Application of the PCR and allele-specific PCR-nucleic acid lateral flow immunoassay for the detection of the *SRY* gene and the G1138A mutation in the *FGFR3* gene for the human DNA

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ABSTRACT

This study aimed to apply the polymerase chain reaction-nucleic acid lateral flow immunoassay (PCR-NALFIA) and AS-PCR-NALFIA assays to detect human DNA and determine human gender based on the human *sex-determining region Y* (*SRY*) gene and to detect G1138A mutation in the *fibroblast growth factor receptor 3* (*FGFR3*) gene causing achondroplasia (ACH), respectively. PCR primers were designed to target the human *SRY* gene. Furthermore, AS-PCR primers were modified to specify the G1138A mutation of the *FGFR3* gene. Primers were labeled with biotin at 5′ end of forward primer and FAM at the 5′ end of reverse primer to detect the target gene, followed by detection of the double labeled amplicon by NALFIA system. The result from PCR could be interpreted within 5 min by the naked eye. The limit of detection of these assays was up to 1 ng of DNA template. These results were also target gene-specific and no cross-contamination with non-target genes was observed. It is an innovative new tool that shows potential for DNA identification and is easily adaptable to other target detection.

1. INTRODUCTION

Nowadays, applying convenient and inexpensive read-out technologies like the nucleic acid lateral flow immunoassay (NALFIA), could simplify the process rather in the reduced time and avoidance of hazardous chemicals [1]. It can avoid using gel electrophoresis (GE). NALFIA using the lateral flow dipstick (LFD) combined with polymerase chain reaction (PCR) employing labeled specific primers was introduced for detecting specific amplified DNA target, so called PCR-NALFIA. PCR-NALFIA is a quick and simple diagnostic technique that is easily incorporated into clinical diagnostics. When compared to conventional detection techniques, the lateral flow strips have advantages in terms of speed and simplicity [2]. Using an antibody capture line and a labeled amplicon, nucleic acids are trapped on the LFD and transformed into an antibody-dependent format. Mechanically, PCR can be used to amplify the desired DNA target by utilizing specific primers labeled biotin and carboxyfluorescein (FAM). The analyte that is contained the biotin-FAM labeled amplicon is identified by binding to an anti-fluorescein antibody previously sprayed on a

nitrocellulose membrane and AuNPs labeled with anti-FAM antibody are used as reporter that enables the colored visualization. According to the analyte concentration, the response is directly proportional [3]. PCR-NALFIA assay is a viable tool for species-specific detection with good accuracy [4,5]. NALFIA has already been utilized as a biosensor for identifying specific amplified target genes [6] and has been broadly applied for point of care (POC) diagnostic tools. At present, this assay has also crossed other research areas [1–3]. PCR-NALFIA may be an alternatively valuable technique for manipulating DNA evidence from biological evidence during a criminal investigation. This technique can easily and quickly facilitate determination of human gender which is crucial in forensic casework such as murder, rape, and missing persons at the crime scene [7]. Sex identification based on DNA analysis is important because forensic and archaeological samples devoid of morphological diagnostic characteristics are ineligible for morphological sex determination [8]. Human sex determination usually relies on two genes that are *sex-determining region Y* (*SRY*) and *amelogenin* (*AMEL*) on nuclear DNA [9-11]. Several PCR-based methods, *SRY* gene has been used as a genetic marker [12,13]. Several methods based on detection of the *AMEL* gene have been reported in forensic science [8,14]. The loop-mediated isothermal amplification (LAMP) assay has been reported for human sex identification using *AMEL* locus [15]. To avoid GE, the LAMP-LFD has been explored for detecting human male DNA based on *SRY* gene [16]. In addition, the *SRY* gene, as an alternative Y-specific marker, is an adjunct with the *AMEL*

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gene. The incorrect identification of types of some males as females due to the frequent occurrence of large area Y chromosomal deletions, which lead to AMELY allelic dropout and inaccurate inferences about the sex of the DNA source [14,17]. Reliable and rapid detection of DNA among molecular analyses is increasingly significant. The advancement of molecular biology and nanotechnology has attracted a great deal of interest in recent years in the field of bioanalytical sciences, and it has been reviewed as having a considerable impact on the ability to accurately and precisely detect a range of target analytes without the use of specialized instrumentation [18,19]. In the medical field, there should only rarely be any diagnostic doubt left following a full clinical and radiologic assessment. When necessary, molecular testing is basic. For example, achondroplasia (ACH), which is the most common inherited skeletal dysplasia caused by the mutation in the *fibroblast growth factor receptor 3* (*FGFR3*) gene. The ACH can be identified by its radiological and clinical characteristics. However, molecular testing for *FGFR3* gene mutations can still be helpful for patients who cannot be separated from hypochondroplasia (HCH). In addition, it is essential for prenatal diagnostic confirmation. Around 98% of persons with ACH contain a c.1138G > A gene change, and 1% or so carry a c.1138G > C mutation [20]. Two mutations in the *FGFR3* gene at nucleotide c.1138 result in a p.Gly380Arg substitution that is responsible for most cases of ACH [21]. However, a significant number of HCH cases has no mutations in *FGFR3*, indicating that other genes may be involved in this phenotype [22]. To distinguish ACH from other types of severe dwarfism, particularly HCH, when prenatal dwarfism is suspected, the detection of the *FGFR3* gene mutations is crucial. These mutations can be identified by a variety of molecular methods. There are several methods for detecting a G1138A mutation in the *FGFR3* gene such as DNA sequencing [23], restriction fragment length polymorphism-PCR (RFLP-PCR) [24], denatured high-performance liquid chromatography (dHPLC) [25], real-time PCR [26], high-resolution melting (HRM) analysis [27], and allele-specific PCR (AS-PCR) [28], Sanger sequencing was employed to confirm these mutations; however, HRM is a quicker, more accurate, and less expensive genotyping assay [29]. Many approaches for identifying target genes have been developed. Nevertheless, conventional screenings are unable to always detect target genes that are present in uncommon conditions. Several DNA-based assays for gene identification have been designed in order to address some of the shortcomings of the conventional approaches. Despite providing strong specificity and sensitivity, they are not frequently used in molecular diagnosis programs due to complicated procedures or lack of funding. The PCR-NALFIA system offers a quick and easy detection technology for target gene verification. This detection format can be read within 5 min on the LFD strip by the naked eye. To the best of our knowledge, the PCR-NALFIA assay has not yet been used to detect human DNA and determine human gender based on human *SRY* gene. Moreover, the AS-PCR-NALFIA assay also has not yet been used to detect G1138A mutation in the *FGFR3* gene for diagnosis of ACH. Therefore, the objective of the present study aimed to detect human DNA and gender from various biological evidence types such as blood, semen, and saliva samples as well as to detect the G1138A mutation in the *FGFR3* gene from DNA samples for diagnosis of ACH that is more rapid and convenient technique than the previous methods.

2. MATERIALS AND METHODS

2.1. DNA Template Preparation

The DNA templates used in this research were provided into two parts for detection of the *SRY* gene and the G1138A mutation in the *FGFR3*

gene. The DNA templates for detection of the *SRY* gene were prepared from blood, semen and saliva samples. Male blood samples (*n* = 5) were kindly obtained from Blood Bank Srinagarind hospital, Khon Kaen University, Khon Kaen, Thailand and stored at 4°C. Semen samples (*n* = 10) were received from Infertility clinic, Srinagarind hospital and temporarily stored at 37°C. Male saliva samples (*n* = 5) were collected from unrelated volunteers and stored at 37°C. Moreover, female blood (*n* = 5) and female saliva (*n* = 5) samples were also provided and used as negative controls for specificity analysis because *SRY* gene cannot be found in female. The genomic DNA was extracted by using GF-1 Blood DNA Extraction Kit (Vivantis, Malaysia) for blood samples and Illustra tissue & cells genomicPrep Mini Spin Kit (GE Healthcare, USA) for semen and saliva samples following to the manufacturer's protocols. For the DNA templates for detection of the G1138A mutation in the *FGFR3* gene, the DNA samples (*n* = 5) used in this research were kindly supplied from genetics and molecular medicine laboratory, Department of Biochemistry, Faculty of Medicine, Khon Kaen university, Khon Kaen, Thailand. Briefly, genomic DNA extracted from peripheral blood of patients with a clinical diagnosis of ACH using isopropanol-fractionation with concentrated NaI and SDS technique. These DNA solutions were used as a template for PCR and allele-specific PCR-NALFIA amplification. The Khon Kaen University Ethics Committee for Human Research had granted ethical approval for the research (HE631576).

2.2. Primer Design and Preparation

The *SRY* primers used in this research were designed using the Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) based on the human *SRY* gene (accession number: JQ811934.1). The specificity of the primers was assessed by using the BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the NCBI database to ensure that no homology exists with other sequences. The ACH primers used in this research were applied from the previous report [28]. The specific forward and reverse primers of both genes were modified by 5′ labeling with biotin and FAM respectively. Primers for PCR are listed in Table 1. All primers were synthesized by Macrogen (Soul, Korea).

2.3. PCR Amplification for PCR-NALFIA

To get the *SRY* and *FGFR3* genes, the labeled specific primers of both genes were performed in the PCR-NALFIA assay. For the SRYF-BIOTIN and SRYR-FAM primers producing fragments of 220 bp, the PCR mixture was carried out in a 25 μL reaction mixture composed of 1× PCR supermix (OnePCR Ultra, Bio-Helix), 0.2 μM of each primer, 600 mM betaine (Sigma-Aldrich, USA), as well as 100 ng of each DNA template and sterilized deionized water to make up a final volume of 25 μL. The thermal cycling was performed using thermal cycler (Prima 96™, Himedia) with the conditions consisted of pre-heating at 94 °C for 5 min, 25 cycles of consecutive incubations at 94 °C for 30 sec, 63 °C for 30 sec, and 72 °C for 30 sec, followed by a final extension at 72 °C for 5 min. For the ACHF-BIOTIN and ACHR-FAM primers producing fragments of 122 bp, the ASPCR reaction was also the same as described above and was cycled under the conditions previously described above. All PCR products were purified by using a PCR purification kit (Invitrogen by Thermo Fisher Scientific, USA).

2.4. PCR-NALFIA

Following PCR amplification, the resultant double labeled PCR product was separated into 2 parts for GE and NALFIA system analysis. For GE system, 10 μL of the double labeled PCR products

Table 1: The primers used in this study.

Gene	Primer	5′-Sequence-3′	Product size (bp)	References
SRY	SRYF-BIOTIN	BIOTIN-AACAGTAAAGGCAACGTCCA	220	Present study
	SRYR-FAM	FAM-ATTTCTCTCTGTGCATGGCC		
	SRYF	AACAGTAAAGGCAACGTCCA		
	SRYR	ATTTCTCTCTGTGCATGGCC		
FGFR3	ACHF-BIOTIN	BIOTIN-GCATCCTCAGCTACA	122	[28]
	ACHR-FAM	FAM-GGAGATCTTGTGCACGGTGG		
	ACHF	GCATCCTCAGCTACA		
	ACHR	GGAGATCTTGTGCACGGTGG		

were examined on 1.5% agarose gel using 0.5× TBE buffer running in voltage 100 V for 30 min stained with fluorescent dye (Smobio, Taiwan) and visualized under the UVITECH gel documentation system. The double labeled PCR products were compared with 100 bp DNA Ladder RTU (GeneDireX, USA). For NALFIA system, 10 μL of the double labeled PCR products were mixed with 100 μL of the HybriDetect assay buffer. Then, the universal LFD (Milenia Genline HybriDetect 1, Giessen, Germany) was dipped into the mixture for 5 min to detect the specific double labeled PCR product. Within 5 min, the results were interpreted by the naked eye on the LFD. Test band and control band are clearly formed the color bands. This indicated that the double labeled PCR amplicon was detected (positive). In contrast, only the control band is visible as the color band. It indicated no double labeled PCR amplicon was detected (negative). In addition, PCR-NALFIA dipsticks were photographed by digital camera (Nikon D5100 with AF-S Micro NIKKOR 105mm f/2.8).

2.5. Sensitivity of PCR-NALFIA Assay

To determine the sensitivity of PCR- and AS-PCR-NALFIA assays for both *SRY* and *FGFR3* genes, different concentrations of the DNA template (100, 50, 25, 10, 5, 1, 0.1, or 0.01 ng/reaction) were prepared and used in PCR and then simultaneously evaluated by the LFD. In both genes, each experiment was repeated three times.

2.6. Specificity of PCR-NALFIA Assay

For *SRY* gene detection, since the *SRY* gene is presented only on male DNA, the specificity of the designed primers in the PCR-NALFIA reaction for *SRY* gene detection was performed to confirm positive and negative results with male and female DNA samples, respectively. Twenty-five nanograms of male or female genomic DNA were used in PCR-NALFIA reactions. For consistency of the experiment, male DNA was extracted from blood, semen and saliva while a sample of the female DNA was extracted from both blood and saliva. Sex determination was also examined. For the detection of G1138A mutation in the *FGFR3* gene for diagnosis of ACH, the specificity of labeled primers was performed to confirm positive and negative results with ACH patient and normal individual DNA samples, respectively. Twenty-five nanograms of DNA sample were used in AS-PCR-NALFIA reactions. Moreover, the PCR products in the present study were analyzed to confirm the PCR product size and sequence by using DNA sequencing (Macrogen Inc., Korea).

3. RESULTS

3.1. PCR-NALFIA

A schematic overview of the conceptual basis of the PCR-NALFIA assay for human *SRY* gene detection is displayed in Figure 1.
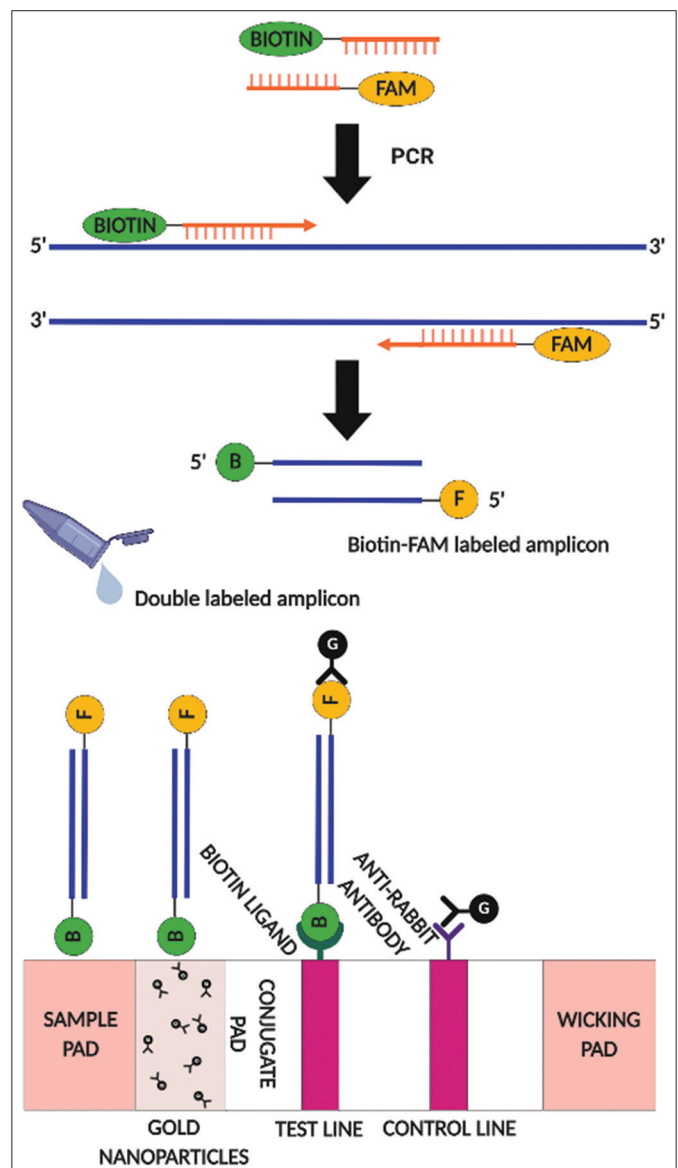

Figure 1: The conceptual basis of the polymerase chain reaction-nucleic acid lateral flow immunoassay assay for human *SRY* gene detection. Modified from [31].

In PCR-NALFIA assay, after the PCR reaction, if the specific double labeled PCR product is present forming a biotin/FAM labeled amplicon. The double-labeled amplicon is mixed with the

HybriDetect assay buffer, and dipped the sample pad of the LFD into the assay buffer. Via capillarity, the amplicon moves toward the direction of the wicking pad. FAM-specific antibodies attached to the membrane of the LFD recognize the FAM molecule. A biotin-specific ligand on the test line of the LFD detects the biotin on the opposite side of the amplicon and blocks it, producing a red-blue band. A control line is colored when an excess of gold nanoparticles is blocked over it by an antibody that is recognized by particular anti-rabbit antibodies. If the test and control bands form the color bands, it indicates that the double-labeled PCR amplicon was detected (positive). In contrast, if only the control band forms visible color bands, it indicates that no PCR amplicon was detected (negative). For *SRY* gene detection, male and female DNA samples were used as a template to amplify the human *SRY* gene. The *SRY* gene is located only on the Y chromosome in males, and there is no *SRY* gene in females. The results showed that the double-labeled PCR products were detected by NALFIA assay but not GE. The universal LFD showed the color at both the test and control lines in the male DNA sample. In contrast, the LFD from female DNA only displayed negative results, showing the color only at the control line. The reactions also included a negative control with no DNA template, whose output was negative, as shown in Figure 2a. On GE analysis, the specific double-labeled amplicon was invisible. However, a positive response was achieved by employing the specific double-labeled amplicon in the NALFIA system as an analyte. In order to verify the existence of the targeted double-labeled amplicon in the PCR-NALFIA reaction, regular primers were used to amplify the human *SRY* gene utilizing the double-labeled amplicon as a template. As expected, the 220 bp products of *SRY* fragments were detectable when using the double-labeled amplicon as a template in the PCR reaction. These results indicated that double-labeled amplicons are presented in the reaction for LFD testing. However, PCR products generated from normal primers only showed the band at the control line on the LFD, although 220 bp fragments of *SRY* gene are presented with male DNA. Moreover, DI (deionized) water was tested in the LFD, and its response was also negative, as shown in Figure 2b. The conceptual basis of the AS-PCR-NALFIA assay

for detecting the heterozygous G1138A mutation in the *FGFR3* gene is presented in Figure 3. In AS-PCR-NALFIA assay, after ASPCR amplification, if a specific double-labeled ASPCR product is present, a biotin/FAM-labeled amplicon will be formed. If the color bands form on the test and control bands, it indicates that the double-labeled ASPCR product was detected (positive). In contrast, if only the color band is visible on the control band, it means that no ASPCR amplicon was detected (negative). Then, the new AS-PCR-NALFIA protocol for detecting the G1138A mutation of *FGFR3* gene was developed as follows. The *FGFR3* gene was amplified by using the set of specific primers were performed for AS-PCR-NALFIA. The DNA samples from patients with ACH (heterozygous for the G1138A mutation) and normal individuals were used as a template to amplify *FGFR3* gene. After ASPCR amplification, the double-labeled PCR product was separated into two parts for GE and NALFIA system analysis. The results showed that the double-labeled ASPCR products were detected only using NALFIA but not by using GE. The LFD showed the color at both the test and control lines in individual heterozygous for the G1138A mutation. In contrast, the LFD from normal individuals was always negative, showing the color only at the control line. The reactions also included a negative control with no DNA template, and its output was negative, as shown in Figure 4a. The double labeled ASPCR product was invisible in the GE assay. However, a positive response was accomplished using the double-labeled ASPCR product in the NALFIA system as an analyte. Thus, to ensure the presence of the double-labeled ASPCR product in the reaction from AS-PCR-NALFIA with labeled primers. Therefore, the *FGFR3* gene was amplified with normal primer using the double-labeled ASPCR product as a template. As expected, the 122 bp products of *FGFR3* fragments were detected when using the double-labeled ASPCR product as the template in the ASPCR reaction. These results indicated that the double-labeled ASPCR products are present in the reaction for LFD testing. However, ASPCR products generated from normal primers showed the band only at the control line on the LFD, although 122 bp fragments of the *FGFR3* gene are present with individual heterozygous for the G1138A mutation. Moreover, DI (deionized) water tested in the LFD also demonstrated a negative response, as shown in Figure 4b.
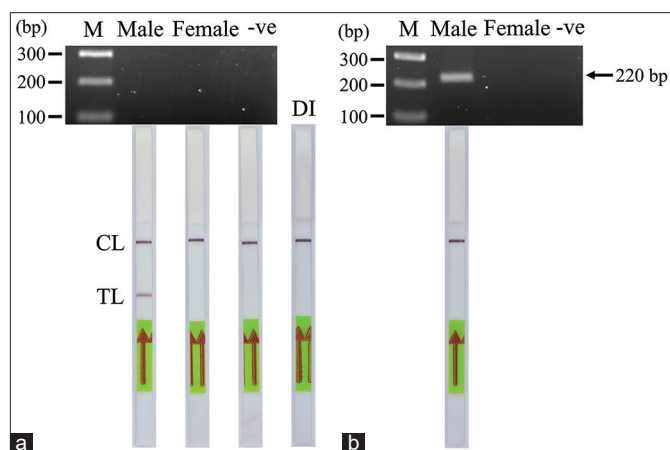
3.2. Sensitivity of NALFIA System

For *SRY* gene detection, to determine the lowest detection limit of this PCR-NALFIA system, PCR was performed using different concentrations of the DNA template ranging from 0.01 to 100 ng. The results showed that the human *SRY* gene amplified up to 1 ng of DNA template in PCR-NALFIA assay. Visual detection of a faint test line on DNA templates as low as 1 ng was seen with the LFD, as shown in Figure 5. However, as described above, the double-labeled amplicon was invisible on the agarose gel. Thus, there is no data shown. Meanwhile, this study's detection limit of PCR-NALFIA system was 1 ng/reaction. For the detection of G1138A mutation in *FGFR3* gene, to identify the detection limit of this AS-PCR-NALFIA system, ASPCR was performed using different concentrations of the DNA template ranging from 0.01 to 100 ng. The results showed that the *FGFR3* gene amplified 1 ng of DNA template in AS-PCR-NALFIA assay. With the LFD, a faint test line was visually detected on DNA templates as low as 1 ng in concentration, as shown in Figure 6. ASPCR-based GE was also performed simultaneously, but the double-labeled ASPCR product was invisible on the agarose gel, as described above. Therefore, the data are not shown here. Thus, the detection limit of AS-PCR-NALFIA system in this study was 1 ng/reaction.
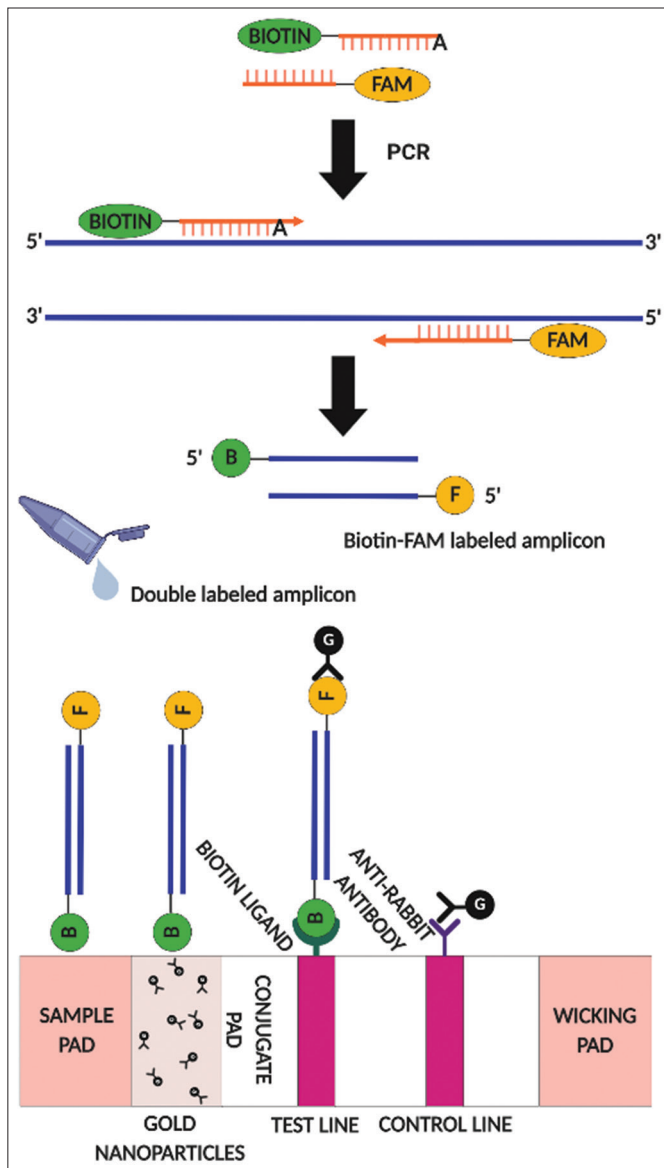

Figure 2: The polymerase chain reaction-nucleic acid lateral flow immunoassay (PCR-NALFIA) assay for detection of the human *SRY* gene. (a) PCR using primers SRYF/SRYR labeled with biotin and FAM, respectively, for human *SRY* gene detection. (b) PCR using unlabeled primers SRYF/SRYR for the detection of human *SRY* gene. Upper part: Agarose GE, Lower part: PCR-NALFIA assay, CL: Control line, TL: Test line. (M: 100 bp DNA marker and -ve: Negative control).

Figure 3: The conceptual basis of the AS-polymerase chain reaction-nucleic acid lateral flow immunoassay for detection of the heterozygous G1138A mutation in the *FGFR3* gene. Modified from [31].

3.3. Specificity of NALFIA System

The specificity of the PCR-NALFIA assay for *SRY* gene detection was performed under the optimal PCR conditions with the genomic DNA concentration of 25 ng obtained from both males and females. After the PCR amplification was completed, the LFD was visually inspected. The applicability of DNA target amplification was confirmed by the appearance of the test band on the LFD, which was observed by the naked eye. The double-labeled amplicon of the *SRY* gene was detectable using NALFIA when using a male DNA as the template in the PCR-NALFIA assay. In contrast, no positive result was observed when using a female DNA as a template. The negative result is also present in the negative control (no DNA template), as shown in Figures 7-9, respectively. The result confirmed the specificity of the PCR-NALFIA assay for *SRY* detection. To verify the specificity of the AS-PCR-NALFIA assay, ASPCR was carried out using the genomic DNA concentration of 25 ng obtained from patients with ACH, and an ordinary individual's DNA was used as a template to amplify *FGFR3*

gene. The ASPCR products, obtained by employing the methods described above, were then introduced to the NALFIA system, and the LFD was visually examined and observed by the naked eye. The results showed that the LFD revealed the color at both the test and control lines in positive control for heterozygous G1138A mutation and patients with ACH (heterozygous for the G1138A mutation). On the other hand, the LFD from an ordinary individual always demonstrates negative results, showing the color only at the control line. In addition, no DNA template was used as the negative control in the experiments, and its outcome was also negative, as shown in Figure 10.

4. DISCUSSION

This research investigated the development of PCR-NALFIA assay based on human *SRY* gene for sex determination and AS-PCR-NALFIA assay for detecting heterozygous G1138A mutation in the *FGFR3* gene for the diagnosis of achondroplasia (ACH). In this research, the PCR-NALFIA assay was designed to detect male DNA and assessed for its property to identify human *SRY* gene in human blood, semen, and saliva samples. Moreover, AS-PCR-NALFIA assay for detecting heterozygous G1138A mutation in the *FGFR3* gene was also successfully identified in the human DNA sample. The NALFIA system combines both PCR amplification and universal LFD format. The result can be interpreted on the LFD within 5 min with the naked eye. Compared to conventional amplicon detection methods, the LFD system does not need special tools, gel electrophoresis equipment, or post-amplification processing. As a result, the NALFIA system can probably be considered a promising new molecular tool for DNA detection in a moderately well-equipped laboratory with standard thermal cycler apparatus and refrigeration.

For PCR-NALFIA assay used to detect human *SRY* gene, specific primers for *SRY* gene, including SRYF and SRYR labeled with biotin and FAM at the 5′ ends, were used for PCR-NALFIA. Male and female DNA samples were used as a template to amplify human *SRY* gene. The specific double-labeled PCR amplicons achieved with the SRYF/SRYR primers were also detected using the NALFIA. The LFD provided positive results when using male DNA. In contrast, the LFD displayed negative results when using female DNA as a template for PCR reactions. Although the specific double-labeled amplicon was invisible in GE method, a positive response was accomplished using the specific double-labeled amplicon as an analyte in NALFIA system. Then, the purified double-labeled amplicon was quantified by using Qubit™ 4 fluorometer (Invitrogen by Thermo Fisher Scientific, USA), QFX fluorometer (DeNovix, USA), and DS-11 spectrophotometer (DeNovix, USA). It revealed that the concentration of the purified double-labeled amplicon was below the limit. This solution indicated that the sensitivity of the PCR-NALFIA assay was higher than agarose GE for the visualization of double-labeled PCR amplicons. However, PCR products generated from regular primers showed the band only at the control line on the LFD, although 220 bp fragments of *SRY* gene are present with male DNA. For PCR-NALFIA assay used to detect G1138A mutation in *FGFR3* gene, specific primers, including ACHF and ACHR labeled with biotin and FAM at the 5′ end, were used for AS-PCR-NALFIA. DNA samples from patients with ACH (heterozygous G1138A mutation) and normal individuals were used as a template to amplify *FGFR3* gene. The double-labeled ASPCR amplicon was invisible when the GE method was performed. However, a positive response was accomplished using the double-labeled ASPCR product as an analyte in the NALFIA system, providing the same result as the PCR-NALFIA. However, ASPCR products generated from regular primers showed the band only at the control line on the LFD, although 122 bp
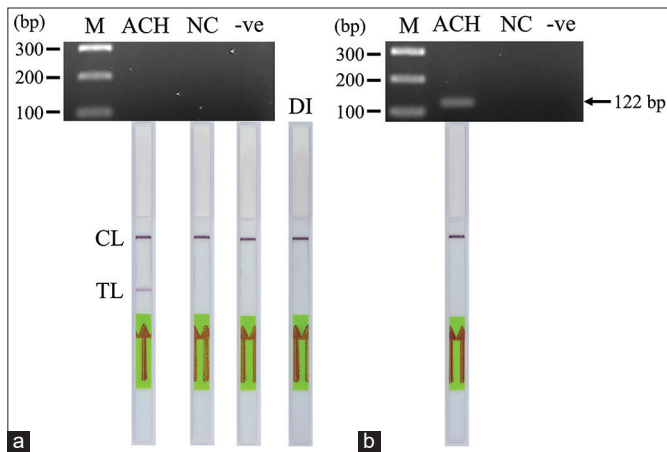
Figure 4: The AS-polymerase chain reaction-nucleic acid lateral flow immunoassay (PCR-NALFIA) assay for detection of the heterozygous G1138A mutation in the *FGFR3* gene. (a) PCR using primers ACHF/ACHR labeled with biotin and FAM, respectively, for *FGFR3* gene detection. (b) PCR using unlabeled primers ACHF/ACHR for the detection of the *FGFR3* gene. Upper part: Agarose GE, Lower part: AS-PCR-NALFIA assay, CL: Control line, TL: Test line. (M: 100 bp DNA marker, ACH: Achondroplasia (heterozygous for the G1138A mutation), NC: Normal individual; -ve: Negative control).
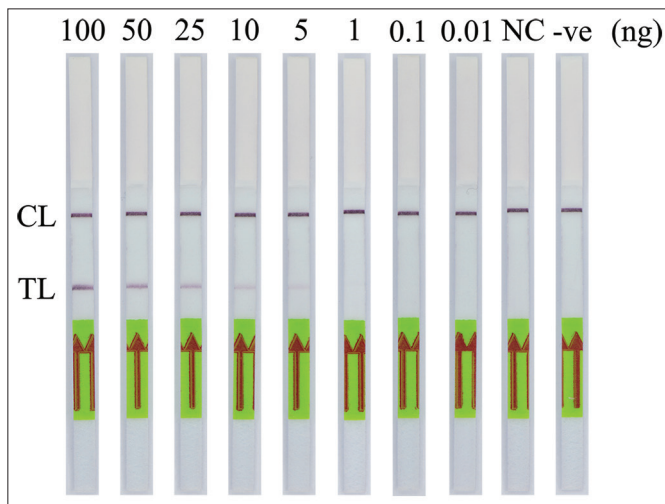
Figure 5: The sensitivity of polymerase chain reaction-nucleic acid lateral flow immunoassay assay for detection of the human *SRY* gene. CL: Control line, TL: Test line (NC: Female DNA template; -ve: Negative control).

Figure 6: The sensitivity of AS-polymerase chain reaction-nucleic acid lateral flow immunoassay assay for detection of the heterozygous G1138A mutation in the *FGFR3* gene. CL: Control line, TL: Test line (NC: Normal individual; -ve: Negative control).

Figure 7: The specificity of polymerase chain reaction-nucleic acid lateral flow immunoassay assay for detection of the human *SRY* gene from blood samples. CL: Control line, TL: Test line. (1–5: Male blood samples, 6–10: Female blood samples; -ve: Negative control).

fragments of the *FGFR3* gene are present with individual heterozygous G1138A mutation. Moreover, the sequencing analysis of the specific purified PCR/ASPCR products from PCR/ASPCR reactions from both regular primers confirmed the expected size and sequence.

These outcomes confirm the high detection sensitivity of the PCR- and AS-PCR-NALFIA systems developed in this study. When the PCR amplification utilizes a DNA target and specific primers labeled with biotin and FAM at the 5′ end, the sensitivity level does not decrease. This investigation supports that the sensitivity of both the PCR- and AS-PCR-NALFIA assays is higher than that of PCR-GE, which is in accordance with other works [30,31]. As previously reported, PCR-NALFIA assay sensitivity was over 1000 times greater than PCR-GE [31].
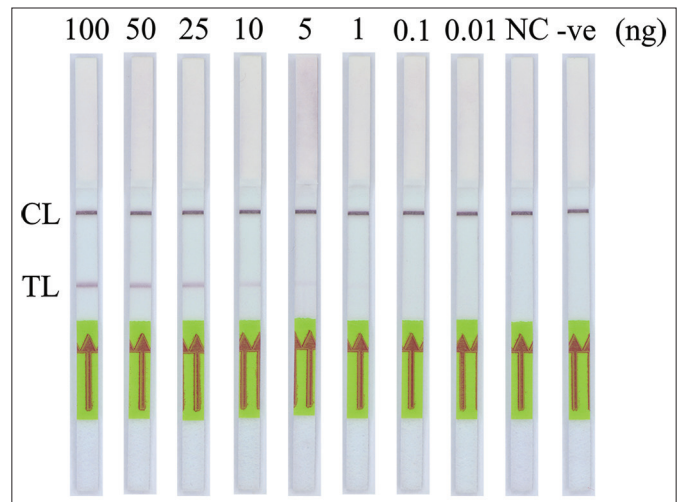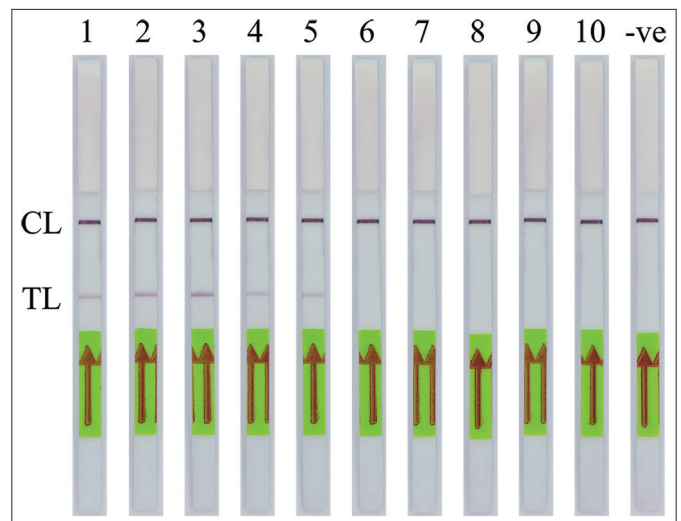
The detection limit of both PCR- and AS-PCR-NALFIA assays was assessed by using different levels of concentration of the DNA template. The detection limit of both the PCR- and AS-PCR-NALFIA systems developed in this study was 1 ng, where a faint test line was still visible on the LFD in the NALFIA system. PCR-GE was also performed simultaneously, but the double-labeled PCR product was invisible on the agarose gel. These outcomes confirmed that the employed universal LFD had a higher sensitivity. Regarding the limits of detection, PCR-NALFIA was superior [32]. According to a previous study, the ribosomal DNA intergenic spacer sequences used in the NALFIA assay to detect the fungus *Macrophomina phaseolina* in agricultural soil samples had a detection limit of 17.3 fg [31]. Moreover, as previously reported, the detection limit for the verification of sheep-specific PCR products was 0.01 pg based on the mitochondrial gene sequences of sheep [30]. It could be stated that the higher copy number of both areas
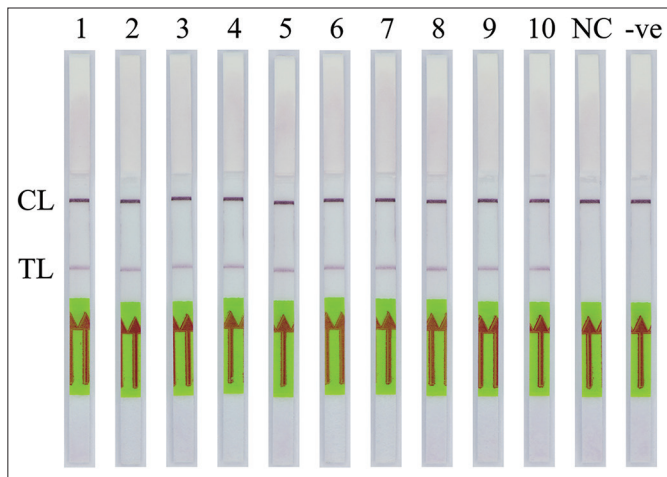
Figure 8: The specificity of polymerase chain reaction-nucleic acid lateral flow immunoassay assay for detection of the human *SRY* gene from semen samples. CL: Control line, TL: Test line. (1–10: Semen samples, NC: Female DNA template; -ve: Negative control).
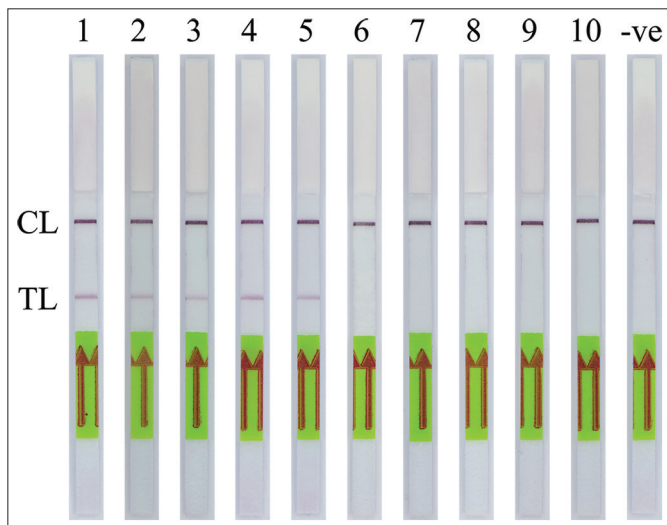

Figure 9: The specificity of polymerase chain reaction-nucleic acid lateral flow immunoassay assay for detection of the human *SRY* gene from saliva samples. CL: Control line, TL: Test line (1–5: Male saliva samples, 6–10: Female saliva samples and -ve: Negative control).

Figure 10: The specificity of AS-polymerase chain reaction-nucleic acid lateral flow immunoassay assay for detection of the heterozygous G1138A mutation in the *FGFR3* gene. CL: Control line, TL: Test line. (ACH: Positive control for heterozygous G1138A mutation, 1–5: Patients with ACH (heterozygous G1138A mutation), NC: Normal individual; -ve: Negative control).

increases the sensitivity of PCR-based approaches. Therefore, this may be the reason why differs from our results because both target genes used in PCR- and AS-PCR-NALFIA assays have a lower number of copies. From our results, it could be determined that the universal LFD system achieves a high detection sensitivity as a sensitive molecular technology. However, there is a chance that aerosols may have contaminated the sample, thus producing false-positive results [30]. This issue is considered indispensable in PCR-NALFIA with the universal LFD because the method is even more sensitive than PCR-GE. However, the LFD still has some benefits besides its greater sensitivity compared to GE detection, such as the quick test that could be completed within 5 min. It is considerably easy to carry out and does not require expensive equipment or trained personnel. Moreover, the interpretation method is simple. PCR-NALFIA is a specific and sensitive method that requires only about 1.5–2 h from DNA extraction to detection of the target genes [32]. Due to the necessity for a quick
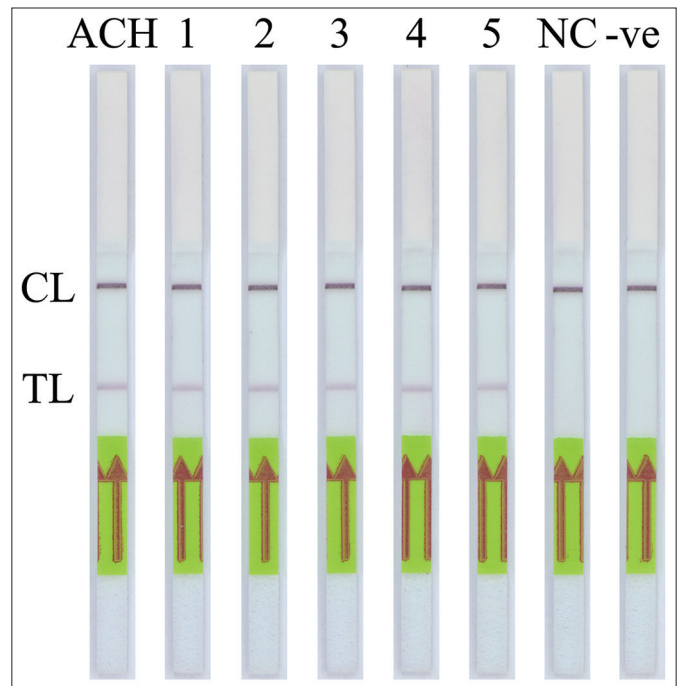
and sensitive detection technique that utilizes a POC testing strategy, the utilization of the LFD system for organism detection is gradually becoming more common in several scientific areas [31].

5. CONCLUSION

From the results found in this study, it could be stated that the developed PCR- and AS-PCR-NALFIA assays in the study were suitable for identifying target gene in DNA samples. The detection limit of both PCR- and AS-PCR-NALFIA assays was determined to be 1 ng for template DNA. The PCR-NALFIA assay was specific for human *SRY* gene detection, and cross-contaminations were not detected with any non-target genes. In addition, AS-PCR-NALFIA assay was also specific for detecting heterozygous G1138A mutation in the *FGFR3* gene to diagnose achondroplasia (ACH). This NALFIA system can be quickly altered and used with different target genes. Therefore, it might be practical for gene detection in various contexts as a simple-to-use system. Furthermore, this procedure could become a practical alternative for advancing rapid diagnostic kits for the target gene.

6. ACKNOWLEDGMENTS

7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in

drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study was approved by the Khon Kaen University Ethics Committee for Human Research (approval no. HE631576).

11. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

12. PUBLISHER'S NOTE

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