Metabolomics Profiling for Amino Acidopathies of Infants in Hospital Based Settings in Bangladesh and Development of Easy-to-Use Kit for Disease Diagnosis and Monitoring

PhD Thesis

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DEPARTMENT OF GENETIC ENGINEERING AND BIOTECHNOLOGY
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CERTIFICATE

This is to certify that Md. Shawkat Hossain (Registration Number: 15/2016-17) conducted his PhD thesis work entitled **"Metabolomics profiling for amino acidopathies of infants in hospital based settings in Bangladesh and development of easy-to-use kit for disease diagnosis and monitoring"** under my supervision for the fulfillment of the degree of "Doctor of Philosophy in Genetic Engineering and Biotechnology' from University of Dhaka-1000, Bangladesh. Furthermore his works are also co-supervised by Dr. Abu Ashfaqur Sajib, Department of Genetic Engineering and Biotechnology, University of Dhaka, Bangladesh. The thesis work in part or whole has not been submitted elsewhere for any other degree.

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DECLARATION

I hereby declare that this thesis entitled **"Metabolomics profiling for amino acidopathies of infants in hospital based settings in Bangladesh and development of easy-to-use kit for disease diagnosis and monitoring"** contains no material which has been accepted for the award of any other degree or diploma in any university or equivalent institution. To the best of my knowledge, the thesis contains no material previously published or being considered for publication, except where due reference is made in the text of the thesis.

……..

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DEDICATION

Dedicated to

My Parents, Wife, and Daughters (Nuzhat & Mahnoor),

Teachers, Mentors, Beloved Incepta,

For the love, guidance, and wisdom,

And to my Country Bangladesh, a source of pride.

ABSTRACT

This study aimed to address the challenges associated with identifying inborn errors of metabolism (IEM) in resource-constrained environments, focusing on amino acid and galactose metabolism disorders in the Bangladeshi infant population. Liquid Chromatography Mass Spectrometry (LC-MS) is a robust diagnostic tool but faces limitations due to cost, complexity, and accessibility. Therefore alternative approaches, including enzyme assays, genetic testing, and metabolite profiling, were explored for effective IEM detection. In pursuit of an affordable diagnostic solution, a low-cost kit was developed for early detection and intervention of amino acid and galactose-related disorders. A validated HPLC-based method was established for accurate quantification of amino acids. The method exhibited strong linearity (R2 = 0.9999) and high accuracy (99.10% average recovery), establishing its suitability for amino acid analysis. Precision was confirmed through repeatability and intermediate precision tests, with low RSD values (0.30% to 1.53% and 0.23% to 0.66%, respectively). Amino acid cutoff ranges were determined for the Bangladeshi population using the validated HPLC method. Noteworthy variations in amino acid counts were observed in positive samples, emphasizing the importance of accurate cutoff values. A lateral flow paper-based diagnostic kit was designed for phenylalanine detection, offering a cost-effective means for phenylketonuria (PKU) monitoring. The kit demonstrated a minimum detection limit of 2 mg/dL, catering to PKU therapy needs. The study also concentrated on Galactosemia as a model disorder, underlining the necessity of early intervention. Molecular analysis of the GALT gene unveiled specific mutations in the Bangladeshi population, contributing to Galactosemia's molecular diversity. Evaluation of silent mutations' impact on splicing shed light on potential gene expression and protein function alterations, influencing Galactosemia outcomes. An easy-to-use lateral flow diagnostic kit for Galactosemia was developed, showing specificity for galactose and delivering rapid results within 5 minutes. Stability assessment revealed promising results at 2-8°C storage over a year, with signal degradation at higher temperatures, emphasizing proper storage conditions. The study highlights the potential of the HPLC method for amino acid detection, paving the way for accessible and cost-effective amino acidopathy detection facilities. The Galactosemia kit, with further refinement, holds promise for broader use. Insights into alternate GALT gene splicing patterns provide avenues for therapeutic exploration. Future research should fine-tune kits and establish precise phenylalanine and galactose cutoff ranges for the Bangladeshi population, contributing to improved IEM management.

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CHAPTER 1: INTRODUCTION

1.1 Metabolic disorders

Metabolic disorders are fascinating yet complex conditions that can affect individuals differently. These disorders arise when normal metabolism, which involves converting food into energy at a cellular level, is disrupted. The process of metabolism is intricate, with thousands of enzymes working together in various metabolic pathways to break down proteins, carbohydrates, and lipids. Unfortunately, metabolic diseases can impair the cell's ability to perform critical biochemical reactions that involve processing or transporting these molecules. While metabolic disorders are typically hereditary, affected individuals may not display any symptoms for a prolonged period. However, symptoms usually surface when the body's metabolism is stressed, such as during a febrile illness or after an extended fasting period. These symptoms can manifest in various ways, depending on the specific disorder. Although individual metabolic diseases are rare, they are relatively common when considered a group. The commonness of specific metabolic disorders varies with an approximate occurrence 1 in 500 (or even higher in isolated populations) to less than 1 in 1,000,000. Metabolic disorders affect around 1 in 1,000 individuals, making them a significant public health concern. Understanding the underlying causes of these conditions and developing new treatments approach is essential for improving the lives of individuals living with metabolic disorders **[1].**

1.2 Genetic Foundation of Inborn Error Metabolism (IEM)

1.2.1 Historical Context of IEM

"Inborn error of metabolism" (IEM) was introduced by a British physician Sir Archibald Garrod in 1908. His pioneering work in medical genetics led him to postulate that certain inherited disorders, such as alkaptonuria and albinism, were caused by a deficiency or total absence of enzymes that played crucial roles in specific biochemical pathways. Garrod's groundbreaking identification and classification of inborn errors of metabolism represented a significant conceptual breakthrough in medical genetics in the 20th century. It was a major

step forward in understanding the genetic causes of inherited disorders and paved the way for further research. In simple terms, IEM refers to a group of rare diseases caused by inherited congenital disabilities. These defects alter the body's ability to extract energy from nutrients, leading to many symptoms and complications. The impact of IEM can vary from person to person, and the severity of symptoms can range from mild to life-threatening condition **[2].**

Arthur Garrod's deep interest in chemical pathology propelled him to explore urinary chemistry as a reflection of complete disease and metabolism. Commencing with a handful of families grappling with the enigmatic alkaptonuria, Garrod's investigation, coupled with the emerging insights into Mendelian inheritance, led to a pioneering revelation: numerous perplexing diseases could be elucidated as inherited disorders of metabolism. Expounding Mendel's principles in the context of alkaptonuria, Garrod unveiled "The Incidence of Alkaptonuria: A Study of Chemical Individuality" in 1902. **[3]**.

Detailing his evolving comprehension of the condition and delving into speculative causes, Garrod draws intriguing comparisons between alkaptonuria and albinism regarding their inheritance in the paper. Notably, in this exploration, Garrod underscores the concept of "chemical individuality" in the absence of prior knowledge about genes, articulating, Alkaptonuria, an uncommon hereditary ailment impacting amino acid metabolism, notably Phenylalanine and Tyrosine, manifests by causing urine to transition from yellow to brown to black when exposed to air. In later stages, individuals with this condition develop arthritis marked by brown pigment deposition in joint cartilage and connective tissue. Examining repetition patterns in multiple families, it becomes evident that alkaptonuria adheres to an autosomal recessive inheritance pattern, attributed to a gene mutation affecting the metabolism of alkapton compounds. Over the subsequent decade, Garrod further delves into the type of inherited metabolic diseases, elucidating the recessive inheritance of most enzyme defects. He expands his metabolic inquiries to encompass cystinuria, pentosuria, and albinism. These three inborn errors, along with alkaptonuria, are collectively known as “Garrod's tetrad” a testament to his groundbreaking contributions to the field of chemical pathology and the understanding of inherited metabolic disorders **[4].**

1.2.2 IEM and Hereditary Metabolic Diseases

Inborn errors of metabolism (IEM) are the underlying causes of hereditary metabolic diseases (HMD), typically arising from the absence or reduced activity of specific enzymes or

disruptions in protein transportation. These conditions often entail the accumulation of substances usually present in minute quantities, the insufficiency of vital intermediary products, the scarcity of specific end products, or even the toxic accumulation of byproducts from alternative metabolic pathways **[5].**

The fundamental molecular underpinnings of biochemical disorders in hereditary metabolic diseases (HMD) can be attributed to genetic mutations in the loci responsible for crucial enzymatic processes. These mutations can exert their influence by impairing the functioning of activator proteins or co-factors essential for enzymatic activity. Furthermore, disruptions may arise in the intricate mechanisms governing protein transportation, including carrier systems and recognition markers vital for efficient cellular trafficking. These intricate genetic abnormalities lie at the core of the pathophysiology of HMD, contributing to the perturbation of normal biochemical pathways and the subsequent manifestation of metabolic dysfunctions **[6].**

1.2.3 Hallmark feature of IEM

Inborn errors of metabolism (IEM) encompass a spectrum of disorders that can manifest at birth or later in life, such as phenylketonuria, albinism, lactose intolerance, Gaucher disease, and Fabry disease, among others. IEM refers to a condition wherein the body's metabolic processes are perturbed due to underlying genetic disorders. These disorders arise from mutations occurring in genes that code for enzymes, synthesizing defective or dysfunctional enzymes. Consequently, the affected enzymes fail to fulfill their functions within the intricate metabolic pathways. The hallmark feature of IEM is the abnormal accumulation or storage of specific metabolites in various tissues, organs, and the bloodstream, giving rise to a spectrum of health-related ailments. Over the past few decades, researchers have identified hundreds of distinct IEMs, each with unique genetic and biochemical characteristics. While most IEMs are rare, it is important to note that some can pose life-threatening conditions. Despite the prevalence of these disorders, many individuals remain unfamiliar with the concept of inherited metabolic disorders and may have never encountered the term **[7].**

The genetic foundation of inborn errors of metabolism resides in the occurrence of mutations within individual genes. Mutations represent alterations in the human genome, specifically the genes that encode vital biological information. These genetic modifications can arise from

exposure to various factors, including viruses, pathogenic bacteria, ultraviolet radiation, unhealthy dietary choices, and environmental influences. As a result of these mutations, genetic abnormalities emerge, leading to disruptions in normal gene function, giving rise to inherited human diseases. Thus, it is through the intricate interplay of genetic mutations and their subsequent impact on gene integrity that the complex web of inborn errors of metabolism unfolds **[8**].

1.3 Genetic Disorders

The majority of inborn errors of metabolism (IEM) are inherited disorders that arise due to genetic alterations, which can manifest in two primary patterns: autosomal recessive inheritance, affecting non-sex chromosomes, and X-linked recessive inheritance, affecting sex-linked chromosomes. Autosomal recessive disorders necessitate the presence of two copies of the mutated gene, one from each parent, for the condition to manifest. In the case of X-linked recessive disorders, the mutation occurs on the sex chromosomes, particularly the X chromosome. These disorders predominantly affect males, who possess one X and one Y chromosome, while females have two X chromosomes. Additionally, genetic disorders can be inherited in either a dominant or recessive mode in human beings. Dominant inheritance occurs when a single copy of the mutated gene is sufficient to cause the disorder, whereas recessive inheritance necessitates the presence of two copies of the mutated gene for the disorder to manifest. This intricate interplay of different modes of inheritance contributes to the diverse landscape of genetic disorders observed in human populations **[9]**.

1.3.1 Role of Inheritance Patterns

Inheritance patterns play a crucial role in the development of genetic disorders. Recessive inheritance implies that the disorder arises when an individual possesses two disease-causing copies of a gene, one inherited from each parent. If a person carries only one disease-causing allele, they are considered a "carrier" and have an increased risk of passing the disorder to their offspring. For a child to be affected by the disorder, both parents must either be carriers or already affected by the condition. In most cases of recessive inheritance, the disorder occurs due to non-functional copies of a gene. This means that either no protein is produced

from the gene or the protein product is altered, rendering it unable to perform its intended function. Individuals with one working gene copy can sometimes produce enough functional protein to remain unaffected. However, the absence of any functional copies of the gene results in the manifestation of the disorder. It is important to note that many carriers may be unaware of a genetic disorder in their family, as genetic testing is often not pursued until the decision to have children is made. On the other hand, dominant inheritance indicates that a person will have the genetic disorder if they possess just one disease-causing copy of the gene. The term "dominance" refers to the function of the specific product encoded by the gene rather than how the gene is inherited. In cases of dominant inheritance, the genetic disorder is passed from an affected parent to an affected child. These inheritance patterns highlight the intricate mechanisms underlying the transmission of genetic disorders and emphasize the importance of genetic counseling and testing to assess the risks associated with family planning **[10]**.

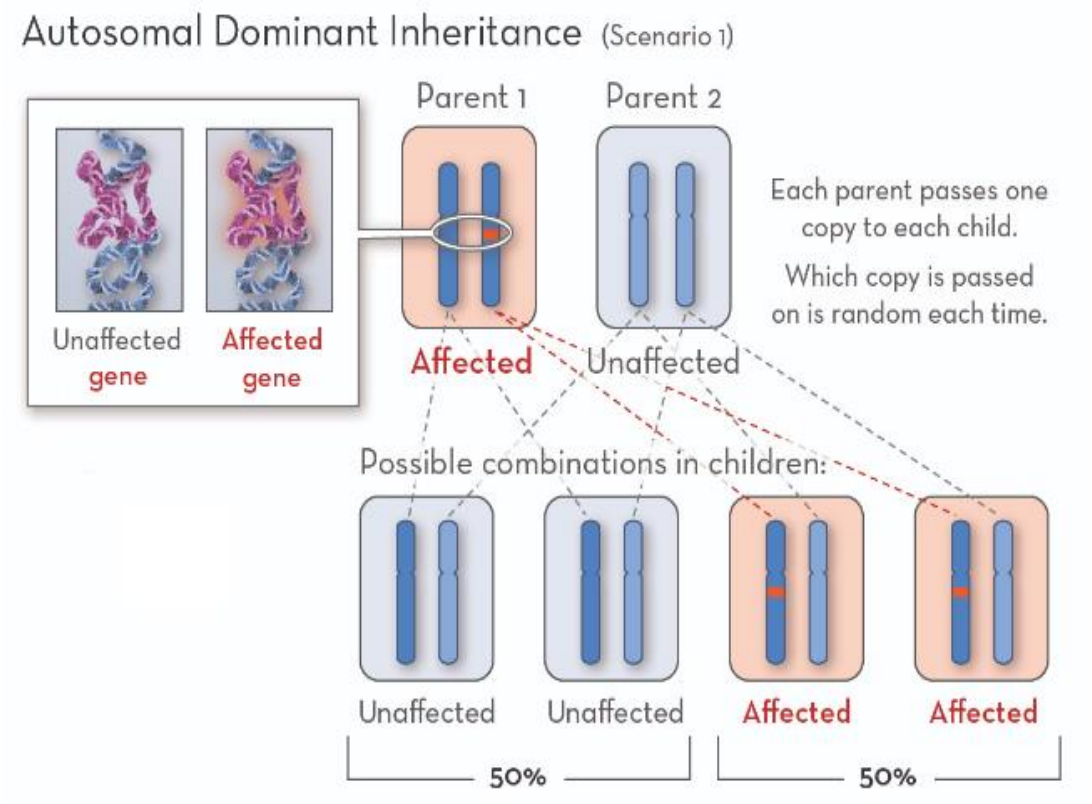


Figure 1.1. Illustration of Parental Transmission of genetic disorder to Offspring in a Family (Scenario-1)

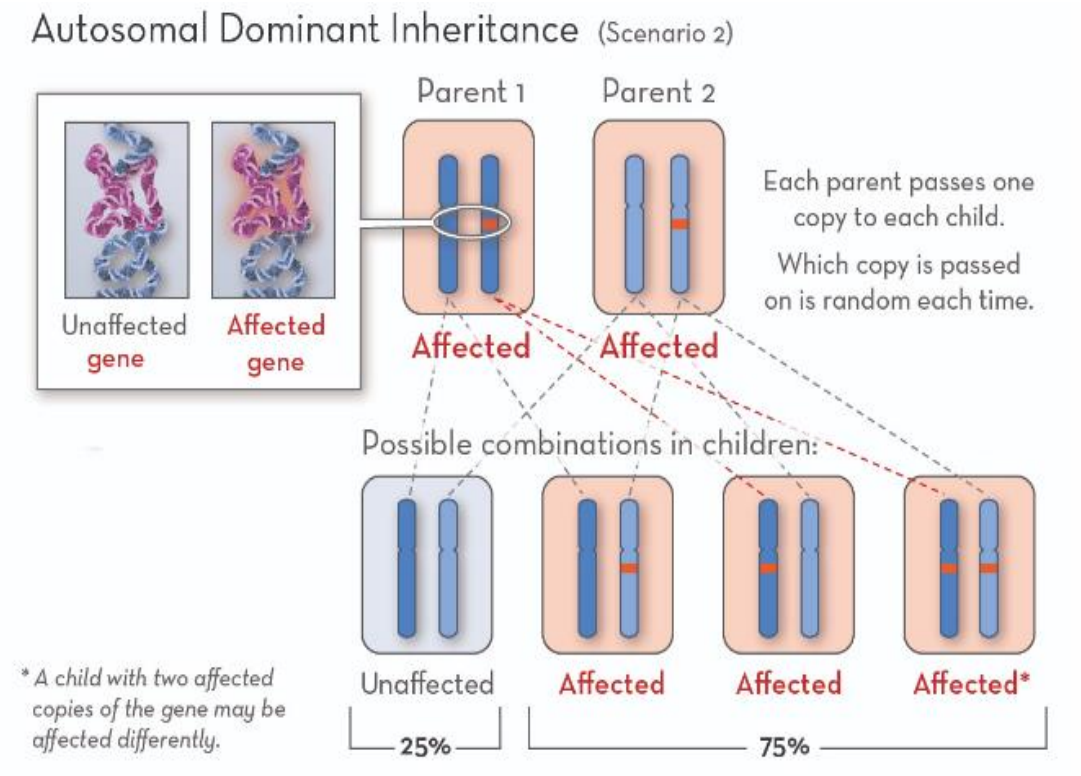


Figure 1.2. Demonstration of Parental Transmission of genetic disorder to Offspring in a Family (Scenario-2)

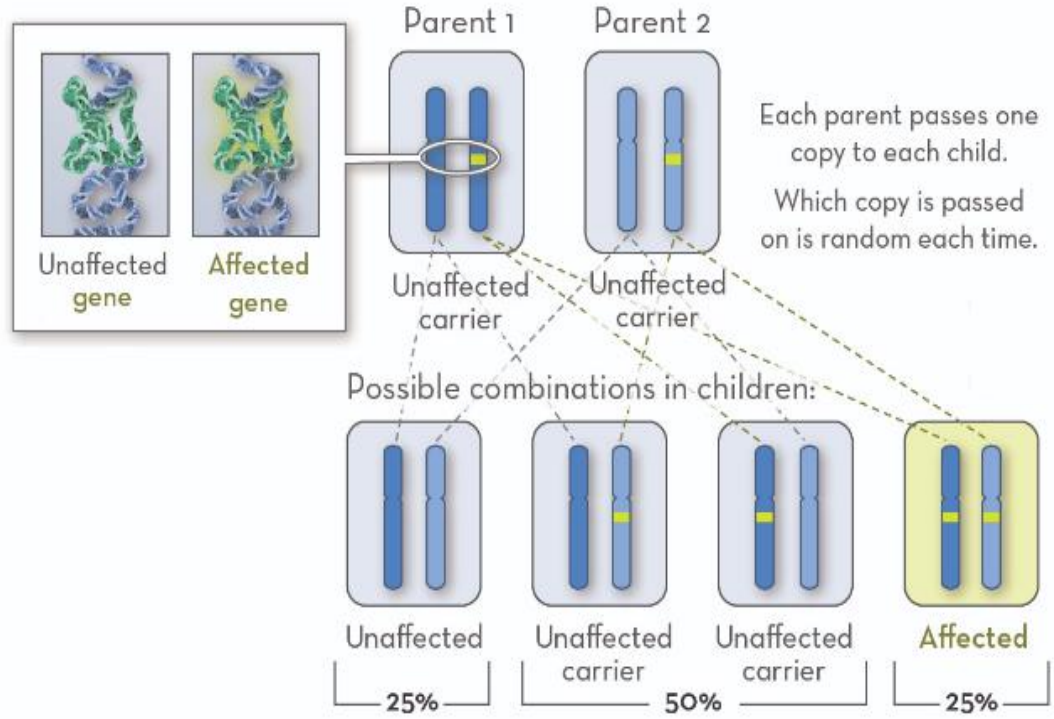


Figure 1.3. Autosomal Recessive Inheritance: Manifestation of Carrier Genes in a Family's Offspring

Individuals who possess one functional copy and one non-functional copy of a specific gene are commonly referred to as carriers. In the context of recessive genetic disorders, these disorders are determined by the presence of two carrier genes, one inherited from the father and the other from the mother. Consequently, the likelihood of inheriting carrier genes in an autosomal inheritance pattern can be summarized as follows:

1. Child (A) has a 25% chance of inheriting two functional copies of the gene and will therefore be unaffected by the disorder, exhibiting normal health.

2. Children (B and C) have a 50% chance of inheriting one functional copy and one non-functional copy of the gene, making them carriers like their parents. Although carriers typically do not manifest symptoms of the disorder, they can potentially pass the non-functional gene to their offspring.

3. Child (D) has a 25% chance of inheriting two non-functional copies of the gene, manifesting the inherited disorder. This occurs when the individual receives the same abnormal gene for the specific trait from both parents.

This inheritance pattern underscores the probabilities associated with the transmission of genes in autosomal recessive disorders and highlights the potential outcomes for each child regarding their genetic status and the likelihood of developing the related diseases.

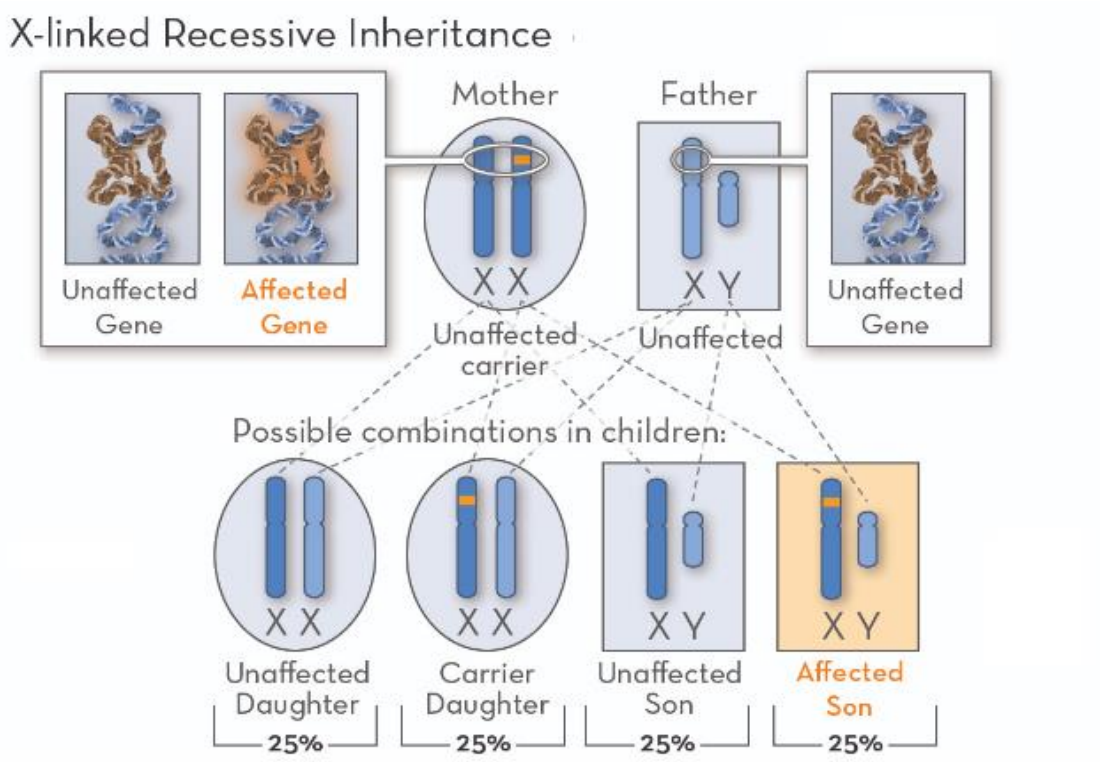


Figure 1.4. X-Linked Inheritance Pattern: Illustration of Familial Transmission of X-Linked Genetic Disorders.

Genetic disorders exhibiting an X-linked inheritance pattern demonstrate distinct effects on males and females. Like autosomal disorders, X-linked genetic disorders can manifest through dominant or recessive inheritance patterns. However, most X-linked genetic diseases tend to follow a recessive way. In the case of X-linked recessive diseases, males are significantly more susceptible to being affected by the disorder than females. This discrepancy arises due to the sex chromosome composition, where males possess one X Males possess one Y chromosome (XY) while females have two X chromosomes (XX). As a result, the presence of a single copy of the non-functioning gene on the X chromosome in males can lead to the expression of the disorder, as there is no second X chromosome to compensate for the abnormality.

Conversely, females require two copies of the non-functioning gene, one on each X chromosome, to express the disorder. Thus, the X-linked inheritance pattern underscores the dissimilarities in disease manifestation between males and females, highlighting the significance of genetic factors and the sex chromosome composition in the inheritance of X-linked genetic disorders **[10]**.

1.4 Metabolic Consequences

The human body relies on the intricately orchestrated series of chemical reactions within cells to efficiently produce and utilize energy, maintain essential biological processes, and sustain overall health. These interconnected chemical reactions, collectively known as metabolic pathways, are fundamental for maintaining homeostasis and supporting the proper functioning of various bodily systems. Critical to the functionality of metabolic pathways are enzymes, specialized proteins that act as catalysts. Enzymes are pivotal in accelerating biochemical reactions, enabling them to occur at a rate necessary for the efficient metabolism of substances within the body. Importantly, enzymes themselves remain unchanged and unaffected by the reactions they facilitate. In metabolism, enzymes are involved in the breakdown, transformation, and utilization of macronutrients such as proteins, fats, and carbohydrates. They enable the conversion of complex molecules into simpler forms that can be utilized for energy production, cellular growth, and the synthesis of vital biomolecules. However, when there is a deficiency or error in the function of specific enzymes, the average metabolic process can be disrupted. This disruption can manifest as a metabolic abnormality characterized by imbalances in the concentrations of metabolites and the impaired synthesis or breakdown of essential substances. Such abnormalities can lead to a wide range of metabolic disorders, affecting various organs and systems within the body. Understanding enzymatic defects and their impact on metabolic pathways is crucial in diagnosing and managing inherited metabolic disorders. By unraveling the intricate biochemical basis of these defects, researchers and healthcare professionals can gain insights into the underlying mechanisms of these disorders, paving the way for improved diagnostic techniques, targeted treatments, and potential therapeutic interventions **[11]**.

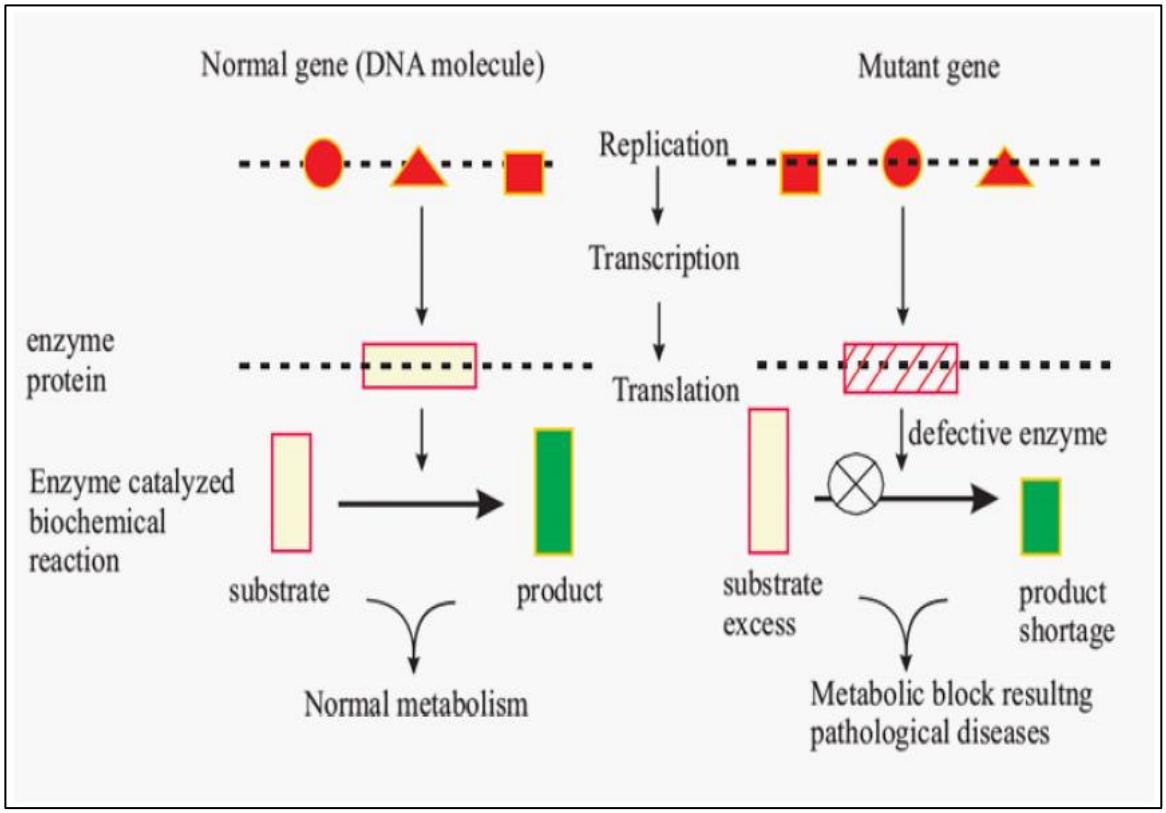


Figure 1.5. Impaired enzyme activity leads to metabolic disruptions, characterized by the accumulation of substrates and deficiencies of products within cells, contributing to the dysregulation of specific metabolic pathways **[1]**.

1.4.1 Accumulation of a substrate

In the realm of inborn errors of metabolism (IEM), a range of potential metabolic consequences may arise, including the buildup of specific substrates, which are processed or transformed within metabolic pathways. Due to enzyme deficiencies or defects, these substrates may not be adequately broken down or utilized, resulting in their accumulation within cells or body tissues.

1.4.2 Accumulation of intermediate metabolites

Within metabolic pathways, numerous intermediate metabolites are produced as intermediaries during the step-by-step breakdown or synthesis of substances. In cases of IEM,

impaired enzyme function can impede the normal progression of these pathways, causing the accumulation of these intermediate metabolites, which may have toxic or disruptive effects on cellular processes.

1.4.3 Lack of an essential product

Certain metabolic disorders may hinder synthesizing or producing actual end products within specific metabolic pathways. Insufficient enzyme activity or altered enzymatic function can disrupt the normal progression of reactions, leading to a deficiency or shortage of critical end products required for normal physiological functioning.

1.4.4 Interference with normal metabolic function

IEM can interfere with the overall functioning of metabolic processes, disrupting the delicate balance and coordination of biochemical reactions. Enzyme deficiencies or defects can impede the conversion of substances, alter energy production and utilization, and perturb the regulation of metabolic pathways, thereby impacting the overall efficiency and homeostasis of the body's metabolic system. These metabolic consequences highlight the diverse range of disturbances in individuals affected by IEM, underscoring the importance of identifying and addressing these abnormalities to mitigate potential health complications and optimize overall well-being.

1.5 Indications of IEM

The physical irregularities related with severe onset of inborn errors of metabolism can provide indications or strong possibilities of an IEM:

1. Acute illness in infants follows a period of customary performance and nourishing, occurring within short period of time.
2. Neonates are presenting with confiscations and hypotonia.
3. Infants with an unusual redolence.

1.5.1 Strong possibilities of IEM:

1. Persistent or recurrent vomiting.
2. A condition known as failure to thrive is characterized by an inability to acquire weight or significant weight reduction.
3. Apnea or breathing agony.
4. Enlarged liver.
5. Lethargy.
6. Intermittent coma.
7. Mysterious blood loss.
8. Ancestral cases of neonatal deaths or similar illnesses in family members.
9. Parental consanguinity (related by blood).
10. Sepsis, particularly with *E. coli* infection.

These symptoms can serve as important indicators for healthcare professionals in identifying the possibility or substantial likelihood of an inborn error of metabolism in individuals experiencing acute onset physical anomalies.

1.5.2 Other physical anomalies

Some other physical anomalies associated with acute onset inborn errors of metabolism, along with the possible corresponding IEM:

1. Ambiguous genitalia: Congenital adrenal hyperplasia.
2. Hair or Skin problems (alopecia, dermatitis): Multiple carboxylase deficiency, biotinidase deficiency, arginosuccinic aciduria.
3. Structural brain abnormalities (agenesis of the corpus callosum, cortical cysts): Pyruvate dehydrogenase deficiency.
4. Macrocephaly: Glutaric aciduria, type I.
5. Renal cysts, facial dysmorphia: Glutaric aciduria, type II; Zellweger syndrome.
6. Facial dysmorphia: Peroxisomal disorders (Zellweger syndrome).
7. Cataract: Galactosemia, Lowe syndrome.
8. Retinopathy: Peroxisomal disorders.

9. Lens dislocation, seizures: Sulfite oxidase deficiency, Molybdenum cofactor deficiency.
10. Facial dysmorphia, congenital heart disease, vertebral anomalies: 3-OH-isobutyric CoA deacylase deficiency.

1.5.3 Clinical manifestations of inborn errors of metabolism (IEM)

The clinical manifestations of inborn errors of metabolism (IEM) that commonly present in the neonatal period. These symptoms can aid in the identification of potential metabolic disorders:

1. Signs related to neurology: Poor draw, tiredness (developing blackout), abnormalities in muscle character, reduction of reactions, and seizures indicate neurologic involvement in IEM.
2. Gastrointestinal signs: Poor feeding, vomiting, and diarrhea are gastrointestinal symptoms often observed in neonates with IEM.
3. Respiratory signs: Hyperpnea (abnormally rapid breathing) and respiratory failure can be respiratory manifestations associated with certain metabolic disorders.
4. Organomegaly: Enlargement of organs, particularly the liver, and heart, can be observed in neonates affected by certain metabolic disorders.

These clinical manifestations provide valuable insights for healthcare professionals in recognizing the likelihood of underlying metabolic disorder in neonates presenting with these symptoms. Prompt identification and diagnosis of IEM are crucial for appropriate management and intervention to optimize the outcomes for affected individuals **[12, 13]**.

1.6 Types of IEM

Inborn errors of metabolism (IEM) have the potential to impact any organ system and often exhibit manifestations that range from severe lethal conditions to sub-acute advancing deteriorating syndromes. These disorders can be categorized as follows:

1. Syndromes associated with protein absorption: This category includes conditions such as aminoacidopathies, organic acidopathies, and defects in urea cycle, which involve impairments in the metabolism of amino acids and peptides.
2. Syndromes associated with carbohydrate absorption: disorders of carbohydrate uptake, glycogen metabolism syndromes, and conditions of gluconeogenesis and glycogenolysis fall into this category, affecting the metabolism of carbohydrates.
3. Conditions related to the metabolism of fatty acids and ketone bodies: These disorders involve abnormalities in the breakdown and utilization of fatty acids and ketone bodies.
4. Complaints of energy metabolism: This category encompasses disorders that affect the body's overall energy production and utilization processes.
5. Metabolic sicknesses related to purines, pyrimidines, and nucleotides: Conditions in this category involve disturbances in the metabolism of these vital components of nucleic acids and cellular energy molecules.
6. Complaints of the metabolism of sterols: This category includes conditions that disrupt the metabolism of sterols; these are crucial constituents of cell membranes and serve as precursors to other substances essential molecules such as hormones.
7. Illnesses of porphyrin and heme uptake: Porphyrin and heme metabolism disorders involve impairments in synthesizing and degrading these compounds, which play crucial roles in oxygen transport and enzymatic reactions.
8. Complaints of lipid and lipoprotein absorption: Conditions such as fatty acid oxidation defects (e.g., medium-chain acyl dehydrogenase deficiency) and sphingolipidoses are classified within this category.
9. Congenital disorders of glycosylation and other protein modification diseases involve abnormalities in the processes that modify and attach sugar molecules to proteins.
10. Lysosomal complaints: Lysosomal storage illnesses, like Gaucher's syndrome and Niemann-Pick's illness occur due to deficiencies in specific enzymes responsible for breaking down substances within lysosomes.

11. Peroxisomal disorders: This category comprises disorders such as Zellweger syndrome and adrenoleukodystrophy, characterized by peroxisome function and metabolism impairments.
12. Complications of neurotransmitter: These disorders affect the synthesis, breakdown, and regulation of neurotransmitters, which are crucial for proper neuronal communication.
13. Metabolic disorders related to organic compounds and cofactors: Conditions in this category involve impairments in the digestion of essential vitamins and cofactors required for various biochemical reactions.
14. Metabolic disorders involving trace elements and metals: Conditions such as Menke's kinky hair problem and Wilson's disease result from disturbances in the uptake of specific trace elements and metals.
15. Metabolic complications related to xenobiotics: This category encompasses disorders related to the metabolism of foreign substances and chemicals in the body.

These distinct categories illustrate the diverse range of metabolic pathways and processes that can be affected by IEM, underscoring the complexity and breadth of these genetic disorders **[14]**.

1.7 Global Prevalence of IEM

Individual inborn errors of metabolism (IEM) are considered sporadic sicknesses, typically with incidences of less than 1 per 100,000 births. Though, when viewed together, the overall frequency of IEM can range from approximately 1 in 800 to 2500 births. Incidence rates of specific classes of disorders can vary across different populations, as demonstrated by the two reviews provided.

In a study carried out in British Columbia, where the majority of the population is Caucasian, the estimated incidences of numerous inborn errors of metabolism (IEM) were observed. For instance, amino acid disorders (excluding phenylketonuria) were noted at a rate of 7.6 per 100,000 births, phenylketonuria at 7.5 per 100,000 births, organic acidemias at 3.7 per 100,000 births, urea cycle diseases at 1.9 per 100,000 births, glycogen storage diseases at 2.3 per 100,000 births, lysosomal storage diseases at 7.6 per 100,000 births, peroxisomal disorders at 3.5 per 100,000 births, and mitochondrial diseases at 3.2 per 100,000 births. In

contrast, another study conducted in the West Midlands of the United Kingdom, with around 11% of the population consisting of individuals from black and ethnic minority groups, reported different frequencies of selected IEM during the period from 1999 to 2003. Notably, amino acid disorders (excluding phenylketonuria) were documented at 18.7 per 100,000 births, phenylketonuria at 8.1 per 100,000 births, organic acidemias at 12.6 per 100,000 births, urea cycle diseases at 4.5 per 100,000 births, glycogen storage diseases at 6.8 per 100,000 births, and lysosomal storage diseases at 19.3 per 100,000 births, peroxisomal disorders at 7.4 per 100,000 births, and mitochondrial diseases at 20.3 per 100,000 births.
These data emphasize the rarity of individual IEM, with variations in incidence rates observed across different populations and classes of disorders. Understanding the frequency and distribution of IEM is vital for healthcare professionals involved in diagnosis, and management can provide genetic counseling services for individuals and families affected by genetic disorders **[12]**.

The most recent estimations suggest that the incidence rates of inborn errors of metabolism (IEM) indicate a prevalence of approximately one in 800 to 2500 live births. While individual disorders are being diagnosed more frequently, it is worth noting that these conditions remain relatively rare and demonstrate variations in occurrence across different countries and regions. In a recent pioneering effort by Ferreira et al., they developed a comprehensive classification system, taking into account the prevalence of each category of IEMs (as illustrated in Figure 1). Employing stringent criteria, they aimed to establish the initial formal nosology of IEMs, with a commitment to regularly updating this classification to reflect evolving knowledge and understanding. This remarkable initiative significantly contributes to our comprehension of IEMs, providing a valuable resource for clinicians, researchers, and healthcare professionals engaged in diagnosing, managing, and ongoing investigation of these intricate disorders **[15].**

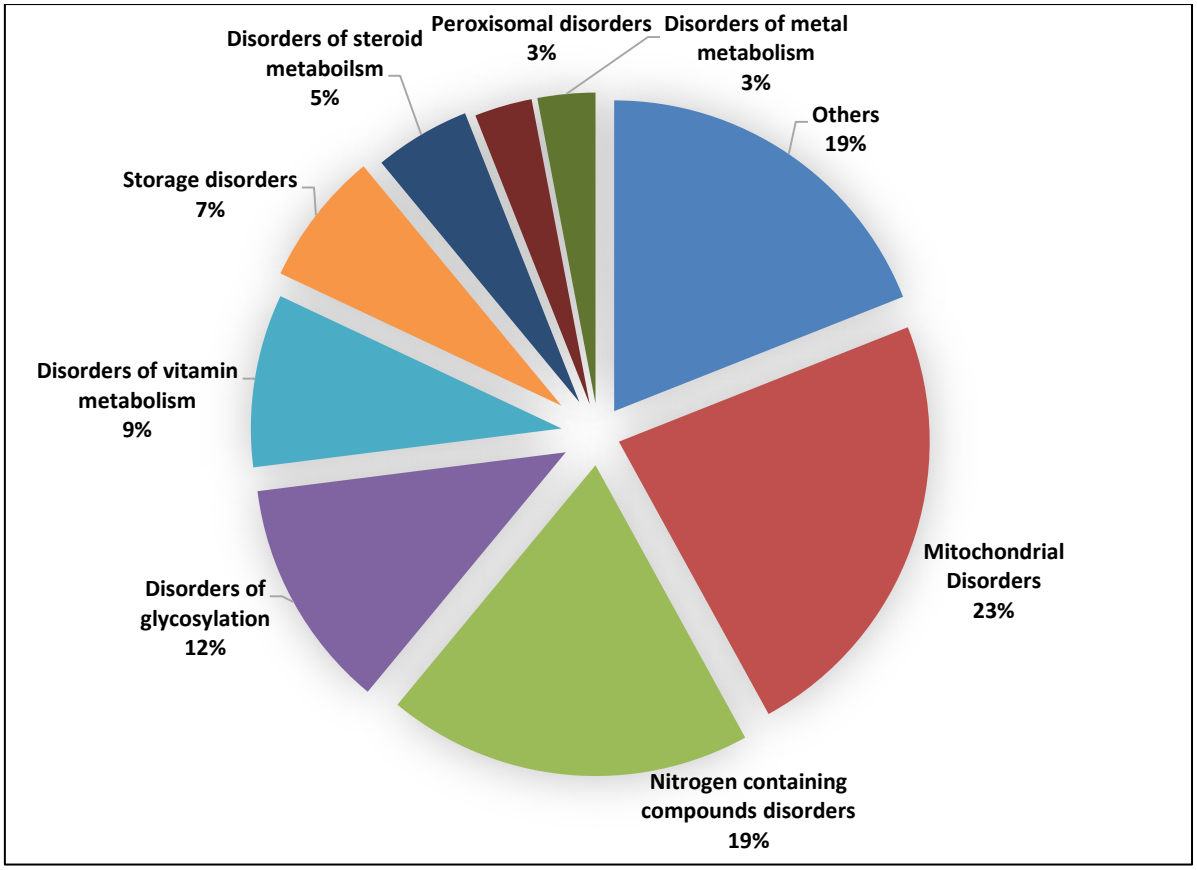


Figure 1.6. Distribution of IEMs by category based on the specified inclusion criteria **[16]**.

As illustrated in the accompanying Figure, findings from diverse global investigations have unveiled notable disparities in the documented incidence rates of inborn errors of metabolism (IEM) diseases. These variations manifest not only in the overall incidence of IEM but also in specific categories encompassing organic acid disorders, lysosomal storage disorders, fatty acid metabolism disorders, mitochondrial disorders, urea cycle disorders, amino acid metabolism disorders, and carbohydrate metabolism disorders. Nonetheless, the underlying causes of these incidence rate discrepancies and the observed divergences across different regions worldwide have yet to be comprehensively elucidated. Countries or areas with a higher prevalence of consanguineous marriages have exhibited significantly elevated incidence rates, sometimes exceeding 50-fold. This observation suggests that consanguinity plays a substantial role in the increased occurrence of IEMs within these populations. Moreover, regional disparities in genetic diversity, coupled with heightened rates of inbreeding and larger family sizes, contribute to the varying incidences observed in the

overall prevalence of IEMs. These complex interdependencies create a diverse landscape of IEM occurrences across distinct population groups **[17].**

Furthermore, understanding overall IEM incidence rates is further complicated by differences in research methodologies and durations employed across various countries when investigating the same disease. These methodological variances can introduce additional disparities in the reported incidence rates, impeding the establishment of consistent and comparable global data. Comprehensive investigations encompassing diverse populations and standardized research methodologies are imperative to enhance our comprehension of IEM incidence rates and the factors contributing to these disparities. Such endeavors will provide crucial insights into the epidemiology and underlying determinants of these intricate disorders, ultimately facilitating the development of more effective strategies for prevention, diagnosis, and management on a global scale **[16]**.

Global Prevalence of Inborn Errors of Metabolism (IEM) Diseases and Regional Variations: (Incidence Rates per 100,000 Live Births.) **[15]**

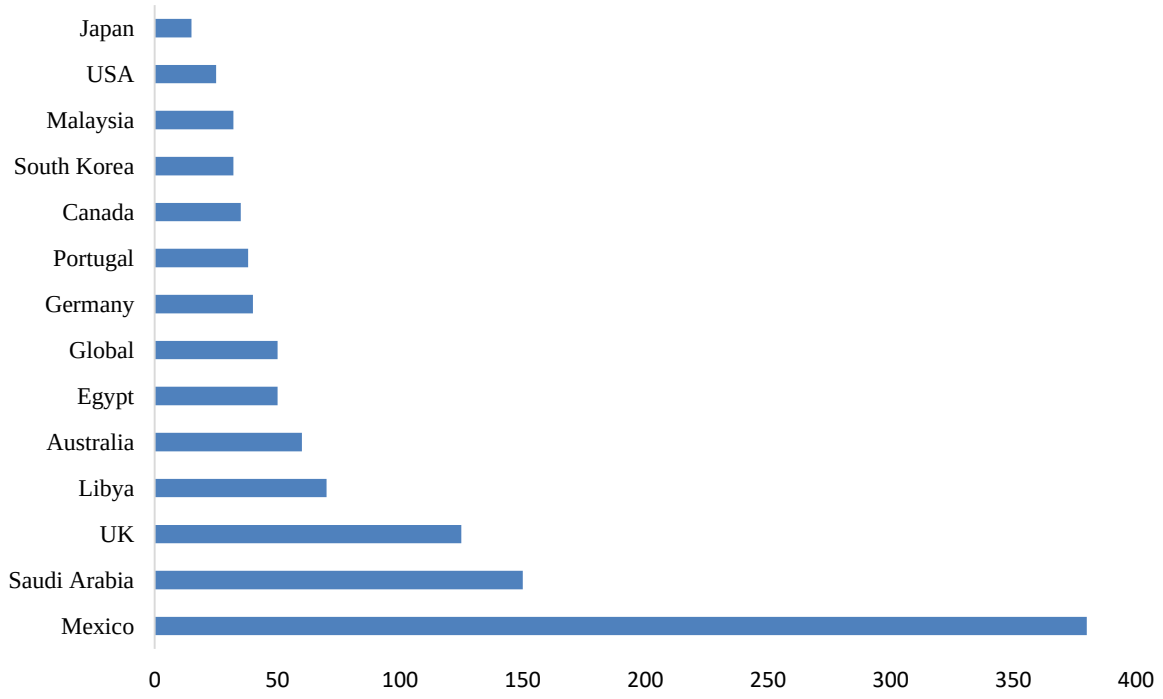


Figure 1.7. Overall incidence of IEM per 100,000 live births

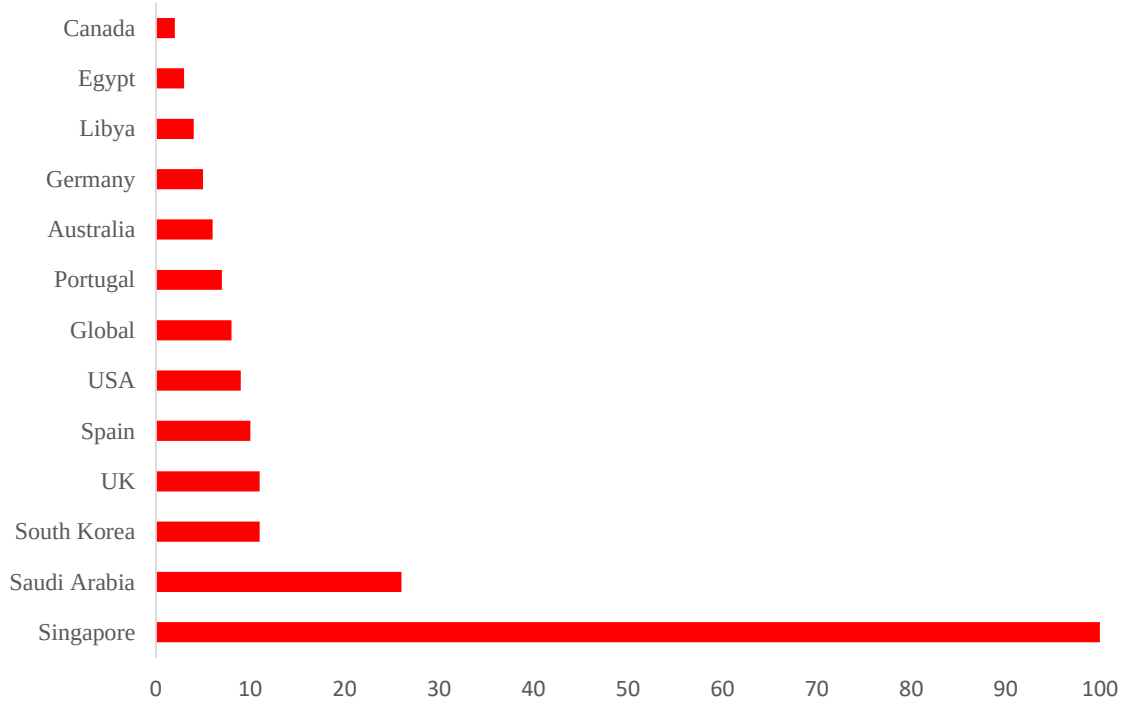


Figure 1.8. Incidence of organic acid disorder per 100,000 live births

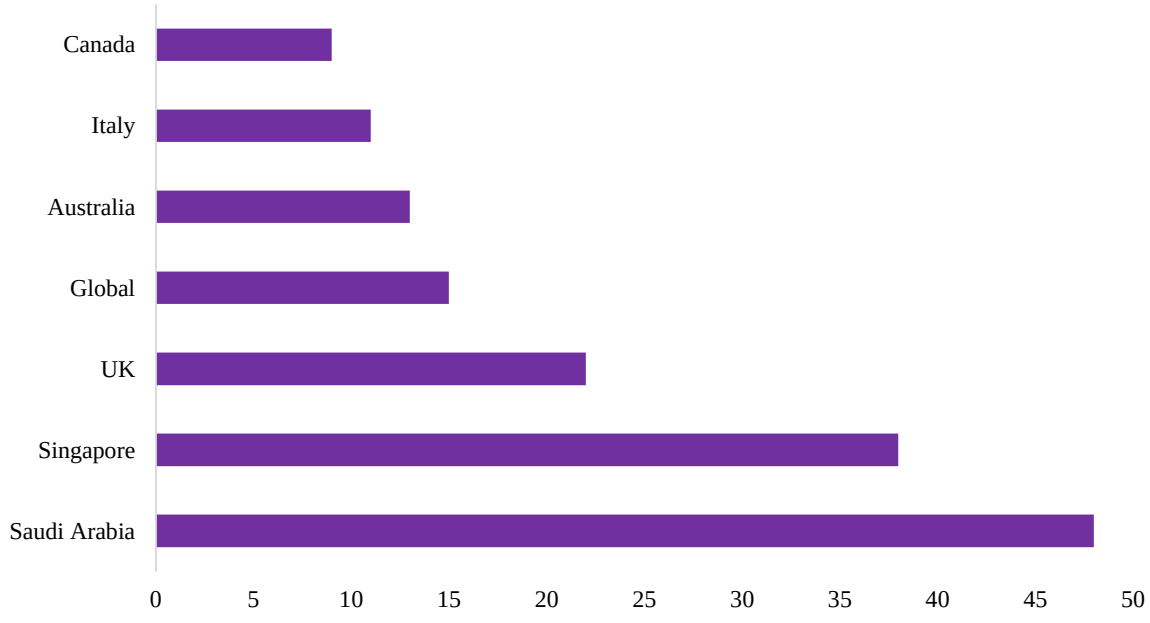


Figure 1.9. Incidence of lysosomal disorder per 100,000 live births

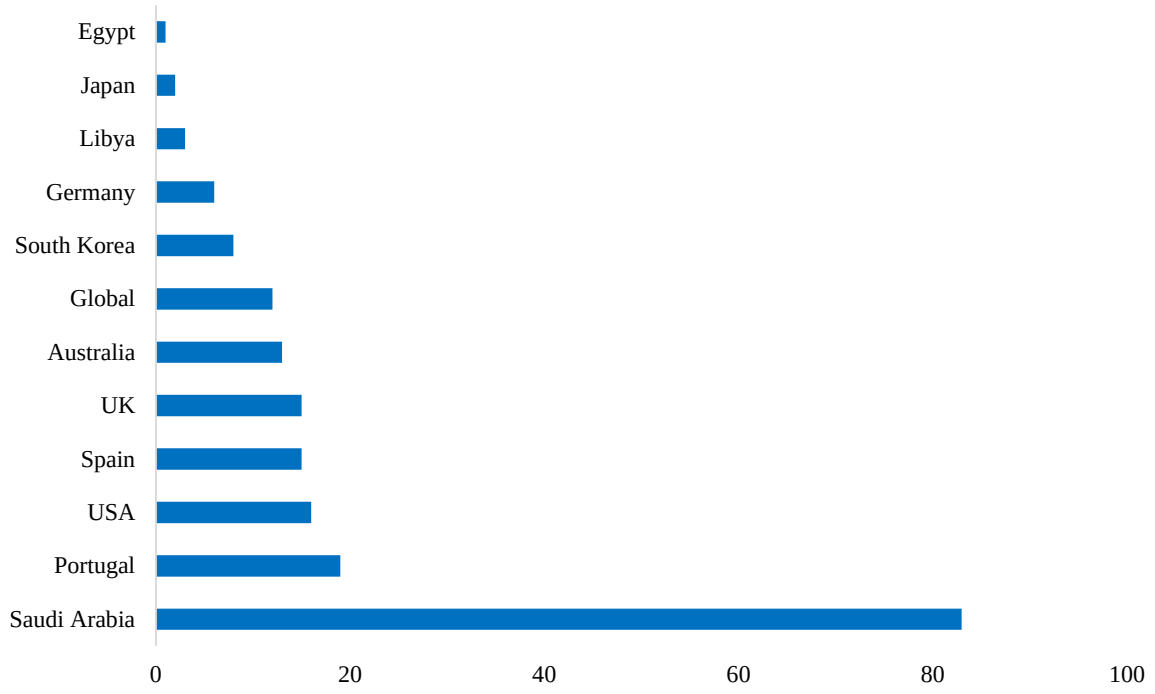


Figure 1.10. Incidence of fatty acid metabolism disorder per 100,000 live births

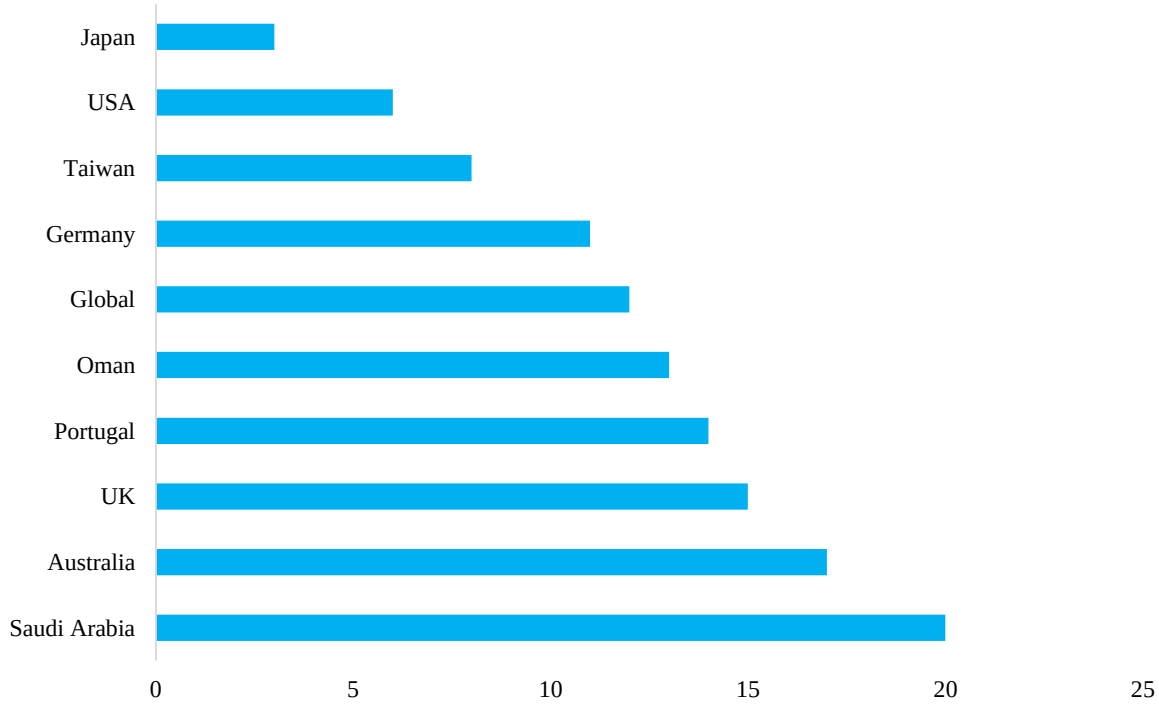


Figure 1.11. Incidence of amino acid metabolism disorder per 100,000 Live Births

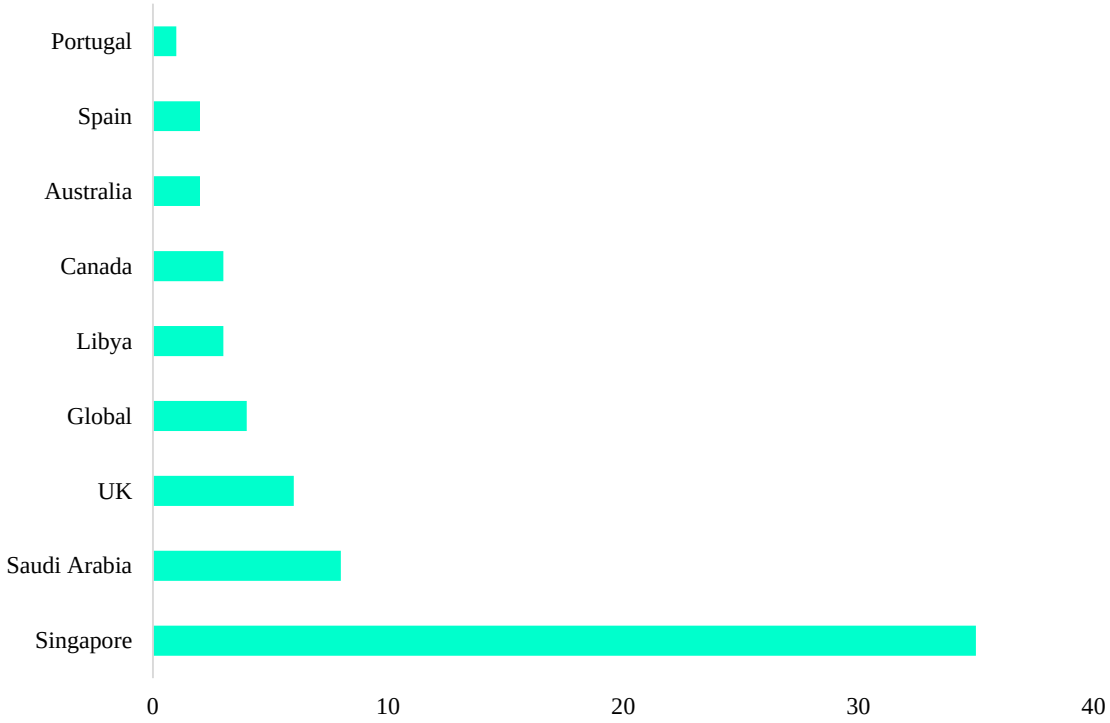


Figure 1.12. Incidence of urea cycle disorders per 100,000 live births

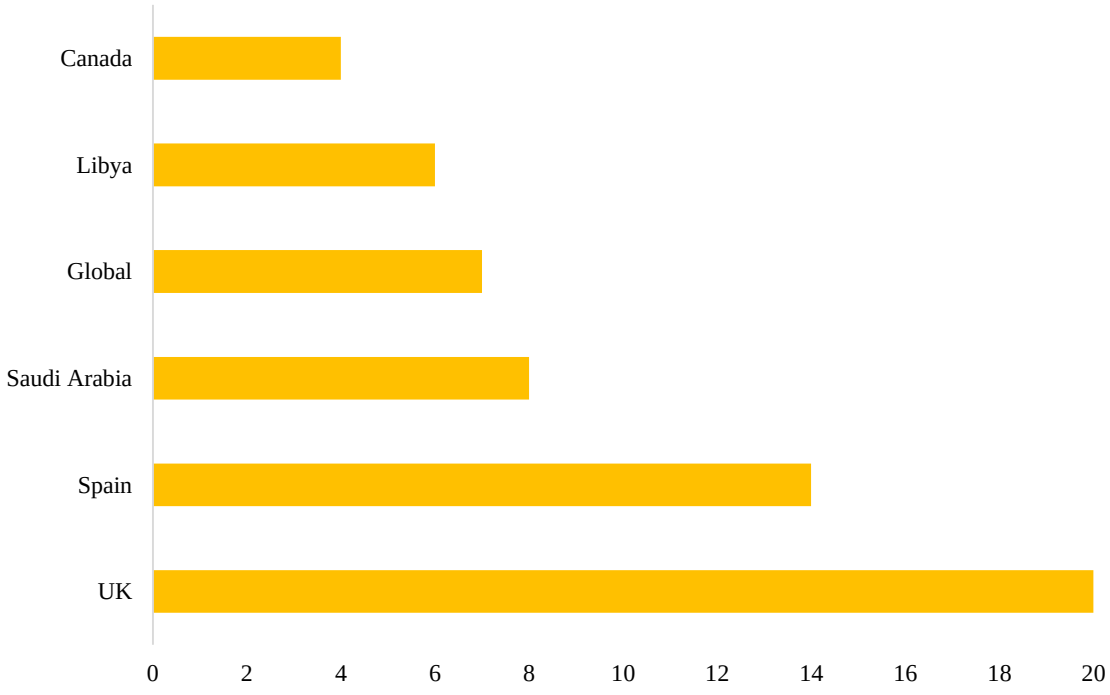


Figure 1.13. Incidence of mitochondrial disorders per 100,000 live births

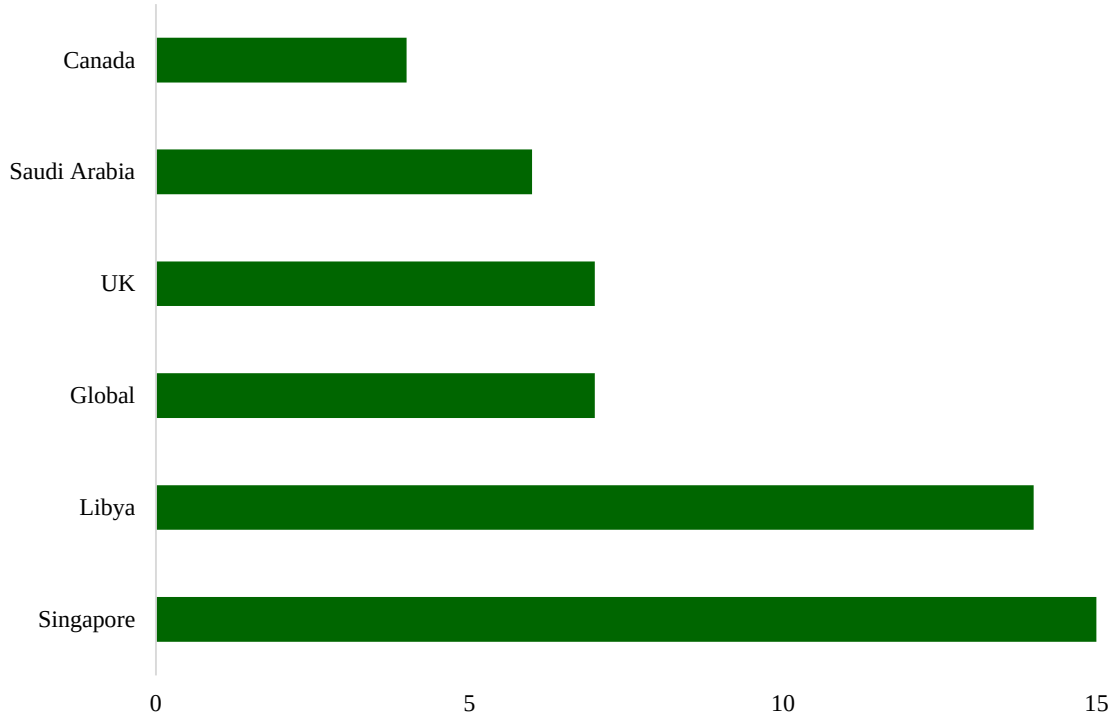


Figure 1.14: Incidence of carbohydrate metabolism disorder per 100,000 live births

1.8 Prevalence of IEM in Bangladesh

In Bangladesh, a nation grappling with numerous healthcare challenges, one area of concern that has remained inadequately explored is the prevalence of inborn errors of metabolism (IEM) among newborns. The scarcity of published studies on this critical topic has created a significant gap in understanding, hindering efforts to gauge the true extent of the issue. This lack of comprehensive data underscores the urgent need for establishing a nationwide newborn screening program, a vital initiative that could potentially transform the landscape of infant healthcare in the country. Amidst this prevailing dearth of knowledge, a ray of hope emerged through a study which meticulously conducted in a tertiary care hospital-based setting, marked a significant milestone in the quest to unravel the mysteries of IEM among Bangladeshi newborns. Focusing on 50 sick neonates, this study employed advanced Tandem Mass Spectrometry (TMS) testing, a sophisticated analytical technique, to delve deep into the intricate world of metabolic disorders **[18].**

The outcomes of this study were nothing short of astonishing, revealing a startling reality: 64% of the neonates under scrutiny, a total of 32 cases, exhibited abnormal results in TMS testing. This alarming revelation served as a stark reminder of the pressing nature of the problem within the Bangladeshi population. Furthermore, the study unveiled a complex tapestry of IEM, identifying ten distinct types of metabolic disorders affecting these vulnerable newborns. This diversity showcased the multifaceted challenges faced by healthcare professionals in diagnosing and managing these conditions effectively. A particularly noteworthy aspect of the study was the discovery that 34% of the neonates examined had consanguineous relationships. This genetic factor adds a layer of complexity to the prevalence of IEM, indicating a potential area for further research and genetic counseling. Understanding the genetic underpinnings of these disorders is paramount, as it can inform strategies for early detection, counseling, and potentially, preventive measures in high-risk families. The implications of this findings are profound and far-reaching. This serves as a clarion call to action, emphasizing the urgency and importance of implementing a comprehensive nationwide screening program. Such a program, beyond providing essential data to comprehend the actual prevalence and distribution of IEM among newborns, holds the key to early detection and intervention. By identifying these disorders at their nascent stages, healthcare professionals can initiate timely and appropriate interventions, significantly improving the health outcomes of affected infants. Therefore the need for concerted efforts and strategic investments in healthcare infrastructure and research to address the challenges posed by IEM comprehensively and must galvanize these stakeholders into prioritizing the establishment of a robust and widespread newborn screening initiative. By doing so, they can pave the way for a healthier, brighter future for Bangladesh's youngest citizens, ensuring that every newborn has the opportunity to thrive, unencumbered by the burden of undetected metabolic disorders.

1.9 Diagnosis of IEM

Uncovering metabolic disorders presents several diagnostic challenges due to the following:

1. The intermittent nature of metabolic illness: Metabolic disorders often exhibit episodic symptoms, making it challenging to identify and diagnose them during periods of everyday health.

2. The broad spectrum of clinical symptoms: Metabolic disorders can present with various clinical manifestations. Presentations, which can also overlap with symptoms seen in more common conditions. This overlap complicates the diagnostic process and increases the risk of misdiagnosis.
3. The relatively low incidence of these disorders: Metabolic disorders are rare, resulting in limited exposure and experience among healthcare professionals. This lack of familiarity can hinder timely and accurate diagnosis.
4. The specialized knowledge required: Diagnosis and management of metabolic disorders necessitate technical expertise and access to specific diagnostic tests, such as genetic or biochemical assays. These tests are essential for identifying metabolic abnormalities and confirming the presence of a disorder.
5. The need for collaboration among healthcare professionals: Due to the complexity of metabolic disorders, a multidisciplinary approach involving various medical specialties, geneticists, and specialized laboratories are often required for accurate diagnosis and comprehensive care.

Addressing these diagnostic challenges requires increased awareness, improved access to specialized testing, and enhanced collaboration among healthcare professionals. By promoting education, research, and the development of diagnostic tools, we can enhance the early detection and effective management of metabolic disorders, improving the outcomes and quality of life for individuals who are impacted by these conditions **[12].**

The diagnostic challenge posed by patients with inherited metabolic disorders is distinct in clinical practice. Despite harboring strong suspicions of an underlying metabolic anomaly, the extensive array of diseases and the diverse clinical features pose challenges in selecting appropriate diagnostic tests. The clinical phenotypes associated with inborn errors of metabolism (IEM) exhibit broad and often nonspecific characteristics, resembling more common conditions and symptoms spanning from fetal development to adulthood. Although most IEM traditionally present in acutely ill newborns or young infants, advances in tandem mass-spectrometry-based newborn screening have mitigated the frequency of acute episodes and subsequent complications for many identified conditions. It's crucial to recognize, however, that screening cutoff values may overlook milder mutations and subtle biochemical phenotypes. Therefore, a standard newborn screen should not dismiss the possibility of an

inherited metabolic anomaly in a patient whose clinical presentation suggests such a condition. While the study of clinical and biochemical phenotype of inborn errors of metabolism together in offspring has a long-established past, the recognition of delayed-onset or adult-onset types of these disorders has historically lagged. However, recent ages have witnessed a growing acknowledgment of adult-onset types, often presenting as trifling phenotypes. These cases, characterized by less severe symptoms in juvenile stage, repeatedly result in delayed diagnosis. The clinical features of delayed-onset types may significantly diverge from the classical manifestations associated with the underlying illness, adding complication to the diagnostic process. Navigating this diagnostic challenge necessitates a judicious selection of tests, guided by a combination of the patient's clinical presentation and the results of repetitive laboratory valuations. Characteristic laboratory findings signaling a metabolic defect encompass hypoketotic hypoglycemia, lactic acidosis, metabolic acidosis, ketosis, hyperammonemia, or a combination of metabolic acidosis and hyperammonemia. The analysis of these blood and urine test results in conjunction with the patient's clinical presentation aids in narrowing down potential subsets of metabolic disorders. Specific clinical scenarios can also heighten suspicion of an underlying inborn error of metabolism (IEM), such as critically ill neonates experiencing deterioration post an uncomplicated pregnancy, episodes of illness with fluctuating symptoms like lethargy or neurological manifestations triggered by intercurrent illness or stress, multisystem involvement, failure to thrive, developmental delay, progressive neurological signs, or atypical neurological symptoms with or without psychological issues—especially in adults after ruling out other common causes. **[19].**

In the pursuit of diagnosing inherited metabolic disorders (IEM), while biochemical genetic and molecular genetic tests remain essential for confirmation, basic laboratory tests play a crucial role by providing initial clues to the underlying condition. In many cases, a comprehensive assessment of medical and family history, clinical symptoms, and basic laboratory investigations is sufficient to classify an IEM and initiate preliminary treatment, particularly in emergencies.

When there is a suspicion of an IEM, specific routine laboratory tests should always be considered:

- Complete blood count with differential count

- Liver function tests
- Renal function tests
- Blood Ammonia
- Blood gases
- Blood Anion gap
- Blood Glucose
- Blood Lactate
- Urine and blood ketones

The selection of appropriate laboratory investigations to investigate the possibility of an IEM begins by determining whether the condition is likely attributed to defects in small molecule metabolism (such as amino acid disorders, organic acid disorders, purine and pyrimidine disorders, urea cycle disorders, mitochondrial energy metabolism) or defects in organelle metabolism (such as lysosomal or peroxisomal disorders). Minor molecule disorders often present as acute illnesses requiring urgent intervention, whereas organelle metabolism disorders commonly manifest with neurological and neuromuscular symptoms, organomegaly, and hepatic dysfunction, with or without dysmorphism. However, it is essential to note that conditions affecting organelle function can also present with metabolic crises involving hypoglycemia or metabolic acidosis, requiring immediate intervention. Additionally, it is crucial to recognize that certain IEMs may offer without life-threatening metabolic problems but rather exhibit atypical symptoms such as skin blistering after sunlight exposure or neurological manifestations (e.g., cryptogenic abdominal pain, paresthesia or psychotic episodes), as seen in most porphyrias. Exceptions include congenital erythropoietic porphyria (CEP), which can lead to severe liver disease, and acute intermittent porphyria (AIP), which can result in severe lactic acidosis **[20].**

1.10 Basic Biochemical Laboratory Tests for IEM Study

Initial screening tests play a significant role in the evaluation of metabolic disorders. Although less commonly employed nowadays, these preliminary tests can provide valuable guidance for metabolic studies, particularly in emergencies.

1.10.1 Reactive Strips

Commercially available reagent strips offer a quick and qualitative analysis of various urine intermediates, including protein, blood, leukocytes, nitrite, glucose, ketones (acetoacetic acid), pH, specific gravity, creatinine, bilirubin, and urobilinogen. Other strips enable rapid determination of reducing substances such as glucose, lactose, fructose, pentoses, and sulfite. The interpretation of results can be done through visual observation.

1.10.2 Qualitative Tests

These assays complement the reactive strip tests and provide additional information regarding potential metabolic errors. Results from most qualitative tests can be interpreted visually, except for the Ehrlich test and Bratton-Marshall reaction, which require a spectrophotometer.

Number of manufacturers are developing different paper based commercial ready to use kits like-
urine reagent strips for urinalysis from a commercial manufacturer from Germany- Ulti Med Products (Deutschland) GmbH can able to detect multiple components from urine samples- glucose, bilirubin, ketone (acetoacetic acid), protein, urobilinogen, nitrite, leukocytes and ascorbic Acid.

Figure 1.15. Urine reagent strips **[21]**

Some companies are currently developing paper based kits to diagnosis infectious diseases like Mologic and Abingdon Health. Mologic is known for developing lateral flow assays for various applications, including detection of metabolites and biomarkers. Abingdon Health is specializes in the development and manufacture of lateral flow assay tests for fertility, health and well-being, infectious disease and drug testing

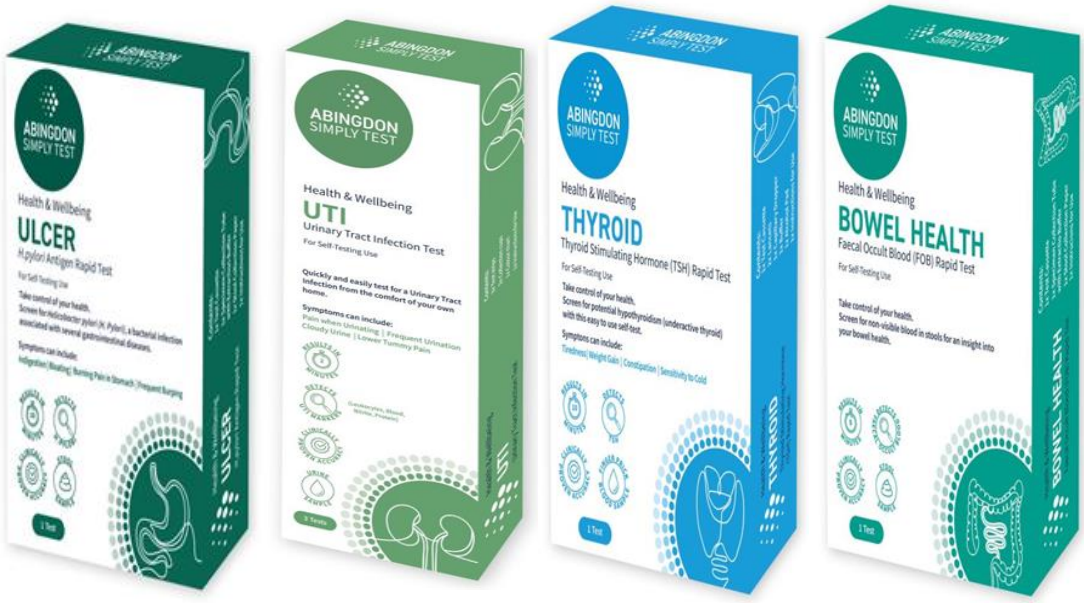


Figure 1.16. Ready to use kit for the detection of ulcer, urinary tract infection, thyroid and bowl health **[22]**

But most of the available kits are immunological and for infectious diseases; for assay purpose of blood metabolites liquid reaction based assay kits are available.

For example some standard screening tests include:

- Benedict test
- 2,4-Dinitrophenylhydrazine
- Nitroprusside test
- Nitroso naphthol test
- Ferric chloride test
- Ehrlich test
- Urine thiosulfate measurement

- Toluidine blue spot test
- Electrophoresis and thin-layer chromatography (TLC)
- Bratton-Marshall test

These screening tests, whether reactive strip tests or qualitative assays, serve as initial steps to help guide further metabolic investigations. They provide valuable insights into potential metabolic abnormalities and contribute to the diagnostic process in cases of suspected metabolic disorders **[23]**.

1.11 Techniques to Study Metabolic Disorders

1.11.1 Spectrophotofluorometric Studies

A primary objective of these studies is to assess the overall concentration of specific metabolites and measure the activity of particular enzymes. Several enzymatic activities, such as chitotriosidase, can serve as screening markers, as their increased levels in both plasma and dried blood spots (DBS) have been observed in various lysosomal storage disorders (LSDs). To ensure accurate interpretation of biochemical assays, creatinine, and protein determinations are typically performed as reference parameters. Other biochemical analyses, including organic acids, glycosaminoglycans (GAGs), sialic acid, and enzyme assays in tissues or leukocytes, often refer their results to creatinine or protein concentrations. Additionally, creatinine concentration is vital in investigating disorders related to creatine metabolism. By incorporating these measurements, comprehensive metabolic investigations strive to provide valuable insights into the specific metabolite concentrations and enzyme activities associated with various metabolic disorders.

1.11.2 Gas Chromatography and MS

Exploring the world of metabolic disorders, the organic acid assay, specifically the total ion chromatogram (TIC), emerges as an exceptionally informative tool. This assay can quickly investigate various inborn metabolic errors (IEM). By analyzing the organic acid profile, valuable insights can be gained, allowing for identifying specific products associated with enzyme deficiencies or detecting crucial "flags" that provide guidance for further investigations. Notably, the organic acid profiles may exhibit variations between clinically ill

and asymptomatic patients. As the realm of metabolic disorders expands, novel metabolites are being identified, prompting the revisiting of old profiles, even those obtained years earlier. Furthermore, the organic acid profile offers intriguing information concerning enriched diet patterns, drug usage, bacterial or plastic contaminants, and other relevant factors. Through the organic acid assay, researchers and clinicians delve into a wealth of metabolic data, unraveling crucial insights into the underlying mechanisms of various IEMs and their associated clinical manifestations.

1.11.3 Additional specific organic acid detection by GC and MS (simple ion monitoring, SIM)

Exploring the domain of metabolic analysis, mass spectrometry allows for a comprehensive assessment of organic acids. By configuring the mass spectrometer to target specific ions, metabolites in minute concentrations can be identified and quantified. Key metabolites that are typically examined include:

- Succinylacetone (associated with tyrosinemia type 1).
- Orotic acid (linked to urea cycle disorders).
- Guanidinoacetoacetate (indicative of defects in creatine metabolism).
- Methylmalonic acid (characteristic of methylmalonic acidemia and disorders of cobalamin metabolism).
- Mevalonic acid (associated with mevalonic aciduria).
- Acetyl-N-aspartate (indicative of Canavan disease).

In addition to organic acid analysis, evaluating very long-chain fatty acids is easily accomplished through gas chromatography-mass spectrometry (GC/MS). This methodology enables screening of saturated and unsaturated fatty acids, plasmalogens, pristane, and phytanate. Furthermore, the profiling of sterols is performed via GC-MS, utilizing total ion chromatogram (TIC) and selected ion monitoring (SIM) modes. The comprehensive analysis of sterols allows for identifying various pathologies, especially those associated with cholesterol synthesis pathways before or after squalene.

To ensure smooth laboratory operations, skilled technicians proficient in sample preparation, assay performance, and instrument operation are crucial. Implementing micro techniques,

such as multi wheel plate systems or microfluid-based enzyme assays, is recommended, promoting efficient use of resources while enhancing analytical capabilities. Automation tools like autosamplers for GC/MS or other robotic devices contribute to cost savings and increase overall efficiency. Well-organized laboratories optimize time, human resources, and chemical supplies. The expertise of professionals well-versed in inherited metabolic diseases is paramount in evaluating results, confirming positive cases, and promptly initiating appropriate patient management and therapy.

Establishing a metabolic analysis laboratory is relatively cost-effective, particularly compared to the expense associated with sophisticated equipment like MS/MS. This affordability is especially significant in areas where specialized centers for these studies may be lacking. Once the basic laboratory infrastructure is in place, expansion of the pathology investigations becomes feasible, incorporating additional equipment such as amino acid analyzers, high-performance liquid chromatography (HPLC), MS/MS instruments, and tools for molecular genetic testing and cell culture. Consideration of local or regional incidence of IEM and the availability of therapies is vital in planning and executing such expansion endeavors **[24].**

1.12 Importance of Early Diagnosis

The manifestation of clinical symptoms in inherited metabolic disorders (IEM) exhibits considerable variability, both among different IEMs and within variant forms of the same disorder. The timing of symptom presentation is influenced by the accumulation of toxic metabolites or the deficiency of essential substrates. Disorders related to carbohydrate or protein metabolism and disturbances in energy production typically emerge during the neonatal period or early infancy, displaying an unrelenting and rapidly progressive course.

On the other hand, milder variants of these diseases tend to present later in infancy or childhood, often characterized by episodic episodes. In contrast, fatty acid oxidation disorders, glycogen storage disorders, and lysosomal storage disorders generally have a subtle and insidious onset during infancy or childhood. IEMs that primarily manifest with fine neurologic or psychiatric features often remain undiagnosed until adulthood, further highlighting these disorders' complexity and diverse clinical presentations **[25].**

Several important factors come into play when considering the implementation of screening programs for disorders. These considerations help assess the feasibility and effectiveness of screening efforts. They include:

1. Identifying diseases that may not be clinically evident at birth makes early detection through screening crucial for timely intervention.
2. Evaluating the frequency of the disorder within the population to determine the potential impact and benefits of implementing a screening program.
3. Recognizing the potential for irreversible harm or adverse outcomes if there is a delay in diagnosing the condition emphasizes the need for early detection through screening.
4. Assessing the availability of a straightforward and reasonably reliable test that can be utilized for screening purposes.
5. Weighing the presence of effective treatments or interventions that can significantly improve outcomes if the disease is detected early through screening.

Considering these factors helps guide decision-making processes regarding the implementation of screening programs, ensuring that the chosen disorders are suitable for screening based on their clinical impact, prevalence, potential harm, and the availability of effective interventions **[26].**

Screening plays a fundamental role in diagnosing suspected cases of inborn metabolic diseases, employing simple, cost-effective techniques such as paper chromatography, thin-layer chromatography (TLC), and specific biochemical tests. However, it is essential to note that a definitive diagnosis should not be solely based on the results of screening tests. For comprehensive analysis and quantification of amino acids, organic acids, and metabolites in biological fluids, high-performance liquid chromatography (HPLC) is considered a reliable technique.

To diagnose metabolic disorders, advanced methods like TMS and GC/MS are used. These sophisticated techniques provide enhanced sensitivity and specificity in identifying metabolic abnormalities. One such valuable marker is the measurement of acylcarnitines in blood, which reflects the accumulation of primary mitochondrial acyl-CoA metabolites in disorders related to fatty acid and amino acid catabolism. By utilizing advanced technologies like TMS,

an acylcarnitine "profile" can effectively identify the majority of defects in these metabolic pathways, aiding in accurate diagnosis and subsequent management of patients with inborn metabolic disorders. With the advancement of chemical diagnostic techniques, gas chromatography/mass spectrometry (GC/MS) has emerged as a crucial component of routine diagnostic services. This powerful analytical tool, in combination with an amino acid analyzer, has enabled the diagnosis of approximately 115 inherited organic and amino acid disorders. Since its inception in 1978, this methodology has identified over 910 cases and 80 inherited amino acid metabolic diseases. Among these disorders, lactic acidosis has been the most commonly detected inborn organic metabolic error, accounting for approximately 40% of the total cases. This highlights the significant contribution of GC/MS in unraveling the complexities of metabolic disorders and aiding in their accurate diagnosis **[12].**

1.12.1 Importance of Easy-to-Use Kits for IEM Detection and Monitor

The need for laboratories dedicated to the comprehensive study of inborn errors of metabolism (IEM) poses a significant challenge worldwide. The limited availability of such specialized facilities hampers the efficient detection and treatment of affected individuals. As a consequence, many patients remain undiagnosed, or their conditions are identified at a stage when optimal intervention becomes challenging. Compounding the issue, transporting biological samples over long distances to reach suitable laboratories introduces additional delays and potential degradation of specimens, further prolonging the diagnostic process. To address these limitations, there is a pressing need to establish more laboratories equipped to perform IEM screening and diagnosis. Increasing the number of specialized centers would significantly enhance the accessibility of diagnostic services and facilitate timely interventions for patients. By decentralizing these facilities, individuals residing in distant areas could benefit from quicker and more efficient diagnoses, thereby minimizing the risk of prolonged suffering and adverse health outcomes. Efforts should be directed towards expanding laboratory infrastructure and expertise in IEM, fostering collaboration between healthcare institutions, and promoting knowledge-sharing among professionals. Establishing regional centers with well-trained personnel and state-of-the-art technologies would enable accurate and timely identification of metabolic disorders, ensuring prompt initiation of appropriate therapies. By addressing these challenges and improving the availability and

accessibility of specialized laboratories, we can significantly enhance the outcomes and quality of life for individuals affected by IEM worldwide **[24].**

1.12.2 Benefits of Easy-to-Use Kits

When considering the implementation of a screening test, several key factors come into play. First and foremost, the test should be cost-effective, ensuring that it is affordable and within the budgetary constraints of a developing country. This is particularly important as limited economic resources must be strategically allocated to address various social and health challenges, including but not limited to malnutrition, improving access to quality public services, enhancing primary education, tackling unemployment, and managing international external debts. Consequently, diseases related to inborn errors of metabolism (IEM) may not always receive the necessary prioritization. Furthermore, the geographical location of a country can heavily influence its health priorities. In regions where infectious diseases pose significant threats to the population, the allocation of resources and attention toward combating these diseases becomes a primary concern. The urgent need to prevent, control, and treat infectious illnesses often precede the less prevalent IEM-related disorders. However, despite these challenges, it is crucial to recognize the long-term benefits of implementing effective screening programs for IEM. By identifying and addressing these disorders early on, significant health and economic burdens can be alleviated. Investing in developing and utilizing screening tests that are both sensitive and specific will enable the timely detection of individuals at risk of IEM, allowing for appropriate interventions and management strategies to be implemented. Additionally, raising awareness among policymakers, healthcare providers, and the general public about the importance of screening for IEM can help advocate for the necessary resources and support to prioritize these conditions alongside other health challenges.

1.13 Importance of Studying the Amino Acidopathies and Carbohydrate-Related Syndromes

The essential roles of amino acids in our body are diverse and vital. Acting as fundamental building blocks of proteins, amino acids contribute to the structural integrity of various tissues. Additionally, they serve as precursors for synthesizing crucial molecules such as

neurotransmitters, porphyrins, and nitric oxide. Moreover, dietary proteins provide amino acids that can help as an energy source through their catabolism, generating organic acids that contribute to replenishing the Krebs cycle and ammonia, which is eliminated through the urea cycle. However, disruptions in the intricate pathways responsible for amino acid metabolism can lead to a group of disorders known as amino acid disorders or amino acidopathies. These conditions arise from inherited defects that impair the proper functioning of these metabolic pathways.

Consequently, the normal metabolism of amino acids is disrupted, leading to the accumulation of toxic substances or the deficiency of essential molecules. Amino acid disorders encompass various genetic conditions, each characterized by a specific defect in amino acid metabolism. These disorders can affect various aspects of amino acid processing, including their uptake, synthesis, degradation, and conversion to other molecules. The resulting imbalances in amino acid levels and the accumulation of toxic intermediates can profoundly affect overall health and development. It's important to note that the symptoms and severity of amino acid disorders vary depending on the specific metabolic pathway affected and the degree of impairment. Some diseases may present in early infancy with acute symptoms, while others may manifest later in life with milder or more intermittent symptoms. Prompt diagnosis and appropriate management of these disorders are essential to prevent or minimize the potential long-term complications associated with amino acid imbalances **[27].**

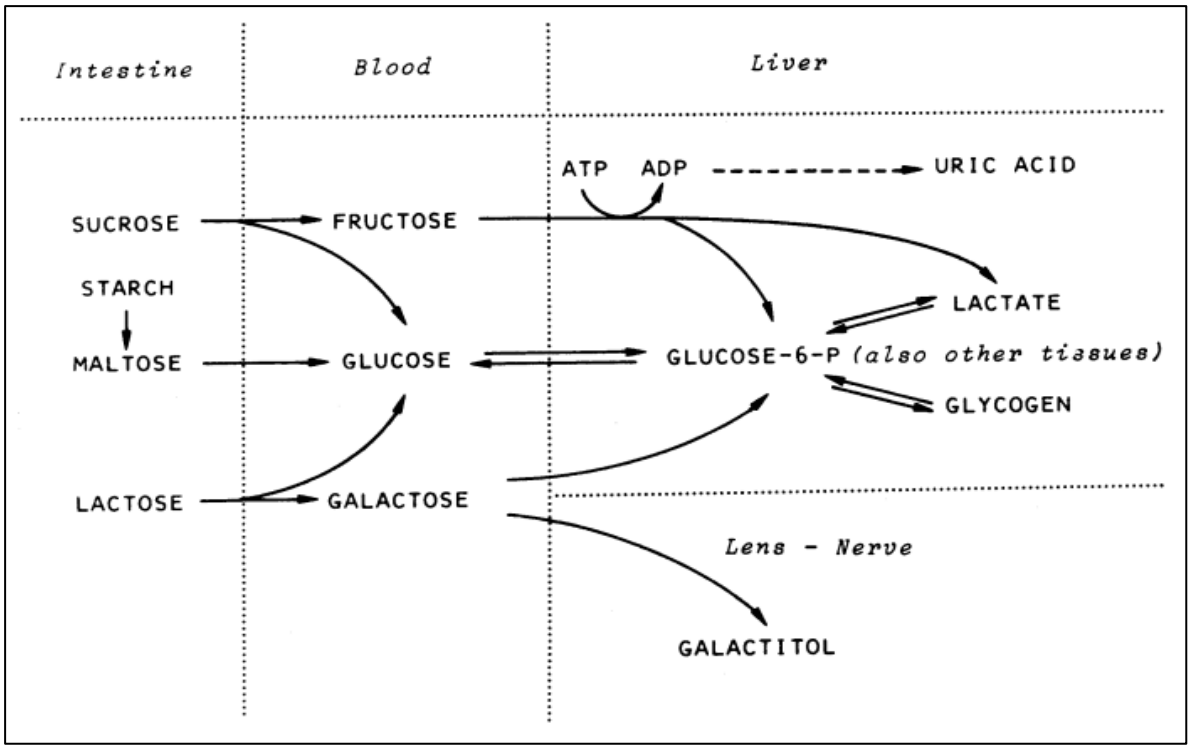


Figure 1.17. Carbohydrate metabolism and regulation **[28]**

Protein and carbohydrates are essential nutrients and energy sources derived from dietary intake. These nutrients are crucial in supporting growth and overall health, particularly during early developmental phases. Proteins are metabolized into amino acids, while carbohydrates are broken down into fructose/glucose, ultimately entering the "Krebs Cycle" to produce energy. In early childhood, lactose and lactose-containing foods are the primary dietary sources of carbohydrates for infants and children. The breakdown of lactose yields glucose and galactose, facilitating normal metabolism. However, inherited metabolic disorders can disrupt the enzymatic conversion process, leading to lactose intolerance or galactosemia conditions.

Similarly, during amino acid metabolism, any hereditary enzymatic disorder can result in the excessive accumulation of amino acids or their derivatives. Although proteins and carbohydrates are crucial during early development, excess amino acids or galactose accumulation can hinder optimal mental and physical growth. Therefore, early diagnosis plays a vital role in identifying the presence of excessive amounts of these nutrients due to inherited metabolic disorders. The progressive developmental failure associated with these disorders can be mitigated through dietary management. By detecting and addressing the

imbalances caused by inherited metabolic disorders early, healthcare professionals can implement appropriate dietary interventions to ensure proper nutrient utilization and support optimal growth and development.

1.14 Types of Amino Acid Disorders

1.14.1 Phenylketonuria

Phenylketonuria (PKU) is a genetic disorder in many foods when the body cannot break down phenylalanine, an amino acid. It affects approximately 1 in 13,500-19,000 births in the United States. The phenylalanine hydroxylase is crucial in converting phenylalanine to tyrosine, an amino acid necessary for various physiological processes. PKU is categorized into different phenotypes based on the levels of phenylalanine in the plasma. Severe PKU is characterized by phenylalanine levels exceeding 1200 μmol/L, while mild PKU ranges from 600 to 1200 μmol/L. Hyperphenylalaninemia refers to phenylalanine levels above the normal cutoff but below 600 μmol/L. Clinically, PKU can present with various symptoms, including growth failure, global developmental delay, severe intellectual disabilities, and neurological complications. Elevated phenylalanine levels during pregnancy can have teratogenic effects on the developing fetus, leading to developmental abnormalities and cognitive impairments. This condition is known as maternal PKU. Notably, phenylalanine accumulation can also occur in disorders affecting the biosynthesis and regeneration of the biopterin cofactor, which is essential for the function of phenylalanine hydroxylase. The management of PKU primarily focuses on nutritional interventions to prevent excessive phenylalanine accumulation. This involves restricting natural protein intake while ensuring an adequate intake of other essential nutrients. Phenylalanine-free protein substitutes are used to provide the necessary amino acids without contributing to phenylalanine levels. Early and consistent management of PKU is crucial to prevent the long-term complications associated with phenylalanine accumulation and to support optimal growth and development. Regularly monitoring phenylalanine levels and adherence to dietary restrictions are essential components of the treatment approach for individuals with PKU **[29, 30]**.

1.14.2 Disorders of tyrosine metabolism

The tyrosine metabolic pathway is a series of enzymatic reactions primarily occurring in hepatocytes and renal proximal tubules. Tyrosinemia I, the most severe inherited disorder of tyrosine metabolism, is caused by a deficiency of fumarylacetoacetate hydrolase, the final enzyme in the tyrosine catabolic pathway. This disorder has a high incidence in the French Canadian ethnicity and is associated with hepatorenal dysfunction. A deficiency of hepatic tyrosine aminotransferase characterizes Tyrosinemia II and presents with symptoms including mental retardation and other severe manifestations. Tyrosinemia III, a rare disorder, results from a deficiency in the activity of 4-hydroxyphenylpyruvate dioxygenase and is characterized by mild mental retardation or convulsions. All three disorders are biochemically identified by elevated plasma tyrosine levels (hypertyrosinemia) and the excretion of downstream tyrosine metabolites in urine. Additionally, elevated plasma tyrosine can be observed in vitamin-C-responsive transient tyrosinemia cases during the neonatal period. These disorders highlight the importance of proper tyrosine metabolism and the significance of biochemical markers in their diagnosis and management **[31, 32].**

1.14.3 Maple syrup urine disease (MSUD)

The metabolic disorder known as maple syrup urine disease (MSUD) results from a deficiency in the branched-chain α-keto acid dehydrogenase complex, which is responsible for the metabolism of branched-chain amino acids. This condition presents various clinical phenotypes based on the age of onset, symptom severity, and response to thiamine supplementation [9]. Biochemically, MSUD is characterized by elevated levels of branched-chain amino acids (leucine, isoleucine, valine, and allo-isoleucine) in the plasma, along with an abnormal ratio of these amino acids (valine: isoleucine: leucine/3.5:1:2 is the normal ratio). Dietary management involves restricting leucine intake and monitoring all branched-chain amino acids and allo-isoleucine. The classic form of MSUD, associated with minimal or no residual enzyme activity, manifests with developmental and neurological delays, encephalopathy, feeding difficulties, and the distinctive odor of maple syrup in urine. Effective management strategies involving dietary interventions are crucial for individuals with MSUD to prevent complications and promote long-term health **[33]**.

1.14.4 Urea cycle illnesses

In the process of protein breakdown, the carbon skeleton of amino acids undergoes metabolism to produce gluconeogenic and/or ketogenic precursors, while the nitrogen group is converted to ammonia through deamination. To eliminate the toxic ammonia derived from amino acids and other metabolic sources, it enters the urea cycle and is converted to urea, a nontoxic and readily excreted substance. This vital cycle primarily occurs in the liver, and any deficiency in the enzymes or transporters involved can lead to the accumulation of ammonia (hyperammonemia), which exerts highly toxic effects on the central nervous system. It is estimated that 1 in every 8,000 individuals suffer from urea cycle disorders. Most urea cycle disorders follow an autosomal recessive inheritance pattern, except for ornithine-transcarbamylase deficiency (OTCD), which is X-linked. Plasma citrulline serves as a critical amino acid in the biochemical diagnosis of urea cycle defects, aiding in evaluating and identifying these metabolic disorders **[27].**

1.14.5 Nonketotic hyperglycemia

The glycine metabolism is disrupted in a severe disorder known as nonketotic hyperglycemia (NKH). Glycine is normally catabolized through the action of a four-peptide cleavage complex consisting of P-protein, T-protein, H-protein, and L-protein. These proteins play essential roles in various reactions involved in glycine metabolism. In NKH, the disorder is characterized by the elevation of glycine levels in plasma and cerebrospinal fluid (CSF). The severity of NKH often leads to a high mortality rate within the first few months of life or significant intellectual disabilities in surviving individuals **[27].**

1.14.6 Types of Carbohydrate Metabolism Disorders

Carbohydrate metabolism disorders encompass a range of inborn errors, with glycogen storage diseases (GSD) and disorders of galactose and fructose metabolism being the most prevalent. This comprehensive review focuses on the current understanding of the clinical symptoms, diagnostic approaches, and treatment strategies for these conditions. GSDs affecting the liver, such as type I, III, and IX, commonly present with hepatomegaly and

hypoglycemia. Diagnosis typically involves noninvasive methods like mutation analysis. For GSD I, the primary treatment involves a carbohydrate-balanced diet with frequent meals, nocturnal continuous tube feeding, or uncooked corn starch to prevent hypoglycemia. In cases of severe cirrhosis, liver transplantation might be an option. Many countries include Classical galactosemia in newborn screening programs and affected neonates can benefit from lactose-free infant formula. In the case of hereditary fructose intolerance, a strict fructose-restricted diet is essential for management **[34].**

Disorders in carbohydrate metabolism involve the impaired breakdown and utilization of carbohydrates in the body. An illustrative instance is lactose intolerance. Carbohydrates form a substantial part of our dietary intake and undergo transformation into Glucose, Galactose, and Fructose — three essential monosaccharides. Inborn errors of carbohydrate metabolism arise when the body fails to efficiently process and utilize these sugars, leading to a range of metabolic disorders.

1.14.7 Galactosemia

Classical Galactosemia stands out as the most prevalent monogenic disorder in newborns, a condition of carbohydrate metabolism with an incidence of 1 in 55,000 births. Stemming from transferase deficiency, it is attributed to mutations in the gene encoding galactose-1-phosphate uridyl transferase (GAL-1-P Uridyl Transferase). Notably, around 70% of Galactosemia-causing alleles exhibit a singular missense mutation in exon 6. The consequential reduction in GAL-1-P uridyltransferase activity hampers the efficient conversion of Galactose to Glucose. This inefficiency leads to the alternative metabolism of Galactose into Galactitol and Galactonate, resulting in the accumulation of Galactose and its metabolites in tissues.

Galactosemia, a disorder of Galactose metabolism, manifests with various signs and long-term disabilities. Common symptoms include failure to thrive, hepatic insufficiency, cataracts, and developmental delay. Long-term disabilities encompass poor growth, mental retardation, and ovarian failure in females. Screening for Galactosemia involves measuring GAL-1-P Uridyl Transferase activity. Early detection enables timely treatment, primarily focused on eliminating Galactose from the diet. Galactosemia can also result from gene mutations responsible for Galactokinase or Uridine Diphosphate Galactose-4-epimerase.

Classical Galactosemia is included in newborn screening programs in many countries. Lactose-free infant formula can be lifesaving for affected neonates, while hereditary fructose intolerance necessitates a strict fructose-restricted diet. A deficiency of Galactokinase primarily causes cataracts but does not lead to growth failure, mental retardation, or hepatic disease. Dietary reduction of Galactose serves as the treatment, though less severe than in classical Galactosemia. This deficiency can occur systemically or be limited to red blood cells and leukocytes **[35, 36]**.

1.14.8 Metabolic Condition Related to Fructose

Mutations in the gene encoding hepatic fructokinase lead to the most common autosomal recessive disorder associated with fructose metabolism. This enzyme is crucial in the first step of fructose metabolism, and its deactivation leads to asymptomatic fructosuria. Another autosomal recessive disorder is hereditary fructose intolerance (HFI), characterized by poor feeding, failure to thrive, hepatic and renal insufficiency, and potential fatality. HFI is caused by a deficiency of fructose1,6-bisphosphate aldolase in the liver, kidney cortex, and small intestine. Symptoms in HFI individuals are only triggered upon ingestion of fructose or sucrose. A hepatic fructose 1,6-bisphosphatase (FBPase) deficiency leads to impaired gluconeogenesis, hypoglycemia, and severe metabolic acidemia. However, with adequate support beyond childhood, affected individuals can achieve average growth and development. Managing fructose and sucrose intake is crucial to prevent complications associated with these disorders.

1.14.9 Metabolic Condition Related to Glucose

Abnormalities of glucose metabolism are the most common errors of carbohydrate metabolism. The causes are heterogeneous and include both environmental and genetic factors. Disorders associated with elevated levels of plasma glucose have been classified into three categories:

- Type-1 Diabetes Mellitus
- Type-2 Diabetes Mellitus
- Maturity-Onset Diabetes in Young (MODY)

Within the realm of diabetes mellitus (DM), the principal categorizations embodied by Type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM). T1DM unfolds predominantly in the formative years of children and adolescents, stemming from deficiencies in insulin secretion. Conversely, T2DM aligns itself with the demographic of middle-aged and elderly individuals, whose protracted exposure to hyperglycemia results from lifestyle and dietary choices detrimental to insulin function. The pathogenic trajectories of T1DM and T2DM diverge significantly, giving rise to distinctive origins, clinical presentations, and therapeutic paradigms for each variant. On a separate trajectory, maturity-onset diabetes of the young (MODY) emerges as a multifaceted condition marked by the early onset of diabetes that does not necessitate insulin dependence, typically identified before the age of 25. Its hereditary pattern manifests as autosomal dominance, distinctly deviating from the autoimmune involvement observed in T1DM. The intricate tapestry of MODY involves a myriad of genetic influences, notably including mutations in hepatocyte nuclear factor-1-alpha (HNF1A) and the glucokinase (GCK) gene, prevalent in 52 to 65 percent and 15 to 32 percent of MODY cases, respectively. **[37]**

1.14.10 Metabolic Condition Related to Lactose

The ability to metabolize glucose depends partly on the activity of an intestinal brush border enzyme called lactase-phlorizin hydrolase (LPH). In most mammals, LPH activity diminishes after infants are weaned from maternal milk. However, the persistence of intestinal LPH activity is shared on autosomal recessive traits in human populations with a frequency of 5% to 90%. The geographic distribution of lactase persistence is concordant with areas of high milk intake. The persistent ability for adults to use dairy products as a source of vitamin D may have had a selective advantage in this population. Lactase non-persistence is common in tropical and subtropical countries. Individuals with lactase non-persistency may experience nausea, bloating, and diarrhea after ingesting lactose. LPH is encoded by the lactase gene on chromosome 2.

1.14.11 Metabolic Condition Related to Glycogen

Carbohydrates are most commonly stored as glycogen in humans. Consequently, enzyme deficiencies that lead to impaired synthesis or degradation of glycogen are also considered

disorders of carbohydrate metabolism. The two organs most commonly affected are the liver and the skeletal muscle. Glycogen storage disorders that affect the liver typically cause hepatomegaly and hypoglycemia. Those that affect skeletal muscle cause exercise intolerance, progressive weakness and cramping **[34, 38].**

The relative incidence of carbohydrates disorder **[39, 40, 41]** -

1. GSD- 1:20,000 live births
2. Galactosemia- 1:16,000 live births
3. Fructose intolerance- 1:18,000 live births

1.15 The Significance of PKU and Galactosemia Research in a Global and Bangladesh Context

Inborn errors of metabolism affect approximately 1 in 800 live births, making them a significant health concern. Among these disorders, Phenylketonuria (PKU) is the most frequently observed amino acid metabolism disorder, with an incidence rate of 1 in 15,000. While these conditions typically manifest in infancy, they can also manifest later in adulthood. Galactosemia, another prominent disorder, exhibits molecular heterogeneity, with over 100 mutations identified in the GALT gene. However, the specific prevalence and mutation data for Galactosemia in Bangladesh is currently unavailable.

Percent	Frequency	Population
60-70	Q188R	America
62-64	Q188R	Germany
85	Q188R	Ireland
60	Q188R	Australia
46	Q188R	Czech
0	Q188R(2.0 N318D	**Japan**
40	N314D(2.7Q188R)	**India**
14.5	N314D(3.6Q188R)	**Pakistan**
----------	----------	----------Bangladesh ?

Table 1.1. GALT gene mutation in different population **[42]**

1.16 The Challenges of Current Diagnosis Process: Expense, Complexity, and Implementation in Rural and National Settings

In many developed countries, implementing national screening programs has proven invaluable in detecting metabolic disorders at an early stage. Liquid chromatography, often coupled with mass spectrometry, is commonly employed for mass-scale screening. The required testing methods and analysis time can vary based on the instrument and analyte type. The following information has been compiled to offer pertinent details on instrumentation and analysis time related to amino acid detection. Various analytical methods have been employed for amino acid analysis, each with its advantages and considerations. Based on the review outcome described in **[43]** different analytes, analysis times, and the corresponding instrumentation used, some facts about amino acid analysis are revealed. For the analysis of 23 amino acids, HPLC with fluorescence detection (FLD) and OPA derivatization is employed, requiring a total analysis time of 17 minutes.

On the other hand, the analysis of 38 amino acids using ion exchange coupled with LC-UV requires derivatization with ninhydrin reagent and an extensive analysis time of 115 minutes. Ion pair chromatography combined with LC/MS/MS allows for the analysis of 16 amino acids in 15 minutes. A similar ion pair approach is utilized to analyze 26 amino acids in plasma and 22 amino acids in urine using Ion Pair UHPLC, with a total analysis time of 30 minutes. HILIC LC/MS/MS enables the separation of isomers for 16 amino acids but requires a more extended analysis time of 60 minutes. Monolithic Silica - FLD, with derivatization using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), offers a fast method with a total analysis time of 10 minutes, though it is yet to be validated for biological samples. CE-MS enables the analysis of 32 amino acids without the need for derivatization; however, it does involve a more extended analysis time. UHPLC-UV&CD with derivatization using 4-fluoro-7-nitro-2, 1, 3-benzoxadiazole (NBD-F) offers a fast analysis time of 5.5 minutes, but its validation for biological samples is limited to food samples. Finally, GC/MS with derivatization using propyl chloroformate presents an alternative approach, but it requires different instrumentation and derivatization **[43].**

1.17 The rationale of the research work

Amino acid accumulation resulting from metabolic pathway-related disorders can harm an individual's physical and mental development. Early diagnosis is crucial in initiating timely treatment to prevent irreversible damage. While many developed countries have established national screening programs for the early detection of prevalent metabolic disorders, such programs are lacking in most Asian countries, including densely populated South Asian countries like Bangladesh. Implementing a comprehensive screening program would involve identifying suspected individuals and conducting specific tests for further identification. However, challenges arise due to the large population size, the complexity of diagnosis, and limited resources. Despite the benefits of early diagnosis in improving life expectancy and preventing neurological issues, current testing methods such as LC/MS, CE-MS, GC/MS, UHPLC-UV CD, and HPLC FLD pose financial constraints. The installation and maintenance of the necessary equipment are costly, and the required reagents and chemicals are expensive.

Moreover, skilled human resources are needed to operate and analyze the results. Additionally, the analysis time can vary significantly, ranging from 5 minutes to 115 minutes, and certain methods may not be validated for biological samples. Hence, in a developing country like Bangladesh, implementing such analysis methods in remote areas nationally presents significant challenges. Overcoming these challenges requires carefully considering financial resources, accessibility, and personnel training.

This study focused on developing an easily accessible and cost-effective method for detecting amino acids and carbohydrates, specifically galactose. The main objective is to deploy the technique in rural areas of Bangladesh where resources and infrastructure may be limited. This study chooses amino acids and galactose as key metabolites for analysis due to their abundance in daily food consumption. Amino acid metabolism disorders are among the most common metabolic diseases, and dietary management is crucial in treatment. Therefore, routine monitoring of specific amino acid levels in body fluids is essential.

Regarding carbohydrates, galactose was chosen to represent lactose, found in milk and milk-related dietary sources during early development. Galactose is converted into glucose in the lactose metabolic pathway. The choice of galactose over glucose is influenced by the widespread use of glucose measurement devices, such as glucometers, which are quick,

affordable, and accessible in our country. However, measuring increased levels of galactose, which typically occur in Galactosemia, requires using urinary or blood samples in laboratory diagnosis to assess the presence of enzymes needed for converting galactose into glucose and developing an easy-to-use method for detecting amino acids and galactose aimed to improve the diagnosis and monitoring of metabolic disorders in rural areas of Bangladesh, where access to specialized laboratory facilities may be limited.

1.18 The objectives

1. Development of a metabolic screening method using High-Performance Liquid Chromatography (HPLC) for the detection of amino acidopathies in infants. This method aims to provide a reliable and efficient approach to identifying and diagnosing prevalent amino acid disorders.
2. Creation of an easy-to-use kit for the detection of prevalent amino acid disorders. The aim is to create a simple and efficient diagnostic tool for healthcare professionals to assess amino acid metabolism issues in patients.
3. Genetic analysis of Galactosemia, a carbohydrate disorder. This objective involves studying the genetic components and variations associated with Galactosemia, explicitly focusing on the genes related to galactose metabolism. The goal is to deepen our comprehension of the disorder's molecular foundation.
4. Development of an easy-to-use diagnosis and monitoring kit for Galactosemia. This objective aims to create a practical and accessible tool for diagnosing and monitoring Galactosemia. The kit will provide healthcare professionals with a reliable method to detect and track galactose levels in blood samples, enabling effective management of this disorder.

By accomplishing these objectives, this study seeks to advance diagnostic and monitoring methods for amino acid disorders and Galactosemia, leading to improved early detection, treatment, and management of these conditions.

CHAPTER 2: MATERIALS & METHODS

2.1. Sample Collection

The study enrolled 100 healthy samples and 100 suspected cases of patients aged between 2 and 18. The participants were selected from the National Institute of Nuclear Medicine and Allied Sciences (NINMAS) clinical care settings and the Department of Endocrinology at Bangabandhu Sheikh Mujib Medical University (BSMMU) in Dhaka. Additionally, 14 suspected samples from the Institute for Developing Science and Health Initiatives (ideSHi) were explicitly obtained for GALT mutation study, as these samples exhibited high levels of reducing substrate (non-glucose).

2.1.1. Ethical Approval and Study Population

The study protocol was reviewed and approved by the National Ethics Review Committee (NERC) of the Bangladesh Medical Research Council (BMRC), Dhaka, Bangladesh (BMRC/NREC/2013-2016/990). Parents provided written consent prior to the event. Blood specimens from patients with IEMs and healthy participants were collected.

2.1.2. Inclusion Criteria

To be included in the study, participants had to exhibit various symptoms, including lethargy, irritation, poor feeding, tachypnea, seizures, persistent vomiting, toe-walking, unexplained developmental delay, abnormal movement, language retardation, a history of unexplained death of a previous sibling, positive family history of metabolic disorders, and parental consanguinity. These patients were also subjected to further investigations for signs of metabolic disorders, such as metabolic acidosis with an increased anion gap, persistent or recurrent hypoglycemia, hypotonia, hyperammonemia, splenomegaly, and abnormal imaging and electrophysiologic findings. **[44, 45, 46, 47].**

However, patients with the aforementioned symptoms who had a history of perinatal brain injury, central nervous system infection, or chromosomal abnormalities were excluded from

the study **[46] [47]**. Suspected patients with inherited metabolic disorders meeting the inclusion criteria were referred by pediatric clinicians from the National Institute of Neurosciences & Hospital, Bangabandhu Sheikh Mujib Medical University, and Dhaka Shishu Hospital in Bangladesh. Prior to enrollment, all patients underwent thorough examinations by expert clinicians. Consent was obtained from parents/legal guardians prior to participation.

2.1.3. Specimen Collection and Storage

Blood plasma samples were prepared from infants after collecting whole blood specimens after 4-hour fasting using the standard venipuncture method after drawing blood (3 mL) from study participants in Heparin containing vacutainer plastic tubes. The tubes were mixed several times by Up-down. Centrifuge the tube of plasma for 20 minutes at 2000g speed and 4-6°C temperature. Take out the supernatants (serum) and prepare 500 µL of aliquots of serum in 1.5 mL sterile tubes and stored tubes at -70°C until the analysis was performed.

2.2. Metabolic screening method development in HPLC

2.2.1. Materials

1. Sodium Phosphate Anhydrous
2. Sodium Tetraborate
3. Sodium Azide
4. Acetonitrile
5. Methanol
6. Sodium Tetraborate
7. 9-Fluorenylmethoxycarbonyl Chloride
8. O-Phthalaldehyde
9. Phosphoric Acid
10. 3-mercaptopropionic acid (3-MPA)
11. Water
12. Column: Acclaim RSLC 120 C18
13. Instrument: Dionex UHPLC system

2.2.2. Procedure

Before going to run the prepared samples in the instrument following reagents were prepared as per developed protocol-

2.2.3. Preparation of mobile phase A

Dibasic Sodium Phosphate anhydrous of 1.42 g, Sodium Tetraborate of 3.81g and Sodium Azide of 0.0325 g was taken in a 1000 mL volumetric flask. 600 mL of water added to dissolve and finally reached volume up to 1000 mL. Solution pH was adjusted to 7.8±0.01 with diluted Phosphoric Acid. Filtered through filter having a nominal pore size not greater than 0.2 µm. Finally, solution was degassed in an ultrasonic bath.

2.2.4. Preparation of mobile phase B

Acetonitrile, Methanol and water was mixed at a ratio of 45:45:10. Filtered through filter having a nominal pore size not greater than 0.2 µm. Finally degassed the mixture in an ultrasonic bath.

2.2.5. Derivatization reagents

2.2.5.1. Reagent A

0.1 M Borate Buffer, pH 9: 0.953 g of Sodium Tetra Borate was taken in a 25 mL volumetric flask, dissolved and reached volume up to the mark with water. pH was adjusted to 9 by diluted Hydrochloric Acid solution.

2.2.5.2. OPA solution (50mg/mL)

0.500 g of O-Phthalaldehyde was taken in a 10 mL volumetric flask. Then dissolved and reached volume up to the mark with Methanol.

2.2.5.3. Reagent C

0.025 g of 9-Fluorenylmethoxycarbonyl Chloride was taken in a 10 mL volumetric flask. Then dissolved and reached volume up to the mark with Acetonitrile.

2.2.5.4. Reagent B

0.8 mL 0.1 M borate buffer, 0.2 mL OPA solution and 20 µL MPA solution was taken in a 2 mL vial with slotted septum.

2.2.5.5. Reagent D

0.5 mL of 85% Phosphoric Acid was taken in a 50 mL volumetric flask and reached volume up to the mark with mobile phase A.

2.2.6. Preparation of standard solution

Reference standard of 18 amino acids (Glycine, Alanine, Tyrosine, Cystine, Isoleucine, Leucine, Valine, Methionine, Histidine, Threonine, Proline, Phenylalanine, Serine, Arginine, Aspartic acid, Glutamic acid, Lysine and Tryptophan) procured from Sigma; 10 mg were taken in a 250 mL volumetric flask and 150 mL of 0.1N Hydrochloric Acid added to dissolve. Finally, 0.1N Hydrochloric Acid was added to reach volume up to 250 mL and mixed well. Filtered through a syringe filter having a nominal pore size not greater than 0.2 µm, first 10 mL of filtrate was discarded. Finally, the standard solution was collected in a glass bottle.

2.2.7. Preparation of Sample Solution

Plasma sample (100 µL) was pipetted into 2.0 mL micro-centrifuge tube and 10 lL of DTT (for reduction of disulphide bonds and to release bound cysteine and homocysteine from proteins) was added to it. Then the solution was vortexed for 30 s. After that an equal volume (100 µL) of 10 % sulfosalicylic acid was added to precipitate the proteins, followed by vortex mixing for 2 min. Then the samples were centrifuged at 25,000 rpm for 10 min at 4°C yielding a clear supernatant ready to be derivatized **[48].**

2.2.8. Instrumentation and UHPLC Analysis

Instrument used for the method development purpose was (DIONEX – ULTIMATE 3000) equipped with quaternary gradient pump, Thermo Scientific Dionex ACC-3000 autosampler column compartment and diode array detector.

2.2.8.1. Chromatographic conditions

Column : Acclaim RSLC 120 C18, 2.2 µm, 100 mm x 2.1mm

Detector : 338 nm from 0.00 to 7.9 min and 263 nm from 7.9 to end.

Injection volume : 1 mL

Flow rate : 0.722 mL/min

Column temperature : 40 °C

Sample temperature : 25 °C

Run time : 15 minutes

Time(min)	Mobile phase A(% v/v)	Mobile phase B(% v/v)
0.000	95	5
0.210	95	5
7.210	47	53
7.800	0	100
9.420	0	100
9.530	95	5
12.00	95	5

Table 2.1. Gradient program for elution

1	Draw water (10 mL;Udp Draw)
2	Draw Air (1 mL;Udp Draw)
3	Draw 0.1M borate buffer pH9.0 (5 mL; Udp Draw)
4	Draw sample (1 mL; Udp Draw)
5	Draw Air (6 mL;Udp Draw)
6	Mix in needle 3x (6 mL; Udp Move Syringe Load and Unload)
7	Wait 15 s (Udp Mix Wait)
8	Needle Wash (100 mL; Udp Mix Needle Wash).
9	Draw OPA/MPA reagent with borate buffer pH9.0 (1 mL; Udp Draw)
10	Draw Air (7 mL;Udp Draw)
11	Mix in needle 6x (7 mL; Udp Move Syringe Load and Unload)
12	Wait 15 s (Udp Mix Wait)
13	Needle Wash (100 mL; Udp Mix Needle Wash)
14	Draw FMOC solution (1 mL;Udp Draw)
15	Draw Air (8 mL; Udp Draw)
16	Mix in needle 6x (8 mL; Udp Move Syringe Load and Unload)
17	Needle Wash (100 mL; Udp Mix Needle Wash)
18	Wait 15 s (Udp Mix Wait)
19	Draw injection diluent (3 mL; Udp Draw)
20	Draw Air (11 mL;Udp Draw)
21	Mix in needle 4x (11 mL; Udp Move Syringe Load and Unload)
22	Draw water (10 mL;Udp Draw)
23	Inject (Total derivatization mixture is injected; Udp Inject Marker)

Table 2.2. Injection Programming

2.2.8.2. Calculation

Calculate the amount of all amino acids in g per 100 mL by using the following equation:

$$\frac{\text{As}}{\text{Astd}} \times \text{Cstd} \times \frac{100\ \text{mL}}{\text{Vs}} \times 100\ \text{mL}$$

Where,

As = peak area of respective amino acid in sample solution

Astd = peak area of respective amino acid in standard solution

Vs = volume of sample (mL)

Cstd = conc. of respective amino acid in standard solution.

2.2.9. Method Validation

Validation of the method and performance of the DIONEX – ULTIMATE 3000 were done using three levels of reference standards (low, medium, and high). Reference standards were prepared using aforementioned process and performance of the method was evaluated in terms of intra-assay and inter-assay accuracy and precision, linearity, limit of detection (LOD) or functional sensitivity, limit of quantitation (LOQ), and recovery. The method validation analysis was done for Glycine, Alanine, Tyrosine, Cystine, Isoleucine, Leucine, Valine, Methionine, Histidine, Threonine, Phenylalanine, Serine, Arginine, Aspartic acid, Glutamic acid, Lysine and Tryptophan.

2.3. Phenylalanine Detection Kit Development

To develop a low costing and easy to use diagnosis kit to identify elevated phenylalanine in blood sample following reaction was adopted from **[49]** -

$$Phe + NAD^{+} \xrightarrow{PheDH} NADH + PhePyr + NH_4^{+}$$

$$NBT + NADH + mPMS \longrightarrow Formazan\ product + NAD^{+}$$

Enzyme solution was prepared with Phenylalanine dehydrogenase (Sigma), in water pH 7.0. $NAD^{+,}$ NBT (Nitro blue tetrazolium) and methoxyphenazine methosulfate (mPMS) was also prepared in pH 7.0 water.

2.3.1. Paper Based Kit Development

To transfer the chemical reaction on to paper, multiple types of paper were evaluated based on following criteria-

1. Fixation outcome
2. Reaction time
3. Reproducibility
4. Stability

To do the transformation of liquid assay in to paper, in this study following paper materials were evaluated first:

1. Nitrocellulose membrane 0.45 µm
2. Nylon 0.45 µm
3. Whatman paper
4. Glass fiber pad
5. Cellulose pad

Initially evaluation was made on Nitrocellulose, Nylon and Whatman paper sequentially to find out where fluidic flow is acceptable in terms colorimetric reaction with time.

2.3.2. Assessment of liquid flow on Paper

Sequentially analyzed buffer flow on different paper materials. Same length of different paper and glass material was prepared, soaked with buffer materials of 220 mM bis-tris propane buffer pH 9.3 then dried in drying chamber. Then NAD^+ and Phenylalanine Dehydrogenase (PheDH) combination applied on dried paper an absorbent paper attached in the edge of the paper .Finally combination of 220 mM bis-tris propane buffer pH 6.3, NBT, mPMS and substrate Phenylalanine applied on the sample pad and wicking process observed on different paper considering time.

2.3.3. Kit Assembly

Two glass fiber pads used to make the final assembly of the kit. Two pads were initially soaked with two different pH buffers (220 mM bis-tris propane buffer pH 9.3 and 220 mM bis-tris propane buffer pH 6.3). Enzyme containing pad kept in bottom position (Glass Pad-1) which was soaked with pH 9.3 buffer. Top position (Glass Pad-2) which was soaked with 6.3 buffer contained NBT and mPMS. After drying, Glass Pad-2 placed on Glass Pad-1 and stacked together to apply the substrate.

2.3.4. Phenylalanine Standard Solution Preparation

L-Phenylalanine standard was obtained from Sigma Aldrich, freshly dissolved in RO water to prepare standard solution and kept in room temperature before use.

2.3.5. Assessment of Kit Performance

After assembling the kit, phenylalanine substrate applied and check the appearance of color through reaction with time measurement.

2.3.5.1. Drying temperature and time impact assessment

Pads were soaked with two different pH buffer and dried in drying chamber at different temperature of 30ºC, 40º C and 60º C for 60, 120 and 180 min to check the drying efficiency and kit performance. It was found that drying at 60º C for 180 min give better result.

2.3.5.2. Enzyme Concentration Fixation

Variable enzyme concentration was tried to determine better color reaction with minimal amount of enzyme usage. Phenylalanine concentration was checked from (2mg/dL to 6 mg/dL) along with buffer solution used as blank.

2.3.5.3. Developed Kit Reproducibility Check

Kit performance was evaluated by using blank buffer, water and standard phenylalanine.

2.4. Metabolic disease (Galacetosemia) mutational analysis of Bangladeshi population

2.4.1. Primer Design for Mutational Analysis

Galactose-1-Phosphate Uridylyl Transferase (GALT) gene size is 4,010 bp consisting of total 11 exon. From literature study it was found that most of the mutational variabilities reside in between the region of exon 6 and exon 10. Based on that mutational hot spot region GALT gene following primers have been designed.

GALT 1F 5-CAGGAGGGAGTTGACTTGGTGT-3
GALT1R 5-GAGCCTGACTCTCACTGGAATCA-3
GALT 2F 5-GTCCTAACCAGTGAGCACTGGT-3
GALT2R 5-CTTGTGCAATGACTGGAGCA-3

2.4.2. DNA extraction

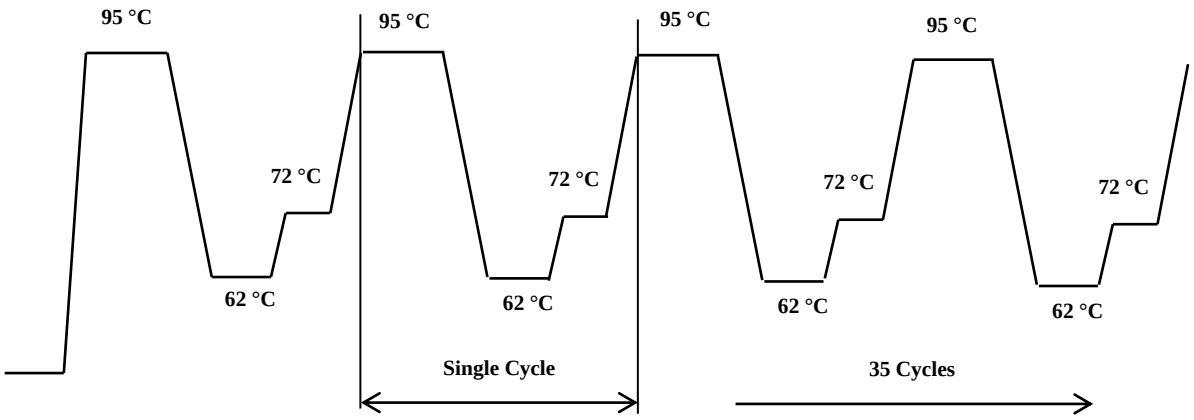
DNA extraction from 14 suspected patient samples have been done by using Flexigene DNA extraction kit (Qiagen). From each sample 100 µL blood taken, 750 µL FG1 kit buffer added, mixed by inversion 10 times, centrifuged for 30 sec at 10000xg, discarded the supernatant completely, 150 µL FG2 kit buffer added then vortexed immediately, spin down and placed in a heating block at 65° C for 5 minutes, 150 µL isopropanol (100%) added to precipitate DNA, finally centrifuged for 5 min at 10000xg. Supernatant was discarded and 150 µL ethanol (70%) added, vortexed immediately, then centrifuged for 5 minutes at 10000xg, supernatant discarded, dried the DNA pellet and finally dissolved in 50 µL dH_2O for PCR process development.

2.4.3. PCR method development and Optimization

DreamTaq PCR Master Mix (2X) was procured from Thermoscientific consisting of ready-to-use solution containing DreamTaq DNA Polymerase, optimized DreamTaq buffer, MgCl2, and dNTPs. Following reaction condition has been followed-

10X dream buffer	: 2.5µl
dNTP (10mM)	: 1.25µl
P-F (GALT 1_F)	: 0.5µl
P-R (GALT 1_R)	: 0.5µl
PCR-H20	: 19.05µl
Template	: 1µl
Dream Taq	: 0.2µl
Total	: 25µl

Following reaction program has been followed -



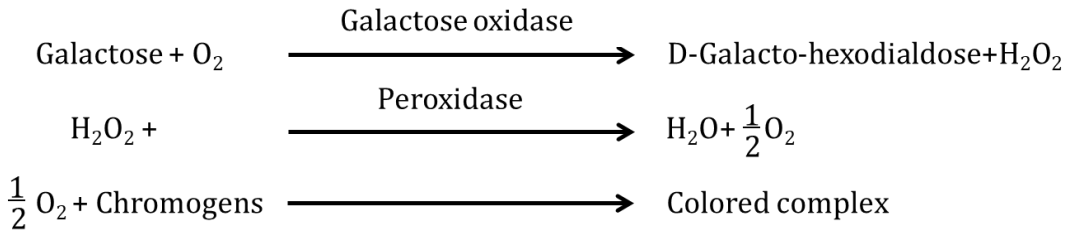
Elongation temperatures optimized considering temperature ranging from 55°-62° C.

2.4.4. Amplified GALT gene PCR product Sequencing

Optimized amplified product from PCR reaction was purified by PCR product purification kit (Qiagen). Big Dye X terminator kit from Applied Biosystem used for cycle sequencing purpose. After cycle sequencing products were purified and analyzed in 3130 Genetic Analyzer. Acquired sequenced data had been analyzed by splicing site comparison software (Splice Site Prediction by Neural Network).

2.5. Galactose Identification Kit Development from Blood

Following reaction was adopted to develop the method-



This methodology was followed to detect galactose in dairy milk **[50].**

Enzyme solution was prepared with Galactose oxidase (Sigma), horseradish peroxidase (Sigma) in phosphate buffer pH 7.0. For color reaction development, four chromogen solutions were prepared in phosphate buffer (pH 7.0) using individually ODN (o-dianisidine)/ABTS (2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)/OPD (o-Phenylene Diamine) /TMB (3,3', 5,5; -tetramethylbenzidine). Prepared solutions were checked and comparative analysis were done to identify better intensified color reaction. Absorbance spectrum of individual solutions had been checked by using scanning UV spectrometer (Schimadzu).

2.5.1. Different Substrate Color Reaction Time Measurement

Different chemical substrates color reaction change with time has been observed before transfer into paper-based kit generation process. - ABTS (2, 2- Azino-Bis-(3-ethylbenzThiazoline-6-Sulphonate), TMB (3, 3, 5, 5- Tetramethylbenzidine), OPD (O-Phenylene Diamine) and ODN (3, 3-Dimethoxybenzidine).

2.5.2. Enzyme Concentration Fixation

Variable enzyme concentration was tried to determine better color reaction with minimal amount of enzyme usage. Galactose concentration was checked from (5mg/mL to 50 mg/mL) along with buffer solution used as blank.

2.5.3. Galactose Standard Solution Preparation

D- (+)-Galactose standard was obtained from Sigma Aldrich, freshly dissolved in RO water to prepare standard solution and kept in room temperature before use.

2.5.4. Paper Based Kit Development

To transfer the chemical reaction on to paper, multiple types of paper was evaluated based on following criteria-

1. Fixation outcome
2. Reaction time
3. Flow rate time
4. Reproducibility
5. Stability

During the development process following types of paper was evaluated-

1. Nitrocellulose membrane 0.45 µm
2. Nitrocellulose membrane 135
3. Nylon 0.22 µm
4. Nylon 0.45 µm
5. Whatman paper

For the initial transfer process evaluation, manual dispensing of enzyme-chromogen solution was made on individual paper, cut into small pieces and dried in room temperature. Galactose standard solutions (5mg/mL) were tried on primary paper arrangement and evaluated the paper performance.

Following equipment and material used for the process-

1. Rapid test dispenser (HM3030)
2. Guillotine cutter (CT300)
3. Guillotine Cutter (ZQ2000)
4. Sample pad (Glass fiber)
5. Absorbent pad (Cellulose)

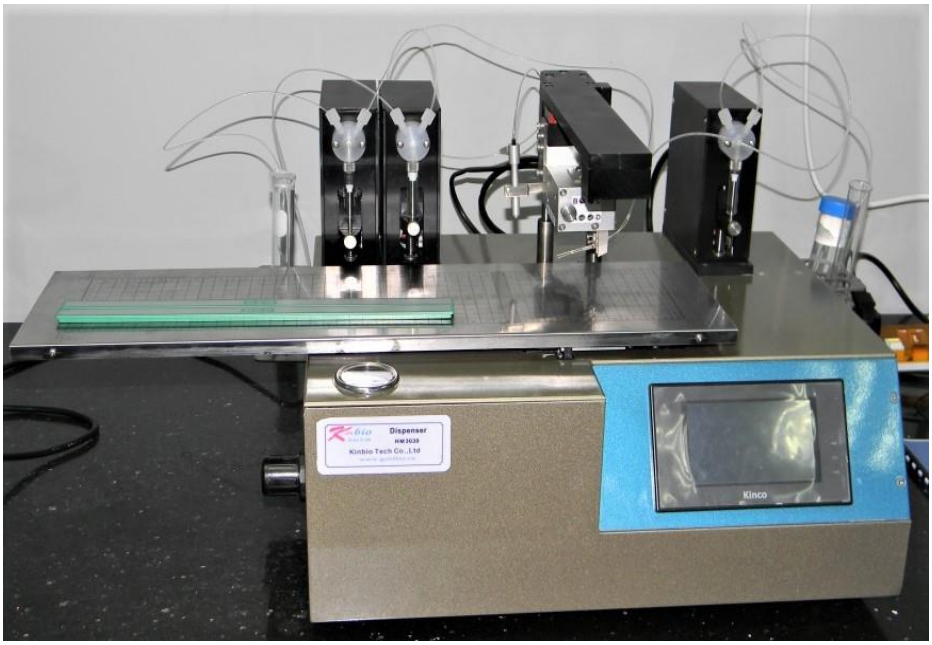


Figure 2.1. Rapid test Dispenser (HM3030)

Figure 2.2. Guillotine cutter (CT300 and ZQ2000)

Figure 2.3. Large cutter

For kit assembly purpose fabricated plastic device (curtesy: Incepta Pharmaceuticals Ltd) was used and evaluated the final selected paper with water, buffer solution, Galactose standard and human serum spiked with 5mg/mL standard Galactose.

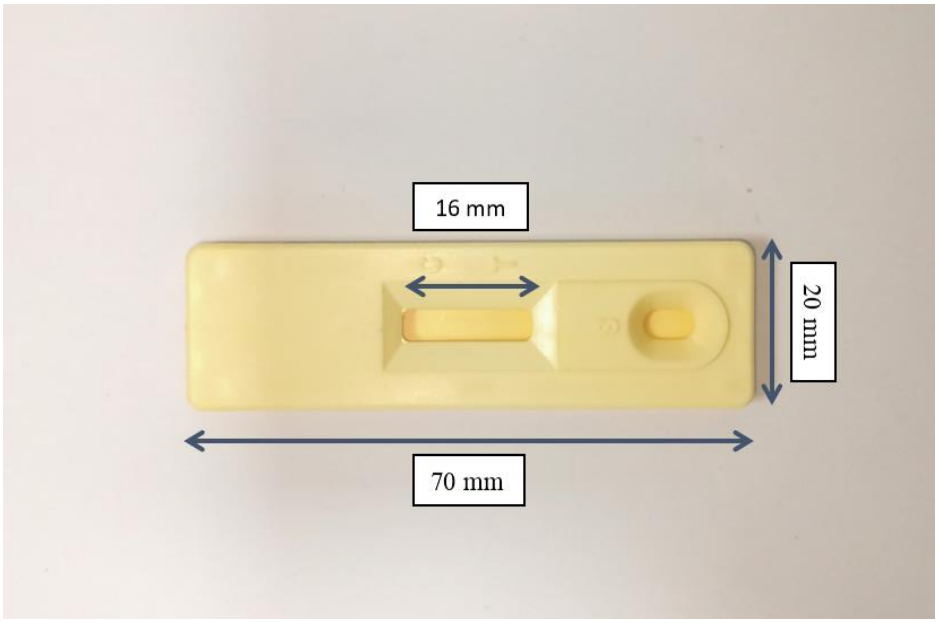


Figure 2.4. Plastic Cassette to hold kit

2.5.4.1. Control Line Setup

To develop a chemical-based control line in this study multiple chemical indicators were investigated. As chemical indicators change their appearance in different pH condition therefore, they would be a good candidate for test line, so that in neutral and in slight basic condition change of color can indicate the performance of the kit **[51]**. Following chemical indicators were investigated-

1. Bromothymol blue
2. Phenophthelin
3. Bromophenol blue

Basis of chemical selection was evaluated based on changing color in serum pH level. Final arrangement was made in fabricated plastic case and checks the performance with water, buffer and serum with spiked galactose standard.

2.5.4.2. Developed Kit Stability Evaluation

Stability of kit evaluation was conducted for both real time and accelerated condition.

CHAPTER 3: RESULTS

3.1 Method Standardization

To fix up the retention time standard amino acid mixture solution was run three times using the same methodology following same process condition. Following data processing the average retention time for each amino acid was measured. Consecutive run results and chromatogram have been given -

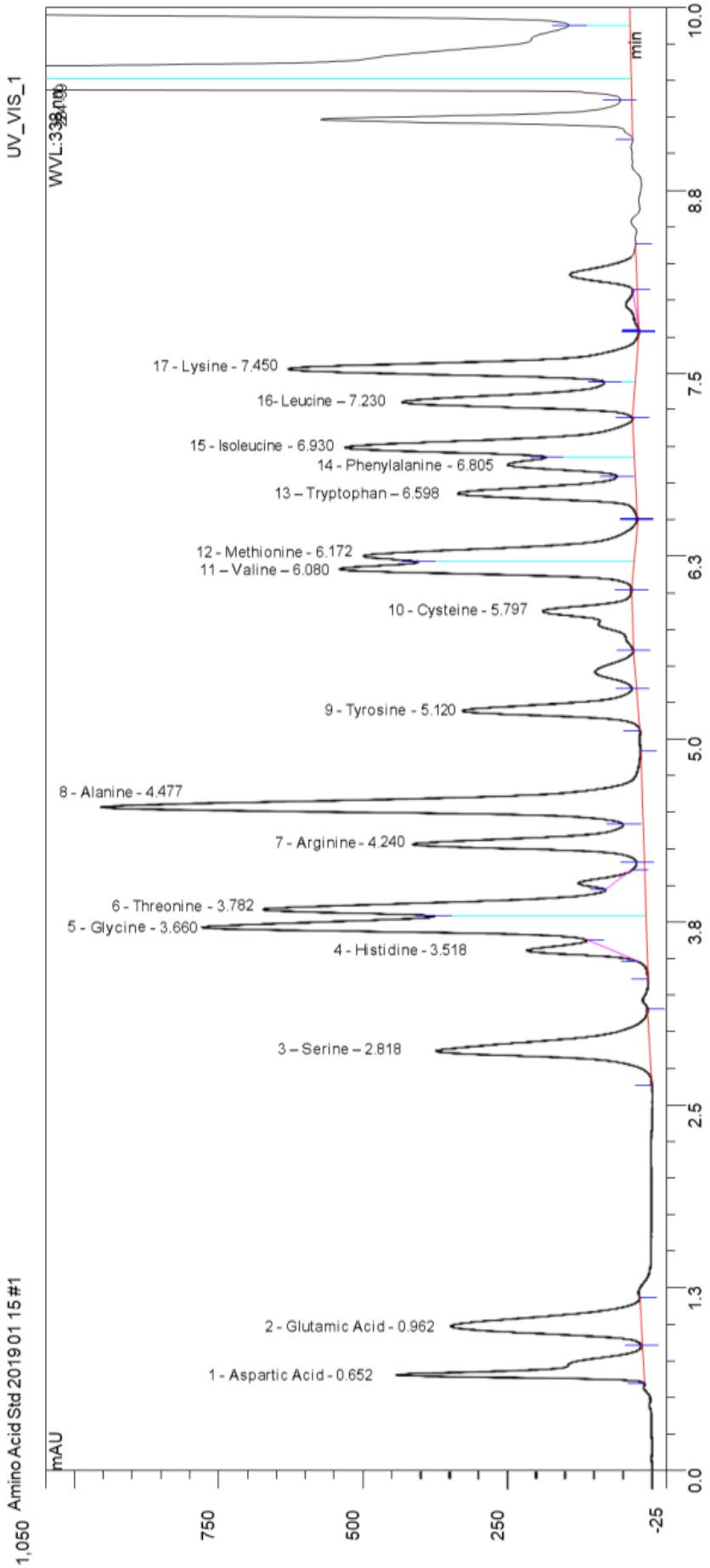


Figure 3.1. Standard Mix Run Chromatogram-1

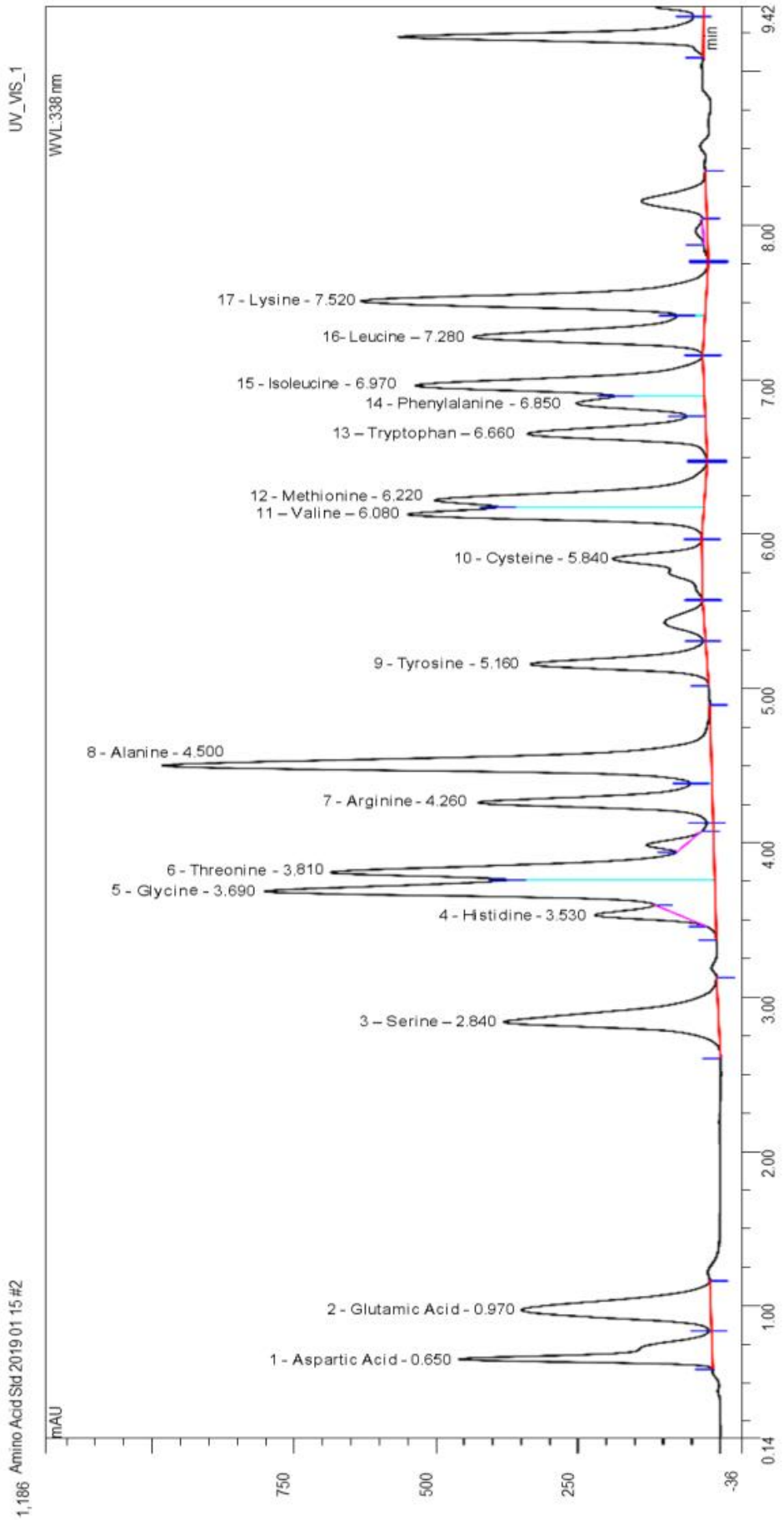


Figure 3.2. Standard Mix Run Chromatogram-2

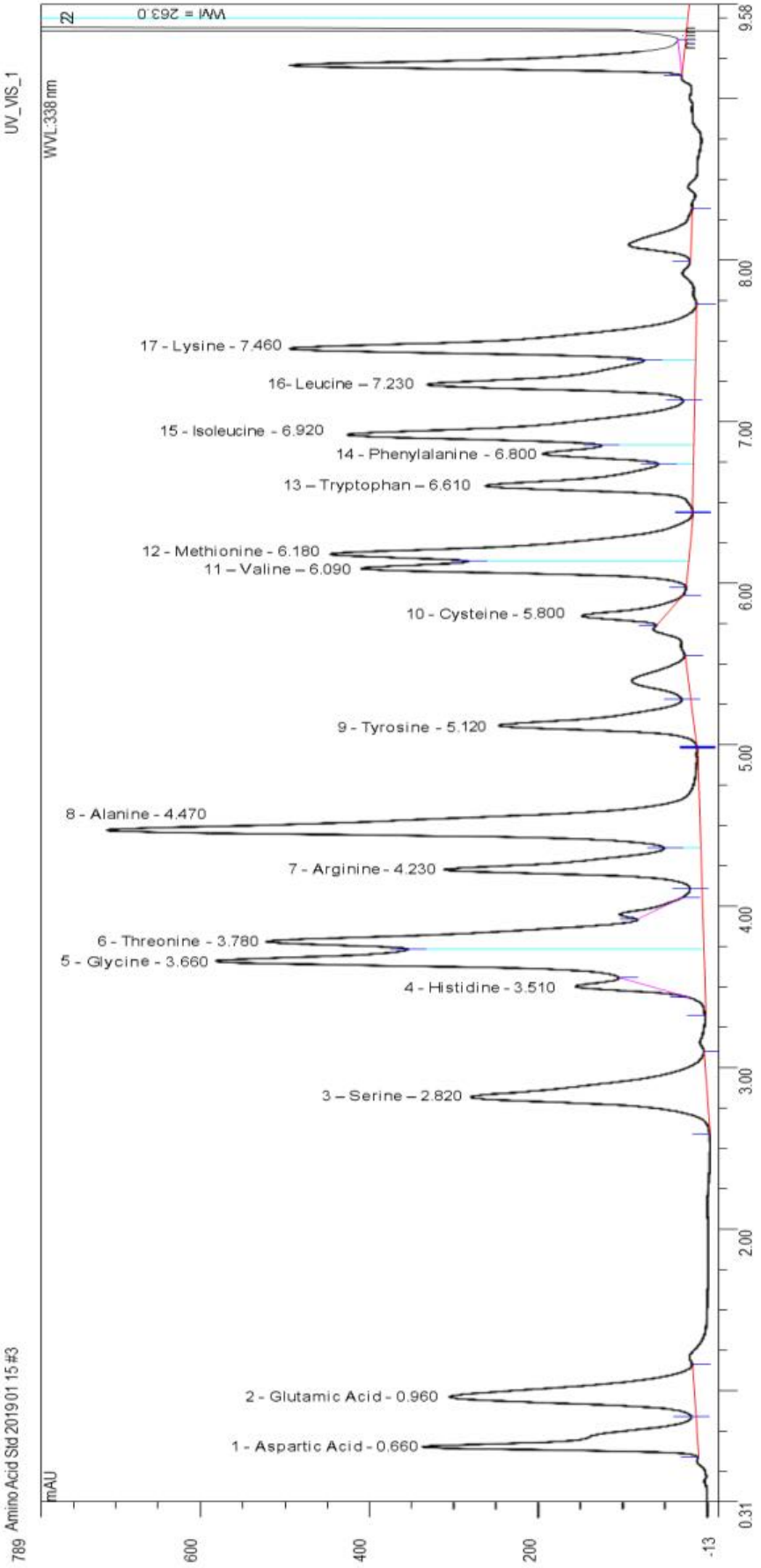


Figure 3.3. Standard Mix Run Chromatogram-3

3.1.1 Specificity Analysis

For specificity evaluation retention times of multiple consecutive standard sample run was measured and relative standard deviation (RSD) were calculated for each amino acid shown in Table-1 and RSD of individual amino acid shown in Figure 3.4.

Table 3.1. Relative Standard Deviation analysis of the standard amino acids

Sl. No.	Amino Acid	STD 1	STD 2	STD 3	Avg	RSD
1	Aspartic Acid	0.652	0.650	0.660	0.654	0.66%
2	Glutamic Acid	0.962	0.970	0.960	0.964	0.45%
3	Serine	2.818	2.840	2.820	2.826	0.35%
4	Histidine	3.518	3.530	3.510	3.519	0.23%
5	Glycine	3.660	3.690	3.660	3.670	0.39%
6	Threonine	3.782	3.810	3.780	3.791	0.36%
7	Arginine	4.240	4.260	4.230	4.243	0.29%
8	Alanine	4.477	4.500	4.470	4.482	0.29%
9	Tyrosine	5.120	5.160	5.120	5.133	0.37%
10	Cysteine	5.797	5.840	5.800	5.812	0.34%
11	Valine	6.080	6.130	6.090	6.100	0.35%
12	Methionine	6.172	6.220	6.180	6.191	0.34%
13	Tryptophan	6.598	6.660	6.610	6.623	0.41%
14	Phenyl Alanine	6.805	6.850	6.800	6.818	0.33%
15	Iso Leucine	6.930	6.970	6.920	6.940	0.31%
16	Leucine	7.230	7.280	7.230	7.247	0.33%
17	Lysine	7.450	7.520	7.460	7.477	0.41%

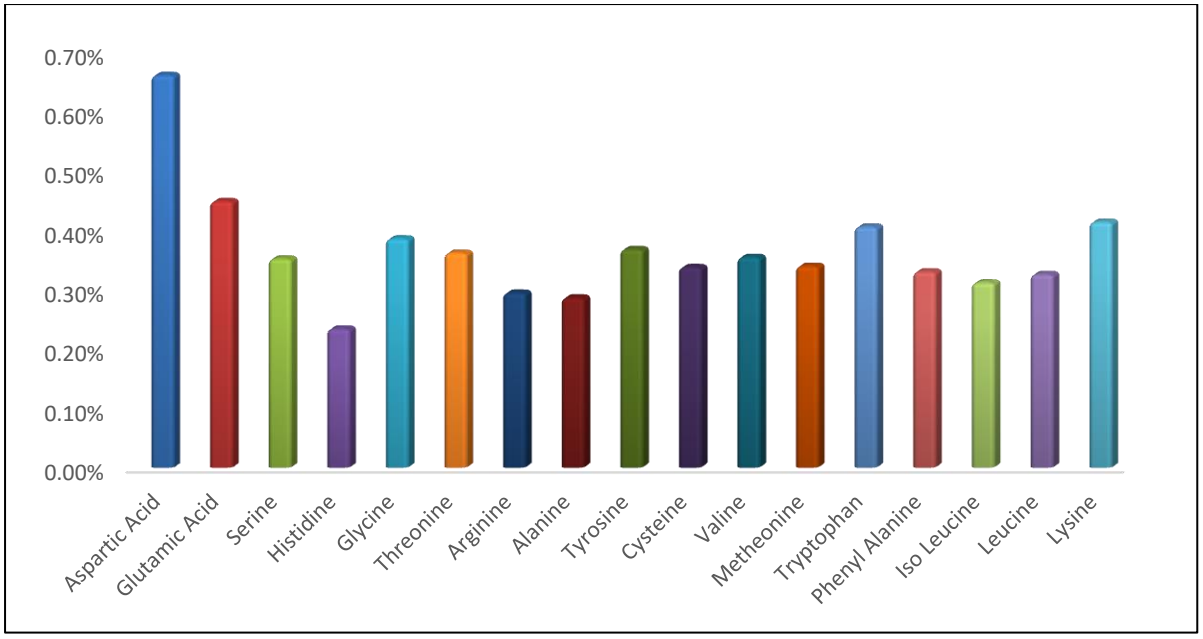


Figure 3.4. Relative standard deviation of standard amino acids in specificity analysis

The variances in average retention times between the commercial samples and the standard amino acid mixture are detailed in Table 3.2.

Table 3.2. Illustration of the disparities in average retention times between the commercial samples and the standard amino acid mixture

Sl. No	Amino Acid	STD Avg. R. Time	DMEM Avg. R. Time	Difference %
1	Aspartic Acid	0.66	0.55	-10.7
2	Glutamic Acid	0.97	0.97	0.0
3	Serine	2.84	2.83	-1.7
4	Histidine	3.53	3.54	0.3
5	Glycine	3.69	3.70	1.3
6	Threonine	3.81	3.81	-0.3
7	Arginine	4.26	4.26	-0.3
8	Alanine	4.50	4.62	12.0
9	Tyrosine	5.16	5.14	-2.3
10	Cysteine	5.84	5.74	-10.3
11	Valine	6.13	6.11	-2.0
12	Methionine	6.22	6.19	-2.7
13	Tryptophan	6.65	6.52	-13.0

14	Phenyl Alanine	6.84	6.82	-2.3
15	Iso Leucine	6.96	6.94	-2.7
16	Leucine	7.27	7.24	-3.3
17	Lysine	7.50	7.46	-4.3

3.1.2 Linearity analysis

The linearity was established using five standard solutions containing 10, 25, 100, 250 and 1000 nmol cm^{-3} of each amino acid. The data of peak area vs. amino acid concentration were treated by linear least squares regression analysis. The values of the slope, intercept and the coefficient of determination of the calibration curve for amino acids are given in Table 3.3.

Table 3.3. Linearity data analysis of the standard amino acids

Sl. No.	Amino Acid	Slope×10^3	Intercept×10^4	R^2
1	Aspartic Acid	1.574	0.6	0.9975
2	Glutamic Acid	0.508	5.05	0.9999
3	Serine	1.212	-0.12	0.9999
4	Histidine	1.132	-0.11	0.9989
5	Glycine	0.877	-0.13	0.9999
6	Threonine	1.395	-0.58	0.9999
7	Arginine	1.995	-1.04	0.9999
8	Alanine	1.006	-2.63	0.9999
9	Tyrosine	2.198	-0.87	0.9999
10	Cysteine	1.512	-0.63	0.9984
11	Valine	1.228	0.26	0.9999
12	Methionine	1.674	0.97	0.9999
13	Tryptophan	1.815	0.87	0.9988
14	Phenyl Alanine	1.946	12.35	0.9999
15	Iso Leucine	1.537	1.15	0.9999
16	Leucine	1.494	0.93	0.9999
17	Lysine	1.588	17.35	0.9999

3.1.3 Accuracy Analysis

Accuracy may be defined as the agreement between the found value and the true value of the reference material provided by a reference laboratory, and can be presented as the percent recovery. This validation parameter was estimated by analyzing the reference material of commercial media. The analysis underwent three repetitions on distinct days, encompassing the entire analytical process. Post-analysis, percent recovery was computed for each well-recovered amino acid, and the findings are detailed in Table 3.4. Notably, serine demonstrated the highest percent recovery (99.06%), with threonine reaching the peak (108.51%) and aspartic acid recording the lowest (90.92%) percent recovery. The overall average percent recovery, encompassing all amino acids, stood at 99.10%, falling within the acceptable range of 90-110%.

Table 3.4. Accuracy analysis of the method

Amino Acid	DMEM-1	DMEM-2	DMEM-3	DMEM Avg. (µmol/L)	Actual Conc. (µmol/L) [51]	Recovery (%)
	Conc. µmol/L	Conc. µmol/L	Conc. µmol/L			
Aspartic Acid	95.117	26.019	15.140	45.43	49.9624	90.92
Glutamic Acid	64.373	48.283	34.7273	49.13	49.9558	98.34
Serine	248.38	245.43	248.51	247.44	249.7859	99.06
Histidine	197.24	196.59	193.67	195.83	202.8873	96.52
Glycine	237.6	233.39	239.45	236.81	249.7669	94.81
Threonine	488.15	489.27	483.19	486.87	448.7072	108.51
Arginine	840.23	838.94	833.12	837.43	846.7279	98.90
Alanine	48.56	49.79	47.14	48.50	49.9495	97.09
Tyrosine	295.1	296.11	291.92	294.38	307.9088	95.61
Cysteine	257.18	258.19	235.44	250.27	258.2663	96.90
Valine	434.91	439.1	433.16	435.72	451.131	96.58
Methionine	118.54	120.1	119.58	119.41	115.5419	103.34

Tryptophan	47.59	49.61	45.32	47.51	44.1659	107.56
Phenyl Alanine	179.43	178.76	178.1	178.76	173.7257	102.90
Iso Leucine	418.24	402.29	406.32	408.95	415.231	98.49
Leucine	449.31	433.6	447.75	443.55	450.1448	98.54
Lysine	625.7	628.14	629.33	627.72	624.1877	100.57

3.1.4 Precision Analysis

In this work, the precision was estimated by measuring the repeatability and intermediate precision. In the case of repeatability, the same standard was derivatized and injected 6 times in a row and then the RSD was calculated for each amino acid. The obtained RSD values were in the range of 0.30% – 1.53 % (Table 3.5).

Table 3.5. Precision analysis of the method

Sl. No	**Amino Acid**	**1**	**2**	**3**	**4**	**5**	**6**	**Avg.**	**RSD**
1	Aspartic Acid	0.652	0.650	0.660	0.670	0.670	0.670	0.662	1.29%
2	Glutamic Acid	0.962	0.970	0.960	0.960	0.970	0.960	0.964	0.47%
3	Serine	2.818	2.840	2.820	2.760	2.770	2.730	2.790	1.39%
4	Histidine	3.518	3.530	3.510	3.490	3.490	3.470	3.501	0.57%
5	Glycine	3.660	3.690	3.660	3.630	3.630	3.730	3.667	0.95%
6	Threonine	3.782	3.810	3.780	3.750	3.750	3.920	3.799	1.53%
7	Arginine	4.240	4.260	4.230	4.230	4.230	4.220	4.235	0.30%
8	Alanine	4.477	4.500	4.470	4.440	4.440	4.420	4.458	0.60%
9	Tyrosine	5.120	5.160	5.120	5.090	5.090	5.080	5.110	0.53%

10	Cysteine	5.797	5.840	5.800	5.740	5.740	5.730	5.775	0.70%
11	Valine	6.080	6.130	6.090	6.010	6.020	6.010	6.057	0.76%
12	Methionine	6.172	6.220	6.180	6.110	6.110	6.100	6.149	0.73%
13	Tryptophan	6.598	6.660	6.610	6.540	6.540	6.530	6.580	0.72%
14	Phenyl Alanine	6.805	6.850	6.800	6.720	6.720	6.710	6.768	0.79%
15	Iso Leucine	6.930	6.970	6.920	6.830	6.830	6.820	6.883	0.85%
16	Leucine	7.230	7.280	7.230	7.130	7.130	7.120	7.187	0.87%
17	Lysine	7.450	7.520	7.460	7.370	7.370	7.360	7.422	0.80%

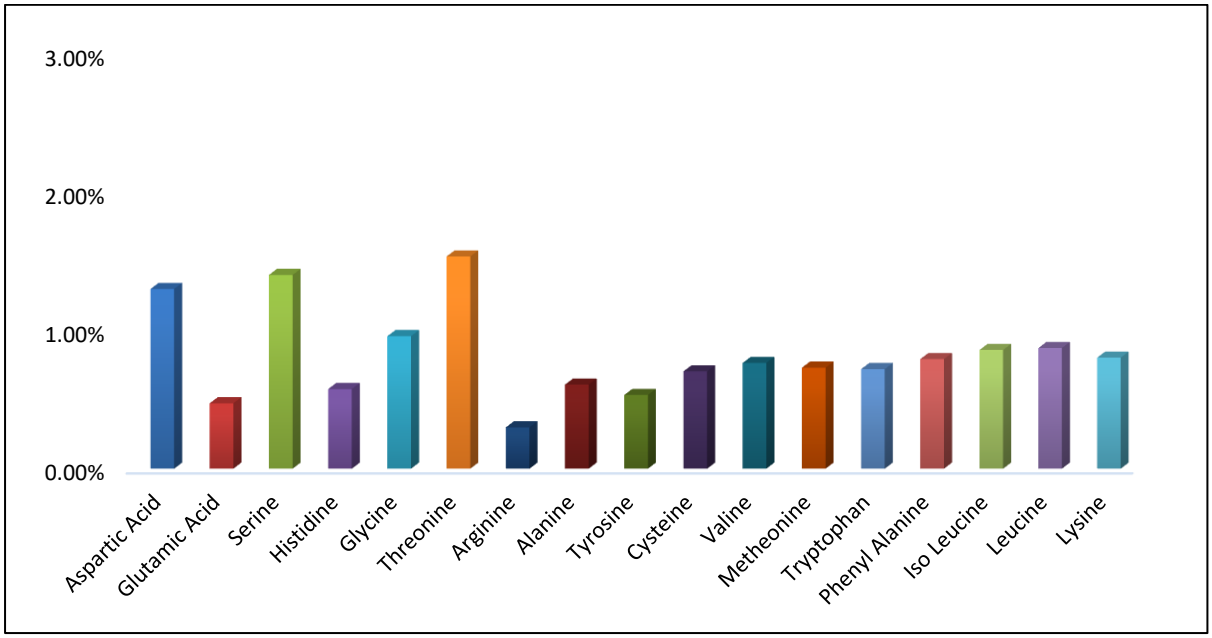


Figure 3.5. Relative standard deviation of the standard amino acids in precision analysis

3.1.5 Intermediate Precision Analysis

The intermediate precision was estimated by repeating the whole analytical procedure on three different days. The same standard sample was hydrolyzed three times, derivatized, and injected, separately on three different days. As can be seen from Table 6, the RSD was less than 0.7 %, and, being below 10 %.

Table 3.6. Intermediate precision RSD of the standard amino acids

Sl. No	Amino Acid	STD 1	STD 2	STD 3	Avg.	RSD
1	Aspartic Acid	0.652	0.650	0.660	0.654	0.66%
2	Glutamic Acid	0.962	0.970	0.960	0.964	0.45%
3	Serine	2.818	2.840	2.820	2.826	0.35%
4	Histidine	3.518	3.530	3.510	3.519	0.23%
5	Glycine	3.660	3.690	3.660	3.670	0.39%
6	Threonine	3.782	3.810	3.780	3.791	0.36%
7	Arginine	4.240	4.260	4.230	4.243	0.29%
8	Alanine	4.477	4.500	4.470	4.482	0.29%
9	Tyrosine	5.120	5.160	5.120	5.133	0.37%
10	Cysteine	5.797	5.840	5.800	5.812	0.34%
11	Valine	6.080	6.130	6.090	6.100	0.35%
12	Methionine	6.172	6.220	6.180	6.191	0.34%
13	Tryptophan	6.598	6.660	6.610	6.623	0.41%
14	Phenyl Alanine	6.805	6.850	6.800	6.818	0.33%
15	Iso Leucine	6.930	6.970	6.920	6.940	0.31%
16	Leucine	7.230	7.280	7.230	7.247	0.33%
17	Lysine	7.450	7.520	7.460	7.477	0.41%

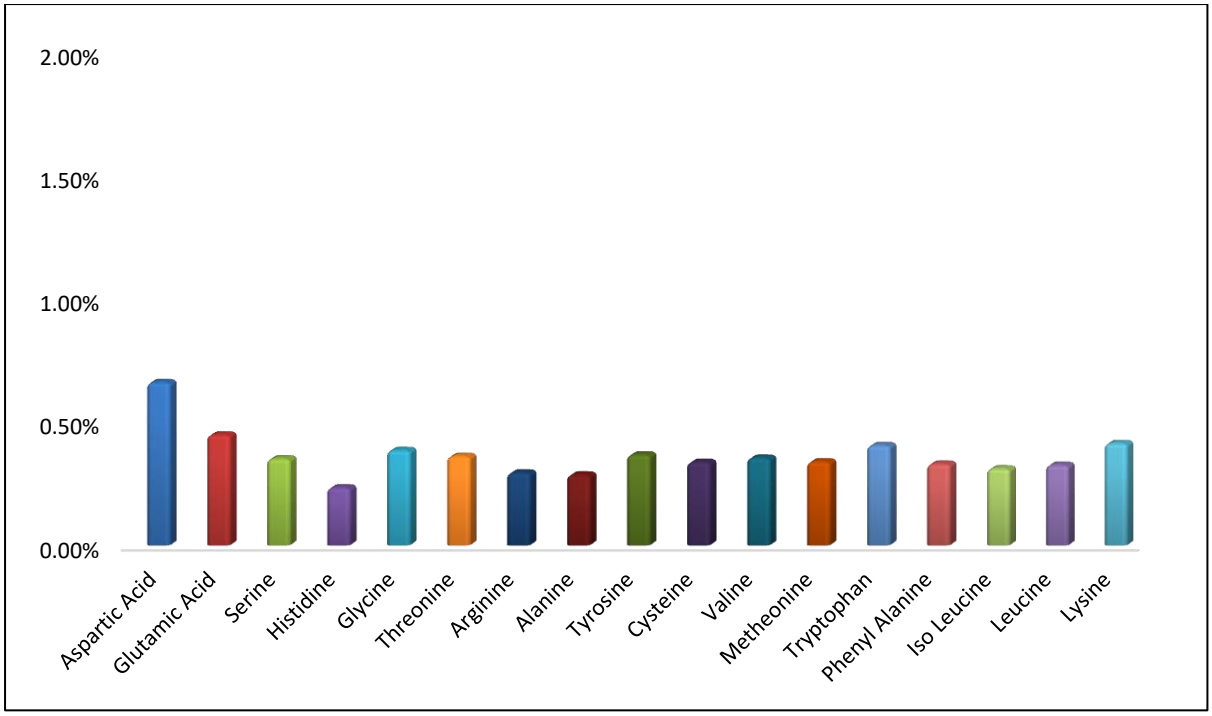


Figure 3.6. Interassay variability distribution of standard amino acids in intermediate precision analysis

3.1.6 Retention Time Order

Retention time order of the standard amino acids were fixed while method development.

Table 3.7. Retention time order

Sl. No	Amino Acid	R. Time
1	Aspartic Acid	0.66
2	Glutamic Acid	0.97
3	Serine	2.84
4	Histidine	3.53
5	Glycine	3.69
6	Threonine	3.81

7	Arginine	4.26
8	Alanine	4.50
9	Tyrosine	5.16
10	Cysteine	5.84
11	Valine	6.13
12	Methionine	6.22
13	Tryptophan	6.65
14	Phenyl Alanine	6.84
15	Iso Leucine	6.96
16	Leucine	7.27
17	Lysine	7.50

3.2 Healthy Range Generation

To fix the healthy range among Bangladeshi population, samples from 100 subjects were collected. Serums from collected samples were tested through the developed method. Minimum and maximum outcomes are compiled in Table 3.8.

Table 3.8. Healthy range outcome from HPLC test

Sl. No	Amino Acid	Min µmol/L	Max µmol/L
1	Glutamic Acid	33.98	142.73
2	Serine	47.57	209.344
3	Glycine	66.6	626.08
4	Threonine	8.39	151.1
5	Arginine	5.74	114.81
6	Alanine	89.79	830.62
7	Tyrosine	11.03	82.78
8	Valine	110.96	307.29
9	Tryptophan	9.79	63.65
10	Phenyl Alanine	18.16	60.53

11	Iso Leucine	7.62	114.34
12	Leucine	22.86	129.59
13	Lysine	34.2	143.64

3.3 Suspected Patients Sample Analysis

For investigation purpose 100 IEM suspected patients samples were collected from Bangladeshi infants who were enrolled in this study. Positive samples distribution is given in Table 3.9 and Figure 3.7.

Table 3.9. Positive samples distribution from the study

Amino Acid	Number of Positive Sample
Glutamic Acid	17
Serine	6
Arginine	3
Tyrosine	3
Phenyl Alanine	20
Leucine	1
Lysine	6

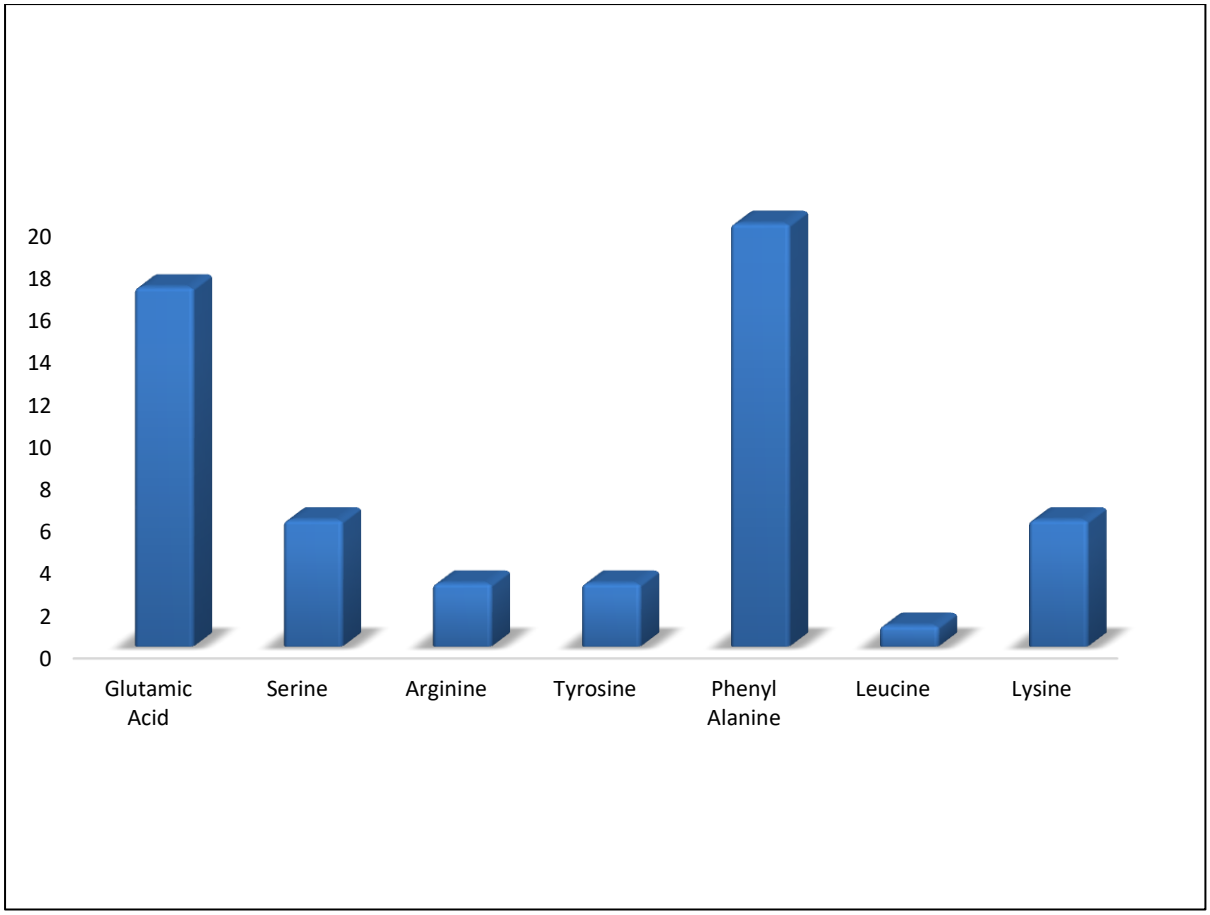


Figure 3.7. Positive samples distribution graph

3.4 Paper based diagnosis kit development for Phenylketonuria detection

Colorimetric enzymatic reaction was evaluated to fix minimum enzyme concentration as well as the minimum substrate concentration. As per selected enzymatic process (Reference Number has to add) chemical reaction was carried out.

3.4.1 Enzyme Concentration Fixation

To evaluate the enzyme concentration variable enzyme concentration was tried (10 U/mL, 20U/ml, 40U/mL). Result shown in the Figure 3.8.

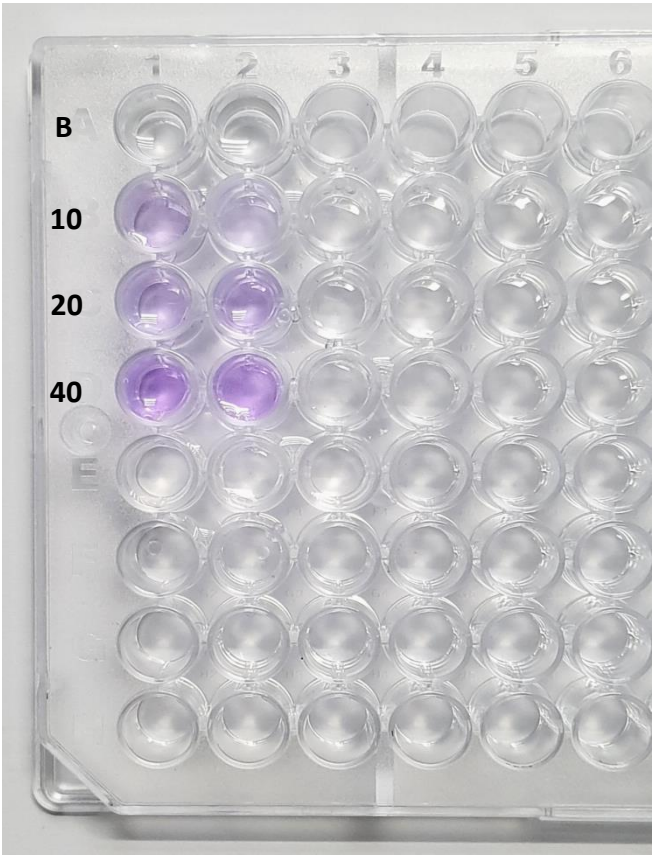


Figure 3.8. Variable enzyme concentration evaluation; Blank, 10 U/mL, 20U/ml and 40U/mL

3.4.2 Substrate color reaction check with time

Variable substrate concentration 10 mg/dL (605.3635 µmol/L) 30 mg/dL, (1816.0906 µmol/L) 50mg/dL (3026.8176 µmol/L) 70mg/dL (4237.5446 µmol/L) was investigated and result shown in fig-9. In all cases reaction time observed up to 10 minutes.

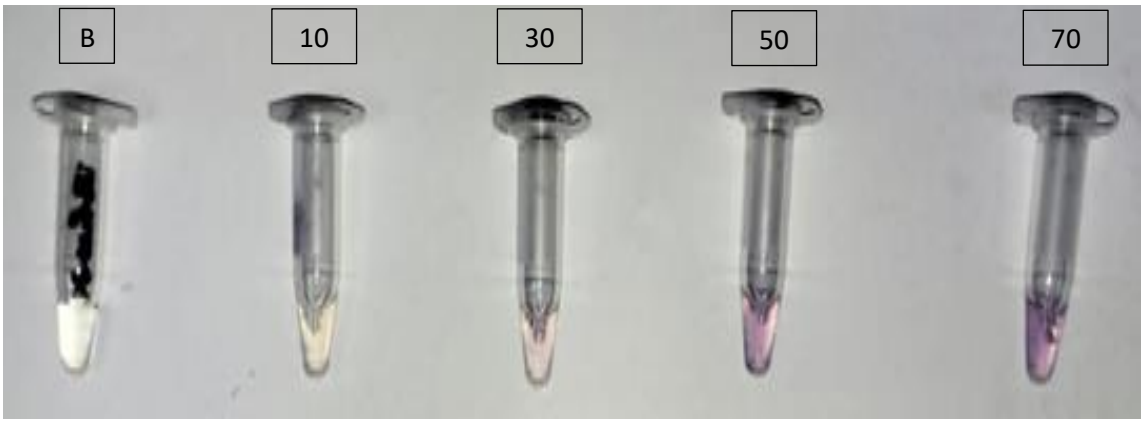


Figure 3.9. Variable substrate concentration evaluation

To determine absorbance maximum, all samples scanning absorbance were determined and obtained result shown in Figure 3.10.

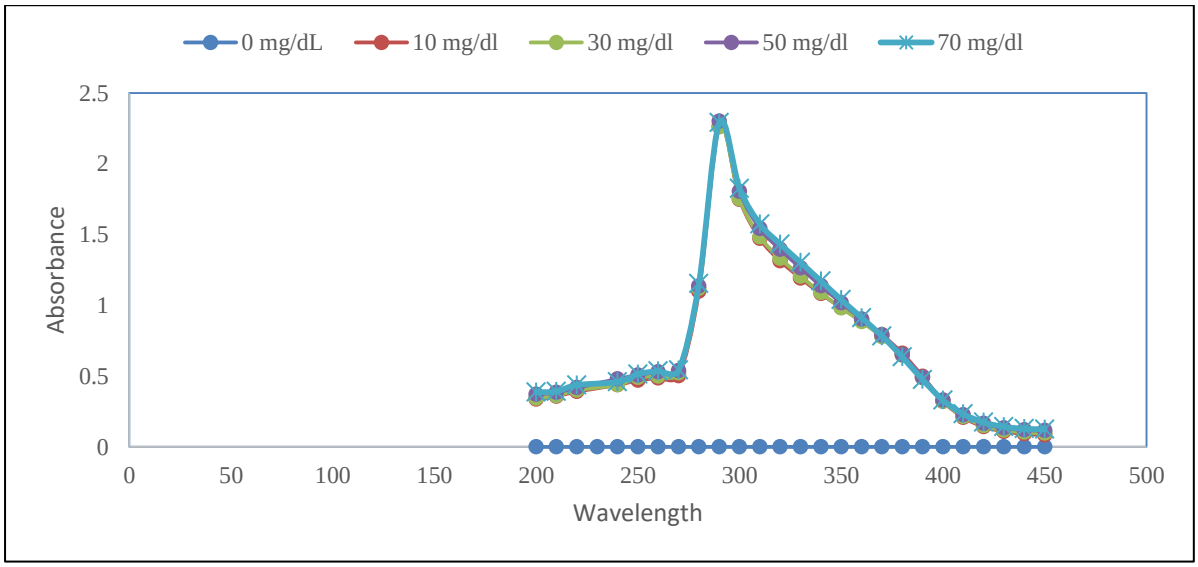


Fig 3.10. Absorbance maximum measurement of samples

3.4.3 Transform liquid reaction in to paper-based kit

3.4.3.1 Assessment of liquid flow on Paper

Sequentially analyzed buffer flow on different paper materials. It was found buffer flow and response generation through flow process is not suitable for any of the paper materials. Color reaction and color intensity was not good in any paper material except glass fiber. In glass fiber pad flow is minimal but reaction response and color generation was found satisfactory.

3.4.3.2 Kit Assembly

General assembly of the glass fiber pad is shown in Figure 3.11. Two layers of glass fiber pad used where upper layer was soaked with (220 mM bis-tris propane buffer pH 9.3) and bottom layer was soaked with (220 mM bis-tris propane buffer pH 6.3). Initially trial was conducted without baking the layers and second trial was made with 37º C baking for 20, 40 and 60 min.

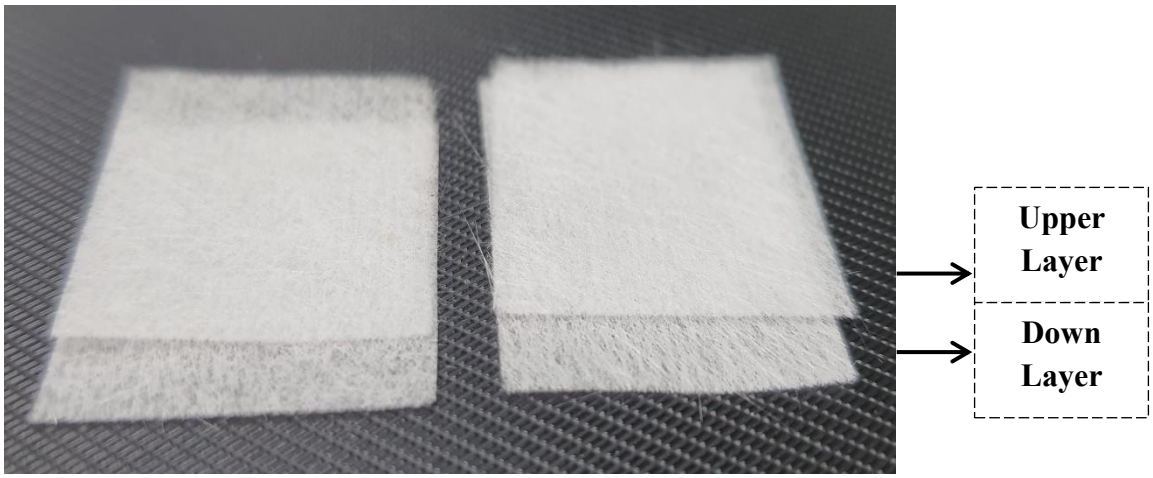
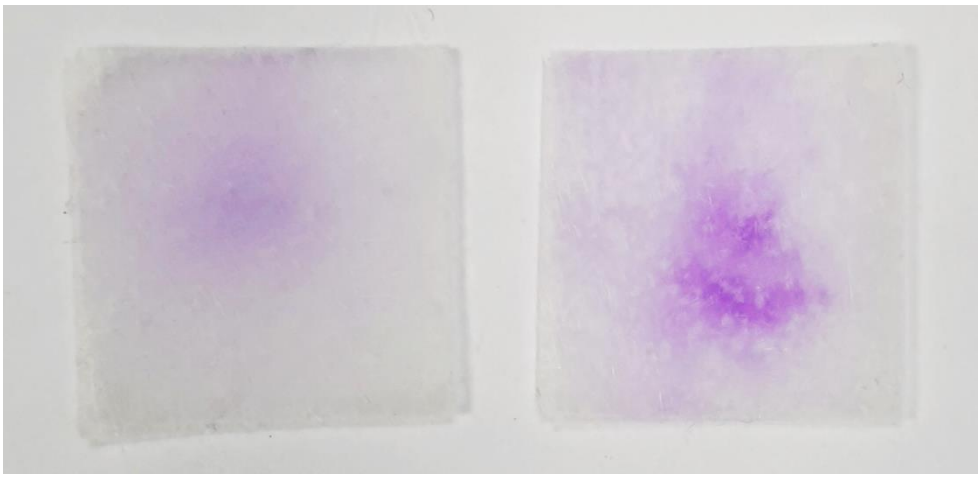


Figure 3.11. Two layers of glass fiber pads were used to harbor two buffers with different pH.

3.4.3.3 Drying temperature and time impact assessment

Different drying temperatures (40º C and 60º C) were applied to find out the optimum drying condition (Figure 3.12). Both the kit assembly was dried for 180 min.



40° C **60° C**

Figure 3.12. Drying temperature impact assessment

3.4.3.4 Limit of Detection (LOD) and Detection Time Fixation

Different enzyme concentrations ranging from 2 mg/dL to 6 mg/dL were evaluated with detection time observations from 5 min to 15 min. Obtained results are shown in Figure 3.13 and figure 3.14.

Figure 3.13. Blank, 2 mg/dL, 4mg/dL and 6 mg/dL for 5 min

Figure 3.14. Blank, 2 mg/dL, 4mg/dL and 6 mg/dL for 15 mins

3.5 Metabolic disease (Galacetosemia) mutational analysis of Bangladeshi population

3.5.1 PCR Method Development

In this study using the designed primer based on the hot spot exon 6 to exon 10 region of GALT gene a total of 14 samples were examined from individuals suspected to have Galactosemia. The PCR product size was determined 942bp (Figure 3.15 and 3.16) and PCR products were purified (Figure 3.17)

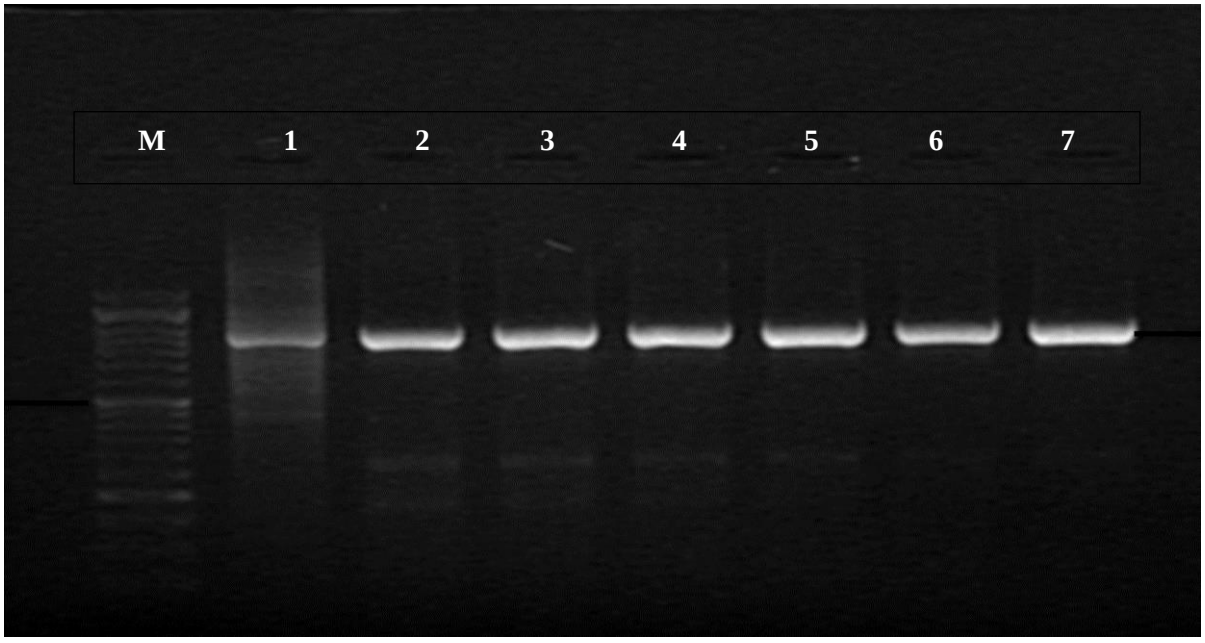


Figure 3.15. PCR gel run result of method standardization process; Marker (M) and Sample (1-7)

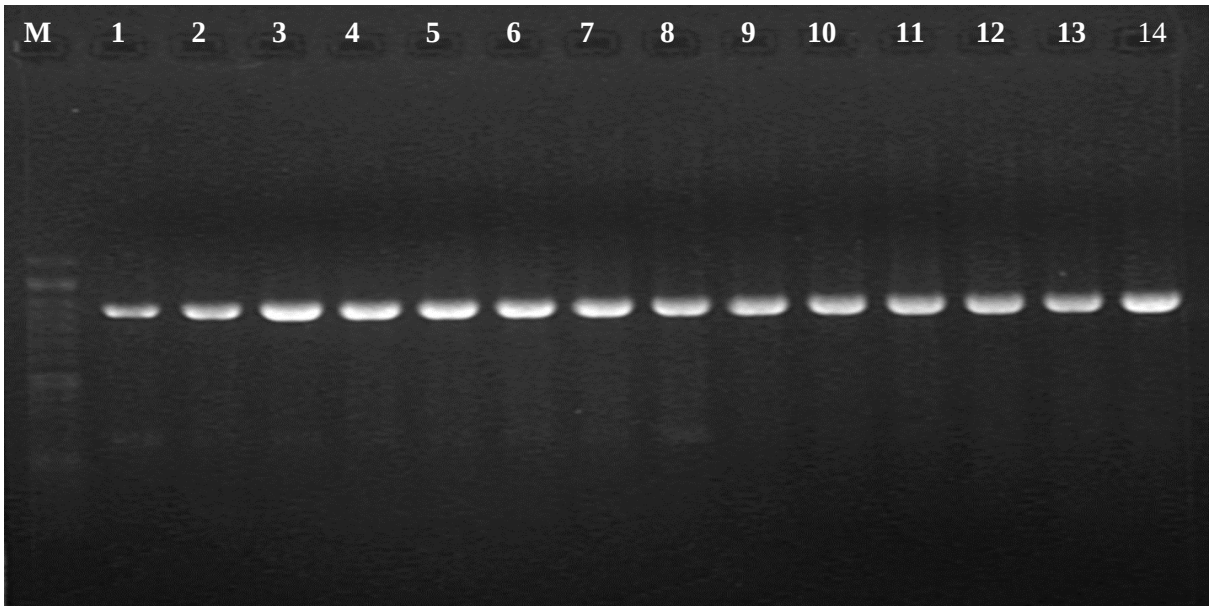


Figure 3.16. PCR gel run of suspected 14 samples. Marker (M) and Sample (1-14)

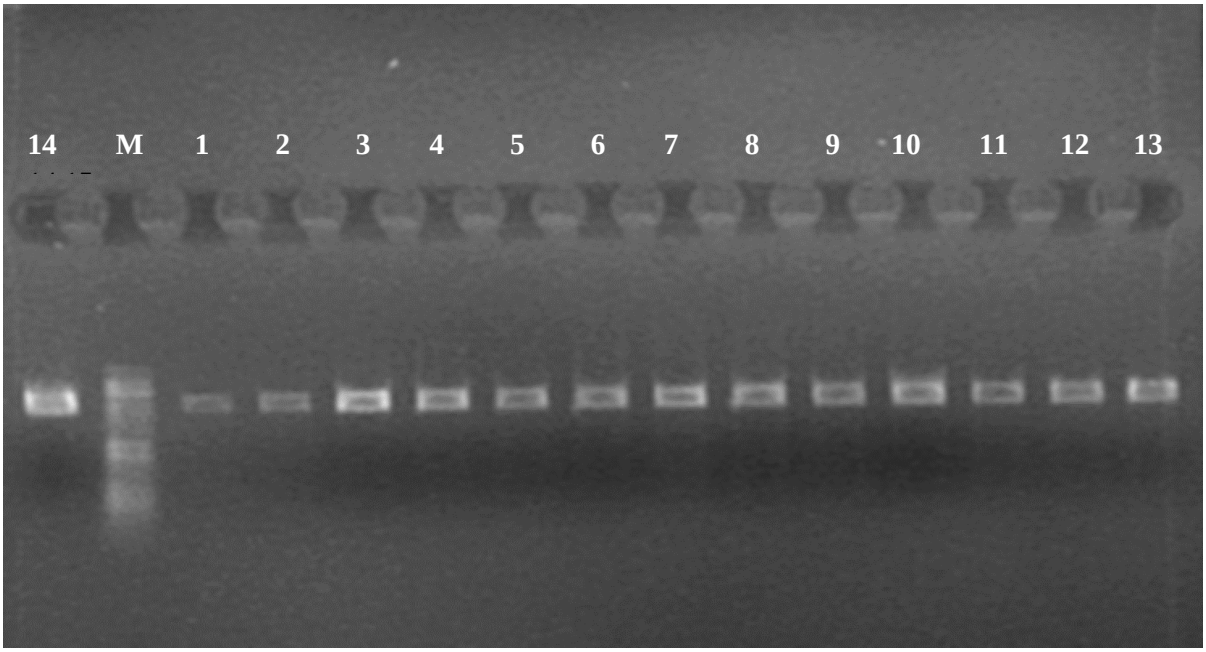


Figure 3.17 Column purified PCR product of suspected samples of Galactosemia. Sample-14, Marker (M) and Sample (1-13).

3.5.2 Genetic analysis of suspected samples

After PCR optimization of this product the mutational analysis of fourteen suspected sample whose reducing substrate was high (non-Glucose) of Bangladesh population was performed by genetic analyzer 310. The GALT gene sequencing result found the silent mutation (Exon 7 c.652 C>T, p.L218L) in the exon 7 of the GALT gene of four samples out of fourteen suspected samples (Figure 3.18 and 3.19). To perform mutational analysis following forward primer was used-

GALT1_F: CAGGAGGGAGTTGACTTGGTGT

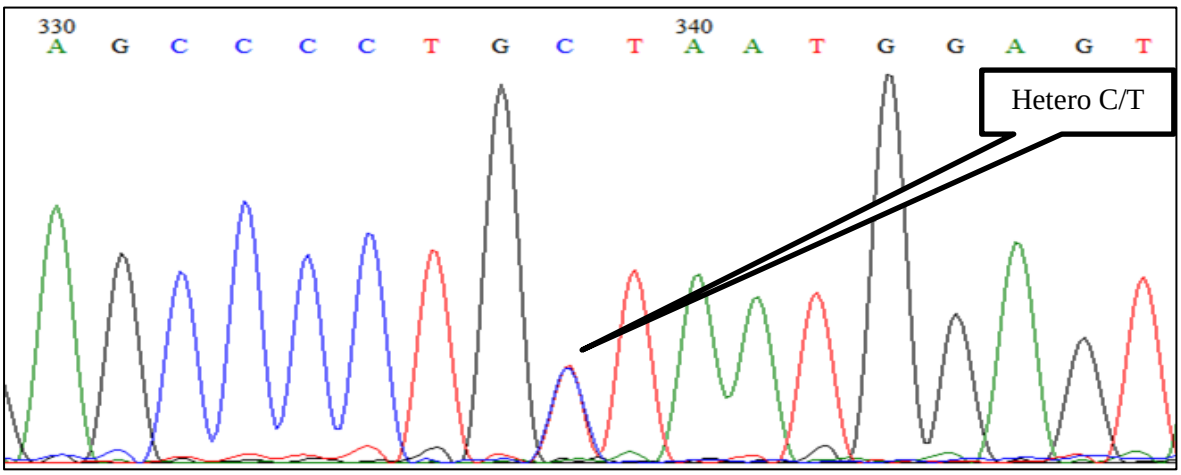


Figure 3.18 Silent mutation (Exon 7 c.652 C>T, p.L218L) in the exon 7 of the GALT gene

Homo sapiens chromosome 9, GRCh38.p7 Primary Assembly
Sequence ID: NC_000009.12Length: 138394717Number of Matches: 1
Related Information
PubChem BioAssay-bioactivity screening
Map Viewer-aligned genomic context
Range 1: 34648138 to 34648747GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1127 bits(610)	0.0	610/610(100%)	0/610(0%)	Plus/Plus

Features:
galactose-1-phosphate uridylyltransferase isoform 1galactose-1-phosphate uridylyltransferase isoform 2

```
Query  1         GATGGGCTGTTCTAACCCCCACCCCCACTGCCAGGTAAGGGTGTCAGGGGCTCCAGTGGG  60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  34648138  GATGGGCTGTTCTAACCCCCACCCCCACTGCCAGGTAAGGGTGTCAGGGGCTCCAGTGGG  34648197

Query  61        TTTCTTGGCTGAGTCTGAGCCAGCACTGTGGACATGGGAACAGGATTAATGGATGGGACA  120
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  34648198  TTTCTTGGCTGAGTCTGAGCCAGCACTGTGGACATGGGAACAGGATTAATGGATGGGACA  34648257

Query  121       GAGGAAATATGCCAATGATGTGGAGGCTTGGAGGTAAAGGACCTGCCTGTTCTTCTCTGC  180
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  34648258  GAGGAAATATGCCAATGATGTGGAGGCTTGGAGGTAAAGGACCTGCCTGTTCTTCTCTGC  34648317

Query  181       TTTTGCCCCTTGACAGGTATGGGCCAGCAGTTTCCTGCCAGATATTGCCCAGCGTGAGGA  240
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  34648318  TTTTGCCCCTTGACAGGTATGGGCCAGCAGTTTCCTGCCAGATATTGCCCAGCGTGAGGA  34648377
                                                          Hetero C/T
Query  241       GCGATCTCAGCAGGCCTATAAGAGTCAGCATGGAGAGCCCCTGCTAATGGAGTACAGCCG  300
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Sbjct  34648378  GCGATCTCAGCAGGCCTATAAGAGTCAGCATGGAGAGCCCCTGCTAATGGAGTACAGCCG  34648437

Query  301       CCAGGAGCTACTCAGGAAGGTGGGAGAGAGCCAAGCCCTGTGTCCCCAAGGAGTCCCTAA  360
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  34648438  CCAGGAGCTACTCAGGAAGGTGGGAGAGAGCCAAGCCCTGTGTCCCCAAGGAGTCCCTAA  34648497

Query  361       CTTTCTTATCCCATGAGAGAGGTGTGTAAAGGAGAAAGCTAGAGGTGAACTAGTAGagag  420
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Sbjct  34648498  CTTTCTTATCCCATGAGAGAGGTGTGTAAAGGAGAAAGCTAGAGGTGAACTAGTAGAGAG  34648557

Query  421       agacttgctaggaggccttagcaataatccagtaatctaaaggaaagatgatggtgactt  480
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Sbjct  34648558  AGACTTGCTAGGAGGCCTTAGCAATAATCCAGTAATCTAAAGGAAAGATGATGGTGACTT  34648617

Query  481       agactcgggtggttagtggtagaggtggtgagaagacatcagatcctgggcacattCTTT  540
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Sbjct  34648618  AGACTCGGGTGGTTAGTGGTAGAGGTGGTGAGAAGACATCAGATCCTGGGCACATTCTTT  34648677

Query  541       TCTTCTGCTTCCCTTGCCTATTTGCTGACCACACTCCGGCTCCTATGTCACCTTGATGAC  600
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Sbjct  34648678  TCTTCTGCTTCCCTTGCCTATTTGCTGACCACACTCCGGCTCCTATGTCACCTTGATGAC  34648737

Query  601       TTCCTATCCA  610
                 ||||||||||
Sbjct  34648738  TTCCTATCCA  34648747
```

Figure 3.19 Nucleotide Blast analysis result showing the mutation

3.5.2.1 Mutational Analysis

Mutational analysis of Codon (11 exon) was made and displayed in Figure 3.20.

ATGTCGCGCAGTGGAACCGATCCTCAGCAACGCCAGCAGGCGTCAGAGGCGGAC
GCCGCAGCAGCAACCTTCCGGGCAAACGACCATCAGCATATCCGCTACAACCCG
CTGCAGGATGAGTGGGTGCTGGTGTCAGCTCACCGCATGAAGCGGCCCTGGCAG
GGTCAAGTGGAGCCCCAGCTTCTGAAGACAGTGCCCCGCCATGACCCTCTCAAC
CCTCTGTGTCCTGGGGCCATCCGAGCCAACGGAGAGGTGAATCCCCAGTACGAT
AGCACCTTCCTGTTTGACAACGACTTCCCAGCTCTGCAGCCTGATGCCCCCAGTC
CAGGACCCAGTGATCATCCCCTTTTCCAAGCAAAGTCTGCTCGAGGAGTCTGTAA
GGTCATGTGCTTCCACCCCTGGTCGGATGTAACGCTGCCACTCATGTCGGTCCCT
GAGATCCGGGCTGTTGTTGATGCATGGGCCTCAGTCACAGAGGAGCTGGGTGCC
CAGTACCCTTGGGTGCAGATCTTTGAAAACAAAGGTGCCATGATGGGCTGTTCTA
ACCCCCACCCCCACTGCCAGGTATGGGCCAGCAGTTTCCTGCCAGATATTGCCCA
GCGTGAGGAGCGATCTCAGCAGGCCTATAAGAGTCAGCATGGAGAGCCCCTG**C**T
AATGGAGTACAGCCGCCAGGAGCTACTCAGGAAGGAACGTCTGGTCCTAACCAG
TGAGCACTGGTTAGTACTGGTCCCCTTCTGGGCAACATGGCCCTACCAGACACTG
CTGCTGCCCGTCGGCCATGTGCGGCGGCTACCTGAGCTGACCCCTGCTGAGCGTG
ATGATCTAGCCTCCATCATGAAGAAGCTCTTGACCAAGTATGACAACCTCTTTGA
GACGTCCTTTCCCTACTCCATGGGCTGGCATGGGGCTCCCACAGGATCAGAGGCT
GGGGCCAACTGGAACCATTGGCAGCTGCACGCTCATTACTACCCTCCGCTCCTGC
GCTCTGCCACTGTCCGGAAATTCATGGTTGGCTACGAAATGCTTGCTCAGGCTCA
GAGGGACCTCACCCCTGAGCAGGCTGCAGAGAGACTAAGGGCACTTCCTGAGGT
TCATTACCACCTGGGGCAGAAGGACAGGGAGACAGCAACCATCGCCTGA

Figure 3.20. Codon (11 exon, 1140bp) (4010bp with intron)

3.5.2.2 Analysis of the sequence of amino acids

Analysis of the sequence of amino acid was made to investigate the modified protein sequence shown in Figure 3.21.

>380 AA

MSRSGTDPQQRQQASEADAAAATFRANDHQHIRYNPLQDEWVLVSAHRMKRPWQ
GQVEPQLLKTVPRHDPLNPLCPGAIRANGEVNPQ**Y**DSTFLFDNDFPAL**Q**PDAPSPGPS
DHPLFQAKSARGVCKVMCFHPWSDVTLPLMSVPEIRAVVDAWASVTEELGAQY**P**W
VQI**F**ENKGAMMGC**S**NPH**P**HC**Q**VWASSFLPDIAQREERSQQAY**K**SQHGEPL**L**MEYSR
QELLRKERLVLTSEHWLVLVPFWATWPYQTLLLPRRHVRRLPELTPAERDDLASIM
KKLLT**K**YDNLFETSFPYSMGWHGAPTGSEAGANW**N**HWQLHAHYYPPLLRSATVRK
FMVGYEMLAQAQRDLTPEQAAERLRALPEVHYHLGQKDRETATIA

Figure 3.21. Protein sequence analysis

3.5.2.3 Splicing Site Comparison

Splicing alteration study was made through “Human Splicing Finder” and data shown in Table 3.10, 3.11, 3.12 and 3.13

Table 3.10. Forecasts of the donor site for wild

Start	End	Score	Exon	Intron
246	260	0.78	cggagag	gtgaatcc
517	531	0.60	aacaaag	gtgccatg
558	572	0.97	ctgccag	gtatgggc
700	714	0.49	ctaacca	gtgagcac
710	724	0.96	agcactg	gttagtac

Table 3.11. Forecasts of the acceptor site for wild

Start	End	Score	Intron	Exon
569	609	0.67	gggccagcagtttcctgcc	agatattgcccagcgtgaggag

Table 3.12. Forecasts of the donor site for mutation

Start	End	Score	Exon	Intron
22	36	1.00	ctgccag	gtaagggt
141	155	0.72	cttggag	gtaaagga
184	198	0.96	ttgacag	gtatgggc

307	321	0.53	caggaag	gtgggaga
369	383	0.98	gagagag	gtgtgtaa
495	509	0.47	agaggtg	gtgagaag
631	645	0.49	ctaacca	gtgagcac
641	655	0.96	agcactg	gttagtac
744	758	0.64	cgtgatg	gtcagtct

Table 3.13. Forecasts of the acceptor site for mutation

Start	End	Score	Intron	Exon
8	48	0.90	agccccccacccccctgcc	aggtaagggtgtcaggggctcc
170	210	0.94	tctgcttttgccccttgac	aggtatgggccagcagtttcct
195	235	0.67	gggccagcagtttcctgcc	agatattgcccagcgtgaggag
598	638	0.98	ctatccattctgtcttcct	aggaacgtctggtcctaaccag

3.6 Diagnosis kit development to detect metabolic disease (Galactosemia)

Outline of the detection reaction has given below-

$$\text{Galactose} + O_2 \xrightarrow{\text{Galactose Oxidase}} \text{D-Galactose-hexodialdose} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Galactose Oxidase}} H_2O + \tfrac{1}{2}\, O_2$$

$$\tfrac{1}{2}\, O_2 + \text{Chromogens} \longrightarrow \text{Colored Complex}$$

3.6.1 Absorbance Scan of Different Reagents

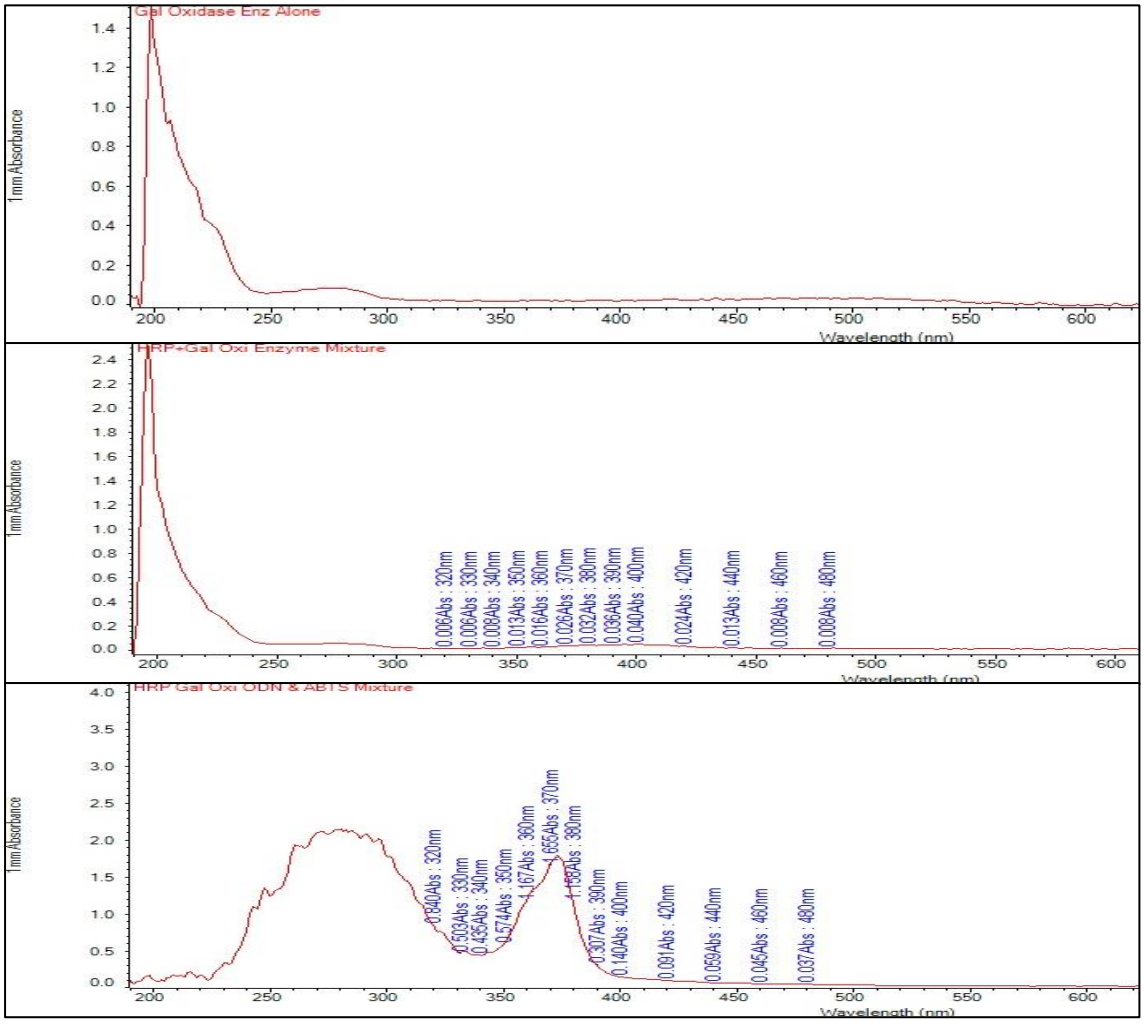

Figure 3.22 Absorbance pattern evaluation of reagents a. Galactose oxidase+ Horseradish peroxidase+ phosphate buffer b. HRP+ Gal. Oxidase Enz. c. HRP+ Gal. Oxidase Enz. + ODN+ ABTS

3.6.2 Liquid reaction evaluation with different substrate

Color reaction outcome evaluation using different substrates- ODN, OPD, ABTS & TMB:

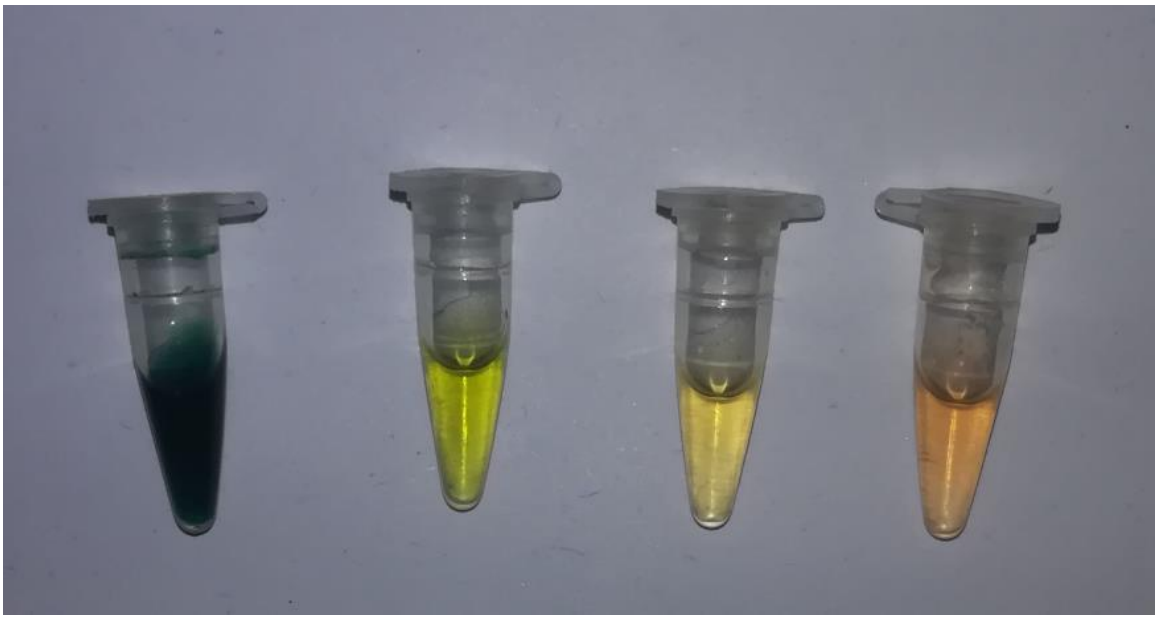

Figure 3.23. Variable color with different chromogen application

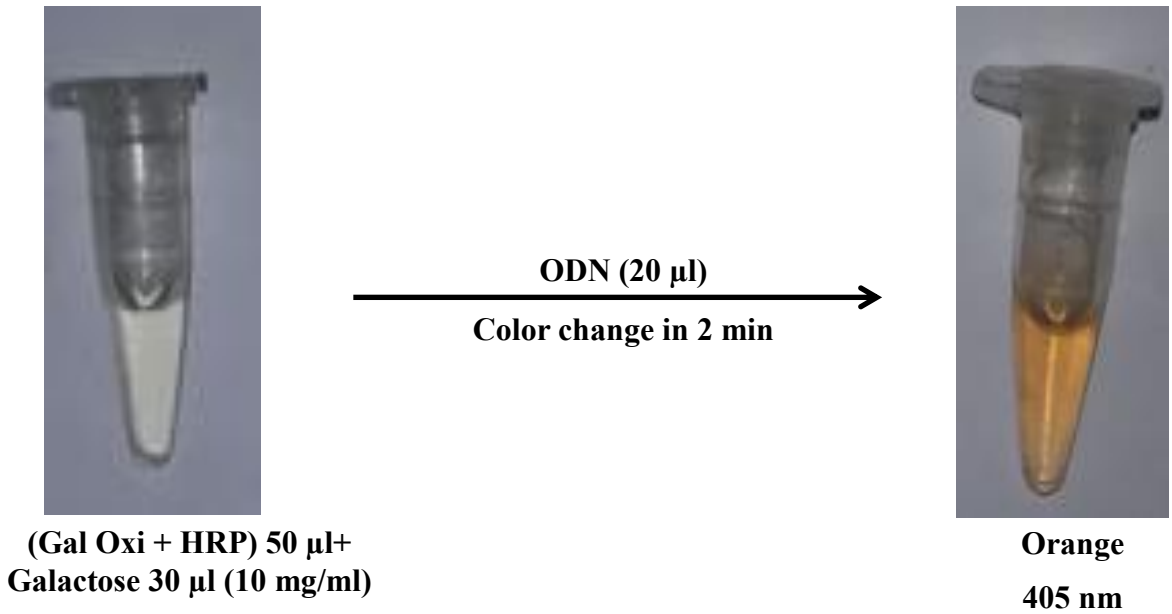

Figure 3.24. ODN (3, 3-Dimethoxybenzidine) chromogenic substrate evaluation

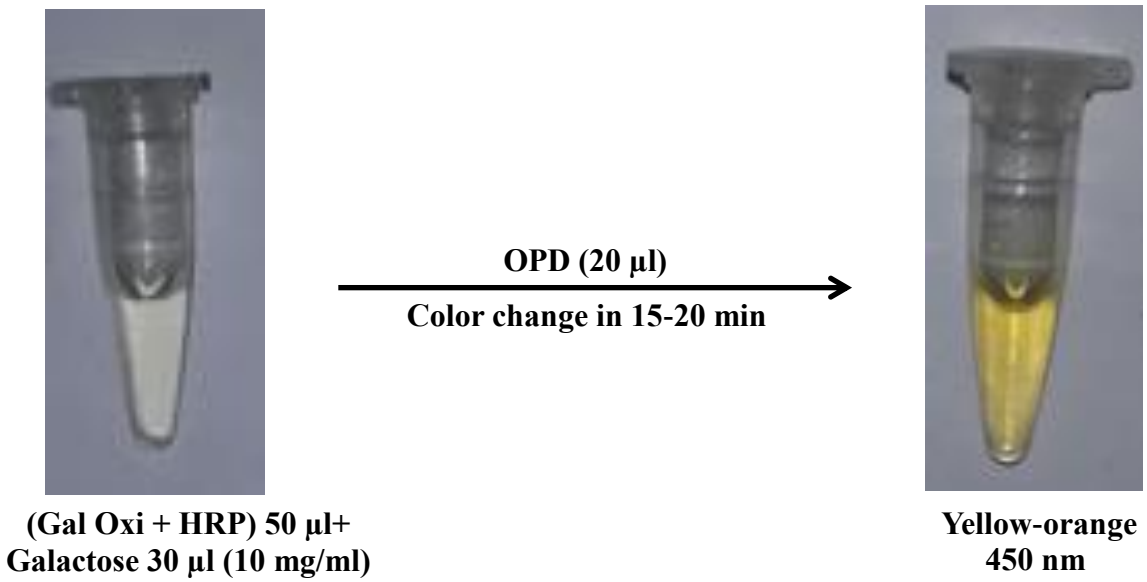

Figure 3.25. OPD (O- Phenylene Diamine) chromogenic substrate evaluation

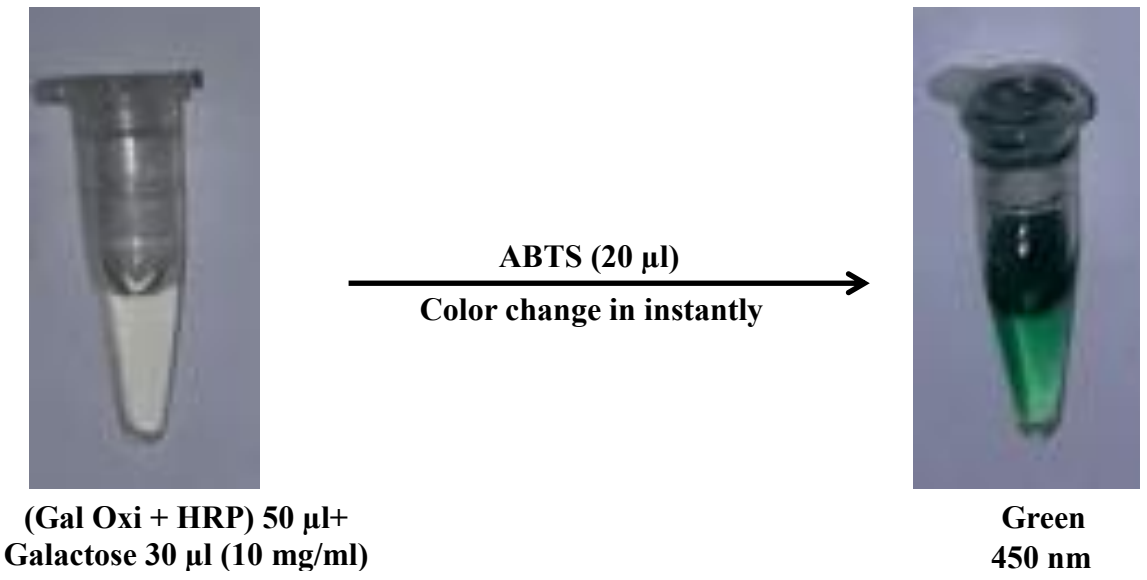

Figure 3.26. ABTS (2, 2- Azino-Bis-(3-ethylbenzo Thiazoline-6-Sulphonate) chromogenic substrate evaluation

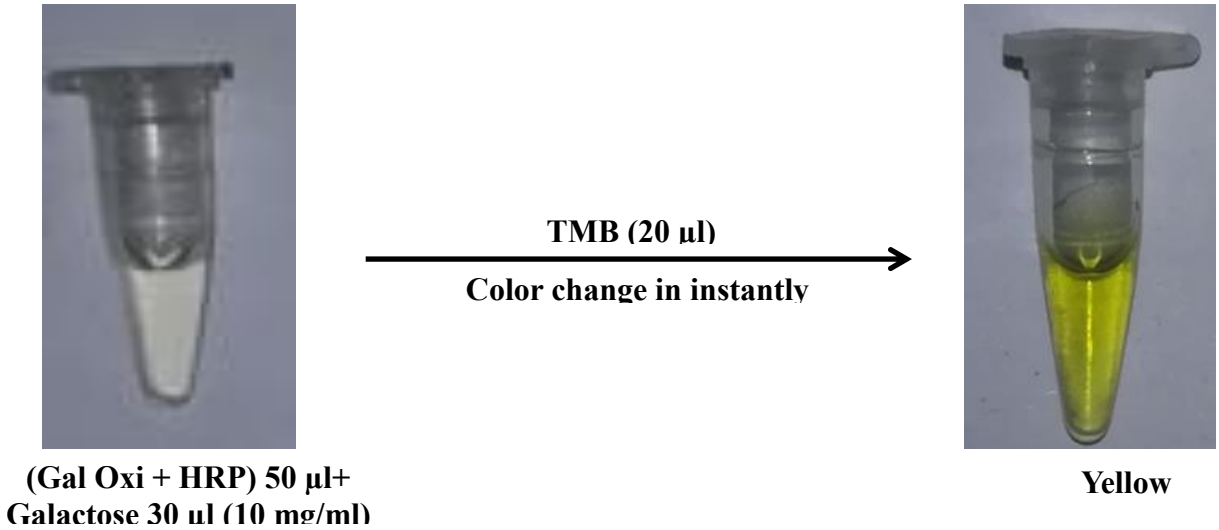

Figure 3.27. TMB (3, 3, 5, 5- Tetramethylbenzidine) chromogenic substrate evaluation

3.6.3 Liquid reaction with variable substrate

Liquid reaction with variable substrate was investigated, results are shown below-

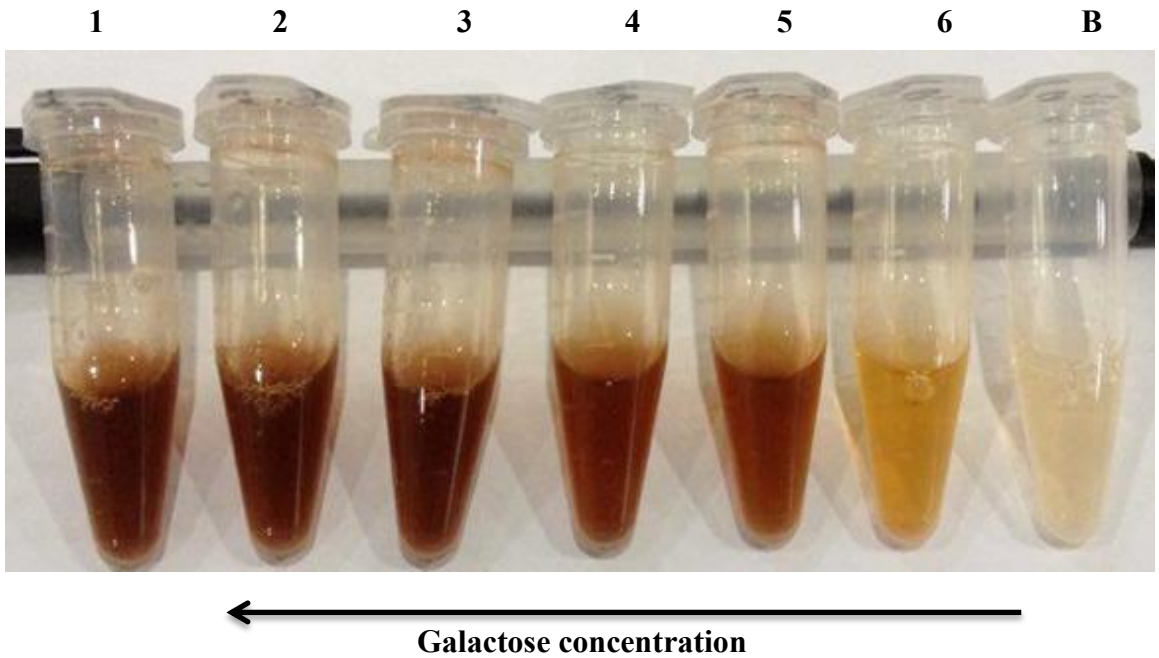

Figure 3.28. The color intensity depends on substrate concentration (50 mg/ mL, 40mg/mL, 30mg/mL, 20mg/mL, 10mg/mL, 5mg/mL and Blank) with enzyme conc. (Galactose oxidase (0.005U/µL) and HRP 5 µL).

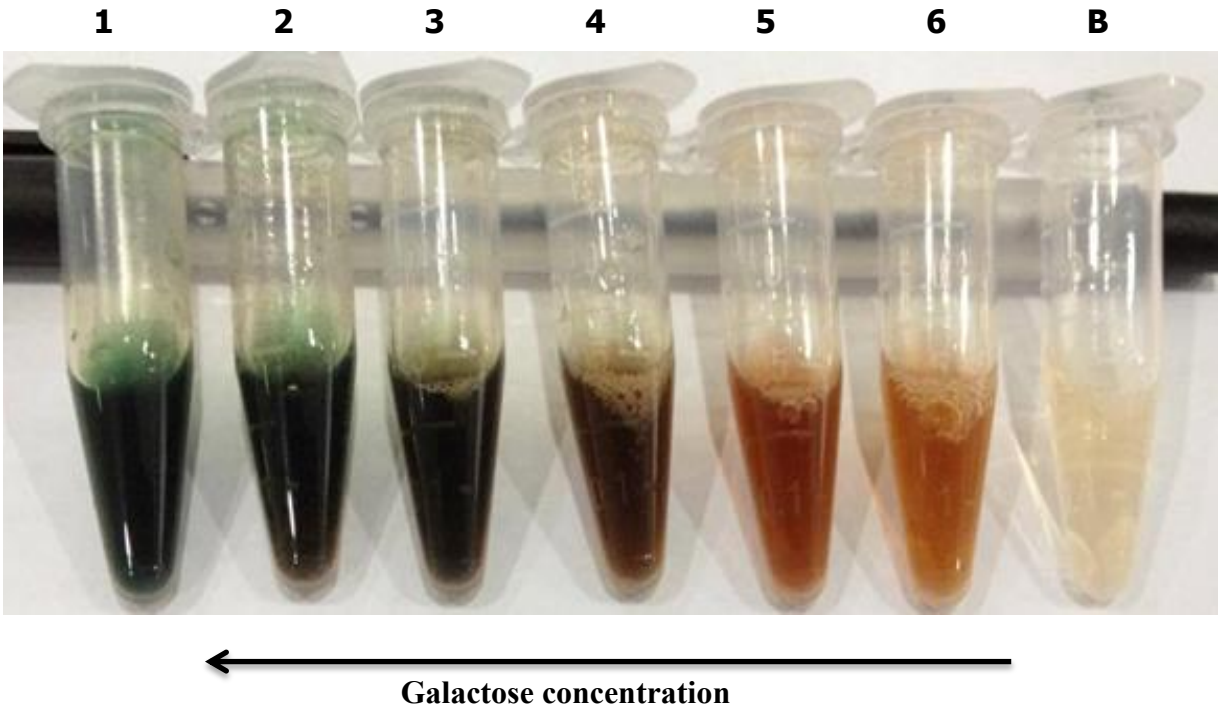

Figure 3.29. The color intensity depends on substrate concentration (50 mg/ mL, 40mg/mL, 30mg/mL, 20mg/mL, 10mg/mL, 5mg/mL and Blank) with enzyme conc. (Galactose oxidase (0.5U/µL) and HRP 5 µL

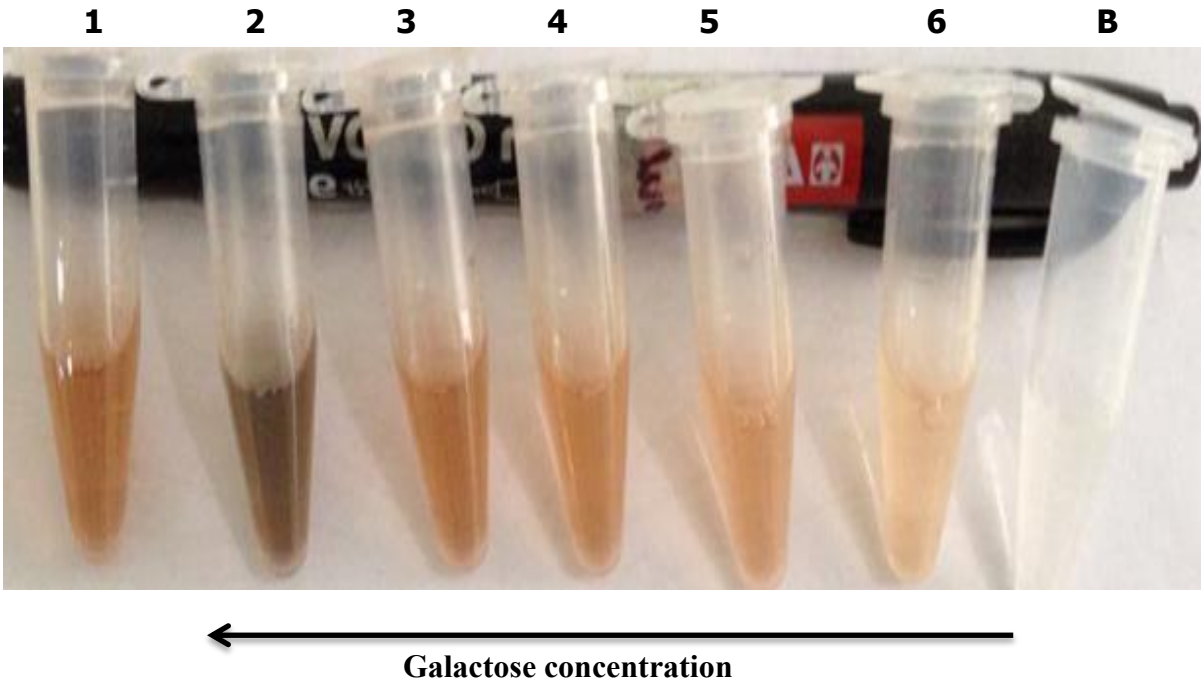

Figure 3.30. The color intensity depends on substrate concentration (50 mg/ mL, 40mg/mL, 30mg/mL, 20mg/mL, 10mg/mL, 5mg/mL and Blank) with enzyme conc. (Galactose oxidase (0.05U/µL) & HRP 5 µL

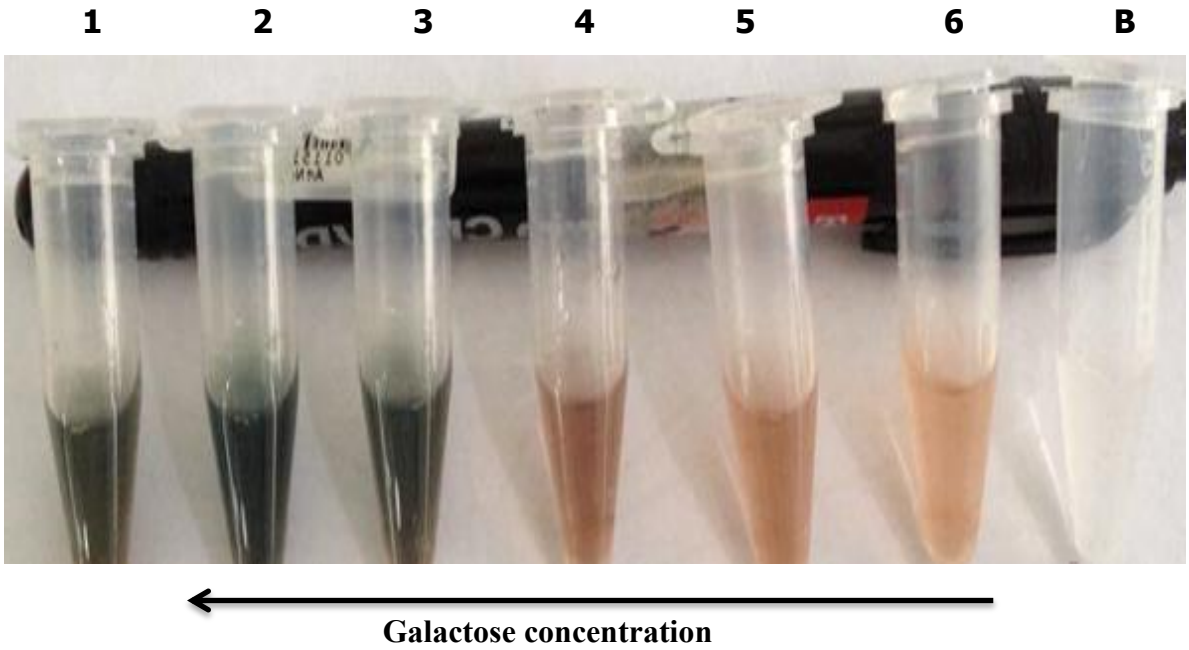

Figure 3.31. The color intensity depends on substrate concentration (50 mg/ mL, 40mg/mL, 30mg/mL, 20mg/mL, 10mg/mL, 5mg/mL and Blank) with enzyme conc. (Galactose oxidase (0.25U/µL) & HRP 5 µL

3.6.4 Variable substrate absorbance measurement

Absorbance scan was evaluated with variable substrate amount:

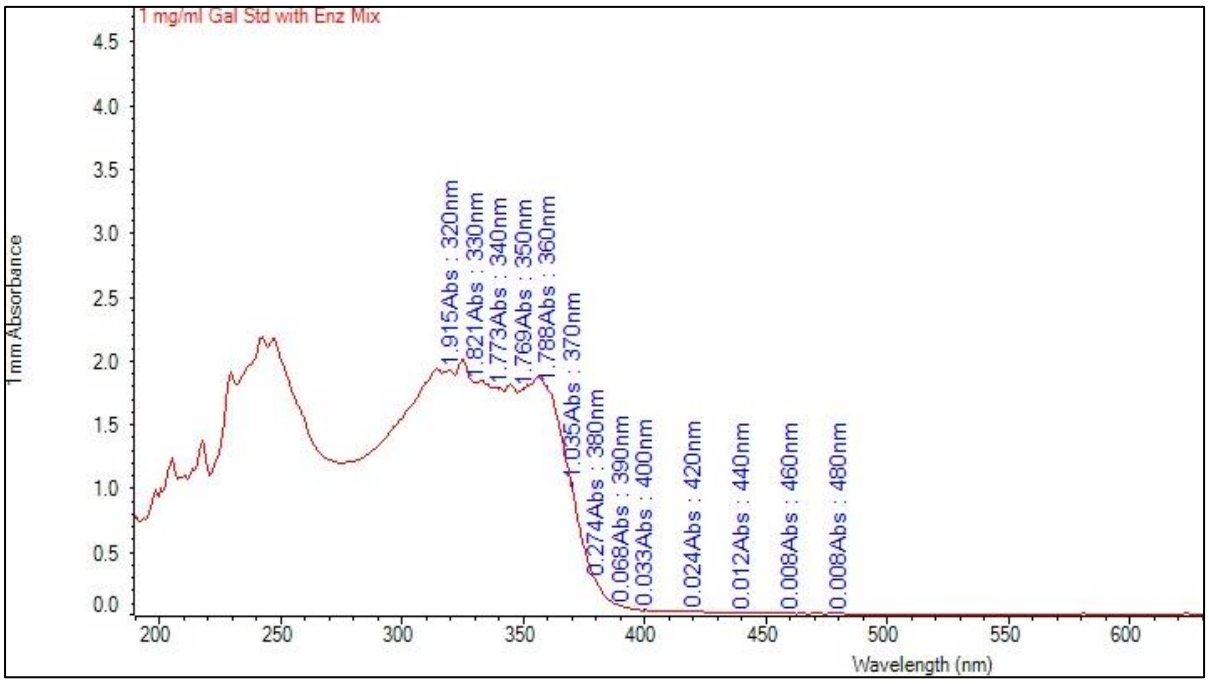

Figure 3.32. 1 mg/mL Galactose standard and enzyme chromogen reaction

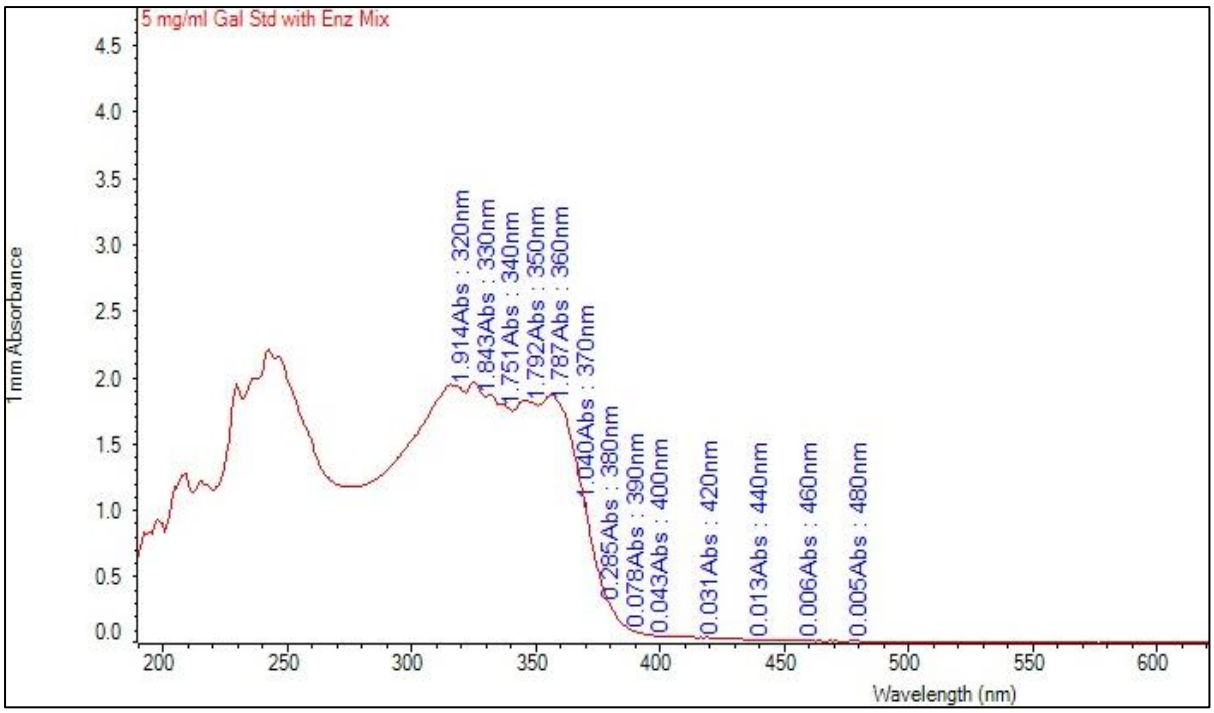

Figure 3.33. 5 mg/mL Galactose standard and enzyme chromogen reaction

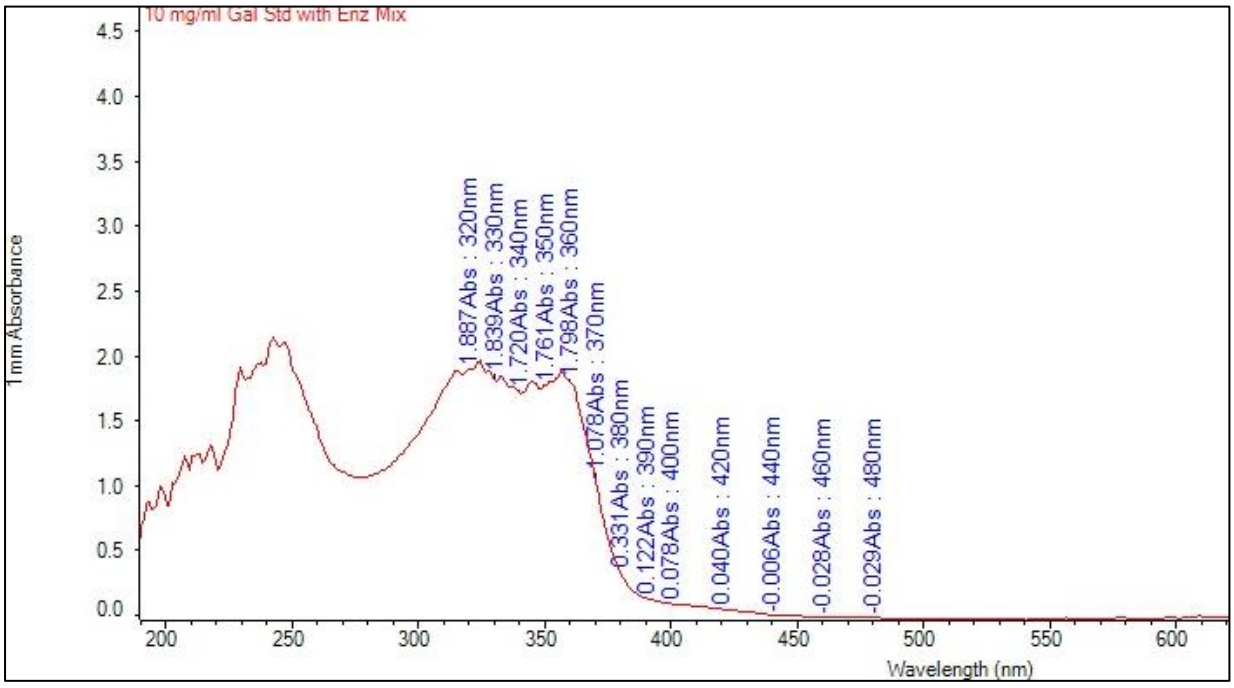

Figure 3.34. 10 mg/mL Galactose standard and enzyme chromogen reaction

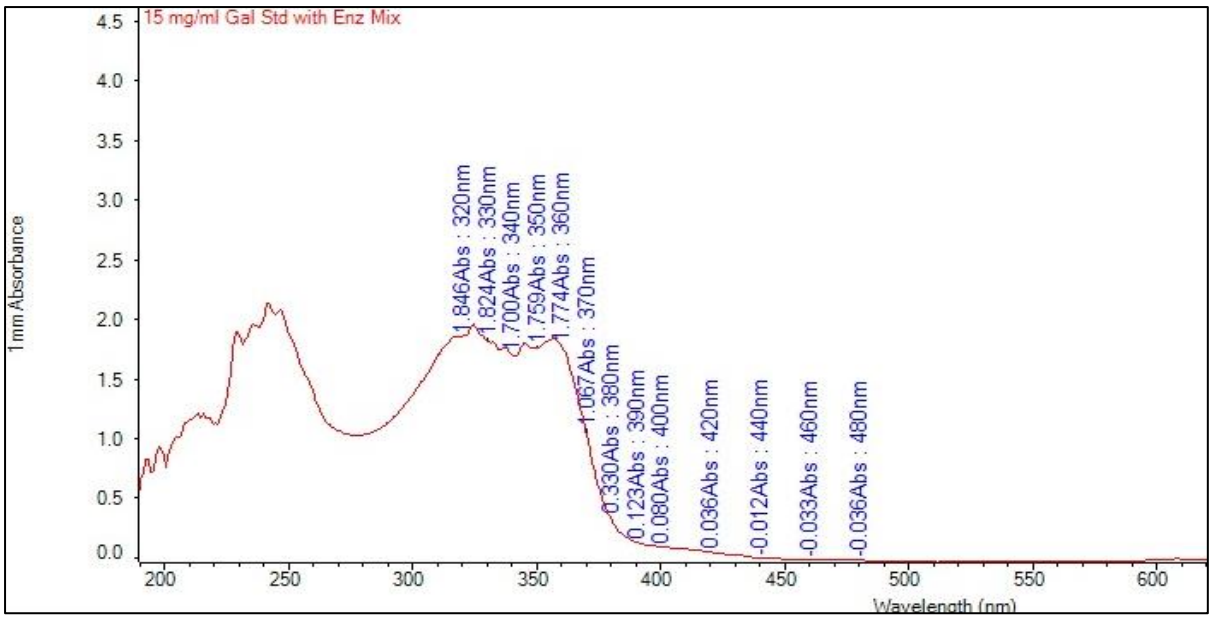

Figure 3.35. 15 mg/mL Galactose standard and enzyme chromogen reaction

3.6.5 Paper kit assembly

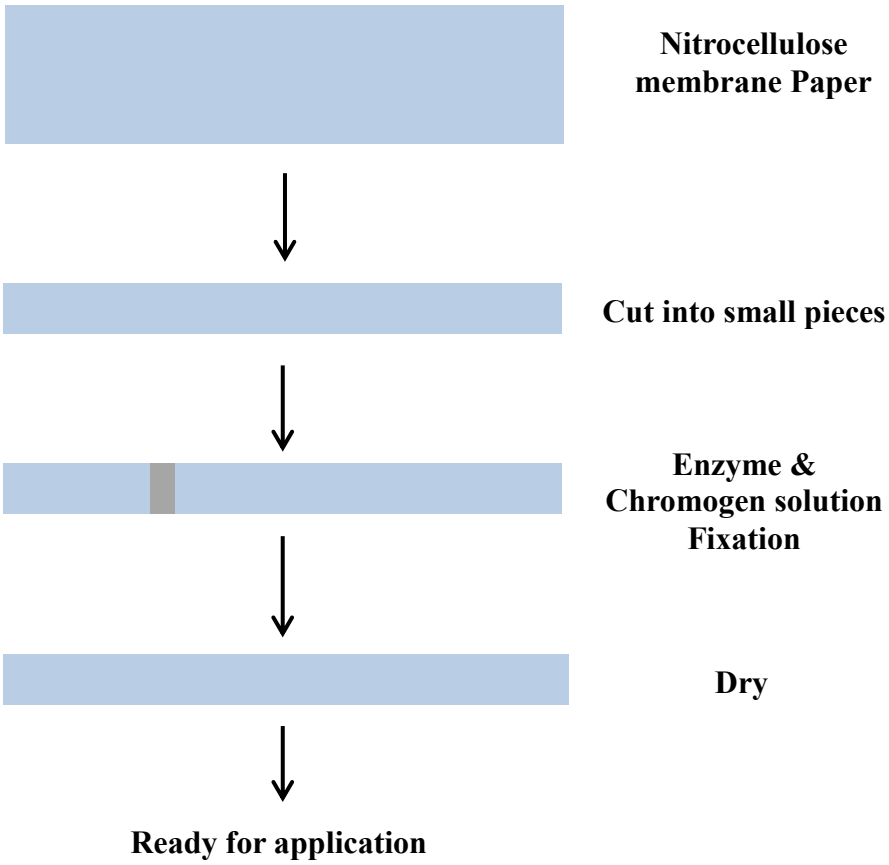

Figure 3.36. Outline of kit assembly with dispensing of Enzyme and Chromogen solution

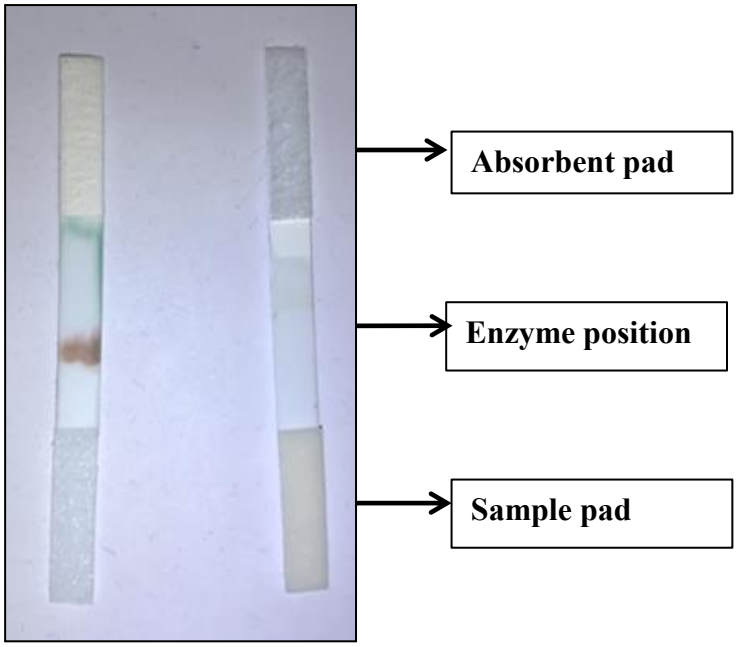

Figure 3.37. Manually casted prototype of kit

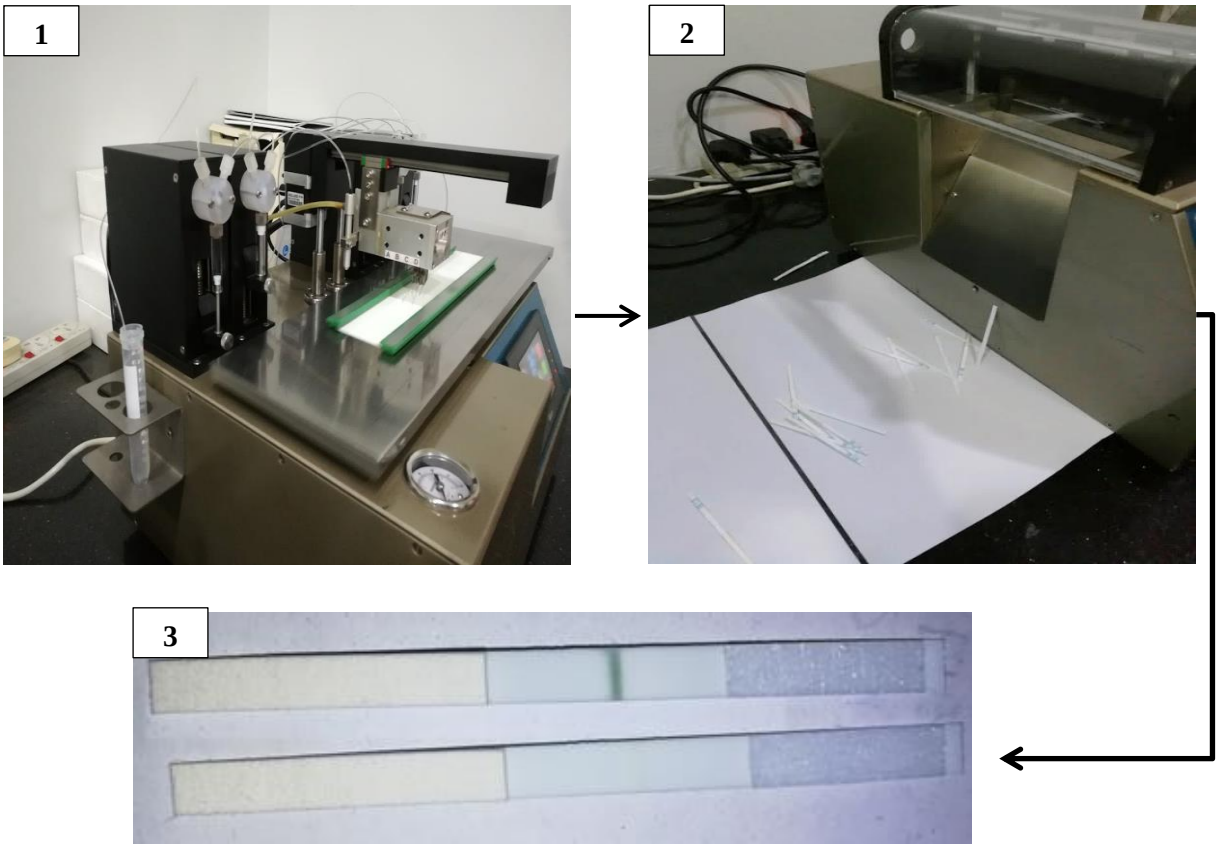

Figure 3.38. Machine casted kit (1.Enzyme line creation 2.cut in specific size with automatic cutter) and tested with standard Galactose.

3.6.6 Assembled kit check with variable concentration of standard Galactose

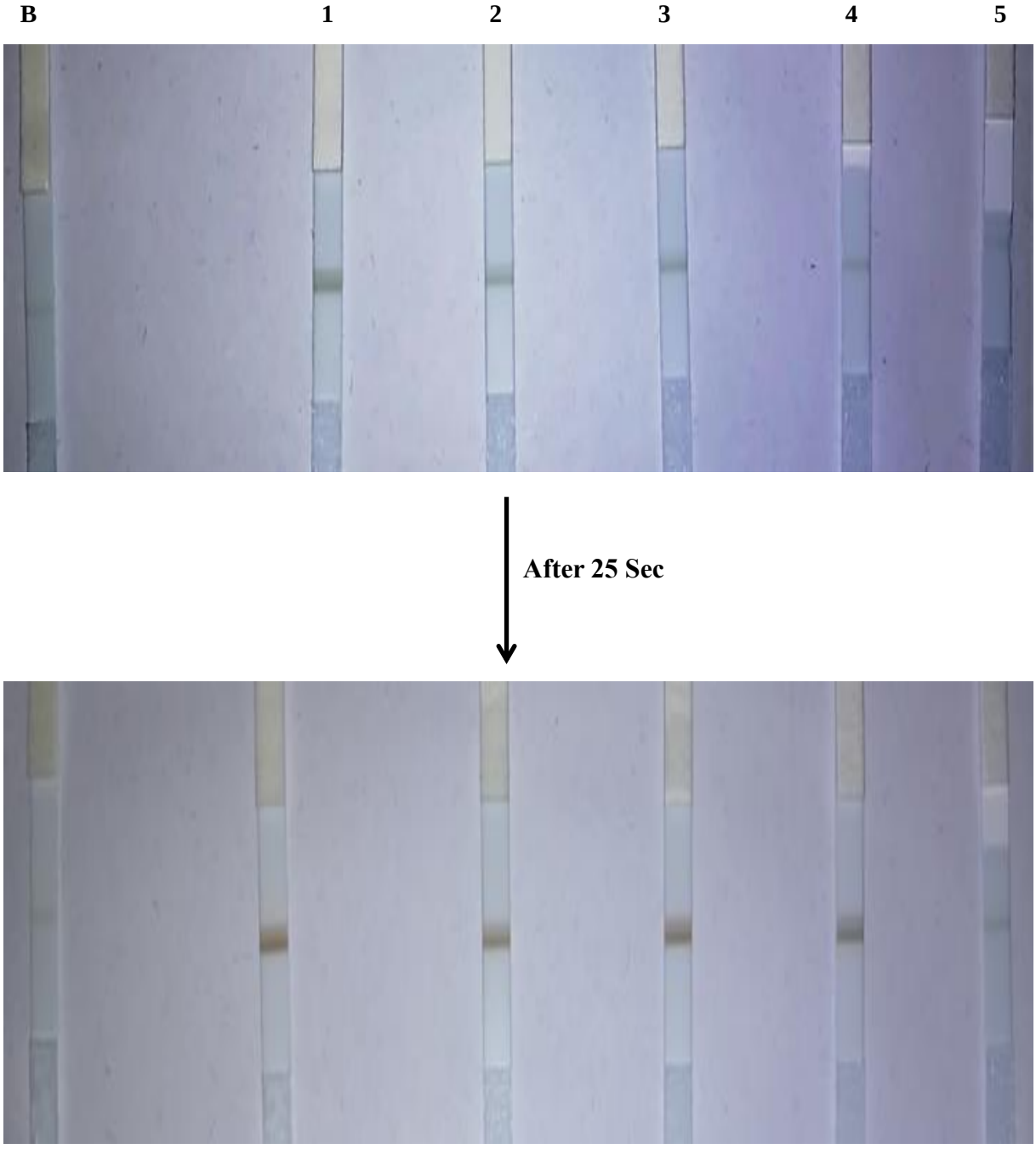

Figure 3.39. Response time measurement with different Galactose standard concentration (Blank, 50 mg/mL, 40 mg/mL, 30 mg/mL, 20 mg/mL and 10 mg/mL)

3.6.7 Paper Selection to get optimum test line

Trialed two types of Nylon Paper and two types of Nitrocellulose paper standard Galactose solution. Prepared enzyme was dispensed 2 hours later of baking at 37° C.

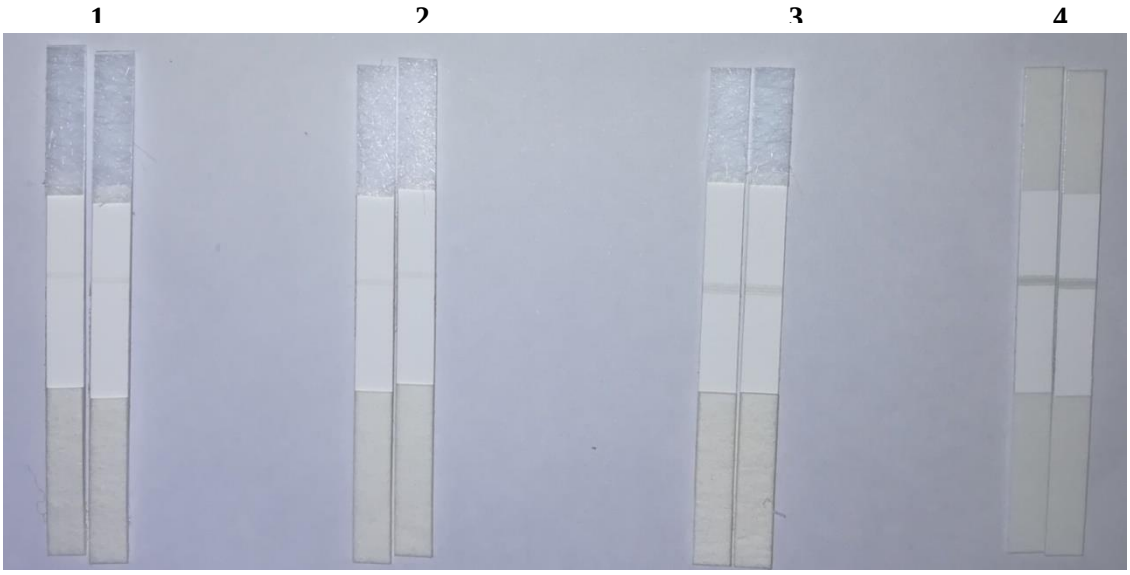

Figure 3.40. Nylon Paper (**1.** 0.22 µm **2.** 0.45 µm) and Nitrocellulose (**3.** 0.45 µm **4.** 135) evaluation

3.6.8 Comparison between 0.22 and 0.45 Nylon Paper

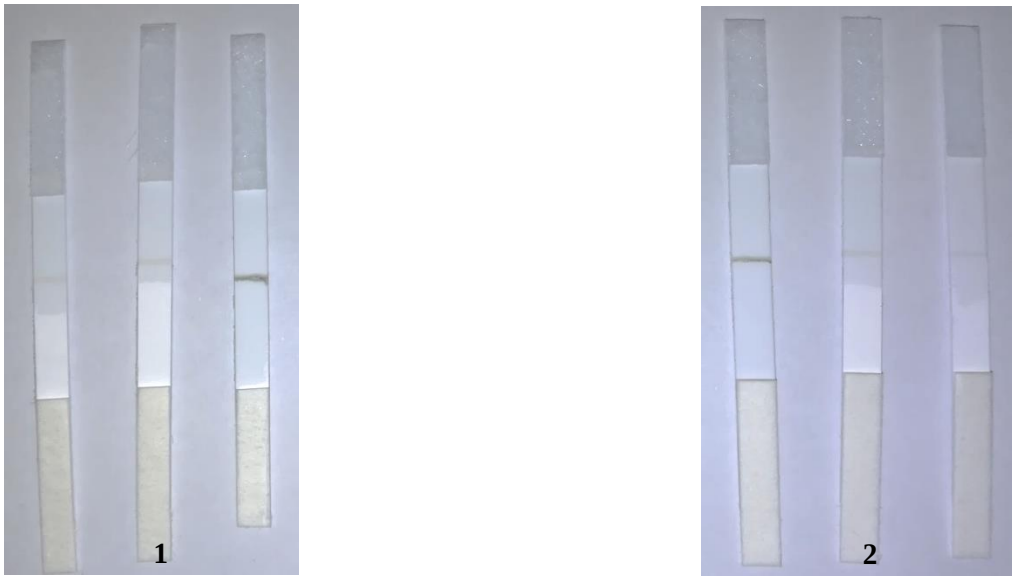

Figure 3.41. Comparison between (1) 0.22 and (2) 0.45 Nylon Paper. In each case Blank, Glucose 10 mg/mL and Galactose 10 mg/mL used.

3.6.9 Assembly of Nylon Kit

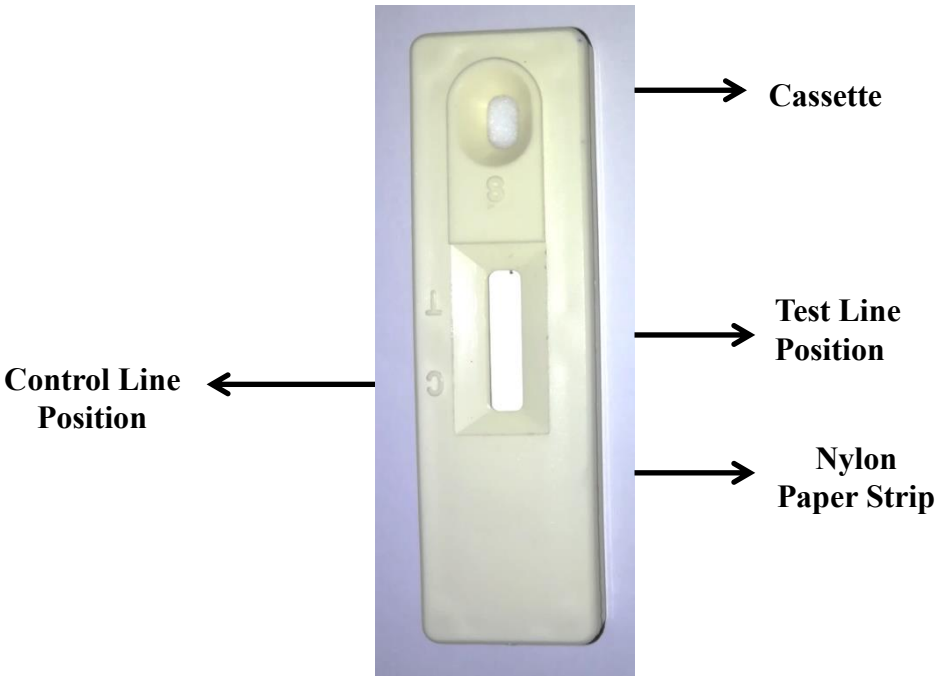

Figure 3.42. Assembled nylon kit in commercial plastic cassette.

3.6.10 Water test for flow rate evaluation

Water travel time through the entire kit area was measured by applying water and dispersed time obtained 4.02 minutes (Figure 3.43.)

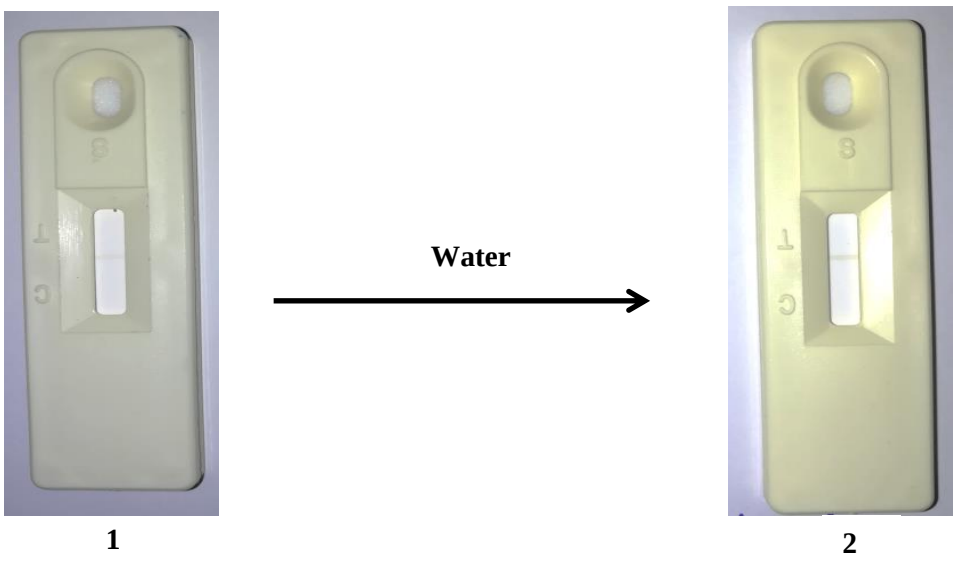

Figure 3.43. Water travel time through the entire kit area measured

3.6.11 Standard Galactose test

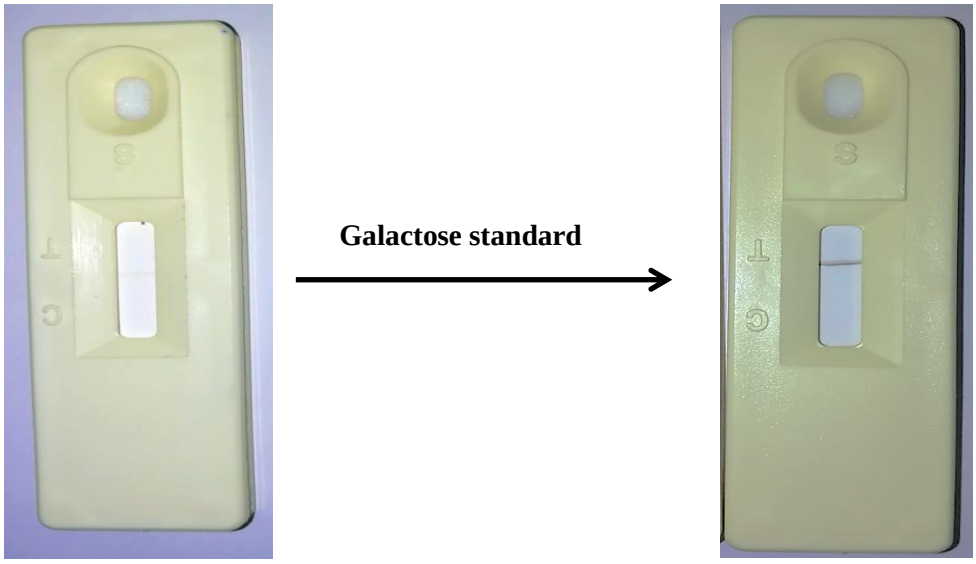

Figure 3.44. Test line evaluation with standard

3.6.12 Human serum with spiked Galactose sample test

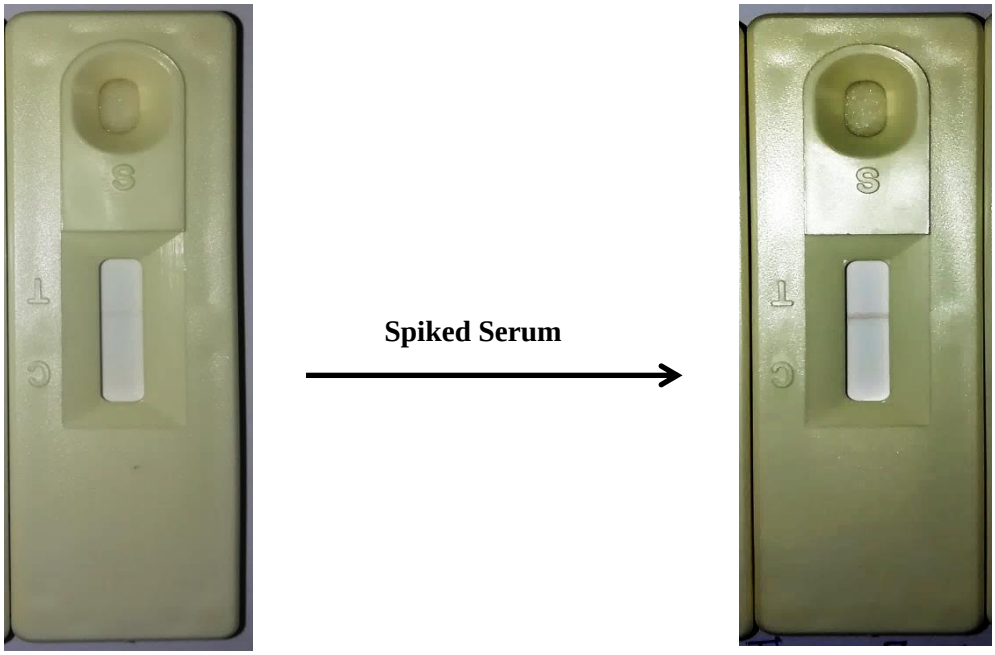

Figure 3.45. Test line evaluation with spiked serum sample

3.6.13 Chemical Indicator Based Control Line

Set up a chemical indicator which can give color change in biological pH (≥7.0). Bromothymol blue, phenolphthalein and Thymol red indicators were trialed for control line establishment.

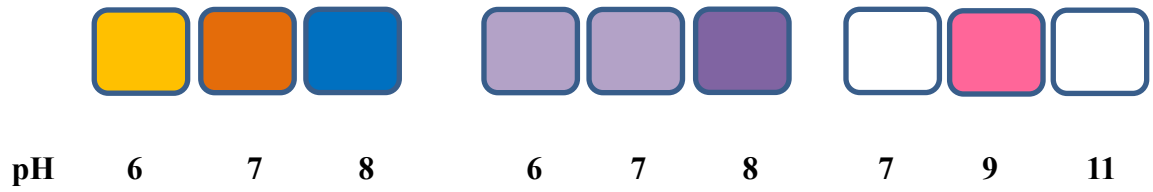

Figure 3.46. Bromothymol blue, Thymol Red and Phenolphthalein color pattern in different pH

3.6.14 Kit assembly with control line and performance check

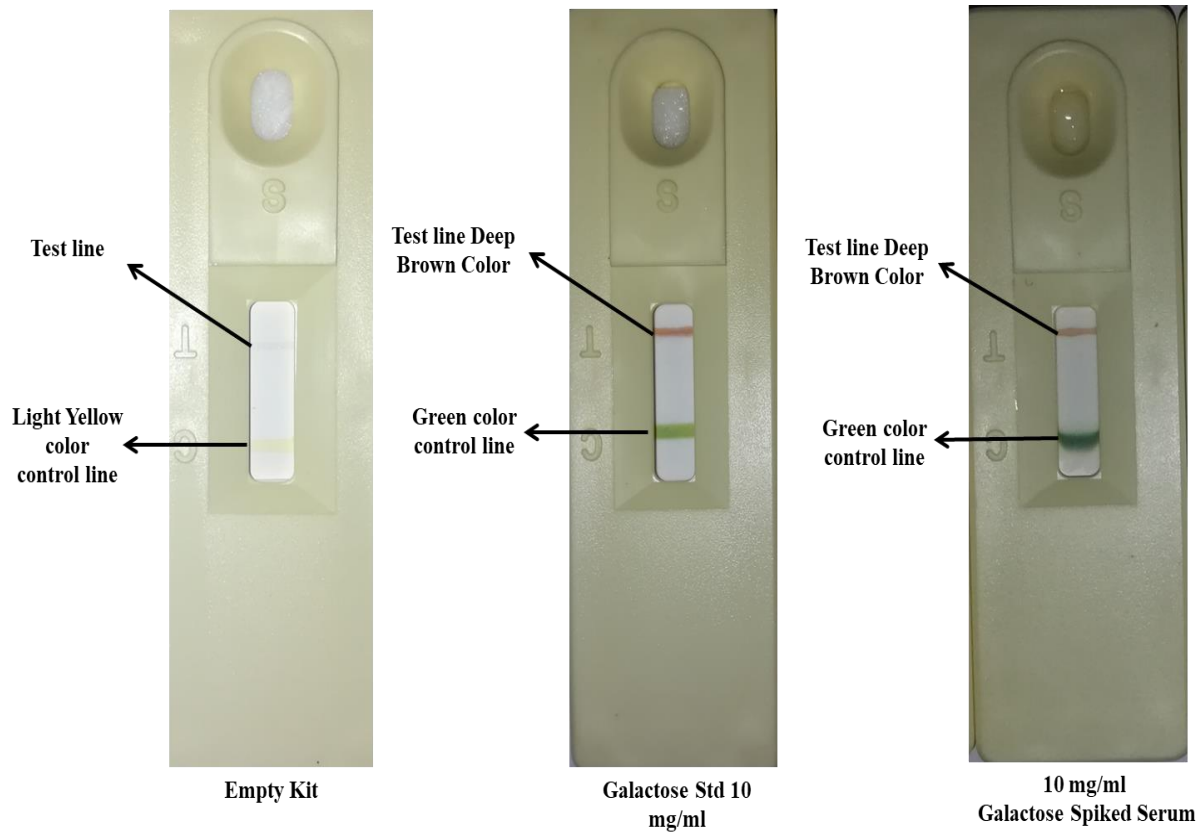

Figure 3.47. Kit evaluation after addition of control line

3.6.15 Empty Final Kit

Final kit was made after dispensing Galactose oxidase enzyme and Bromothymol blue chemical indicator approximately 7 mm apart by auto dispenser machine. Sample and absorbent pad attached with the kit assembled inside the plastic cassette. In the test line very faint green color enzyme line observed and in control line faint yellow line observed.

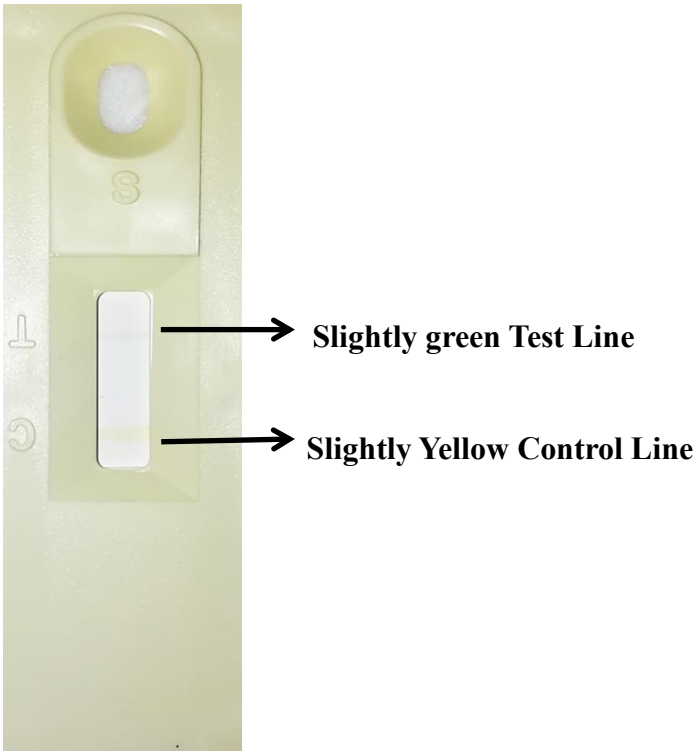

Figure 3.48. Final kit set up with Test line and Control Line

3.6.16 Sample application test

When normal buffer (PBS, pH 7.2) applied test line remained same (2-3 min) and control line became green (20-23 min). When standard Galactose (prepared in PBS, pH 7.2) applied test line became deep green to deep brown color (2-3 min). And control line became green (20-23 min). When serum with spiked Galactose/Galactosemia patient serum sample added, test line became green to brown color (2-3 min) and control line became green (2-3 min).

3.6.16.1 PBS and different concentration of standard Galactose test

Figure 3.49. PBS (1) and variable concentration of Galactose standard [(2) 5 mg/mL, (3) 3 mg/mL and (4) 2 mg/mL] tests

3.6.16.2 PBS & spiked serum Galactose test

Figure 3.50. PBS and spiked serum (5 mg/mL) Galactose test

3.6.17 Stability study of developed kit

After establishing the fundamental parameters, the prototype of Galactosemia kit underwent evaluations to determine their stability. Stability tests were conducted under normal room temperature (25±3 C) conditions and at 2-8°C, as well as through an augmented permanence tests. Performance of the kit at 2-8°C and room temperature (25±3 C) stability assessments took place at intervals of 0th month (initial), 1 month, 3 months, and then every subsequent three months for a total of 12 months. The accelerated stability study involved incubating the kits in a humid environment (60±5%) at temperatures of 37°C at intervals of 0th month (initial), 3 month, and 6 months. Below (table-10, 11 and 12) were the stability test outcome -

Name: Galactosemia Detection Kit

Study Period: June 2022 -June 2023

Test Solution: Galactose spiked serum sample

Interpretation: +++ = Good Intensity, ++ = Less intensity, + = Weak Intensity

Table 3.14. Long Term Stability Data (2-8 ºC)

Test	Stability time points (Months)											
	Long Term Stability Data (2-8 ºC)											
	Initial		1		3		6		9		12	
	T	C	T	C	T	C	T	C	T	C	T	C
Color intensity observation	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++

Table 3.15. Long Term Stability Data (25 ºC ±3ºC; 60RH±5%RH)

Test	Stability time points (Months)											
	Long Term Stability Data (25 ºC ±3ºC; 60RH±5%RH)											
	Initial		1		3		6		9		12	
	T	C	T	C	T	C	T	C	T	C	T	C
Color intensity observation	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++

Table 3.16. Accelerated Stability Data (37 ºC ±3ºC; 60RH±5%RH)

Test						
	Accelerated Stability Data (37 ºC ±3ºC; 60RH±5%RH)					
	Initial		3		6	
	T	C	T	C	T	C
Color intensity observation	+++	+++	+++	++	++	+

CHAPTER 4: DISCUSSION

4.1. Current Methods and Alternate Approaches for Metabolic Profiling

The identification of inborn errors of metabolism (IEM) has long been a challenge, particularly in lower socio-economic conditions where instrumentation and information are scarce. Early diagnosis and treatment of IEMs are critical, as they can help to prevent further complications and provide insights into the underlying molecular mechanisms and pathophysiology. However, traditional methods of IEM identification are often time-consuming and expensive, which can delay the diagnosis and treatment of these conditions. One promising tool for IEM identification is Liquid Chromatography Mass Spectrometry (LC-MS). This technique enables the detection of specific metabolites essential for normal metabolic function, making it an accurate method for identifying inborn errors in metabolism. By using LC-MS, healthcare professionals can quickly and effectively diagnose IEMs and provide timely treatment. Despite its benefits, LC-MS is only sometimes available for clinical use due to its complexity and expense. Challenges associated with sample preparation, instrumentation, and data analysis can also make it difficult to identify IEMs using this technique.

LC-MS use to detect inborn errors of metabolism comes with several drawbacks that need to be considered. One of the main disadvantages is the high cost associated with this technique. LC-MS requires specialized equipment, skilled technicians, and extensive sample preparation, which can make it cost-prohibitive for laboratories and patients in remote areas. Another issue is the complex sample preparation process involved, which includes the extraction, purification, and concentration of the metabolites of interest, making it time-consuming and requiring specialized equipment and expertise. Although LC-MS is highly sensitive, it may not be able to detect low levels of specific metabolites, particularly in patients with mild or intermittent symptoms.

Moreover, the large amounts of complex data generated by LC-MS can be challenging to interpret. They may require significant expertise to analyze accurately; this could lead to inaccurate outcomes, either as a false positive or false negative, if not properly analyzed. Not all laboratories may offer LC-MS testing for inborn errors of metabolism due to the

specialized equipment and expertise required, limiting access to this diagnostic tool for some patients.

Several alternative methods are available, like enzyme assays, genetic testing, and metabolite profiling, which can be employed for this purpose. Enzyme assays involve measuring the activity levels of specific enzymes associated with the metabolic pathway in question. This approach can help to identify enzyme deficiencies that lead to metabolic disorders. Genetic testing, however, involves analyzing the patient's DNA to identify mutations or genetic variations that may be responsible for the metabolic disorder. This technique can be beneficial for identifying inherited metabolic disorders. Metabolite profiling is another alternative method that involves measuring the levels of various metabolites in a patient's biological sample, such as blood or urine. This approach can help to identify patterns of metabolites that are characteristic of specific metabolic disorders. Metabolite profiling can also provide information on the severity of the metabolic disorder and the effectiveness of treatment. The primary objective of this study was to establish an alternative methodology for the analysis of metabolites, specifically amino acids, and galactose, in infants belonging to the Bangladeshi population within a hospital setting. The aim was to explore and determine the cutoff values for amino acids in a healthy population using the developed methodology. Additionally, the study sought to devise a low-cost and user-friendly diagnostic kit capable of detecting diseases related to amino acid and galactose metabolism in the early stages.

By undertaking this research, the intention was to address the need for an improved approach to investigate metabolites in infants, considering the Bangladeshi population's unique characteristics and healthcare context. The development of an alternative methodology holds promise in enhancing the understanding and detection of amino acid and galactose metabolism disorders in this population.

4.2. HPLC Method Development and Standardization

The study focused on establishing the cutoff values for amino acids within a healthy population using the newly developed methodology to accomplish the first objective. This step was crucial in differentiating between normal and abnormal amino acid levels, providing a benchmark for future diagnosis and treatment decisions. By determining these cutoff values, healthcare professionals can better identify potential metabolic disorders and

intervene promptly, thereby mitigating the risk of complications and improving patient outcomes. Moreover, the study aimed to tackle the challenges of cost and accessibility by devising a diagnostic kit that is affordable and easy to use. Developing such a kit was intended to enable early detection of amino acid and galactose metabolism-related diseases in infants, allowing for timely intervention and management. This innovative approach could transform how we diagnose and treat these conditions, especially in resource-limited areas where expensive diagnostic tools may not be easily accessible.

In this study, HPLC-based method development was carried out as an alternate approach to LC-MS. The HPLC method was validated to determine normal amino acid levels in 100 healthy subjects. Subsequently, it was employed to detect amino acidopathies in 100 infants suspected of having inborn errors of metabolism (IEMs). The findings revealed a higher incidence of phenylketonuria (PKU) among the suspected infants, indicating the importance of early detection and intervention for this metabolic disorder. To address this, a PKU diagnostic kit based on phenylalanine dehydrogenase enzyme was developed, ensuring a detection level of 4mg/dL. Additionally, galactose metabolism-related investigation was done at the genomic level, and a galactosemia detection kit was developed using galactose dehydrogenase enzyme. Mutation analysis was conducted on 14 patients suspected of Galactosemia, identifying a missense mutation with potential implications for splicing processes.

Method validation is an essential part of method development, and method validation aims to confirm that the developed method is suitable for its intended purpose as described **[53]**. Therefore, the described method in this study has been extensively validated in terms of system suitability, specificity, linearity, accuracy, precision, and robustness. To investigate the system suitability of the method HPLC system was prepared and ensured that the HPLC system was clean and in proper working condition. Checked the integrity of the column, mobile phase and detector. Chromatographic conditions were set up by following the preparation of the mobile phase and setting up the appropriate flow rate, column temperature, and detection wavelength according to the HPLC method being validated. An individual and combined standard amino acid mixture was used for system suitability purposes, which closely mimics the expected sample matrix **(Figure 3.1, 3.2 and 3.3)**. During system suitability assessment, a 1 mL injection volume was given in each run, and multiple replicate injections were made and assessed that the system was reproducible. Chromatographic parameters were observed and recorded considering retention times, peak shape, width, and

resolution. System suitability parameters like tailing factor, theoretical plates, and resolution were calculated from obtained chromatograms, and found that the developed method is suitable for analytical purposes.

For specificity evaluation purposes, standard amino acid mixture retention times were compared in **Table 3.1** and **Figure 3.4**. The provided data presents the results of a specificity evaluation, comparing the retention times of a standard amino acid mixture. The experiment was conducted three times using the same methodology and process conditions to ensure consistency. The table summarizes the average retention times obtained for each amino acid and the relative standard deviation (RSD) as a measure of precision. The data shows that the retention times for the amino acids vary across the three runs. However, the differences between the individual measurements are relatively small, as indicated by the low RSD values ranging from 0.23% to 0.66%. This suggests that the methodology and process conditions used in the experiment have provided consistent and reproducible results. Analyzing the average retention times, several observations can be made. It was observed that aspartic acid had an average retention time of 0.654, while glutamic acid had a slightly higher average retention time of 0.964. Serine exhibited an average retention time of 2.826, and Histidine followed closely with an average retention time of 3.519. Glycine, threonine, and arginine showed average retention times of 3.670, 3.791, and 4.243, respectively.

Moving further, alanine, tyrosine, and cysteine had average retention times of 4.482, 5.133, and 5.812, while valine, methionine, and Tryptophan exhibited average retention times of 6.100, 6.191, and 6.623, respectively. Phenyl Alanine and isoleucine had average retention times of 6.818 and 6.940, while leucine and lysine showed average retention times of 7.247 and 7.477. It can be concluded that each amino acid in the standard mixture has a specific retention time based on these results. These retention times can be utilized for identification and quantification purposes in analysis. The low RSD values indicate the reproducibility and reliability of the methodology, allowing for consistent retention time measurements. RSD distribution has been showed in **Figure 3.4**. The average retention times of the standard amino acid mixture (STD) with those of commercial samples of DMEM (Dulbecco's Modified Eagle Medium) have been compared and shown in **Table 3.2**. The table also includes the percentage difference between the two sets of retention times. Analyzing the data, it is evident that there are variations in the average retention times between the standard amino acid mixture and the DMEM samples. The following observations are made based on the percentage differences:

1. Aspartic Acid: The DMEM sample showed a lower average retention time (-10.7%) than the standard amino acid mixture.
2. Glutamic Acid: The retention time for Glutamic Acid was the same (0.0%) for both the standard amino acid mixture and the DMEM samples.
3. Serine: The DMEM sample exhibited a slightly lower average retention time (-1.7%) than the standard amino acid mixture.
4. Histidine: The average retention times for Histidine in the standard amino acid mixture and the DMEM samples were similar, with a negligible difference (+0.3%).
5. Glycine: The DMEM sample showed a slightly higher average retention time (+1.3%) than the standard amino acid mixture.
6. Threonine and Arginine: Threonine and Arginine exhibited almost identical average retention times (-0.3%) in both the standard amino acid mixture and the DMEM samples.
7. Alanine: The DMEM sample had a higher average retention time (+12.0%) than the standard amino acid mixture.
8. Tyrosine, Cysteine, Valine, Methionine, Phenyl Alanine, Iso-Leucine, Leucine, and Lysine: All these amino acids showed lower average retention times in the DMEM samples compared to the standard amino acid mixture, with percentage differences ranging from -2.0% to -4.3%. Among them, Tryptophan had the most considerable difference, with a percentage decrease of -13.0%.

Based on that data, it can be evaluated that there are variations in the retention times between the standard amino acid mixture and the commercial samples in DMEM. Several factors, including differences in DMEM composition and interactions with other components, can account for these variations.

Linearity data obtained for a standard amino acid mixture shown in **Table 3.3**. The linearity was established by preparing five standard solutions with varying concentrations of each amino acid and plotting the peak area (y) against the amino acid concentration (x). The data was then subjected to linear least squares regression analysis to obtain each amino acid's slope, intercept, and coefficient of determination (R2). The linearity equation used in this study is $y = bx + a$, where 'a' represents the intercept and 'b' represents the slope. According

to the reference provided, for good linearity, 'a' should be zero within the 95% confidence limits, and the coefficient of determination (R2) should be greater than 0.98 **[53, 54]**. Upon analyzing the results, it is evident that the majority of the amino acids exhibit excellent linearity data. The coefficient of determination (R2) for all the amino acids, except for Cysteine and Tryptophan, is reported as 0.9999, indicating a strong correlation between the peak area and the amino acid concentration. The slope (b) and intercept (a) values for each amino acid represent the relationship between the peak area and the concentration. The slope indicates the change in peak area per unit change in concentration, while the intercept represents the peak area when the concentration is zero. Based on the provided data, the slope values range from 0.508 to 2.198 × 103, and the intercept values range from -2.63 × 104 to 17.35 × 104. These values are specific to each amino acid and reflect the individual response characteristics of the analytical method used. Therefore, the linearity data obtained for the standard amino acid mixture shows a strong correlation between the peak area and the concentration of amino acids. The high coefficient of determination (R2) values, mostly reported as 0.9999, indicate excellent linearity. This suggests that the method is suitable for quantifying the amino acids in samples within the specified concentration range.

The obtained data in **Table 3.4** represents the results of the analytical method's accuracy assessment by analyzing a commercial media reference material. The percent recovery was calculated for each amino acid by comparing the found concentration in the reference material to the actual concentration provided by reference data. The accuracy of an analytical method measures how closely the results align with the true value. It is typically expressed as the percent recovery, indicating the agreement between the found and actual values. In this case, the percent recovery was determined by analyzing the reference material three times in three days and calculating the average percent recovery. Each amino acid shows a different percent recovery upon analysis. The best percent recovery was obtained for serine, with 99.06%, indicating a close agreement between the found and actual concentrations. On the other hand, aspartic acid had the lowest percent recovery of 90.92%, indicating a slight deviation from the actual value.

The average percent recovery considering all the amino acids was 99.10%, which falls within the acceptable range of 90-110% as stated **[53, 54]**. This suggests that the analytical method used in this study demonstrates overall good accuracy, with most amino acids showing satisfactory percent recovery. Notably, percent recovery values above 100% indicate an overestimation of the actual concentration, while values below 100% indicate an

underestimation. In this case, threonine had the highest percent recovery of 108.51%, indicating a slight overestimation of its concentration, while aspartic acid had the lowest percent recovery of 90.92%, indicating an underestimation. Hence, the accuracy assessment of the analytical method indicates that it provides reliable and accurate results for quantifying amino acids in the commercial media reference material. The average percent recovery within the acceptable range suggests that the method can accurately determine the concentrations of amino acids in samples.

The precision of the analytical method was evaluated by measuring both repeatability and intermediate precision. Repeatability refers to the variation observed when the same standard is analyzed multiple times in a row, while intermediate precision accounts for the variation introduced by different factors, such as different days or operators. The repeatability of each amino acid was evaluated by consecutively derivatizing and injecting the standard six times, with the relative standard deviation (RSD) being calculated for each. The obtained RSD values, as shown in **Table 3.5**, ranged from 0.30% to 1.53%. The precision RSD distribution is shown in **Figure 3.5**. For repeatability, it is generally considered acceptable if the RSD is below a certain threshold, often set at 5% **[53, 54]**. In this case, all the RSD values obtained for the amino acids were well below 5%, indicating good repeatability of the analytical method. The RSD values ranged from 0.30% for arginine to 1.53% for threonine, with an average RSD of 0.86% for all amino acids. These low RSD values suggest that the variation in the measurements obtained by repeating the analysis multiple times was minimal. The precision of the method was consistent, and the results were reproducible. The small RSD values indicate that the method is reliable for quantifying amino acids in samples. It is important to note that lower RSD values indicate better precision, as they indicate less variability in the measurements. The obtained RSD values in this study fall within the acceptable range, indicating that the method can provide precise and reproducible results. Based on the repeatability results, it can be inferred that the method demonstrates good precision overall. The method exhibited consistent and reproducible results for the quantification of amino acids. These findings support the reliability of the method and suggest that it can be used confidently to analyze amino acids in samples.

The determination was evaluated by repeating the entire analytical procedure on three different days for intermediate precision assessment of the amino acid. The same standard sample was hydrolyzed, derivatized, and injected separately on each day. The relative standard deviation (RSD) was then calculated for each amino acid to assess the variation

introduced by different days. The RSD values obtained for each amino acid are shown in **Table 3.6**. It can be observed that the RSD values range from 0.23% to 0.66%, with an average RSD of 0.36% for all amino acids. When evaluating intermediate precision, it is generally considered acceptable if the RSD is below a certain threshold, often set at 10% **[53, 54]**. In this case, all the RSD values obtained for the amino acids are well below 10% (**Figure 3.6**), indicating good intermediate precision of the analytical method. The RSD values range from 0.23% for Histidine to 0.66% for aspartic acid. The low RSD values suggest that the variation introduced by different days in the analytical procedure is minimal. The method demonstrates consistent performance across multiple days, indicating its robustness and reliability. The small RSD values indicate that the method can provide precise and reproducible results even when performed on different days. These findings indicate that the method exhibits good intermediate precision, as the variation between different days is minimal. The RSD values obtained are below the acceptable threshold, supporting the method's reliability for determining amino acids.

After validating the developed method, the final retention order was fixed and shown in **Table 3.7**. This developed method was used for the next phase of the study. Blood serum from 100 healthy volunteers enrolled in this study was collected and analyzed through the developed method to generate amino acids cutoff range for the Bangladeshi population. Obtained cutoff data is shown in **Table 3.8**. Except for Histidine, methionine, and aspartic acid, all other 13 amino acids have established minimum and maximum cutoff ranges. IEM suspected 100 patient samples analyzed through the developed method and obtained positive samples distribution shown in **Table 3.9** and **Figure 3.7**. Distribution data analysis of the positive sample distribution reveals the varying degrees of presence and potential significance of different amino acids in the study context. It highlights that glutamic acid, phenylalanine, and serine have relatively higher representation, while leucine, arginine, tyrosine, and lysine exhibit lower counts.

4.3. Phenylalanine detection kit development

As found that the prevalence of phenylalanine was higher in the study population; therefore, an easy-to-use diagnostic kit development was carried out considering the following facts:

1. Phenylalanine is metabolized by an enzyme called phenylalanine hydroxylase, and it is an essential amino acid. However, a genetic condition called phenylketonuria (PKU) may occur where some individuals lack or have a deficiency in this enzyme. This leads to an accumulation of phenylalanine in the body, which can be toxic and cause intellectual disabilities and other health complications. Therefore, studying phenylalanine levels in the population can help identify individuals with PKU and facilitate early intervention and treatment. If an individual cannot metabolize phenylalanine but consumes it through food, unknowingly will cause toxicity in the body. So that regular blood level checkups will be an option to measure the level of phenylalanine in the blood, and an easy-to-use diagnostic kit can solve this problem in regular life.

2. As phenylalanine is a precursor for the synthesis of neurotransmitters, including dopamine, norepinephrine and epinephrine. These neurotransmitters are crucial in brain function, mood regulation, and overall mental well-being. Therefore, early diagnosis of phenylalanine levels in infants will allow physicians to overcome such deficiencies in early life and can prevent irreversible neurological problems.

3. Moreover, by monitoring phenylalanine levels in the population, healthcare professionals can identify individuals at risk for phenylalanine metabolism-related conditions, such as hyperphenylalaninemia, neurodevelopmental disorders, or psychiatric disorders. Identifying and addressing these conditions early can prevent or lessen their impact.

4. For individuals with PKU or other disorders related to phenylalanine metabolism, regular monitoring of phenylalanine levels is essential to assess the effectiveness of treatment interventions, such as dietary modifications or enzyme replacement therapy. Population studies can help identify trends and patterns in treatment outcomes and guide healthcare professionals in optimizing management strategies.

5. Studying phenylalanine in a population provides valuable insights into genetic disorders, dietary patterns, neurotransmitter synthesis, and overall health. It aids in the early identification of individuals at risk, facilitates appropriate interventions, and contributes to developing personalized treatment approaches for metabolic and neurological conditions associated with phenylalanine metabolism.

In the first step of paper-based kit development, liquid reaction stated in [**49]** performance was evaluated and minimal enzyme concentration was checked. Varied enzyme concentration ranging from 10-40 U/mL (**Figure 3.8**) was tried, and varying substrate amounts were analyzed from 10-70 mg/dL (**Figure 3.9**). Absorbance maximum were checked, and all the reactions gave the highest absorbance at 280 nm wavelength (**Figure 3.10**), this indicates that the reaction is suitable for phenylalanine detection purpose. Required buffer flow was checked in different paper materials but was not promising due to the slow flow rate. Therefore, the static reaction model was investigated on glass fiber pads. It was found that glass fiber pad entraps color compound well and can display reaction results more intensely than liquid reaction. To make the kit convenient to use, two layers of glass fiber pad were used (**Figure 3.11**), soaking with the same buffer with two different pH as per the adopted publication [**49].** As per the enzyme data sheet phenylalanine dehydrogenase enzyme's optimum working condition is at higher pH; therefore, top glass fiber pad was fixed in this study to harbor the enzyme in the primary buffer. Whereas in the bottom glass, fiber pad substrates were fixed at lower pH to keep stabilize the substrates in dry condition. The kit assembly developed in this study is simplified from the assembly demonstrated in the adopted publication [**49].** New assembly makes the kit preparation more straightforward and more convenient for use and overcomes the utilization of an expensive laser-cutting machine to prepare the parts for the kit. As per development in this study, typical paper-cutting machines used for an inexpensive diagnostic kit preparation can be utilized to get two layers of glass fibers with minimum intervention.

Temperature-controlled baking was introduced in this study, and it found that 60ºC drying of buffer-soaked glass pads gave better results than 40ºC (**Figure 3.12**) will make the entrapment of buffer and substrate materials in glass fiber pads. During the test run, this dried buffer can be reconstituted with normal water or applied directly with blood serum/plasma. Finally, the developed kit's detection limit was tested by using a spiked buffer sample and serum sample ranging from 2-6 mg/dL. Also, the observation time was investigated and finally fixed for 15 min (**Figure 3.13** and **3.14**). It was found that the kit can detect a minimum 2 mg/dL level of phenylalanine level both in spiked buffer and serum samples. A very faint color was observed for 1 mg/dL level, but the kit can detect phenylalanine well for 2 mg/dL level. In the overall kit development process, 40U/mL enzyme was used. A validated color scale will be the next development phase for the kit to quantify the level of phenylalanine conveniently.

4.4. Mutational Study of Galactosemia disease

Another metabolic disease, Galactosemia study has been conducted in this study. Galactosemia is a genetic disorder that impairs the body's ability to metabolize galactose, and it is a rare condition, a sugar found in milk and dairy products. Undetected and untreated, Galactosemia can lead to severe complications, including liver damage, intellectual disability, and cataracts. Nevertheless, studying Galactosemia in early life allows for early detection through newborn screening programs. This enables prompt intervention and treatment to minimize or prevent the potential health risks associated with the condition. Galactosemia is a model for studying other metabolic disorders and providing insights into broader aspects of human metabolism. Investigating the underlying mechanisms of Galactosemia and its impact on various body systems can contribute to a deeper understanding of metabolic pathways, gene regulation, and potential therapeutic targets. These insights may have implications for developing treatments or interventions for other metabolic disorders. So, in this study, we consider this rare genetic disease as a metabolic study model for the Bangladesh context.

Understanding Galactosemia in early life helps researchers and healthcare professionals optimize treatment strategies. Galactosemia is typically managed through a strict galactose-free diet, which involves avoiding lactose-containing foods and following nutritional alternatives. By studying the condition in early life, researchers can gather data on the effectiveness of different treatment approaches, develop dietary guidelines, and improve the long-term outcomes for individuals with Galactosemia. The prevalence of Galactosemia in Bangladesh is not well-documented, and specific data on the prevalence of Galactosemia in the country is limited. Galactosemia is a genetic disorder that is rare and affects people worldwide. However, its prevalence differs among various populations. With specific prevalence data for Bangladesh, it is easier to accurately estimate the prevalence of Galactosemia in the country. However, it is essential to note that Galactosemia is a genetic disorder, and its occurrence is influenced by factors such as consanguineous marriages, ethnic background, and regional genetic variations. Population-specific studies and genetic screenings would be necessary to determine the exact prevalence of Galactosemia in Bangladesh. To improve our understanding of the prevalence of Galactosemia in Bangladesh, it is essential to promote awareness about the condition among healthcare professionals, implement newborn screening programs, and encourage research initiatives that focus on studying the prevalence and genetic characteristics of Galactosemia within the Bangladeshi population.

Our study aimed to investigate the molecular heterogeneity of Galactosemia by focusing on the GALT gene and specific regions from exon 6 to exon 10. Galactosemia is known to be caused by various mutations in the GALT gene, and some mutations are specific to certain populations. To accomplish this, we designed primers targeting the hot spot region of interest, resulting in a product size 942bp. We optimized the PCR conditions for this product. Subsequently, we performed mutational analysis on fourteen samples from individuals suspected of Galactosemia, as indicated by elevated levels of non-glucose-reducing substrate. The gene product identification process has been developed, and an estimated 942bp products were found in suspected samples (**Figure 3.15, 3.16** & **3.17**). We also sequenced the GALT gene in these samples, and our analysis revealed the presence of a silent mutation (Exon 7 c.652 C>T, p.L218L) in the exon 7 of the GALT gene in four out of the fourteen suspected samples (**Figure 3.18** & **3.19**). This finding suggests the occurrence of a specific mutation within the Bangladeshi population, contributing to the molecular heterogeneity of Galactosemia in this region. Identifying specific mutations in the GALT gene is crucial for understanding the genetic landscape of Galactosemia and its implications for diagnosis, treatment, and genetic counseling. This study sheds light on the molecular mechanisms underlying Galactosemia in Bangladesh and emphasizes the significance of performing targeted mutation analysis in populations with distinct genetic backgrounds.

Investigation into the GALT gene and its splicing site alterations has shown that even silent mutations can significantly affect gene expression and protein function. In our study, we conducted a splicing site comparison between wild-type and mutation models, specifically focusing on the silent mutation (Exon 7 c.652 C>T, p.L218L) found in exon 7 of the GALT gene (**Figure 3.20** and **3.21**). While silent mutations were traditionally considered to be functionally neutral since they do not alter the amino acid sequence of the encoded protein, recent evidence suggests that they can still impact splicing patterns. During gene expression, introns are removed and exons are joined to form the final mRNA transcript through splicing. Mutations near splicing sites can disrupt this process, leading to alternative splicing patterns or aberrant exon inclusion/exclusion. Our investigation revealed that the silent mutation in exon 7 of the GALT gene may induce changes in the splicing site (**Table 3.10, 3.11, 3.12** and **3.13**). These alterations in splicing patterns can ultimately influence the GALT gene and its protein expression. Numerous studies have demonstrated the importance of proper splicing for normal gene function. Splicing site alterations can result in aberrant mRNA transcripts, leading to truncated or non-functional proteins. In the case of Galactosemia, where the GALT

enzyme is crucial for galactose metabolism, any disruption in the splicing process may compromise enzyme activity and functionality. These findings emphasize the significance of evaluating the potential effects of silent mutations on splicing, even if they do not directly alter the protein sequence.

In conclusion, our GALT gene-related study proves that the silent mutation identified in exon 7 of the GALT gene may induce splicing site alterations. Despite the mutation being silent, these alterations can have implications for the GALT gene expression and the resulting GALT protein. Further research is needed to elucidate the precise consequences of these splicing alterations and their impact on GALT enzyme activity and galactose metabolism in individuals with Galactosemia.

4.5. Galactosemia detection kit development

In this study, 14 samples were examined from individuals suspected of Galactosemia, and four galactosemia gene mutations were identified among them. This finding highlights the presence of galactosemia-related metabolic problems within the studied population. However, it is important to note that our sample size was relatively small, and the study was limited to a specific group of individuals. To gain a comprehensive understanding of the prevalence and spectrum of galactosemia-related metabolic disorders in Bangladesh, a large-scale population-based study is warranted. Such a study would allow for identifying a broader range of mutations and provide insights into the overall burden of Galactosemia in the population. This data would be invaluable for healthcare planning, implementing preventive measures, and developing targeted interventions. In light of the challenges associated with population-wide screening in resource-limited settings like Bangladesh, we aimed to develop an easy-to-use lateral flow diagnostic kit for Galactosemia. Such a kit would offer several advantages, including convenience, affordability, and suitability for use in rural areas with limited access to sophisticated laboratory facilities. In our study, we envision the development of a lateral flow diagnostic kit that can accurately and efficiently detect galactosemia-related genetic mutations. The kit should be user-friendly, requiring minimal technical expertise to perform the test.

It should be cost-effective, allowing many healthcare facilities and individuals to access it. Furthermore, the kit should be designed for convenient use in rural areas, considering factors

such as stability under varying environmental conditions and ease of result interpretation. Implementing such a population screening program using an easy-to-use lateral flow diagnostic kit can significantly improve the early detection of Galactosemia and related metabolic disorders in Bangladesh. This would allow for timely intervention and the implementation of appropriate management strategies to mitigate the negative health effects of Galactosemia.

Adopted enzyme-based liquid reaction [**50]** was transferred to paper-based kit assembly. Individual reagents and reagent-enzyme composition absorbance pattern has been observed (**Figure 3.22**). Enzyme solution and HRP-Enzyme solution have no absorbance, but after adding chromogen solution (ODN & ABTS), it has sharp absorption at 380nm. Individual chromogen material response was checked to investigate the color-changing time in liquid reaction (**Figure 3.23**). Color change happened for ODN (3, 3- Dimethoxybenzidine) within 2 minutes (**Figure 3.24**), OPD (O-phenylene Diamine) color change happened within 15-20 minutes (**Figure 3.25**), ABTS (2.2 –Azino-Bis-(3-ethylbenzThiazoline-6-Sulphonate) color change happened instantly (**Fig 3.26**) and TMB (3,3,5,5- Tetramethylbenzidine) color change happened instantly (**Figure 3.27**). As OPD and TMB color change happened instantly and those have similar color outcomes (yellow and yellow-orange) that will interfere with the control line coloring agent, chromogen solution ODN and ABTS were finalized. To find out the optimum Galactose Oxidase enzyme concentration, variable enzyme concentration ranging from 0.005U/ µL to 0.5 U/ µL was investigated (**Figure 3.28, 3.29, 3.30 and 3.31**). From multiple liquid reaction run results, it was finalized that the optimum enzyme concentration was 0.25 U/ µL, which can give variable color patterns based on the amount of substrate (Galactose) applied from 5mg/mL to 50mg/mL (**Figure 3.31**). The absorbance pattern was investigated after finalizing enzyme concentration and chromogen mix. It was found that a similar absorbance pattern (320nm-360nm) was developed for variable substrate concentrations ranging from 1mg/mL to 15mg/mL shown in **Figure 3.32, 3.33, 3.34** and **3.35**.

Generally, for lateral flow kit purposes, a nitrocellulose membrane is used; therefore, in this study, initial work starts with a nitrocellulose membrane and liquid reaction transfer to this membrane and found a positive outcome. Paper kit assembly shown in **Figure 3.36** and the initial response test was performed and the response result shown in **Figure 3.37** using a handmade kit assembled with a sampling pad (glass pad) and absorbent pad. Later machine

was used to dispense, the kits were cut, the hand-assembled kit was prepared, and a repetition test was performed to check the reproducibility of the test (**Figure 3.38**). It was observed that within 25 sec, a color reaction started to appear (**Figure 3.39**). The initial reaction field between the sample pad and the absorbent pad is smaller, so the reaction took place much faster. To make the reaction result more intense on paper, different types of paper (nylon 0.22 µm, nylon 0.45 µm, nitrocellulose 0.45 µm, and nitrocellulose 135) were tried (**Figure 3.40**). Among those paper types, it was found that nylon papers showed sharper bands than nitrocellulose paper. Later given the focus on two types of nylon paper, 0.22 µm, and 0.45 µm, nylon 0.22 µm showed a better intense band than 0.45 µm nylon paper (**Figure 3.41**). Based on multiple trials, nylon 0.22 µm paper was finally fixed for the rest of the development work. As galactose enzyme optimum working temperature is 35ºC **[55]**. Therefore, in this study, paper baking temperature was kept within 37°C. To investigate kit performance for other reducing sugar like glucose and galactose was tested together in two types of nylon paper, 0.22 µm, and 0.45 µm, and it was found that the kit is specific for galactose and not applicable for glucose (**Figure 3.41**).

A suitable plastic casing is used to harbor the machine-cut paper kit. The selected cassette is a commercially available plastic product which a length of approximately 70mm, a width of 20 mm, and a test window of around 16 mm (**Figure 3.42**). After assembling the paper kit in the cassette, normal water flow was checked, and the full water travel time was around 4 minutes (**Figure 3.43**). Later standard galactose was applied to check the kit's performance. In the test line position, a deep green band appeared which, after 5 minutes, turned into a deep brown color that indicated the kit could detect galactose (**Figure 3.44**). Kit performance was checked with a spiked human blood serum sample with 10mg/mL galactose, as the serum flow rate is slower than water only (**Figure 3.45**). Therefore, it took around 7 minutes to reach the absorbent pad. To develop the control line in the kit, multiple chemical indicators (bromothymol blue, thymol red, and phenolphthalein) were investigated as per the color change of the indicators in pH of ≥7.0 (**Figure 3.46**). Only bromothymol blue was found to be promising as it can change color to a different color than the test line. Therefore, the user can clearly distinguish the test line from the control line. Bromothymol blue change color from yellow to green when a human serum sample is applied at biological pH but thymol red change color from light yellow to deep yellow, which is hard to distinguish, and phenolphthalein change color from colorless to pink at higher pH than biological pH [**51].** Kit assembly with control line resembles the commercial lateral flow kit device (**Figure 3.47** and

3.48); tested with Galactose standard and Galactose spiked serum sample (**Figure 3.47**). Kit performance was evaluated by application of buffer solution (PBS), standard galactose solution, and spiked human serum sample (**Figure 3.49** and **3.50**). For only buffer application, color change happened only in the control line, indicating the kit performance's authenticity. For standard galactose and serum spiked with galactose samples, in both cases, the color change took place in both the test line and control line. For standard galactose, color change in the control line took a higher time than the serum sample because the optimum buffer pH appearance in the control line position is slower than in the serum sample for the standard Galactose case (**Figure 3.50**). Therefore, in the case of a serum sample kit result can be interpreted within 5 minutes. This faster test outcome will be beneficial for actual field application, reduce test time, and provide an opportunity to handle many samples at a time.

A comprehensive stability evaluation was made for the developed PKU kit for one year; where real-time and accelerated each condition was evaluated. During stability evaluation kits that were stored at 2-8°C, no significant signal decay was observed during the testing period. At room temperature, signal intensity was found to be weaker after the 12th month. However, at 37°C, the signal intensity decreased after the second month and more decreased at six months, resulting in weaker color intensity for samples shown in **Table 3.14, 3.15** and **3.16**.

The advent of an advanced High-Performance Liquid Chromatography (HPLC) method in amino acidopathies has proven to be a significant breakthrough, offering distinct advantages over conventional techniques. In particular, the HPLC method exhibits superior efficiency by markedly reducing analysis time compared to existing methodologies, rendering it a highly appealing choice for amino acid detection. The HPLC method showcases remarkable capability in detecting up to 17 amino acids. Moreover, a promising avenue exists for further refinement of this method, as three additional amino acids can be seamlessly incorporated, culminating in a comprehensive and all-encompassing test package. Such an expanded analytical framework holds tremendous value for clinicians and researchers studying amino acid metabolism. Establishing an amino acidopathies detection laboratory utilizing the HPLC method will be an easily achievable endeavor that is both cost-effective and logistically straightforward compared to the cumbersome and costly Liquid Chromatography/Mass Spectrometry (LC/MS) counterparts. The deployment of an HPLC instrument endowed with auto-dispense capacity adequately fulfills the instrumental requirements for setting up an amino acidopathies detection lab. The initial preparatory steps, entailing the programming of

the test run, prove to be a one-time undertaking, imparting an optimal level of convenience and seamless workflow integration. In addition to its technical advancements, this study contributes significantly to establishing a healthy cutoff range for amino acids within the Bangladeshi population. While the precise calibration of this cutoff range necessitates further refinement through extensive population-based studies, the preliminary findings furnish valuable insights that serve as a solid foundation for improved diagnostic and therapeutic interventions targeting metabolic acidopathies prevalent in the Bangladeshi population. This study's concomitant development of two purpose-designed kits fosters enhanced convenience and affordability, effectively facilitating metabolic acidopathies research. The prototype kit specifically designed to address amino acid metabolic irregularities, namely the Phenylketonuria (PKU) kit, emerges as an economical alternative to its original counterpart, as it solely employs glass fiber-based components, eliminating the need for costly ancillary materials. However, continued efforts to refine and streamline this prototype kit are indispensable to impart a polished commercial appeal. A proposed refinement involves the integration of a color measurement chart, allowing end-users to interpret test outcomes at an individual level seamlessly. Notably, the current iteration of the kit boasts an impressive lower detection limit of 2 mg/mL. However, further refinement is possible to lower the detection level below 1 mg/mL.

On the other hand, Galactosemia, an inherited metabolic disorder, manifests due to mutations in the GALT gene, which encodes the galactose-1-phosphate uridylyltransferase enzyme. The meticulous examination of galactosemia mutations specific to this population assumes paramount importance, as it imparts a profound understanding of the prevalence and spectrum of mutations afflicting individuals of Bangladeshi descent. Indeed, a robust comprehension of the genetic landscape governing galactosemia mutations underpins the accurate diagnosis, genetic counseling, and implementation of tailored management strategies. The comprehensive dissection of galactosemia mutations via a population-based study provides invaluable insights into the specific genetic aberrations fueling the onset of Galactosemia within the Bangladeshi population. The developed galactose detection kit can detect 2 mg/mL; further refinement can reach below 1 mg/mL. Considering population-based investigations, plasma-free galactose levels typically surpass the threshold of 10 mg/dL and, in certain instances, may exhibit a wide range of 90-360 mg/dL (5-20 mmol/L).

Moreover, experimental findings reveal that an elevated concentration of galactose oxidase enzyme enables the detection of concentrations below 100 mg/dL. However, it is essential to

underscore that no comprehensive population-based study has yet been conducted to determine appropriate galactose cutoff ranges tailored to the Bangladeshi population. Consequently, establishing definitive galactose cutoff ranges constitutes an avenue for future exploration. An elaborate examination of the genetic architecture governing Galactosemia is poised to unravel the unknowable realm of alternate splicing within the GALT gene. The profound understanding of the alternate splicing patterns adorning the GALT gene disseminates insights into the underlying molecular reinforcements governing galactosemia pathogenesis. A meticulous dissection of the alternate splicing of the GALT gene in Galactosemia is poised to illuminate the potential consequences of splicing variants on enzymatic function and resultant metabolic instability. By interpreting the details of alternate splicing, researchers can poise to unravel the intricate molecular mechanisms dictating Galactosemia's pathogenic trajectory.

Moreover, such revelatory insights pave the way for developing tailored therapeutic interventions to rectify aberrant splicing patterns, restore regular gene expression, and reinstate metabolic equilibrium. In conclusion, the development of the HPLC method, PKU, and Galactosemia kit impose a substantial advancement in the realm of metabolic disorders. Future research undertakings should concentrate on refining kits, including incorporating a color measurement chart and establishing precise cutoff ranges for phenylalanine and galactose levels explicitly tailored to the Bangladeshi population.

CHAPTER 5: CONCLUSION

The present study represents a significant stride in addressing amino acidopathies in Bangladesh, introducing a cost-effective HPLC method for large-scale application and an innovative paper-based kit for identifying elevated Phenylalanine levels in IEM patients. The mutational analysis of Galactosemia underscores the critical role of alternate splicing regulation in protein functionality. The development of a Galactosemia kit holds promise for enhancing patient management and facilitating widespread population studies through the deployment of both PKU and Galactosemia kits across the country. This comprehensive approach marks a valuable contribution to advancing diagnostic capabilities and improving healthcare outcomes in the context of metabolic disorders in Bangladesh.

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ETHEICAL CLEARANCE

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Professor Dr. Sharif Akhteruzzaman
Department of Genetic Engineering and Biotechnology
University of Dhaka
Dhaka-1000.

Sub: Ethical Clearance of Research Proposal entitled "Innovative approaches for development of diagnostic methods for detecting inborn errors of metabolism and genetic disorders using high-throughput metabolomic profiling as well as monoclonal antibody-based concept (CP-4029)".

Dear Dr. Akhteruzzaman,

I am happy to inform you that your proposal entitled **"Innovative approaches for development of diagnostic methods for detecting inborn errors of metabolism and genetic disorders using high-throughput metabolomic profiling as well as monoclonal antibody-based concept (CP-4029)"** was placed in the Ethical Clearance Certificate for Human Participants Committee meeting held on 16.05.2017 and has been approved for conducting your research project.

I wish for the success of your research project.

Professor Dr. M. Imdadul Hoque
Dean, Faculty of Biological Sciences
University of Dhaka
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paper text:

ABSTRACT This study aimed to address the challenges associated with identifying inborn errors of metabolism (IEM) in resource-constrained environments, focusing on amino acid and galactose metabolism disorders in the Bangladeshi infant population. Liquid Chromatography Mass Spectrometry (LC-MS) is a robust diagnostic tool but faces limitations due to cost, complexity, and accessibility. Therefore alternative approaches, including enzyme assays, genetic testing, and metabolite profiling, were explored for effective IEM detection. In pursuit of an affordable diagnostic solution, a low-cost kit was developed for early detection and intervention of amino acid and galactose-related disorders. A validated HPLC-based method was established for accurate quantification of amino acids. The method exhibited strong linearity (R2 = 0.9999) and high accuracy (99.10% average recovery), establishing its suitability for amino acid analysis. Precision was confirmed through repeatability and intermediate precision tests, with low RSD values (0.30% to 1.53% and 0.23% to 0.66%, respectively). Amino acid cutoff ranges were determined for the Bangladeshi population using the validated HPLC method. Noteworthy variations in amino acid counts were observed in positive samples, emphasizing the importance of accurate cutoff values. A lateral flow paper-based diagnostic kit was designed for phenylalanine detection, offering a cost- effective means for phenylketonuria (PKU) monitoring. The kit demonstrated a minimum detection limit of 2 mg/dL, catering to PKU therapy needs. The study also concentrated on Galactosemia as a model disorder, underlining the necessity of early intervention. Molecular analysis of the GALT gene unveiled specific mutations in the Bangladeshi population, contributing to Galactosemia's molecular diversity. Evaluation of silent mutations' impact on splicing shed light on potential gene expression and protein function alterations, influencing Galactosemia outcomes. An easy-to-use lateral flow diagnostic kit for Galactosemia was developed, showing specificity for galactose and delivering rapid results within 5 minutes. Stability assessment revealed promising results at 2-8°C storage over a year, with signal degradation at higher temperatures, emphasizing proper storage conditions. The study highlights the potential of the HPLC method for amino acid detection, paving the way for accessible and cost-effective amino acidopathy detection facilities. The Galactosemia kit, with further refinement, holds promise for broader use. Insights into alternate GALT gene splicing patterns provide avenues for therapeutic exploration. Future research should fine- tune kits and establish precise phenylalanine and galactose cutoff ranges for the