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Comparison of commercial RNA extraction kits and qPCR master mixes for studying gene expression in small biopsy tissue samples from the equine gastric epithelium

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Gastric tissue biopsy and gene expression analysis are important tools for disease diagnosis and study of the physiology of the equine stomach. However, RNA extraction from gastric biopsy samples is a complex procedure because the samples contain low quantities of RNA and are contaminated with mucous protein and bacterial flora. The objectives of these studies were to compare the performance of RNA extraction methods and to investigate the sensitivity of commercial qPCR master mixes for gene expression analysis of gastric biopsy samples. Three commercial RNA extraction methods (TRIzol™, GENEzol™ and MiniPrep™) and four qPCR master mixes with SYBR® green (qPCRBIO, KAPA, QuantiNova, and PerfeCTa) were compared. RNA qualification and quantitation were compared. Real-time PCR was used to compare qPCR master mixes. The results revealed that TRIzol and GENEzol obtained significantly higher yield of RNA (P<0.01) but that TRIzol had the highest contamination of protein and DNA (P<0.05). Conversely, MiniPrep resulting in a significantly higher purification of RNA (P<0.05) but provided the lowest yield of RNA (P<0.01). For PCR master mixes, KAPA was significantly (P<0.05) more sensitive than other qPCR kits for all amounts of DNA template, particularly at the lowest amount of cDNA. In conclusion, GENEzol is the best method to obtain a high RNA yield and purification and it is more cost-effective than the others as well. Regarding the qPCR master mixes, KAPA SYBR qPCR Master Mix (2x) Universal is superior to the other tested master mixes for studying gene expression in equine gastric biopsies.

Key words: *gastric biopsy, horse, qPCR master mix, RNA extraction*

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Gastric tissue biopsy and gene expression analysis of biopsies are commonly used for disease diagnosis in the equine stomach [5, 13]. They are also important for research, as they enable investigate of the physiology of the normal stomach and pathology of gastric diseases such as ulcer and cancer [7, 10]. A large quantity of high-quality and quantity of RNA is required to perform such qualitative and quantitative analysis of gene expression [9]. However, preparation of RNA from gastric biopsy sample is not easy due to their enrichment with gastric mucous proteins and bacterial flora, as well as the limited amount of available tissue [19]. Moreover, the quantity and quality of purified RNA is critical for

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gene expression analysis methods such as polymerase chain reaction (PCR), microarray, and RNA sequencing [15]. Real-time PCR or quantitative PCR (qPCR) is commonly used to study gene expression of gastric cells and gastric microbes due to it being more accurate and cost-effective than microarray or RNA sequencing [3, 7].

Two common methods used to extract RNA from tissues are the phenol/chloroform-based method and the spin column-based method. The phenol/chloroform-based method uses a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously solubilizes biological material and denatures protein. After adding chloroform, it causes phase separation, where protein, DNA, and RNA are separated [12]. TRIzol Reagent is the most well-known phenol/chloroform-based method for isolating ordinary RNA and small RNAs [12]. Spin column-based nucleic acid purification is a solid phase extraction method used to quickly purify nucleic acids. This method relies on the fact that nucleic acid can bind to the solid phase of silica under certain conditions [17].

Gastric biopsy tissue contains limited amounts of RNA and is often highly contaminated; therefore, the optimal RNA extraction method should obtain a high yield of RNA but with little or no DNA and protein contamination. Furthermore, Taq DNA polymerase and PCR buffer are also critical when studying gene expression using very small samples with limited RNA concentrations, such as gastric biopsy samples. Several studies have shown that individual Taq DNA polymerases and commercial PCR master mixes are different in their amplification efficiency, sensitivity and resistance to PCR inhibitors [1–6]. Our study aimed to compare commercial RNA extraction kits and qPCR master mixes in order to find the most suitable methods and products for studying gene expression in equine gastric biopsies. The results may be useful for studying gastric ulcer and cancer, which can be found in Thailand where the prevalence of gastric ulcer in Thoroughbred horses is 43% [16].

Materials and Methods

Gastroscopic biopsy

This research project was approved by the Animal Care and Use Committee of Faculty of Veterinary Science, Mahidol University. Eighteen thoroughbred horses (5–7 years old) from a polo club were used in these studies. Food and water were withheld from the horses before performing gastroscopic biopsy. Thereafter, the horses were sedated with 0.5–1 mg/kg xylazine hydrochloride intravenously and restrained with a nose twitch. A video gastroscope (model GVE 2100A2, Huger Medical Instrument, Shanghai, China) was inserted through the nose, and then biopsy forceps were inserted via endoscopic biopsy channel. A tissue biopsy sample was collected at the non-glandular part of the stomach, kept on dry ice for transportation, and then stored at −80°C until further analysis. Tissues were individually extracted for RNA collection (six samples for each RNA extraction method).

RNA extraction

Before RNA extraction, tissue biopsy samples were trimmed to produce samples of equal size (approximately $3 \times 3 \times 1$ mm^3). Three commercial products for RNA extraction were compared. ZR RNA MiniPrep™ (catalogue #R1064, Zymo Research, CA, U.S.A.) a spin column-based RNA purification method; TRIzol™ reagent (catalogue #15596026, Invitrogen, ThermoFisher Scientific, Waltham, MA, U.S.A.) and GENEzol™ reagent (catalogue #GZR100, Geneaid Biotech, New Taipei, Taiwan). The latter two are phenol/chloroform-based RNA purification methods.

ZR RNA MiniPrep

Tissues were lysed with 500 *µl* of lysis buffer before centrifugation at 12,000 × g for a min. The lysate was transferred to a column/collection tube to be centrifuged at 8,000 × g for 30 sec, and the flow-through was retained. Thereafter, 0.8 volumes of absolute ethanol were added to the flow-through before it was transferred to the new column, and the column was centrifuged at 12,000 × g for a min. Then the flow-through was discarded and 400 *µl* of RNA Prep Buffer was added to the column. The sample was centrifuged at 12,000 × g for a min, and the flow-through was discarded. Then 800 *µl* of wash buffer was added to the column, centrifuged at 12,000 × g for 30 sec, and the flow-through was discarded. Subsequently, 400 *µl* of wash buffer was added to the column, the column was centrifuged at 12,000 × g for 2 min, and the flow-through was discarded. To elute RNA, 25 *µl* of RNase-free water was added, and the sample was incubated for a min before centrifugation at 10,000 × g for 30 sec.

TRIzol and GENEzol reagent

One thousand microliters of either TRIzol reagent or GENEzol reagent was added to the biopsy samples, which then incubated for 5 min. Then, 0.2 m*l* of chloroform were added and then shaken for 15 sec. Samples were incubated for 2 min and then centrifuged at 12,000 × g for 15 min at 4°C. The aqueous phase was removed and placed in a fresh tube for RNA precipitation. Thereafter 2 *µl* of Pellet Paint Co-Precipitant (EMD Millipore, MA, U.S.A.) and 0.1 volumes of 3 M Na acetate (pH5.2) were added. Then one volume of isopropanol was added, the samples were briefly vortex, and they were incubated for 2 min. They were then centrifuged at 14,000 × g for 5 min. The supernatant was subsequently removed and the pellet was rinsed with 500 *µl*

Table 1. QPCR primer sequences

Name		Sequence
Genomic *GAPDH*	Forward	5′-CATCAAATGGGGCGATGCTG-3′
	Reverse	5′-CATCCACGGTCTTCTGGGTG-3′
Genomic *ACTB*	Forward	5′-GTCACCAACTGGGACGACAT-3′
	Reverse	5′-ATGTCACGCACGATTTCCCT-3′
GAPDH mRNA	Forward	5′-CACTGAGGACCAGGTTGTCT-3′
	Reverse	5′-GGGTCAAGTTGGGACAAGCA-3′
ACTB mRNA	Forward	5′-ATGATGATATCGCCGCGCTC-3′
	Reverse	5′-CCACCATCACGCCCTGG-3′

of 70% ethanol, vortexed, and centrifuged at 14,000 × g for 5 min. Then the supernatant was removed, and the pellet was washed with absolute ethanol and centrifuged at 14,000 × g for 5 min. After the supernatant was removed and the pellet was air-dried for 5 min. Finally, the pellet was resuspended with 25 μl of RNase-free water.

Measurement RNA concentration and contaminations

RNA concentration was measured using a Nanodrop system (ThermoFisher Scientific). The 260/280 ratio calculated from Nanodrop software was also used to evaluate protein contamination. Genomic DNA (gDNA) contamination of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and actin beta (*ACTB*) genes was relatively quantified by real-time PCR using RNA templates. The primers for detecting gDNA of *GAPDH* and *ACTB* were designed to span an intron sequence to allow them to identify the presence of gDNA contamination in the sample.

Comparison of qPCR master mixes

RNA extracted from ZR RNA MiniPrep was used to compare the efficiencies of the four real-time PCR master mixes. Complementary DNA (cDNA) was generated using SuperScript® reverse transcriptase from a SuperScript VILO cDNA synthesis kit (Invitrogen, ThermoFisher Scientific).

Four commercial qPCR master mixes (containing Taq polymerases and buffer) were compared, including qPCRBIO SyGreen Mix (PCR Biosystems, London, U.K.), KAPA SYBR FAST qPCR Master Mix (2x) Universal (KAPA Biosystem, Wilmington, MA, U.S.A.), QuantiNova SYBR Green PCR Kit (Qiagen, Hilden, Germany), and PerfeCTa SYBR Green SuperMix (Quantabio, Beverly, MA, U.S.A.). These commercial kits are commonly used in Thailand. PCR reactions were prepared according to the product recommendations. cDNA was relatively quantified using real-time PCR.

Real-time PCR

In each case, the total reaction volume was 25 μl. A CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, U.S.A.) was used for all reactions with the following PCR amplification program; 3 min at 95°C for activation, 3 sec at 95°C for denaturation, and 25 sec at 60°C for annealing; PCR was performed for 40 cycles. Melting curve analysis was used to identify specific products increasing in 0.5°C increment every 5 sec from 60 to 95°C. Relative quantity (delta Ct) and melting curve analysis were carried out using the Bio-Rad CFX manager 3.1 software (Bio-Rad). PCR Primers were designed using the Primer-BLAST software [20] and are shown in Table 1. Primers for detecting mRNA of *GAPDH* and *ACTB* were designed to span an exon-exon junction as in our previous publication [18].

Statistics

Data are shown as mean ± SD, and the significant difference was calculated using one-way ANOVA and multiple comparison with LSD using SPSS 21 software (IBM, NY, U.S.A.).

Results

RNA concentration and 260/280 ratio

Among the three RNA extraction methods, TRIzol reagent yielded the highest concentration of RNA, and followed by GENEzol and ZR RNA MiniPrep, respectively (Table 2). The RNA yields from TRIzol reagent were very significantly different from those from ZR RNA MiniPrep (P<0.01), but they were not significantly different from those from GENEzol reagent (P>0.05).

The 260/280 ratio is commonly used to evaluate the protein contamination, and a value of 2.0 is considered high purification. The 260/280 ratio of RNA extracted from ZR RNA MiniPrep was highest and closest to 2.0, followed by GENEzol and TRIzol. The 260/280 ratio of each method was significantly different from those of the others (P<0.05) (Table 2).

Genomic DNA (gDNA) contamination

The relative quantities of gDNA contamination in the

Table 2. Comparison of the RNA extraction kits for RNA concentration and 260/280 ratio

Extraction method	RNA concentration (ng/μl)	260/280 ratio
ZR RNA MiniPrep	9.70 ± 3.92[a]	1.98 ± 0.27[c]
TRIzol Reagent	37.5 ± 12.12[b]	1.63 ± 0.04[d]
GENEzol Reagent	28.45 ± 8.68[b]	1.71 ± 0.04[e]

[a, b]Very significant difference (P<0.01). [c–e]Significant difference (P<0.05).

samples are shown in Fig. 1. The gDNA contamination as measured by the *GAPDH* and *ACTB* genes was highest for TRIzol reagent followed by GENEzol reagent and ZR RNA MiniPrep, respectively. The gDNA contamination of the *GAPDH* gene from TRIzol reagent was significantly higher (P<0.01) than that from ZR RNA MiniPrep (approximately 237 times) and GENEzol reagent (about 9.72 times). Similarly, the gDNA contamination of the *ACTB* gene from TRIzol reagent was also significantly higher (P<0.01) than that from ZR RNA MiniPrep (approximately 142 times) and GENEzol Reagent (approximately 16 times).

Comparison among the four qPCR master mixes

RNA extracted from ZR RNA MiniPrep was selected in this experiment because it provided the best quality of RNA. Four commercial real-time qPCR master mixes were used to test the sensitivity of detection of *ACTB* and *GAPDH* gene expression in equine gastric biopsy samples. Use of 500 ng of cDNA template was sufficient to detect *ACTB* gene expression in all qPCR master mixes, but the sensitivity of KAPA was very significantly higher (P<0.01) than the others. At 500 ng of cDNA template, QuantiNova, qPCRBIO, and PerfeCTa were not significantly different in detecting *ACTB* gene expression. After reducing the cDNA template to 100 ng, only the qPCRBIO master mix was unable to detect *ACTB* gene expression. At 10 ng of cDNA template, only QuantiNova and KAPA were able to detect *ACTB* gene expression, but the sensitivity of KAPA was very significantly higher (P<0.01) than that of QuantiNova (17 times higher) (Fig. 2).

For *GAPDH* gene expression, all master mixes were able to detect expression even at the lowest amount of cDNA template (10 ng). At 500 and 100 ng, KAPA was highly significantly different from the others (P<0.01). QuantiNova, qPCRBIO, and PerfeCTa were not significantly different (P>0.05) in detecting *GAPDH* gene expression at 500 and 100 ng of cDNA template. At 10 ng of cDNA template, KAPA was significantly different from QuantiNova (P<0.05), and QuantiNova was also significantly different to qPCRBIO and PerfeCTa (P<0.01) (Fig. 3).

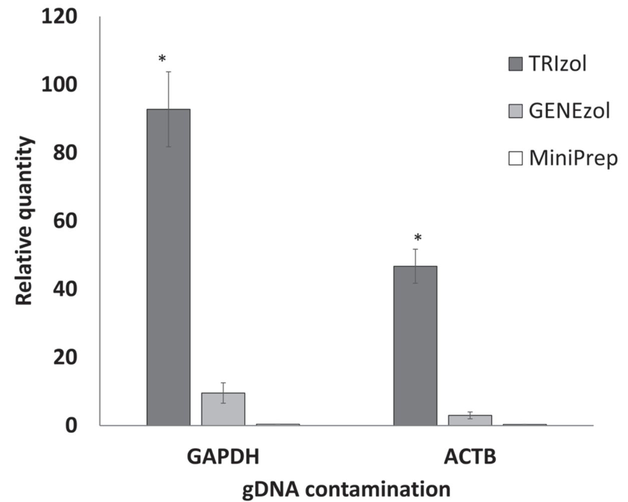

Fig. 1. Relative quantity of gDNA contamination of *GAPDH* and *ACTB* genes in RNA samples extracted using three methods. Comparison between ZR RNA MiniPrep, TRIzol, and GENEzol. *Very significant difference (P<0.01).

Discussions

Our studies revealed differences between commercial RNA extraction kits and qPCR master mixes for studying gene expression in equine gastric biopsy samples. We were able to demonstrate advantages and disadvantages of each RNA extraction method for equine gastric biopsies. The spin column-based method (ZR RNA MiniPrep) provided better RNA quality (purity), whereas phenol/chloroform-based methods (GENEzol and TRIzol) yielded better quantities of RNA. Similarly, previous reports have revealed that each RNA extraction kit for small tissue is different [14, 19], particularly the report of a study that examined methods to extract RNA from human gastrointestinal biopsies, which showed that TRIzol reagent yields a higher RNA concentration compared with a spin column-based method (RNA easy kit, Qiagen) but has a lower RNA purity [19]. A combination of two methods can improve the results, giving higher yields and high purification but with increased costs [19]. Although DNase can be additionally used after RNA extraction to reduce DNA contamination in small samples and can provide a better RNA quality for studying gene expression, it also increases the cost, which can become substantial if a large number of samples are to be analysed [8]. In this study, RNA extracted from TRIzol contained excessive gDNA and protein contamination, and this RNA quality was not good enough to perform RT-qPCR. Therefore, it required further steps such as column-based RNA extraction and/or DNase treatment to enhance the RNA quality. Conversely, the use of ZR RNA MiniPrep and GENEzol resulting in extremely

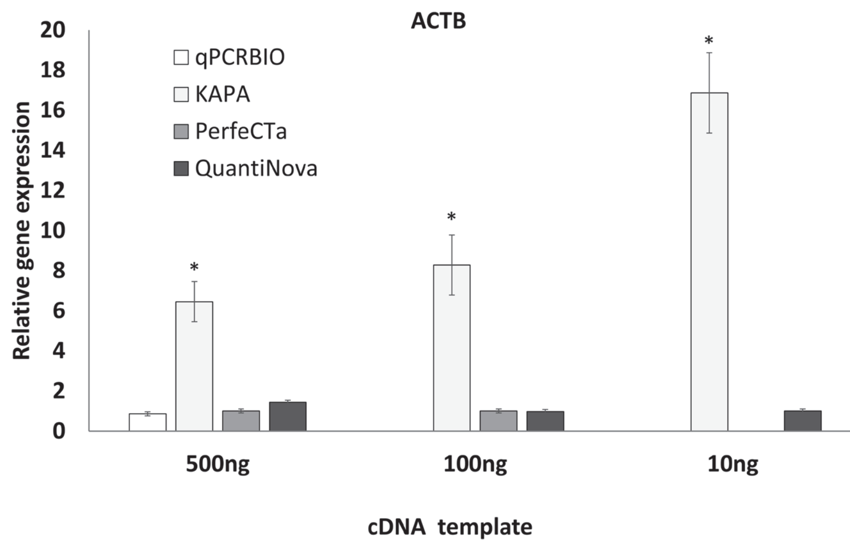

Fig. 2. Relative gene expression of *ACTB* using varying amounts of cDNA template. Comparisons were made between four commercial qPCR master mixes. *Very significant difference (P<0.01).

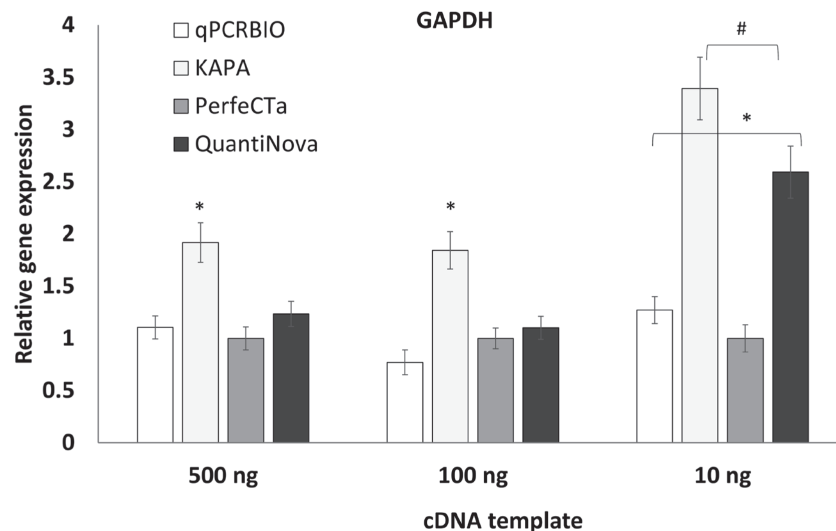

Fig. 3. Relative gene expression of *GAPDH* using varying amounts of cDNA template. Comparisons were made between four commercial qPCR master mixes. *Very significant difference (P<0.01). #Significant difference (P<0.05).

low gDNA contamination; thus, DNase treatment might not be required, but it should be used as an additional complementary treatment to assure there is no gDNA contamination in a sample to interfere with the RT-qPCR reaction.

Although, the TRIzol and GENEzol reagents are basically phenol/chloroform-based solutions for extracting RNA, they are commercially modified and differ from the original reagents [2]. Our results showed that the TRIzol and GENEzol reagents were comparable in term of yielding RNA but that GENEzol performed better in term of RNA purity. Additionally, GENEzol reagent was more economical to use, in terms of cost, than TRIzol reagent and ZR RNA MiniPrep. A disadvantage of a phenol/chloroform-based method to extract RNA from a small amount of tissue is that the RNA pellets are difficult to visualise and can often require co-precipitation in order to facilitate visualisation of

the pellets. A high amount of gDNA contamination from this method requires more steps including addition of DNase and incubation of the samples. Phenol and chloroform are also highly toxic chemicals, requiring specialist waste management, and use of these chemicals may be prohibited in some laboratories. Taken together, the spin column-based method is more convenient and safer than the phenol/chloroform-based method, but it is more expensive.

Our results also revealed a difference in sensitivity among the qPCR master mixes. KAPA master mix was found to be superior in performance to the other qPCR master mixes in this study. Similarly, previous studies have also revealed a substantial difference in detection efficiency using commercial DNA polymerase kits [1, 11]. Furthermore, we previously found KAPA SYBR FAST qPCR Master Mix (2x) Universal to be better for detecting DNA in single sperm analysis than SYBR greenER qPCR SuperMix (Invitrogen, ThermoFisher Scientific) or ViPrimePLUS qPCR Master Mix (Vivantis, Selangor Darul Ehsan, Malaysia) [4]. The difference in sensitivity among the qPCR master mixes may be due to the different types of Taq DNA polymerases and buffers used. Each company develops their own engineered Taq DNA polymerase and buffer, and they are different in term of properties and application.

Based on these observations, we propose that for equine gastric biopsies GENEzol and TRIzol are comparable methods of yielding a high RNA concentration, while ZR RNA MiniPrep is better for RNA purification. Notably, GENEzol provides a more economical option in terms of financial cost, with a high RNA yield and good quality of RNA purification. Taken together, we suggest that GENEzol is the best kit for RNA extraction for equine gastric biopsy. Regarding the qPCR master mixes, KAPA SYBR FAST qPCR Master Mix (2x) Universal is superior to the other tested mater mixes for studying gene expression in equine gastric biopsies.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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