# TECHNIQUE IN RNA ISOLATION OF DIGESTIVE TRACT FROM *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae)

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## ABSTRACT

*Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) or also known as Red Palm Weevil (RPW) is a common pest which cause serious problem in palms. Deep understanding towards physiology of its feeding system might provide meaningful insight in managing this pest infestation. This short note was aimed to isolate good quality RNA from salivary glands, midgut and hindgut of *R. ferrugineus* to be used for gene expression study. A total of four weevils were used to extract RNA from salivary gland while one weevil was used for RNA extraction from midgut and hindgut. Several key measures were taken during tissue harvesting and RNA extraction such as RNAse decontamination of working area, tissue storage in liquid nitrogen and RNA*later*® upon dissection and slight modification during tissue disruption. Total yield for RNA of salivary glands, midgut and hindgut were 6.9 µg, 46.1 µg and 7.2 µg in 20 µL respectively. Quality of the RNA from all tissues were also good with 260/280 ratio being above 2. In addition, Bioanalyzer analysis also gave high RIN number for all RNA. In conclusion, the techniques utilised for RNA extraction from salivary gland, midgut and hindgut of red palm weevil able to yield good quality and quantity of RNA.

**Keywords:** Red Palm Weevil, RNA quality, Insect pest, Invasive species

## ABSTRAK

*Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) atau dikenali sebagai Kumbang Merah Palma (RPW) merupakan perosak utama yang menyebabkan masalah serius di kalangan spesies palma. Kefahaman mendalam terhadap fisiologi sistem pemakanan mungkin memberi maklumat bermakna dalam menangani serangan perosak ini. Nota pendek ini bertujuan memencilkan RNA berkualiti dari kelenjar air liur, usus tengah dan belakang *R. ferrugineus* untuk digunakan dalam kajian pengekspresan gen. Sebanyak empat ekor kumbang digunakan bagi memencil RNA dari kelenjar air liur manakala seekor kumbanv digunakan bagi memencil RNA dari usus tengah dan belakang. Beberapa langkah penting dititikberat semasa pemencilan tisu dan pengekstrakan RNA, seperti dekontaminasi kawasan pemencilan dari RNAse, penyimpanan tisu di dalam cecair nitrogen serta pengubahsuaian kaedah pemecahan tisu. Jumlah RNA bagi kelenjar air liur, usus tengah dan belakang adalah 6.9 µg, 46.1 µg dan 7.2

µg in 20 µL. Kualiti RNA bagi kesemua tisu adalah baik dengam nisbah 260/280 melebihi 2. Selain itu, analisis Bioanalyzer turut memberi nilai RIN yang tinggi bagi kesemua RNA. Kesimpulannya, teknik yang digunakan bagi memencilkan RNA dari kelenjar air liur, usus tengah dan belakang mampu menghasilkan RNA berkualiti dan jumlah yang banyak.

**Kata kunci:** Kumbang Merah Palma, Kualiti RNA, Serangga perosak, Spesies invasif

## INTRODUCTION

Red palm weevil is a serious pest for various palm species (reviewed in Murphy and Briscoe 1999) which had caused severe damages in North-east Peninsular Malaysia mostly in Kelantan and Terengganu (Basari et al. 2011). Integrated Pest Management (IPM) has been recommended as the main approach to control red palm weevil infestation (Abraham et al. 1998). Due to this, further investigation on the weevil physiology, especially the digestive system is very much needed, as it can be exploited for IPM in future (Norzainih et al. 2015; Rosli et al. 2018).

In insect, the salivary glands, midgut and hindgut are important organs in studying regulation of feeding and digestion. Salivary glands (SG) are known to be associated with nutrient intake which involved in digestion and lubrication of food, where the structure and secretions have been modified to meet the different needs and feeding habits of insects (Ribeiro 1995). Midgut is the key player in food digestion as the cells lining the organ are active in enzymes production and secretion, as well as nutrient absorption (Chapman 1998). Deep understanding on genes regulation in these organs will help researchers to identify possible targets which can inhibit the digestion and thus, providing new alternative for red palm weevil biocontrol.

Experiment which involves identification and quantification of gene expression requires isolation of good quality RNA. However, this has become a challenging task for RPW due to its hard chitin exoskeleton and small tissue size which results in low RNA quantity. This problem is further complicated with known facts where intracellular RNA can be easily degraded *ex vivo* due to specific and non-specific nucleases (Takahashi et al. 2013). Since there is growing interest in studying red palm weevil physiology at molecular level, it is critical to document a standard and reliable RNA extraction protocol. Therefore, this technical note is aimed to extract good quality RNA from salivary gland, midgut and hindgut for gene expression analysis. Since there are no current reports on RNA evaluation from RPW insect, our first attempt focused on evaluation of the RNA quality and quantity. This followed by optimization on techniques during sample preparation and RNA isolation which are described in material and methods section.

## MATERIAL & METHODS

Adult red palm weevil was collected from several areas in Terengganu. All samples were maintained in cage with rearing room conditions maintained at 30±2°C, 60-80% relative humidity and fed *ad libitum* with fresh cut sugar cane, as described by Norzainih et al. (2015). All dissecting equipment was prepared as RNAse free (autoclaved and dry heat 80°C for 3 days). All working areas for RNA extraction were cleaned with 70% ethanol and RNAse AWAY (Thermo Scientific, USA) to remove any possible RNAse contamination.

For collection of salivary glands, midgut (MG) and hindgut (HG), red palm weevil was kept in refrigerator (4°C) for 30-40 minutes to immobilize the weevil during dissection procedure. After the weevil became immobile or less aggressive, wings and legs were removed from the body and opening was made on the dorsal surface from the thorax to the abdomen. A cut was also made on the spiracle line on both sides. The body then placed on a dissecting dish and pinned on each side to expose the digestive tract. Salivary glands were first taken out, followed by midgut and hindgut. Two phases of experiment were undertaken. For the first phase (Phase I), body fats, Malpighian tubules were removed in RNAse free PBS and stored in RNA*later*® (Ambion/Thermo Scientific, USA) at 4°C until RNA extraction.

For the second phase (Phase II), sample used was only from midgut and hindgut. Additional techniques were added which include removal of body fats, Malpighian tubules along with gut's content as much as possible by pinching the tissue carefully (without tearing the tissue). These steps were done in RNAse Free PBS. Cleansed tissues were transferred into 1.5 mL tube, flash frozen in liquid nitrogen and stored in -80°C until RNA extraction. Salivary glands from four RPW were pooled into one sample for RNA extraction while midgut and hindgut came from single RPW.

**RNA Extraction**

Salivary glands from four RPW were pooled into one sample for RNA extraction of salivary glands whereas single MG and HG was used each in phase I and phase II of the experiment. RNA extraction was done using InnuPREP RNA mini kit (Analytik Jena, Germany) according to the protocol given with slight modification during lysis step. In this step, the frozen tissue was thawed by the lysis buffer provided and meshed using RNAse free micropestle. The meshed tissue was dissolved in lysis buffer by vigorous vortex. Undissolved particles were removed via centrifugation at 11,000×g for 2 minutes. Subsequent RNA extraction steps were done as described in the protocol given which also included DNA removal using specific DNA removal column provided with the kit.

**RNA Quality**

Quality and quantity of extracted RNA was evaluated using NanoDrop (Thermo Fisher Scientific, USA) and 2100 Expert Bioanalyzer (Agilent, USA). RNA extracted from salivary glands were the only sample underwent short heating step at 55°C and were analysed first. Optimum parameter used in analysing RNA from salivary glands were used as standard for other sample. All RNA was stored in -80°C until use.

## RESULTS & DISCUSSION

Optimisation of RNA extraction was first done on sample from salivary glands. Results showed RNA yield of 345.8 ng/µL with 260/280 ratio above 2.0 (Table 1), indicating intact RNA. Further RNA quality evaluation using Bioanalyser however showed only single peak at 18s for heated salivary gland RNA sample. Insect RNA in general has a different profile in Bioanalyser where recent report showed single peak at 18s if undergo typical heat denaturation step (Fabrick & Hull 2017). Heat denaturation step will break the large subunit rRNA into two fragments, where the sizes are almost similar to 18s. This will cause the fragment to be resolved at similar position with 18s in Bioanalyzer profile (McCarthy et al. 2015; Winneback et al. 2010). From our observation, RNA sample from RPW is also sensitive to heat which causes the disappearance of 28s peak when sample was heated at 50°C while presence of 28s and 18s were distinctly observed when sample was not heated (Figure 1). These findings showed intact RNA was successfully isolated from salivary glands using techniques described above.

Table 1. Quality and quantity evaluation of extracted RNA using NanoDrop for salivary gland

| Sample | Nucleic Acid Concentration [ng/µl] | (260/280) | Total elution volume (µL) | Total RNA obtained (µg) |
|---|---|---|---|---|
| Salivary gland | 345.8 | 2.16 | 20 | 6.9 µg |

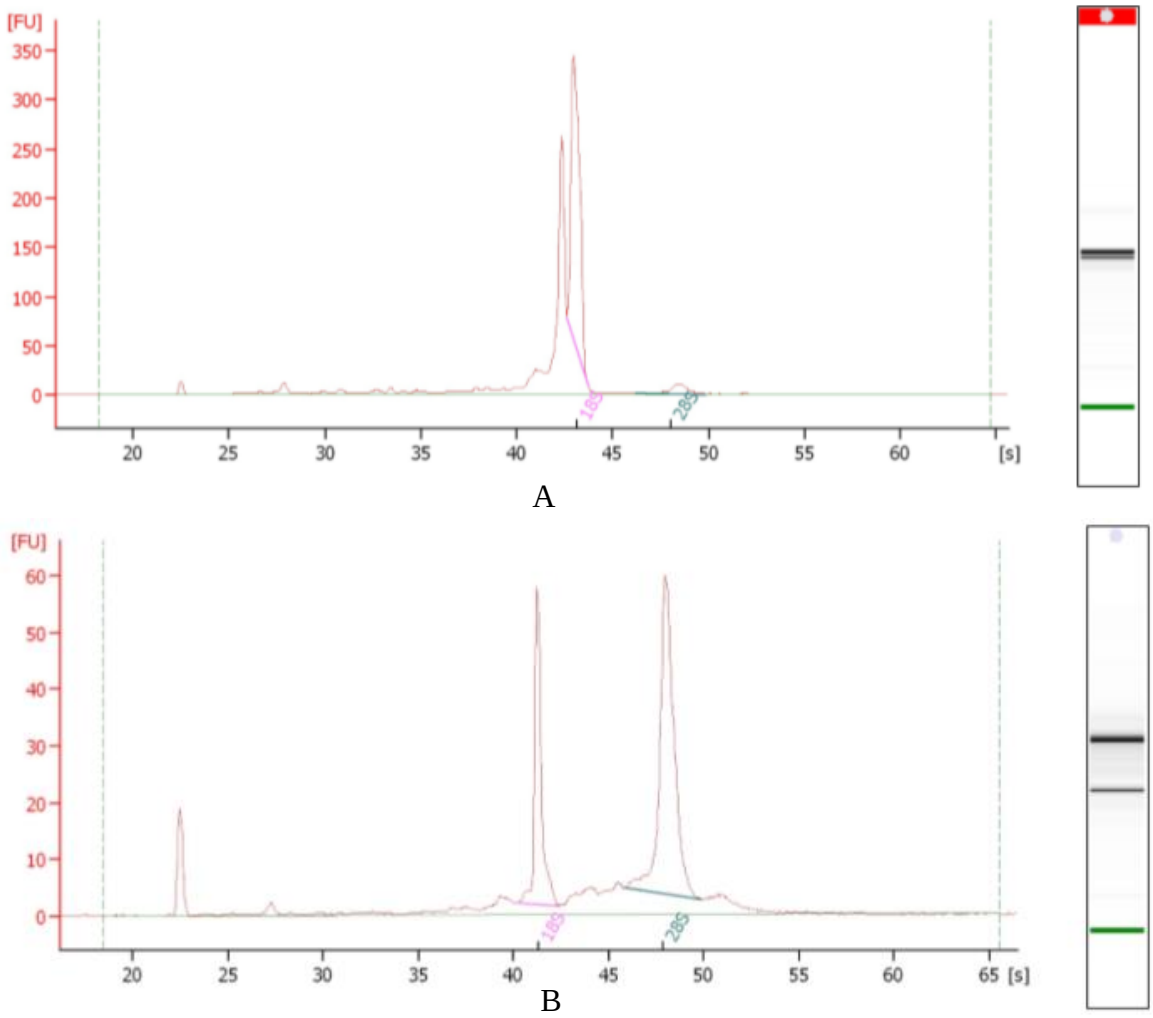


Figure 1. (A) Bioanalyzer electrophoregram and RNA band from salivary gland (heated) showing double peak at 18s. (B) Bioanalyzer electrophoregram and RNA band from salivary gland (non-heated), showing two distinct peaks of 28s and 18s.

High yield of RNA was also obtained for midgut and hindgut by utilising the same techniques used in RNA isolation from salivary glands. A total of 33.7 µg and 6.7 µg of RNA in 20 µL were obtained from midgut and hindgut respectively. Quality evaluation of RNA using 260/280 ratio were also highly satisfied (Table 2). The RNA yield for both midgut and hindgut are considered as good, compared to other studies which obtained a total of 20 µg from 50 mosquito guts (Kukutla et al. 2013). However, low reading of 260/230 ratio in hindgut sample indicates presence of impurities. Absorbance at 230 nm can detect substances such as Tris, EDTA, other buffer salts and proteins (Troutman et al. 2001). Any 260/230 ratio with range lower than 1.8 indicates presence of aforementioned components (Koetsier & Cantor 2019). Further assessment on all RNA isolated was done using BioAnalyser. Although RIN value (RNA Integrity Number) for midgut and hindgut were above 7.0, which is minimum RIN

value if the RNA is going to be used in downstream applications (Exiqon Services 2014), indication of impurities from 260/230 ratio has prompted further optimisation in RNA isolation process.

Table 2. Quality and quantity evaluation of extracted RNA using NanoDrop for midgut and hindgut using RNA*later*® as sample preservative.

| Sample | Nucleic Acid Concentration [ng/µl] | 260/230 | (260/280) | Total elution volume (µL) | Total RNA obtained (µg) |
|---|---|---|---|---|---|
| Midgut | 1684.1 | 1.83 | 2.21 | 20 | 33.7 |
| Hindgut | 336.9 | 1.10 | 2.23 | 20 | 6.73 |

The most common and efficient method for sample preservation before isolation process is by flash freezing in liquid nitrogen or by immersion in aqueous sulphate salt solution, such as RNA*later*®. These techniques able to stop enzymes activities or precipitate the enzymes which allow the RNA to stay intact (Mutter et al. 2004). However, insufficient washing when using RNA*later*® as sample preservation might also be the cause of the impurities observed earlier. Liquid nitrogen was not used in the initial parts of the study due to the small amount of sample to be used. In addition, sample recovery was done carefully to minimise sample loss that might subsequently lead to poor yield. Cryogenic grinding with mortar and pestle was previously correlated with poor yield due to loss of sample on the surface of the mortar (Dutta et al. 2019). However, our RNA yield was improved for midgut with total of 46.1 µg. The purity of RNA was also improved based on 260/230 ratio for midgut and hindgut (Table 3) when liquid nitrogen was used as sample preservative. Extraction with liquid nitrogen involves breaking deep-frozen cells mechanically using a mortar and pestle. Breaking the cell at ultra-low temperature inhibited enzymatic reactions thus proteins and nucleic acid were kept intact. A comparison done by de Heredia and Jansen (2004) on yeast lysis methods for RNA extraction has shown that RNA integrity was increased when using liquid nitrogen. Our finding showed that extraction of RNA utilising this method does not improved integrity as compared to when using RNA*later*® as preservative (Figure 2 & Figure 3). Steps after grinding might be the key to higher RNA integrity for liquid nitrogen based RNA extraction. We used RNAse free PBS to harvest sample before sample was stored in -80°C. Deleterious enzymatic reaction may have caused degradations of RNA while harvesting the sample several times from mortar to ensure thorough collection. In future, lysis buffer with RNAse inhibitor can be used directly to harvest sample from mortar instead of PBS with the aim to immediately inactivate deleterious enzymatic activities before samples fully thawed.

Table 3. Quality and quantity evaluation of extracted RNA using NanoDrop for midgut and hindgut using liquid nitrogen as sample preservative.

| Sample | Nucleic Acid Concentration [ng/µl] | 260/230 | (260/280) | Total elution volume (µL) | Total RNA obtained (µg) |
|---|---|---|---|---|---|
| Midgut | 2304.8 | 2.21 | 2.20 | 20 | 46.1 |
| Hindgut | 362.0 | 1.69 | 2.20 | 20 | 7.24 |

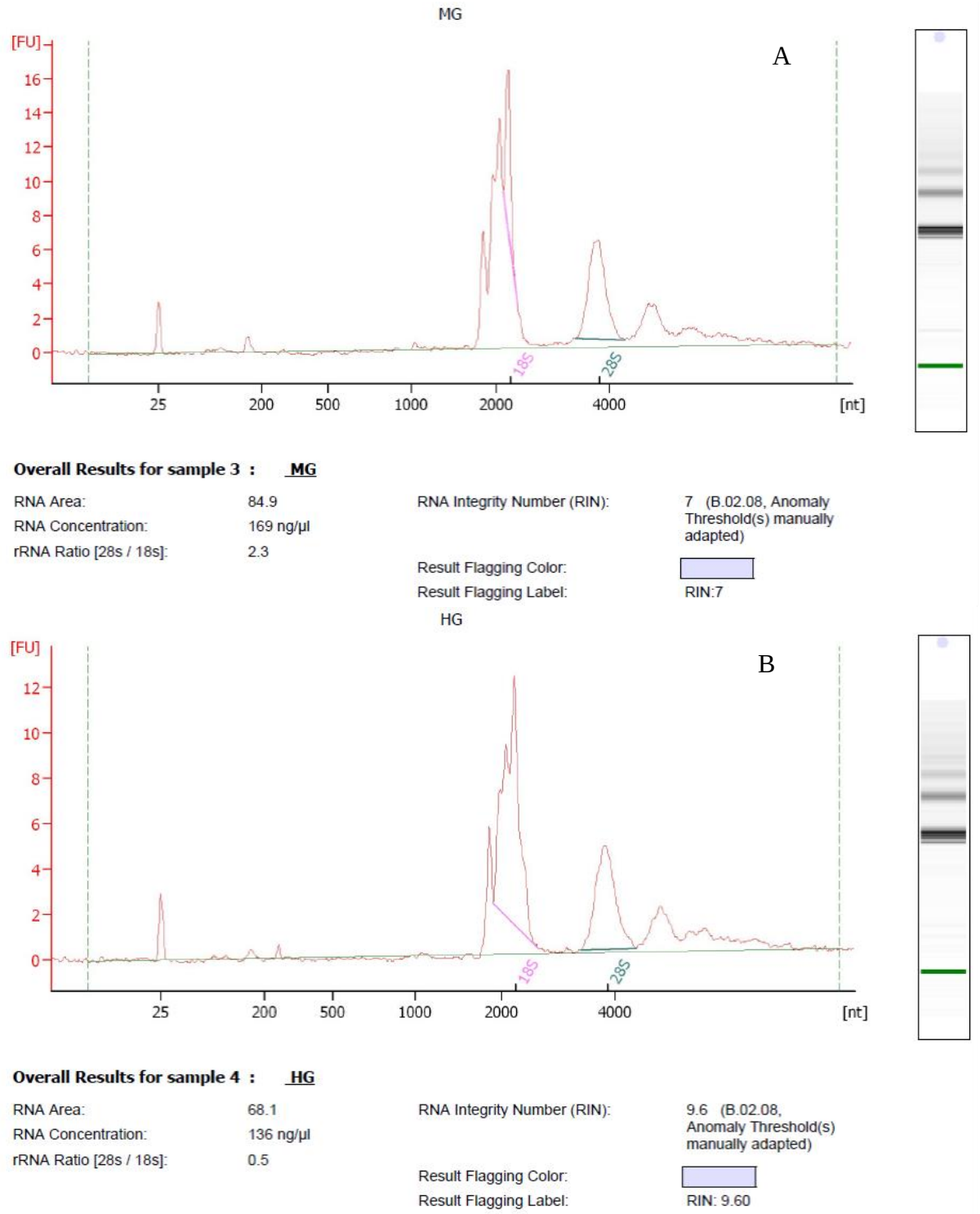


Figure 2. Bioanalyzer electrophoregram and RNA band from (A) Midgut and (B) Hindgut, using RNA*later*® as sample preservative All samples were not-heated prior to bioanalyser analysis.

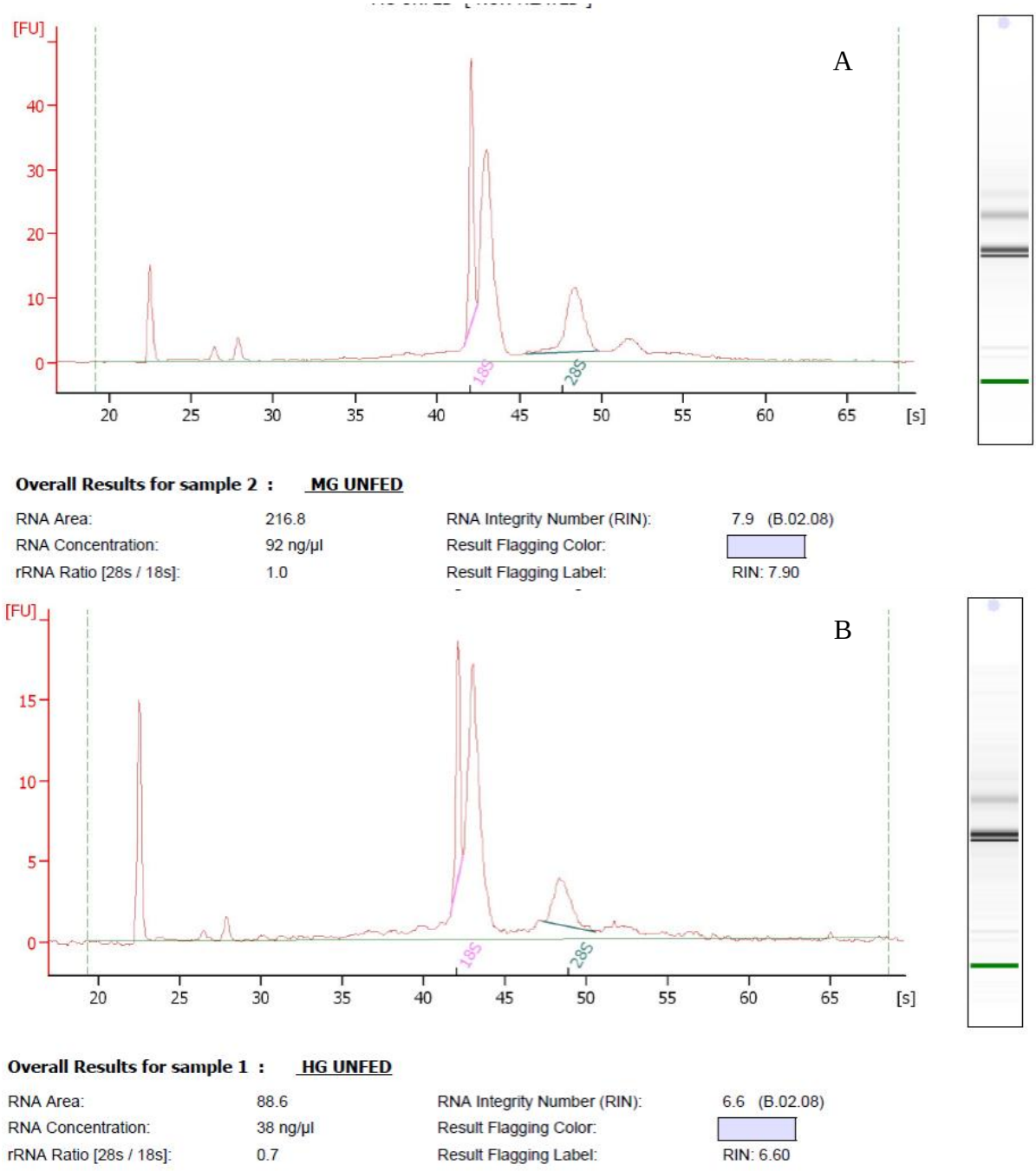


Figure 3. Bioanalyzer electrophoregram and RNA band from (A) midgut and (B) hindgut using liquid nitrogen as sample preservative

In conclusion, RNA extraction from salivary glands, midgut and hindgut of red palm weevil was successfully obtained from small sample number. Utilisation of liquid nitrogen as sample preservative combined with elimination of fats and guts' content able to produce RNA yield with good quality and quantity.

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