DEVELOPMENT OF DATE PALM SEX DETERMINATION LAMP KIT

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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DEVELOPMENT OF DATE PALM SEX DETERMINATION LAMP KIT

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ภาสกร พบพิมาย: การพัฒนาชุดตรวจเพศอินทผลัมด้วยปฏิกิริยาแลมป์ (DEVELOPMENT OF DATE PALM SEX DETERMINATION LAMP KIT) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 78 หน้า

คำสำคัญ: แลมป์/อินทผลัม/ฟีนอลเรด/การทดสอบภาคสนาม/การทำแห้งแบบเยือกแข็ง/เสถียรภาพในระยะยาว

การระบุเพศในระยะต้นกล้าของพืชที่มีเพศผู้และเมียอยู่ต่างต้น เช่น กัญชา (*Cannabis sativa* L.) และอินทผลัม (*Phoenix dactylifera* L.) มีความสำคัญอย่างยิ่งต่อประสิทธิภาพทางการเกษตร เนื่องจากต้นเพศเมียมักมีมูลค่าทางเศรษฐกิจสูงกว่าต้นเพศผู้ วิธีการระบุเพศแบบดั้งเดิมมักใช้เวลานาน ส่งผลให้เกิดความสิ้นเปลืองทรัพยากร งานวิจัยนี้จึงมุ่งพัฒนาเครื่องมือระดับโมเลกุลที่มีความแม่นยำ คุ้มค่า และสามารถใช้งานภาคสนามได้ สำหรับกัญชา ได้มีการพัฒนาเทคนิคการสกัดดีเอ็นเอที่ง่ายและรวดเร็ว เหมาะสำหรับการใช้งานในภาคสนาม และตรวจสอบความถูกต้องของเครื่องหมายดีเอ็นเอ MADC2 โดยใช้เทคนิค Polymerase Chain Reaction (PCR) ซึ่งสามารถจำแนกต้นเพศผู้ (แถบดีเอ็นเอขนาด 390 bp) และต้นเพศเมีย (แถบดีเอ็นเอขนาด 560 bp และ 870 bp) ได้อย่างแม่นยำ สำหรับอินทผลัม ได้มีการพัฒนาเทคนิค Loop-mediated Isothermal Amplification (LAMP) โดยมุ่งเป้าไปที่ลำดับเบสของยีน GPAT3 ที่จำเพาะต่อเพศผู้ เทคนิคนี้สามารถแสดงผลได้ด้วยตาเปล่า โดยใช้ฟีนอลเรด (phenol red) ที่เปลี่ยนสีจากสีแดงเป็นเหลืองเมื่อมีการเพิ่มปริมาณดีเอ็นเอเป้าหมาย ที่อุณหภูมิคงที่ 65°C ภายในเวลา 60–70 นาที โดยไม่ต้องใช้อุปกรณ์ที่ซับซ้อน นอกจากนี้ยังได้พัฒนาชุดตรวจ LAMP แบบแห้ง (lyophilized) ที่มีความเสถียรด้วยการใช้น้ำตาลทรีฮาโลส (trehalose) เป็นสารรักษาความคงตัว ทำให้สามารถเก็บรักษาที่อุณหภูมิ -20°C ได้อย่างน้อย 6 เดือน งานวิจัยนี้ประสบความสำเร็จในการพัฒนาเครื่องมือระดับโมเลกุลที่มีประสิทธิภาพและเข้าถึงได้ สำหรับการระบุเพศในระยะต้นกล้าของกัญชาและอินทผลัม ซึ่งจะช่วยให้เกษตรกรและนักปรับปรุงพันธุ์สามารถจัดการทรัพยากรได้อย่างมีประสิทธิภาพ ลดความเสี่ยงทางเศรษฐกิจ และส่งเสริมการเกษตรแม่นยำด้วยวิทยาศาสตร์และเทคโนโลยี ที่จะให้ผลอย่างยั่งยืน

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา .....

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา .....

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Early sex determination in dioecious crops like *Cannabis sativa* L. (cannabis) and *Phoenix dactylifera* L. (date palm) is vital for efficient agriculture, as females are often more valuable. Traditional methods delay identification and wasting resources. This research developed reliable, cost-effective, field-use molecular tools for early sex determination in Thai cannabis. A simplified, rapid DNA extraction method suitable for field use was developed. The MADC2 Polymerase Chain Reaction (PCR) marker was validated for reliably differentiating male (390 bp amplicon) from female (560 bp and 870 bp amplicons) plants. For date palm, a novel Loop-mediated Isothermal Amplification (LAMP) assay targeting the male-specific GPAT3 sequence was developed. Visual detection using phenol red provides a clear orange-to-yellow color change upon positive amplification at a constant temperature of 65°C within 60-70 minutes. This LAMP assay eliminates complex equipment. A stable, lyophilized LAMP kit using trehalose as a cryoprotectant was also developed. This user-friendly kit demonstrated excellent stability for at least six months, facilitating easy transport and field deployment. This research successfully provides accessible and robust molecular tools for early sex determination of cannabis and date palm. The validated PCR markers for cannabis and the novel, field-ready LAMP kit for date palm empower farmers and breeders with timely information, significantly enhancing resource management, reducing economic risks, and promoting sustainable and profitable cultivation practices.

School of Biotechnology
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Student's Signature .....
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TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

M	=	Molar
mM	=	Milli Molar
mg	=	Milligram
µg	=	Microgram
min	=	Minute
mL	=	Milliliter
g	=	Gram
gDNA	=	Genomic DNA
kg	=	Kilogram
M	=	Mole
mg/ml	=	Milligram per milliliter
ml	=	Milliliter
mM	=	Millimolar
mm	=	Millimeter
ng	=	Nanogram
nt	=	Nucleotide
pmol	=	Picomole
rpm	=	Revolutions per minute
µg/ml	=	Microgram per milliliter
µl	=	Microliter
µm	=	Micrometer
GPAT3	=	Glyceraldehyde-3-phosphate dehydrogenase gene
LOX 5.1	=	Lipoxygenase gene
h	=	Hour

LIST OF ABBREVIATIONS (Continued)

U.S.A.	=	United States of America
dNTPs	=	Deoxyribonucleoside triphosphates
PCR	=	Polymerase chain reaction
LAMP	=	Loop-mediated isothermal amplification

INTRODUCTION

1.1 Introduction

Precision agriculture (PA) involves the integration of advanced technology and data management to enhance the efficiency and accuracy of farming practices. The primary objective of incorporating these technologies is to reduce production costs while enabling farmers to make more informed decisions through accurate predictions of future occurrences. One critical application of PA is in the early sex determination of dioecious crops, such as *Cannabis sativa* L. and *Phoenix dactylifera* L. (date palm), where female plants have higher agriculturally valuable.

Cannabis sativa L., an annual dioecious plant from the Cannabaceae family, is cultivated primarily for its medicinal and industrial uses. Male plants produce pollen necessary for reproduction, while unfertilized female plants produce flowers rich in cannabinoids compounds with significant medicinal value. For optimal cannabinoid yield, cannabis farmers aim for only unfertilized female flowers. However, the plant's sex is typically identifiable only 4–8 weeks after planting, which can lead to inefficiencies in resource use. Cannabis utilizes an XY/XX sex determination system (Mandolino et al., 2002), though alternative mechanisms such as an X-to-autosome balance system have also been proposed (Ming et al., 2007, 2011; Punja & Holmes, 2020; Razumova et al., 2016). Previous studies have focused on the development of male-associated DNA markers, such as the male-associated DNA from Cannabis sativa (MADC2) region, which provides a reliable genomic signature for male plants (Mandolino et al., 1999, 2002). Implementing molecular techniques to identify such markers in local cannabis cultivars, particularly in Thailand, can enable early selection of female seedlings.

Similarly, *Phoenix dactylifera* L., commonly known as date palm, is a dioecious fruit tree in the Arecaceae family, native to the desert regions of the Middle East. In recent years, it has adapted well to the climate in Thailand, producing high yields and

yields and gaining popularity in for fresh and dried forms due to its sweet taste and nutritional value. Only female date palm trees bear fruit, but determining the plant's sex typically takes 3–7 years, depending on the cultivar (Chao et al., 2007; Wellmann et al., 2007). Date palm propagation methods include seed propagation, offshoot division, and tissue culture. While seed propagation is cost-effective and suitable for large-scale planting, it presents a 50:50 chance of producing male or female plants. This uncertainty leads to high investment risks related to planting, fertilization, irrigation, and maintenance before the plant's sex is known. Although tissue-cultured plants offer a more predictable alternative, they are expensive and may involve varieties not well-suited to local growing conditions Thailand (NSTDA, 2022).

To address these challenges, this research aims to develop a rapid, cost-effective, and user-friendly molecular tool for early sex determination in dioecious crops such as cannabis and date palm. The study focuses on applying Loop-mediated Isothermal Amplification (LAMP), a technique that amplifies DNA at 60–70 °C and can increase target genetic material up to a billion times within an hour (Notomi et al., 2000). The reaction's results can be interpreted visually through a color change, using phenol red as an indicator, which shifts from red/orange to yellow upon successful amplification (Quan-Ying et al., 2023). With its high specificity, sensitivity, simplicity, and affordability, the LAMP-based test kit is suitable for field applications, empowering farmers to make wisely planting decisions without reliance on costly equipment or specialized expertise. The success of this research will significantly reduce production costs, minimize risk, and promote sustainable agricultural practices for Thai farmers and beyond.

1.2 Research Objectives

1) To develop simple DNA extraction method for cannabis and date palm leaves
2) To develop method for cannabis sex determination
3) To develop male date palm specific genetic markers for LAMP reaction
4) To develop a suitable reagent for LAMP reactions
5) To create a simple date palm sex determination test (kit) for field use

1.3 Scope of Research

DNA extraction methods for both cannabis and date palm were developed to obtain high-quality genetic material for downstream analysis. A PCR-based method was established for sex determination in cannabis. For date palm, the Loop-mediated Isothermal Amplification (LAMP) technique was employed to identify the sex of known samples. Various components of the LAMP reaction, as well as optimal conditions including the lyophilization of reagent components—were optimized. Furthermore, a simple, field-deployable test kit for early sex determination in date palm was developed to support practical applications in agricultural settings.

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.

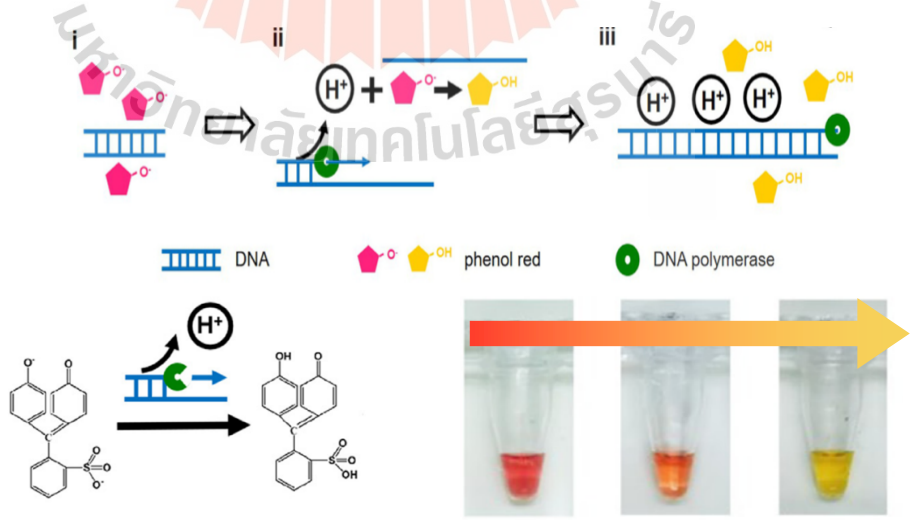

Figure 2.1 Phenol red molecules combine with H+ produced by DNA amplification, resulting in changes in molecular structure.

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 aspolyvinylpyrrolidone (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).

CHAPTER III

RESEARCH METHODOLOGY

3.1 Cannabis

3.1.1 Sample

Thirty cannabis leaf samples of known gender were obtained from the Suranaree University of Technology cannabis Farm (SUT cannabis) in Nakhon Ratchasima (14.871121, 102.025668). The collected samples were stored at -20°C until further analysis.

3.1.2 DNA Extraction

Each cannabis (*Cannabis sativa* L.) leaf sample was first mashed in a mortar with liquid nitrogen until it attained a fine powder consistency, thereafter transferred to a 1.5 mL tube. DNA was extracted using the innuPREP Plant DNA Kit (Analytik Jena, Germany) procedure. The concentration and purity of the extracted DNA was evaluated using absorbance measurements at 260 and 280 nm using a spectrophotometer, as well as via gel electrophoresis. The DNA was further used for amplification by the Polymerase Chain Reaction (PCR).

In a simple and rapid DNA extraction technique (adapted from Wang et al., 1993; Werner et al., 2002; Araujo et al., 2021), 5 mg of young cannabis leaf tissue was ground in an Eppendorf tube with 100 µL of 0.1 mM NaOH using a small pestle for a few seconds. Leaves was also crushed in a mortar using a pestle and after that transferred to the Eppendorf tube. The sample was further neutralized with 100 µL of 0.1 M HCl, and the grinding process continued for an additional 1-2 minutes. Subsequently, 50 µL of the mixture was transferred into a new Eppendorf tube containing 450 µL of 50 mM Tris-HCl, pH 8.0 (diluted 1:10), and vortexed (this technique does not need centrifugation to precipitate big particles). One µL aliquot of the solution was used as DNA template for subsequent analysis.

3.1.3 Specificity Testing of DNA markers for Cannabis sex using PCR

The DNA was amplified with the MADC2 and SCAR119 primers **(Table 3.1)** by PCR reaction in a BIO-RAD thermal cycler (T100™). PCR was performed in a total volume of 25 µL consisting of 0.5 U *Taq* DNA polymerase (homemade), 1X PCR buffer with $MgCl_2$, 0.1 mM of each deoxynucleoside triphosphate (dNTP) mixture (New England Biolabs, Beverly, Massachusetts), 0.3 µM of each primer, and 20-50 ng of template DNA. The same PCR protocols as Mandolino et al. (1999) were used for MADC2 and SCAR119 markers. The PCR started with an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, annealing at the primer's specified annealing temperature for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. Subsequently, the PCR products were analyzed by 2% agarose gel electrophoresis, stained with a 1:20,000 dilution of RedSafe™ (iNtRON Biotechnology, WA, USA). The presence and size of PCR products were visualized over a UV transilluminator (BIORAD® Gel Doc XDR) and analyzed with Quantity One software (BIORAD®, CA, USA).

Table 3.1 Primers used for Cannabis sex determination

Primer	Primer Sequences (5'→ 3')	Amplicon size (bp)
MADC2	F: GTGACGTAGGTAGAGTTGAA R: GTGACGTAGGCTATGAGAG	390 (Male), 560 and 870 (Female)
SCAR119	F: TCAAACAACAACAAACCG R: GAGGCCGATAATTGACTG	119 (Male)

3.2 Date Palm

3.2.1 Sample

Sample of young Date Palm (*Phoenix dactylifera* L.) leaves of both known and unknown sex were collected from 13 cultivars: KL1, Yellow Barhee, Red Barhee, Red India, Um ed Dahan, Khoneizi, Shi Shi, Ghannami, Jozi, Braim, G2, H1, and AP-red1. These samples were obtained from BunMak Date Palm in Nakhon Ratchasima (14.67360, 102.39645) and Phutara Green Park in Buriram (14.69488, 102.50448), Thailand. The collected samples were stored at -20°C until further analysis.

3.2.2 DNA Extraction

Twenty milligrams of date palm leaf samples were mashed in a mortar by protocol modified from Keb-Llanes et al. (2002) with 700 µL of lysis buffer solution containing 100 mM Tris-HCl, 20 mM EDTA, 2% (w/v) cetyltrimethylammonium bromide (CTAB), 4% (w/v) polyvinylpyrrolidone (PVP), 20% (w/v) sodium dodecyl sulfate (SDS), and 10 mM β-mercaptoethanol. The DNA concentration and purity were measured using a Nanodrop spectrophotometer (Thermo Scientific, USA) and visualized by 0.8% agarose gel electrophoresis before being used as a template in PCR (Polymerase Chain Reaction).

3.2.3 Specificity Testing of DNA markers for Date Palm sex using PCR

A total of 40 DNA samples, comprising 20 female and 20 male samples from 13 date palm cultivars, were used as template for LOX 5.1 and GPAT3 gene fragments amplification using PCR for the analysis of a reference gene and male-specific DNA markers, as previously reported (**(Table 3.2)** Jani et al., 2022; Torres et al., 2018). The DNA was amplified using multiplex primer PCR, and the reaction was carried out using a BIO-RAD thermal cycler (T100™). The PCR protocol began with an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of 95°C for 20 seconds, annealing at the primer's specified annealing temperature for 20 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. Subsequently, the PCR products were analyzed under 2% agarose gel electrophoresis, which was stained with a 1:20,000 dilution of RedSafe™ (iNtRON Biotechnology, WA, USA).

Table 3.2 Primer sequences for Date Palm sex determination and their reference

No	Primer name	Sequences (5'→ 3')	Amplicon size (bp)	Reference
1	GPAT3	**F:** AGAAAACCTGATATGCTCTCTG	450	Torres et al., 2018
		R: TGTGATGCACTTGGTAACTACT		
2	LOX5.1	**F:** CTACACCGCAGAGTTTGTCG	156	Jani et al., 2022
		R: AGATTGGACCCATGAGTTGC		

3.2.4 Development of LAMP technique for determining the sex of date palm LAMP primer design

PCR products were purified using a PCR purification kit to remove any impurities and ensure clean sequencing results. The purified PCR products were then sent to a sequencing facility to obtain the nucleotide sequences of the target regions from each cultivar. After sequencing, the quality of the sequencing data was checked to ensure accuracy and completeness. FastQC was used to assess the quality of the sequence reads. Next, the sequences were imported into sequence alignment software that supports ClustalW (e.g., MEGA, BioEdit). Multiple sequence alignment was performed using ClustalW to identify conserved and variable regions among the 13 date palm cultivars. The alignment results were analyzed to identify regions suitable for LAMP primer design. LAMP primers were designed using NEB® LAMP promer design tool software version 1.4.2 (New England BioLabs Co., Ltd.). Parameters for LAMP primer design, including primer length, GC content, and temperature conditions, were optimized to ensure efficient primer performance.

3.2.5 LAMP Reaction and Detection

3.2.5.1 Commercial set Testing (10X *Bsm* buffer)

LAMP reactions were carried out as described previously by Notomi et al. (2000), with some modifications. The primers used were designed as mentioned in 3.4. In brief, each 25 µL reaction mixture contained 1.6 µM of each FIP and BIP primer, 0.8 µM of each LF and LB primer, and 0.2 µM of each F3 and B3 primer (Integrated DNA Technologies (IDT)®, Seoul, South Korea). The reaction was carried out in 1X ThermoPol® Reaction Buffer, which contains 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton® X-100 (pH 8.8 at 25°C), 1.4 mM deoxynucleoside triphosphate (dNTPs) mixture, *Bst* DNA polymerase (New England Biolabs, Beverly, Massachusetts, USA). The mixture also included 0.8 M betaine (Sigma-Aldrich, St. Louis, Missouri, USA), and 1 µL of 30–50 ng DNA template was used. The reaction mixture was incubated at 62°C for 60 minutes. The results were checked visually by observing the formation of white

precipitate, which indicates the accumulation of magnesium pyrophosphate, a by-product of the LAMP reaction. The presence of turbidity in the reaction tube was considered a positive result, as it reflects successful DNA amplification (Mori et al., 2001; Notomi et al., 2000). To confirm the amplification, the reaction products were also analyzed by electrophoresis on a 2% agarose gel stained with RedSafe™ (1:20,000 dilution; iNtRON Biotechnology, Washington, USA).

3.2.5.2 Phenol red preparation

To prepare a 5 mM phenol red solution, 188.2 mg of phenol red sodium salt (molecular weight ≈ 376.38 g/mol) was measured using an analytical balance (Sigma-Aldrich, 2023). The powder was transferred to a clean beaker and dissolved in approximately 80 mL of distilled water with continuous stirring. Once fully dissolved, the solution was poured into a 100 mL volumetric flask, and distilled water was added to make up the final volume. The pH of the solution was checked using a pH meter and adjusted to 8.0 by 2.5 M NaOH (KemAus™, New South Wales, Australia), as phenol red acts as a pH indicator and changes color based on the pH of the solution (Invitrogen, 2008; Rinkenberger & Horwitz, 1993). For experiments requiring sterility, the solution was filtered using a 0.22 µm syringe filter (Freshney et al., 2015). The prepared phenol red solution was stored at 4°C and protected from light to maintain stability (U.S. Pharmacopeia., 2022).

3.2.5.3 2X LAMP with phenol red variation

LAMP reactions were carried out as described previously by Notomi et al. (2000), with some modifications. The primers used were designed as mentioned in 3.4. In brief, a 25 µL reaction mixture containing 1.6 mM of each FIP and BIP primer, 0.8 mM of each LF and LB primer, 0.2 mM of each F3 and B3 primer, 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine (Sigma-Aldrich, St. Louis, Missouri, USA), 0.125 mM phenol red (Loba Chemie, MUMBAI, Maharashtra, India), 1.4 mM dNTPs mixture (New England Biolabs, Beverly, Massachusetts, USA), 3.41 pmol *Bst* DNA

polymerase (homemade), and 1 μL of 30–50 ng DNA template was used. The reaction mixture was incubated at 65°C for 60 minutes. The reaction products were examined visually for a color change in phenol red. A positive result was indicated by a color shift from orange to yellow due to the pH change. Additionally, electrophoresis of the product on a 2% agarose gel stained with a 1:20,000 dilution of RedSafe™ (iNtRON Biotechnology, WA, USA) was performed to confirm amplification.

3.2.6 Lyophilization process

To develop the date palm sex identification kit, the conditions for the LAMP reaction were first optimized, and ready-made solution sets were designed. Prior to the lyophilization process, the buffer solution (containing 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, and 0.125 mM phenol red) was separated from the master mix. This buffer solution was stored at 4°C and later added to reconstitute the reaction with the DNA template. The master mix used for lyophilization consisted of 1.6 mM of each FIP and BIP primer, 0.8 mM of each LF and LB primer, 0.2 mM of each F3 and B3 primer, 1.4 mM dNTPs mixture, and 3.41 pmol *Bst* DNA polymerase (homemade). Sugars were incorporated into the master mix to maintain reaction stability during freeze-drying. Two sugars, 5% D(+)-trehalose dihydrate (ACROS Organics) and sucrose (KemAus™, New South Wales, Australia), were tested for their effectiveness in preserving the reaction components (Jovanović et al., 2006; Kommineni et al., 2022; Zhao et al., 2022). The prepared LAMP master mix was then subjected to a freeze-drying (lyophilization) process. The reaction solution was first prepared and cooled below the freezing point in -80°C freezer (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA) before being placed in a freeze dryer (CHRIST®, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The condenser temperature was lowered to -85°C, and a vacuum of 0.050 mbar was applied. Once the samples were completely dried, they were removed from the freeze dryer.

3.2.7 Testing of lyophilized product

Each lyophilized reagent tube was resuspended with 24.0 µL of buffer, prepared as described in 3.6. After resuspension, 1 µL of 30–50 ng DNA template was added to each tube to initiate the LAMP reaction. The reaction mixture was then incubated at 65°C for 60 minutes to facilitate DNA amplification. The reaction products were examined visually for a color change of phenol red. A positive result was indicated by a color shift from orange to yellow due to the pH change.

3.2.8 Stabilized Freeze-Dried LAMP

The freeze-dried LAMP master mix was tested for stability at 25°C, 4°C, and -20°C over a period of six months. Evaluations were conducted at 7, 14, 21, and 28 days, as well as at 2 and 6 months to assess its performance. The testing procedure followed the method described in 3.7. Additionally, electrophoresis of the amplification product was performed on a 2% agarose gel stained with a 1:20,000 dilution of RedSafe™ to confirm successful DNA amplification.

3.2.9 Properties of Date Palm sex identification kits

Each lyophilized reagent tube was resuspended with 24.0 µL of LAMP buffer and thoroughly mixed using a portable vortex mixer. Then, 1 µL of DNA template (30–50 ng) was added to initiate the LAMP reaction. The reaction was conducted in a small, thermal block at 65°C for 60 minutes, making it suitable for on-site testing.

To facilitate field deployment, the results were evaluated using simple and rapid detection methods. The LAMP reaction mixture contained phenol red, enabling direct visual detection by the naked eye. A positive reaction was indicated by a color change from orange to yellow, confirming successful DNA amplification.

CHAPTER IV
RESULT AND DISCUSSION

4.1 Cannabis

4.1.1 Samples

Thirty cannabis leaf samples of known gender were obtained from the Suranaree University of Technology cannabis Farm (SUT cannabis) in Nakhon Ratchasima (14.871121, 102.025668) **(Figure 4.1)**. The collected samples were stored at -20°C until further analysis.

Figure 4.1 Collection of cannabis leaf samples with known sex from SUT Cannabis for molecular analysis

4.1.2 Comparison of MADC2 and SCAR119 markers

Genomic DNA of thirty cannabis leaf samples from the SUT cannabis farm were extracted. The MADC2 and SCAR119 primers were used in the PCR reaction using total genomic DNA from five selected samples as template. In all-male plants, the MADC2 primers amplification result showed a single DNA band of about 390 bp. In female and monoecious plants showed two bands of about 560 bp and 870 bp, **(Figure 4.2 (A))**. The SCAR119 primers amplification results showed a single DNA band

of 119 bp in only male plants. No bands were seen in female or monoecious plants **(Figure 4.2 (B))**.

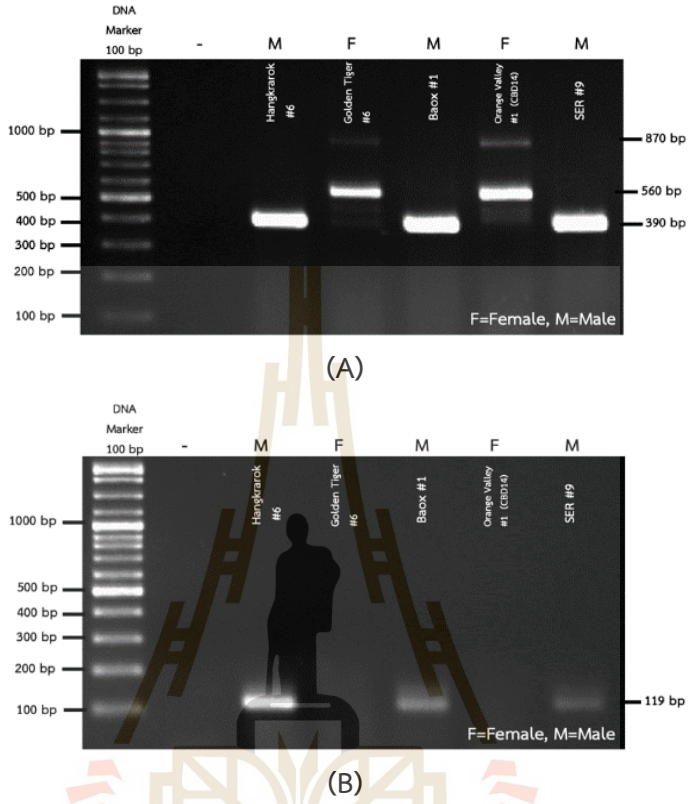

Figure 4.2 Visualization of PCR products for sex determination of cannabis by MADC2 (A) and SCAR119 (B) primers.

The sex determination results using the SCAR119 marker normally agree with those obtained using the MADC2 marker in males. This finding aligns with the research by Faux et al. (2014), which showed that male plants produce a single band of 119 bp. However, since no band is visible in female plant, it is difficult to determine if the absence of a band is the result of a false negative test or whether the plants really are female. Consequently, we chose to apply the MADC2 primers to amplify novel cannabis varieties developed at SUT. These primers provide a more distinct and reliable differentiation between male and female plants, which increases the accuracy of sex determination.

4.1.3 MADC2 marker amplification products

Genomic DNA was extracted from 30 cannabis leaf samples collected from SUT cannabis farm, Suranaree University of Technology. These samples included both established and newly developed cultivars that are now widely cultivated in Thailand due to their high yield and favorable agronomic traits. The extracted DNA was amplified using MADC2-specific primers **(Figure 4.3)**.

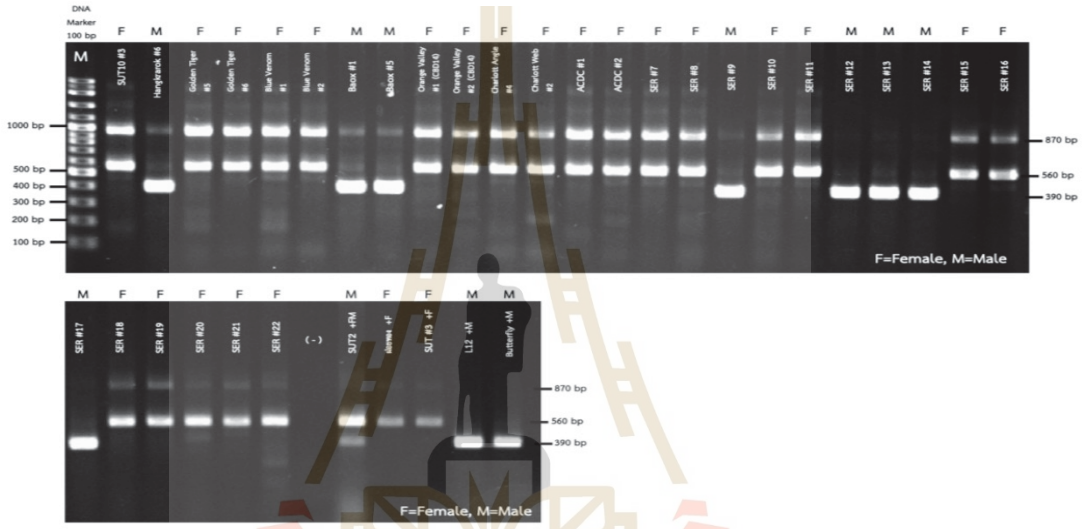

Figure 4.3 Visualization of PCR products for sex determination of cannabis by MADC2 primer

According to Mandolino et al. (1999), MADC2 is a genetic marker that can be amplified by polymerase chain reaction (PCR) to produce a 390 base pair (bp) amplicon from male cannabis DNA. This amplicon is associated with the presence of male phenotypic characteristics in cannabis plants. In this study, the sex of each sample was already known prior to PCR analysis. The PCR results confirmed the accuracy of MADC2 as a sex-linked marker. Samples identified as male showed the expected 390 bp amplicon, while female samples did not. These results were consistent with the known sex of the plants, confirming the reliability of the MADC2 marker for sex identification in Thai cannabis cultivars. These findings were further supported by previous studies, such as those by Techen et al. (2010), who demonstrated the effectiveness of multiple MADC markers in accurately identifying female cannabis plants.

Female cannabis plants are preferred for medicinal and industrial applications because they produce higher concentrations of secondary metabolites, such as cannabinoids, terpenes, and flavonoids. These compounds have various therapeutic and recreational properties (Torres et al., 2022). Therefore, the early identification and selective cultivation of female plants are crucial for maximizing the yield of these valuable compounds.

Previous research has highlighted the importance of early sex determination in cannabis breeding and cultivation. Male plants are often removed from cultivation to prevent pollination and preserve the quality and yield of female flowers (Hermsmeier et al., 2004). Identifying plant sex at an early stage allows for more efficient resource allocation and improved crop management (van Bakel et al., 2011).

The MADC2 genetic marker is a reliable tool for sex determination in Thai cannabis cultivars. Its use aligns with previous research findings and supports the development of more efficient cannabis breeding programs. The application of molecular techniques such as MADC2-based PCR enhances cultivation practices and contributes to the production of high-quality cannabis plants with desirable traits.

4.1.4 Simple and Rapid DNA Extraction

Five miligram of 12 cannabis leaf samples were randomly selected from a total of thirty samples collected from SUT cannabis cultivars. Genomic DNA was extracted using a simple and rapid protocol **(Figure 4.4)**. The extracted DNA was then subjected to PCR amplification using MADC2-specific primers. The amplification results revealed DNA bands consistent with the known sex of each sample **(Figure 4.5)**, thereby confirming the effectiveness of the method in sex identification. Although this simplified extraction technique is fast and easy to perform, it produces DNA of relatively low purity. In several male cannabis samples, three distinct bands 870 bp, 560 bp, and 390 bp were observed. Among these, the 390 bp band serves as the key indicator of male sex. Despite the appearance of non-specific bands, the consistent detection of the 390 bp band supports the reliability of this method for identifying male plants.

The extraction process, including sample grinding, takes approximately 5 to 10 minutes. PCR amplification requires an additional 1.5 to 2 hours, followed by 30 minutes for agarose gel electrophoresis. Compared to conventional extraction techniques, this method is significantly more cost-effective, requiring only three reagents (NaOH, HCl, and Tris-HCl) instead of the 10–15 chemicals used in the method described by Bellstedt et al. (2010). This extraction protocol demonstrated superior performance over other quick methods for plant DNA isolation in terms of success rate, cost efficiency, and processing speed. Notably, this method does not require centrifugation to remove particulate matter, yet it still yields DNA suitable for PCR analysis. Due to its simplicity, low cost, and minimal equipment requirements, this method is well-suited for routine DNA extraction in plant molecular biology studies. Its rapid processing time also makes it particularly useful for high-throughput applications and for laboratories with limited access to specialized instruments.

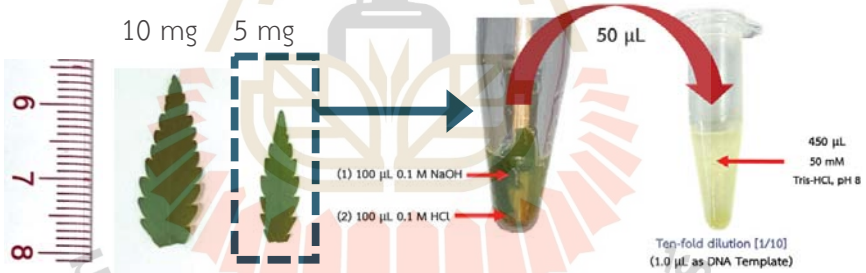

Figure 4.4 Fresh leaf samples from cannabis seedlings along with a ten-fold dilution, prepared used as simple DNA extraction method

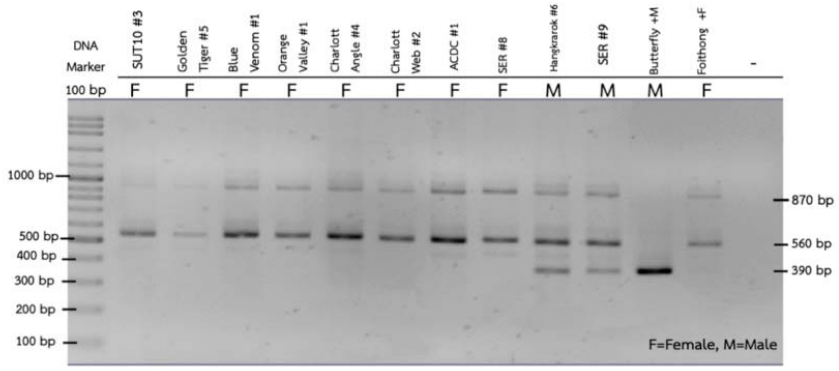

Figure 4.5 Visualization of PCR Products for Sex Determination of Cannabis Using the MADC2 Primers and template from the Rapidly DNA extraction method

4.2 Date Palm

4.2.1 Samples

Samples of young Date Palm (*Phoenix dactylifera* L.) leaves of both known and unknown sex were collected from 13 cultivars: KL1, Yellow Barhee, Red Barhee, Red India, Um ed Dahan, Khoneizi, Shi Shi, Ghannami, Jozi, Braim, G2, H1, and AP-red1. These samples were obtained from BunMak Date Palm in Nakhon Ratchasima (14.67360, 102.39645) and Phutara Green Park in Buriram (14.69488, 102.50448) (**Figure 4.6)**. The collected samples were stored at -20°C until further analysis.

Figure 4.6 Collected sample of young date palm leaves of both known and unknown sex

4.2.2 DNA Extraction

A novel technique for DNA extraction from leaf samples from different date palm cultivars was effectively developed using a modified lysis buffer protocol adapted from Keb-Llanes et al. (2002). This newly created buffer solution successfully replaces traditional hazardous reagents like chloroform, phenol, and liquid nitrogen. The improved formulation contained 100 mM Tris-HCl, 20 mM EDTA, 2% cetyltrimethylammonium bromide (CTAB), 4% polyvinylpyrrolidone (PVP), 20% sodium dodecyl sulfate (SDS), and 10 mM β-mercaptoethanol. These modifications achieved a simplified, more secure, and faster extraction protocol suitable for normal laboratory application.

The DNA extraction process was effectively completed using 20 mg of fresh leaf samples that were hand-mashed in a mortar. The extracted DNA concentrations differed among cultivars, ranging from around 30 to 400 ng/μL, as measured by Nanodrop spectrophotometry (Thermo Scientific, USA) (**Figure 4.7**). The DNA testing demonstrated a consistently high purity, with an average optical density ratio (OD260/280) of 1.95 from all cultivars. This suggests that the DNA is suitable for sensitive downstream applications, primarily PCR-based assays, and that there is minimal protein contamination. These results support other research showing the significance of obtaining an OD260/280 ratio between 1.8 and 2.0 for accurate molecular analysis (Shi et al., 2018; Kumar & Kumar, 2020).

The optimization of the lysis buffer is primarily due to the formation of CTAB and PVP, which have been reported for successfully removing polysaccharides and polyphenolic compounds that are found in date palm leaves. These compounds are known to inhibit enzymatic reactions (Sahu et al., 2012; Healey et al., 2014). The combined use of SDS and β-mercaptoethanol significantly improves cell membrane breakdown and protein denaturation, consequently enhancing DNA purity and yield (Ghaffariyan et al., 2018).

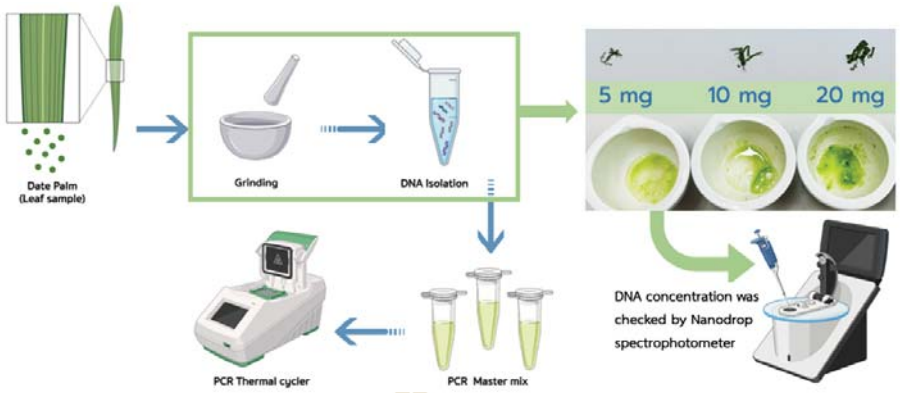

Figure 4.7 DNA extraction process and DNA concentration checking

4.2.3 Specificity Testing of DNA markers for Date Palm sex using PCR

DNA samples were extracted from leaves of various date palm cultivars. The designed primer set specifically targeted male-specific sequences (*Phoenix dactylifera* glyceral-3-phosphate acyltransferase gene, GPAT3) and reference sequence (lipoxygenase gene (LOX5.1)). PCR amplification of GPAT3 revealed successful gender identification. As illustrated in (**Figure 4.8**), a single band of 156 bp in the DNA sample indicated female date palms. In contrast, the presence of two bands of 450 bp and 156 bp, confirmed male samples. These results demonstrated the specificity of the newly designed primers for accurate sex determination in date palms using multiplex PCR.

The results presented here strongly agree with previous studies using PCR-based markers for determining sex in date palm, demonstrating their effectiveness and accuracy (Jani et al., 2022; Torres et al., 2018). Precise identification of gender via molecular markers through early young stages provides significant advantages in practice by enhancing the efficacy of breeding processes and selection methodologies.

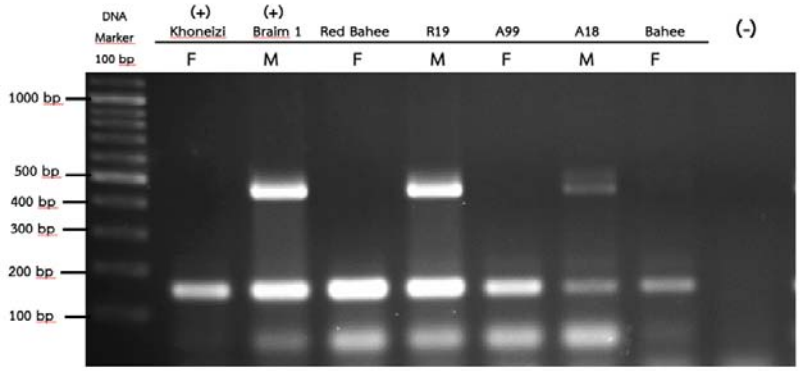

Figure 4.8 Sex verification of Date Palm seedlings using multiplex PCR. DNA samples extracted from seedlings of various cultivars were amplified with primers specific to male date palm sequences. The presence of a single band at 156 bp indicates a female sample. The presence of two bands at 450 bp and 156 bp confirms a male sample.

4.2.4 Development of LAMP technique for determining the sex of date palm LAMP primer design

PCR products from 4.2.3 were successfully purified with a traditional PCR purification kit to remove remaining primers, nucleotides, and other contaminants that may interfere with future sequencing. High-quality data from sequencing was important in precisely identifying the nucleotide sequences of the target regions of the 13 date palm varieties. The quality analysis of the raw sequenced reads was performed using FastQC, confirming the data's accuracy and completeness. The sequences were aligned with ClustalW via MEGA software to allow a comparison between different cultivars. The multiple sequence alignment assisted in the identification of conserved and polymorphic regions, offering significant insight into sequence variation. These findings were important for the subsequent development of LAMP primers. Applying the NEB® LAMP Primer Design Tool (version 1.4.2), a total of three sets of primers were successfully created, including (each set):

1) Forward inner primer (FIP) and Backward inner primer (BIP)

2) Forward outer primer (F3) and Backward outer primer (B3)

3) Forward Loop primer (LF) and Backward Loop primer (LB)

To assure optimal primer efficiency in the specifics of LAMP primer design, primer length, GC content, and temperature conditions were optimized.

4.2.5 LAMP Reaction and Detection

4.2.5.1 Commercial set Testing (10X *Bsm* buffer)

The loop-mediated isothermal amplification (LAMP) methods, performed with a commercial buffer set (10X *Bsm* buffer), demonstrated successful DNA amplification via observation and gel electrophoresis. Significant turbidity was observed in the positive reactions (+M, +F) for the date palm reference gene primers, which can be separated from the clear negative control (-) (**Figure 4.9** (A)). Consequently, the male-specific primers for date palms showed significant turbidity in the positive response (+M), while the negative control (-) and the female reaction (+F) remained clear (**Figure 4.9** (B)). The observed turbidity results from magnesium pyrophosphate, a by-product of DNA synthesis, resulting in showing effective amplified. The turbidity-based detection principle was originally proposed by Mori et al. (2001) as a reaction to the founding LAMP technique developed by Notomi et al. (2000).

Electrophoretic analysis confirmed the visual results, showing unique ladder-like bands for the positive LAMP reactions. The observed bands, which referred to the predicted LAMP amplification profiles resulting from the method introduced by Notomi et al. (2000) and were visualized in a variety of protocols, including those that used alternative detection methods such as calcein, were described by Tomita et al. (2008). The bands ranged from approximately 100 bp to very high molecular weight DNA. The negative control showed absent amplification bands, consequently confirming the assay's specificity and lack of contamination.

The unique mechanism of LAMP is defined by the different banding patterns, which are formed by the anticycling displacement of strands of DNA synthesis driven by *Bst* DNA polymerase, which produces combined products of different lengths. This method was further clarified in research targeting LAMP's application and detection (e.g., Wong et al., 2018) and improved via loop primers developed by Nagamine et al. (2001/2002).

Optimal ionic and buffering conditions, such as those possibly offered by buffers like ThermoPol® Reaction Buffer (compatible with Bst DNA Polymerase, Large Fragment), are crucial for achieving high specificity and yield by maintaining the enzyme's stability and activity. Multiple investigations confirm the overall accuracy and sensitivity of LAMP assays, supporting their use for fast diagnoses, particularly in environments with limited resources.

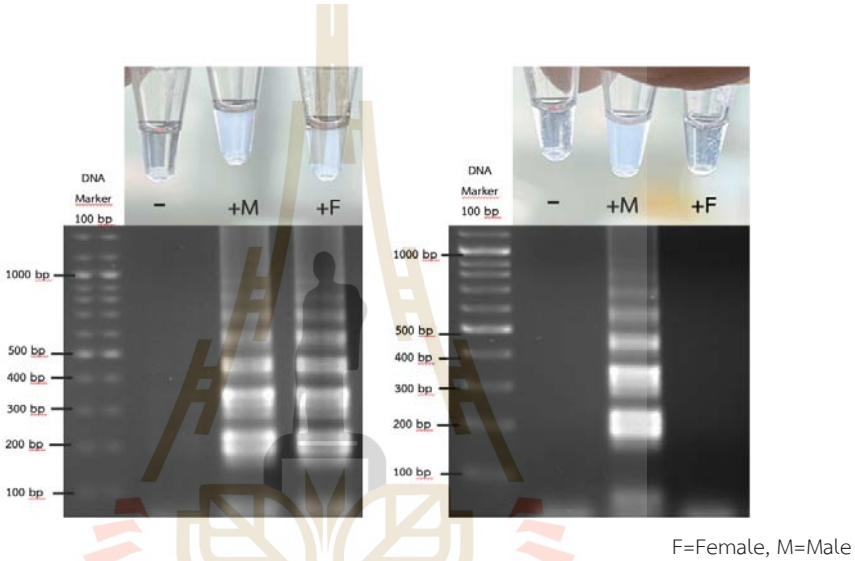

Figure 4.9. LAMP-based Sex Determination of Date Palm using Commercial Buffer Set: **(A)** Amplification with date palm reference gene primers and **(B)** Amplification with male-specific primers for date palm. Positive reactions were identified visually by turbidity and confirmed by gel electrophoresis, which showed characteristic ladder-like patterns indicating successful amplification.

4.2.5.2 2X LAMP with phenol red variation

This study successfully implemented and evaluated a Loop-Mediated Isothermal Amplification (LAMP) assay tailored for the visual detection of target DNA sequences using phenol red as a pH indicator. The assay utilized a custom-prepared 2X LAMP buffer and involved incubation at a constant temperature of 62°C for 60 minutes. Both male-specific and

reference primer sets were employed to assess the assay's performance and specificity, with representative results depicted in **Figure 4.10**.

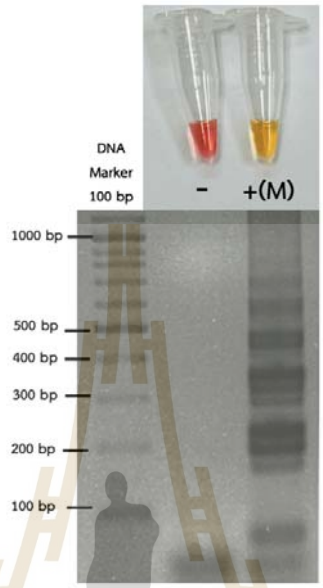

Figure 4.10 Illustrates the colorimetric results of the LAMP reaction following incubation and agarose gel electrophoresis (2%) for male-specific primers of date palm

A key finding, clearly illustrated in **Figure 4.10** (Top), was the effective use of phenol red for real-time, visual monitoring of the amplification process. In reactions where the target DNA (e.g., male-specific target, +M) was present and amplified successfully, a distinct color transition from the initial red/pink of the phenol red indicator to a clear yellow was observed. Conversely, the negative control reaction (-) retained its original red/pink color. This visual change served as a direct, positive indication of amplification. This phenomenon occurs because the extensive incorporation of dNTPs during LAMP by the Bsm DNA polymerase releases a significant quantity of protons (H^+), thereby lowering the pH of the reaction mixture (Tanner et al., 2015; Oscorbin et al., 2017). Phenol red, sensitive to this pH drop, changes color from red/pink (neutral/alkaline) to yellow (acidic, typically < pH 6.8), providing a visual cue for positive reactions (Goto et al., 2009; Tanner et al., 2015). The final concentration of 0.125 mM phenol red

(as used in the experiments shown in **Figure 4.10**) proved optimal for clear visualization within the 60-minute timeframe. The simplicity of this visual detection strategy represents a major advantage for point-of-care diagnostics or resource-limited settings, bypassing the need for complex instrumentation (Kamala et al., 2018; Bhadra et al., 2020).

To corroborate the visual results and confirm the generation of specific LAMP amplification products, all reaction samples were subsequently analyzed using standard agarose gel electrophoresis, as shown directly below the corresponding tubes in Figure 4.10. A strong correlation was observed: the positive sample (+M) that turned yellow (**Figure 4.10**, bottom) displayed the characteristic ladder-like/smeared banding pattern indicative of successful LAMP amplification when resolved on the gel. This pattern results from the complex mixture of stem-loop DNA structures of varying lengths (concatemers) generated during the LAMP process (Notomi et al., 2015; Zhang et al., 2020). In contrast, the negative control (-) that remained red/pink showed no such banding pattern, exhibiting only low molecular weight bands likely corresponding to unused primers or primer-dimers. This congruence confirms the reliability of the phenol red visual detection method for this assay.

Furthermore, the assay's robustness was explored by varying both the initial buffer pH and the Phenol Red concentration, as shown in **Figure 4.11**. Experiments conducted at initial pH values of 7.8 and 8.0, using final Phenol Red concentrations of both 0.125 mM and 0.250 mM, consistently yielded successful results. In all tested positive reactions (+M), a clear red-to-yellow color change was observed, corresponding perfectly with the characteristic LAMP product ladder seen on agarose gel electrophoresis. Negative controls (-) remained red and showed no amplification products on the gel under all conditions. This confirms that the LAMP reaction and the visual detection system function effectively across this range of pH and indicator concentrations, reinforcing the assay's reliability.

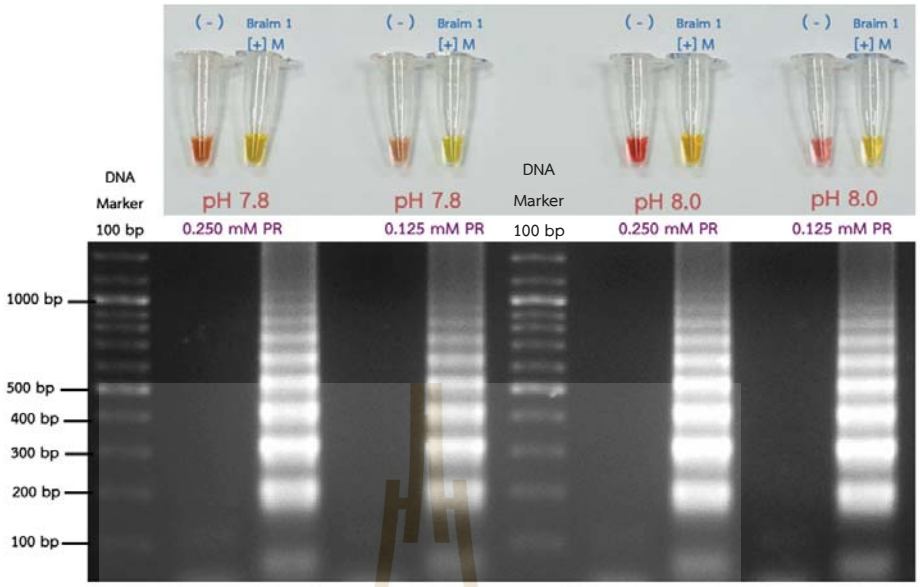

Figure 4.11 Effect of initial at pH 7.8 and 8.0 and varying Phenol Red concentration (0.125 mM and 0.250 mM) on LAMP amplification and visual detection.

The specificity of the primer sets was critically evaluated and is clearly demonstrated in **Figure 4.12**. When using a reference primer set targeting a sequence common to both sexes (**Figure 4.12**, A), both the male (M) and female (F) samples yielded positive results. Both tubes turned yellow and showed strong, characteristic LAMP amplification patterns on the agarose gel, confirming that DNA from both sample types was amplifiable under the assay conditions. In contrast, when the male-specific primer set was used (**Figure 4.12**, B), only the male (M) sample produced a positive result (yellow tube, distinct LAMP ladder/smear on agarose gel). The Female (F) sample, like the negative control (-), remained red/pink and showed no amplification product on the agarose gel, mirroring the negative control lane. This side-by-side comparison definitively confirms the high specificity of the male-specific primers, which selectively amplify target DNA only from male samples, as intended. This specificity is a hallmark of the LAMP technique, attributable to the multiple primer recognition sites required (Notomi et al., 2015; Fu et al., 2017).

The assay consistently demonstrated efficient amplification under isothermal conditions (62°C), highlighting LAMP's suitability for rapid testing without complex equipment (Becherer et al., 2020). Optimized reaction components, including appropriate salt concentrations and the addition of betaine, likely contributed to the robust performance and specificity observed across the experiments shown in Figures 4.10, 4.11 and 4.12 (Wang et al., 2017; Oscorbin et al., 2017; Bhadra et al., 2020).

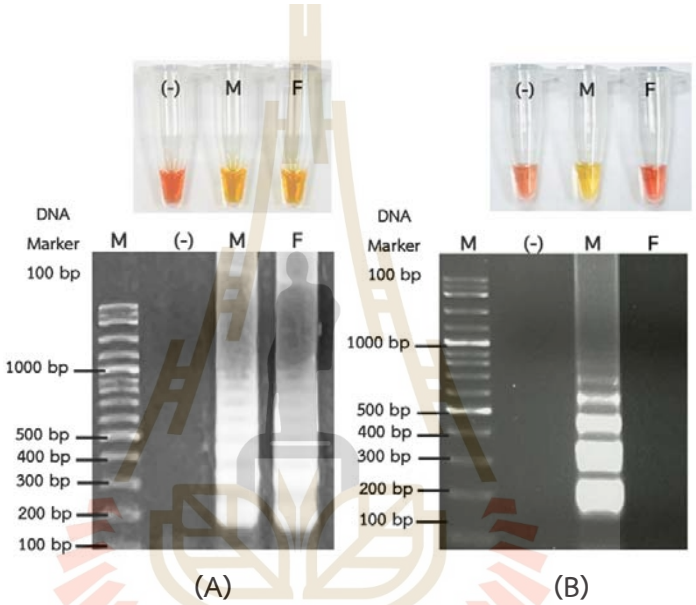

Figure 4.12 Illustrates the colorimetric results of the LAMP reaction following incubation and agarose gel electrophoresis (2%) for both reference gene primers (A) and male-specific primers (B) of date palm

4.2.5.3 Performance Evaluation of Homemade *Bst* DNA polymerase

The experimental results presented in the preceding sections (**Figures 4.9, 4.10, 4.11 and 4.12**) utilized commercially available DNA polymerase enzymes and buffers. However, to facilitate potential commercialization and practical field application of this LAMP assay, cost reduction is crucial, particularly concerning enzyme expenses. Therefore, a homemade *Bst* DNA polymerase large fragment was produced and purified, adapting the methodology described by Paik et al. (2021).

The resulting homemade *Bst* polymerase yielded a concentration of 3.41 pmol/µL. Its performance in the LAMP reaction was subsequently evaluated using 1 µL of the enzyme equivalent to 3.41 pmol per reaction. The results are presented in **Figure 4.13**, which compares the efficiency of the homemade enzyme when used with either a standard commercial buffer (10x buffer (Commercial)) or the custom-prepared 2x buffer containing phenol red (2x buffer with phenol red), assessed at incubation times of 45 and 60 minutes.

Visual inspection (**Figure 4.13, A**) revealed that positive reactions (+M) successfully transitioned from red to yellow at both 45 and 60 minutes, with the yellow color appearing more distinct and intense after 60 minutes. Negative control reactions (-) remained red. These visual results were corroborated by agarose gel electrophoresis (**Figure 4.13, B**). Clear ladder-like patterns, characteristic of successful LAMP amplification, were observed in all positive (+M) lanes for both buffer systems and at both time points. Notably, the amount of amplified product appeared substantially greater at 60 minutes compared to 45 minutes. No amplification products were detected in any negative control (-) lanes.

These findings confirm that the homemade *Bst* polymerase, at a concentration of 3.41 pmol/reaction, functions effectively in the LAMP assay. It is compatible with the developed 2X Phenol Red buffer and provides clear results both visually and by agarose gel electrophoresis within a 60-minute timeframe, comparable to the performance observed with commercial enzymes.

This outcome is highly significant as it demonstrates the feasibility of employing locally produced, cost-effective *Bst* DNA polymerase for this LAMP assay without compromising performance. This substantially enhances the potential for developing this assay into a practical tool for field use or commercial applications.

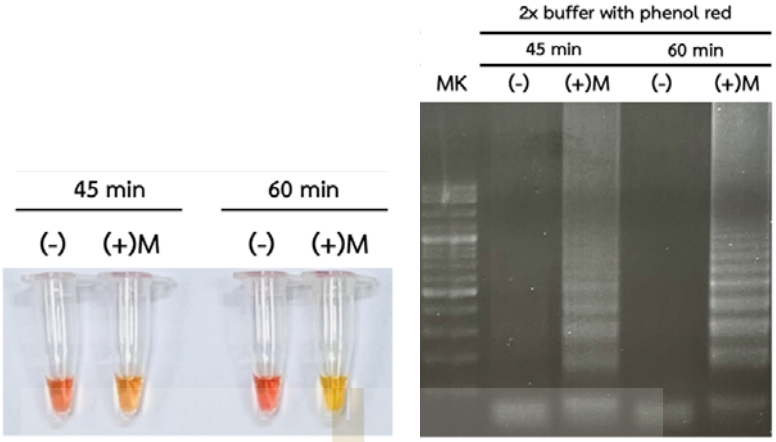

Figure 4.13 Performance evaluation of homemade *Bst* DNA polymerase (3.41 pmol/reaction) in the LAMP assay. **(A)** Colorimetric detection using Phenol Red at 45 and 60 minutes. **(B)** Agarose gel electrophoresis comparing results in commercial 10x buffer and custom 2X Phenol Red buffer at 45 and 60 minutes.

4.2.6 Lyophilization process and testing of lyophilized product

The primary goal of this study was to develop a stable and user-friendly Loop-Mediated Isothermal Amplification (LAMP) kit for the identification of date palm sex, suitable for field applications. A key strategy to enhance stability and eliminate the need for cold-chain storage is lyophilization (freeze-drying) of the reaction components. Lyophilization is a widely adopted strategy not only for molecular diagnostics but also for preserving the stability and extending the shelf life of various complex biological products, including those developed through immunoengineering (Kommineni et al., 2022; Li et al., 2022; Mahmud et al., 2023). This process requires careful optimization, including the use of cryoprotectants to preserve the activity of sensitive reagents like DNA polymerase during the freeze-drying process and subsequent storage (Ahmed et al., 2021; Beattie & Muldrew, 2020).

Initially, the compatibility of potential cryoprotectants with the LAMP reaction chemistry was assessed prior to lyophilization. As per the methodology, the master mix contained essential LAMP reagents, including primers, dNTPs, and *Bst* DNA polymerase. We evaluated the effect of adding either 5% sucrose or 5% D(+)-trehalose dihydrate to this master mix. **Figure 4.14** compares the performance of the LAMP

reaction with these sugars. The results indicated that 5% sucrose can not be used however adding 5% trehalose prior lyophilization can successfully use. In each case, the reaction mixture turned yellow in the presence of male DNA (+M), signifying positive amplification, while remaining red (indicating negative results) with female DNA (+F) and in the no-template control (-). This confirmed that neither 5% sucrose nor 5% trehalose at 5% concentration inhibited the LAMP reaction or interfered with the phenol red indicator system before the lyophilization step. Both sugars are commonly used as lyoprotectants, with trehalose often noted for its superior ability to stabilize biological structures during dehydration (Crowe & Crowe, 2019; Richards et al., 2021). The successful performance of the reaction with these sugars underscores the critical role of lyoprotectants in maintaining the structural and functional integrity of key biological components, such as the polymerase enzyme, during the stresses of freezing and dehydration (Ahmed et al., 2021; Kommineni et al., 2022).

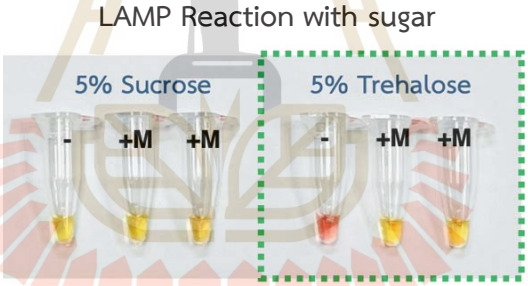

Figure 4.14 Compares the LAMP reaction with 5% sucrose and trehalose and the reaction without added sugars.

Next, we prepared the LAMP master mix with one of the protective sugars and freeze-dried it according to our method. We kept the liquid buffer separate (stored at 4°C). The master mix was frozen at -80°C and then dried under vacuum in the freeze dryer (condenser at -85°C, vacuum 0.050 mbar). To see if the freeze-drying worked, we compared the test results right before and immediately after freeze-drying (Day 0), as shown in **Figure 4.15**. Before freeze-drying, the test correctly showed yellow for male DNA and red for female no DNA. Importantly, after freeze-drying and adding back the buffer and DNA, the test gave the exact same correct results.

Following this compatibility check, the complete LAMP master mix, formulated with an appropriate cryoprotectant (based on the results shown in **Figure 4.14**), was subjected to the lyophilization process as described. This involved separating the buffer solution (which was stored at 4°C), freezing the master mix at -80°C, and subsequently drying it in a freeze dryer under controlled conditions (condenser at -85°C, vacuum at 0.050 mbar). The effectiveness of this process was evaluated by comparing the LAMP reaction performance immediately before and after lyophilization (Day 0), as shown in **Figure 4.15**. The pre-lyophilization reaction (using 2X buffer concentration relative to the final reaction volume) clearly showed the expected colorimetric results: yellow for male DNA and red for female DNA and the negative control. Crucially, upon reconstitution of the dried pellet with the 1X reaction buffer and addition of the respective DNA templates, the post-lyophilization reaction yielded identical results. The tubes containing male DNA turned yellow, while those with female DNA or no template remained red.

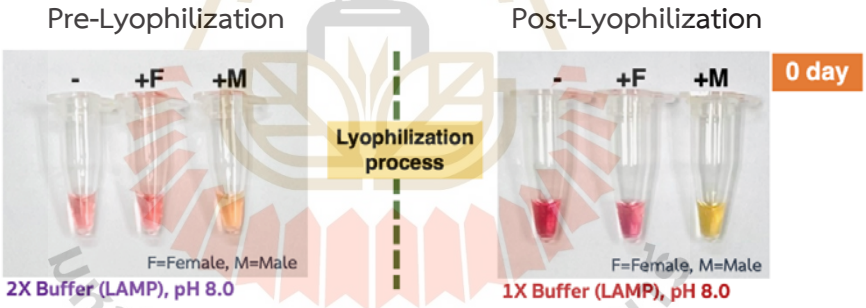

Figure 4.15 The results of the LAMP reaction before and after lyophilization

To further confirm the reliability and specificity of the developed LAMP assay, we tested it on multiple individual date palm samples. **Figure 4.16** shows the results of testing 20 different samples (alternating male M and female F, lanes 1-20), along with positive controls (+F, +M) and a negative control (-). Looking at the bottom gel, which shows the LAMP products, a clear ladder-like pattern indicating successful amplification is visible in all lanes corresponding to male samples.

The visual results in the reaction tubes, shown between the top and bottom gels in **Figure 4.16**, matched the gel results perfectly. All male samples resulted in a

yellow color, indicating a positive reaction, while all female samples and the negative control remained red or orange, indicating a negative reaction. These results demonstrate that the LAMP assay is highly specific for male date palm DNA and performs reliably across different individual plant samples, confirming the accuracy of the simple visual color readout.

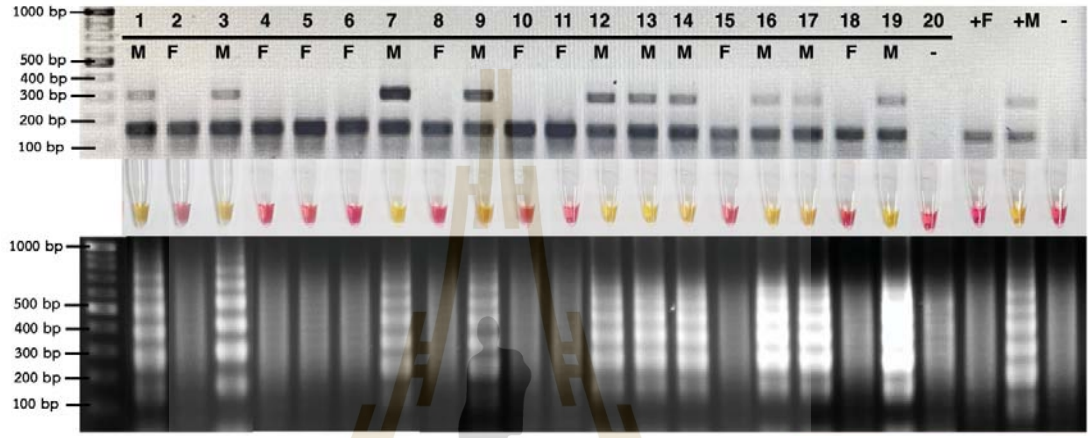

Figure 4.16 Testing of the date palm sex identification assay on multiple individual samples.

This result demonstrates that the selected lyophilization protocol, including the separation of the buffer and the incorporation of a cryoprotectant sugar, successfully preserved the functional integrity of the LAMP reagents, including the Bst DNA polymerase and primers. Achieving this successful preservation validates the chosen protocol, as improper lyophilization can lead to loss of activity due to factors like ice crystal damage or residual moisture, challenges that are actively addressed in the preservation of sensitive biologics (Kommineni et al., 2022; Silva et al., 2022). The ability to maintain enzyme activity and reaction specificity after freeze-drying is essential for creating a reliable point-of-need diagnostic tool (Carter & Weitz, 2023; Kaminski et al., 2022). The development of such a lyophilized kit offers significant advantages for date palm cultivation. It eliminates the dependence on cold-chain transport and storage, facilitating distribution and use in diverse field conditions, including resource-limited settings where date palms are economically important (El-Hadrami & Al-Khayri, 2020; Jia et al., 2021). Accurate and early sex determination allows

growers to manage their plantations more efficiently by identifying and removing excess male seedlings, optimizing resource allocation, and potentially increasing overall yield (Al-Mssallem et al., 2019; Hazzouri et al., 2020). The ready-to-use format, requiring only the addition of the separately stored buffer and the sample DNA, simplifies the workflow for the user (Lau & Botella, 2021; Silva et al., 2022).

4.2.7 Stabilized Freeze-Dried LAMP

The stability of the developed freeze-dried LAMP assay for date palm sex identification was evaluated over time at various storage temperatures (-20°C, 4°C, and room temperature (RT)). The results, assessed at 7, 14, 21, 28 days, 2 months and up to 6 months, demonstrate the robustness and reliability of the lyophilized kit, particularly for its intended visual detection method.

Across all tested time points and storage conditions shown (7, 14, 21, 28 days, 2 months and 6 months), the visual assay using phenol red indicator provided consistent and accurate results. The reaction tubes containing male DNA template (+M) consistently turned yellow, indicating successful amplification and the resulting pH drop, while tubes with no template (-) or female DNA template (+F) remained orange/pink, correctly indicating a negative result (**Figure 4.17**). This demonstrates that the core reaction components, including the *Bst* polymerase and primers, remained sufficiently active after lyophilization and storage. The stability, especially the functionality observed after storage at room temperature for 21 days, highlights the effectiveness of incorporating cryoprotectant sugars (trehalose), which are known to preserve enzymatic activity and reagent integrity during freeze-drying and subsequent storage (Jovanović et al., 2006; Hardinge & Murray, 2019; Kaminski et al., 2021).

Gel electrophoresis analysis was performed to further confirm the amplification results. In agreement with the visual assay, strong, characteristic ladder-like banding patterns, typical of positive LAMP reactions (Notomi et al., 2000), were consistently observed in the male DNA lanes (+M) across all storage conditions and time points depicted (**Figure 4.17**). This confirms specific and efficient amplification of the target male sequence. The stability assessment indicated reliable performance for

at least 21 days even at RT, with optimal stability expected at lower temperatures (-20°C, 4°C) for long-term storage (up to 6 months as tested per methods).

An important observation from **Figure 4.17, 4.18 and 4.19** are that the incubation time for the test was increased. At 7 days, the test was run for 60 minutes. However, at 14 days to 6 months, the time was increased to 70 minutes. This change likely happened because the chemicals in the kit might start to work slightly slower after being stored for two weeks or more, especially if kept at room temperature. Even with protective sugars, the activity of the copying enzyme (*Bst* polymerase) can decrease a little over time. By adding 10 extra minutes, the test ensures that even slightly slower reactions have enough time to copy plenty of DNA. This helps make sure the color change from orange/pink to yellow is clear and easy to see for the male samples, keeping the test reliable as the kit gets older.

Interestingly, faint bands were observed in both the negative control (-) lanes (containing no DNA template) and the female DNA (+F) lanes on the agarose gels across the different storage conditions and time points. The presence of bands in the negative control (-) suggests potential low-level non-specific amplification. LAMP's isothermal nature and use of multiple primers can sometimes lead to primer-primer interactions (primer-dimers) or spurious amplification, especially in the absence of a specific target or during prolonged incubation (Gadkar et al., 2018; Tanner et al., 2015). While efforts are made to optimize primer design and reaction conditions to minimize this, faint background amplification can occasionally be detected by sensitive methods like gel electrophoresis (Becherer et al., 2020).

The similar faint banding pattern observed in the female DNA (+F) lanes could arise from the same non-specific amplification mechanisms (e.g., primer-dimers). Alternatively, it might indicate very low-level non-specific binding of the primers to partially complementary sequences within the female date palm genome under the assay conditions (65°C). Given that the LAMP primers were presumably designed against male-specific sequences, significant amplification from female DNA is not expected, and the observed bands are indeed much fainter than the male positive control.

Crucially, this low-level non-specific amplification detected by gel electrophoresis did *not* generate sufficient proton release (a byproduct of nucleotide

incorporation) to cause the significant pH drop required to change the phenol red indicator's color (Tanner et al., 2015). Visual detection methods based on pH change inherently have a higher threshold of detection compared to the endpoint visualization on an agarose gel (Zhang et al., 2020; Becherer et al., 2020). This difference in sensitivity explains why the visual assay remained accurate and did not produce false-positive results for the (-) and (F) samples. Therefore, while gel analysis reveals minor non-specific products, the visual assay is robust for its intended purpose of distinguishing male from female date palms using this stable, lyophilized kit.

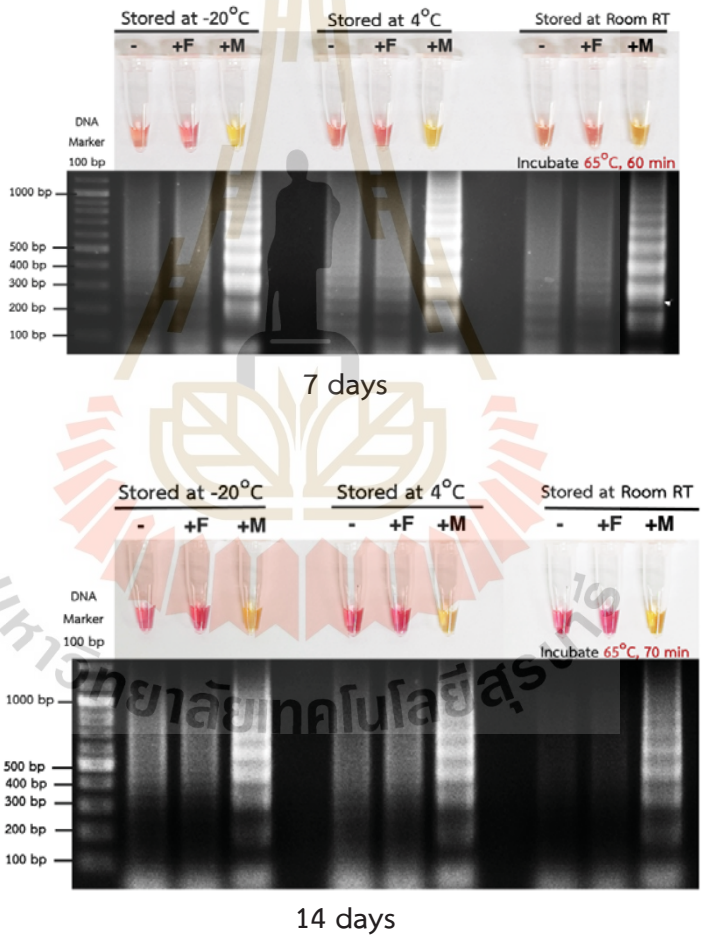

Figure 4.17 Shows the results of testing the freeze-dried date palm sex identification kit after storing it at different temperatures (-20°C, 4°C, and Room Temperature) for 7 and 14 days.

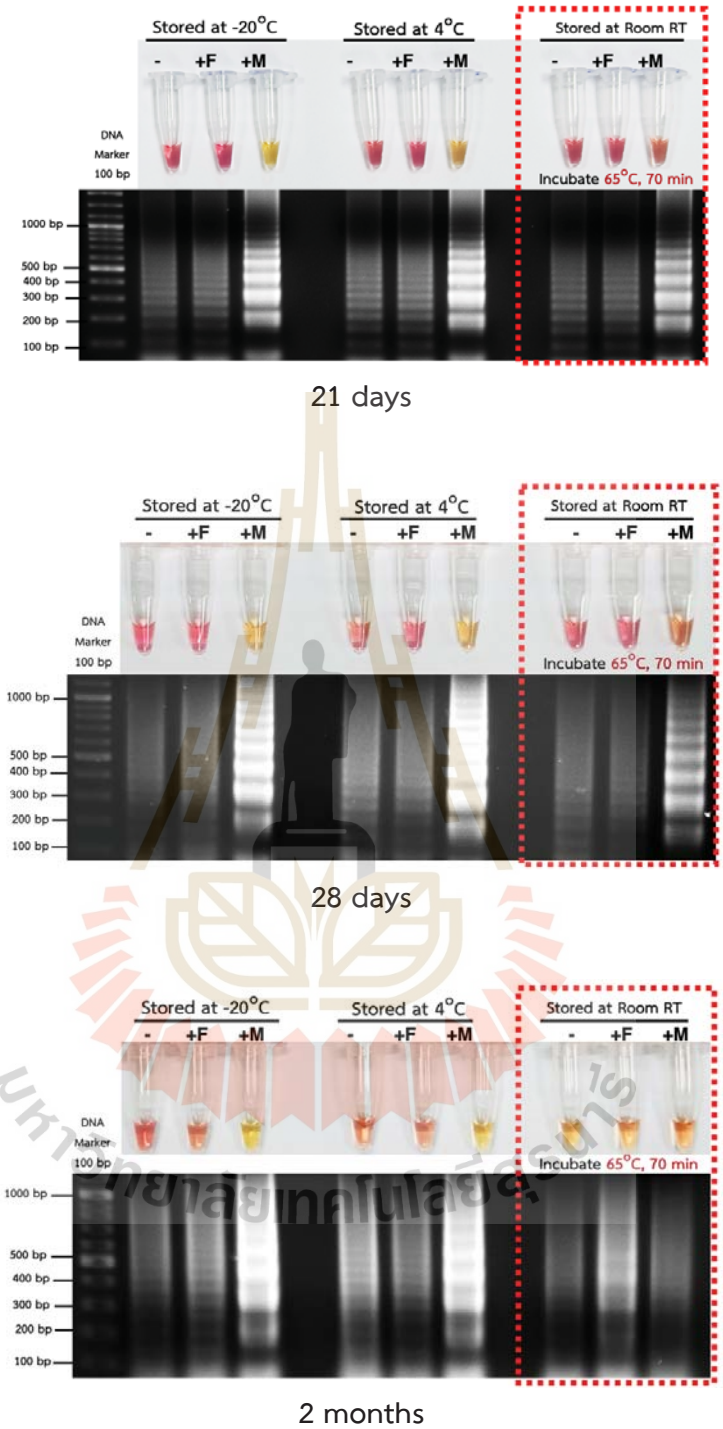

Figure 4.18 Shows the results of testing the freeze-dried date palm sex identification kit after storing it at different temperatures (-20°C, 4°C, and Room Temperature) for 21, 28 day and 2 months

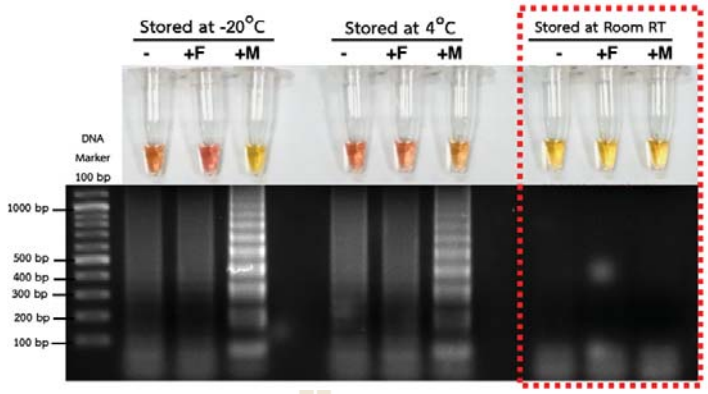

Figure 4.19 Shows the results of testing the freeze-dried date palm sex identification kit after storing it at different temperatures (-20°C, 4°C, and Room Temperature) for 6 month

4.2.8 Properties of Date Palm sex identification kits

The results demonstrate the successful development and functioning of a simple, visually detectable LAMP assay for date palm sex identification suitable for on-site applications (**Figure 4.20**). The key advantage highlighted is the incorporation of phenol red, which facilitates a straightforward, naked-eye interpretation of the results (orange to yellow color change upon positive amplification). This eliminates the need for complex and often expensive laboratory equipment, such as gel electrophoresis apparatus or real-time PCR machines, for result detection.

Furthermore, the assay's ability to operate at a single, constant temperature (65°C) using a basic thermal block, combined with a relatively rapid reaction time (60 minutes), underscores its suitability for field deployment. This portability and simplicity allow for potentially rapid sex determination of date palm samples directly at the point of cultivation or collection, which significantly benefits breeding programs and commercial cultivation by enabling early identification and selection of desired plants.

In recognition of its innovation and potential impact in agricultural biotechnology, the MANDate date palm gender test kit was presented at the Kaohsiung International Invention and Design EXPO (KIDE), held in Kaohsiung, Taiwan (**Figure 4.21**). The kit earned international acclaim by winning two prestigious gold awards the ACE

Gold Award and the Gold Medal highlighting the significance and excellence of the innovation in a competitive global forum.

Figure 4.20 Date palm sex determination and fieldwork by using the "Date Palm Sex Determination LAMP Kit"

Figure 4.21 The MANDate date palm gender test kit was honoured with two gold awards at the Kaohsiung International Invention and Design EXPO (KIDE)

CHAPTER V

CONCLUSION

The research validated the efficacy of the MADC2 PCR marker for reliable sex differentiation in Thai cannabis cultivars, with distinct and consistent band patterns enabling early selection of economically valuable female plants. For date palm, a novel Loop-mediated Isothermal Amplification (LAMP) assay was developed and optimized, providing a rapid, field-friendly alternative to conventional sex determination methods. The inclusion of phenol red allowed straightforward, visual detection of male-specific amplification, eliminating the need for sophisticated laboratory equipment. Significantly, this study achieved the development of a stable, lyophilized LAMP kit employing trehalose as a stabilizer. This user-friendly kit demonstrated excellent shelf stability for at least six months -20°C storage facilitating easy deployment in field conditions. Overall, this research makes substantial contributions by providing accessible, reliable, and cost-effective molecular tools for early sex determination in cannabis and date palm cultivation. These innovations empower farmers and breeders to make informed, timely decisions, significantly optimizing resource management, reducing economic risks, and enhancing agricultural sustainability and profitability within Thailand and globally.

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BIOGAPHY

Passakorn Phobphimai was born on October 10, 1997, in Nakhon Ratchasima, Thailand. In 2015, he finished high school at Sungnoen School, Nakhon Ratchasima. In 2021, he graduated with a bachelor's degree from the School of Crop Production Technology at Suranaree University of Technology, Nakhon Ratchasima. He spent 1 year as the research assistant (RA) of Assoc. Prof. Dr. Mariena Ketudat-Cairns in the School of Biotechnology, learning about PCR techniques, methods for DNA extraction, and LAMP techniques. He started his master degree in the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, with the "SUT Graduate Degree Scholarship for Undergraduate Students with Academic Achievement" in 2023.

He presented poster presentations, including:

1) A poster presented at the 35th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2023), Nakhon Ratchasima, Thailand: **Validation of Loop-Mediated Isothermal Amplification for Date Palm (*Phoenix dactylifera* L.) Sex Identification**.

2) The 8th SUT International Colloquium on Agricultural Technology 2025, Nakhon Ratchasima, Thailand: **Advancing Date Palm Sex Determination: Development of a Stabilized Freeze-Dried LAMP Assay Protocol**.

Part of this thesis work was published in the Suranaree Journal of Science and Technology (2)2025 under the title: **SIMPLE DNA EXTRACTION AND VALIDATION OF MOLECULAR MARKERS FOR EARLY SEX DETERMINATION IN NEW THAI *Cannabis sativa* L. CULTIVARS.**

His innovation, the "**MANDate date palm gender test kit"**, won two gold awards **(ACE Gold Award and Gold Medal)** at the 2023 Kaohsiung International Invention and Design EXPO (KIDE 2023) in Taiwan.

He is **the CTO and Co-founder of Gen-A-Tech Co., Ltd.,** where he plays a key role in technology transfer, turning academic research into practical biotech tools for agriculture.