DEVELOPMENT OF PLASMA MATERIAL PREPARATION FOR RAPID ANTI-HEPATITIS C TEST

WIPAWEE THANYACHARERN

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Biomedical Sciences - (Type A 1)
2025

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By Wipawee Thanyacharern

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Biomedical Sciences - (Type A 1) of Naresuan University

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ABSTRACT

Quality control samples play a vital role in ensuring the validity of laboratory results during quality control analysis. This study assessed the effectiveness of different quality control sample preparations for hepatitis C testing. The samples included liquid forms with and without stabilizers, as well as lyophilized samples with and without trehalose. The objective was to develop and optimize plasma materials for use with hepatitis C rapid diagnostic test (RDT) kits by evaluating preparation methods, creating a linearity panel for analytical validation, and examining the homogeneity and stability of the plasma materials. The analysis demonstrated that the prepared plasma materials were commutable and could be effectively utilized across all five rapid test kits. The concentrations of these materials were suitable for monitoring quality control in testing, and the plasma materials exhibited consistent performance. Stability studies indicated that the plasma materials remained stable for up to six months when stored at temperatures of 2-8°C and 25 ± 5°C, and for up to 28 days at 37°C and 45°C.

In conclusion, the plasma materials developed for hepatitis C antibody testing were prepared using lyophilization with trehalose. Three levels of quality control samples were created: strong positive, weak positive, and negative. Furthermore, the linearity panel materials for Elecsys Anti-HCV II were prepared at six different concentrations to validate and ensure the accuracy of analytical systems in HCV diagnostics. The prepared plasma materials demonstrated homogeneity and stability, maintaining consistency for up to six months at temperatures between 2-8°C and 25 ± 5°C, and for up to 28 days at 37°C and 45°C.

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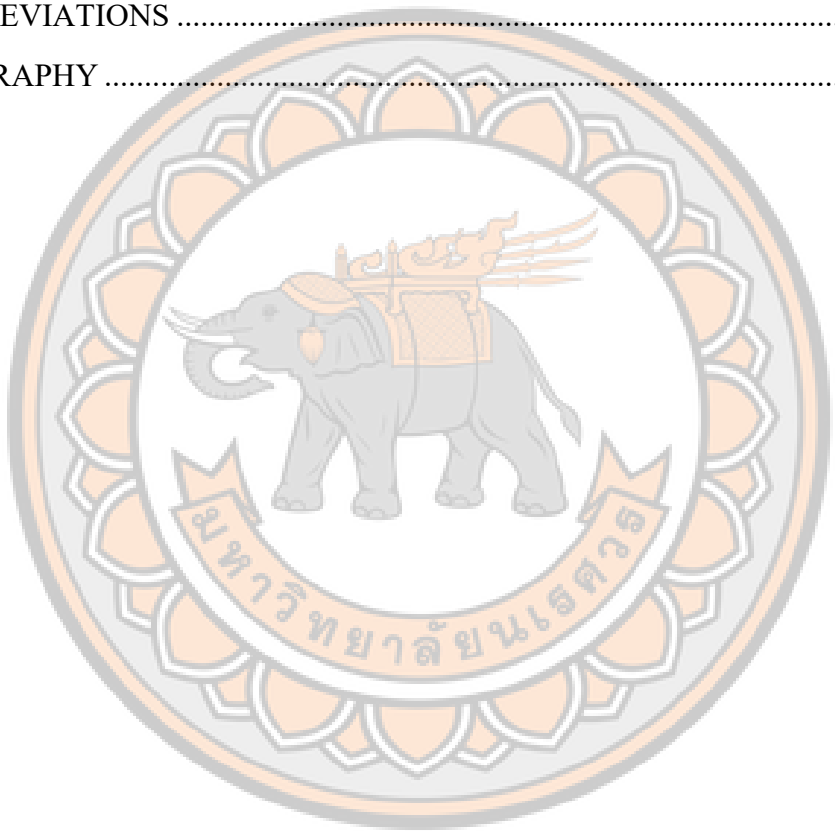

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CHAPTER I
INTRODUCTION

Background and Significance of the Study

Hepatitis C virus (HCV) infection presents a significant global health challenge, resulting in chronic hepatitis, cirrhosis, and potential progression to hepatocellular carcinoma. The consequences of HCV on mortality and morbidity are substantial and are experienced worldwide. This infection is a leading contributor to global illness and death, especially in regions where access to antiviral treatments and healthcare resources is limited. According to the World Health Organization (WHO), approximately 58 million individuals globally are living with chronic HCV infection, leading to around 290,000 deaths each year due to HCV-related liver complications, including cirrhosis and liver cancer. The impact of HCV extends beyond individual health concerns, placing significant pressure on public health systems and economies, which must allocate considerable resources for the management and treatment of complications related to HCV infection. Therefore, prioritizing the prevention and treatment of HCV infection is critically important. Effective screening strategies to identify infections in their early stages, along with improved access to appropriate antiviral therapies, are essential elements of a comprehensive response to this public health issue. The development of efficient screening methods and the implementation of quality control in HCV testing are vital steps in reducing transmission rates and, consequently, in lowering the associated morbidity and mortality linked to this infection.

In Thailand, the Department of Disease Control, through the Viral Hepatitis Coordination Center, has formulated comprehensive guidelines titled "Guidelines for Screening and Treatment of Hepatitis C Virus Infection to Improve Access to Antiviral Therapy." These guidelines are integral to addressing the challenge of Hepatitis C Virus (HCV) infection within the country. Their primary objective is to provide structured recommendations that facilitate the implementation of effective strategies for combating HCV infection in Thailand. Screening for Hepatitis C Virus is conducted by detecting Anti-HCV using immunological principles, utilizing two recognized methodologies.

1. Laboratory-based immunoassays:

Examples of these include Chemiluminescence immunoassay (CLIA), Electrochemiluminescence assay (ECL), and Enzyme immunoassay (EIA), These laboratory tests are standardized methods, but they come with certain challenges and are relatively more expensive compared to rapid test kits.

2. Rapid tests

Rapid tests are quick and simple, requiring minimal equipment. They are also more cost-effective compared to laboratory-based reagent tests and can be performed at service points. Using rapid test kits is an effective option for expanding screening capacity to reach a larger population. Ensuring quality control in testing with rapid test kits is essential and should follow quality assurance practices, such as the routine use of quality control samples or internal quality control. Accurate and reliable results from rapid test kits for screening depend on the consistent use of these quality control samples.

To increase the capacity for screening, Rapid test kits is useful for screening tests to reach a larger number of individuals. Ensuring quality control in testing with

Rapid test kits is essential, following Quality Assurance practices such as routine use of Quality Control Sample or Internal Quality Control. Using Rapid test kits for screening requires quality control samples to obtain accurate and reliable test results.

Quality control testing is an essential component of procedures designed to ensure the quality and reliability of laboratory testing. This process is critical for monitoring the accuracy and validity of results. Dependable control samples should yield data that is consistent and statistically accurate. This consistency and reliability in testing procedures help to prevent the recurrence of uncertain errors during the assay.

The Transfusion Transmitted Pathogens Section of the National Institute of Health of Thailand (NIH) provides quality control samples for the Rapid Anti-hepatitis C test that are used to support the laboratories in Thailand. These quality control samples are specific to each test kit as a single product used per test kit brand. This study is designed to develop the plasma material preparation for rapid Anti-hepatitis C test, so it can be used with at least five rapid anti-hepatitis C test kits.

The use of quality control samples in current practice presents challenges due to variability between production lots and the diverse range of brands available in the market. This variability is particularly significant with low-concentration samples, as different brands or production lots often produce inconsistent results. To mitigate this issue, it is essential to prepare quality control samples specifically tailored for each test kit. Considering these variations, we conducted a study aimed at determining the optimal concentration for preparing quality control samples, with the goal of ensuring the accuracy and reliability of test results.

Rapid diagnostic tests can be valuable alternatives to standard serological assays for detecting the hepatitis C virus (HCV) in resource-limited settings(Mahajan et al., 2019).Rapid diagnostic tests could help identify HCV-infected individuals more expeditiously, and thus the availability of high-performing, quality-assured RDTs is essential in the process of scaling up HCV screening efforts (Mane et al., 2019).Rapid test was conducted in an independent laboratory by three technicians using a blind evaluation structure. The study did not include any specific controls other than the internal controls integrated into each assay. This lack of controls has clearly indicated a high risk of obtaining false results from the Rapid test due to the staff's failure to adhere to quality assurance guidelines (Fondjo, Ngoupo, Ngono, Plantier, & Njouom, 2018).

This study aims to develop preparation methods for the application of plasma material for the rapid anti-hepatitis C test. Our researchers have created innovative plasma materials that can be customized to different concentrations. These materials will be used to manufacture a solution suitable for serving as a sample quality control in the rapid anti-hepatitis C test. Although this plasma material is specific to one brand, it will be designed to be compatible with three different brands of rapid test anti-HCV assays. And study aims to develop preparation methods for the linearity panel is significant in the evaluation and testing of medical devices. It focuses on assessing the device's ability to measure and display results across a range of different standard values. By using samples that closely approximate standard values, it provides users with confidence that the obtained results are accurate and reliable within the desired range.

Linearity panel for Automate is a set of samples or materials with known concentrations that is used to assess the linearity or linear range of an analytical instrument or assay. It is crucial for evaluating how accurately the instrument or assay can measure and display results across a range of different concentrations. The production of a linearity panel is essential for several reasons. The use of linearity panel

helps confirm that a medical device is precise and adheres to established standards. This is crucial for ensuring accuracy and reliability in medical services and diagnostics. linearity panel is essential for verifying the accuracy, assessing the linear range, and maintaining the quality and reliability of analytical instruments and assays in laboratory and diagnostic settings. It is a critical component of quality assurance and helps ensure the credibility of scientific and medical testing.

Purposes of the Study

1. To optimize the condition of anti-HCV plasma materials.
2. To develop the linearity panel materials preparation protocol for Elecsys Anti-HCV II.
3. Study of the homogeneity and stability of plasma control materials.

Statement of the Problems

The accurate and reliable detection of the Hepatitis C Virus (HCV) is critical for effective medical diagnosis and treatment. However, the preparation of plasma materials intended for use as quality control samples across different brands of rapid HCV test kits presents a considerable challenge. Each brand has distinct components and specific material preparation requirements, which complicates the efforts to ensure consistency and quality in the prepared materials. According to data from the Food and Drug Administration (FDA) as of November 2024, 81 rapid anti-hepatitis C test kits have been authorized ((FDA), 2024). The extensive range and considerable number of HCV rapid test kits present challenges in developing plasma materials that can be consistently and reliably utilized across various brands. A fundamental issue arises from the differences among these test kits, which can affect both the stock and concentration of the prepared plasma materials. This variation may lead to inconsistent and unreliable test results. Additionally, the lack of standardized methods for preparing plasma materials that are compatible with multiple HCV rapid test kit brands adversely impacts Internal Quality Control (IQC) within diagnostic testing. Consequently, this can diminish the overall credibility of laboratory testing processes.

This study aims to address the challenge of preparing plasma material that is suitable for use with five different Hepatitis C Virus (HCV) rapid test kits. The challenge arises from the distinct variations among these test kits, including differences in composition and the potential variations introduced during preparation, which may impact both the stock and concentration of the prepared material. The primary objective of this research is to develop methods for the preparation of plasma material that is specifically tailored to meet the requirements of both the rapid anti-hepatitis C test and the Elecsys anti-HCV II test. The importance of quality control samples in this context cannot be overstated, as they are critical for ensuring the accuracy of laboratory results. This study also aims to evaluate the quality control samples utilized in hepatitis C testing. The plasma materials examined in this research play a significant role in the Internal Quality Control of HCV rapid diagnostic tests. The study focuses on developing cultivation techniques for preparing plasma materials that are compatible with a minimum of five test kits. The aim is to produce plasma material that demonstrates both homogeneity and stability. The data analysis indicates that the commutable plasma materials may be independently utilized with three test kits, and the concentrations of

these materials are deemed appropriate for effectively monitoring quality control in the analysis.

The objective of this study is to develop customized plasma materials that align with various levels of anti-hepatitis C. This initiative is designed to ensure these materials are suitable for use as quality control samples in rapid anti-hepatitis C testing. Furthermore, the study aims to produce plasma materials that are compatible with five distinct brands of rapid anti-hepatitis C tests. Customizing plasma materials for linearity panels is essential for verifying the accuracy of tests, assessing the linear range, and maintaining the quality and reliability of analytical instruments and assays used in laboratory and diagnostic environments. This undertaking is a critical component of quality assurance and plays a significant role in upholding the integrity of scientific and medical testing.

In summary, the study addresses the need to prepare plasma material for multiple HCV rapid test kits, considering their differences and ensuring that the material meets specific requirements for quality control and linearity panel purposes. Additionally, it aims to make this material compatible with five different test kit brands.

Scope of the Study

1. Plasma Material Preparation Optimization: This research focuses on the development of optimal methods for preparing plasma material. The primary objectives in this aspect include:
    - Optimizing plasma material levels to the specific requirements of five commonly used anti-HCV rapid test kits in laboratory settings and for the linearity panel.
    - Optimizing the most suitable preservation conditions to ensure the stability and integrity of the plasma material.
2. Homogeneity Study: The study will include an analysis of the homogeneity of the prepared plasma material. This analysis is essential to assess the uniformity and consistency of the material across different batches
3. Stability Assessment: The research will investigate the accelerated and real-time stability of the plasma material. This evaluation will consider factors such as:
    - The impact of different storage conditions on the stability of the plasma material.
    - The effects of transportation conditions on the material's integrity during transit.
    - An assessment of the shelf life of the plasma material to determine its durability and suitability for use over time.

The study's overall objective is to optimize the preparation of plasma material, ensure its homogeneity, and assess its stability under various conditions.

This comprehensive approach is crucial to guarantee the material's reliability and suitability for use in anti-HCV rapid test kits and linearity panels within laboratory settings.

Hypotheses of the Study

1.Plasma material can be used as internal quality control material materials for five rapid anti-hepatitis C test kit and linearity panel for Elecsys anti-HCV II.

2.The produced developed plasma material is homogeneous and stability.

CHAPTER II
LITERATURE REVIEW

Basic Assumption

Internal Quality Control (Westgard, 2003)

It is a process used in medical and laboratory settings to monitor and verify the accuracy and reliability of medical testing or laboratory analysis. IQC is primarily used in clinical laboratories and medical centers. The main goal is to ensure that the test results or medical analyses conducted in the lab or medical facility are accurate and reliable.

There are various methods for conducting IQC, but the key is to use known samples as a baseline for checking the accuracy of the lab process or medical instrument. If IQC test results do not match the expected values, it may necessitate adjustments, or a review of the processes or instruments used in testing to ensure accurate and reliable results in medical testing.

Internal Quality Control (IQC) level

The term "IQC level" typically refers to different levels or concentrations used in Internal Quality Control (IQC) processes in laboratories or medical testing facilities. These levels are used to assess the accuracy and reliability of testing procedures and equipment. IQC levels involve the use of control materials with known values at different concentrations or levels.

Linearity panel

In laboratory and analytical contexts refers to a set of samples or materials with known concentrations that are used to evaluate the linearity or linear range of an analytical instrument or assay. It is essential to assess how accurately the instrument or assay can measure and display results across a range of different concentrations.

Rapid test" or "rapid diagnostic test"

Rapid test" or "rapid diagnostic test" is a medical test designed to quickly provide results, typically within a short period, often in a matter of minutes. These tests are designed for speed and convenience, making them valuable tools in various medical, clinical, and diagnostic settings. And automate is the automation of testing refers to the use of automated processes and equipment to conduct diagnostic tests. Automated testing is an important aspect of clinical laboratories and medical facilities.

Related Works and Studies

8.1 Hepatitis C virus (HCV)

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus classified in the *Hepacivirus* genus within the *Flaviviridae* family. The Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus classified under the *Hepacivirus* genus within the Flaviviridae family, which also includes viruses such as yellow fever, West Nile, and dengue. HCV particles are spherical and vary in size, typically ranging from 40 to 80 nm in diameter. Currently, 7 genotypes and 84 subtypes of HCV have been identified. The intact HCV particle is commonly associated with lipoproteins, which contribute to its structure and infectivity (Dubuisson & Cosset, 2014; Lindenbach & Rice, 2013; Piver et al., 2017; Smith et al., 2014).

HCV belongs to the *Hepacivirus* genus within the *Flaviviridae* family, along with *Pestiviruses* and Flaviviruses. Its genome consists of a positive, single-strand RNA molecule containing two untranslated regions at the 5' and 3' ends and a large open reading frame that encodes a polyprotein of approximately 3010 to 3030 amino acids. This polyprotein undergoes post-translational processing, where it is cleaved into structural and non-structural proteins by host and viral enzymes (Figure 1) (Giannini & Brechot, 2003).

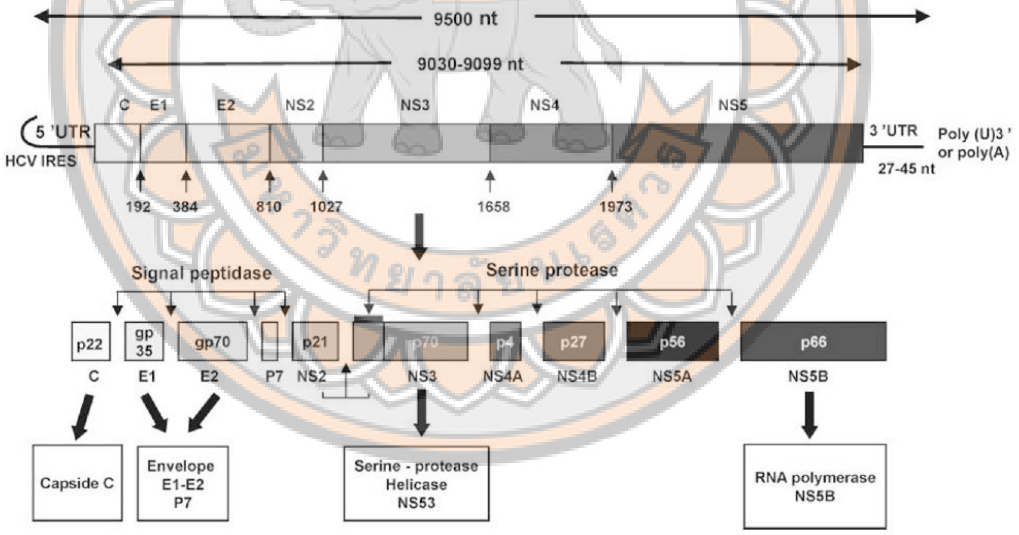


Figure 1 HCV genome organization and processing of viral polyprotein

Source : (Giannini & Brechot, 2003)

Hepatitis C is an infectious disease caused by the hepatitis C virus (HCV), an RNA virus belonging to the Flaviviridae family. HCV infection can result in acute hepatitis C, and following the acute phase, 50–80% of patients progress to chronic hepatitis C. Chronic HCV infection triggers an ongoing inflammatory process that can lead to liver fibrosis, cirrhosis, hepatocellular carcinoma, and ultimately, death. In many

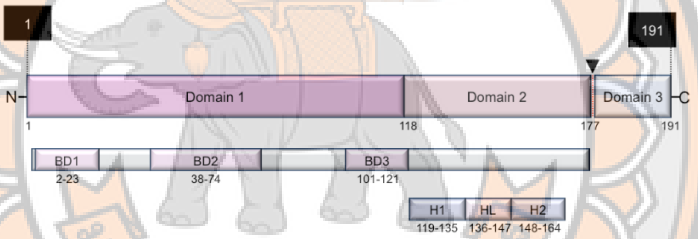
parts of the world, hepatitis C is the primary indication for liver transplantation (Manns et al., 2017).

The HCV genome encodes three structural proteins (core, E1, and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B)

CORE

The HCV core protein serves a crucial role as a structural protein, playing a part in the formation of the viral capsid – a spherical protective structure encasing the viral genomic RNA. It's noteworthy that the core protein is the initial protein to be synthesized from the HCV genome. Together with the envelope glycoproteins (E1 and E2), these three proteins constitute the structural components of HCV.

In the nucleocapsid structure, the mature core protein exhibits properties that facilitate its attachment to the lipid membrane derived from the host and the HCV RNA. Particularly, the N-terminal region of the core protein is responsible for binding to the HCV genomic RNA (Eng et al., 2018; Gawlik & Gallay, 2014; Kao, Yi, & Huang, 2016; Klein, Polyak, & Lingappa, 2004; Rupp & Bartenschlager, 2014; Strosberg, Kota, Takahashi, Snyder, & Mousseau, 2010).



Note: BD = Basic Domain; H = Helix; HL = Hydrophobic Loop

Figure 2 Protein Domain Structure of HCV Core Protein

Source : **(Online)**

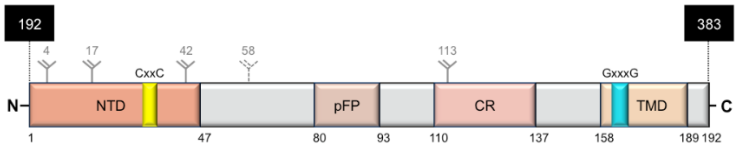
E1

The envelope glycoprotein E1 is a heavily glycosylated transmembrane protein that forms a tight association with the E2 glycoprotein, collectively constituting the envelope glycoprotein E1-E2 heterodimer. E1 serves a pivotal role in various stages of the HCV replication cycle, encompassing attachment to the host cell, fusion with endosome-lipid membranes, and assembly processes.

In terms of attachment, E1 might engage with host apoproteins or potentially CD36. Furthermore, E1 contributes to the binding of E2 with host receptors by maintaining a favorable E1-E2 structural conformation that facilitates receptor interaction. After the HCV particle undergoes endocytosis, the acidic conditions within the endosome trigger conformational changes in both E1 and E2. These changes lead to the fusion of the endosomal membrane with the viral envelope, enabling the release of HCV RNA into the cytoplasm.

Recent research suggests that the E1 protein also engages with several other viral proteins, thereby coordinating the intricate process of HCV assembly (El Omari et al.,

2014; Haddad et al., 2017; Law et al., 2013; Mazumdar, Banerjee, Meyer, & Ray, 2011; Moustafa et al., 2018; Tong, Lavillette, Li, & Zhong, 2018; Yost, Wang, & Marcotrigiano, 2018).



Note: the gray numbers and symbols indicate glycosylation sites; HVR = Hypervariable Region; NT = N-terminal; IgVR = Intergenotypic Variable Region; TM = Transmembrane

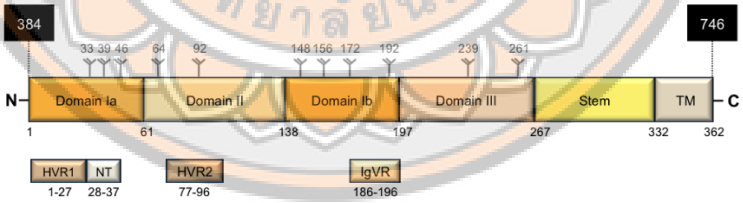
Figure 3 Domain Structure of HCV E1 Envelope Protein

Source: Online HC. HCV Biology [https://www.hepatitisc.uw.edu/page/hcv/biology]

E2

The envelope glycoprotein E2 is a highly glycosylated transmembrane protein that binds with the E1 envelope glycoprotein, creating E1-E2 heterodimers. These heterodimers are integrated into the lipid membrane derived from the host and collectively form the HCV envelope. E2 has an elongated structure and serves key functions in host receptor binding, entry, and fusion with the endosomal membrane.

E2 interacts with various host cell surface receptors, including CD81 and scavenger receptor class B type 1 (SR-B1)(Albecka et al., 2011; Drummer, 2014; Fauvelle, Felmlee, & Baumert, 2014; Freedman, Logan, Law, & Houghton, 2016; Khan et al., 2014; Kong et al., 2013; Prentoe, Velázquez-Moctezuma, Foung, Law, & Bukh, 2016; Sabahi, Uprichard, Wimley, Dash, & Garry, 2014; Vieyres, Dubuisson, & Pietschmann, 2014).



Note: the gray numbers and symbols indicate glycosylation sites; HVR = Hypervariable Region; NT = N-terminal; IgVR = Intergenotypic Variable Region; TM = Transmembrane

Figure 4 Domain Structure of HCV E2 Glycoprotein

Source: Online HC. HCV Biology [https://www.hepatitisc.uw.edu/page/hcv/biology]

P7

The p7 protein is a small, hydrophobic transmembrane protein that plays a role in viral assembly and release. It can form ion channels in host cell membranes by oligomerizing into hexamers. Due to this characteristic, p7 is classified as a viroporin protein.

While p7 has a structural function within hepatocytes, it is not a component of the viral particle itself. During the assembly process, p7 proteins collaborate with other viral proteins within the endoplasmic reticulum. This collaboration facilitates the transfer of core proteins from lipid droplets for the purpose of capsid assembly and membrane envelopment.

The channel activity of p7 is involved in neutralizing the low pH environment of the cellular secretory compartment, thereby safeguarding viral glycoproteins from inactivation (Atoom, Taylor, & Russell, 2014; Chandler, Penin, Schulten, & Chipot, 2012; Denolly et al., 2017; Gentzsch et al., 2013; Khaliq, Jahan, & Hassan, 2011; Madan & Bartenschlager, 2015; OuYang et al., 2013).

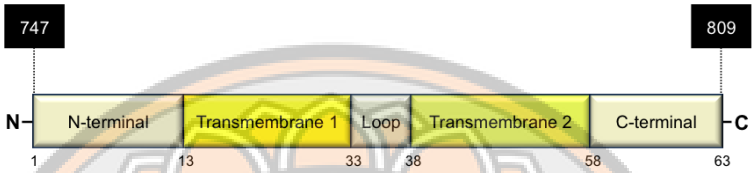


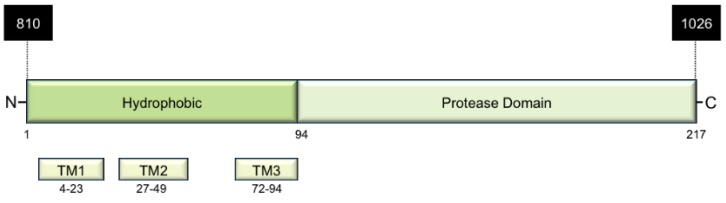
Figure 5 Domain Structure of HCV p7 Protein

Source: Online HC. HCV Biology [https://www.hepatitisc.uw.edu/page/hcv/biology]

NS2

The nonstructural protein 2 (NS2) serves a dual role in the HCV life cycle, acting as both a cysteine protease and a cofactor in the assembly process. The protease domain of NS2, working in collaboration with the N-terminal region of NS3, functions as a cysteine protease responsible for catalyzing a single cleavage event between the NS2 and NS3 proteins. This cleavage is crucial for releasing NS2 from NS3 and is essential for RNA replication.

While NS2 is not necessary for HCV RNA replication, it plays a significant role in coordinating viral assembly when combined with several other viral proteins. Notably, NS2 appears to colocalize with E1, E2, NS3, and NS5A near the core proteins and lipid droplets during the viral assembly process (Jirasko et al., 2008; Jones, Murray, Eastman, Tassello, & Rice, 2007; Lorenz, 2010; Lorenz, Marcotrigiano, Dentzer, & Rice, 2006; Popescu et al., 2011; Yi, Ma, Yates, & Lemon, 2009).



Note: TM = Transmembrane

Figure 6 Domain Structure of HCV NS2 Protein

Source: Online HC. HCV Biology [https://www.hepatitisc.uw.edu/page/hcv/biology]

NS3

The nonstructural 3 (NS3) protein is a multifunctional enzyme with both serine protease and helicase activities. The serine protease domain of NS3 is responsible for initiating most of the cleavage events within the viral polyprotein, leading to the liberation of nonstructural proteins. Additionally, NS3 plays a crucial role in evading the host's innate immune response by enzymatically deactivating various host cell factors that could hinder viral replication.

The helicase region of NS3 is believed to be involved in unwinding the viral RNA and supporting the process of viral replication. A significant milestone in the advancement of direct-acting antiviral therapy was achieved by determining the crystal structure of the NS3 protease. This breakthrough enabled the design of targeted NS3/4A protease inhibitors, marking a significant stride in antiviral drug development (Brass et al., 2008; De Francesco, Pessi, & Steinkühler, 1999; Love et al., 1996; Morikawa et al., 2011; Raney, Sharma, Moustafa, & Cameron, 2010; Rupp & Bartenschlager, 2014).

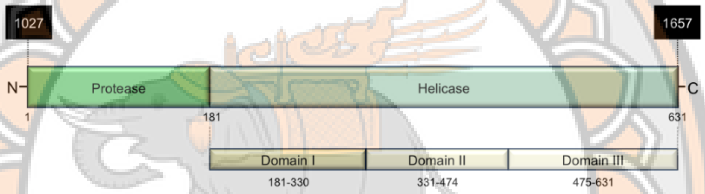


Figure 7 Domain Structure of HCV NS3 Protein

Source: Online HC. HCV Biology [https://www.hepatitisc.uw.edu/page/hcv/biology]

NS4A

The nonstructural protein 4A (NS4A) is the smallest among the nonstructural HCV proteins. NS4A serves multiple functions in the HCV life cycle, Its functions include (1) anchoring the NS3-4A complex to the outer membranes of the endoplasmic reticulum and mitochondria, (2) serving as a cofactor for the NS3 serine protease, (3) enhancing the helicase activity of NS3, and (4) regulating NS5A hyperphosphorylation and viral replication.

The interactions between NS4A and NS4B have a role in controlling genome replication, and the interplay between NS3 and NS4A contributes to virus assembly dynamics (Brass et al., 2008; Lin, Wu, Hsiao, & Su, 1997; Lindenbach et al., 2007; Morikawa et al., 2011; Phan, Kohlway, Dimberu, Pyle, & Lindenbach, 2011; Zhu & Briggs, 2011).

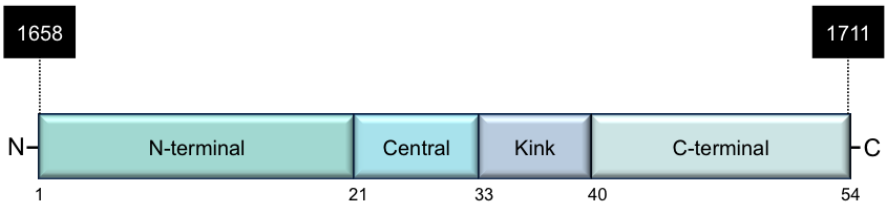
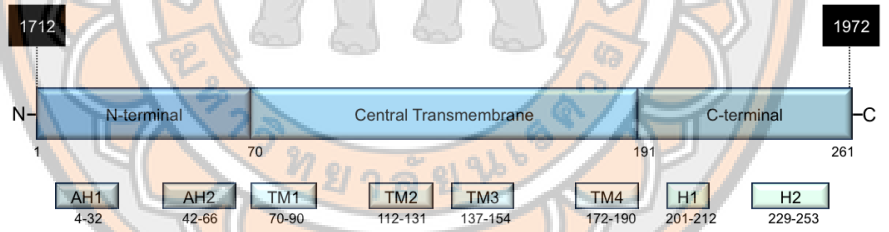


Figure 8 Domain Structure of HCV NS4A Protein

Source: Online HC. HCV Biology [https://www.hepatitisc.uw.edu/page/hcv/biology]

NS4B

The nonstructural protein 4B (NS4B) is a crucial component that induces alterations in the cytoplasmic membrane and facilitates virus-host interactions. This protein colocalizes within the endoplasmic reticulum, along with other nonstructural proteins, to form the replication complex. NS4B is among the key viral elements necessary for the creation of the membranous web, a specialized microenvironment within the cytoplasm that plays a vital role in supporting viral replication (Esser-Nobis et al., 2013; Gouttenoire, Penin, & Moradpour, 2010; Lundin, Lindström, Grönwall, & Persson, 2006; Paul, Hoppe, Saher, Krijnse-Locker, & Bartenschlager, 2013; Rai & Deval, 2011).



Note: AH = Amphipathic Helix, H = Helix; TM = Transmembrane

Figure 9 Domain Structure of HCV NS4B Protein

Source: Online HC. HCV Biology [https://www.hepatitisc.uw.edu/page/hcv/biology]

NS5A

The nonstructural protein 5A (NS5A) is a key component of the HCV replication complex, interacting with essential viral elements (NS4B, NS5B, RNA) and host cell proteins (such as cyclophilin A and kinases) to precisely regulate viral replication and assembly. NS5A plays a vital role in forming double-membrane vesicles (DMVs) derived from the endoplasmic reticulum, which significantly enhance viral replication efficiency. Beyond its role in replication, NS5A contributes to HCV pathogenesis, modulates cell signaling pathways, aids in virus propagation, and influences the interferon response via the interferon-alpha sensitivity-determining region. Importantly, NS5A is a target for direct-acting antiviral agents known as NS5A inhibitors (Appel et

al., 2008; Berger et al., 2014; Bukh, 2016; Dustin & Rice, 2007; Morozov & Lagaye, 2018; Romero-Brey et al., 2015; Yin, Goonawardane, Stewart, & Harris, 2018; Zayas, Long, Madan, & Bartenschlager, 2016).

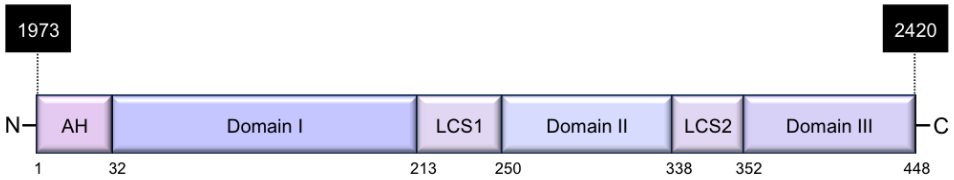
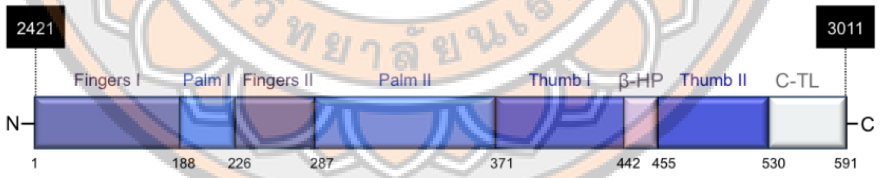


Figure 10 Domain Structure of HCV NS5A Protein

Source: Online HC. HCV Biology [https://www.hepatitisc.uw.edu/page/hcv/biology]

NS5B

The nonstructural protein 5B (NS5B) functions as an RNA-dependent RNA polymerase (RdRp) and holds a pivotal role in HCV replication. This enzyme's primary function is to facilitate the polymerization of ribonucleoside triphosphates (rNTP) during the process of viral RNA replication. NS5B polymerase inhibitors are divided into two main subclasses: (1) nucleotide analogs, which imitate the natural substrate and cause chain termination upon incorporation into the new RNA, and (2) non-nucleotide inhibitors, which bind to allosteric sites on the enzyme and disrupt its functionality (Ago et al., 1999; Appleby et al., 2015; Boyce et al., 2014; Lesburg et al., 1999; Lohmann, Roos, Körner, Koch, & Bartenschlager, 2000; Love et al., 2003; Mosley et al., 2012; Soriano et al., 2013; Thueng-in et al., 2014).



Note: β-HP = β-Hairpin; C-TL= C-Terminal Linker

Figure 11 Domain Structure of HCV NS5B Protein

Source: Online HC. HCV Biology [https://www.hepatitisc.uw.edu/page/hcv/biology]

The World Health Organization (WHO) advocates a two-step approach for the testing and diagnosis of Hepatitis C Virus (HCV) infection.

1. Anti-HCV Antibody Testing: The initial step involves conducting a serological test to detect the presence of anti-HCV antibodies. This assessment is critical for identifying individuals who have been infected with the virus. A positive result indicates either a current or past HCV infection.

2. Confirmation of Chronic Infection: Following a positive serological test for anti-HCV antibodies, a nucleic acid test to measure HCV ribonucleic acid (RNA) is necessary to confirm the presence of chronic infection and evaluate the need for treatment. This confirmation is particularly important, as approximately 30% of individuals infected with HCV can naturally clear the virus through a robust immune response without requiring medical intervention, though they will continue to test positive for anti-HCV antibodies.

This structured approach ensures accurate diagnosis and appropriate management of HCV infection. The nucleic acid test detects the presence of HCV RNA in the blood, confirming active viral replication (Figure 2) (Getchell et al., 2013).

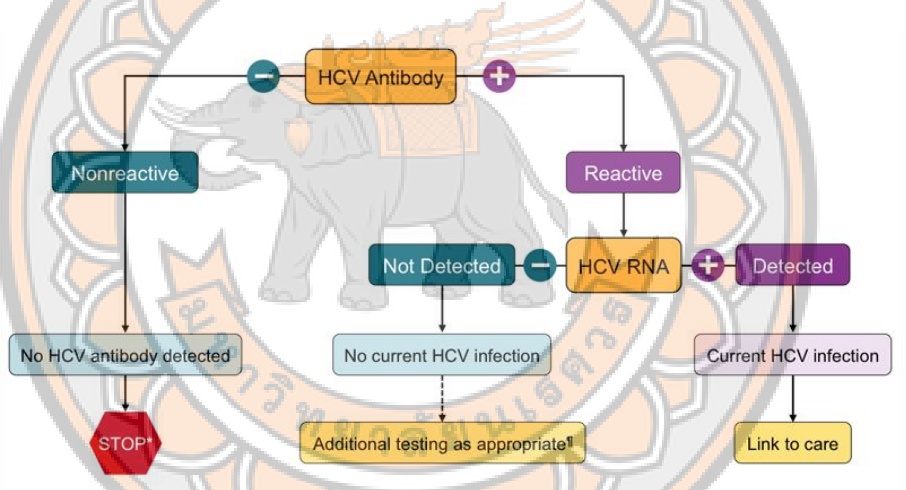


Figure 12 HCV testing sequence recommended by the Centers for Disease Control and Prevention in May 2013 (Getchell et al., 2013)

Source: (Getchell et al., 2013)

8.2 Hepatitis C Virus (HCV) Infection and Immune Response

The Hepatitis C virus (HCV) is an RNA virus characterized by a high mutation rate, which enables it to evade the body's immune response effectively (Organization, 2023; Prevention).

Upon entry into the body, the virus initiates infection through a series of steps, as outlined below:

1. Entry and Initial Infection in the Liver

HCV typically enters the body via blood-to-blood contact, commonly through shared needles, unscreened blood transfusions, or shared personal items such as razors or toothbrushes. Once in the bloodstream, the virus is transported to the liver, where it begins replication. The liver provides an optimal environment for HCV replication, as hepatocytes (liver cells) contain the necessary cellular machinery for the virus to reproduce.

2. Viral Replication within Liver Cells

Upon reaching the liver, HCV attaches to specific receptor proteins on hepatocyte surfaces, allowing it to enter the cell. Once inside, the virus releases its RNA, which serves as a template for creating new viral particles.

The replication process relies on the host cell's machinery to produce viral proteins and assemble new virus particles. These viral components include structural proteins that form the viral envelope and non-structural proteins essential for immune evasion. Following replication, new viruses are released from the liver cell, spreading to infect additional hepatocytes, thus establishing widespread infection within the liver.

3. Immune Response to HCV Infection

When the body detects HCV, the immune system initiates a multi-layered response, primarily through innate immunity and adaptive immunity:

Innate Immunity: As the body's first line of defense, innate immunity responds rapidly in a non-specific manner. Key immune cells, including macrophages and neutrophils, engage in neutralizing and ingesting the virus. Additionally, liver cells release cytokines—chemical signals that activate and recruit other immune cells to assist in combating the infection.

Adaptive Immunity: Adaptive immunity generates a targeted response specific to HCV and involves B-cells and T-cells. These cells work in tandem to produce antibodies and target virus-infected cells directly.

- B-cells produce antibodies that bind to viral proteins on the surface of HCV, thereby neutralizing the virus and marking it for destruction by other immune cells.
- T-cells, particularly cytotoxic T-cells, play a crucial role in directly destroying infected hepatocytes, thus preventing further viral replication, and spread.

4. Chronic Infection and Long-Term Implications

HCV's high mutation rate allows it to evade the immune response, which frequently leads to chronic infection.

Chronic HCV infection results in sustained liver inflammation, prompting the accumulation of scar tissue (fibrosis) in the liver. Over time, this fibrosis can progress to cirrhosis, significantly increasing the risk of hepatocellular carcinoma (liver cancer).

5. Treatment Protocols

The current standard treatment for HCV involves direct-acting antivirals (DAAs), which effectively eradicate the virus over a course of 12-24 weeks. DAAs function by inhibiting viral proteins crucial for replication, thereby halting the spread of HCV within the liver. These therapies offer a high likelihood of cure and substantially reduce the risks of cirrhosis and liver cancer.

8.3 HCV Diagnosis Guidelines in Thailand (Department of Disease Control, 2023)

The Hepatitis C Virus (HCV) diagnostic guidelines in Thailand, established by the Thai Association for the Study of the Liver and the Department of Disease Control under the Ministry of Public Health, are a comprehensive framework aimed at ensuring accurate and efficient diagnosis and management of HCV infections. These guidelines are essential for maintaining high standards of care across healthcare facilities in Thailand, promoting standardized diagnostic practices that contribute to improving patient outcomes and supporting global efforts to eliminate HCV. The diagnostic process is divided into several stages, each carefully designed to address specific aspects of HCV infection.

Initial Screening

The initial screening stage involves testing for anti-HCV antibodies in the patient's serum or plasma, a process commonly performed through enzyme immunoassays. This test detects antibodies that indicate whether the patient's immune system has previously encountered the virus. A positive result here signifies prior exposure to HCV, but it does not differentiate between past and current infections, nor does it confirm if the virus is still active. Therefore, while a negative result generally suggests no prior exposure to the virus, a positive result necessitates further testing to ascertain whether the infection is ongoing. This approach ensures that individuals are not misdiagnosed and emphasizes the importance of additional confirmatory tests for those with positive anti-HCV results.

Confirmation of Active Infection

The second stage focuses on confirming active infection, a critical step in the diagnostic process. For individuals who tested positive for anti-HCV antibodies, an HCV RNA test is conducted to detect viral RNA in the bloodstream, providing definitive confirmation of an active infection. This test, typically performed through Real-time PCR, is highly sensitive and specific, capable of detecting even low levels of viral RNA. The confirmation of active infection is vital not only for determining who requires treatment but also for identifying patients who may pose a risk of transmitting the virus to others. This precise confirmation step prevents unnecessary treatment for individuals with past, resolved infections and allows healthcare providers to prioritize resources for patients who need intervention.

Assessment of Disease Severity

Once an active infection is confirmed, assessing the severity of the disease is the next critical step. This assessment begins with liver enzyme tests, which measure levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) to evaluate the extent of liver inflammation or damage. Elevated enzyme levels can be indicative of liver stress or injury, providing important insights into the impact of the infection on the liver. In addition to enzyme testing, imaging studies such as ultrasound and Fibro Scan are recommended. Fibro Scan is a non-invasive technology that measures liver stiffness, helping to assess the level of fibrosis or scarring within the liver. This comprehensive evaluation is crucial for understanding the progression of the disease and for determining the most appropriate treatment plan, as patients with advanced liver damage may require more intensive and carefully monitored therapy.

Genotype Testing

The final stage in the diagnostic process involves genotype testing, which identifies the specific genetic variation, or genotype, of the HCV infection. Different HCV genotypes can respond differently to antiviral treatments, making this step essential for selecting the most effective treatment regimen. In Thailand, genotype 3 is the most common, but other genotypes may also be present, each with unique treatment considerations. Knowing the patient's HCV genotype allows healthcare providers to tailor the treatment plan, optimizing the likelihood of successful viral eradication. This approach aligns with international treatment guidelines, which emphasize genotype-based therapy for achieving higher cure rates and improving overall treatment outcomes.

Together, these guidelines provide a structured, step-by-step approach to HCV diagnosis that ensures each patient receives accurate, targeted, and effective care. By following this systematic protocol, healthcare providers in Thailand can improve diagnostic accuracy, avoid unnecessary treatments, and develop treatment plans tailored to each patient's unique condition. Furthermore, the adoption of these guidelines across healthcare settings supports Thailand's commitment to HCV elimination, enhancing the quality and consistency of HCV care nationwide and reducing the prevalence and transmission of the virus in the population. This standardized approach not only enhances individual patient care but also strengthens public health efforts, contributing to the broader goal of eliminating HCV as a significant health threat in Thailand.

8.4 Rapid test Anti-HCV

Rapid tests, also known as rapid diagnostic tests (RDTs), are simple, easy-to-use tests that provide results quickly, usually within 20 minutes or less. Unlike most standard tests that must be sent to a laboratory, rapid tests are conducted and provide results directly at the point of care, where the patient is receiving treatment.

The point of care refers to the location where healthcare services are provided. Rapid tests often utilize a lateral flow assay, which is conducted on a membrane strip composed of several key parts: an application pad, conjugate pad, nitrocellulose membrane, and an adsorption pad, all assembled on a plastic backing. Typically, the nitrocellulose membrane has two lines, one for detecting the target analyte and one as an internal control.

Lateral Flow Assay (LFA)

Lateral flow assays (LFAs), also known as immuno-chromatographic assays (ICA), have recently gained significant interest due to their low cost, simplicity, speed, and compatibility with portable detection devices. LFAs are conducted on a membrane strip comprising several components, including an application pad, conjugate pad, nitrocellulose membrane, and adsorption pad, all assembled on a plastic backing (Figure 3) (Koczula & Gallotta, 2016; Warkad, Song, Pal, & Nimse, 2019).

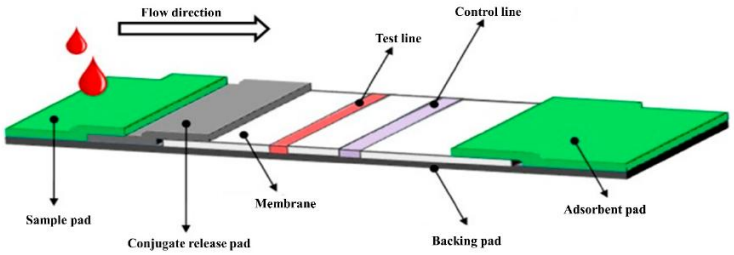


Figure 13 A typical structure of a lateral flow immunoassay test strip

Source: Koczula & Gallotta, 2016

Lateral flow assays (LFA) are a technique used for the detection and measurement of chemical substances or biological components in a sample. LFA has a basic structure composed of the following components:

Sample Pad: This is the layer where the sample is applied to the test device. The sample pad helps filter and prepare the sample for testing.

Conjugate Pad: This layer contains the detection reagents (conjugates) that are linked to the target analyte for detection. The detection reagents in this layer are often colored or marked with specific indicators.

Nitrocellulose Membrane: It is a network-like membrane used for the separation and differentiation of the detection substances. The nitrocellulose membrane has small lines called Test Lines and Control Lines, which indicate the presence or absence of detection substances.

Absorbent Pad: This layer is used to absorb the sample or the detection substances that pass through the nitrocellulose membrane and assist in the flow of the sample.

Housing: It is the protective casing that prevents light exposure and maintains the integrity of the LFA components. The housing is often made of plastic or other materials to protect against damage and moisture from the external environment.

Rapid test Kits consist of five product studies:

1. STANDARD Q HCV Ab (SD Biosensor Inc, Korea) (W. P. P. Report, 2023)

- The STANDARD Q HCV Ab Test is a rapid chromatographic immunoassay designed for the qualitative detection of specific antibodies to HCV in human serum, plasma, or whole blood
- Performance: sensitivity of 98.9% and a specificity of 100%.
- Principle of the test:

According to SD Biosensor Inc's assay description, the STANDARD Q HCV Ab Test contains two pre-coated lines on the nitrocellulose membrane: a "C" control line and a "T" test line. Both lines are invisible in the result window before any specimen is applied. The control line region is coated with monoclonal anti-NS3 and monoclonal anti-Core, while the test line region is coated with monoclonal anti-human IgG. Four recombinant HCV antigens derived from the core, NS3, NS4, and NS5 regions are conjugated with colloidal gold particles and used as detectors for HCV antibodies.

During testing, if HCV antibodies are present in the specimen, they interact with the recombinant HCV antigens conjugated with colloidal gold particles, forming an antibody-antigen-gold particle complex. This complex then migrates along the membrane by capillary action to the test line, where it is captured by monoclonal anti-human IgG. If HCV antibodies are present, a violet line will appear in the result window, with the intensity of the line varying based on the concentration of HCV antibodies in the specimen. If no HCV antibodies are present, no color appears on the test line. The control line serves as a procedural control and should always be visible if the test is performed correctly.

Figure 14 STANDARD Q HCV Ab (SD Biosensor Inc, Korea

Source: Biosensor, 2024

2. Bioline™ HCV Hepatitis C Virus test kit (Abbott, Germany) (W. P. Report, 2020)

- Bioline™ HCV test is an immunochromatographic rapid test for the qualitative detection of antibodies specific to HCV in human serum, plasma, or whole blood.
- Performance: Sensitivity 99.3 %, Specificity 98.1 %
- Principle of the test:

According to the assay description from Abbott Diagnostics Korea Inc., the Bioline HCV test contains a nitrocellulose membrane strip pre-coated with recombinant HCV capture antigens (core, NS3, NS4, and NS5) in the test line region (T). During testing, the protein A-colloid gold conjugates and the specimen move chromatographically along the membrane to the test region. Here, the antigen-antibody-protein A gold particle complex forms a visible line, providing high sensitivity and specificity.
The test device is marked with the letter's "T" and "C" on the case, representing the "Test Line" and "Control Line." Both lines are invisible in the result window before the specimen is applied. The control line serves as a procedural control and should appear if the test is performed correctly.

Figure 15 Bioline™ HCV Hepatitis C Virus test kit (Abbott, Germany)

Source: Care, 2024

3. One Step Hepatitis C Virus Whole Blood/Serum/Plasma Test (Wondfo, China) (Guangzhou Wondfo Biotech Co., 2022)

- Wondfo One Step Hepatitis C Virus Whole Blood/Serum/Plasma Test is intended for use by healthcare professionals and as a qualitative screening in vitro diagnostic test for the detection of Hepatitis C antibodies in whole blood, serum, or plasma.
- Performance: sensitivity of 99.0% and a specificity of 99.8%
- Principle of the test:

Wondfo One Step Hepatitis C Virus Whole Blood/Serum/Plasma Test is a rapid immunochromatographic test designed for the visual detection of hepatitis C antibodies in whole blood, serum, or plasma samples, aiding in the diagnosis of hepatitis C virus infection. The test utilizes recombinant antigens corresponding to highly immunoreactive regions of HCV, including the core, NS1, NS2, NS3, NS4, and NS5 proteins.

When the specimen is added, it is absorbed by capillary action, mixes with the protein-dye conjugate, and flows across the pre-coated membrane. If the HCV antibody level in the specimen meets or exceeds the detection limit, the HCV antibodies bind to the antigen-dye conjugate and are captured by immobilized antigens in the test region (T), producing a visible colored band that indicates a positive result.

If the HCV antibody level in the specimen is zero or below the detection threshold, no colored band appears in the test region (T), indicating a negative result. As a procedural control, a colored line will appear in the control region (C) if the test has been performed correctly.

Figure 16 One Step Hepatitis C Virus Whole Blood/Serum/Plasma Test

Source: Wondfo, 2024

4. Onsite HCV Ab Plus Rapid test (strip) (CTK Biotech,USA) (CTK Biotech, 2014)

- Performance: Sensitivity 98.7 %, Specificity 99.6 %
- Principle of the test:

OnSite HCV Ab Plus Rapid Test is a double-antigen lateral flow chromatographic immunoassay. The test cassette includes (1) a burgundy-colored conjugate pad containing recombinant HCV fusion antigens (core, NS3, NS4, and NS5) conjugated with colloidal gold (HCV Ag conjugates) and a control antibody, and (2) a membrane strip with a test line (T line) and a control line (C line), both coated with recombinant HCV fusion antigens and a control antibody.

When an adequate volume of test specimen is dispensed into the sample well, it migrates across the cassette by capillary action. If HCV antibodies are present in the specimen, they will bind to the HCV Ag conjugates. This immunocomplex is then

captured on the membrane by the pre-coated, non-conjugated HCV fusion antigen, forming a burgundy-colored T line that indicates a positive or reactive result for HCV antibodies. The absence of a T line suggests a negative result.

The test includes an internal control (C line), which should display a burgundy-colored line formed by the immunocomplex of control antibodies, regardless of the presence of color on the T line. If the C line does not appear, the test result is invalid, and the specimen should be retested using a new device.

Figure 17 Onsite HCV Ab Plus Rapid test (strip) (CTK Biotech, USA)

Source: CTK Biotech, 2024

5. HCV Rapid test Cassette (ACRO BIOTECH, INC, USA) (ACRO BIOTECH, 2018)

- Performance: Sensitivity 98.7 %, Specificity 99.6 %
- Principle of the test:

HCV Rapid Test Cassette (Serum/Plasma) is a qualitative test designed for detecting HCV antibodies. The membrane is pre-coated with recombinant HCV antigen in the test line region of the cassette. During testing, the serum or plasma specimen reacts with recombinant HCV antigen conjugated with colloidal gold. This mixture then migrates upward on the membrane by capillary action, reacting with the recombinant HCV antigen and producing a colored line. The presence of this colored line indicates a positive result, while the absence of a line indicates a negative result.

As a procedural control, a colored line appears in the control line region, confirming that the proper volume of specimen has been added and that membrane wicking has occurred correctly.

In this study, five rapid anti-hepatitis C test kits were used, represented by the numbers 1, 2, 3, 4, and 5, respectively shown in Table 1.

Table 1 The detail of selected HCV rapid test kit & lab Automate

Detailed	Automate	Rapid test kits				
	Elecsys Anti-HCV II	1	2	3	4	5
HCV capture antigen	core, NS3 and NS4	core, NS3, NS4 and NS5	core, NS3, NS4 and NS5	Core, NS1, NS2, NS3, NS4, NS5	core, NS3, NS4 and NS5	not available
Sensitivity	100%.	98.9%	99.3 %	99.0%	98.7 %	98.7 %
Specificity	99.66%.	100%.	98.1 %	99.8%	99.6 %	99.6 %
Specimen type	serum and plasma (potassium EDTA, lithium heparin, sodium heparin, and sodium citrate).	serum, plasma, or whole blood.	serum, plasma (heparin, EDTA and sodium citrate) or whole blood.	Whole Blood, Serum or Plasma	Whole Blood, Serum or Plasma	serum, plasma

8.5 ISO 17034:2016; General requirements for the competence of reference material producers (ISO 17034, 2016).

ISO 17034:2016, titled "General requirements for the competence of reference material producers (RMPs)," is a standard published by the International Organization for Standardization (ISO). It establishes the requirements and guidelines for organizations involved in the production of reference materials (RMs).

Reference materials play a crucial role in ensuring the accuracy and comparability of measurements worldwide. They are used for various purposes, including calibration, quality control, proficiency testing, method validation, and assigning values to other

materials. Certified reference materials (CRMs) are particularly important for establishing or confirming metrological traceability to conventional scales.
However, it's worth noting that ISO 17034:2016 does not specifically address statistical methods for assessing the homogeneity and stability of reference materials for qualitative properties. This means that the standard focuses primarily on the general requirements and competencies of reference material producers, rather than the specific statistical approaches for qualitative property assessment.
If you require guidance on the assessment of homogeneity and stability for qualitative properties of reference materials, it may be necessary to consult other sources or standards that specifically cover these aspects.

8.5.1 Reference materials (RMs)

Reference materials (RMs) are essential tools used throughout the measurement process for method validation, calibration, quality control, and interlaboratory comparisons. RMs are materials that possess specified properties and are sufficiently homogeneous and stable. They are used to assess the accuracy and performance of measurement methods, establish traceability, monitor quality control, and compare results between laboratories. Homogeneity ensures representative sampling and accurate measurement, while stability ensures that the material's properties remain consistent over time. RMs provide a reliable basis for calibration, method validation, quality control, and proficiency testing, contributing to the accuracy and comparability of measurements across different entities and industries.

8.5.2 Certified reference material (CRM)

A certified reference material (CRM) is a type of reference material that has undergone additional certification processes to ensure its properties are reliable. It is characterized using valid metrological procedures for specified properties and accompanied by an RM certificate. The certificate includes the value of the specified property, its associated uncertainty, and a statement of metrological traceability.

CRMs are distinguished by their independent evaluation and certification, which verifies their homogeneity, stability, and traceability. This certification provides a higher level of confidence in the CRM's properties and allows it to be used as a benchmark or standard in calibration, method validation, quality control, and proficiency testing.

The CRM certificate provides comprehensive information about the measured value, uncertainty, and traceability of the specified property. This enables users to rely on the CRM as a trustworthy reference in various measurement processes. Overall, CRMs play a crucial role in ensuring the accuracy and reliability of measurements by providing certified values with associated uncertainties and traceability statements.

8.6 ISO Guide 35:2017; Guidance for characterization and assessment of homogeneity and stability (ISO Guide 35, 2017).

" Reference materials - Guidance for characterization and assessment of homogeneity and stability," is a standard published by the International Organization for Standardization (ISO). This guide provides guidance and recommendations to produce reference materials, specifically focusing on the assessment of homogeneity and stability, as well as other important aspects related to the characterization and value assignment of properties.

Key concepts and approaches covered in ISO/Guide 35:2017:

Assessment of Homogeneity: The guide provides guidance on how to assess the homogeneity of a reference material. Homogeneity refers to the uniformity of the material's properties within a given batch or unit. The standard outlines various statistical and experimental approaches for determining the degree of homogeneity.

Assessment of Stability: ISO/Guide 35:2017 offers guidance on assessing the stability of reference materials. Stability refers to the ability of a material to maintain its properties over a defined period. The guide provides approaches for evaluating and managing the risks associated with potential stability issues that could affect the properties of interest in the reference material.

Characterization and Value Assignment: The standard discusses the characterization of reference materials, which involves determining the properties or characteristics of the material that are relevant for its intended use. It also provides guidance on assigning values to these properties, including certified values, which are values with known uncertainties.

Evaluation of Uncertainty: ISO/Guide 35:2017 addresses the evaluation of uncertainty associated with the certified values assigned to reference materials. Uncertainty quantifies the range within which the true value of a property is expected to lie. The guide provides guidance on estimating and expressing uncertainties associated with the certified values.

8.7 ISO 15189:2022 Medical laboratories: Requirements for quality and competence (*ISO 15189*, 2022)

"Medical laboratories - Requirements for quality and competence." It specifies the quality management system requirements for medical laboratories to demonstrate their competence and ability to provide reliable laboratory services. The standard focuses on ensuring the accuracy, reliability, and quality of laboratory testing, which is crucial for patient care and treatment.

key points and requirements outlined in ISO 15189:2022:

Quality management system: The standard emphasizes the implementation of a robust quality management system (QMS) within the laboratory. The laboratory must establish policies, procedures, and processes to ensure quality, safety, and continuous improvement.

Organization and management: The standard address the need for effective laboratory management, including defining the laboratory's organizational structure,

roles, responsibilities, and management of resources. It also emphasizes the importance of maintaining appropriate documentation and records.

Personnel competence: ISO 15189:2022 emphasizes the competence of laboratory personnel. This includes ensuring that staff members possess the necessary education, training, skills, and experience to perform their assigned tasks competently. Competency assessment and ongoing professional development are also vital aspects.
Pre-analytical, analytical, and post-analytical processes: The standard outlines requirements for the entire laboratory testing process, including pre-analytical (sample collection, transportation, and preparation), analytical (testing and analysis), and post-analytical (result validation, reporting, and interpretation) processes. It focuses on accuracy, traceability, and minimizing errors throughout these stages.

Equipment and facilities: ISO 15189:2022 specify requirements for equipment, instrumentation, and facilities used in the laboratory. This includes calibration, maintenance, and verification of equipment to ensure accurate and reliable results. Adequate facilities and infrastructure, including safety measures, are also addressed.

Internal and external quality control: The standard highlights the importance of implementing internal quality control measures to monitor the accuracy and precision of test results. It also emphasizes participation in external quality assessment programs to compare the laboratory's performance with other laboratories and ensure ongoing proficiency.

8.8 Quality Assurance Programs, or Quality Management Systems (QMS)

Quality Assurance Programs, also known as Quality Management Systems (QMS), are systematic approaches implemented by organizations to ensure that their products or services consistently meet or exceed customer expectations. These programs aim to monitor and improve the quality of processes, procedures, and outputs, ultimately leading to enhanced customer satisfaction.
Key elements and activities within Quality Assurance Programs include:

Quality Policies and Objectives: Establishing clear quality policies and objectives that align with the organization's overall goals and customer requirements.

Documentation and Standard Operating Procedures (SOPs): Developing and maintaining documented procedures and work instructions that outline specific quality-related activities, ensuring consistency and standardization.

Risk Management: Identifying and assessing potential risks to quality, implementing measures to mitigate those risks, and establishing contingency plans.

Training and Competence: Providing adequate training and development opportunities for employees to ensure they possess the necessary skills and knowledge to perform their tasks effectively and maintain quality standards.

Process Control: Implementing measures to control and monitor key processes, including document control, change management, and recordkeeping.

Monitoring and Measurement: Regularly monitoring and measuring performance indicators and quality metrics to assess the effectiveness of processes and identify areas for improvement.

Internal Audits: Conducting internal audits to evaluate compliance with quality standards, identify non-conformities, and initiate corrective actions.

Corrective and preventive Actions (CAPA): Establishing procedures for addressing non-conformities, initiating corrective actions to address immediate issues, and implementing preventive actions to avoid future occurrences.

Continuous Improvement: Encouraging a culture of continuous improvement by seeking opportunities for enhancing quality, efficiency, and customer satisfaction through regular reviews, data analysis, and implementation of improvement initiatives.

External Assessments and Certifications: Participating in external assessments and certifications by recognized accreditation bodies to validate adherence to specific quality standards and demonstrate the organization's commitment to quality.

8.9 Trehalose

Trehalose is a type of non-reducing disaccharide, consisting of two glucose molecules linked together in a 1,1-glycosidic bond. Among the three possible anomers of trehalose (a, b-1,1-, b, b-1,1-, and a, a1,1-), only the a, a-trehalose (shown in Figure 1) has been found in living organisms. This naturally occurring disaccharide is widely distributed in the biological world. Trehalose has been known to be present in lower plant species for many years, with the first report dating back to 1832 in ergot of rye.

Trehalose is commonly found in yeast and fungi, where it is present in spores, fruiting bodies, and vegetative cells. For instance, *Dictyostelium mucoroides* spores and macrocysts have been found to contain as much as 7% trehalose on a dry-weight basis. Overall, trehalose is a versatile sugar with diverse functions in different organisms. Its unique properties make it an essential molecule for survival and adaptation in various environments. The widespread presence of trehalose across different biological systems highlights its importance and potential applications in biotechnology, food science, and medicine (Clegg & Filosa, 1961; Elbein, 1974).

8.10 StabilZyme™ SELECT Stabilizer

StabilZyme™ SELECT Stabilizer is a comprehensive stabilizing solution specifically formulated to maintain the structural integrity of antibody/antigen conjugates, antibody-coated particles, and unmodified proteins, all of which are commonly utilized in immunoassays. Its unique composition helps preserve the functional conformation of these components, which is crucial for maintaining assay sensitivity and specificity over extended storage periods.

When conjugates or proteins are mixed directly with SELECT Stabilizer, they achieve a stable, ready-to-use concentration, eliminating the need for further dilutions. This feature simplifies preparation workflows and reduces handling errors, ensuring reliable performance across various assay conditions. Additionally, SELECT Stabilizer's stabilizing properties are effective under a range of temperature and storage conditions, making it an ideal choice for immunoassays that require consistent quality and performance stability (Lee et al., 2014).

Figure 18 StabilZyme™ SELECT Stabilizer

Source: Surmodics, 2024

8.11 Elecsys Anti-HCV II (Roche, Germany) (Diagnostics, 2015 V 0.5)

Test principle

The assay follows the sandwich principle and has a total duration of 18 minutes.

- 1st incubation: A sample of 50 µL is mixed with a reagent containing biotinylated HCV-specific antigens (55 µL) and a reagent containing HCV-specific antigens labeled with a ruthenium complex (55 µL). This mixture reacts to form a sandwich complex.
- 2nd incubation: Streptavidin-coated microparticles are added to the mixture, causing the sandwich complex to bind to the solid phase through the interaction between biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell, were the microparticles are magnetically captured on the surface of the electrode. Unbound substances are then removed using Procell/ProCell M.
- A voltage is applied to the electrode, inducing chemiluminescent emission, which is measured by a photomultiplier.

Results are automatically determined by the software by comparing the electrochemiluminescence signal obtained from the reaction product of the sample with the cutoff value signal previously obtained through calibration.

Figure 19 Elecsys Anti-HCV II (Roche, Germany)

Source: Diagnostics, 2024

8.12 Fresh frozen plasma (FFP)

Fresh Frozen Plasma (FFP) is a type of plasma derived from human blood donations, containing essential proteins critical for various medical treatments, especially for replacing blood clotting factors. FFP is obtained through the following processes:

Plasma Separation: FFP can be prepared by separating plasma from whole blood donations or through a process called plasmapheresis. In plasmapheresis, plasma is separated and collected, while the remaining blood components are returned to the donor. This process yields a higher volume of plasma, allowing for more effective collection.

Timely Freezing: After plasma is separated, it must be frozen within a specified timeframe (usually within 6-8 hours after collection) to preserve the integrity of essential proteins and clotting factors. Freezing typically occurs at temperatures below -18°C, with long-term storage at -30°C or lower, which helps to maintain protein quality over extended periods (Department of Health, 2002).

Key Components of FFP: Coagulation Proteins: FFP contains vital clotting factors, including Factor VIII, Factor IX, and fibrinogen, which are essential for blood clotting. Acute-Phase Proteins: These proteins respond to inflammation or injury, helping the body manage infections and trauma. Immunoglobulins: These antibodies support the immune system by helping to identify and neutralize pathogens.
Albumin: The primary protein in plasma, albumin maintains fluid and electrolyte balance and helps regulate blood pressure (Wallace, 2003).

Clinical Uses: FFP is used to treat bleeding disorders due to clotting factor deficiencies in patients with acute liver failure, Disseminated Intravascular Coagulation (DIC), or massive blood loss from trauma. It is also utilized in patients with genetic clotting disorders.
It is beneficial for patients who require urgent clotting factor replacement before undergoing surgery or invasive procedures and for those who cannot produce enough plasma proteins on their own.

Preparation and Usage: Before administration, FFP must be thawed at 30-37°C and used within 24 hours of thawing to ensure protein quality. Proper thawing and handling are essential to avoid contamination and maintain patient safety (Department of Health, 2002).

8.13 Xenon Test Chamber Model Q-Sun Xe-1 Brand Q-LAB (Corporation, 2024)

The Q-SUN Xe-1 Xenon Test Chamber, Model Xe-1 by Q-LAB, reproduces the damage caused by full-spectrum sunlight and rain. In just a few days or weeks, the Q-SUN tester can replicate the damage that would typically occur over months or years outdoors. The Q-SUN Xe-1 tester is a tabletop chamber for lightfastness, colorfastness, and photostability testing. It features a single xenon arc lamp and offers optional water spray and chiller. Its compact design is ideal for labs with a limited budget or only occasional testing needs. Installation of the Xe-1 tester is simple, and specimens can be easily mounted horizontally on its flat specimen tray. The Q-SUN Xe-1 tester is the simplest, most reliable, and easiest-to-use xenon arc test chamber available

Figure 20 Xenon Test Chamber Model Q-Sun Xe-1 Brand Q-LAB

Source: Corporation, 2024

8.14 Phosphate-Buffered Saline (PBS) (Scientific, n.d.-a; Sigma-Aldrich, n.d.)

Phosphate-Buffered Saline (PBS) is a water-based salt solution that is commonly used in biological research. PBS is made up of sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate (Na_2HPO_4), and potassium phosphate (KH_2PO_4). This buffer solution is designed to maintain a constant pH (typically around 7.4), which is like that of human body fluids, making it particularly suitable for handling biological samples.

Detailed Composition and Properties

Components and Their Roles:

Sodium Chloride (NaCl) and Potassium Chloride (KCl): These salts provide essential ions that mimic the osmolarity and ion concentration of body fluids, helping to maintain cellular integrity and prevent osmotic stress in biological samples.

Sodium Phosphate (Na_2HPO_4) and Potassium Phosphate (KH_2PO_4): These buffering agents help stabilize the pH of the solution. A stable pH is critical when working with biological samples, as fluctuations in pH can denature proteins, affect enzyme activities, and alter cellular processes.

Typical Uses of PBS in Research

Cell Culture: PBS is often used to rinse cells during culture to remove any residual media before processing cells for assays, staining, or other experimental procedures.

Sample Washing: It's also used in immunology and molecular biology for washing samples in techniques like ELISA (Enzyme-Linked Immunosorbent Assay), Western blotting, and immunohistochemistry. PBS provides a stable environment that helps prevent non-specific binding and reduces background signal.

Dilution and Reconstitution: In laboratory settings, PBS is used as a diluent to prepare reagents or reconstitute lyophilized samples. The physiological pH and salt concentration help preserve the activity of proteins and enzymes during dilution.

Preparation of PBS

PBS can be prepared by dissolving precise amounts of NaCl, KCl, Na_2HPO_4, and KH_2PO_4 in distilled water and adjusting the pH to 7.4. The solution is often sterilized by autoclaving for use in sterile cell culture and other biological applications.

Advantages of Using PBS

pH Stability: The buffering capacity of PBS ensures that the pH remains stable, which is essential for maintaining the structure and function of biological molecules.

Isotonic Solution: PBS's salt concentration is designed to be isotonic, meaning it prevents cells from shrinking or bursting due to osmotic pressure, making it ideal for

cell handling. PBS is widely applicable across numerous laboratory procedures, from tissue and cell rinsing to acting as a diluent in assays, making it a fundamental solution in biological research.

8.15 Tween 20 (Polysorbate 20) (Scientific, n.d.-b)

Tween 20 (Polysorbate 20) is a non-ionic surfactant widely used in biological and medical research laboratories. It helps to effectively disperse substances that are insoluble in water, making it invaluable in various applications. Tween 20 is synthesized from polyethylene oxide and sorbitan, specifically through the esterification of lauric acid and polysorbate. It is considered a highly safe substance and is typically used in low concentrations.

Properties and Benefits of Tween 20 can reduce surface tension, allowing it to disperse insoluble substances, such as proteins or biological compounds, evenly in aqueous solutions. This property is especially beneficial for preparing samples that require consistent distribution for accurate analysis. As a non-ionic surfactant, Tween 20 does not interact with charged molecules or ions, allowing it to be used in a wide variety of solutions and biological samples without altering their chemical properties. Tween 20 is stable across a range of pH levels and temperatures, making it suitable for applications under diverse conditions, such as varying temperatures and in solutions with different acidity or alkalinity.

Applications in Research and Medicine: Tween 20 is used to disperse proteins in aqueous solutions, facilitating accurate testing and analysis. In ELISA and Western blot tests, which require high precision, Tween 20 is used in washing steps for samples and membranes to prevent unwanted binding and reduce background noise, enhancing the clarity of results. Tween 20 is used to stabilize emulsions, ensuring the mixture of water and oil-based components in products such as creams and topical ointments. This helps the products maintain consistency and stability over time.

Precautions and Handling: Although Tween 20 is considered highly safe, it should be used in appropriate quantities, as excessive amounts may alter the properties of biological samples. It should also be stored in suitable conditions to preserve its effectiveness.

Literature Review

Rapid diagnostic tests (RDTs) can be viable alternatives to standard serological assays for detecting hepatitis C virus (HCV) in settings with limited resources. The objective of this study was to assess the performance of three RDTs manufactured in India in comparison to chemiluminescent microparticle immunoassay (CLIA) for HCV infection screening (Mahajan et al., 2019).

Performance evaluation of three rapid screening assays for the detection of antibodies to the hepatitis C virus in Cameroon was conducted. The study involved 200 plasma samples, including 100 positive and 100 negative samples, which were stored at -80 °C at the Centre Pasteur of Cameroon (CPC). These samples were screened for anti-HCV antibodies at the same laboratory using an automated chemiluminescent microparticle immunoassay called the Architect anti-HCV assay by Abbott Diagnostics, Wiesbaden, Germany. The performance of three different rapid tests, namely the Multisure-HCV Antibody Assay, First Response® HCV Card Test, and Toyo® Anti HCV Test, was evaluated. The tests were carried out by three independent laboratory technicians who were blinded to the sample status. The study did not include any specific control other than the internal control incorporated in each assay. The results of the study indicated that none of the evaluated rapid tests met the European Union standards, which require a sensitivity and specificity of ≥ 99%. These standards are based on studies conducted in Europe, where the conditions for performing the assays differ significantly from those in resource-limited countries in terms of adherence to quality assurance practices (such as supply chain reliability and implementation of standardized logbooks). The World Health Organization (WHO) has clearly demonstrated the high probability of obtaining false results when rapid tests are not conducted in accordance with quality assurance measures (Fondjo et al., 2018).

Rapid diagnostic test kits (RDTs) are valuable tools for quickly identifying individuals infected with the hepatitis C virus (HCV), and the availability of high-quality RDTs is crucial for expanding HCV screening efforts. The purpose of this study was to assess the performance of five anti-HCV RDTs. The results of the evaluation revealed that Alere Truline, SD Bioline, and OraQuick RDTs met the sensitivity and specificity criteria established by the Drug Controller General, India, the national regulatory authority. These RDTs also exhibited excellent inter-observer agreement and demonstrated superior operational characteristics. Based on our findings, certain HCV RDTs show favorable performance and can be a valuable tool for efficiently screening HCV infections (Mane et al., 2019).

The diagnosis of hepatitis C virus (HCV) infection involves testing for antibodies to HCV (anti-HCV), hepatitis C core antigen (HCV Ag), and HCV RNA. To ensure quality control (QC) and quality assurance (QA), proficiency panels are provided by reference laboratories and international organizations. However, shipping these panels with dry ice can be expensive and challenging, especially for laboratories in resource-limited countries. This study explored alternative methods of specimen preservation and transport to improve diagnostics and reduce costs. Plasma samples positive for anti-HCV and HCV RNA was either dried using the dried tube specimens (DTS) method or lyophilized at different time and temperature intervals. The DTS method resulted in the loss of anti-HCV reactivity in low-positive samples and insufficient volume for HCV RNA testing. On the other hand, lyophilized samples maintained positive results for anti-HCV even after storage at 4°C and 25°C for 12

weeks. Additionally, HCV RNA remained detectable in all samples stored at 4°C, 25°C, 37°C, and 45°C over the course of 12 weeks. In conclusion, lyophilization of specimens preserved the integrity of plasma samples for testing HCV infection markers and can be an effective method for sharing proficiency samples without the need for costly shipping with dry ice, addressing challenges faced by donor and recipient laboratories (Poe, Duong, Bedi, & Kodani, 2018).

The laboratory diagnosis of HCV infection typically relies on the detection of circulating antibodies. To ensure accurate and reliable testing, laboratories performing anti-HCV tests should have a well-established quality assurance program. Quality control (QC) materials, including Internal Quality Control (IQC) and External Quality Assessment Scheme (EQAS), play a crucial role in this process. Creating IQC samples is a cost-effective method for QC material. This study aimed to investigate the impact of temperature on the stability of anti-HCV antibodies in plasma during storage. Sensitive and specific ELISA assays designed for detecting anti-HCV antibodies were used to evaluate stability. Anti-HCV positive samples were subjected to incubation at three different temperatures (37°C, 48°C, and 65°C) for 7 days and 14 days, and the antibodies were measured using two ELISA kits. The results clearly indicate that temperature influences the stability of anti-HCV antibodies in plasma samples. Elevated temperatures significantly reduce the E-ratios compared to the initial values observed immediately after transitioning the samples from -80°C to room temperature. Therefore, it is essential to store IQC samples under ideal conditions to maintain their stability (Charu Mehra Kamal* & Sharma, 2015).

Developing a highly stable Dried Tube Specimen (DTS) for use in quality assurance programs and training related to rapid HIV testing. This DTS was specifically designed as a quality control material for testing recent HIV infections. A key innovation in this DTS is the addition of trehalose, a sugar known for its properties in stabilizing proteins and cells. By adding trehalose to the sample before drying, the DTS achieved remarkable stability, maintaining its properties at temperatures as high as 37°C for up to 12 weeks without requiring cold storage. The study found that DTS samples treated with trehalose retained their infection classification consistently across various temperatures, including 4°C, 25°C, and 37°C, throughout the study period (up to 12 weeks). In contrast, DTS samples without trehalose tended to change infection classification when stored at higher temperatures. The trehalose-treated DTS showed the ability to preserve its testing characteristics without the need for a cold chain, reducing costs and logistical complexity in transport and storage, especially in resource-limited settings. The findings suggest that trehalose-stabilized DTS has potential as a quality control material for rapid HIV testing in point-of-care settings and resource-constrained areas. Additionally, it is suitable for use in proficiency testing programs in field sites lacking temperature-controlled storage, offering an effective tool for field-based quality assurance (Di Germanio et al., 2023) .

Evaluating the performance of 13 rapid diagnostic tests (RDTs) for detecting Hepatitis C Virus (HCV) antibodies across different populations, including both HIV-infected and HIV-uninfected individuals. Conducted in diverse settings—Nigeria, Georgia, Cambodia, and Belgium—the study utilized archived plasma samples to assess the sensitivity and specificity of each test, with the aim of identifying tests suitable for use in low- and middle-income countries (LMICs) where HCV prevalence is high, and resources are limited. Findings indicate that in HIV-uninfected samples, most RDTs demonstrated high sensitivity (≥98%) and specificity (≥99%), meeting World Health

Organization (WHO) performance criteria. However, in HIV-infected samples, while specificity remained relatively high, sensitivity decreased significantly, with only one RDT achieving greater than 95% sensitivity. This reduced sensitivity in HIV-positive samples is thought to be due to lower anti-HCV antibody levels in individuals co-infected with HIV. Importantly, samples with detectable HCV viral load or core antigen (cAg) showed improved sensitivity, suggesting that active HCV infection in co-infected individuals may enhance test performance. Operationally, the RDTs exhibited consistent results across multiple readers and between different production lots, with very low rates of invalid tests, underscoring the reliability and reproducibility of these RDTs under varying conditions. The study highlights the impact of HIV coinfection on HCV RDT sensitivity, emphasizing the necessity for these tests to be evaluated in HIV-endemic populations to ensure accuracy (Vetter et al., 2022).

CHAPTER III
RESEARCH METHODOLOGY

1.Research Methodology

This research consists of 4 main studies. Overview of methodology of this research is on Figure 4

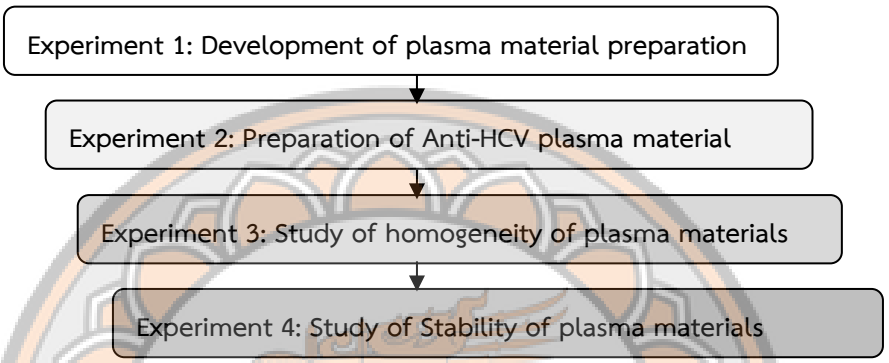


Figure 21 Overview of conducting research

2. Research materials

2.1 Chemicals and reagents
- StabilZyme™ SELECT Stabilizer (Surmodics, Inc., United States)
- PBS (Phosphate Buffered Saline)
- Tween 20 (Polysorbate 20) (HiMedia Laboratories LLC, USA)
- Bronidox (BASF SE, Germany)
-Trehalose (Thermo Fisher Scientific, United States)

2.2 Instruments and Equipment
- Autopipette (Eppendorf, Germany)
- Biosafety cabinet (Thermo Fisher Scientific, USA)
- Cobas e 411 analyzers (Roche Diagnostics, Switzerland)
- ScanVac CoolSafe Freeze Dryer (LaboGene ApS, Denmark)
- Hot air oven (Memmert, Germany)
- Magnetic Stirrer and magnetic bar (IKA, Germany)
- Pipette aid and Seropipette (BrandTech Scientific, Germany)
- Q-Sun Model Xe-1 (Q-Lab Corporation, United States)
- Pipette and Tip (AxyPet™, Axygen, USA)

3.Experiment

Experiment 1: Development of plasma material preparation for quality control plasma materials and the linearity panel materials

1.1 Preparation of plasma material

1.1.1 Plasma samples were obtained from excess fresh frozen plasma collected at a blood bank. The selected samples were classified as HCV positive, or HCV negative based on results from blood donors. Comprehensive reference results were provided for both nucleic acid tests and serological tests.

1.1.2 Screening was conducted using the Cobas e 411 analyzer with the Elecsys Anti-HCV II assay (Roche, Germany). Plasma stock samples with results ≥ 1.00 COI were considered reactive. Samples with high values were collected for dilution purposes, creating a plasma sample pool for preparing plasma materials.

1.1.3 Dilute the anti-HCV stock pool using a two-fold dilution with HCV Negative FFP diluent solution. To prepare a two-fold serial dilution from a plasma sample pool, follow these steps:

- The initial dilution at a 1:2 ratio was prepared by mixing 1.0 ml of the plasma sample with 1.0 ml of the HCV Negative FFP, achieving a 1:2 dilution. Precise measurements were essential to maintain consistency and accuracy across all dilution steps.
- Proper mixing is ensured for homogeneity by thoroughly combining the plasma sample and the diluent. The solution is mixed by inverting the tube or using a vortex mixer until it appears uniformly mixed and homogeneous, which is essential for accurate and reliable test results in subsequent dilutions.
- The 1:2 diluted mixture is then transferred carefully into a clean, labeled tube or vial designated for dilution purposes to avoid contamination. Each tube or vial used is sterile and free from residual substances.
- To create further dilutions, 1.0 ml of the 1:2 dilution mixture is combined with 1.0 ml of the diluent to achieve a 1:4 dilution. This process is repeated by transferring 1.0 ml from each subsequent dilution and mixing it with 1.0 ml of the diluent, continuing until the desired dilution levels are reached (e.g., 1:8, 1:16, 1:32, and so forth).
- The dilution series is completed by repeating the process until the final dilution reaches the target concentration of 1:1024 for the rapid anti-hepatitis C test and 1:16384 for the Elecsys Anti-HCV II test. Each tube is accurately labeled with the corresponding dilution factor to prevent mix-ups during testing.
- Finally, all tubes are visually inspected to ensure no phase separation or inconsistencies in mixing. The diluted samples are stored under appropriate conditions until further use, following laboratory guidelines.

1.1.4 Anti-HCV rapid test procedure

- Materials were prepared by ensuring the test kit and plasma samples were at room temperature.
- The plasma sample was added by using a pipette to place the specified amount into the sample well.
- The buffer solution was then added by dropping the recommended amount into the same well.
- Results were waited for 15-30 minutes, following the manufacturer's instructions to ensure accuracy.
- Results were interpreted as follows: if only the control line appears, the result is negative (indicating no antibodies detected). If both the control and test lines appear, the result is positive (indicating HCV antibodies detected). If no control line appears, the result is invalid, and the test must be repeated with a new kit.
- The reading of test results for the rapid anti-hepatitis C test was categorized based on the intensity of the test line, which reflected the concentration or presence of the target analyte. Reactive intensity levels were classified into four levels: 4+, 3+, 2+, and 1+, with 4+ indicating the highest reaction intensity, suggesting a strong presence of the analyte, and 1+ indicating a weak but detectable presence. For non-reactive samples, where no visible test line appeared, the result was recorded as 0, indicating the absence of the target analyte. Proper interpretation of the test line intensity helped determine the level of analyte concentration in each sample, ensuring accuracy in clinical evaluations. If no control line appears, the result is invalid, and the test must be repeated with a new kit. Detailed instructions on how to interpret these test results were provided in Table 2 and Figure 6.

1.1.5 Elecsys Anti-HCV II test procedure

- Samples and reagents are prepared by ensuring all reagents and controls are at the correct temperature and ready for use.
- The Cobas e411 analyzer is loaded by placing the reagents, calibrators, and controls in their designated slots.
- Plasma or serum samples are added by using the automated sample loading system or manually loading the samples into the analyzer.
- The test is run by selecting the Elecsys Anti-HCV II protocol on the system interface and starting the process.
- Results are interpreted automatically by the system, which displays quantitative or qualitative results after processing. The manufacturer's instructions are followed precisely to ensure accurate outcomes. Detailed instructions on how to interpret these test results were provided in Table 2 and Figure 6.

Table 2 Intensity levels of test band and descriptions for Interpreting results of rapid anti-hepatitis C test

Reaction intensity level	Test band intensity description
0	The test line is absent or visible only as a faint shadow without detectable coloration.
1+	The test line is less than half as intense compared to the control line.
2+	The test line is half as intense compared to the control line.
3+	The test line is more than half as intense compared to the control line.
4+	The test line is as intense as the control line.

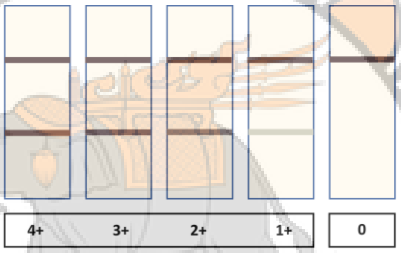


Figure 22 Band Intensity levels and descriptions for Interpreting results of rapid anti-hepatitis C test

1.1.7 Select three dilutions that correspond to the following test result levels: concentration of preparing QC Samples for rapid anti-hepatitis C test, select 3 levels of concentration

- **Level 1 Strong Positive:** These samples show reactive test results with a reaction intensity close to the control line.
- **Level 2 Weak Positive:** These samples show reactive test results with a different reaction intensity compared to the control line.
- **Level 3 Negative:** These samples show non-reactive test results, indicating no reaction (From HCV negative).

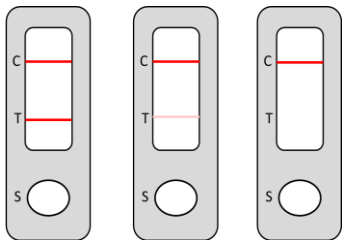


Figure 23 Concentration of preparing QC Samples of rapid anti-hepatitis C test

1.1.8 The selection of six concentration levels for the preparation of the Elecsys Anti-HCV II Linearity Panel is a critical step in verifying both the accuracy and linearity of the system's response across the designated sample concentration range. It is essential that the preparation appropriately encompasses the required concentration range for the assay, defined as follows:Beginning with a high COI (≥1.00 COI) HCV-positive plasma sample, which serves as the initial sample obtained from the Elecsys Anti-HCV II measurement, this sample will be subjected to a 2-fold serial dilution using an HCV-negative FFP diluent. This method will yield a range of six concentrations, which includes High Concentration, Medium to Low Concentration, Very Low Concentration, and Negative Control.

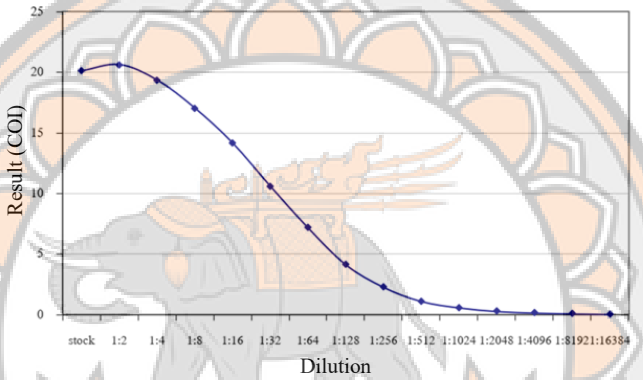


Figure 24 Test results of samples with Elecsy Anti-HCV

1.1.9 Prepare plasma material follow dilution suitable both liquid Sample and lyophilized sample. Type of plasma material shown in Table 3.

Liquid Sample

- Frozen plasma samples were thawed by placing them at 2-8°C until they are completely thawed.
- The samples were inactivated by incubating them in a water bath at 56°C for 40 minutes (Song et al., 2010).
- The samples were centrifuged at 10,000 rpm at 4°C for 10 minutes, after which the supernatant is removed.
- The samples were filtered using filter paper with a pore size of 0.8 µm.
- Bronidox was added to a final concentration of 0.05% (Sigma-Aldrich, St. Louis, MO, USA) (Huh et al., 2023).
- Plasma material was prepared by following the appropriate dilution protocol for liquid samples. Samples were aliquoted in 1 mL volumes per tube. Three concentrations of plasma materials were tested using a rapid anti-hepatitis C test, and six concentrations of plasma materials were tested using a laboratory-based immunoassay, the Elecsys Anti-HCV II.
- Liquid samples were prepared with the addition of StabilZyme™ SELECT Stabilizer and Negative FFP.

Lyophilized sample

- Frozen plasma samples were thawed by placing them at 2-8°C until they were completely thawed.
- The samples were inactivated by incubating them in a water bath at 56°C for 40 minutes (Song et al., 2010).
- The samples were centrifuged at 10,000 rpm at 4°C for 10 minutes, after which the supernatant was removed.
- The samples were filtered using filter paper with a pore size of 0.8 µm.
- Bronidox was added to a final concentration of 0.05% (Sigma-Aldrich, St. Louis, MO, USA) (Huh et al., 2023).
- Plasma material was prepared by following the appropriate dilution protocol for lyophilized samples. Samples were aliquoted in 1 mL volumes per tube. Three concentrations of plasma materials were tested using a rapid anti-hepatitis C test.
- Lyophilized samples were prepared with the addition of 250 mM trehalose and Negative FFP.
- Freezing involved rapidly lowering the temperature of the sample to below -40°C to ensure that water molecules turned into solid ice. This quick-freezing process helped prevent the formation of large ice crystals, which could damage the structure of substances in the sample.
- Drying, or sublimation in a vacuum, began once the sample was frozen. The sample was placed in a lyophilized (freeze dryer) that carefully controlled temperature and pressure. The lyophilized lowered the pressure to a very low level, causing the ice to sublimate directly into vapor without passing through the liquid phase. This process typically took 24 hours.
- Once the lyophilization process was complete, the sample was removed from the lyophilized and placed in a moisture-proof container, such as a glass vial or test tube with a moisture-resistant cap. The sample was then stored in a dry, cool environment, typically in a freezer (Rey & May, 2004; Wang, 2000).
- The lyophilized sample was reconstituted with 1 ml of PBS + 0.1% Tween 20.

Table 3 Type of plasma material

Type of plasma material	
Liquid	**Lyophilization**
1.HCV Positive diluted with HCV Negative	1.HCV Positive diluted with HCV Negative
2. HCV Positive diluted with StabilZyme™ SELECT Stabilizer in HCV Negative	2. HCV Positive diluted with trehalose in HCV Negative
3.Negative Plasma	3.Negative Plasma

1.2 Accelerated stability studies of plasma material

After preparing the plasma material, it will be incubated at various temperatures to study accelerated stability. Every week, over a period of 28 days, the samples from each temperature will be transferred to -20ºC for comparison with samples that have been continuously stored at -20ºC. The timeline for collecting plasma material for the accelerated stability study is shown in Table 4.

Table 4 Sample collection for stability accelerated testing of liquid and lyophilized Samples at -20°C and 45°C using simulation machine

keep to -20 °C	45 ºC Temperature, including by Simulation machine	
	Liquid Sample (tube)	Lyophilized Sample (tube)
Day 0	10	10
Day 7	5	5
Day 14	5	5
Day 21	5	5
Day 28	5	5

1.3 Data analysis

The data collecting the sample was stored for the longest time and at the highest temperature compared to samples stored at -20 ºC on day 0 by Elecsys Anti-HCV II, using the One-Way ANOVA criteria (T < T critical). The sample was stored at the highest temperature and for the longest duration, in comparison to the samples stored at -20 ºC on day 0. This was using One Step Hepatitis C Virus Serum/Plasma Test analysis for qualitative data, with a 100% positive and negative accuracy rate.

Experiment 2: Preparation of Anti-HCV plasma material

Prepare appropriate dilution samples suitable for both liquid and lyophilized samples. Three levels of anti-HCV antibody QC samples were tested using five different test kits, and six levels of anti-HCV antibody samples for the linearity panel were tested using the Cobas e 411 (Elecsys Anti-HCV II).

2.1 Plasma material

Plasma material sample for five rapid anti-hepatitis C test dilute the plasma in a two-fold dilution with diluent solution and select three anti-HCV antibody level.

2.2 Linearity panel

Preparing linearity panel for Elecsys Anti-HCV II dilute the plasma in a two-fold dilution with diluent solution and select six anti-HCV antibody level.

2.3 Data analysis

The results obtained from the assays were visually interpreted by two independent readers. All operators involved were trained in performing and interpreting the HCV rapid diagnostic test (RDT) assays.

Experiment 3: Study of homogeneity of plasma materials

3.1 Homogeneity test

Plasma materials were prepared by selecting stable samples from accelerated stability studies and testing their homogeneity. The samples were randomly distributed, with the number of units determined in accordance with the recommendations of ISO Guide 35.

Homogeneity was checked to ensure that all tubes were consistent. The sampling strategy selected for the homogeneity study was simple random sampling. A random sample of 10 tubes was selected from the entire batch. Afterward, the random samples were tested using the Elecsys Anti-HCV II and a rapid test assay.

3.2 Data analysis

The homogeneity of the plasma materials for qualitative data was analyzed with a 100% positive and negative accuracy rate. Between-unit homogeneity was analyzed using one-way ANOVA, while within-unit homogeneity was assessed using an F-test ($F < F$ critical), based on ISO Guide 35. The results indicated that the plasma materials demonstrated statistically significant homogeneity (Guide, 2017).

Experiment 4: Study of Stability of plasma materials

4.1 Stability test

Real-time stability: The plasma materials were stored at temperatures of 2-8°C and 25 ± 5°C for a period of 6 months to investigate the storage duration of the materials. A sample control was kept at -20°C.

Transport stability: The plasma materials were stored at temperatures of 37°C and 45°C for a period of 28 days to investigate the storage duration of the materials. A sample control was kept at -20°C.

Table 5 Real-time stability studies of plasma material

keep to -20 °C	Temperature (°C)	
	2-8	**25 ± 5**
Day 0	10	10
1 Month	5	5
2 Months	5	5
3 Months	5	5
4 Months	5	5
5 Months	5	5
6 Months	5	5

4.2 Data Analysis

The stability of the quality control (QC) sample for qualitative data was analyzed with a 100% positive and negative accuracy rate and a T-test. If the material was deemed stable, the T < T critical criterion was employed to determine if the slope was significantly different (Guide, 2017).

CHAPTER IV
RESULTS AND DISCUSSION

Experiment 1: Development of plasma material preparation for quality control plasma materials and the linearity panel materials

1.1 Concentration for preparation plasma material

The test results for the positive HCV sample were obtained by conducting a two-fold serial dilution of the plasma using an HCV-negative diluent. The diluted plasma was subsequently analyzed with Kit 3 and the Elecsys Anti-HCV II assay. From these analyses, three optimal concentrations were identified for use as quality control (QC) samples, and six optimal concentrations were selected for the linearity panel.

The optimal concentrations for preparing QC samples included all three levels. level 1 (Strong Positive) was selected at a 1:4 dilution, with test results showing 4+ for test kit 1, 3+ for test kit 2, 2+ for test kit 3, 3+ for test kit 4, and 3+ for test kit 5. level 2 (Weak Positive) was selected at a 1:25 dilution, with test results showing 3+ for Kit 1, 1+ for Kit 2, 1+ for Kit 3, 1+ for Kit 4, and 2+ for Kit 5. Level 3 (Negative) used HCV negative samples and testing with all five brands of Rapid Test kits in 0 (non-reactive) results, confirming the absence of reactivity, as shown in Table 6 and Appendix A.

Table 6 Test results of sample dilution series and reactivity results for Elecsys Anti-HCV II and five rapid anti-hepatitis C tests

Dilution	Elecsys Anti-HCV II (COI)	Test Kit 1	Test Kit 2	Test Kit 3	Test Kit 4	Test Kit 5
HCV Liquid Stock	74.07	4+	4+	3+	3+	3+
HCV Liquid 1:2	104.80	4+	4+	3+	3+	3+
HCV Liquid 1:4	153.2	4+	3+	2+	3+	3+
HCV Liquid 1:8	245.30	4+	3+	2+	2+	4+
HCV Liquid 1:16	237.90	4+	2+	2+	1+	3+
HCV Liquid 1:25	204.80	3+	1+	1+	1+	2+
HCV Liquid 1:32	172.70	3+	1+	1+	1+	2+
HCV Liquid 1:64	101.60	3+	1+	1+	1+	2+
HCV Liquid 1:128	55.48	1+	0	1+	0	1+
HCV Liquid 1:256	28.50	1+	0	0	0	0
HCV Liquid 1:512	14.70	0	0	0	0	0
HCV Liquid 1:1024	7.53	0	0	0	0	0
HCV Liquid 1:2048	3.78	NA	NA	NA	NA	NA
HCV Liquid 1:4096	1.88	NA	NA	NA	NA	NA
HCV Liquid 1:8192	0.971	NA	NA	NA	NA	NA
HCV Liquid 1:16384	0.536	NA	NA	NA	NA	NA

Note: Reaction intensity levels 1+, 2+, 3+, and 4+ = Reactive; 0 = Non-Reactive; NA = Not Analyzed.

The optimal concentrations for preparing linearity panel samples were carefully determined using the Elecsys Anti-HCV II test kit. The results were subsequently plotted on a graph to identify the most suitable concentrations, as depicted in Figure 8. The selected concentrations include Level 1 at 1:16, Level 2 at 1:32, Level 3 at 1:128, Level 4 at 1:512, Level 5 at 1:1024, and Level 6, which serves as the Negative control. Detailed test results are presented in Table 7 for further reference.

Table 7 Concentration of results (COI) for preparation linearity panel by Elecsys Anti-HCV II test kit

Concentration	Dilution	Results (COI)
Level 1	1:16	237.90
Level 2	1:32	172.70
Level 3	1:128	55.48
Level 4	1:512	14.70
Level 5	1:2048	3.78
Level 6	Negative FFP	0.088

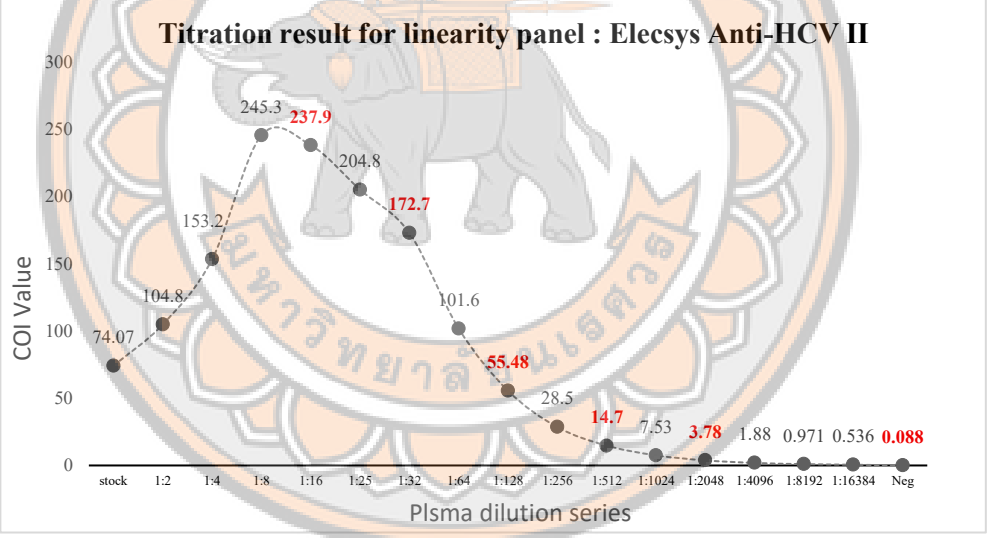


Figure 25 Titration results of dilution plasma with the Elecsys Anti-HCV II test kit

1.3 Accelerated Stability Studies of Plasma Material

Plasma materials were prepared according to the optimal dilutions for both liquid and lyophilized samples. Three concentrations were designated as quality control (QC) samples and were tested using Kit 3 and the Linearity Panel. All six concentrations were analyzed using the Elecsys Anti-HCV II test kit.

The samples were stored using a Q-Sun Model Xe-1 at 45°C for 28 days, and Isochronous Stability Testing was conducted by collecting samples on Day 7, Day 14, Day 21, and Day 28. The collected samples were tested with kit 3 and the Elecsys Anti-HCV II. The results were compared with those of samples stored at -20°C.

The Accelerated stability test of samples was conducted using kit 3. The results obtained from the assays were visually interpreted by two independent readers. The test results for Level 1 and Level 2 samples were as follows: Liquid – HCV Positive without Stabilizer showed 2+/1+, Liquid – HCV Positive with Stabilizer showed 2+/1+, Lyophilized – HCV Positive without Trehalose showed 2+/1+, and Lyophilized – HCV Positive with Trehalose showed 2+/1+. The developed plasma material remained stable at 45°C for at least 28 days. The qualitative test analysis, with a 100% positive accuracy rate, showed in Table 8 and Appendix B.

Table 8 Stability test results of different types of plasma materials at 45ºC over time

Types of plasma materials	Stability Test Results at 45ºC (Day)									
	0	7	14	21	28	0	7	14	21	28
	Strong Positive					Weak Positive				
Liquid										
HCV Positive without Stabilizer	2+	2+	2+	2+	2+	1+	1+	1+	1+	1+
HCV Positive with Stabilizer	2+	2+	2+	2+	2+	1+	1+	1+	1+	1+
Statistical stability analysis	100% Positive accuracy rate									
Lyophilized										
HCV Positive without trehalose	2+	2+	2+	2+	2+	1+	1+	1+	1+	1+
HCV Positive with trehalose	2+	2+	2+	2+	2+	1+	1+	1+	1+	1+
Statistical stability analysis	100% Positive accuracy rate									

The Accelerated stability test of samples was conducted using the Elecsys Anti-HCV II test kit .The test results for Level 1 and Level 2 samples are as follows: Liquid - HCV Positive without stabilizer showed stability for 28 days and 21 days, respectively; Liquid - HCV Positive with stabilizer showed stability for 28 days and 14 days; Lyophilized - HCV Positive without Trehalose showed stability for 21 days and 28 days; and Lyophilized - HCV Positive with Trehalose showed stability for 28 days for both levels. The developed plasma material remained stable at 45°C for at least 28 days. The results were analyzed using One-Way with T < T critical showed in Table 9.

Table 9 Accelerated stability test results of plasma material using Elecsys Anti-HCV II test kit over time

Type	**Liquid Sample**				**Lyophilized Sample**			
Time (Days)	HCV Positive without Stabilizer		HCV Positive with Stabilizer		HCV Positive without trehalose		HCV Positive with trehalose	
	Strong positive	Weak positive	Strong positive	Weak positive	Strong positive	Weak positive	Strong positive	Weak positive
0	173.35	179.25	165.50	209.55	213.20	164.40	175.28	165.88
7	171.95	159.10	163.95	175.45	201.65	157.15	164.90	161.15
14	171.70	133.60	163.35	160.75	198.80	153.60	163.35	159.10
21	171.05	136.70	163.85	150.75	197.10	158.85	167.45	164.40
28	172.43	125.2	163.25	134.13	188.15	156.28	164.53	156.28
t cal	0.770	3.488	2.358	4.357	3.106	1.207	1.379	0.603
t crit	3.182	4.303	3.182	12.706	4.303	3.182	3.182	3.182
p-value	0.497	0.073	0.100	0.144	0.090	0.314	0.262	0.589
stable day (T cal < T crit)	**28**	**21**	**28**	**14**	**21**	**28**	**28**	**28**

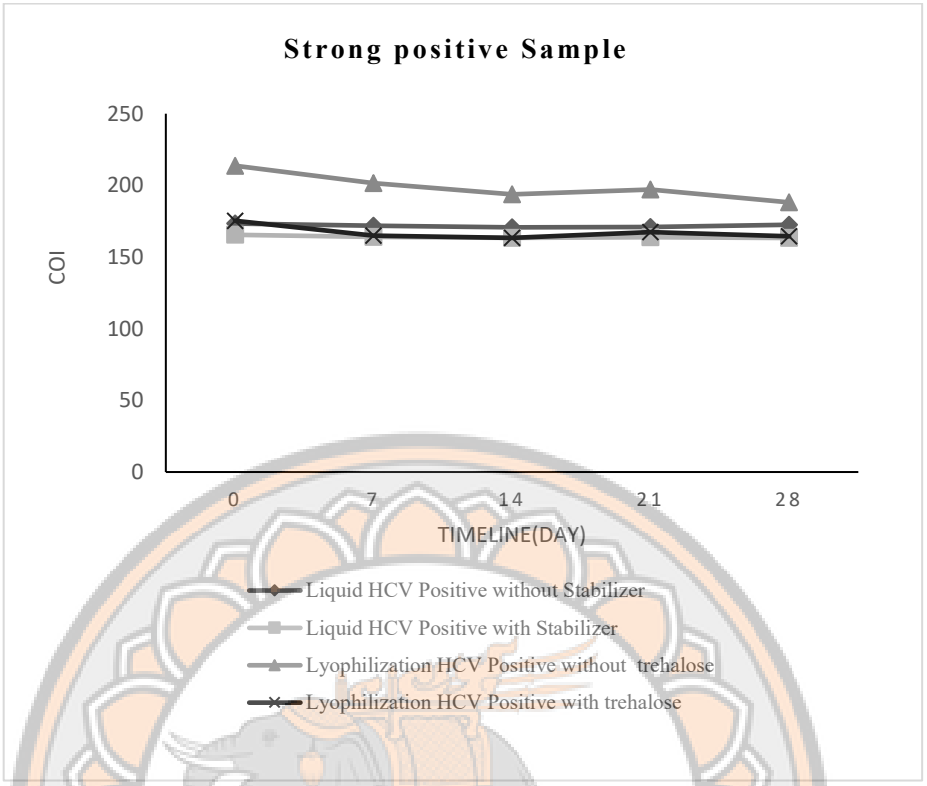


Figure 26 Stability trends of strong positive plasma materials at 45°C

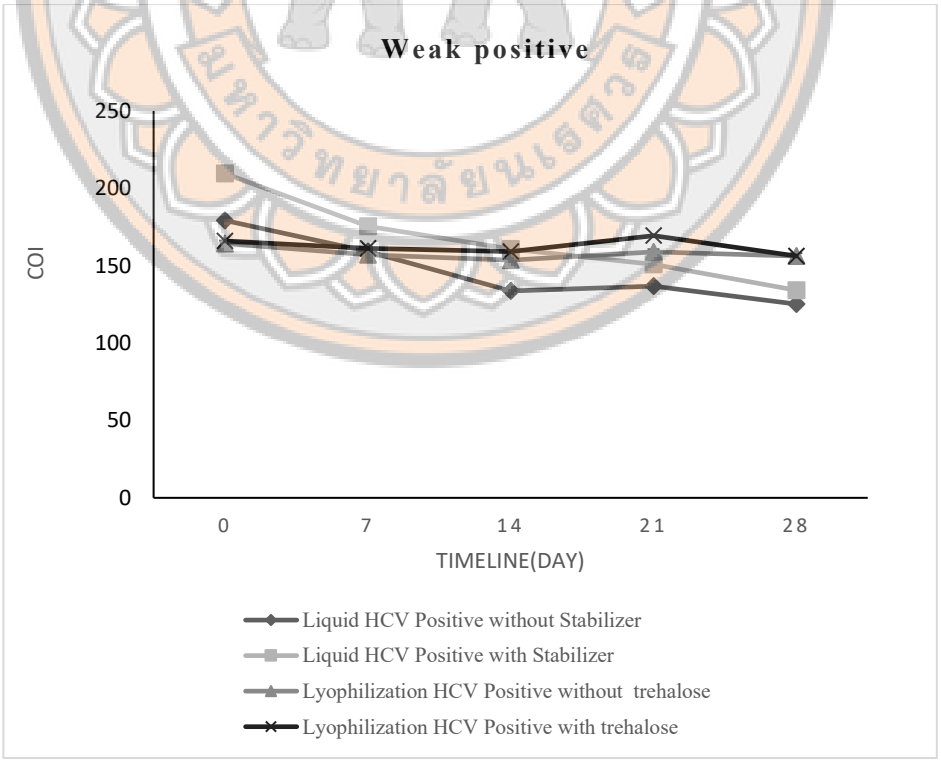


Figure 27 Stability trends of weak positive plasma materials at 45°C

Experiment 2: Preparation of Anti-HCV plasma material

2.1 Characteristic of plasma material

Prepare samples according to the selected optimal concentrations. The sample preparation includes a QC sample for all three optimal concentrations and a linearity panel with six optimal concentrations. Use formula $C_1 V_1 = C_2 V_2$ to calculate the required volumes for preparation. Ensure that the calculated volume covers testing sample characteristics, stability, and homogeneity.

Test the characteristics of the diluted samples. The samples should be suitable for both liquid and lyophilized forms. The plasma material will be tested at three anti-HCV antibody levels using five rapid Anti-HCV test kits, and at six anti-HCV antibody levels using the Cobas e 411 (Elecsys Anti-HCV II) system.

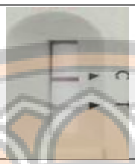
The test results for plasma materials show that for strong positive samples, all kits consistently provided high-intensity results, with Kit 1 showing 4+ across all plasma types, both liquid and lyophilized, Kit 2 showing results between 3+ and 4+, Kit 3 showing consistent 2+ results, Kit 4 showing 2+ to 3+, and Kit 5 maintaining 3+ across all types; for weak positive samples, the kits displayed some variability, with Kit 1 showing consistent 3+ results across all materials, Kit 2 showing results ranging from 1+ to 2+, with better performance in plasma stabilized with Stabilizer, Kit 3 maintaining stable 1+ results, Kit 4 showing 1+ to 2+, performing better with lyophilized samples, and Kit 5 consistently showing 2+ across all types; finally, for negative samples, all kits showed 0 (non-reactive) results for both liquid and lyophilized HCV-negative plasma showed in Table 10 and **11.**

Table 10 Characteristic test results of plasma material at different levels with various stabilizers across rapid anti-hepatitis C test

Kit	Plasma materials level									
	Strong positive				Weak positive				Negative	
	Liquid		Lyophilized		Liquid		Lyophilized		liquid	Lyophilized
	HCV Positive without Stabilizer	HCV Positive with Stabilizer	HCV Positive without Trehalose	HCV Positive with Trehalose	HCV Positive without Stabilizer	HCV Positive with Stabilizer	HCV Positive without Trehalose	HCV Positive with Trehalose	HCV Negative plasma	HCV Negative plasma
1	4+	4+	4+	4+	3+	3+	3+	3+	0	0
2	3+	4+	3+	3+	1+	2+	1+	1+	0	0
3	2+	2+	2+	2+	1+	1+	1+	1+	0	0
4	3+	2+	3+	3+	2+	1+	2+	2+	0	0
5	3+	3+	3+	3+	2+	2+	2+	2+	0	0

Note: Reaction intensity levels 1+, 2+, 3+, and 4+ = Reactive; 0 = non-Reactive.

Table 11 Test results of plasma materials characteristics at three concentrations using the five Rapid anti-hepatitis C test kit

Kit	Plasma materials level		
	Strong positive	Weak positive	Negative
1.			
Result	4+	3+	0
2.			
Result	3	1+	0
3.			
Result	2+	1+	0
4.			
Result	3+	2+	0
5.			
Result	3+	2+	0

2.2 Characteristic of linearity panel samples

Linearity panel is a structured set of samples encompassing six distinct concentrations, aimed at assessing the linear relationship between the concentration of an analyte and the measurement outcomes obtained from an instrument or testing methodology. For the preparation of a linearity panel for the Elecsys Anti-HCV II, it is essential to perform a two-fold dilution of the plasma using an appropriate diluent solution alongside negative plasma. It is recommended to select six levels of anti-HCV antibodies and conduct ten tests for each concentration level. The accompanying graph illustrates the Cut-Off Index (COI) values obtained from each test across the various dilution levels, as depicted in Figure 28. Following this, please calculate the average values for each of the six concentrations based on the ten test results, and subsequently plot a graph to present the linearity range, as shown in Figure 29.

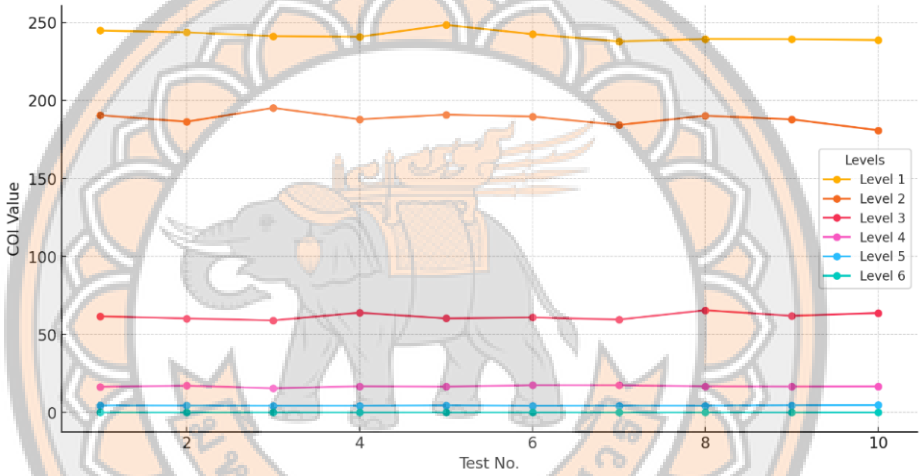


Figure 28 Six Level of linearity panel

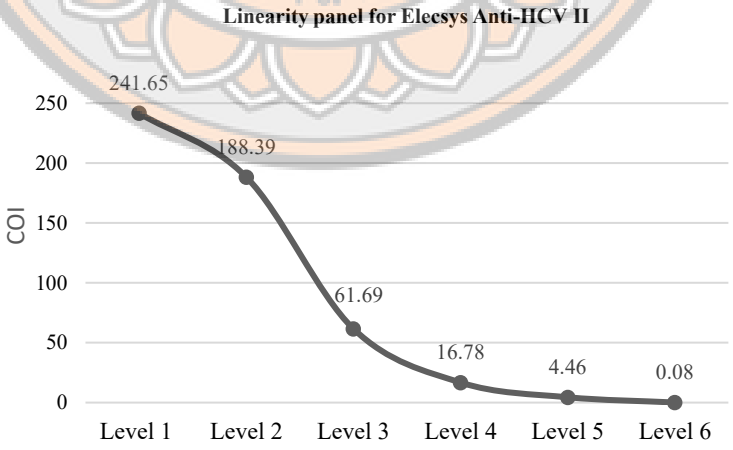


Figure 29 Six Level dilution of linearity panel

Experiment 3: Study of homogeneity of plasma materials

Homogeneity has been checked to ensure that all tubes are homogeneous. Select sampling strategy for a homogeneity study is simple random sampling (ISO Guide 35, 2017). Random sample of 10 tubes was selected from the entire sample. After the random samples were tested using the Elecsys Anti-HCV II and rapid test assay.

The homogeneity test of plasma material samples was done by simple random sampling, and 10 tubes were randomly selected from all concentrations for QC Sample preparation. The test was done with rapid anti-hepatitis C (kit 3). The test results for Level 1 Strong Positive concentration, the homogeneity result is the Strong Positive sample is the same in all 10 tubes, it means that the sample is homogeneous, 100% positive accuracy rate, Level 2 weak Positive concentration is the same in all 10 tubes, it means that the sample is homogeneous, 100% positive accuracy rate and Level 3 Negative sample, the test results of all 10 tubes, it means that the sample is homogeneous, 100% negative accuracy rate showed in table 12 and Appendix C.

Table 12 Homogeneity of Prepared plasma material tested with rapid anti-hepatitis C test (Kit 3)

Tube No.	Result		
	Strong positive	Weak positive	Negative
1	2+ (Reactive)	1+ (Reactive)	0 (Non-Reactive)
2	2+ (Reactive)	1+ (Reactive)	0 (Non-Reactive)
3	2+ (Reactive)	1+ (Reactive)	0 (Non-Reactive)
4	2+ (Reactive)	1+ (Reactive)	0 (Non-Reactive)
5	2+ (Reactive)	1+ (Reactive)	0 (Non-Reactive)
6	2+ (Reactive)	1+ (Reactive)	0 (Non-Reactive)
7	2+ (Reactive)	1+ (Reactive)	0 (Non-Reactive)
8	2+ (Reactive)	1+ (Reactive)	0 (Non-Reactive)
9	2+ (Reactive)	1+ (Reactive)	0 (Non-Reactive)
10	2+ (Reactive)	1+ (Reactive)	0 (Non-Reactive)

The homogeneity test of the plasma material at the strong positive concentration (dilution 1:4) was conducted using the Elecsys Anti-HCV II test kit, where the within-sample variation check confirmed that the calculated C_{exp} value of 0.430 was less than the Cochran critical value of 0.602, indicating that the variation within the sample was acceptable; furthermore, the One-Way ANOVA F-test showed no significant differences among the test objects in each randomly selected tube, as the F value of 2.417 was less than the critical F value of 3.020 showed in Table 13.

Table 13 Homogeneity of Prepared Plasma Material tested with Elecsys Anti-HCV II at Strong Positive (Dilution 1:4)

No.	Tube no.	Result		$D^2 = (x_1 - x_2)^2$
		Portion 1	Portion 2	
1	9	143.60	137.50	37.210
2	13	137.60	138.50	0.810
3	30	142.30	145.00	7.290
4	31	135.60	138.40	7.840
5	48	146.00	137.20	77.440
6	58	139.30	141.80	6.250
7	63	135.00	140.70	32.490
8	73	136.10	138.80	7.290
9	83	134.40	132.60	3.240
10	97	133.70	133.40	0.090
			$\sum D_i^2$ =	179.950

Anova: Single Factor

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	195.7225	9	21.74694444	2.416998549	0.0927	3.02038
Within Groups	89.975	10	8.9975			
Total	285.6975	19				

The homogeneity test of the plasma material at the weak positive concentration (dilution 1:25) was conducted using the Elecsys Anti-HCV II , where the within-sample variation check confirmed that the calculated C_{exp} value of 0.478 was less than the Cochran critical value of 0.602, indicating that the variation within the sample was acceptable; furthermore, the One-Way ANOVA F-test showed no significant differences among the test objects in each randomly selected tube, as the F value of 0.841 was less than the critical F value of 3.020 showed in Table 14.

Table 14 Homogeneity of prepared plasma material tested with Elecsys Anti-HCV II (Roche, Germany) at Weak positive (Dilution 1:25)

No.	Tube no.	Result		$D^2 = (x_1 - x_2)^2$
		Portion 1	Portion 2	
1	9	170.20	163.80	40.960
2	13	158.60	157.90	0.490
3	30	165.10	168.60	12.250
4	31	162.50	167.40	24.010
5	48	170.80	157.70	171.610
6	58	169.70	165.50	17.640
7	63	180.20	163.80	268.960
8	73	163.50	167.30	14.440
9	83	163.00	166.40	11.560
10	97	167.00	166.40	0.360
			$\sum D_i^2$ =	562.280

Anova: Single Factor

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	213.042	9	23.67133333	0.841976714	0.5971	3.02038
Within Groups	281.14	10	28.114			
Total	494.182	19				

Experiment 4: Study of stability of plasma materials

Sample collection was conducted to study the stability of plasma samples using the real-time stability method over a period of 6 months. Comparative results of strong positive plasma materials stored at 2-8°C and 25°C were compared with those stored at -20°C. The results indicated that the prepared plasma materials had a shelf life of 6 months.

4.1 Real-time stability of plasma materials

The stability test of plasma material with trehalose at strong positive using five rapid anti-hepatitis C test kits and the Elecsys Anti-HCV II panel showed that after 6 months at 2-8°C and 25 ± 5 °C. At 2-8°C, the stability test yielded a calculated t value (t cal) of 0.988, which is below the critical t value (t crit) of 2.570 confirming the material's stability for at least 6 months. At 25 ± 5°C, the stability test resulted in a t cal of 0.317, which is also below the t crit of 2.570, indicating that the material remained sufficiently stable for 6 months. Additionally, the p-values at 2-8°C (0.368) and 25 ± 5°C (0.764) were both greater than 0.05, indicating no significant degradation over 6 months. The detailed findings are shown in Table 15 and in Appendix D.

Table 15 Stability results of plasma material with trehalose at strong positive

Temperature (°C)	Time (Months)	Test kits					
		Elecsys Anti-HCV II (COI)	1	2	3	4	5
2-8	0	158.40	4+	3+	2+	3+	3+
	1	157.25	4+	3+	2+	3+	3+
	2	161.40	4+	3+	2+	3+	3+
	3	163.60	4+	3+	2+	3+	3+
	4	159.95	4+	3+	2+	3+	3+
	5	164.55	4+	3+	2+	3+	3+
	6	158.80	4+	3+	2+	3+	3+
Statistical stability analysis	**t cal**	0.988					
	t crit	2.570	100% Positive accuracy rate				
	p-value	0.368					
	Conclusion	sufficiently stable					
25 ± 5	0	158.40	4+	3+	2+	3+	3+
	1	176.30	4+	3+	2+	3+	3+
	2	176.30	4+	3+	2+	3+	3+
	3	161.55	4+	3+	2+	3+	3+
	4	176.9	4+	3+	2+	3+	3+
	5	163.75	4+	3+	2+	3+	3+
	6	161.35	4+	3+	2+	3+	3+
Statistical stability analysis	**t cal**	0.317					
	t crit	2.570	100% Positive accuracy rate				
	p-value	0.764					
	Conclusion	sufficiently stable					

The stability test of plasma material with trehalose at weak positive using five rapid anti-hepatitis C test kits and the Elecsys Anti-HCV II panel showed that after 6 months at 2-8°C and 25 ± 5 °C, at 2-8°C, the stability test yielded a calculated t value (t cal) of 0.586, which is below the critical t value (t crit) of 2.570 confirming the material's stability for at least 6 months. at 25 ± 5 °C, the stability test resulted in a t cal of 0.318, which is also below the t crit of 2. 570 confirming the material's stability for at least 6 months. Additionally, the p-values at 2-8°C (0.584) and 25 ± 5°C (0.763) were both greater than 0.05, indicating no significant degradation over 6 months. The detailed findings are shown in Table 16 and in Appendix D.

Table 16 Stability results of plasma material with trehalose at weak positive

Temperature (°C)	**Time (Months)**	**Test kits**					
		Elecsys Anti-HCV II (COI)	1	2	3	4	5
2-8	0	169.65	2+	1+	1+	2+	2+
	1	168.00	2+	1+	1+	2+	2+
	2	165.80	2+	1+	1+	2+	2+
	3	166.35	2+	1+	1+	2+	2+
	4	166.60	2+	1+	1+	2+	2+
	5	166.40	2+	1+	1+	2+	2+
	6	168.85	2+	1+	1+	2+	2+
Statistical stability analysis	**t cal**	0.586	100% Positive accuracy rate				
	t crit	2.570					
	p-value	0.584					
	Conclusion	sufficiently stable					
25 ± 5	0	169.65	2+	1+	1+	2+	2+
	1	172.95	2+	1+	1+	2+	2+
	2	164.45	2+	1+	1+	2+	2+
	3	166.30	2+	1+	1+	2+	2+
	4	170.50	2+	1+	1+	2+	2+
	5	160.15	2+	1+	1+	2+	2+
	6	173.25	2+	1+	1+	2+	2+
Statistical stability analysis	**t cal**	0.318	100% Positive accuracy rate				
	t crit	2.570					
	p-value	0.763					
	Conclusion	sufficiently stable					

4.2 Transport Stability of plasma materials

The stability study of samples stored at 37°C and 45°C, conducted to simulate transportation conditions, was performed using kit 3. Samples were collected at intervals of 7, 14, 21, and 28 days and compared with control samples stored at -20°C. The samples were tested using both kit 3 and the Elecsys Anti-HCV II The test results for samples stored at 37°C and 45°C demonstrated 100% stability with a positive accuracy rate. Based on the analysis using One-Way ANOVA criteria, the calculated t-value (t cal) being less than the critical t-value (t crit) indicates that the samples remained stable during transportation for up to 28 days shown in Table 17.

Table 17 Transport Stability results of plasma material with trehalose

Temperature (°C)	Time (Days)	Result of Transport Stability of plasma materials			
		Strong positive		Weak positive	
		Elecsys Anti-HCV II (COI)	Kit 3	Elecsys Anti-HCV II (COI)	Kit 3
37	0	126.15	2+	151.4	1+
	7	127.20	2+	137.60	1+
	14	123.85	2+	135.45	1+
	21	129.25	2+	137.55	1+
	28	131.20	2+	135.75	1+
Statistical stability analysis	**t cal**	1.604	100% Positive accuracy rate	1.906	100% Positive accuracy rate
	t crit	3.182		3.182	
	p-value	0.207		0.153	
	Conclusion	sufficiently stable		sufficiently stable	
45	0	175.28	2+	165.88	1+
	7	164.90	2+	161.15	1+
	14	163.35	2+	159.10	1+
	21	167.45	2+	164.40	1+
	28	164.53	2+	156.28	1+
Statistical stability analysis	**t cal**	1.379	100% Positive accuracy rate	0.603	100% Positive accuracy rate
	t crit	3.182		3.182	
	p-value	0.262		0.589	
	Conclusion	sufficiently stable		sufficiently stable	

CHAPTER V

DISCUSSION AND CONCLUSION

Preparation of plasma materials for use as quality control samples is essential for testing with Rapid Anti-HCV test kits, as rapid assays are widely used in diagnosing HCV infection, especially in developing countries. Rapid tests offer several advantages, such as ease of use, no need for expensive equipment or highly trained personnel, and the ability to provide immediate results. These features enhance flexibility and accessibility, making rapid tests suitable for various clinical settings (Cha et al., 2013).Therefore, the use of quality control samples for tracking the accuracy of analytical tests in daily work is of great importance. Purposes of the study consist of optimization of condition Anti-HCV level to prepare plasma materials, to develop the linearity panel materials preparation protocol for Elecsys Anti-HCV II and study of the homogeneity and stability of plasma control materials.

Discussion

The use of three internal quality control sample levels is essential for diagnostic tests to effectively differentiate between strong positive, weak positive, and negative samples. This approach aligns with the recommendations from the World Health Organization (WHO) (Organization, 2011) and the Clinical and Laboratory Standards Institute (CLSI) regarding internal quality control (IQC) for serological assays.

Incorporating both strong and weak positive controls is crucial for evaluating the sensitivity of test kits and identifying potential false negatives, especially for weakly reactive samples. This three-level QC system—comprising strong positive, weak positive, and negative controls—provides an effective method that complies with WHO standards to ensure test accuracy, sensitivity, and reliability in HCV rapid diagnostic testing.

The optimal concentration for quality control (QC) samples of five HCV rapid test kits was evaluated based on test line intense reactions. Two independent readers noted variability among the samples. At the Level 1 (Strong Positive) concentration, Kit 1 stood out as the most effective, exhibiting the darkest and most pronounced test line intense reactions (4+). Following closely were Kits 2, 4, and 5, each demonstrating a moderate test line intense reaction (3+). In contrast, Kit 3 was less responsive, showcasing the lightest test line intense reactions (2+). When assessing the Level 2 (Weak Positive) concentration, Kit 1 continued to excel, again displaying the darkest intensity (3+), with Kit 5 closely trailing at (2+). Kits 2,3 and 4 also showed variability at this level.

At Level 1 selection with a 1:4 dilution, the test results from Kit 3 showed an intensity of 2+, which was closer to the weak positive range. When tested at a less diluted concentration of 1:2, Kit 3 test line intense reactions to 3+. However, preparing samples at a 1:2 concentration requires a large amount of stock pool sample to achieve the desired concentration, which could result in rapid depletion of the stock pool. This would necessitate frequent selection and preparation of new stock pools, leading to increased costs and time associated with the sample preparation process.

Additionally, samples with higher concentrations may exhibit increased viscosity, making them more challenging to handle during transport and storage. This increases the risk of degradation or contamination during handling. This study indicates that a lower concentration, such as 1:4 (153.20 COI), is more suitable as it enhances quality

control, reduces resource waste, and ensures ongoing usability for diagnostic testing applications.

Level 2 selection with a 1:25 dilution (204.80 COI), the test results from Kit 1 demonstrated an intensity of 3+, which was closer to the strong positive range. It was not feasible to increase the dilution further to achieve a stronger test line intensity, as this could negatively impact the performance of the other test kits. Balancing compatibility across multiple kits while maintaining suitable test line intensity was a critical consideration in the selection process.

However, some kits displayed less intense reactions, each registering a light intensity (1+). The variations in test line intense reactions among the five HCV rapid test kits can be attributed to several factors, particularly differences in antigen sensitivity and the specific designs of antigens used in each kit. Each test kit employs distinct antigens or unique combinations of antigens—such as Core, NS3, NS4, and NS5—that interact with HCV antibodies in different ways. According to the information provided in the package inserts of the rapid test kits, Kit 1, Kit 2, and Kit 4 utilize capture antigens from Core, NS3, NS4, and NS5, while Kit 3 includes Core, NS1, NS2, NS3, NS4, and NS5 antigens. The kit 3 captures a greater number of antigen types compared to the other test kits. This could impact the concentration of the test line, potentially resulting in a weaker signal. This occurs because the amount of each individual antigen present at the test line is lower than in kits with fewer antigen types. Variations in the composition of antigens play a significant role in determining both the sensitivity and specificity of rapid tests. Manufacturers use varied methods for calibration and assembly, which can affect product performance and test accuracy (López-Martínez et al., 2019). The way antigens are designed is essential for ensuring the accuracy of HCV tests (Tang et al., 2017). This study shows that these manufacturing differences can significantly impact diagnostic tests. Different antigens affect how effectively the tests interact with various types of HCV antibodies, but this is particularly critical for kits designed to detect low antibody levels for early diagnosis, as inconsistencies can result in false-negative results, especially when testing plasma with low antibody concentrations.

The optimal concentrations for the linearity panel in the Elecsys Anti-HCV II Test Kit are designed to verify the sensitivity, accuracy, and consistency of the system across a range of HCV antibody levels. The selected concentrations are as follows: Level 1 at 1:16, Level 2 at 1:32, Level 3 at 1:128, Level 4 at 1:512, Level 5 at 1:1024, and Level 6 serves as a negative control. These concentrations were chosen to provide a broad detection range that ensures reliable diagnostic performance. It is essential to select concentrations that do not fall within the Hook Effect range (Diagnostics, 2015 V 0.5). The Hook Effect is a phenomenon observed in immunoassays that occurs when high analyte concentrations saturate the binding sites of antibodies. This saturation prevents the formation of complete antigen-antibody complexes, leading to a significant decrease in signal intensity, an unreasonably low coefficient of influence (COI), and reduced accuracy of test results.

The accelerated stability test was conducted on HCV-positive plasma materials to assess their stability under high-temperature conditions. This evaluation utilized both a rapid test kit (Kit 3) and the Elecsys Anti-HCV II system. The study compared two preparation forms: liquid and lyophilized. Each form was tested both with and without a stabilizing agent under accelerated stability conditions at temperatures up to 45°C.

The stability evaluation began with a qualitative rapid test, followed by a detailed analysis using COI values as a semi-quantitative measure. Statistical comparisons were made between the calculated t-values and the critical t-thresholds to determine if there was significant degradation in the samples. The results indicated that various sample formats, including both liquid and lyophilized samples with stabilizers like StabilZyme™ SELECT Stabilizer and trehalose, remained stable at 45°C for 28 days. The study emphasized that the addition of trehalose and the use of lyophilization effectively preserved sample integrity, particularly under heat stress.These findings align with previous research, which demonstrates that trehalose protects protein structures by stabilizing water molecules and maintaining their three-dimensional shape, thus enhancing stability at high temperatures (Poe et al., 2018).

Accelerated stability testing using Kit 3 demonstrated that all sample types exhibited similar test line reactions (2+/1+) at both Level 1 (strong positive) and Level 2 (weak positive) after 28 days at 45°C. However, quantitative testing with the Elecsys Anti-HCV II system revealed differences in stability among the sample types. Liquid HCV-positive samples without stabilizers remained stable for 28 days at Level 1 but exhibited reduced stability at Level 2, lasting only 21 days. In contrast, lyophilized HCV-positive samples containing trehalose showed the best stability, maintaining their integrity for the entire 28-day period at both levels. These findings are consistent with previous studies, which indicate that lyophilization helps reduce moisture and prevents biochemical reactions, effectively preserving protein stability and minimizing degradation in HCV plasma samples (Wang, 2000).

Additionally, research supports using lyophilization to improve the stability (Wang, 2000). This aligns with findings that lyophilization with trehalose enhances the stability of HCV plasma samples, making them well-suited for storage in high-temperature environments. Trehalose serves as a protein stabilizer by trapping water molecules and preserving the three-dimensional structure of proteins, thereby reducing degradation under high-temperature condition (Ohtake & Wang, 2011).Trehalose also reduces protein aggregation, a common issue when proteins are exposed to heat, helping maintain sample quality and stability better than samples without additives. Lyophilized HCV samples with trehalose support both high-temperature (วงเล็บอุณห)storage and real-time transport, making them an ideal choice for stabilizing plasma materials.

Lyophilized HCV Positive samples that have been diluted with trehalose demonstrate superior stability due to trehalose's ability to preserve protein structure and prevent degradation. The lyophilization process effectively reduces moisture content, further stabilizing the protein. These samples demonstrate superior stability at 45°C, maintaining consistent COI values over a period of 28 days. Consequently, the combination of trehalose and lyophilization is effective method for preserving plasma quality during storage and transport under heat-sensitive conditions.

The homogeneity and stability assessment of HCV plasma samples has revealed a high level of consistency across various sample levels, affirming their reliability for HCV testing protocols. Qualitative tests demonstrated 100% consistency among strong positive, weak positive, and negative samples, thus ensuring accurate reactivity. Additionally, quantitative analysis utilizing the Elecsys Anti-HCV II test confirmed stability, with acceptable variations observed in both strong and weak positive concentrations. The statistical evaluation indicated that for strong positive samples, the F-test value (2.416) was lower than the F-critical value (3.020), suggesting that there

were no significant differences among the samples. Similarly, for weak positive samples, the F-test value (0.842) was also below the F-critical value (3.020), further confirming that there were no significant differences among these samples. Overall, these findings substantiate the use of these plasma samples as standardized quality control materials, guaranteeing precise and reproducible results in HCV diagnostics.

The real-time stability tests for HCV diagnostic samples demonstrated consistent reliability under various storage and transport conditions, confirming their suitability for diagnostic applications. The tests simulated standard storage conditions by holding samples at 2-8°C (refrigerated) and 25 ± 5°C (room temperature) for six months. The results indicated that both level 1 (strong positive) and level 2 (weak positive) samples remained stable throughout the testing period. Specifically, when tested using the Elecsys Anti-HCV II test kit and analyzed via One-Way ANOVA, the T-cal values for strong positive samples consistently remained below the T-crit for both storage conditions, indicating no significant degradation over the six months. Likewise, weak positive samples showed acceptable stability, with T-cal values staying below the T-crit at both temperatures. Furthermore, the assessments of transport stability confirmed the strong reliability of samples stored under different conditions, including real-time evaluations. Samples maintained at 2-8°C and 25 ± 5°C demonstrated stable performance for six months, and stability testing using the Elecsys Anti-HCV II further validated these results. These findings highlight that HCV samples are well-suited for extended storage under both refrigerated and room temperature conditions without compromising their integrity.

The transport stability of lyophilized HCV-positive samples with trehalose was tested under conditions that typical transport scenarios, where temperature control may be limited. To simulate these conditions, samples were stored at temperatures of 37°C and 45°C for 28 days, reflecting potential fluctuations during transport. This aligns with the 2024 temperature forecasts from the Thai Meteorological Department, which predict highs of 43.0°C to 44.5°C from mid-March to early May, particularly in northern regions. The stability of the samples was assessed using Kit 3 and Elecsys Anti-HCV II. For Level 1 strong positive samples, the calculated temperature values (T-cal) at both 37°C and 45°C remained below the critical threshold (T-crit), indicating no significant degradation. Similarly, level 2 weak positive samples also demonstrated stability, with T-cal values consistently below T-crit at both temperatures. These results indicate that lyophilized samples containing trehalose are stable and can endure temporary exposure to temperatures of 45°C without affecting their reliability. Lyophilization effectively preserves the integrity of HCV samples, providing a practical and cost-efficient solution for sharing quality control and linearity panels. This method also addresses the challenges related to shipping on dry ice, making it suitable for transferring samples between donor and recipient laboratories (Poe et al., 2018).

Conclusion

The study aimed to develop pooled plasma materials as quality control (QC) samples for HCV rapid test kits. The findings confirmed that QC plasma materials for hepatitis C antibody testing were successfully prepared using lyophilization with trehalose. The selected concentrations for QC samples included strong positive, weak positive, and negative.

For the linearity panel, all six selected concentrations were effectively utilized to validate and ensure the accuracy of analytical systems in HCV diagnostics. These panels enhance laboratory confidence in test reliability and support accurate clinical decision-making.

In conclusion, the quality control plasma materials were prepared using lyophilization with trehalose, with strong positive, weak positive, and negative concentrations. The linearity panel, consisting of six selected concentrations, effectively validated analytical systems in HCV diagnostics, ensuring result accuracy and reliability. Plasma materials prepared with trehalose demonstrated superior stability, maintaining homogeneity, and remaining stable for up to six months at 2-8°C and 25 ± 5°C, and up to 28 days at 37°C and 45°C.

Limitations of the study

This study presents several limitations that warrant careful consideration. Firstly, the analysis relied on a single lot of the Rapid Test Anti-HCV kits for testing the plasma samples, which introduces the possibility that results may vary with different lots. Furthermore, the evaluation of real-time stability was confined to a period of six months, potentially limiting our insights into prolonged stability. Finally, according to data from the Food and Drug Administration (FDA) as of November 2024, a total of 81 rapid anti-hepatitis C test kits ((FDA), 2024) have been authorized; however, this study evaluated only five of these kits.

Recommendations for future research

This study showed that quality control (QC) samples remained stable for up to six months. However, more research is needed to evaluate their stability for 12 months or longer under various storage conditions. Furthermore, future studies should focus on collecting data from real-world applications in medical laboratories to assess the practical usability and suitability of QC samples for routine diagnostic testing.

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APPENDIX

APPENDIX A

Results of sample dilution series and reactivity results for five rapid anti-hepatitis C test

Figure 30.1 Kit 1

Stock 1:2 1:4 1:8 1:16 1:32 1:64 1:128 1:256 1:512 1:1024

Figure 30.2 Kit 2

Stock 1:2 1:4 1:8 1:16 1:32 1:64 1:128 1:256 1:512 1:1024

Figure 30.2 Kit 3

Stock 1:2 1:4 1:8 1:16 1:32 1:64 1:128 1:256 1:512 1:1024

Figure 30.2 Kit 4

Stock 1:2 1:4 1:8 1:16 1:32 1:64 1:128 1:256 1:512 1:1024

Figure 30.2 Kit 5

Figure 30 Results of HCV positive dilution test

APPENDIX B

Results from Accelerated Stability Studies at 45°C

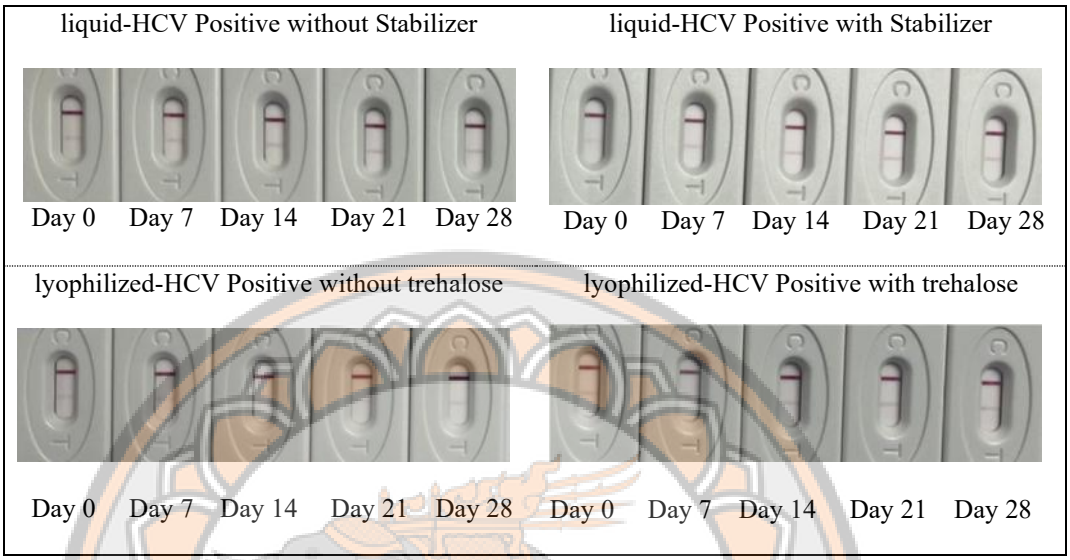

Figure 31 Results of accelerated stability studies for strong positive samples at 45°C using Kit 3

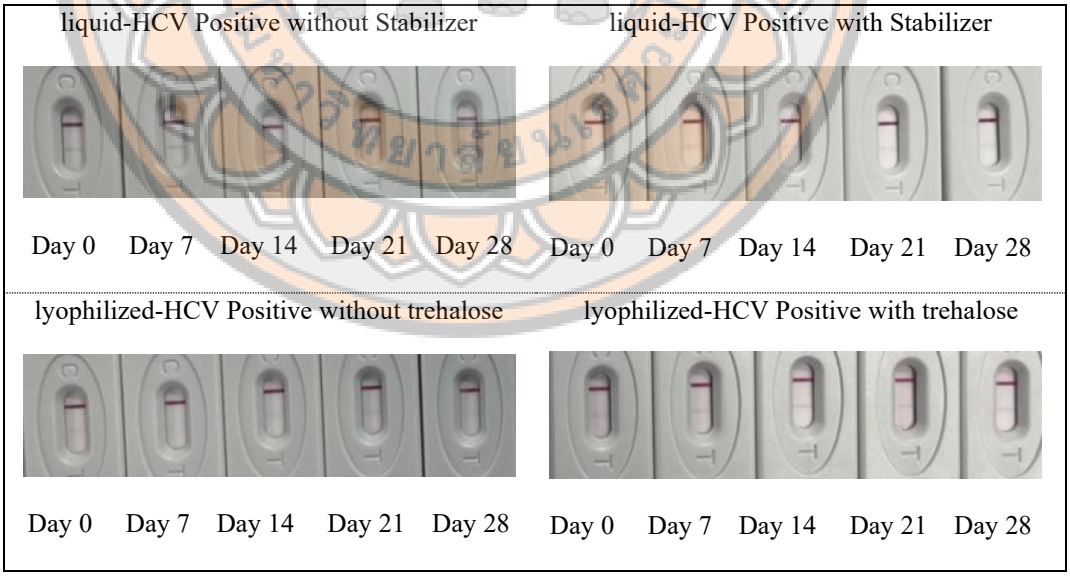

Figure 32 Results of accelerated stability studies for weak positive samples at 45°C using Kit 3

APPENDIX C

Results of homogeneity test

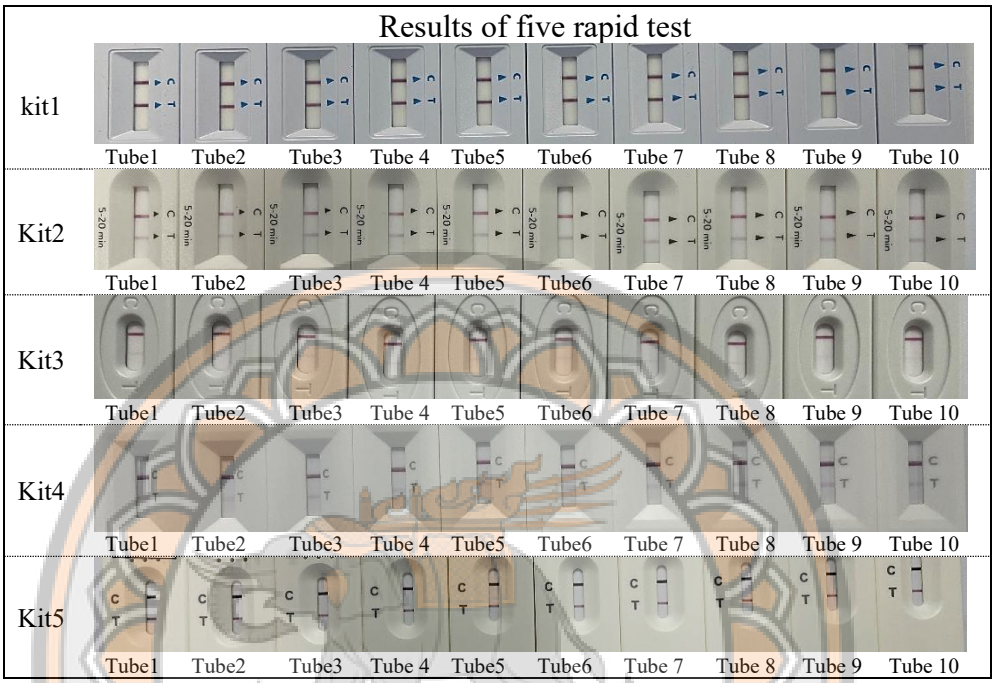

Figure 33 Homogeneity test results of plasma material with trehalose at strong positive with five rapid test kits

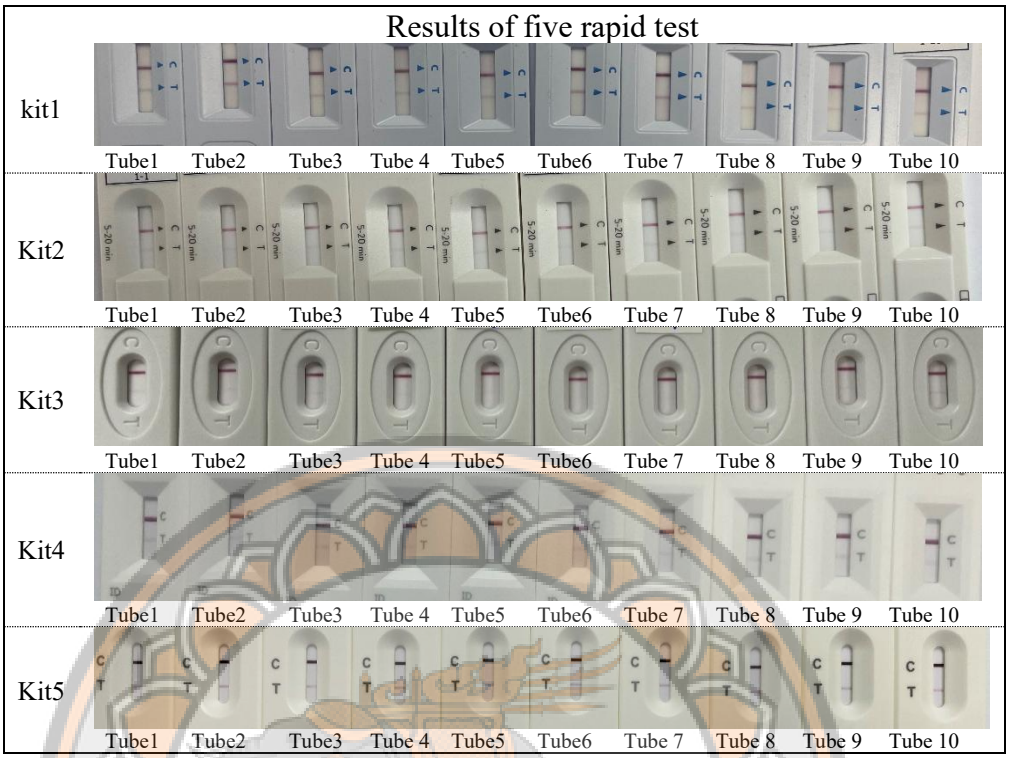

Figure 34 Homogeneity test results of plasma material with trehalose at weak positive with five rapid test kits

APPENDIX D

Results from real-time stability Studies

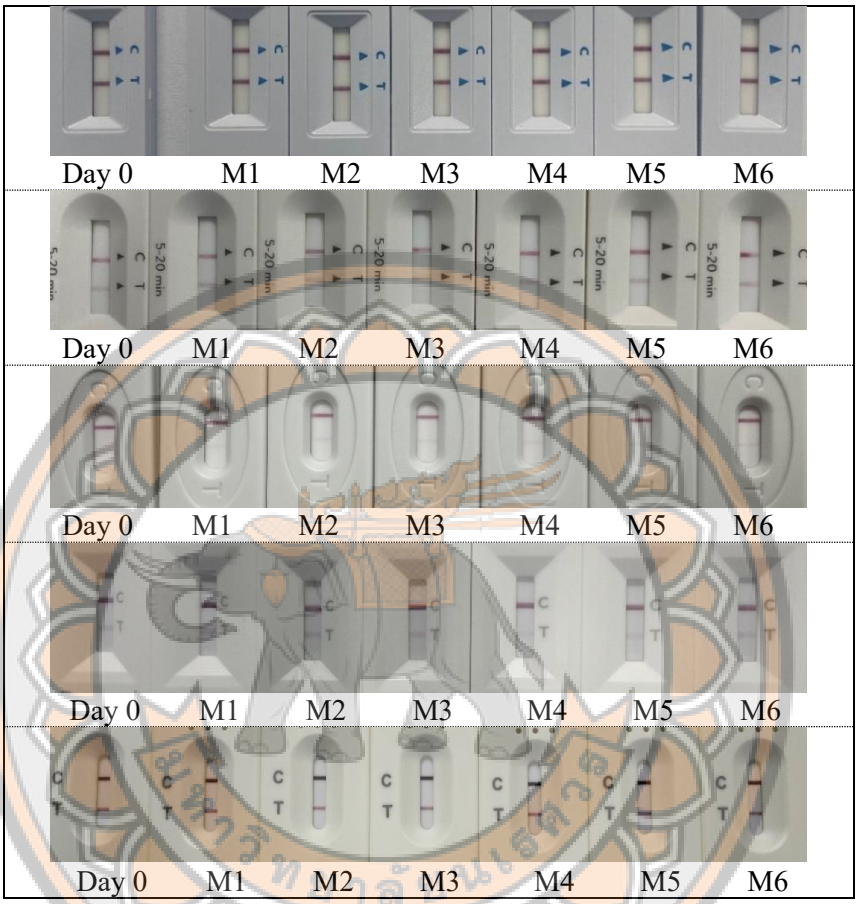

Figure 35 Real-time stability results of strong positive plasma material with trehalose stored at 2-8°C for 6 months across 5 rapid test kits

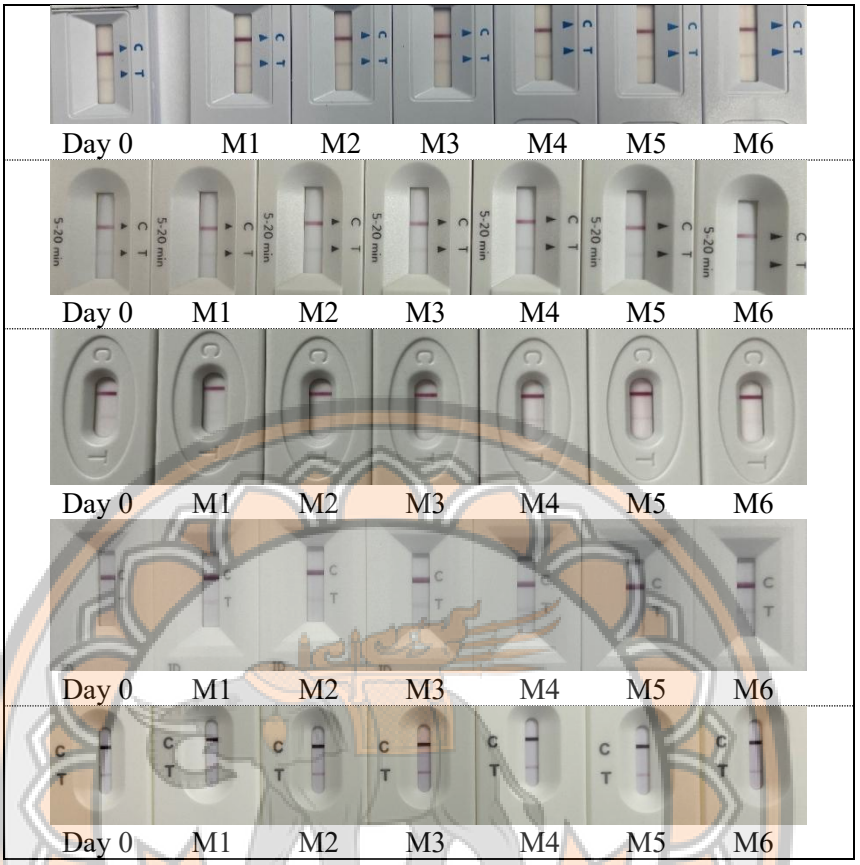

Figure 36 Real-time stability Results of weak positive plasma material with trehalose stored at 2-8°C for 6 months across 5 rapid test kits

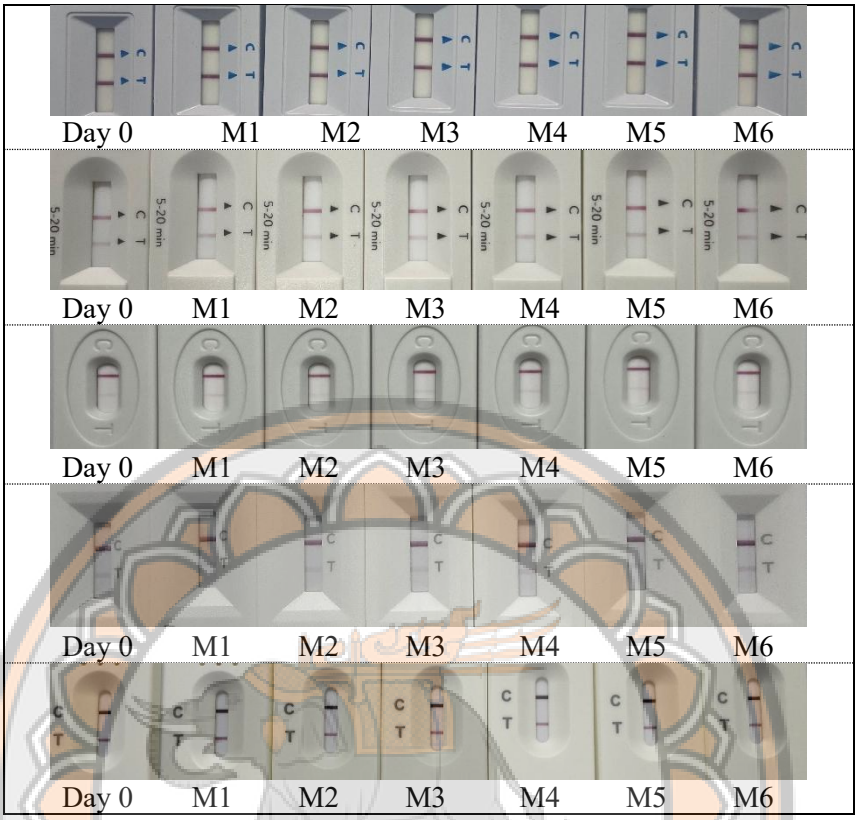

Figure 37 Real-time stability results of strong positive plasma material with trehalose stored at 25 ± 5 °C for 6 months across 5 rapid test kits

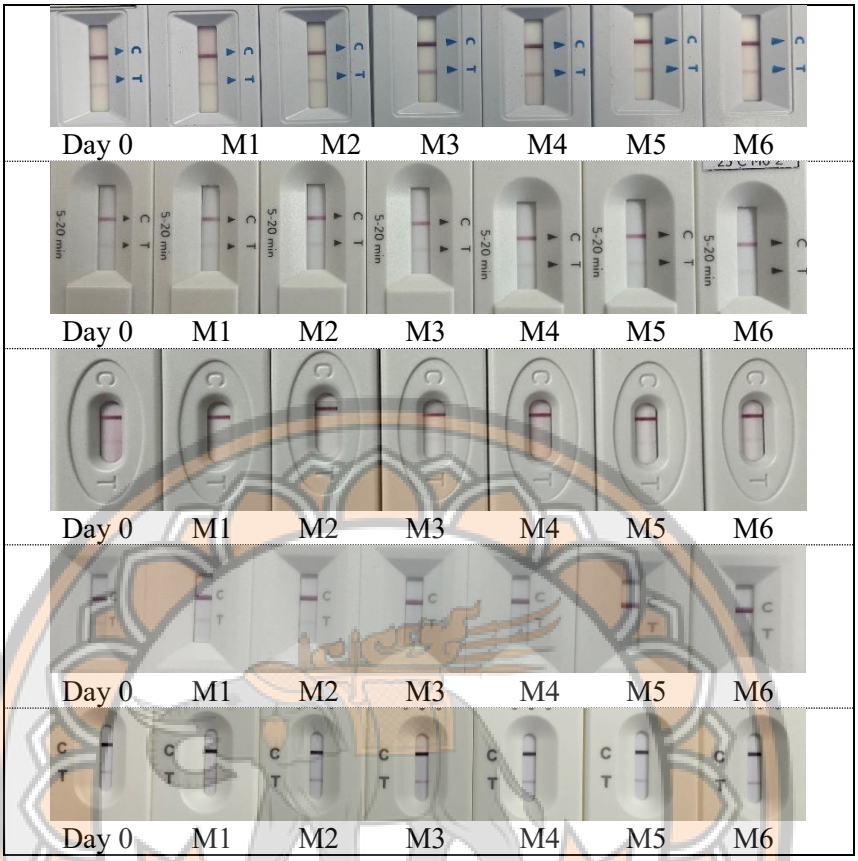

Figure 38 Real-time stability results of weak positive plasma material with trehalose stored at 25 ± 5 °C for 6 months across 5 rapid test kits

ABBREVIATIONS

CAPA	=	Corrective and preventive Actions
CLIA	=	Chemiluminescence immunoassay
CRM	=	Certified reference material
DTS	=	Dried tube specimen
ECL	=	Electrochemiluminescence assay
EIA	=	Enzyme immunoassay
FFP	=	Fresh frozen plasma
HCV	=	Hepatitis C virus
HIV	=	Human immunodeficiency virus
IQC	=	Internal quality control
LFA	=	Lateral flow assay
PBS	=	Phosphate buffered saline
QC	=	Quality control
QMS	=	Quality assurance programs, or Quality management systems
RDT	=	Rapid diagnostic tests

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Publication	
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มหาวิทยาลัยนเรศวร