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Optimization of RNA Extraction from *Aedes aegypti* and *Aedes albopictus*

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ABSTRACT

RNA extraction is the critical initial stage in analyzing certain gene expressions, further analysis using Real Time PCR technology, and performing virus detection. However, the process of extracting RNA is often hampered by the risk of contamination, resulting in low concentrations of RNA and low purity of RNA. This is often an obstacle in extracting mosquito RNA especially detecting Dengue Virus (Den-V). Dengue virus (Den-V) can cause dangerous diseases in humans such as Dengue Fever (DHF) which is transmitted through the bites of Aedes aegypti and Aedes albopictus mosquitoes. This study aims to find out the effective steps for extracting RNA from Aedes aegypti and Aedes albopictus mosquitoes. The method being compared is a commercial RNA extraction kit with modification (addition of β-mercaptoethanol) and without modification. The results showed that the best DNA concentration and purity were obtained in mosquito samples from modified process. The purity ratio of RNA extracted without modification was 1.971 (0.021 ± 0.800) while with modification it was 2.003 (0.011 ± 0.112). Aedes aegypti had a better average concentration of 7.146 µg/ml for unmodified RNA and 7.613 µg/ml for modified RNA. This research is expected to be a reference for further studies on viruses in Aedes aegypti and Aedes albopictus.

Keywords: *RNA Extraction, Aedes aegypti, and Aedes albopictus*

INTRODUCTION

RNA extraction is the process of extracting or separating RNA from other biomolecules present in cells, such as proteins and DNA. RNA extraction is performed for research or diagnostic purposes. This process can be carried out by various methods, such as the phenol-acid method, the guanadinium buffer method or by commercially available kit and systems. The factors of a successful RNA extraction refer to: 1. Quality of materials used, 2. Method chosen, 3. Solubility of materials, 4. Processing speed, 5. Use of stabilizing agents, 6. Storage conditions during extraction, and 7. Effectiveness of immersion and wash cycle.

RNA Extraction is known as a crucial process, therefore researchers frequently optimize RNA extraction protocols. Among the researchers who did this included Rodriguez et al., 2015; Norollahi et al., 2018; Zhao et al., 2020; dos Santos Lucena-Leandro et al., 2022. Unfortunately, optimization of RNA extraction was not found in mosquito samples, especially *Aedes aegypti* and *Aedes albopictus*. The RNA extraction method becomes more varied if modifications are made to the method. Modification of RNA extraction is often done in the sample destruction process. The varied composition of the sample constituents causes the difference of destructive compounds. For example, the addition of β-mercaptoethanol in the sample preparation (destruction) process. The addition of β-mercaptoethanol is expected to produce a higher concentration of RNA isolates with a better purity ratio. This research is expected to be a reference in the study of *Aedes aegypti* and *Aedes albopictus,* which are known as Dengue virus (DenV) vectors. Dengue Virus (DenV) is a type of arbovirus of the genus Flavivirus, Family Flaviviridae, which causes dengue fever that mostly occurs in tropical and subtropical regions, with transmission by *Ae. aegypti* as the main vector and *Ae. albopictus* as a co-vector (Isna and Sjamsul 2021). This is because the *Ae. aegypti* prefer to be around residential areas so they will have more contact with humans (Sari et al., 2012). Optimization of this RNA extraction was used as the first step in our research on Dengue Virus Identification and Barcoding. The virus barcoding that is found will be useful as a step to prevent the spread of the virus and appropriate treatment measures.

METHOD

Materials and Tools

The sample used in this research are isolates of RNA taken from *Aedes* sp. The tools and materials used were insect nets, *cooler* boxes, plastic cups, cotton, gauze, rubber, ice gel, petri dishes, pin needles, insect tweezers, stereo microscopes, vials, mosquito samples, small corks, centrifuge, micro pipette, vortex mixer, analytics, microwave, 1.5 mL propylene tube, 0.5 mL PCR tube, permanent marker, water, molecular grade water (RNAse/DNase free), 70% ethanol, cotton, label paper, wipes, Eppendorf tube 1.5 cc. DNA extraction using

the Zymo RNA Extraction kit, personal protective equipment (masks, lab coats, gloves).

Sampling Method

Sampling of *Aedes* sp. carried out for 4 days from 28 June to 1 July 2022 at several points in UIN Sunan Ampel Surabaya. The points taken were in front of the twin tower building, in front of the library, multimedia building, and in front of the Faculty of Science and Technology building from the afternoon to evening. Sampling process is equipped with insect nets, when the mosquito was caught with the net then put it in a plastic cup covered with gauze and tied with rubber. *Aedes* spp mosquito samples which obtained were stored in a *cooler* box.

Mosquitoes Identification

The mosquito samples used were female *Aedes aegypti* and *Aedes albopictus*. Identification of the sex and species of mosquitoes was carried out using a stereomicroscope. The male type of mosquito has dense antennae, while the female has the opposite. Species identification can be seen through the pattern of the scutum, legs, and mesepimeron lines. *Aedes aegypti* has two curved lines, while *Aedes albopictus* only has one. The legs of *Aedes aegypti* have lines, while Aedes albopictus does not. There are two separate white dots of Mesepimeron on *Ae. aegypti*, whereas in *Ae. albopictus* the shape of the white dots on the mesepimeron tends to be irregular and combined to form the letter V. This identification step refers to Rueda (2004).

RNA Extraction

Reagent preparation was carried out by adding 96 ml of 100% ethanol (104 ml of 95% ethanol) to 24 ml of RNA Wash Buffer or concentrate (R1057) or 192 ml of 100% ethanol (208 ml of 95% ethanol) to 48 ml of Wash Buffer RNA concentrate (R1058), then added 275 µl DNase/RNase-Free Water per vial for slow inversion and then stored in the freezer at -20°C.

Sample preparation was carried out by preparing the sample, the sample was weighed and crushed, then 200 µl DNA/RNA shield (1x)3 was added. The sample was transferred to a micro tube and 300 µl DNA/RNA shield (1x)3 was added, then 50 µl PK Dig6estion Buffer solution was added, with addition of 25 µl proteinase-K. Then vortexed and incubated at 55°C for 30 minutes, vortexed again and then centrifuged at 10,000-16,000 x G for 2 minutes, transfered the supernatant to a new RNAse-free tube, added 500 µl of RNA lysis Buffer solution and vortexed. **Modifications** are made at this stage. The modification was carried out by adding 5 µl β-mercaptoethanol to the microtube along with the process of adding 50 µl PK Digestion Buffer solution and 25 µl proteinase-K.

RNA purification was carried out by transferring the sample into the Spin-Away Tube Filter (yellow) + microtubes and

centrifuging at 16,000 x G for 30 seconds. After that a supernatant (Flow through) will be produced, and 250 µl ethanol (95-100%) is added to the supernatant (Flow through). The supernatant (Flow through) is transferred to the Zymo-Spin IIICG Column' (green) + Collection Tube and centrifuged at 16,000 rpm for 30 seconds, then the supernatant (Flow through) was discarded, Zymo-Spin IIICG Column' (green) was added 400 µl RNA Wash Buffer and centrifuged at 16,000 x G for 30 seconds then the supernatant (Flow through) was discarded. After that, a microtube containing 5 µl DNase 1 (1 U/l) and 75 µl DNA Digestion Buffer was prepared and then vortexed. The solution was put into the Zymo-Spin IIICG Column' (green) + Collection Tube. Incubated at room temperature (20-30°C) for 15 minutes. 400 µl of RNA Prep Buffer was added to the Zymo-Spin IIICG Column' (green) and centrifuged at 16,000 x G for 30 seconds then the supernatant (flow through) was discarded. 700 µl RNA Wash Buffer was added to the Zymo-Spin IIICG Column' (green) and centrifuged at 16,000 x G for 30 seconds then the supernatant (flow through) was discarded. 400 µl RNA Wash Buffer was added to the Zymo-Spin IIICG Column' (green) and centrifuged at 16,000 x G for 2 minutes, discard the flow through. Carefully transfer the Zymo-Spin IIICG Column' (green) into the RNase-free tube. Add 100 µl DNase/RNase-Free Water directly to the matrix tube and centrifuge at 16,000 x G for 30 seconds. The eluted RNA can be used immediately or stored in the freezer (minus 70°C).

Spectrophotometry

Spectrophotometry was performed using a Biodrop spectrophotometer. In this study using a wavelength of λ 260/280 nm to see the concentration and purity of RNA in the sample.

RESULTS AND DISCUSSION

RNA extraction plays an important role in determining the success of further analysis. Detection of Dengue Virus in mosquito samples may be hampered due to the non-optimum RNA extraction process. The process which is not optimal can be seen from the low concentration of whole genome RNA produced, and the ratio of purity which is not at 1.8-2.0. The quality of the extracted total RNA was then measured using a spectrophotometer to determine its concentration and purity (Adiputra et al., 2012). Table 1 below shows the results of RNA extraction carried out on 2 samples of mosquitoes which are the main vectors of Dengue Virus, *Aedes albopictus* and *Aedes aegypti*.

Table 1. Isolate of RNA Spectrophotometry Result using Zymo KIT

Location	Sample	Identification Result	Purity λ260/280		Concentration (μg/ml)	
			Without Modification	Modified	Without Modification	Modified
1.1	1	*Ae. Aegypti*	1,000	1,803	7,451	7,924
1.2	2	*Ae. albopictus*	2,063	2,032	0,269	1,292
2.1	3	*Ae. Aegypti*	2,021	2,011	7,840	8,232
2.2	4	*Ae. albopictus*	2,041	2,021	9,019	9,470
3.1	5	*Ae. aegypti*	1,687	1,844	0,295	1,310
3.2	6	*Ae. albopictus*	2,089	2,045	1,381	1,450
4.1	7	*Ae. aegypti*	2,051	2,026	4,761	4,888
4.2	8	*Ae. albopictus*	2,224	2,112	4,507	4,732
5.1	9	*Ae. aegypti*	2,048	2,024	19,77	20,758
5.2	10	*Ae. albopictus*	2,063	2,035	7,451	7,924
6	11	*Ae. albopictus*	2,063	2,032	20,800	21,840
7	12	*Ae. albopictus*	2,072	2,036	1,623	1,704
8.1	13	*Ae. aegypti*	2,109	2,055	8,290	8,705
8.2	14	*Ae. albopictus*	1,807	1,904	1,075	1,129
9.1	15	*Ae. aegypti*	2,051	2,026	4,761	4,888
9.2	16	*Ae. albopictus*	2,166	2,083	1,858	1,851
10.1	17	*Ae. Aegypti*	2,109	2,055	4,002	4,202
10.2	18	*Ae. albopictus*	1,807	1,904	1,808	1,989
		Lowest Deviation	0,021	0,011		
		Highest Deviation	0,800	0,112		
		Average Deviation	0,116	0,034		

Source: Private document, 2023

The results of Table 1. show that the addition of β-mercaptoethanol gives better results than in the absence of β-mercaptoethanol. The purity ratio of the extracted RNA without modification was 1.971 (0.021 ± 0.800) while the extracted RNA with the modification was 2.003 (0.011 ± 0.112). The deviation value obtained in the RNA extraction without the addition of β-mercaptoethanol is quite large, reaching 0.800. The ratio of RNA purity is optimal in the range of 1.8-2.0.

The process and results of RNA extraction are also influenced by the condition of the tissue of origin, the age of the sample, the initial handling, the extraction technique chosen, the ability to pre-prepare, and the quality of the reagents used. The state of the tissue of origin is important because it will determine the amount of RNA available for extraction and its stability. The age of the sample is crucial because it can affect the amount and purity of the RNA. Preliminary treatments such as tissue milling, solvents, cutting tools, and other procedures are necessary to reduce the contact between proteolytic enzymes and RNA. Extraction techniques such as extraction with phenol-chloroform, detergent foam, or foam-based methods of electrophoresis are key factors in RNA extraction. Preparatory ability is essential to remove additional materials such as proteins, lipoproteins, and salts thereby increasing the purity of RNA. The quality of the

reagents used such as buffers, enzymes, and salts is crucial to ensure that RNA is not degraded during extraction.

This study compared the RNA extraction protocols used without modification and with modifications. Modification as a differentiator used is the addition of β-mercaptoethanol. The protocol used in this study was the **Spin Column Separator Extraction Technique**: This technique uses a special column filter to separate various types of biomolecules from biological samples. Usually used to purify and bind DNA polymerase in the synthesis of single-chain DNA polymerase (RT-PCR).

Extraction of RNA can be done in various ways, including: **Trichloroacetate Extraction:** Trichloroacetate Extraction is a method used to extract or separate RNA from other components such as protein, DNA, and other metabolites. This method is carried out by giving a trichloroacetic compound to the sample to be extracted. (Lecturio, 2023). **Guanidinium carboxylate extraction**: Guanidinium carboxylate extraction is a method used to extract and purify RNA from biological samples. This method is carried out by giving guanidinium carboxylate to the sample to be extracted. (Bioforum, 2023). **Magnetic Column Extraction**: Magnetic Column Extraction is a method used to purify RNA from biological samples. This method is carried out by using magnetic particles that are flowed by biological samples. Magnetic particles will interact with components such as RNA and will be suspended above the surface of the column. Then, the RNA can be separated from the other components with an eluent (Vector, 2023). **Tri Extraction Reaction**: This method is used to extract RNA by using lysosomal proteins and ionic liquids. **Chloroform Eluent**: This process is used to extract various types of RNA including mRNA, rRNA and tRNA. With this technique, RNA is separated from the DNA component contained inside it. **Sac-Lakes method**: This is one of the most popular methods for extracting mRNA. This method requires a thermolabile phosphatase enzyme which helps remove phosphoric acid from the RNA chain to allow later separation. **DNase I method**: This is the best method when it comes to extracting mRNA or tRNA from eukaryotic cells. The DNase I enzyme is used to break down the DNA chain so as to allow the separation of RNA from the other components. **Phenol-Chloroform Method**: This method is commonly used to extract various types of RNA including mRNA, sRNA, tRNA, and ribosomal RNA. This technique also reduces DNA contamination in the mixture. **Tri-reagent Technique**: This technique involves mixing, precipitation and purification to extract total RNA from cellular extracts. This is known as the 'triumvirate' method of dilution with alkalis (Thermofisher, 2023). **Extraction of RNA without Tannins**: This technique uses special chemicals that bind nucleotides and proteolytic (breakdown of proteins) to break down the cell membrane, allowing RNA to be

easily absorbed into the solution. (Thermofisher, 2023).

Table 2. Average Value of DNA Purity and Concentration from 2 Species

Species	Purity λ260/280		Concentration (μg/ml)	
	Wihout Modification	With Modification	Wihout Modification	With Modification
Ae. aegypti	1,885	1,981	7,146	7,613
Ae. albopictus	2,040	2,020	4,979	5,338

Source: Private document, 2023

Based on Table 2, it can be concluded that *Aedes aegypti* has a better average purity ratio than *Aedes albopictus*. The concentration values obtained also showed similar results. *Aedes aegypti* had a better average concentration of 7.146 μg/ml for unmodified RNA and 7.613 μg/ml for modified RNA. It is not yet clear what the different content between the two species makes the extraction of *Aedes agypti* easier to do than *Aedes albopictus*. Identification of *Aedes agypti* and *Aedes albopictus* was carried out as Rueda (2004) by looking at the pattern of the scutum, legs and mesepimeron lines. *Aedes aegypti* has 2 curved lines, while *Aedes albopictus* only has 1. The legs of *Aedes aegypti* have lines, while *Aedes albopictus* does not. There are two separate white dots in Mesepimeron on *Ae. aegypti*, whereas in *Ae. albopictus* in the form of white dots on mesepimeron tend to be irregular and combined to form the letter V. The differentiating compounds contained in the two species have not yet been found, so the identification of the two species was not carried out biochemically.

The reason of the superior RNA extraction results following the addition of β-mercaptoethanol is because β-mercaptoethanol is used in RNA extraction to help remove nucleoprotein pulling agents that prevent the release of RNA fragments. It can also increase the stability and reduce the damage caused by the excess amount of sulfhydryl bases in nucleoproteins, thereby increasing the purity and yield of the extracted RNA fragments.

CONCLUSION

The results showed that the best concentration and purity were obtained in mosquito samples that were modified in the process. The purity ratio of RNA extracted without modification was 1.971 (0.021 ± 0.800) while with modification it was 2.003 (0.011 ± 0.112). *Aedes aegypti* had a better average concentration of 7.146 μg/ml for unmodified RNA and 7.613 μg/ml for modified RNA. This research is expected to be a reference in the study of *Aedes aegypti* and *Aedes albopictus* as Dengue virus (DenV) vectors.

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