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**Cost-effectiveness analysis of alkaline lysis, MagNA Pure,
and phenol-chloroform DNA extraction methods
followed by measurement of single gene copy
number using quantitative real-time PCR for
Dirofilaria immitis microfilaria**

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Sungpradit S, Nuchprayoon S, Chatsuwana T. Cost-effectiveness analysis of alkaline lysis, MagNA Pure, and phenol-chloroform DNA extraction methods followed by measurement of single gene copy number using quantitative real-time PCR for *Dirofilaria immitis* microfilaria. Chula Med J 2010 Nov - Dec; 54(6): 549 - 61

Objective : *Dirofilariasis or heartworm disease, a mosquito-borne parasitic disease, continues to be a serious problem in canine (dogs) and feline (cats) species. The disease is caused mainly by the filarial nematode, Dirofilaria immitis. In canine dirofilariasis, the adult worms, present in the pulmonary arteries and the right ventricle, causing damages and leading to pulmonary hypertension and right-sided congestive heart failure. In addition, acute death in feline dirofilariasis causes lung injury resulting in respiratory distress. Being critical to transmission and complex disease pathology, the microfilarial stage is useful in laboratory diagnosis; however, the microfilaria identification process is laborious, difficult and time-consuming. Development of a molecular diagnostic will provide an efficient alternative. The present study analyzes relative efficiencies of three different methods of DNA extraction from D. immitis microfilariae for quantitative real-time PCR (qPCR).*

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- Methods** : Three different microfilaria DNA extraction methods: alkaline lysis, MagNA Pure and phenol-chloroform were studied. DNA extracted from 1, 10, and 100 *D. immitis* microfilariae were analyzed by qPCR, targeting the single copy of filarial glutathione-S-transferase (*gst*) gene.
- Results** : We were able to detect as low as 0.025-microfilaria by qPCR following an alkaline lysis extraction. The alkaline lysis method requires the least amount of time to complete (20 min) and it is also the least expensive, less than \$0.05 per sample.
- Conclusion** : The alkaline lysis DNA extraction method appears to be the most sensitive, least time-consuming, and most cost-effective method for *D. immitis* microfilariae. This technique is likely to provide the most suitable platform for improved detection and measurement of microfilarial copy number and 'free' filarial DNA. In particular, this technique will advance diagnosis of feline dirofilariasis that involves characteristically low circulating microfilariae and amicrofilaremia.
- Keywords** : microfilaria, *Dirofilaria immitis*, alkaline lysis, MagNA Pure, phenol-chloroform, quantitative real-time PCR

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ศิวะพงษ์ สังข์ประดิษฐ์, สุรางค์ นุชประยูร, ธนิษฐา ฉัตรสุวรรณ. การวิเคราะห์ประสิทธิภาพ
ของวิธีการสกัดสารพันธุกรรมชนิดดีเอ็นเอ ด้วยวิธี alkaline lysis, MagNA Pure และ phenol-
chloroform ของไมโครฟิลาเรียของพยาธินอนหัวใจชนิด *Dirofilaria immitis* และตรวจวัด
ดีเอ็นเอด้วยวิธี quantitative real-time PCR. จุฬาลงกรณ์เวชสาร 2553 พ.ย. - ธ.ค.; 54(6):
549 - 61

วัตถุประสงค์ : โรคพยาธินอนหัวใจ (heartworm disease หรือ dirofilariasis) ซึ่งเป็นโรคที่มี
ยุ่งเป็นพาหะ ยังคงเป็นปัญหาที่สำคัญในสุนัขและแมว โรคนี้มีสาเหตุมาจาก
พยาธิตัวกลมชนิด *Dirofilaria immitis* ในสุนัขที่เป็นโรคพยาธินอนหัวใจ
จะพบพยาธิตัวเต็มวัยในเส้นเลือด pulmonary artery และหัวใจห้องล่างขวา
ทำให้เกิดกลุ่มอาการที่เรียกว่า pulmonary hypertension และ right-sided
congestive heart failure นอกจากนี้ แมวที่เป็นโรคพยาธินอนหัวใจ จะมีผล
ต่อปอดเกิดอาการที่เรียกว่า respiratory distress และตายอย่างเฉียบพลัน
ตัวอ่อนระยะไมโครฟิลาเรียมีบทบาทเกี่ยวข้องกับการแพร่กระจายโรค การเกิด
พยาธิสภาพที่ซับซ้อน และเป็นระยะที่ใช้ในการตรวจวินิจฉัยทางห้องปฏิบัติการ
อย่างไรก็ตาม การตรวจแยกชนิดของไมโครฟิลาเรียใช้แรงงาน เวลา และยุ่งยาก
การพัฒนาวิธีการวินิจฉัยโดยใช้วิธีทางโมเลกุลจึงเป็นทางเลือกหนึ่ง การศึกษา
เป็นการวิเคราะห์ประสิทธิภาพของวิธีการสกัดสารพันธุกรรมชนิดดีเอ็นเอที่
แตกต่างกันสามวิธี ในการสกัดดีเอ็นเอของไมโครฟิลาเรีย และนำมาตรวจวัด
ด้วยวิธี quantitative real-time PCR (qPCR)

วิธีการ : วิธีการสกัดดีเอ็นเอของไมโครฟิลาเรียสามวิธี ได้แก่ วิธี alkaline lysis, MagNA
Pure และ phenol-chloroform โดยสกัดดีเอ็นเอจากไมโครฟิลาเรียชนิด *D. immitis*
จำนวน 1, 10 และ 100 ตัว โดยนำดีเอ็นเอที่ได้ ไปวิเคราะห์ด้วยวิธี qPCR
โดยใช้ยีนเป้าหมายเป็นยีนที่มีหนึ่งยีนในเซลล์ ได้แก่ ยีน glutathione-S-
transferase (gst)

ผลการทดลอง : วิธี alkaline lysis ร่วมกับวิธี qPCR สามารถตรวจวัดดีเอ็นเอของไมโครฟิลาเรีย
ได้น้อยที่สุดจำนวน 0.025 ตัว วิธีสกัดดีเอ็นเอวิธีนี้ใช้เวลาอย่างน้อยที่สุด คือ 20 นาที
และเป็นวิธีที่ประหยัดที่สุดคิดเป็นเงิน 0.05 ดอลลาร์ต่อหนึ่งตัวอย่าง

- สรุปผล** : วิธี *alkaline lysis* เป็นวิธีที่มีความไว ประหยัดเวลา และมีประสิทธิภาพในการสกัดดีเอ็นเอของไมโครฟิลาเรียชนิด *D. immitis* มากที่สุด วิธีดังกล่าวสามารถนำไปประยุกต์ใช้ในการตรวจวินิจฉัยโรคพยาธิหนอนหัวใจ วัดปริมาณยีนของไมโครฟิลาเรียและวัดปริมาณดีเอ็นเออิสระของพยาธิฟิลาเรีย วิธีนี้สามารถพัฒนาเพื่อใช้ในการตรวจวินิจฉัยโรคพยาธิหนอนหัวใจในแมว ซึ่งมีจำนวนไมโครฟิลาเรียในกระแสเลือดในระดับที่ต่ำหรือไม่มีไมโครฟิลาเรียในกระแสเลือด
- คำสำคัญ** : *Microfilaria, Dirofilaria immitis, Alkaline lysis, MagNA Pure, Phenol-chloroform, Quantitative real-time PCR.*

Canine and feline heartworm diseases (Dirofilariasis) are caused by filarial nematode, *Dirofilaria immitis* which are transmitted by mosquitoes (*Aedes* spp., *Anopheles* spp., *Culex* spp., and *Armigeres* spp.). Heartworm infections in dogs and cats have been reported worldwide.^(1,2) In most cases, signs of the disease will appear within 1 - 2 years after infection. Typical signs include coughing, labored breathing, weakness, exercise intolerance, symptoms which cause severe cardiopulmonary problems and even death.⁽³⁾ Dirofilariasis is also a zoonotic filariasis that causes human pulmonary dirofilariasis. Accidental infections with *D. immitis* in humans have been reported.^(4,5) Human pulmonary dirofilariasis develops when the immature parasites die in the right ventricle, embolized, disseminated to the lungs, and then located in small branches of the pulmonary arteries. Chest radiography exhibits noncalcified or calcified nodules of 1 - 3 cm in diameter, called coin lesions.^(6,7) The lesions are often inaccurately diagnosed as primary or metastatic lung cancer. Partial lung resection followed by histopathological examination is therefore necessary for definitive diagnosis.⁽⁸⁾

In feline dirofilariasis, however, the diagnosis of this disease is more difficult than the canine heartworm. Most feline heartworm infections are light and consist of less than six adult worms, where usually only one or two worms are present, and approximately one-third of these cases include worms of the same sex. Microfilaremia is seldomly observed in infected cats. The chances of finding microfilariae are ameliorated by using modified Knott's or Millipore filter concentration techniques.⁽²⁾ However, these methods are difficult, laborious, time consuming, and have low sensitivity.

For the detection of the human filarial nematode (*Wuchereria bancrofti* and *Brugia malayi*) DNA in the patient blood and mosquito samples, the specific quantitative real-time PCR (qPCR) methods have been developed.⁽⁹⁻¹⁵⁾ qPCR can be as sensitive as one microfilaria of *W. bancrofti* and *B. malayi* by using multiple copies of the filarial genes such as *SspI*⁽¹³⁾ and *HhaI*⁽¹²⁾ were selected for amplification of the targeted gene.

In this study, we compare three different DNA extraction methods for their efficiency to extract *D. immitis* microfilarial DNA. The efficiency of the extraction was evaluated by using a *D. immitis*-specific qPCR assay which targets the nematode single copy gene for glutathione-S-transferase (*gst*).⁽¹⁶⁾ We believe that this extraction method will be helpful to amplify other genes with high sensitivity and specificity.

Materials and Methods

Animals

D. immitis microfilariae were kindly provided by Dr. Sonthaya Tiawsirisup (Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Thailand).

Parasites

Blood was drawn from the cephalic veins of an infected dog and kept in vacutainer EDTA tubes (Becton, Dickinson and Co., Franklin Lakes, NJ). Microfilariae were isolated by filtration of the blood with isotonic phosphate buffered saline (PBS [pH 7.4]; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄) through a 5.0 μm pore-size polycarbonate membrane (Millipore; Billerica, MA).⁽¹⁷⁾ The filter membrane, carrying microfilariae, was

immersed in a 35 x 10 cm petri dish (Becton, Dickinson and Co.) containing PBS. Microfilariae were picked up individually for 1, 10, and 100 microfilariae (duplicated sets) under a stereo microscope using modified insect pin No. 6.

DNA extraction methods

- Alkaline lysis extraction:

DNA extraction was modified from a previously instruction⁽¹⁸⁾ Briefly, individual microfilariae were suspended in a 0.2 ml thin-wall PCR tube containing 5 μ l lysis buffer (0.05% SDS and 0.025N NaOH). Samples were incubated in a heat block at 95 °C for 15 min, spun down, and resuspended in 195- μ l sterile deionized water. All DNA extractions were stored at -20 °C until use.

- MagNA Pure extraction:

Individual microfilariae were suspended and digested in a 1.5 ml microcentrifuge tube containing 90 μ l lysis buffer (20 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM MgCl₂, 0.5% Tween 20, 150 μ g/ml Proteinase K). Samples were then incubated in a 65 °C water bath for 3 h and enzyme activity was inactivated by incubation in a heat block at 90 °C for 10 min. DNA extraction was performed using MagNA Pure LC DNA Isolation Kit II (tissue) and the MagNA Pure automated extractor (Roche Diagnostics, Indianapolis, IN) according to manufacturers' protocol. In brief, the isolation procedure is based on magnetic-bead technology. The samples were lysed by incubation with 300 μ l of a special lysis buffer containing a chaotropic salt and 100 μ l of proteinase K. Magnetic glass particles were added, and total nucleic acids contained in the sample were bound to their surface. Unbound substances were removed

with several washing steps: then the purified total nucleic acid was eluted with 200 μ l of low-salt buffer.⁽¹⁹⁾ All DNA extractions were stored at -20 °C until use.

- Phenol-chloroform extraction:

Individual microfilariae were lysed and enzymatic activities inactivated, as described above. DNA was extracted with equal volumes of phenol : chloroform : isoamyl alcohol (25:24:1) by centrifugation at 15,300 g for 5 min. The aqueous phase (upper phase) was transferred into a fresh tube, and sequentially extracted using one volume of chloroform. DNA was precipitated by the addition of 2.5 volume of ice-cold absolute ethanol and 0.1 volume of 3 M sodium acetate pH 5.2 at -70 °C for 30 min. The precipitate was centrifuged at 18,000 g (4 °C) for 30 min. The pellet was washed with cold 70% ethanol, allowed to air dry, and resuspended in 200 μ l of sterile deionized water.⁽²⁰⁾ All DNA extractions were stored at -20 °C until use.

The *D. immitis* microfilarial DNA samples from 1, 10, and 100 microfilariae were prepared in 200- μ l final volume and 5 μ l of DNA samples (equivalent to 0.025, 0.25, 2.5 microfilariae per reaction, respectively) were used in qPCR.

Quantitative real-time PCR

Specific *D. immitis* glutathione-S-transferase (*gst*) primers, *gstDiro-F* (5' GAA TGG TGA AAA TAA TGC GGA AAC 3') and *gstDiro-R* (5' ATA AGC CTC ATA GAT CAT TCT TGT G 3') (Invitrogen, Carlsbad, CA) were designed from *D. immitis*, *W. bancrofti*, and *B. malayi*'s *gst* mRNA (accession number U14753, AY195867, and Y12788, respectively)⁽²¹⁾ -using CLUSTAL X⁽²²⁾, and Primer3 programs.⁽²³⁾ The set of

primers can bind to the exon of the *D. immitis* *gst* mRNA (Figure 1). Plasmids containing the inserts of the *D. immitis* *gst* gene were prepared for use as standards in qPCR. The PCR products (100 bp) were ligated into the vector pGEM-T easy (3,018 bp) (Promega, Madison, WI) and cloned into DH5- α cells. Positive colonies containing *gst* encoded plasmid were sequenced (Macrogen, Seoul, Korea) to verify that the correct sequences had been amplified. Plasmid DNA was quantified by spectrophotometry (BioRad Laboratories, Irving, CA), and 10-fold dilutions were prepared between 0.01 femtogram/microliter ($\text{fg}\mu\text{l}^{-1}$) and $100 \text{ fg}\mu\text{l}^{-1}$ (equivalent to about 2.97×10^0 to 2.97×10^4 copies).⁽²⁴⁾

Samples and the PCR master mixture were contained in 20 μl glass capillaries. Sample detection was based on SYBR Green I dye incorporation with

the intended PCR products. Samples were assayed for the presence of this signal in real time during each PCR cycle, and the cycle number at which the first signal detected was correlated to the original concentration of DNA. The specificity of amplification was confirmed by melting curve analysis. In each experiment the 10 μl volume in each capillary tube contained 2 μl of 5X LightCycler DNA Master SYBR Green (Roche Diagnostics), 0.5 μl of each primer at 10 μM , 2 μl of sterile distilled water, and 5 μl of DNA samples. After one pulse of centrifugation to allow mixing and to drive the mix into the distal end of each tube, the glass capillaries were placed in LightCycler instrument (Roche Diagnostics). The amplification program included an initial denaturation step for 1 cycle at 95 $^{\circ}\text{C}$ for 10 min and 45 cycles of denaturation at 95 $^{\circ}\text{C}$ for 10s, annealing at 60 $^{\circ}\text{C}$ for 10s, and

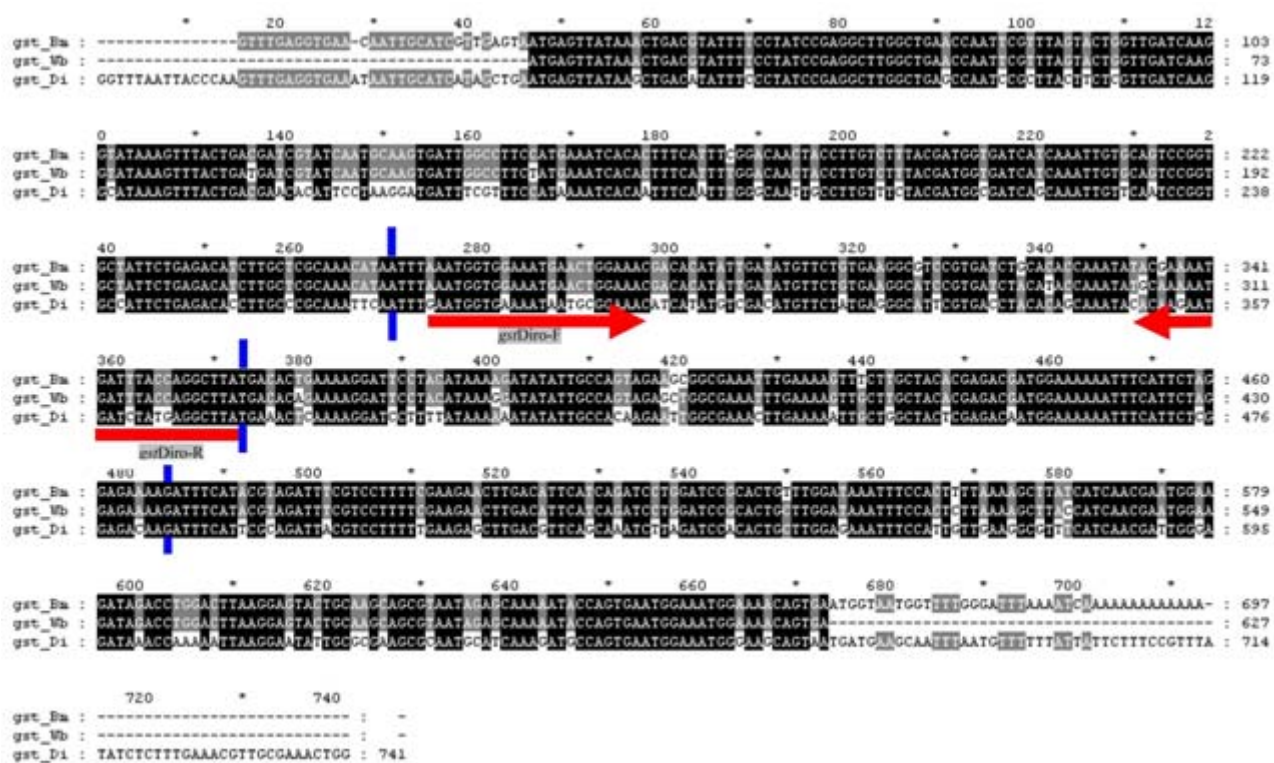


Figure 1. Alignment of glutathione-S-transferase mRNA of *W. bancrofti*, *B. malayi* and *D. immitis*: exon splice sites indicated by the vertical bars.

extension at 72 °C for 5s. Melting curve analysis was done at 65 to 90 °C (temperature transition, 20 °C/s) with stepwise fluorescence acquisition by real-time measurement of the fluorescence directly in clear-glass capillary tubes. Sequence-specific standard curves were generated by using 10-fold serial dilutions of a standard concentration of *gst* encoded plasmid. The number of copies of each sample transcript was then calculated from the standard curve with LightCycler software (Roche Diagnostics).⁽¹⁰⁾

Cost analysis

The cost per extraction was calculated for the commercially available kits by dividing up the cost of the kit by the number of extraction that could be performed with the kit. The costs of alkaline lysis and phenol-chloroform were calculated based on the cost

of the consumable supplies required for each extraction.⁽²⁵⁾

Results

Sensitivity and specificity of qPCR

The sensitivity of the qPCR SYBR green assay was capable of detecting 10-fold dilution series of *D. immitis* *gst*-plasmid DNA between 0.01 fg μ l⁻¹ and 100 fg μ l⁻¹ (equivalent to about 2.97x10⁰ to 2.97x10⁴ copies). This set of primers could amplify both *D. immitis* *gst* DNA and mRNA templates since they were designed from an exon. The primers were specific for *D. immitis* *gst* but not those of *B. malayi* and *W. bancrofti* as they did not amplify the *B. malayi* and *W. bancrofti* *gst* genes (Figure 2). The amplification plots of the sample, *gst*-plasmid control and negative control were used to compare with the created standard curve (Figure 3).

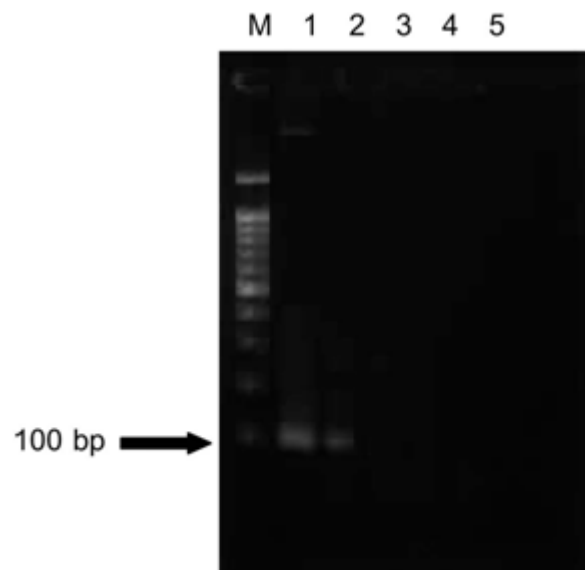


Figure 2. Ethidium bromide staining patterns of the PCR products on a 2% agarose gel. Arrow indicates 100-bp *D. immitis*-specific band. Lane 1, PCR products obtained from positive control plasmid; Lanes 2, PCR products obtained from *D. immitis* microfilariae; Lane 3, PCR products obtained from *B. malayi* microfilariae; Lane 4, PCR products obtained from *W. bancrofti* microfilariae; Lanes 5, negative control containing no DNA; Lane M, DNA size markers (1.5 kb plus DNA ladder).

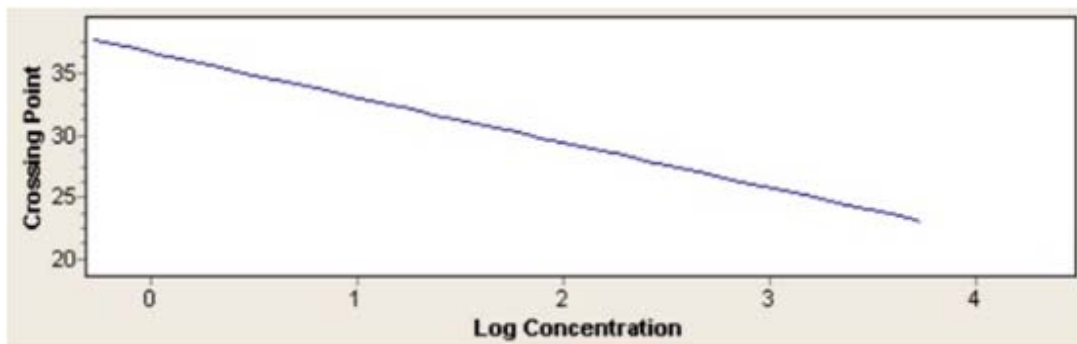


Figure 3. Amplification plots of crossing point (y-axis) versus log concentration (x-axis) show the analytical sensitivity of the *gst* real-time PCR for detecting *D. immitis* microfilarial DNA. Plasmid DNA template between 0.01 $\text{fg}\mu\text{l}^{-1}$ and 100 $\text{fg}\mu\text{l}^{-1}$ from left to right, equivalent to about 2.97×10^0 to 2.97×10^4 copies, were used to construct the standard curve.

Comparison of microfilaria DNA extraction methods

We compared the effectiveness of three different microfilaria DNA extraction methods by using *D. immitis* microfilarial DNA as a template to detect *gst* gene. The Light Cycler PCR SYBR green assay combined with melting curve analysis of the qPCR product were selected for the microfilaria gene detection. The melting curve analysis is shown (Figure 4): the mean melting temperature \pm SD averaged 79.98 ± 0.035 .

Three methods (alkaline lysis, MagNA-Pure, and phenol-chloroform) have different efficiency in microfilaria DNA extraction. The average crossing point (CP) values (correlate inversely with the copy

number of starting template), copy number of microfilariae, and *gst* copy number/microfilaria of each method are demonstrated (Table 1).

One microfilaria's CP of the alkaline lysis method (33.41 ± 1.07) was significantly ($P = 0.03$) lower than those of the MagNA Pure method (38.01 ± 0.29). One hundred microfilariae's CP of the alkaline lysis method (26.61 ± 0.03) and MagNA Pure method (27.34 ± 0.29) were significantly ($P = 0.007$ and 0.01) lower than those of the phenol-chloroform method (32.52 ± 0.75). These results suggested that a high concentration of microfilarial DNA could be obtained from the alkaline lysis method.

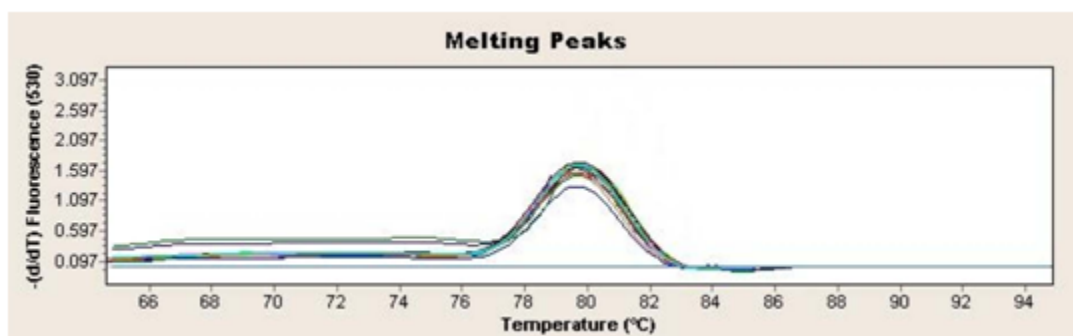


Figure 4. The melting curve analysis of the amplification products of *gst* DNA from *D. immitis* microfilaria.

Table 1. Efficiency of DNA extraction methods as evaluated by a *D. immitis* *gst* real-time PCR on the Roche LightCycler.

Extraction method	Average crossing point and copy number of different number of microfilariae per reaction						Cost (\$) (Baht)*	Time used (min)
	Crossing point (CP)			<i>gst</i> copy number				
	0.025	0.25	2.5	0.025	0.25	2.5		
Alkaline lysis	33.41	30.53	26.61	8.77	49.1	597	0.05 (1.65)	20
MagNA Pure	38.01	33.08	27.34	0.43	11.5	377	2.40 (79.39)	90
Phenol-chloroform	ND	35.51	32.52	ND	2.07	14.7	0.12 (3.97)	120

*** 1 US \$ = 33.08 bahts (January 19, 2010)

ND = Not determined

Cost and time analysis

Alkaline lysis and phenol-chloroform methods were manual, whereas MagNA Pure was automated. The cost per sample for the MagNA Pure method was the most expensive (\$2.40) and required 90 min to complete. On the other hand, the alkaline lysis method requires the short length of time to complete (20 min) and it is also the least expensive (less than \$0.05 per sample for 0.2 ml thin-wall PCR tube). The phenol-chloroform method requires two hours and cost the same as the alkaline lysis method (\$0.12 per sample) (Table 1). In contrast, the phenol-chloroform method is more time-consuming than the alkaline lysis method by six folds.

Discussion

Quantitative real-time PCR (qPCR) using either SYBR green^(16,26) TaqMan probes^(9,11-13) or fluorescence resonance energy transfer (FRET) hybridization probes^(10,14) have been used previously for detection of DNA from filarial parasites in human and mosquito samples. Multiple copies^(12,13) of the

filarial genes, such as *Sspl* and *Hhal*, were selected for the species-specific, *W. bancrofti* and *B. malayi*, respectively. In this study, the specific single-copy *D. immitis* *gst* gene, located on an exon was used to detect the copy number of heartworm microfilariae, using SYBR green assay. This system could be used to study the gene copy numbers per microfilaria⁽¹⁶⁾ and *gst* gene expression in *D. immitis* using real-time reverse transcription PCR technique.

The alkaline lysis DNA extraction method combined with qPCR SYBR green assay used in this study has a better sensitivity than QIAamp DNA extraction kit followed by the Eclipse minor groove binding probe (MGB) qPCR assay tested in *B. malayi*. From this study, the qPCR could detect as little as 0.025-microfilaria using the alkaline lysis technique. The detection limit of this method was 3.77 - 5.97 copies (0.025 microfilaria multiples by 150.8-238.8 copies per microfilaria) or about 0.01 fg whereas the Eclipse MGB qPCR assay with DNA extraction kit has a detection limit of 0.1 fg, or approximately 22 *Hhal* copies.⁽¹²⁾

The CP results of one microfilaria found in our study, (alkaline lysis method, 33.41 ± 1.07 and MagNA Pure method, 38.01 ± 0.29 , $P = 0.03$) and CP results for 100 microfilariae (alkaline lysis method, 26.61 ± 0.03 and MagNA Pure method, 27.34 ± 0.29 , $P = 0.007$) showed a statistically significant difference. The previous literature described that DNA recovery was reduced when using the MagNA Pure LC total nucleic acid isolation kit. This extraction assay may have a structurally impaired DNA recovery, resulting in a loss of sensitivity in molecular diagnostic testing.⁽²⁷⁾

Circulating *D. immitis* microfilariae are seldomly found in infected cats. When microfilariaemias do develop in cats, they appear only about one week later than in dogs (195 days postinfection at the earliest), but seldomly persist beyond 228 days postinfection. The absence of microfilariae may be due to low or single-sex worm burdens or immune clearance of microfilariae and perhaps a reversible suppression of microfilariae production.^(2,28) The female filarial 'free' DNA, which is released when they deliver a number of microfilariae, and/or the microfilarial 'free' DNA that is released during interaction with the host immune response⁽²⁸⁾ should be used as a novel target for canine and feline dirofilariasis diagnosis.

Previously, cases in human pulmonary dirofilariasis patients were surgically mistaken for lung cancer.⁽²⁹⁾ The coin lesion of this disease was differentially diagnosed from primary or metastatic neoplasia, fungal infections, and tuberculosis using lung biopsy followed by histopathological examination.⁽³⁰⁾ The human pulmonary dirofilariasis' lung parenchyma DNA extraction, combined with the

qPCR assay, could be of benefit for the zoonotic dirofilariasis definitive diagnosis.

In conclusion, we demonstrate that the alkaline lysis DNA extraction method, a simple, rapid, reproducible, and economical in-house method is a useful approach for the diagnosis and species differentiation of animal filarial parasites.

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