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))**.

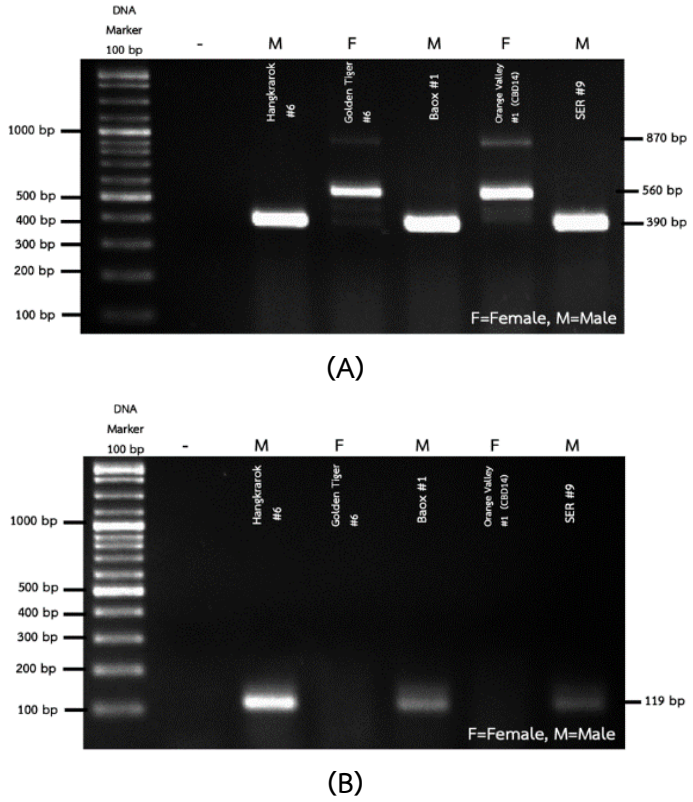


Figure 4.2 Visualization of PCR products for sex determination of cannabis by MADC2 (A) and SCAR119 (B) primers.

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)**.

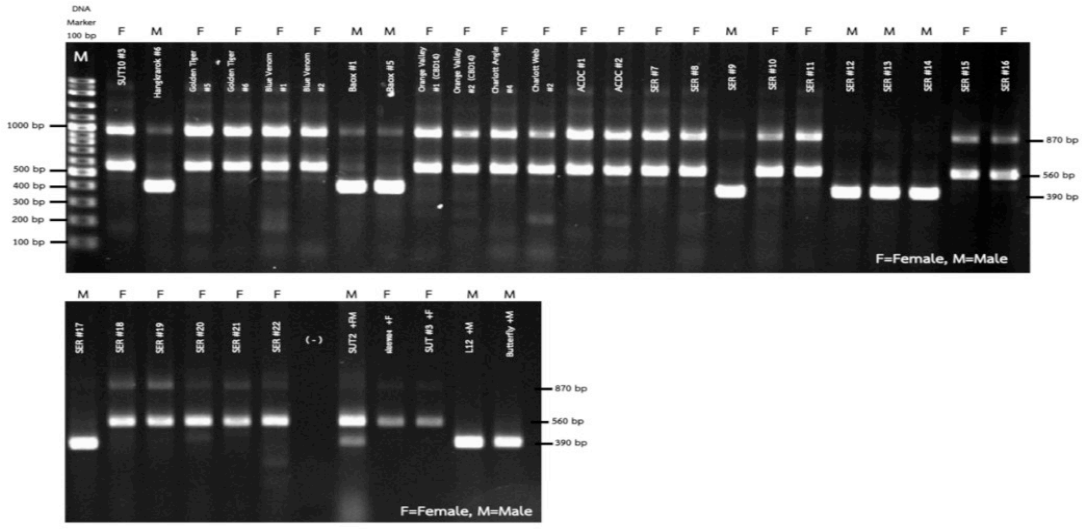


Figure 4.3 Visualization of PCR products for sex determination of cannabis by MADC2 primer

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.

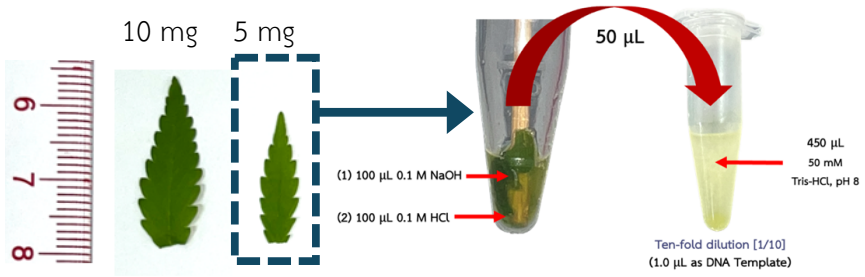


Figure 4.4 Fresh leaf samples from cannabis seedlings along with a ten-fold dilution, prepared used as simple DNA extraction method

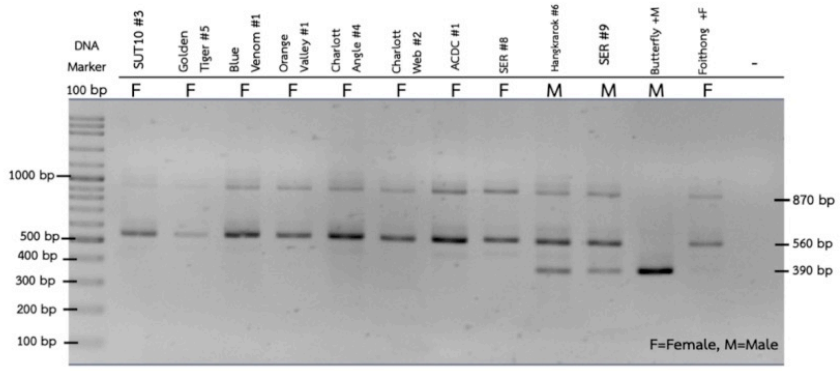


Figure 4.5 Visualization of PCR Products for Sex Determination of Cannabis Using the MADC2 Primers and template from the Rapidly DNA extraction method

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).

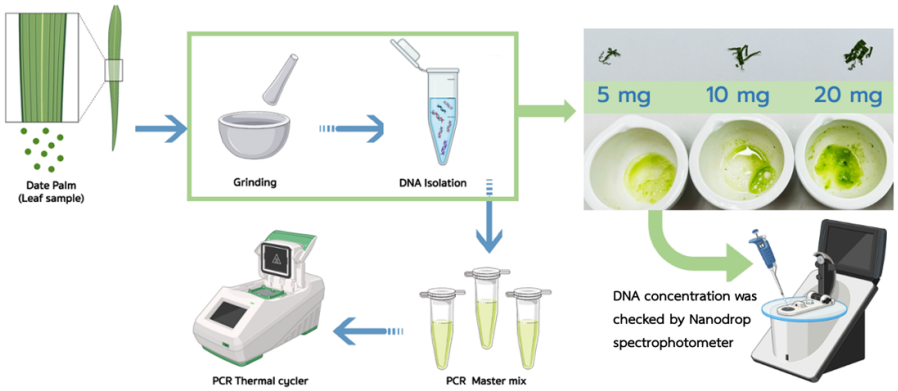


Figure 4.7 DNA extraction process and DNA concentration checking

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.

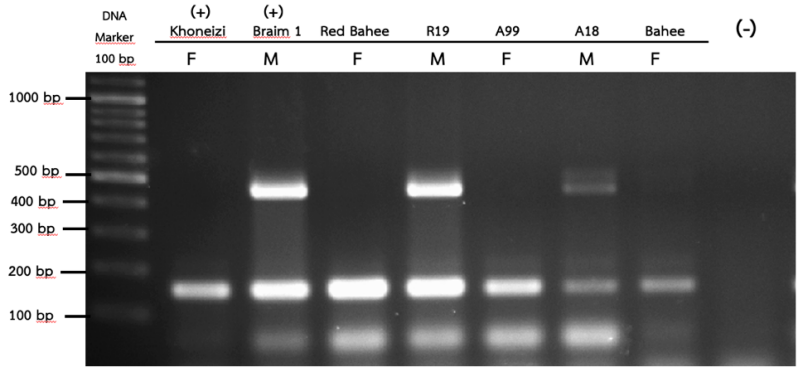


Figure 4.8 Sex verification of Date Palm seedlings using multiplex PCR. DNA samples extracted from seedlings of various cultivars were amplified with primers specific to male date palm sequences. The presence of a single band at 156 bp indicates a female sample. The presence of two bands at 450 bp and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

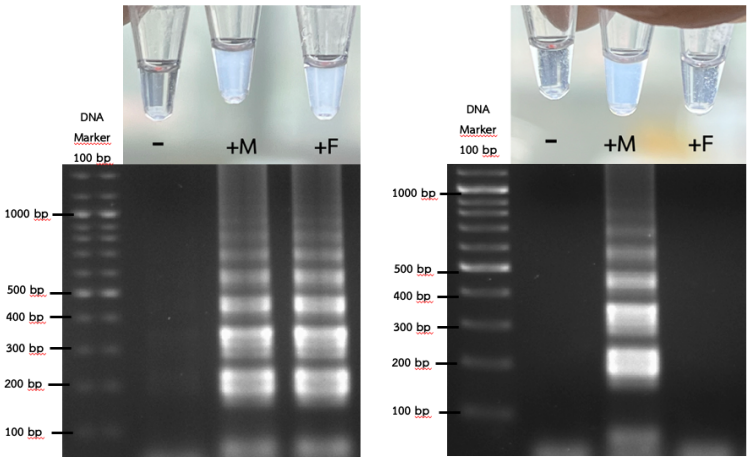
4.2.5.1 Commercial set Testing (10X *Bsm* buffer)

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.



F=Female, M=Male

(A) **(B)**

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**.

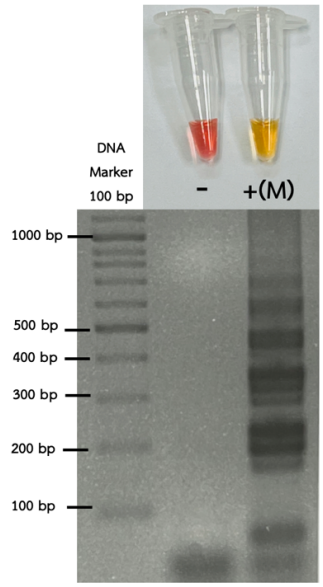


Figure 4.10 Illustrates the colorimetric results of the LAMP reaction following incubation and agarose gel electrophoresis (2%) for male-specific primers of date palm

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.

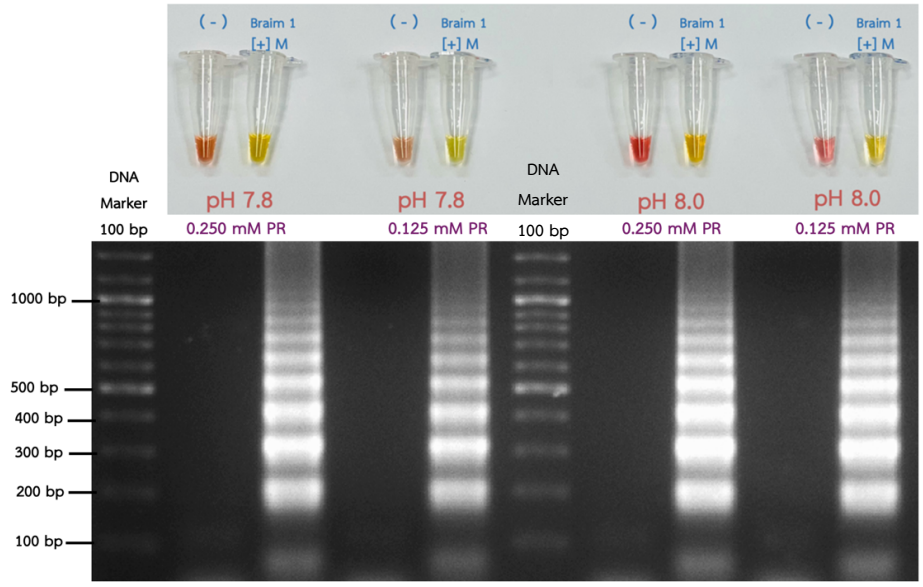


Figure 4.11 Effect of initial at pH 7.8 and 8.0 and varying Phenol Red concentration (0.125 mM and 0.250 mM) on LAMP amplification and visual detection.

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).

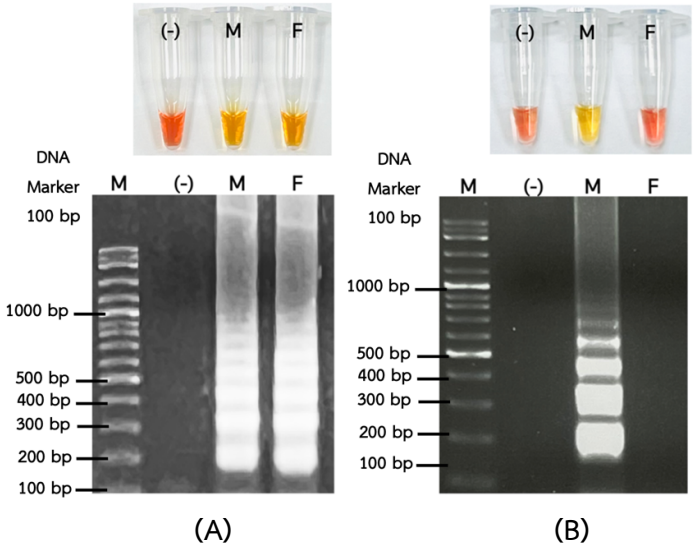


Figure 4.12 Illustrates the colorimetric results of the LAMP reaction following incubation and agarose gel electrophoresis (2%) for both reference gene primers (A) and male-specific primers (B) of date palm

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.

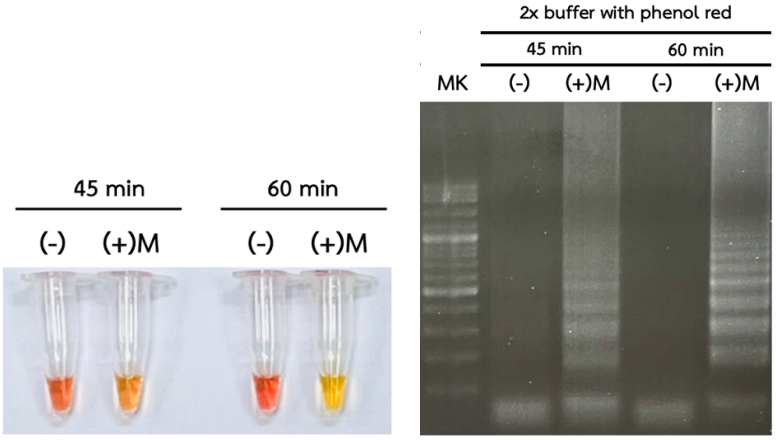


Figure 4.13 Performance evaluation of homemade *Bst* DNA polymerase (3.41 pmol/reaction) in the LAMP assay. **(A)** Colorimetric detection using Phenol Red at 45 and 60 minutes. **(B)** Agarose gel electrophoresis comparing results in commercial 10x buffer and custom 2X Phenol Red buffer at 45 and 60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

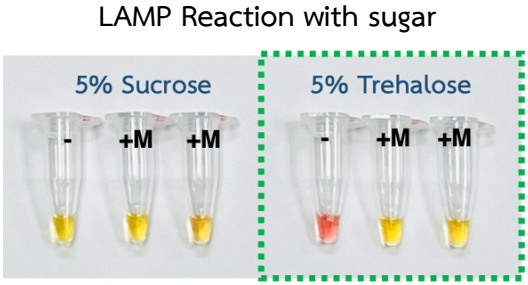


Figure 4.14 Compares the LAMP reaction with 5% sucrose and trehalose and the reaction without added sugars.

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.

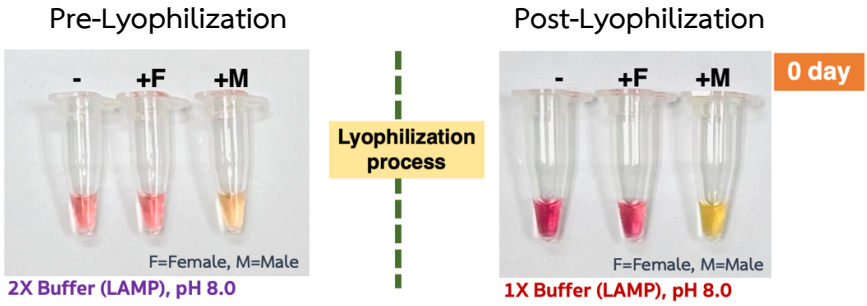


Figure 4.15 The results of the LAMP reaction before and after lyophilization

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.

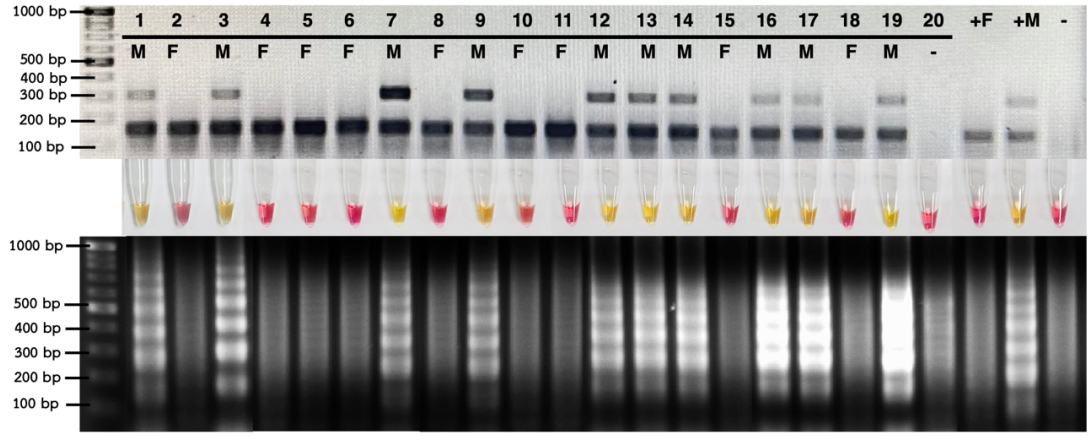


Figure 4.16 Testing of the date palm sex identification assay on multiple individual samples.

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.

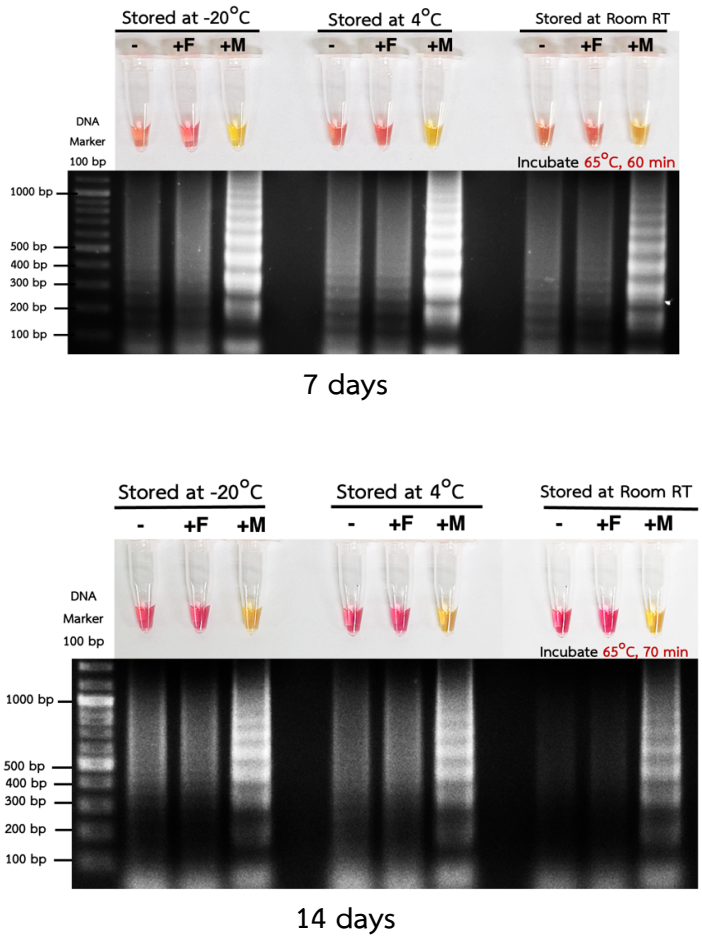


Figure 4.17 Shows the results of testing the freeze-dried date palm sex identification kit after storing it at different temperatures (-20°C, 4°C, and Room Temperature) for 7 and 14 days.

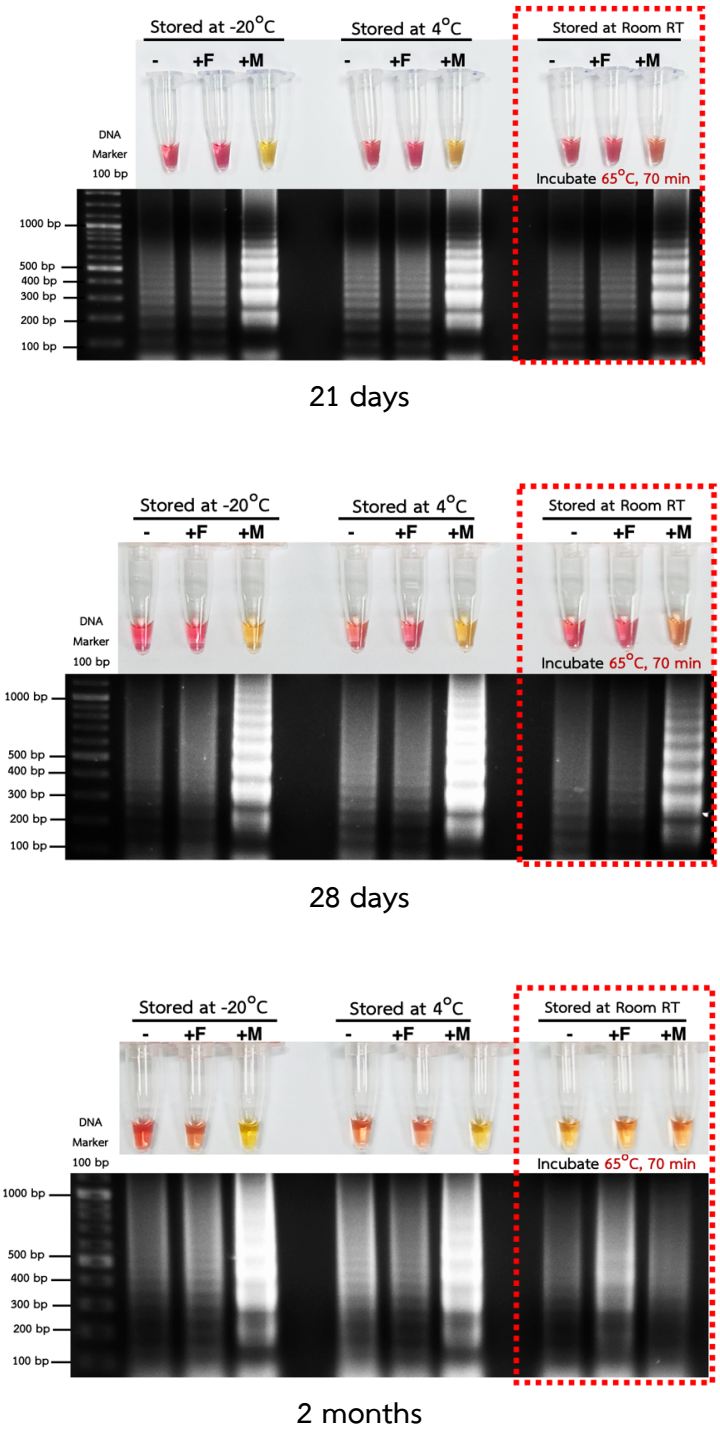
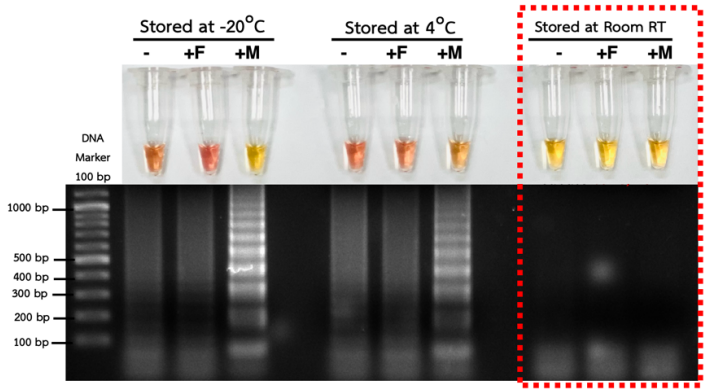


Figure 4.18 Shows the results of testing the freeze-dried date palm sex identification kit after storing it at different temperatures (-20°C, 4°C, and Room Temperature) for 21, 28 day and 2 months



6 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)