was the smallest value of kit, it was near other intermediate results. In mean that sensitivity of kit should be increased.

Another experiment set was tested in Düzen laboratory. In this set 5 patients and one blank card was used. Each sample was studied three times. Results of study were given in Table 3-9 and Figure 3-10

Table 3-9 Prepared kit was tested in Düzen Laboratory with six anonymized real samples. One of the samples was control and one sample was known as galactosemic. According to kit results, kit was caught galactosemic value. But it gave nearly same result for other values.

Patients	Values	Düzen Laboratory Values
Control	29.3	0
1	267.7	>55mg/dL
2	35.3	7.9 mg/dL
3	34.0	6.7 mg/dL
4	28.7	4.7 mg/dL
5	2.6	<1.9 mg/dL

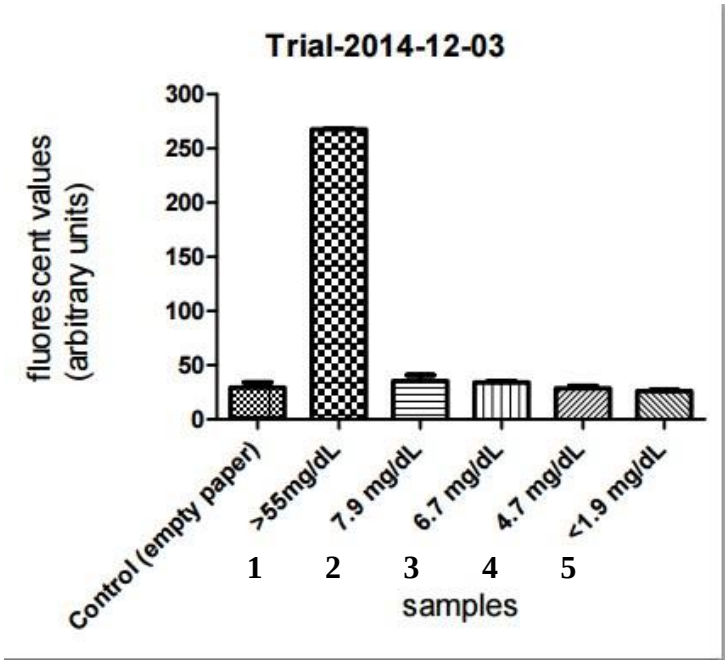


Figure 3-10 Graph of Düzen Laboratory test's result

3.4 Strips Results

Fluorometric reading is a relatively expensive method due to the equipment cost. In order to minimize this, in this thesis, studies on paper strips were done. Paper strips' studies were first performed on punched filter papers. Amount of solution that was put on to the paper was chosen according to diameter of punch by allowing it to uniformly cover the surface With these parameters, 5 to 10 µl solutions were put on papers. 10 µl enzyme solutions was added and dried. After desiccation, 8 µl solution II was added and re-dried. After adding solution II, paper must be dried at 23°C in dark. Then a different concentration of galactose solution with Tris-HCl was put into these papers. They incubated at 37 °C 1 hour. However, no color change color observed through this protocol, probably due to the enzyme degradation during drying process.

To protect the enzyme, in the next trial we did not put the enzyme solution on to the paper. Rather, only 8 µl of solution II was dropped on to the paper. The paper was then put in to the tubes and dried at 23 ° C for 2 to 3 hours. An important point here, tubes were covered with aluminum foil to protect from light. Simultaneously, 20 µl enzyme solutions were added to 40 µl different concentrations of galactose solutions and they were incubated at 37 °C 1 hour. After incubation, dried papers put in to the tubes and incubated at 23°C 10 minutes. Figure 3-11 was shown after drying of paper. Figure 3-12 showed color changes of paper. With increasing galactose mM, color became darker pinky-red.

Figure 3-11 Dried strips after addition solution II.
No color changes were observed

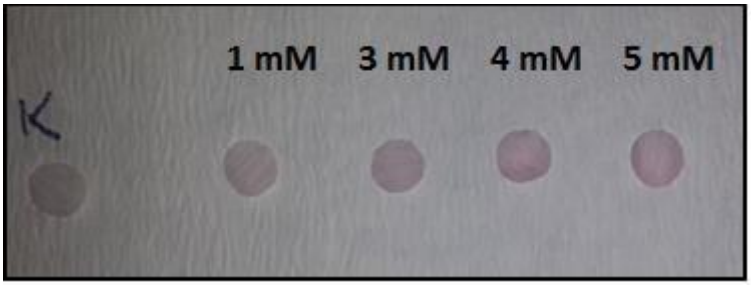


Figure 3-12 Color changes after incubating with different galactose concentrations and enzyme solutions. With increasing galactose concentration pinky-red color was became more visible

Other trials were also performed using strips instead of filter papers. These strips were bigger and thicker than filter paper strips. Therefore, strips were divided in to two. And solutions amount was bit increased. In the strips studies, firstly both of enzyme solution and solution II were dropped and dried at room temperature. After this step, strips were incubated at 37 °C 1 hour with different concentrations of galactose solutions which prepared with Tris-HCl. Yet, there was no positive result at the end of the experiment. Enzyme solution was fragile solution and it loses its ability with temperatures changes.

If enzyme solution and solution II were dropped simultaneously or one dried and other dropped, the solutions became inactive. In means that there were no results at the end. Therefore, enzyme solution could be prepared separately as always and solution II was dropped on the paper and dried. With this method, both fluorometric results were handled and both strips result were.

Because of thickness and size, solution II amount were increased to 10 µl. Strips were placed on to the heat block. Temperature was arranged to 23° C. They were dried 2 – 3 hours. Pay attention to the medium should be kept dark. Galactose and enzyme solutions were added on the strips gave more accurate result because strips were thick and long and its all part did not contact with solution II. After dropping galactose-enzyme solution they incubated 10 min at 23° C at dark. Figure 3-13 showed, dried strips color and Figure 3-14 results of experiment. Control strips had no color change. Strips color changed occurring from edge to middle. Edge sides of strips had darker pinky-red color.

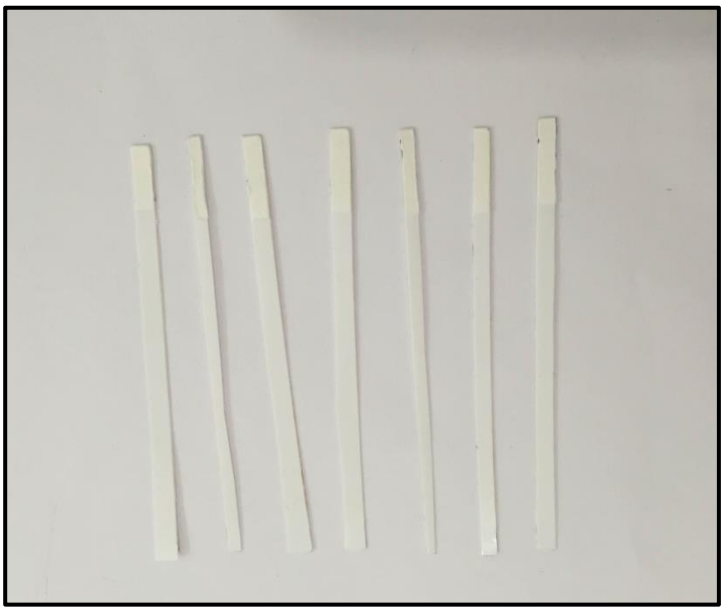


Figure 3-13 Dried strips after addition solution II. No color changes were observed

Figure 3-14 Color changes after incubating with different galactose concentrations and enzyme solutions. With increasing galactose concentration pinky-red color was became more visible

Another trial on the strips, were using entire size of the strip. 16 µl solutions II were put on the strips. Because of size, it was important to every place should be reached solution II. All other process was same with above procedures. Galactose concentrations in that trial again 1 mM, 3 mM, 5 mM, 7 mM and 10 mM (Figure 3-15 and Figure 3-16).

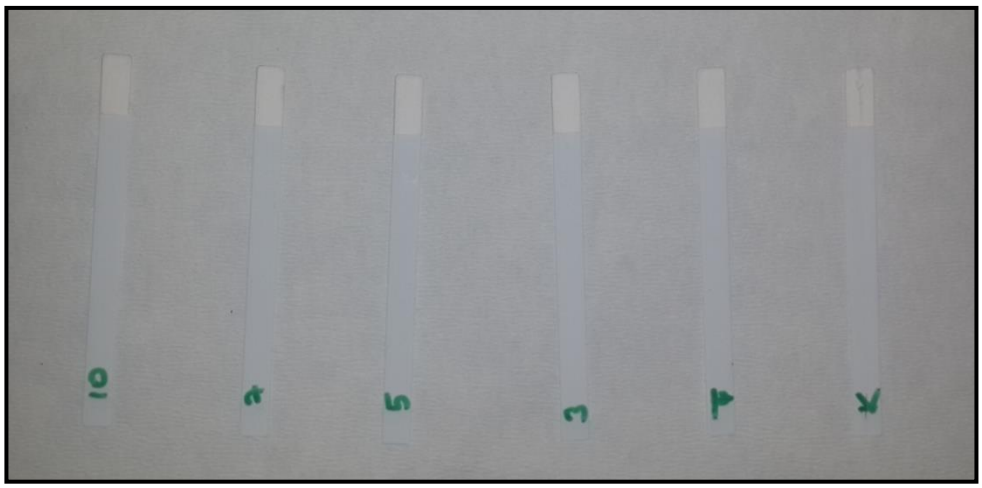


Figure 3-15 Dried strips after addition solution II. No color changes were observed. Thicker strips were used. And solution II amount was increased 16 µl.

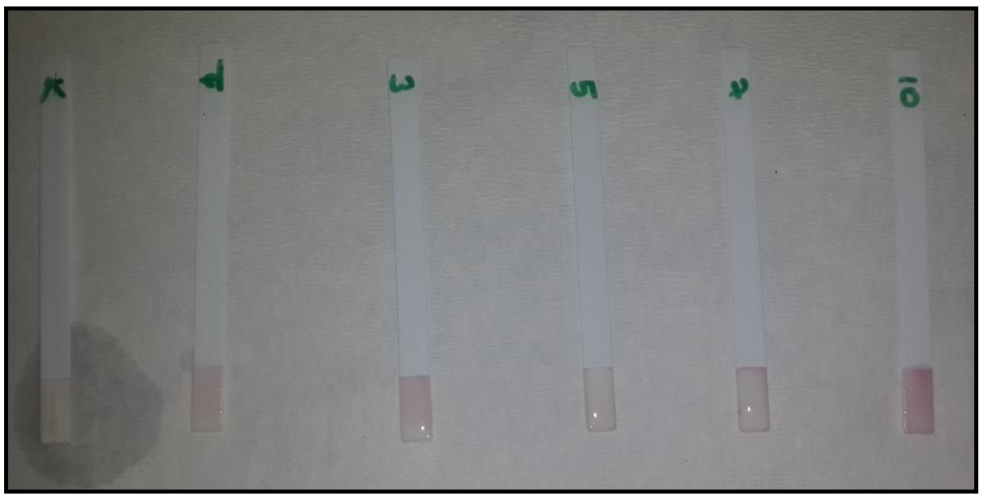


Figure 3-16 Color changes after incubating with different galactose concentrations and enzyme solutions. With increasing galactose concentration pinky-red color was became more visible.

To test accuracy of strips, control studies were done. 4 different control groups were formed.

Each group contains different thesis chemical.

Control 1 group: Iodonitrotetrazolium + buffer 2 + galactose

Control 2 group: Diaphorase + buffer 2 + galactose

Control 3 group: Solution 2 + galactose

Control 4 group: Solution 2 + Enzyme Solution

In control 1 group, Iodonitrotetrazolium and buffer 2 (buffer that used in order to solution II) were prepared and dropped on the strips. It dried 23° C at dark 2 – 3 hour. After drying process only 5.0 mM galactose – Tris-HCl solution was dropped. There was no enzyme activity. So there was no color change.

In control 2 group, diaphorase and buffer 2 were prepared and dropped on the strips. It dried 23° C at dark 2 – 3 hour. After drying process only 5.0 mM galactose – Tris-HCl solution was dropped. There was no enzyme activity. So there was no color change.

In control 3 group, solution II was prepared and dropped on the strips. It dried 23° C at dark 2 – 3 hour. After drying process only 5.0 mM galactose – Tris-HCl solution was dropped. There was no enzyme activity. So there was no color change.

In control 4 group, solution II was dropped on the strips. . It dried 23° C at dark 2 – 3 hour. After drying process only enzyme solution was added. In the absence of galactose or galactose – 1 – phosphate, there was no reaction. Figure 3-17 show dried strips. On strips was prepared to done normal experiment procedure. Figure 3-18 showed, there was no color changes in ant strips, expect normal study strip. To conclude, strip worked on the presences of both enzyme solution and its substrate.

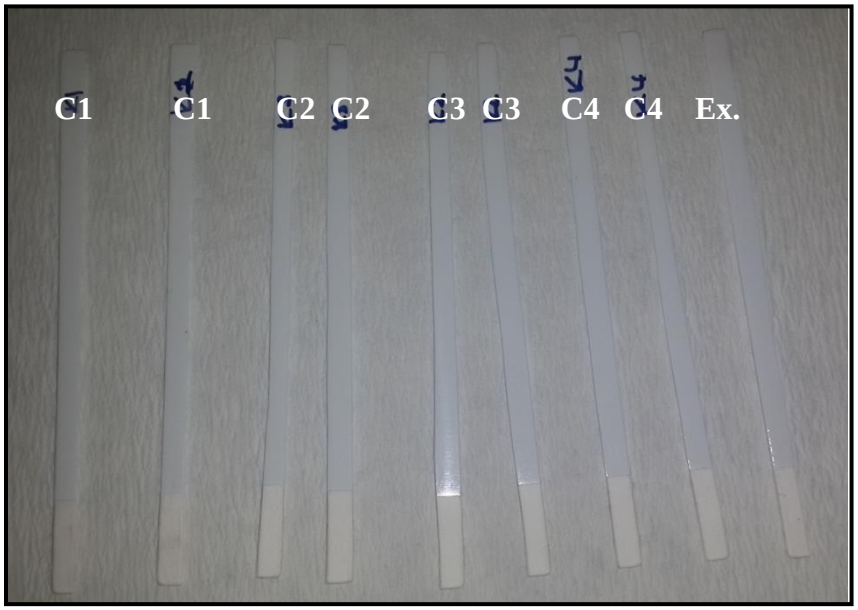


Figure 3-17 Dried strips after addition solution II. Four control group and one experiment strip were studied.

Figure 3-18 Control groups did not give any color changes. Only color changed observed in the presences of substrate, enzyme and solution II association

Another control experiment was done. In this control test, again same experiment which was described above was repeated. Results were overlapped each other. Figure 3-19 shown 4 control group. Each control group studied two times. Figure 3-20 showed experiment result. Only, normal experiment procedure strip gave pinky-red color.

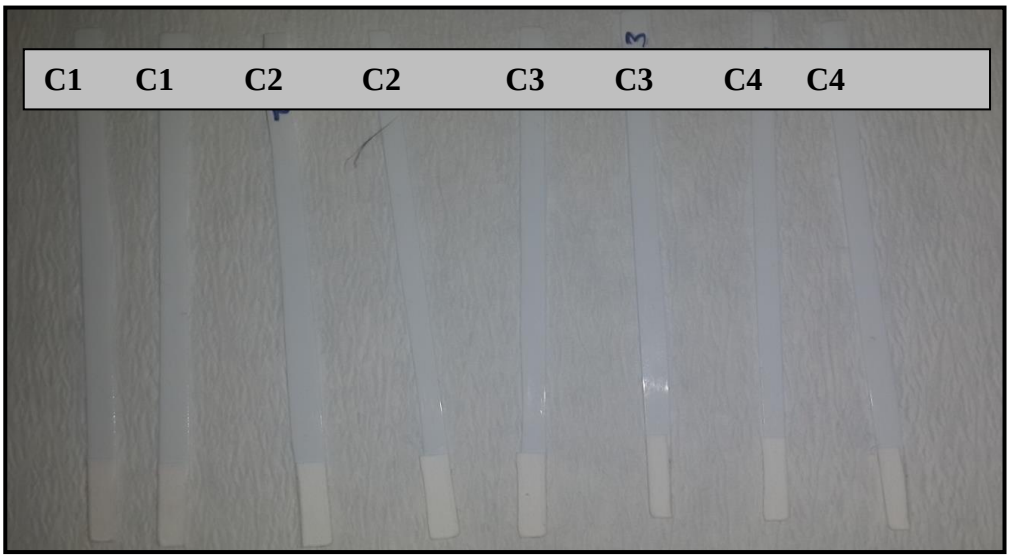


Figure 3-19 Another study was done in control experiment. Dried strips after addition solution II. Four control group and one experiment strip were studied.

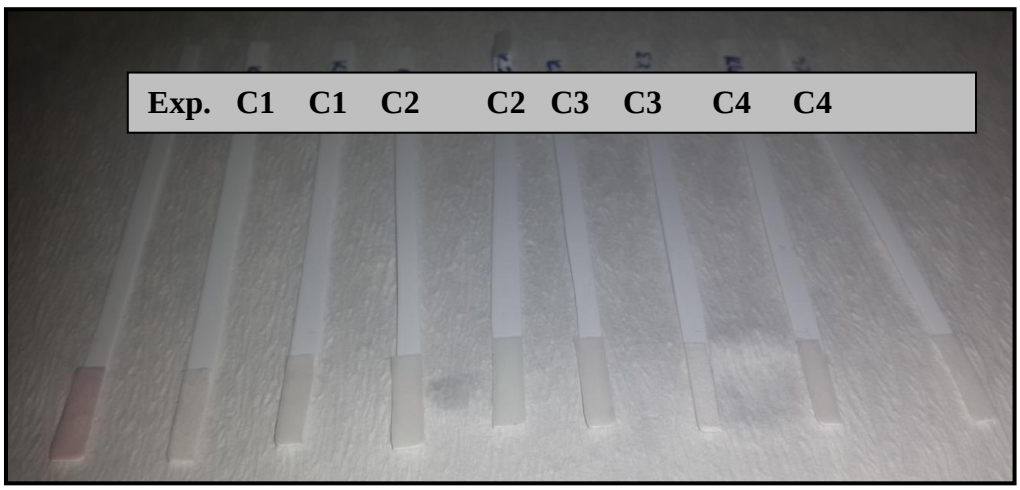


Figure 3-20 Control groups did not give any color changes. Only color changed observed in the presences of substrate, enzyme and solution II association

After control group experiments were tested, and it was proved that strips gave pinky-red color in the presences of enzyme + substrate + solution II. Blood galactose and galactose – 1 – phosphate experiments were done. Same experiment procedure was applied.

Five different galactose – 1 – phosphate concentrations were studied. These are 1.0 mM, 3.0 mM, 5.0 mM, 7.0 mM and 10.0 mM. Figure 3-21 pointed dried strips, no color changed was observed during drying process. Figure 3.-22 showed color changes. Until 5 mM concentrations, strips did not reflect any color, but in 5 mM, it gave tiny color. After 5.0 mM, it gave pinkish color (figure 3-21&3-22).

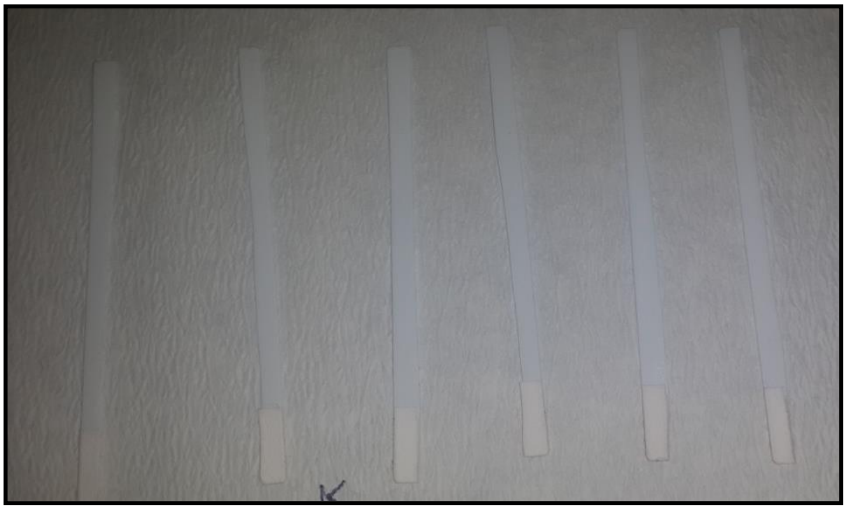


Figure 3-21 Dried strips after solution II addition.

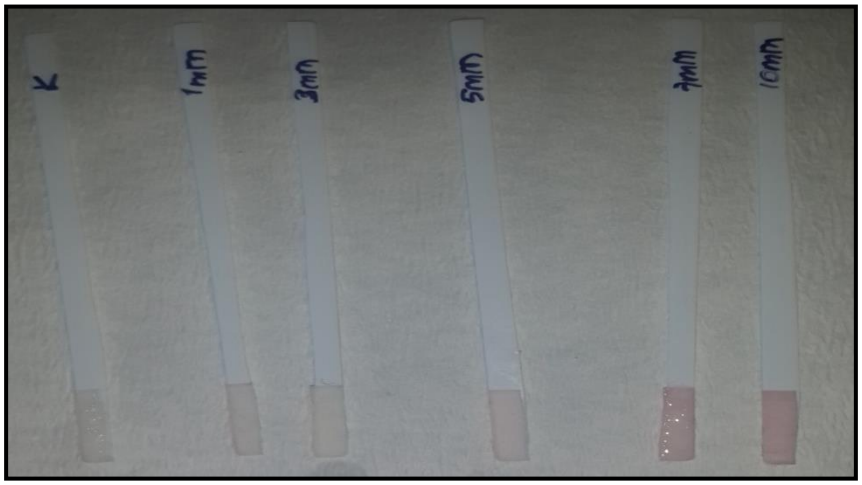


Figure 3-22 Color changes in the presences of galactose-1-phosphate. With increasing concentration in galactose-1-phosphate, strips became more pinky-red color. Until 5 mM, pink red color on the strip did not early detectable

3.5 Validation Results

Validation studies were done in order to increase sensitivity in the small concentrations of galactose and galactose – 1 - concentrations.

Four conditions of enzyme solutions were prepared and tested in small amount of galactose – 1 – phosphate concentrations. These concentrations were 0.1 mM, 0.5 mM and 1.0 mM. In 1st condition was only GADH enzyme was duplicated. In normal condition, three of concentration's value was close each other. In 1st condition, again there were exact differences between result of 0.1 mM and 0.5 mM. Only thing was 1.0 mM concentration value two times of 0.5 mM value. In 2nd condition only, AP was duplicated. This result was much more significant than both condition 1 and normal condition. The values were separate each other. But in order to increase sensitivity, values of concentration should be exactly separating. In 3rd condition both AP and GADH amount were duplicated. Results separated more exact lines (Table 3-10 and Figure 3-23).

Table 3-10 Four different enzyme solution were prepared. According to these enzyme solutions, the most sensitive results were taken in 3rd condition in smaller values of concentration

Galactose- 1-phosphate Concentration [mM]	Normal Condition	1st Condition	2nd Condition	3rd Condition
0.1 mM	174	144	85	82
0.5 mM	271	185	224	327
1.0 mM	231	328	436	515

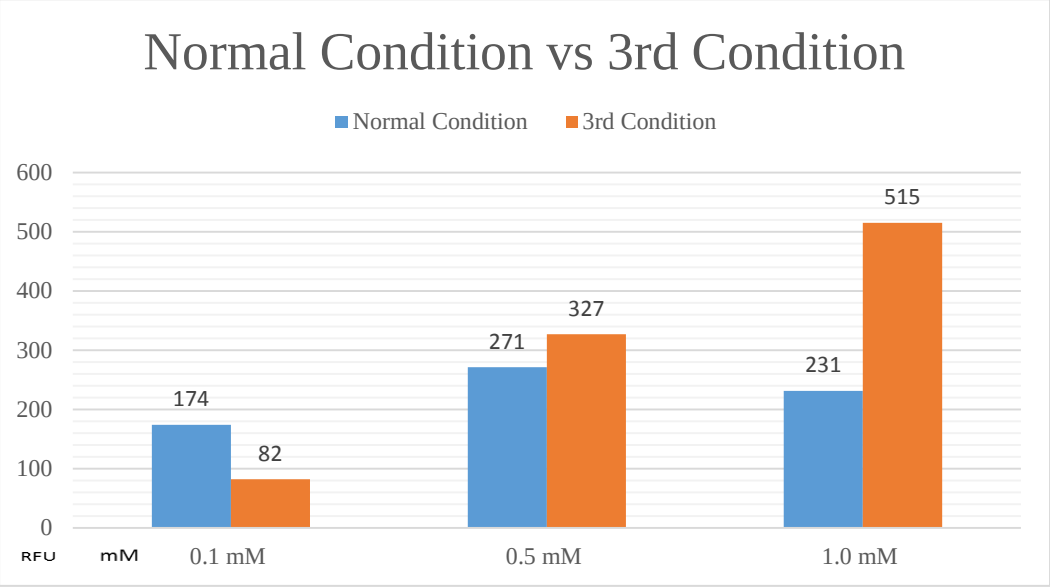


Figure 3-23 Graph of comparison between 3rdcondition vs normal condition of enzyme solution. In 3rdcondition enzyme amounts were duplicated. In smaller value of galactose – 1- phosphate concentration high enzyme amount gave more sensitive result.

After these studies, new concentrations of galactose-1-phosphate were added. These concentrations were 1.5 mM, 2.5 mM, 3.0 mM. This study was done with both enzyme concentrations were duplicated. Each tube studied duplicated. Table 3-11 shown normal condition and 3rd condition results. Comparing normal condition vs 3rd condition, in normal condition small concentration's values were

very close to each other. For example 0.1 mM and 0.5 mM gave almost same result although 0.5 mM was 5 times bigger than 0.1 mM. On the other hand, in 3rd Condition only 0.5 mM and 1.0 mM results were close each other. But others gave more clear result. Figure 3-24 and Table 3-11 were showed results of the experiment.

Table 3-11 Six small concentration of galactose-1-phosphate were prepared. These concentrations were tested with both normal and 3rd condition. The most sensitive results were taken in 3rd condition in smaller values of concentration

Galactose- 1-phosphate Concentration [mM]	**Normal Condition (RFU)**	**3rd Condition (RFU)**
0.1 mM	202	175
0.5 mM	208	601
1.0 mM	838	767
1.5 mM	955	1145
2.5 mM	1707	1884
3.0 mM	1598	2882

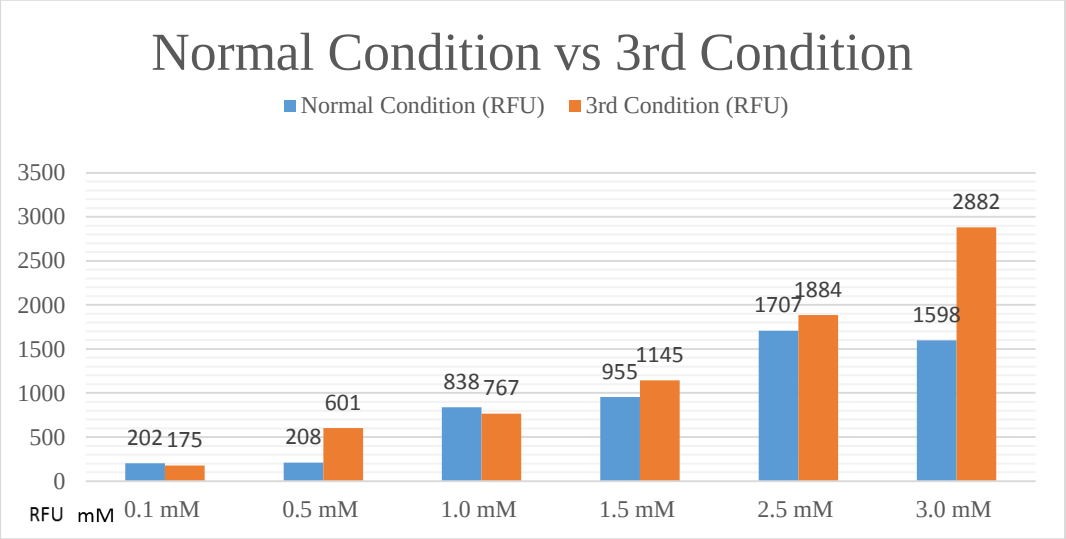


Figure 3-24 Graph of comparison between 3rd condition vs normal condition of enzyme solution. In 3rdcondition enzyme amounts were duplicated. In smaller value of galactose – 1- phosphate concentration high enzyme amount gave more sensitive result.

Another validation study was done with halving enzyme amounts in enzyme solution. In this study showed that within halved amount of enzymes, values were getting closer to each other and did not reflect true result (Table 3-12 and Figure 3-25).

Table 3-12 4th condition and normal condition was compared. In smaller amount of galactose-1-phosphate concentration, halved of enzyme amount did not increase sensitivity of enzyme solution.

Galactose- 1-phosphate Concentration [mM]	4th Condition (RFU)	Normal Condition(RFU)
0.1 mM	92	229
0.5 mM	122	256
1.0 mM	196	500
1.5 mM	336	1129
2.5 mM	479	1719
3.0 mM	555	1692
4.0 mM	891	2194
5.0 mM	965	2946

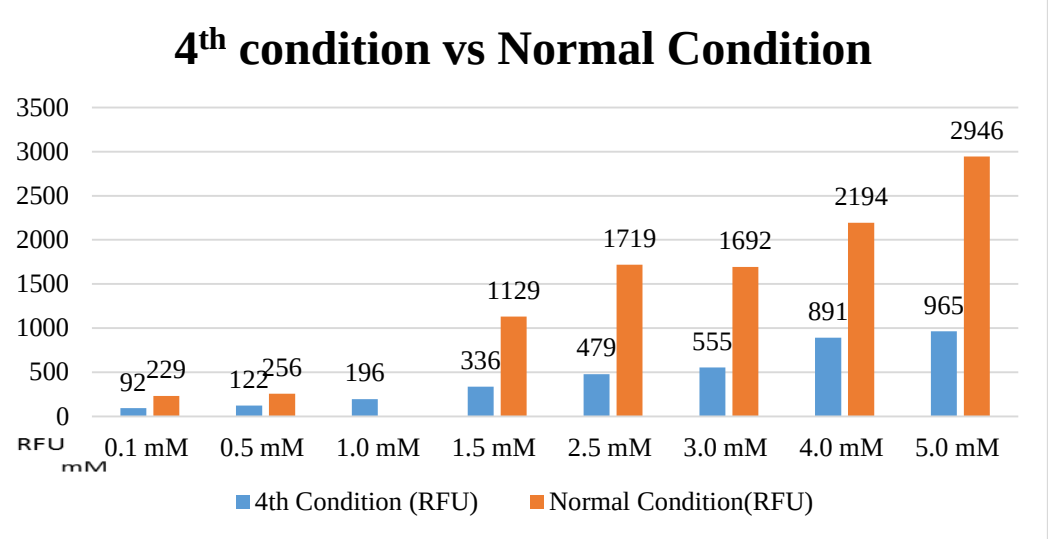


Figure 3-25 Graph of comparison between 4th condition and normal condition. According to results were given in the graph, when enzyme amounts were halved, sensitivity of enzyme solution decreased.

Another trials were done on the incubation time. Incubation time became halved. In this experiment 0.1 mM, 0.5 mM, 1.5 mM, 2.5 mM, 3.0 mM, 4.0 mM and 5.0 mM galactose – 1 – phosphate were used. Table 3-15 and Figure 3-26 showed results of experiment.

Table 3-13 5th condition and normal condition was compared. In smaller amount of galactose-1-phosphate concentration, when incubation time was halved, enzyme sensitivity did not increase.

Galactose- 1- Phosphate Concentration [mM]	**5th Condition**
0.1 mM	289
0.5 mM	374
1.5 mM	500
2.5 mM	636
3.0 mM	1231
4.0 mM	1665
5.0 mM	1922

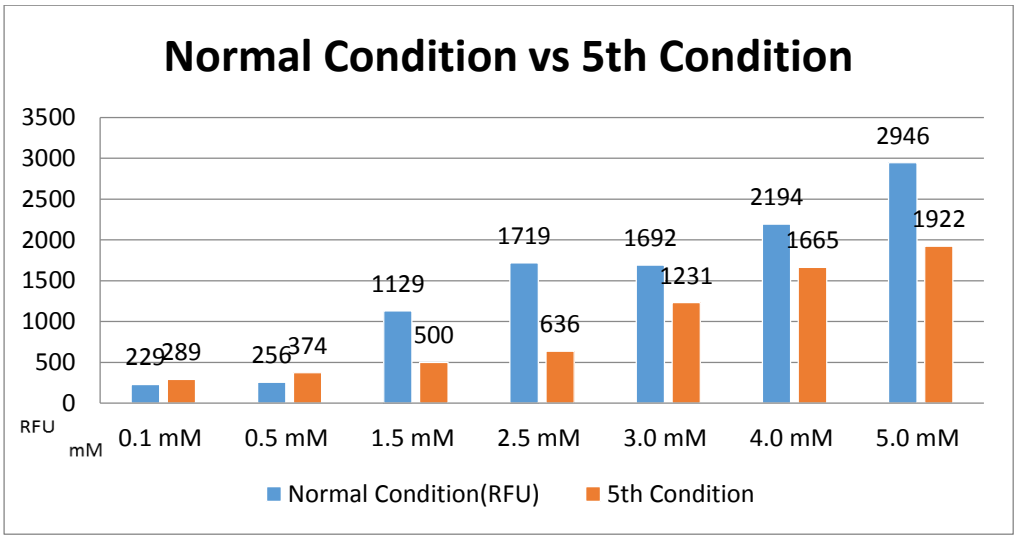


Figure 3-26 Graph of comparison between 5th condition and normal condition. According to results were given in the graph, when incubation time was halved, sensitivity of enzyme solution decreased.

As a result of validation studies, best condition was handled in when both enzyme amounts were duplicated. Decreasing incubation time and enzyme amount did not improve the sensitivity of enzyme activity. Also other validation studies should be done. For example both incubation temperatures should be adjusted again. Below and upper temperatures should be tested. All conditions enzyme amount, incubation time and temperature must be retried in order to obtain the most accurate enzyme sensitivity.

CHAPTER 4

CONCLUSION

Leloir Pathway includes three main enzymes GALK, GALE and GALT. Any mutations that occur in these three enzymes cause Galactosemia which is a recessive genetic disorder. New-born with galactosemia show no effect until drink milk and milk based formula food. Acute complications for examples, sepsis, shock, liver damage, cataracts, etc. are seen after drink milk or milk based formula food. In order to prevent these acute syndromes, galactosemia screening test should be done after birth.

In Turkey, there is no local production of neonatal galactosemia screening kit. Here we have provided the first prototype of such a galactosemia kit for newborns.

After drawing base-line studies, prepared kit was tested on real samples in Düzen Laboratories. According to this study, kit detected galactosemia patient and gave almost same result with the reference value.

In parallel to these studies, shelf life studies were also performed. In these studies we have observed a decrease in enzyme activity over weeks.

One of the most important studies of this thesis was strips studies. In strip studies, strips was saturated to solution II and dried. After desiccation, it incubated with substrate-enzyme solution and results were observed. Strips gave only pink-red color in the presences of substrate+ enzyme+ solution II combination.

Strip studies make the kit more applicable for rural places and it decreases cost of the kit.

In this thesis, final studies included the validation studies. Aim of validation study increase sensitivity of enzyme solution and accuracy of kit. According to these studies duplication of enzyme amount increased enzyme sensitivity and gave more accurate results.

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