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# Optimization of RNA Extraction Method of Banana Leaf Using Li Buffer and GENEzol Reagent

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*Abstract* – **High quality extracted RNA from plant tissues is used in downstream transcriptomic application that needs high quantity for multiple analysis. Attaining high quality and high yield for banana plant tissue is a challenge with its high number of secondary metabolites becoming contaminants, effecting the purity of RNA extracted. A procedure to extract banana plant RNA with high quality and quantity in this study is developed to be simple, robust, affordable and accessible using basic lab equipment and materials. This study optimizes RNA extraction from the leaf of juvenile Pisang Susu (*Musa spp.*) using a combination of Li buffer (modified CTAB) and GENEzol reagent. Treatment differs in the use of β-mercaptoethanol and the duration of sample precipitation with isopropanol. NucleoSpin RNA Plant extraction kit was made as comparison. Measurement of RNA quantity used quantus fluorometer, and quality measured by spectrophotometer. Results showed that the addition of β-mercaptoethanol in Li buffer is vital with samples left to precipitate overnight providing best results. The total RNA obtained had a higher yield compared to the commercial kit with 108 – 211 ng/µl and 0.35 – 0.37 ng/µl respectively. Light absorbance $A_{260/280}$ indicating sample purity between method has similar RNA quality of 0.917 – 1.084 and 0.843 – 1.026. This study proved that modification of CTAB buffer using Li buffer for RNA extraction resulted in better quality and quantity of RNA compared to the commercial RNA extraction kit.**

***Keywords*** *– Banana, Optimization, RNA Extraction.*

## INTRODUCTION

Advancement in biotechnology enables the production of Genetically Modified Organism that can supplement nutritional values needed by people or to make crops have certain weather tolerance and resistance to diseases. This process is possible through gene editing, in targeting specific genes with known expressions [1]. The analysis of gene expression is needed to determine the regulating gene and to understand the organism response towards its environment at a molecular level. Information on gene expression is understood through quantification of the expression level of the genes. Thus, RNA quality is an important factor for obtaining a valid gene expression measurement, such as qPCR analysis [2, 3, 4] and high-throughput transcriptome sequencing analysis [5]. High quality RNA with sufficient quantity is needed for further downstream transcriptomic application, to provide meaningful data. However, the presence of secondary metabolites in plants becomes a contaminant that effects the purity of extracted RNA and hindering cDNA synthesis [6, 7].

Banana makes up a part of the rich culinary in Indonesia, with varieties in banana desserts that provides livelihood for the people. One of the bananas that can be found in Indonesia is Pisang Susu of the *Musa acuminata*, AA. A challenge faced in farming bananas is the wilt disease from the *Fusarium* bacteria. In understanding the effects of *Fusarium* on the banana plant, its gene expression has been analyzed by several studies [8, 9]. Both studies and other similar ones, do not mention their extracted RNA quality nor quantity. Meanwhile, banana plants have high numbers of secondary metabolites amongst the 105 metabolites identified in juvenile banana plants [10]. It can be suspected that not mentioning the total amount of RNA

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obtained from extraction is due to the low quality and/or quantity of RNA, but the yielded RNA must continue to be used for analysis at the transcriptomic level due to limited research time.

A study on ripening banana fruit has been conducted [11] to formulate a simple procedure for RNA isolation with results of high-quality RNA suitable for RT-PCR that yielded 80-150 µg of total RNA per gram of fresh tissue. However, the method has not yet been applied to other banana plant tissues. Methods of RNA extraction varies, including the use of commercial kits, as well as buffers and its modification that may be more effective in certain plant species [12, 13, 14] depending on the contents as possible contaminants. The use of commercial kits becomes prohibitively expensive to low-resource labs when paired with additional equipment such as TissueLyser [15] and refrigerated centrifuge [16].

It is needed to develop a simple procedure for extracting RNA from banana plant tissue that procures high yield with high-quality, which is affordable and not time consuming. Existing modified method with the use of CTAB buffer will further be modified and combined with the reagent GENEzol, adjusting to the use of non-refrigerated centrifuge and down scaled from ml to µl. The use of easy to attain and affordable materials, along with basic lab equipment in low-resource settings will make future studies in the analysis of gene expression accessible to all. Thus, the purpose of this study is to develop an optimized RNA extraction method of banana plant that is simple, robust, affordable and accessible using basic lab equipment and materials.

## METHODOLOGY

### Materials and Tools

The materials used in this study are the leaf of juvenile Pisang susu (*Musa spp*.), NucleoSpin RNA Plant extraction kit (Macherey-Nagel), GENEzol reagent (Geneaid), Li buffer (3% CTAB, 2% PVP, 2.0 M NaCl, 20 mM EDTA, 100 mM Tris pH 8.0, added with 2% β-mercaptoethanol when being used), HyperLadder 1kb (Bioline), Quantifluor RNA system kit (Promega) for RNA quantification measurement, Tetro cDNA synthesis kit (Bioline), MyTaq HS Red Mix (Bioline) for PCR, and ACTIN gene as the housekeeping gene. The tools used for RNA quantification measurement are quantus fluorometer (Promega), and BK-UV1800PC spectrophotometer (Biobase).

### RNA Extraction Method

The method of RNA extraction used is a combination of the Li buffer and the GENEzol reagent, where an RNA extraction kit is used as comparison. Two different treatment was used on the optimization of the RNA extraction. The first is without the use of β-mercaptoethanol and precipitating the sample with isopropanol for 2 hours, labeled as sample E. The second is with the use of β-mercaptoethanol and sample precipitation with isopropanol left overnight, as sample F. Each sample treatment has 6 repetitions, and total RNA of 40 µl. Whereas the RNA extraction kit used is NucleoSpin RNA Plant from Macherey-Nagel that becomes sample G with 2 repetitions and total RNA of 50 µl following the procedure of the kit.

Each microtube was filled with 500 mg of banana leaf that was grinded with liquid nitrogen and added with 800 µl Li buffer and 16 µl β-mercaptoethanol that is vortexed together. The sample is then incubated at 65$^{o}$C for 20 minutes. As much as 0.6 volume (480 µl) of chloroform was added and vortexed, continued by centrifugation at 13,000 g for 10 minutes. The supernatant was moved to a new microtube and given 1 volume of chloroform that were vortexed together and centrifuged again at 13,000 g for 10 minutes. The supernatant is taken into a new microtube, precipitated with 1 volume of isopropanol, and left in the freezer of -40$^{o}$C overnight. After that, the sample is centrifuged at 13,000 g for 10 minutes and the acquired pellet is air dried before being resuspended in 100 µl Nuclease Free Water (NFW). The extraction is continued with the addition of 800 µl GENEzol and 160 µl chloroform, with the tube shaken vigorously for 10 seconds and then centrifuged at 13,000 g for 15 minutes. The supernatant is transferred to a new tube and given 1 volume of isopropanol. It is incubated at room temperature for 10 minutes, continued by centrifugation at 13,000 g for 10 minutes. The acquired pellet is washed with 70% ethanol as much as 800 µl by vortex, and centrifuged at 13,000 g for 5 minutes. The supernatant is disposed of using a pipette, whereas the pellet is air dried and resuspended in 40 µl NFW. During the work of extracting RNA, if not mentioned otherwise, the samples has always been kept on shredded ice.

### Data Analysis

We measured the quantity and quality of the extracted RNA. The quantity of RNA was measured

using a Quantus fluorometer (Sigma-Aldrich), while the quality of RNA was analysed not only through the Quantus fluorometer measurements at $A_{260/280}$ but also by visualising the PCR electrophoresis gel using ACTIN gene primers.

Extracted RNA was first viewed through electrophoresis and UV visualization, then quantified using quantus fluorometer with the kit QuantiFluor RNA system from Promega. The quality of RNA is rated by its purity, observed by the absorbance of light wave from 260 nm, 280 nm and 230 nm using a spectrophotometer. Two highest and lowest sample quantity of RNA was then made into cDNA using the kit Tetro cDNA synthesis (Bioline) with its formulation consisting of 1 µg total RNA sample (10 µl), 2 µl NFW, 4 µl 5x RT buffer, 1 µl dNTP mix 10mM total, 1 µl random hexamer primer mix, 1 µl RNase inhibitor, and 1 µl Reverse Transcriptase. The attained cDNA was then amplified by PCR, and was also re-PCR.

## RESULT AND DISCUSSION

Result shows that sample F has both high quantity and quality of total RNA (table 1). Visually, RNA is prominent in sample F than sample E (figure 1) and there is no visualization in sample G. The quality of RNA obtained is similar between the optimized method and the extraction kit used as seen in $A_{260/280}$ light absorbance. However, despite the expensive price of the kit, the RNA quantity is far superior in both treatment of optimized method with 14 to 602 times more yielded RNA than sample G. Even so, RNA extraction using kit is done approximately under 1 hour, whereas the optimized method will take up to 2 days. Compared to the protocol developed by [17], their method is spread out over 3 days making this study's method faster by a day.

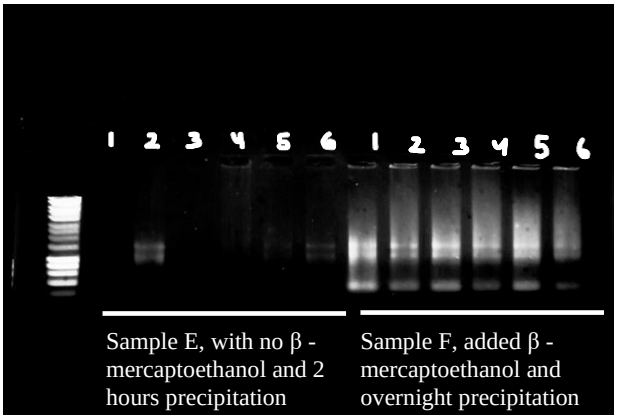


Figure 1. Electrophoresis Result of RNA Isolation from *Musa spp.* Leaf, with Bioline HyperLadder 1 kb

Table 1. Result of Total RNA Isolation of The Leaf of Juvenile *Musa spp*

| Sample | Quantity (ng/µl) | $A_{260/280}$ | $A_{260/230}$ |
|---|---|---|---|
| E1 | 0.024 | 0.763 | 1 |
| E2 | 28.6 | 1.125 | 1 |
| E3 | 5.9 | 0.776 | 0.437 |
| E4 | 5 | 0.996 | 0.903 |
| E5 | 5.8 | 0.929 | 1.182 |
| E6 | 13 | 1.122 | 1.17 |
| F1 | 203 | 0.958 | 0.349 |
| F2 | 199 | 1.05 | 0.984 |
| F3 | 108 | 1.084 | 1.037 |
| F4 | 211 | 0.918 | 1.062 |
| F5 | 142 | 0.958 | 0.861 |
| F6 | 157 | 0.917 | 1.4 |
| G1 | 0.37 | 1.026 | 1.079 |
| G2 | 0.35 | 0.843 | 1.3 |

*description: E. 1st treatment, F. 2nd treatment, G. MN Kit.

Sample downsizing in this study using half of [18] protocol is a success. Following [19] version of modified protocol, less sample used is still able to yield high quantity RNA (table 2). Using this study optimized method with 1.5 g of sample will be able to yield approximately the same RNA quantity from [19] of 5 g sample. Thus, the optimized method is suitable to use in studies with limited sample.

Table 2. Sample Comparison of Total RNA Yielded Between Optimized Method [19]

| Sample | Tubes | Quantity (ng/µl) | Dissolved in NFW |
|---|---|---|---|
| 2 – 5 g | 15 ml | 391 – 782 | 100 µl |
| 500 mg | 2 µl | 108 – 211 | 40 µl |

Extracted RNA instead of resuspended in 0.2 M NaCl as the original protocol, was resuspended in NFW and purified using GENEzol reagent to optimize the purity amount of RNA extracted. [19] compared extraction using ATP Biotech Kit, Qiagen Kit and GENEzol with yielded RNA concentration only readable in GENEzol of 264 – 451 ng/µl and the rest having less than 100 ng/µl. Meanwhile, [12] compared the use of GENEzol with Ambion Kit and GeneAll Kit for oil palm plant tissue RNA extraction. GENEzol has the cheapest price amongst the three, but its yielded RNA concentration fares better than the GeneAll kit with 572.3 – 999.2 ng/µl from extracting oil palm leaf.

The role of β-mercaptoethanol itself is vital in RNA extraction as seen between sample E and F (Figure 1). It prevents the oxidation of phenolic compounds and degrades plant protein, thus suppressing high numbers of secondary metabolite possible to be produced from cell wall lysis. Meanwhile, the

incubation time during precipitation also effects the quantity and purity of nucleic acids extracted [15].

RNA extracted using the optimized method compared to the MN kit was able to be used for cDNA synthesis. Sample F had adequate quantity of 1 µg total RNA, whereas sample G did not and was instead used 12 µl RNA as the maximum quantity possible to be added for cDNA synthesis. The total RNA required of 1 µg for sample G1 with 0.37 ng is 2702 µl, which is 54 times more than the amount of 50 µl RNA acquired from the kit extraction. cDNA was then amplified by PCR and re-PCR using ACTIN housing gene. Visualization by electrophoresis showed results in the PCR and re-PCR of sample F and G (figure 2, figure 3). It can be understood that the obtained total RNA can be used to the next level of analysis. However, in the case of MN kit, the RNA needed for cDNA has to be in a larger quantity thus the need to use more kit preparations.

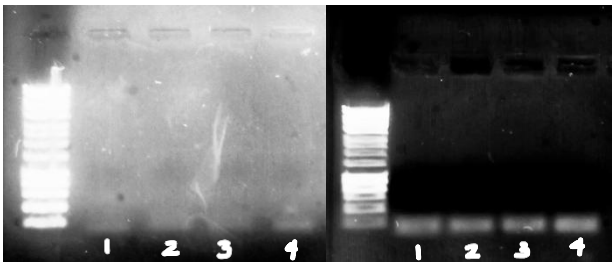


Figure 2. Electrophoresis Result of Sample F PCR (left) and re-PCR (right) using ACTIN Gene with Bioline HyperLadder 1 kb.

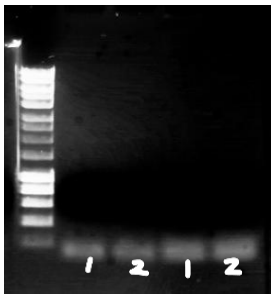


Figure 3. Electrophoresis Result of Sample G PCR (left) and re-PCR (right) using ACTIN Gene with Bioline HyperLadder 1 kb

A side finding of DNA was found during the second centrifugation after the lysis of cell walls. The supernatant containing RNA was extracted to be precipitated in isopropanol, where the white interphase containing DNA was also extracted and given three different treatments. Sample 1 was directly suspended in NFW, with sample 2 washed with 70% ethanol before being resuspended in NFW, and sample 3 being suspended in sodium citrate. However, respectively the DNA quantity was low at 6.7 ng/µl, 4.37 ng/µl, and 2.97 ng/µl along with a light absorbance of $A_{260/280}$ 0.979, 1.036, and 1.051.

## CONCLUSION

This study demonstrates the modification of the CTAB buffer using Lithium buffer as the extraction buffer in the total RNA isolation of plant samples, resulting in better total RNA in terms of quality and quantity compared to the use of commercial RNA extraction kits. (Macherey-Nagel RNA extraction kit). However, further optimization is needed for RNA extraction on a larger scale (medium or large) to achieve optimal results.

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