CHAPTER II

LITERATURE REVIEW

2.1 Overview of Date Palm (*Phoenix dactylifera* L.) and situation of date palm in Thailand and Cannabis

1) Date palm

Date Palm (*Phoenix dactylifera* L.) is a dioecious plant, meaning that individual plants are either male or female. Flowering, which occurs between 3-7 years after planting, is the stage at which the sex of the plant becomes identifiable (Wellmann et al., 2007). Farmers typically aimed to cultivate five female plants for every male plant. Currently, 1,000 households are engaged in date palm farming, covering a planted area of 14,000 rai. The annual harvest is 5,000 tons, with an average selling price between 200-700 Bahts per kilogram (Agricultural Production Information System Online, Department of Agricultural Extension, 2024)

Date palm propagation can be achieved through three primary methods: seed germination, shoot separation from the mother plant, and tissue culture. Each method presents distinct advantages and disadvantages. Seed propagation is straightforward and cost-effective, allowing for rapid and large-scale multiplication. However, this method results in an approximately equal ratio of male to female plants which sometimes even more male plants. The sex of the plants remains unknown until flowering. This uncertainty necessitates a 3-7-year investment in planting, maintenance, and infrastructure before determining plant sex, posing financial risks to farmers (Chao et al., 2007; Wellmann et al., 2007).

2) Cannabis

Cannabis (*Cannabis sativa* L., *C. indica*, *C. ruderalis*) is typically a dioecious species, meaning individual plants are either male or female. While female plants are most commonly cultivated, both sexes occur naturally. The plant's sex generally becomes identifiable during the pre-flowering or early flowering stage,

which occurs several weeks to months after germination, depending on genetic factors (Clarke & Merlin, 2013). Unlike dioecious crops such as date palms, which require a balanced ratio of male to female plants for pollination, cannabis cultivators seeking cannabinoid-rich flowers prioritize the growth of unpollinated female plants. Male and hermaphroditic plants are systematically removed upon identification to prevent pollination and preserve flower quality (Cervantes, 2006).

Following the legalization of cannabis in June 2022, Thailand experienced a surge in cannabis cultivation and retail operations. However, this growth has been largely unregulated. Ongoing regulatory discussions and potential restrictions on recreational cultivation have contributed to a lack of comprehensive national data regarding the number of cultivators, cultivated area, and overall yield (Reuters, 2024; *Bangkok Post*, 2023).

Cannabis is propagated through three primary methods: seed germination, cloning (cuttings from a mother plant), and tissue culture. Each method offers distinct advantages and drawbacks. Seed propagation is straightforward but results in an approximately 1:1 ratio of male to female plants. Because the plant's sex is not evident until flowering, growers must invest considerable time, space, and resources before removing undesirable males (Leafly Staff, 2022). The unpredictability and genetic variation inherent in seed propagation present challenges for those aiming to cultivate high-quality female flowers. As a result, many producers prefer cloning or tissue culture techniques, which ensure genetically consistent, female-only crops (Cervantes, 2006).

2.2 Biology and Reproductive System of Date Palm / Cannabis

1) Date palm

The date palm is a long-lived, perennial monocot native to arid and semi-arid climates (Chao & Krueger, 2007). It displays characteristic monocot features, including a single cotyledon, fibrous roots, and parallel venation (Soliman & Al-Obeed, 2013). The unbranched trunk supports a terminal crown of large pinnate leaves.

Reproductive System: The date palm is strictly dioecious. Male and female flowers are borne on separate trees in large, branched inflorescences called spadices,

which emerge from spathes among the fronds (Chao & Krueger, 2007; Soliman & Al-Obeed, 2013).

Male Flowers: These bear numerous small, whitish flowers with six stamens, producing large quantities of light, powdery pollen (Chao & Krueger, 2007). One male palm can pollinate many females.

Female Flowers: Female inflorescences also contain numerous flowers, typically with three carpels, although usually only one develops into a fruit a drupe known as a date after fertilization (Soliman & Al-Obeed, 2013). The stigmas remain receptive for only a short period.

Pollination and Fruit Development: While wind pollination is possible, it is inefficient for commercial cultivation. Artificial pollination is therefore widely practiced, with pollen manually transferred from male to female trees to ensure reliable fruit set and high yields (Chao & Krueger, 2007; El-Kosary, 2009). Upon fertilization, a single carpel matures into the edible date fruit containing one seed.

2) Cannabis

Cannabis is an annual, herbaceous dicot native to Central Asia (Small, 2015). Its leaves are palmate-compound, and its female flowers are rich in resinous trichomes that produce a diverse array of phytochemicals, including cannabinoids (Andre et al., 2016; Small, 2015).

Reproductive System: Cannabis is primarily dioecious, though monoecious individuals also occur (Small, 2015; Spitzer-Rimon et al., 2019).

Male Flowers: Male plants develop loose panicles of staminate flowers, each comprising five sepals and five stamens that efficiently release pollen into the wind (Small, 2015).

Female Flowers: Female plants produce pistillate flowers in dense clusters ("buds" or "colas"), each with an ovule protected by bracts and two stigmas designed to intercept airborne pollen (Spitzer-Rimon et al., 2019). These structures are the primary sites of cannabinoid biosynthesis (Andre et al., 2016).

Pollination and Seed Development: Naturally, cannabis relies on wind (anemophily) for pollination. Male plants produce abundant, lightweight pollen that is

carried by the wind to the receptive stigmas of female flowers (Small, 2015). However, in cultivation aimed at maximizing cannabinoid yield (found primarily in female flowers), this natural pollination process is often intentionally prevented. Growers achieve this by removing male plants before they release pollen. This results in unpollinated, seedless female flowers, known as "sinsemilla," which typically develop higher concentrations of cannabinoids compared to pollinated flowers (Small, 2015; Spitzer-Rimon et al., 2019).

Both date palms and cannabis utilize dioecy and wind pollination, strategies that rely on airborne pollen transfer between spatially separate male and female individuals. Male plants of both species produce abundant, lightweight pollen, while females have specialized structures for efficient pollen capture. Despite these shared traits, their life cycles and cultivation goals diverge significantly. The date palm, a perennial species, requires artificial pollination to ensure consistent fruit production (El-Kosary, 2009), while cannabis, an annual plant, is often grown under conditions that prevent pollination to enhance secondary metabolite yield (Small, 2015).

2.3 Date Palm Sex Determination

Identifying the sex of date palms at an early stage offers substantial economic and logistical benefits. It allows growers to eliminate unwanted male seedlings early, optimizing the allocation of resources to productive female plants (Al-Dous et al., 2011). This selection process significantly reduces cultivation costs and maximizes land use efficiency, leading to higher overall productivity in date palm orchards (Cherif et al., 2013). Furthermore, early sex identification facilitates breeding programs by enabling breeders to select specific male and female parents at a younger age, accelerating the development of improved cultivars (Elmeer & Mattat, 2012).

Early attempts to distinguish sexes involved morphological and physiological comparisons, but these proved unreliable before flowering (Abdel-Hamid et al., 2020). Cytogenetic studies also explored chromosome differences, but identifying distinct sex chromosomes in date palms has been challenging (Siljak-Yakovlev et al., 1996). The advent of molecular biology provided powerful tools for sex identification. Researchers began searching for DNA markers linked to the sex determination region(s) of the date

palm genome. Various molecular marker techniques have been employed, including Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), and Sequence Characterized Amplified Regions (SCAR) (Al-Dous et al., 2011; Elmeer et al., 2011; Zhao et al., 2012).

2.3.1 Specific Markers: LOX5.1 and GAPT3

Among the various markers developed, several Sequence Characterized Amplified Region (SCAR) markers have gained attention for their potential utility in sex determination. Two such markers are often discussed in the literature, sometimes referred to by codes related to the genes or sequences they target or are derived from, such as those related to Lipoxygenase (LOX) and Glyceraldehyde-3-phosphate dehydrogenase (GPAT3) genes.

2.3.1.1 LOX5.1 (Reference Marker): Various genetic markers have been employed for sex identification, including Random Amplified Polymorphic DNA (RAPD), Sequence Characterized Amplified Region (SCAR), Simple Sequence Repeats (SSRs), and Single Nucleotide Polymorphisms (SNPs). SCAR markers, in particular, are popular due to their high specificity, reliability, and ease of application in standard laboratories (Osman et al., 2014). Numerous studies have aimed to identify gene loci or genomic regions linked to sex determination to develop precise and rapid sex-specific markers. LOX5.1 Marker and Its Role in Sex Determination. Research into female-associated markers has led to the identification of LOX5.1, a marker that has garnered significant attention. Osman et al. (2014) developed SCAR markers based on RAPD fragments associated with female sex determination, and LOX5.1 was among the markers continuously investigated. The LOX5.1 primer can also serve as a reference primer or positive control in PCR analyses. In this context, the LOX5.1 primer is designed to amplify a DNA fragment from the Lipoxygenase (LOX) gene, a gene family that plays fundamental and crucial physiological roles in plants, such as stress response and growth (Joo & Oh, 2012). Consequently, the LOX gene, or a part of the LOX5.1 gene, is often present in the genomes of both male and female plants, allowing it to produce a DNA band in samples from both sexes. Using LOX5.1 as a reference primer or positive control is highly beneficial for quality control in PCR experiments, especially when

using sex-specific markers that rely on the presence/absence of DNA bands. The consistent amplification of a LOX5.1 band in all samples, regardless of sex, helps to confirm that DNA extraction procedures were effective and that the PCR reaction proceeded successfully in every sample (Bendiab et al., 2024). The DNA band produced by LOX5.1 in this scenario is typically homomorphic (i.e., appears with similar characteristics, such as size, in both sexes), thus serving as an important reference point for comparison with the DNA bands of the actual sex-determining markers.

2.3.1.2 GAPT3 (Male-Specific Marker): Research has also focused on identifying male-specific markers. Markers derived from regions linked to male development, potentially near genes like GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), have been reported. GAPT3 is often cited as a male-specific SCAR marker (or derived from a male-specific RAPD band). Studies suggest that primers designed for GAPT3 consistently amplify a specific DNA fragment only in male date palms, with no corresponding amplification in females (Saleh & El-Hadad, 2018; Osman et al., 2014). This male-specific nature makes GAPT3 a potentially robust marker for identifying male seedlings, complementing female-associated markers. Combining a reliable female-associated marker and a male-specific marker like GAPT3 could significantly increase the accuracy of sex determination assays (Younis et al., 2020).

2.3.1.3 Challenges and Future Directions Despite progress, challenges remain. The genetic basis of sex determination in date palms is complex and not fully elucidated, although recent genomic studies suggest an XY system with a relatively small male-specific region on the Y chromosome (Hazzouri et al., 2019; Torres et al., 2018). The reliability of molecular markers can sometimes vary across different date palm cultivars due to genetic diversity (Al-Mahmoud et al., 2012). Recombination events between the marker and the actual sex-determining locus could also potentially lead to misidentification, although markers tightly linked to the locus minimize this risk.

Future research should validate existing markers like LOX5.1 and GAPT3 across various cultivars and geographical origins. Fine-mapping the sex-determination region and identifying the causal genes will provide the ultimate solution for developing foolproof markers (Hazzouri et al., 2019). Integrating genomic and

transcriptomic data holds great promise for uncovering the precise mechanisms and genes involved in date palm sex determination (Zhao et al., 2012).

Early sex determination is critical for optimizing date palm cultivation. Molecular markers, particularly SCAR markers, have emerged as valuable tools for identifying the sex of date palms at the seedling stage. Markers such as LOX5.1 (showing female-associated polymorphism) and GAPT3 (showing male-specific amplification) represent significant advancements in this field. While requiring further validation and refinement, these markers offer a practical means to improve the efficiency and productivity of date palm agriculture by allowing for early selection of desired female plants. Continued research into the genetics and genomics of sex determination will further enhance the accuracy and reliability of these diagnostic tools.

2.4 Polymerase Chain Reaction (PCR): Principles and Limitations

The Polymerase Chain Reaction (PCR) is a cornerstone technique in molecular biology, enabling the in vitro amplification of specific DNA sequences by orders of magnitude (Mullis & Faloona, 1987; Saiki et al., 1988). Developed by Kary Mullis in the 1980s, for which he received the Nobel Prize in Chemistry in 1993, PCR revolutionized genetic analysis, diagnostics, and biotechnology (Bartlett & Stirling, 2003; Mullis, 1990). Its power lies in its ability to generate millions to billions of copies of a target DNA segment from a minute starting sample. This review will explore the fundamental principles underlying the PCR process and discuss its inherent limitations.

2.4.1 Principles of PCR

The PCR process relies on thermal cycling, consisting of repeated cycles of temperature changes, to amplify a specific DNA target sequence. Each cycle typically involves three key steps: denaturation, annealing, and extension (Saiki et al., 1988; Erlich, 1989).

Denaturation: The reaction mixture, containing the template DNA, primers, DNA polymerase, deoxynucleotide triphosphates (dNTPs), and buffer, is heated to a high temperature, typically 94-98°C. This heat breaks the hydrogen bonds holding the double-stranded DNA template together, separating it into two single strands (Bartlett & Stirling, 2003; Garibyan & Nersisyan, 2013).

Annealing: The temperature is lowered, usually to 50-65°C, allowing short, synthetic DNA sequences called primers to bind (anneal) to complementary sequences on the single-stranded template DNA. Two primers are used: a forward primer and a reverse primer, flanking the target region to be amplified (Innis & Gelfand, 1990; Rychlik et al., 1990). The specific annealing temperature is crucial and depends on the primer length and sequence composition.

Extension (Elongation): The temperature is raised again, typically to 72°C, which is the optimal temperature for the DNA polymerase enzyme. The most used enzyme is Taq polymerase, isolated from the thermophilic bacterium Thermus aquaticus, which can withstand the high denaturation temperatures (Chien et al., 1976; Saiki et al., 1988). The polymerase binds to the primer-template complex and synthesizes a new DNA strand complementary to the template strand, extending from the primer by adding dNTPs (Garibyan & Nersisyan, 2013).

These three steps constitute one cycle. Repeating this cycle 25-40 times results in an exponential amplification of the target DNA sequence located between the two primers (Mullis & Faloona, 1987). The amount of target DNA approximately doubles with each cycle, leading to a massive increase in the number of copies.

2.4.2 Limitations of PCR

Despite its widespread utility, standard PCR has several limitations that researchers must consider:

Requirement for Sequence Information: Primer design necessitates prior knowledge of the DNA sequences flanking the target region. PCR cannot be used to amplify completely unknown sequences (Innis & Gelfand, 1990; Lorenz, 2012).

Sensitivity to Contamination: PCR's high sensitivity makes it extremely susceptible to contamination by extraneous DNA, either from other samples, previous PCR products (carry-over contamination), or the environment. This can lead to false-positive results, requiring stringent laboratory practices and controls (Kwok & Higuchi, 1989; Borst et al., 2004).

Inhibition: Various substances present in biological or environmental samples (e.g., heme from blood, humic acids from soil, certain reagents) can inhibit the DNA polymerase activity, leading to reduced amplification efficiency or false-

negative results (Wilson, 1997; Opel et al., 2010). Sample preparation methods are often required to remove these inhibitors.

Primer Specificity and Optimization: Primers may sometimes bind to non-target sequences (non-specific binding) if conditions are not optimal, leading to the amplification of undesired DNA fragments. Careful primer design and optimization of annealing temperature and magnesium concentration are critical for specificity (Rychlik et al., 1990; Lorenz, 2012).

Size Limitation: Standard PCR protocols using *Taq* polymerase are generally most efficient for amplifying DNA fragments up to a few kilobases (kb) in length. Amplifying very long fragments (>10-20 kb) can be challenging and often requires specialized polymerases and modified protocols (Cheng et al., 1994).

Polymerase Fidelity: *Taq* polymerase lacks significant proofreading activity, meaning it can incorporate incorrect nucleotides during synthesis, albeit at a relatively low rate. While often acceptable, this can be problematic for applications requiring high sequence accuracy, such as cloning or mutation detection. High-fidelity polymerases with proofreading capabilities are available but may be more expensive or require different reaction conditions (Eckert & Kunkel, 1991; Cline et al., 1996).

Quantification: Standard endpoint PCR is generally considered qualitative or semi-quantitative. Determining the precise starting quantity of target DNA is difficult because the reaction eventually reaches a plateau phase. Quantitative PCR (qPCR or real-time PCR) techniques were developed to overcome this limitation, allowing for accurate measurement of DNA concentration during the amplification process (Higuchi et al., 1993; Bustin, 2000).

The Polymerase Chain Reaction is a powerful and versatile technique based on the principles of DNA denaturation, primer annealing, and enzymatic extension, allowing for the exponential amplification of specific DNA sequences. Its invention has profoundly impacted biological research, diagnostics, forensics, and numerous other fields. However, users must be aware of its inherent limitations, including the need for prior sequence knowledge, susceptibility to contamination and inhibition, potential for non-specific amplification, limitations on fragment size, enzyme fidelity issues, and the qualitative nature of standard endpoint analysis. Understanding these principles and

limitations is crucial for the effective application of PCR and the accurate interpretation of its results. Ongoing developments, such as qPCR and high-fidelity polymerases, continue to address some of these constraints, further expanding the capabilities of this essential molecular tool.

2.5 Principles and Mechanisms of LAMP

The rapid and accurate detection of specific nucleic acid sequences is fundamental to molecular diagnostics, genetics, and various life science research areas. While the Polymerase Chain Reaction (PCR) has long been the gold standard, its requirement for thermal cycling limits its application in resource-limited settings or for point-of-care testing. Loop-mediated Isothermal Amplification (LAMP), first described by Notomi et al. (2000), emerged as a powerful alternative technique that amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions, typically between 60-65°C. This review focuses on the fundamental principles and intricate mechanisms underlying the LAMP reaction.

2.5.1 Core Principles of LAMP

The cornerstone of LAMP is its ability to operate at a single, constant temperature, eliminating the need for sophisticated thermal cyclers (Notomi et al., 2000; Parida et al., 2008). This isothermal nature is primarily enabled using a DNA polymerase with high strand displacement activity, most commonly the *Bacillus stearothermophilus* (*Bst*) DNA polymerase large fragment (Nagamine et al., 2002; Gill & Ghaemi, 2008). Unlike the *Taq* polymerase used in standard PCR, *Bst* polymerase can separate DNA strands as it synthesizes a new strand, obviating the need for a high-temperature denaturation step. Another defining principle is the use of a unique primer set, typically comprising four to six primers that recognize six to eight distinct regions on the target DNA sequence, contributing significantly to the reaction's high specificity (Notomi et al., 2000; Zhang et al., 2020).

2.5.2 Mechanism of Amplification

The LAMP mechanism is complex but highly efficient, relying on auto-cycling strand displacement DNA synthesis. It can be broadly divided into initial steps leading

to the formation of a dumbbell-like structure and subsequent cycling amplification (Notomi et al., 2000; Nagamine et al., 2002).

Initiation Phase: The process begins with the binding of the Forward Inner Primer (FIP) and Backward Inner Primer (BIP) to the target DNA. The FIP contains sequences complementary to the F1c region (complementary to F1) and the F2 region within the target. Synthesis initiates from the 3' end of the F2 region of the FIP, displacing the complementary strand. This is followed by the binding and extension of the Forward Outer Primer (F3), which displaces the FIP-linked complementary strand (Notomi et al., 2000). A similar process occurs symmetrically on the opposite strand using the BIP (containing B1c and B2 regions) and the Backward Outer Primer (B3). This results in the formation of single-stranded loops at the ends of the newly synthesized strands due to the complementary F1/F1c and B1/B1c sequences within the FIP and BIP, respectively (Parida et al., 2008; Becherer et al., 2020).

Dumbbell Structure Formation and Elongation: The released strand containing the FIP sequence forms a stem-loop structure at one end. This structure serves as a template for synthesis initiated by the BIP, followed by B3 primer extension, which displaces the strand. This displaced strand now possesses complementary sequences derived from both FIP and BIP at its ends, allowing it to rapidly form a dumbbell-like structure with stem-loops at both the 5' and 3' ends (Notomi et al., 2000; Yan et al., 2014). This dumbbell structure serves as the starting point for the exponential amplification phase.

Cycling Amplification: The core of LAMP's efficiency lies in the subsequent cycling amplification phase. The dumbbell structure undergoes self-primed DNA synthesis. The FIP primes synthesis from the loop region, extending and displacing the downstream strand. This process continually opens new single-stranded loop regions, which then serve as annealing sites for subsequent FIP and BIP priming events (Nagamine et al., 2002; Gill & Ghaemi, 2008). Each priming event initiates strand displacement synthesis, leading to the elongation of the original structure and the release of new dumbbell structures or inverted repeats of the target sequence concatenated together.

Role of Loop Primers: To accelerate the reaction, two additional primers, Loop Forward (LF) and Loop Backward (LB), can be included (Nagamine et al., 2002). These primers anneal to the single-stranded loop regions generated during the cycling amplification phase, providing additional starting points for DNA synthesis. This significantly shortens the time required to achieve detectable levels of amplification (Nagamine et al., 2002; Tanner et al., 2015).

2.5.3 Product Formation and Detection

The amplification process results in the accumulation of a large quantity of DNA structures that are concatemers of the target sequence, forming stem-loop structures of various lengths (Notomi et al., 2000). This massive amplification (up to 10^9 copies in under an hour) facilitates simple detection methods. A common byproduct of the reaction is magnesium pyrophosphate, which precipitates and increases the turbidity of the reaction mixture, allowing real-time or endpoint detection by visual inspection or turbidimetry (Mori et al., 2001). Alternatively, fluorescent intercalating dyes (e.g., SYBR Green) or pH-sensitive dyes (detecting proton release during dNTPs incorporation) can be used for colorimetric or fluorescent detection (Tanner et al., 2015; Zhang et al., 2020).

2.5.4 Specificity

The high specificity of LAMP is attributed to the use of four to six primers recognizing multiple distinct regions on the target sequence (Notomi et al., 2000; Foudeh et al., 2016). The probability of all primers annealing correctly to a non-target sequence under isothermal conditions is extremely low. Furthermore, the strand displacement activity of the *Bst* polymerase contributes to the reaction's robustness, making it less susceptible to inhibitors often found in clinical or environmental samples compared to PCR (Kaneko et al., 2007; Francois et al., 2011; Njiru, 2012).

LAMP operates on the principles of isothermal strand displacement DNA synthesis facilitated by *Bst* polymerase and a unique set of four to six primers targeting multiple regions of the DNA sequence. The mechanism involves the formation of a dumbbell-like DNA structure that enables self-priming and auto-cycling amplification, leading to the rapid accumulation of large quantities of target DNA concatemers. The inclusion of loop primers can further accelerate the reaction. These principles and

mechanisms confer on LAMP its characteristic features of high specificity, sensitivity, rapidity, and operation under isothermal conditions, making it a valuable tool, particularly for point-of-care molecular diagnostics and field applications.

2.6 Diagnostic Applications and Performance of LAMP

LAMP assays have been developed and applied across a wide spectrum of diagnostic fields:

2.6.1 Infectious Diseases: This is the most extensive area of LAMP application. Assays have been successfully developed for detecting various pathogens, including:

2.6.1.1 Viruses: Such as Dengue virus (Parida et al., 2005), Influenza virus (Poon et al., 2006), SARS-CoV-2 (Augustine et al., 2020; Dao Thi et al., 2020), and HIV (Curtis et al., 2018).

2.6.1.2 Bacteria: Including Mycobacterium tuberculosis (Iwamoto et al., 2003; Boehme et al., 2010), *Salmonella* spp. (Hara-Kudo et al., 2005), and Vibrio cholerae (Yamazaki et al., 2008).

2.6.1.3 Parasites: Such as Plasmodium species causing malaria (Poon et al., 2006; Njiru et al., 2008a), Leishmania species (Takagi et al., 2009), and *Trypanosoma brucei* (Njiru et al., 2008b).

2.6.2 Veterinary Diagnostics: LAMP is used for rapid detection of animal pathogens like Avian Influenza virus, Foot-and-Mouth Disease virus, and various bacterial infections affecting livestock (Wang et al., 2017).

2.6.3 Food Safety and Agriculture: It provides a tool for detecting foodborne pathogens (e.g., *Listeria monocytogenes*, *E. coli* O157:H7) in food matrices and identifying plant pathogens (Niessen & Vogel, 2010; Fall et al., 2007).

2.6.4 Point-of-Care Testing (POCT): The simplicity and minimal equipment requirements make LAMP highly suitable for POCT, particularly in resource-constrained areas where access to sophisticated labs is limited (Wong et al., 2018; Niemz et al., 2011). Integration with microfluidic devices and simple readout systems further enhances its POCT potential (Augustine et al., 2020).

2.7 Phenol Red indicator

Mori et al. (2001) initially described the use of pH indicators, including phenol red, in LAMP reactions to facilitate visual detection without the need for complex equipment. The incorporation of phenol red, a pH-sensitive colorimetric indicator, has further enhanced the LAMP technique by allowing simple, visual detection of amplification products. Phenol Red Indicator is a pH-sensitive dye commonly used in cell culture media to monitor pH changes. In LAMP reactions, phenol red serves as a colorimetric indicator that visually signifies the occurrence of DNA amplification. The principle behind this lies in the production of protons during DNA synthesis, which lowers the pH of the reaction mixture. As the pH drops, phenol red changes color from red (neutral pH) to yellow (acidic pH) (**Figure 2.1**), providing a clear visual indication of a positive LAMP reaction. The use of phenol red in LAMP offers several advantages. Firstly, it simplifies the detection process, eliminating the need for sophisticated equipment such as fluorescence detectors. This makes the technique accessible and practical for field applications and resource-limited settings. Additionally, the color change is easy to interpret, allowing for quick and reliable detection of amplification products (Tanner et al., 2015; Mori et al., 2001). Additionally, Goto et al. (2009) explored the application of phenol red in LAMP assays for pathogen detection, demonstrating its reliability in identifying bacterial and viral targets in clinical samples.

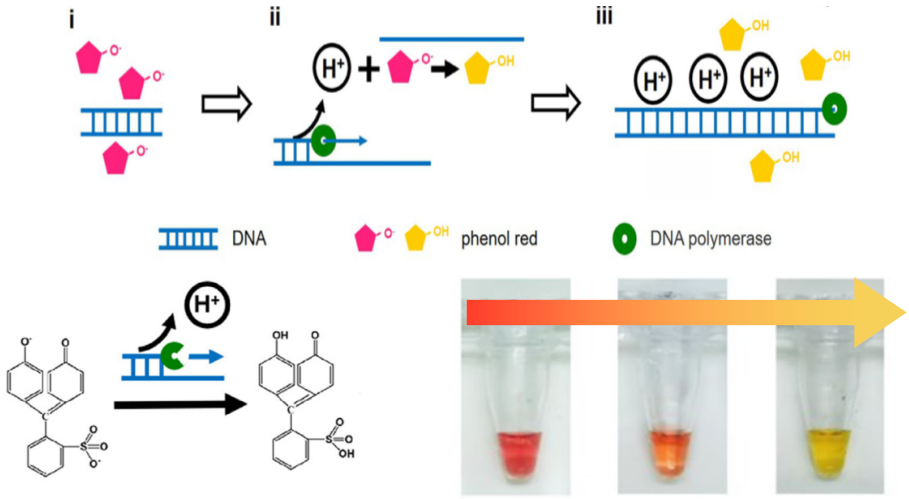

Figure 2.1 Phenol red molecules combine with H+ produced by DNA amplification, resulting in changes in molecular structure.

However, the use of phenol red is not without challenges. One major limitation is its sensitivity to initial pH conditions and the buffering capacity of the reaction mixture. Calvo et al. (2012) highlighted that variations in the initial pH could affect the reliability of the color change, potentially leading to false-positive or false-negative results. This necessitates careful optimization of reaction conditions to ensure consistent and accurate outcomes when using phenol red as an indicator.

Further research by Parida et al. (2010) examined the integration of phenol red in multiplex LAMP assays, demonstrating its effectiveness in simultaneously detecting multiple targets. This study reinforced the versatility of phenol red in complex diagnostic applications, where the need for simultaneous detection of various pathogens is crucial.

Additionally, studies by Wong et al. (2018) and Singh et al. (2019) explored the application of phenol red in environmental monitoring and food safety testing, respectively. Both studies underscored the broad applicability of phenol red in LAMP assays beyond clinical diagnostics, highlighting its potential in diverse fields such as environmental science and food safety.

2.8 Cryoprotectants and Stabilizers in Freeze-Drying

Loop-mediated isothermal amplification (LAMP) has emerged as a powerful molecular diagnostic tool due to its rapidity, specificity, sensitivity, and operation under isothermal conditions, eliminating the need for sophisticated thermal cyclers (Notomi et al., 2000). These characteristics make LAMP particularly suitable for point-of-care (POC) testing and applications in resource-limited settings. However, the liquid format of LAMP reagents, particularly the enzymes like *Bst* DNA polymerase, poses challenges for long-term storage and transportation, especially under ambient or fluctuating temperatures (Francois et al., 2011). Freeze-drying (lyophilization) offers a promising solution by removing water, thereby significantly reducing degradation pathways and potentially enabling ambient temperature stability (Jennings, 1999). Achieving successful lyophilization of complex biological mixtures like LAMP assays requires the inclusion of specific excipients, namely cryoprotectants and stabilizers, to preserve the

integrity and activity of sensitive components during the freezing, drying, and subsequent storage phases.

The primary challenge during freeze-drying is the stress imposed on biological macromolecules, especially enzymes. Freezing stress can arise from ice crystal formation and shifts in pH and solute concentration, while drying stress results from the removal of essential hydration shells (Carpenter & Crowe, 1988). Cryoprotectants primarily protect biomolecules during the freezing step, while lyoprotectants offer stabilization during the drying phase, although these terms are often used interchangeably as many compounds serve dual roles (Wang, 2000). Sugars, particularly non-reducing disaccharides like trehalose and sucrose, are widely recognized as highly effective cryo- and lyoprotectants. Their efficacy is attributed to mechanisms such as the "water replacement hypothesis," where sugar molecules hydrogen-bond with biomolecules in place of water, maintaining their native conformation, and "vitrification," where they form a highly viscous, glassy amorphous matrix that restricts molecular mobility and inhibits degradative reactions (Crowe et al., 1998; Allison et al., 1999). Numerous studies have demonstrated the successful use of trehalose and sucrose in stabilizing freeze-dried LAMP assays for detecting various pathogens, maintaining amplification efficiency after storage (Hayashida et al., 2015; Carter et al., 2017; Wee et al., 2017). For instance, Gadkar et al. (2018) developed a field-deployable, freeze-dried LAMP assay for malaria diagnosis using a trehalose-based formulation that remained stable for months at ambient temperature. Similarly, Aonuma et al. (2010) utilized trehalose to stabilize LAMP reagents for *Plasmodium falciparum* detection, showing stability for at least 9 months at 30°C.

While disaccharides are effective, they can sometimes crystallize during storage, particularly under high humidity, which can compromise their protective effect. Combining sugars with other excipients or using alternative stabilizers can mitigate this and enhance overall stability. Polyols like mannitol are often added as bulking agents to provide structural integrity to the lyophilized cake and prevent collapse, although mannitol can crystallize and may offer less direct protection to enzymes compared to amorphous sugars (Wang, 2000; Kim et al., 1998). Polymers such aspolyvinylpy-rrolidone (PVP) and polyethylene glycol (PEG) have also been investigated. PVP can

act as a cryoprotectant and help prevent enzyme aggregation, while also contributing to the glassy matrix (Francois et al., 2011; Mensink et al., 2017). Some studies combine trehalose with PVP or other additives to optimize LAMP reagent stability (Chen et al., 2019; Njiru et al., 2012). For example, Njiru et al. (2012) combined trehalose with bovine serum albumin (BSA) to successfully lyophilize LAMP reagents for detecting human African trypanosomiasis.

Freeze-drying is a critical enabling technology for translating the potential of LAMP assays into practical, stable diagnostic tools suitable for diverse settings. The successful application of this technology relies heavily on the rational selection and optimization of cryoprotectants and lyoprotectants. Disaccharides, especially trehalose and sucrose, remain the cornerstone of LAMP stabilization due to their proven effectiveness in preserving enzyme activity. However, optimal formulations often benefit from the inclusion of other excipients like bulking agents (mannitol) or polymers (PVP) to enhance cake structure, prevent crystallization, and further improve long-term stability under challenging environmental conditions. Continued research into novel excipient combinations and tailored formulations remains essential for developing robust, shelf-stable LAMP assays for global health applications.

2.9 Role of Sugars in Lyophilization of Molecular Kits

Lyophilization, or freeze-drying, is a widely adopted technique for preserving sensitive biological materials by removing water through sublimation under vacuum. This process significantly enhances the stability and shelf-life of products, particularly molecular diagnostic kits which often contain delicate components like enzymes, antibodies, and nucleic acids (Carpenter et al., 2002; Pikal, 2002). Ambient temperature storage and transport become feasible, reducing reliance on cold chains. However, the stresses encountered during freezing and dehydration can damage these biomolecules, compromising kit performance (Wang, 2000). Sugars, particularly disaccharides, are commonly incorporated into formulations as key excipients to protect these components during lyophilization and subsequent storage. This review examines the critical roles sugars play in stabilizing molecular kits during the freeze-drying process.

2.9.1 Stresses During Lyophilization and the Need for Stabilizers

Molecular kits rely on the functional integrity of their biological components, such as DNA polymerases, reverse transcriptase, antibodies, and oligonucleotides (primers and probes). These molecules are susceptible to damage during the distinct phases of lyophilization. During freezing, the formation of ice crystals can cause mechanical stress and lead to detrimental concentration increases of solutes and pH shifts in the unfrozen fraction (Chang et al., 1996; Pikal, 2002). The subsequent drying phase removes water, including essential hydration shells surrounding biomolecules, which can lead to conformational changes, denaturation, and aggregation (Allison et al., 1999; Wang, 2000). Furthermore, stability during storage is critical, requiring protection against residual moisture, temperature fluctuations, and chemical degradation pathways (Constantino & Pikal, 2004). Sugars act as crucial stabilizers (cryoprotectants during freezing and lyoprotectants during drying and storage) to mitigate these stresses.

2.9.2 Mechanisms of Sugar-Mediated Stabilization

Sugars employ several mechanisms to protect biomolecules during lyophilization and storage:

2.9.2.1 Water Replacement Hypothesis: During drying, as water is removed from the surface of biomolecules, sugars like sucrose and trehalose can form hydrogen bonds with polar groups on the biomolecule surface. This interaction effectively substitutes for the lost water molecules, helping to maintain the native structure and prevent unfolding or aggregation (Carpenter & Crowe, 1989; Allison et al., 1999). The effectiveness of a sugar often correlates with its ability to mimic the interaction of water.

2.9.2.2 Vitrification Hypothesis (Glass Dynamics): Upon drying, certain sugars (especially amorphous ones like sucrose and trehalose) form a highly viscous, non-crystalline glassy solid. This glassy matrix physically entraps the biomolecules, severely restricting their molecular mobility (Franks et al., 1991; Slade & Levine, 1991). By immobilizing the active components, the glass inhibits diffusion-

controlled processes like unfolding, aggregation, and chemical degradation reactions. A key property of this glassy state is the glass transition temperature (Tg), the temperature above which the glass transitions to a more rubbery, less viscous state with increased molecular mobility. For optimal long-term storage stability, the formulation's Tg must be significantly higher than the storage temperature (Pikal, 2002; Constantino & Pikal, 2004).

2.9.2.3 Cryoprotection During Freezing: While less pronounced than their lyoprotective effects, sugars can also offer some protection during the freezing step. By increasing the viscosity of the solution and interacting with water, they can modify ice crystal growth and reduce the concentration of solutes in the unfrozen phase, lessening freeze-concentration stress (Carpenter et al., 2002).

2.9.3 Common Sugars Used in Lyophilization

Different sugars exhibit varying properties and stabilization efficiencies:

2.9.3.1 Trehalose and Sucrose: These non-reducing disaccharides are considered the gold standard lyoprotectants for proteins, enzymes, and other biomolecules (Crowe et al., 1996; Leslie et al., 1995). They are highly effective at forming stable amorphous glasses with high Tg values (around 115°C for trehalose and 75°C for sucrose, anhydrous) and efficiently participate in water replacement (Colaco et al., 1992; Lins et al., 2019). Trehalose is often slightly preferred due to its higher Tg and potentially superior stabilization in some systems, possibly related to its structural flexibility and interaction with water (Jain & Roy, 2009; Olsson et al., 2016).

2.9.3.2 Mannitol: This sugar alcohol often crystallizes during freezing or annealing steps in the lyophilization cycle. While crystalline mannitol does not provide lyoprotection via vitrification or water replacement, it serves as an excellent bulking agent, providing structural support to the lyophilized cake, preventing collapse, and

potentially improving drying efficiency (Kim et al., 1998; Johnson et al., 2002). It is frequently used in combination with amorphous sugars like sucrose, where mannitol provides bulk and sucrose provides molecular stabilization (Jiang et al., 2017).

2.9.3.3 Other Sugars and Polyols: Monosaccharides like glucose generally have lower Tg values and are more hygroscopic, making them less suitable as primary stabilizers. Other polyols like sorbitol remain amorphous but possess low Tg values, limiting their utility for room-temperature stable formulations unless combined with high-Tg excipients (Constantino & Pikal, 2004). Polymers like dextran or PVP are sometimes added alongside sugars to further modulate Tg or improve cake properties (Mensink et al., 2017).

2.9.4 Formulation Considerations for Molecular Kits

The optimal sugar or combination of sugars for a molecular kit depends heavily on the specific components being stabilized (e.g., polymerase activity, antibody binding, nucleic acid integrity) and the desired product attributes (e.g., reconstitution time, cake appearance, target shelf-life and storage temperature). Careful formulation development is required, often involving screening various sugar types and concentrations, potentially alongside other excipients like buffers, amino acids, or polymers, to achieve maximum stability and functionality of the final lyophilized kit (Jain & Roy, 2009; Mensink et al., 2017). The interaction between different excipients must also be considered, as some combinations can lead to undesirable effects like phase separation or depression of Tg (Pikal, 2002).

Sugars play an indispensable role in the successful lyophilization of molecular kits. Primarily through the mechanisms of water replacement and vitrification, amorphous disaccharides like trehalose and sucrose protect sensitive biomolecules from damage during freezing, drying, and storage. They maintain molecular structure by substituting for structural water and immobilize components within a stable glassy matrix, characterized by a high glass transition temperature (Tg). While crystalline sugars like mannitol contribute primarily as bulking agents,

combinations of amorphous and crystalline sugars are often employed to optimize both molecular stability and the physical properties of the lyophilized product. The careful selection and optimization of sugar excipients are critical for developing robust, stable, and reliable molecular kits with extended shelf-lives suitable for diverse applications.

2.10 Trehalose: Structure, Protective Mechanisms, Benefits, and Enzyme Stabilization in LAMP assay

Trehalose is a naturally occurring, non-reducing disaccharide found in various organisms, including bacteria, yeast, fungi, insects, invertebrates, and plants (Elbein et al., 2003). These organisms often synthesize and accumulate trehalose in response to environmental stress conditions such as dehydration, heat, cold, and osmotic stress.

2.10.1 Structure of Trehalose

Chemically, trehalose consists of two alpha-glucose units linked together by an alpha,alpha-1,1-glycosidic bond (Richards et al., 2002). This unique linkage distinguishes it from other common disaccharides like sucrose or maltose. The 1,1-glycosidic bond makes trehalose exceptionally stable, as both anomeric carbons are involved in the linkage, rendering it non-reducing (Ohtake & Wang, 2011). This stability contributes to its resistance to acid hydrolysis and enzymatic degradation by common glycosidases, and prevents it from participating in Maillard reactions (browning reactions) with amino acids or proteins, which is a significant advantage in preservation contexts (Crowe et al., 1998).

2.10.2 Protective Mechanisms of Trehalose

The protective effects of trehalose on biomolecules (proteins, lipids) and whole cells during stress, particularly dehydration:

2.10.2.1 Water Replacement Hypothesis (WRH): Proposed initially by Crowe and colleagues, this hypothesis suggests that during dehydration, trehalose molecules directly replace water molecules that normally hydrate biomolecules, forming hydrogen bonds with polar residues on proteins and lipid headgroups (Crowe et al., 1984; Crowe et al., 1998). This

interaction helps maintain the native structure and prevents denaturation or fusion of membranes upon water removal and subsequent rehydration.

2.10.2.2 Vitrification Hypothesis (Glass Formation): Trehalose has a high glass transition temperature (Tg) compared to other sugars (Levine & Slade, 1988). Upon drying, trehalose solutions can form a stable, amorphous, glassy (vitrified) state. This highly viscous matrix immobilizes biomolecules, drastically slowing down diffusion-limited degradative reactions and preventing structural rearrangements like protein unfolding or aggregation (Franks et al., 1991; Ohtake & Wang, 2011).

2.10.2.3 Preferential Exclusion/Hydration: In aqueous solutions, trehalose is preferentially excluded from the surface of proteins. This phenomenon increases the chemical potential of the protein, thermodynamically favoring the compact, native state over the unfolded state, as unfolding would expose more surface area to the unfavorable solvent environment (Timasheff, 2002; Kaushik & Bhat, 2003). This mechanism contributes to protein stabilization even in solution, not just during drying.

2.10.2.4 Chemical Stability: As mentioned earlier, the non-reducing nature of trehalose prevents detrimental Maillard reactions with proteins, which can occur with reducing sugars during storage or processing, especially at elevated temperatures (Ohtake & Wang, 2011).

2.10.3 General Benefits and Applications of Trehalose

Beyond its fundamental role in anhydrobiosis (life without water), trehalose's protective properties have been harnessed in various fields:

2.10.3.1 Food Industry: Used as a stabilizer, texturizer, and sweetener with low carcinogenicity, improving the shelf life and quality of processed foods, especially dried or frozen products (Ohtake & Wang, 2011; Schiraldi et al., 2002).

2.10.3.2 Cosmetics: Incorporated into formulations to protect skin cells from dehydration and environmental stress (Richards et al., 2002).

2.10.3.3 Pharmaceuticals and Biotechnology: Employed as a cryoprotectant and lyoprotectant for stabilizing therapeutic proteins, antibodies, vaccines, and cells during freezing, lyophilization (freeze-drying), and long-term storage (Crowe et al., 1998; Ohtake & Wang, 2011; Allison et al., 1999). It helps maintain the structure and biological activity of sensitive molecules.

2.10.4 Enzyme Stabilization in LAMP Assays

Trehalose has emerged as a key excipient for stabilizing LAMP reagents. Its protective mechanisms, particularly water replacement and vitrification, are highly effective in preserving enzyme activity during lyophilization or air-drying of LAMP reaction mixtures (Hsieh et al., 2011; Toubanaki et al., 2016). By incorporating trehalose, the core enzymes and other components can be dried into a stable format that can be stored for extended periods at room temperature without significant loss of activity (Chen et al., 2011; Hayashida et al., 2015). Upon rehydration with the sample, the enzymes regain their function, allowing the LAMP reaction to proceed efficiently. This stabilization facilitates the development of ready-to-use, field-deployable diagnostic kits, eliminating the need for a cold chain (Kamau et al., 2019; Carter et al., 2020). Studies have demonstrated that trehalose, often in combination with other stabilizers, successfully maintains the sensitivity and reliability of LAMP assays for detecting various pathogens after prolonged storage of the dried reagents (Francois et al., 2011; Tanner et al., 2016).