
The Effect of Sample Weigh, Incubation Duration And Isolation Methods on The Quality of *Phalaeonopsis amabilis* root DNA

Etty Handayani¹⁾, Innaka Ageng Rineksane¹⁾, Bambang Heri Isnawan¹⁾, Haris Anggita Setiawan¹⁾, Agus Manto²⁾

¹⁾Agrotechnology Study Program, Agriculture Faculty, Universitas Muhammadiyah Yogyakarta

²⁾Akademi Komunikasi Perkebunan Yogyakarta

*Corresponding author: etty@umy.ac.id

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ABSTRACT

Orchid *Ph. amabilis* is one of the plants designated as "Indonesian National Flower" and designated as "Puspa Pesona Indonesia". Orchid *Ph. amabilis* has a diversity that is not widely known, so that it is necessary to identify it molecularly. DNA isolation is the first step in molecular testing. This study aims to determine the sample weight, incubation time and the appropriate isolation method for DNA isolation from samples of *Ph.amabilis* orchid roots. In this study, the DNA isolation stage used two experimental methods, namely the CTAB method (Murray and Thompson) and the mini kit. The research method used was a single factor experiment with a combination of sample weight and incubation time consisting of 6 treatments with 3 replications, so 18 experimental units were obtained. The experimental treatment used was from the roots of the orchid *Ph. amabilis* which has a sample weight of 0.25 g, 0.5 g, and 0.75 g and each sample weight treatment was incubated for 30 minutes and 60 minutes. In addition, DNA isolation using the kit method had a sample weight of 0.25 g, 0.5 g and 0.75 g and an incubation period of 10 minutes. The results showed that the DNA isolation of *Ph. amabilis* with a sample weight of 0.75g, incubation time of 30 minutes using the CTAB method showed the best treatment in producing DNA with a concentration of 384 ng/μl and a purity of 1.9. This study is expected to provide information on sample weight, incubation time and the appropriate method for DNA isolation of *Ph. amabilis* orchid roots.

Keywords: *Ph.amabilis* Orchid, DNA Isolation, Root Sample Weight, Incubation Time, Isolation Method, DNA Quality

INTRODUCTION

Indonesia is a country with high biodiversity, according to Indonesia National Geographic, (2019) ranks 2nd after Brazil in the world. One of the biological resources owned by Indonesia is orchids, Orchid plants are flowering ornamental plants as well as superior horticultural commodities with high economic value due to the beauty of their flowers, one type of orchid plant is (*Phalaenopsis amabilis* (L.) Blume) or better known as white moon orchids, the flowers of this orchid plant have been designated by the government as "Puspa Pesona Indonesia" in 1993. More than 4,000 species of orchids can be found in Indonesia, mainly on the islands of Java, Borneo, Sumatra, and Papua, consisting of 46-60 species, 22 of which grow naturally in Indonesia.

According to the *List of Endangered Plants in Indonesia Plant Biodiversity*, almost 70% of orchid species including moon orchids have been included in the threatened species. The orchid bulb *Ph. amabilis* has different characteristics in each region. This difference is found in the characteristics of the size of the flower and the shape of the labellum (Rukmana, 2000). However, the level of diversity of *Ph. amabilis* orchids in Indonesia is not widely known and there has been no research on the diversity or kinship of *Ph. amabilis*. to find out the level of diversity of *Ph. amabilis* orchids, that molecular research can be carried out. One of the efforts to determine the level of diversity of *Ph. amabilis* orchids can be done molecular research. This can help in providing information about the proximity of *Ph. amabilis*, so that the genetic distance can be known to be used as a reference in crosses between relatives (Sundari, 2018). Therefore, genetic information is needed, so that we can know the genes that play a role in the growth, development and metabolism of plants (Arditti, 1992)

High accuracy of genetic information can be achieved by optimizing DNA isolation techniques to support the success of the resulting DNA. The DNA Isolation stage is the first step that must be done in molecular analysis before the next stage.

The main principle of isolating DNA from tissues is to extract and lyse plant tissue so that cell extracts consisting of tissue cells, DNA, and then cell extracts are purified so that pellets containing total DNA are produced. Success in DNA isolation can be seen from the results of high quality and quantity of DNA. This can be done by optimizing DNA which is influenced by several factors, including sample weight and incubation time. Handayani, (2008) states that the weight of the sample used in DNA isolation in orchid plants can result in significantly different concentrations of DNA. The length of the incubation time also affects the high or low concentration of DNA. If it is incubated for too long it can damage DNA and if it is too short it cannot destroy cell membranes and tissues. Therefore, temperature and time must be well regulated so that DNA is obtained with the appropriate concentration. Based on research conducted by Langga & Kuswinanti, (2012). The incubation treatment at a temperature of 65°C for 30 minutes maximized the release of DNA from the cell and degraded proteins from the cell wall optimally compared to other incubation treatments resulting in a fairly high purity level of 1.78. While based on research Syarifuddin, (2011) incubation at a temperature of 65°C for 1 hour is able to optimize the work of the extraction buffer added to the sample.

In this experiment, the DNA isolation of *Ph. amabilis* will use the modified *Cetyl Trimethyl Ammonium Bromide* (CTAB) isolation method. This method is widely used in the DNA isolation of plant genomes containing many polysaccharides and polyphenol compounds (Lumaret *et al*, 1998). There are three main steps in DNA extraction, namely cell wall destruction (lysis), separation of DNA from solid materials such as cellulose and proteins, and DNA purification (Surzycki, 2000). DNA isolation protocols have been widely developed at this time, but they are still universal for plants or animals and microorganisms. Some of the DNA isolation protocols in the CTAB DNA extraction kit package for plants are equipped with polysaccharide cleaning materials and columns (Porebski, *et al*, 1997). Along with the

development of science and technology, DNA isolation techniques can be used. DNA isolation using kits is more practical because in one kit package there is a ready-to-use isolation solution that saves work time but is relatively more expensive (Sari, *et al*, 2014) Universal DNA purification kits for isolation purposes are considered faster, simpler and more effective than conventional isolation methods. The use of the roots of adult *Ph. amabilis orchid plants* as a sample is expected to be able to optimize the DNA

isolation process, this is because the roots are part of the meristematic plant so that the cells contained in the roots are easy to extract so that they can support the success of DNA isolation.

This study is expected to provide information about the appropriate method of DNA isolation from the roots of *Ph. amabilis* so that the accuracy of genomic information in *Ph. amabilis orchid plants* is obtained as genetic information to support the development of plant biotechnology.

MATERIALS AND METHODS

The research was carried out in the *Invitro Culture* laboratory room, Faculty of Agriculture, University of Muhammadiyah Yogyakarta, as well as the Integrated Testing Laboratory and Plant Breeding Laboratory, Gadjah Mada University from April to June 2021. The test was carried out by isolating DNA using the roots of the *Ph. amabilis* orchid plant, then the isolation results were analyzed to test the quality of the DNA. The materials used in this study are *Ph amabilis* orchid root sample material, chemicals used in DNA isolation and purification techniques include: *Cetyl Trimethyl Ammonium Bromide* (CTAB) buffer, *Polyvinyl Pyrrolone* (PVP), *Genomic DNA Mini*

kit, *ependrof tube*, *collection tube*, *filter column*, *GD Column*, *blue tip*, *TE*, *70% ethanol* and *Cholorofoam Isoamylalcohol* (CI), then the materials used for the detection and measurement of DNA concentration and purity are *agarose*, *sterile aquadest*, *DNA loading*, *Tris EDTA (TE)* and *ethidium bromide*. *Smobio DNA marker*, The tools used in this experiment consist of *glassware*, *analytical scales*, *ependorf tubes*, *micropastle*, *mortal*, *scapel*, *incubator*, *shaker*, *waterbath*, *microcentrifuge*, *microwave*, *electrophoresis device*, *spectrophotometer*, *NanoPhotometer™*, *UV-Illuminator*, *camera marker*, and *stationery*

RESULTS AND DISCUSSION

DNA Concentration of *Ph. amabilis* Using the CTAB Method

The concentration value of the isolated DNA is used as a measure of the degree of contamination of a sample and determines a good sample for further analysis. According to Fatchiyah, (2012).

Good quality DNA based on tests has concentrations above 100 ng/ul.

Hasil penelitian identifikasi lama inkubasi dan berat sampel akar terhadap kualitas dan The quantity of DNA from the root of the *Ph.amabilis* orchid using the CTAB isolation method produced the results of the DNA concentration analysis presented in Table 1.

Tabel 1. Effect of sample weight treatment and incubation duration on the concentration of *Ph. amabilis* root DNA using the CTAB method

Treatment	Deuteronomy	Concentration (ng/μl)	Average (ng/μl)
0,25 g 30 minute	1	188,6	113,5a
	2	68,3	
	3	88,6	
0,25 g 60 minute	1	379	406,6a
	2	505,0	
	3	336	
0,5 g 30 minute	1	76,6	323,0a
	2	270,6	
	3	622	
0,5 g 60 minute	1	502	415a
	2	369	
	3	375	
0,75 g 30 minute	1	83,0	384a
	2	526	
	3	543	
0,75 g 60 minute	1	744	356,2a
	2	211,5	
	3	113,1	

Description : The concentration value of DNA isolation results from the root of the *Ph. amabilis* orchid at wavelengths of 260 nm and 280 nm.

The notation of the same letter shows that there is no effect of singnifikan on each treatment.

Based on the results of the measurement of concentration values from 18 DNA samples of the *Phaleopnosis amabilis* orchid (Table. 1) The average value of the highest concentration was shown at the sample weight treatment of 0.5g, the incubation period of 60 minutes with a concentration of 415 ng/μl, then followed by the sample weight treatment of 0.25 g of incubation time of 60 minutes with a concentration value of 406.6 ng/μl, the sample weight of 0.75 g of incubation time of 30 minutes with a concentration value of 384 ng/μl, Sample weight 0.75 g incubation duration 60 minutes with a concentration value of 356.2 ng/μl. The result of the highest concentration value is suspected to be with the ideal sample weight and the optimal incubation time so that in this treatment the lysis buffer can degrade proteins from the cell wall optimally and the release of DNA will be

maximized so that high concentration results are obtained. This is in line with the research conducted by (Langga & Kuswinanti, 2012) on plants (*Vitex cofassus Reinw*). The results of the study showed that the average concentration of DNA extracted was highest in the incubation treatment of 60°C for 60 minutes, and had the best average DNA purity rate.

Table 1 data shows that the treatment of 0.25 g of incubation time of 30 minutes produced the lowest concentration value when compared to the average value of DNA concentration from other treatments, namely with an average DNA concentration value of 113 ng/μl. This can be caused by the treatment of the smallest sample weight and the minimum treatment of the incubation period causing the DNA extraction process to be suboptimal.

DNA Concentration of *Ph. amabilis* Using the Mini Kit Method

The data from the research on the modification of the length of incubation and the weight of the root sample on the results of the DNA

concentration value from *the roots of the Ph.amabilis* orchid using the mini kit method is presented in Table 2.

Table 2. Concentration of DNA of *the root of Ph. amabilis* using the Mini Kit method

Treatment	Concentration (ng/μl)		
	U1	U2	Average ng/μl
0,25 g 10 minute	-	-	-
0,5 g 10 minute	1.10	0,90	1,4
0,75 g 10 minute	-	-	-

Description : The concentration value was calculated from the DNA isolation of *the root of the Ph. amabilis* orchid at wavelengths of 260 nm and 280 nm.
(-) : Unidentified DNA concentration values

The data in table 2 shows the isolation data using the *Phal.amabilis* orchid root DNA concentration mini kit method using a relatively low concentration of 0.90 - 0.10 ng/μl (Table 2). The low value of DNA concentration is suspected to be due to the weight of the sample used (0.25 g, 0.5 g, 0.75 g) it is suspected that the use of the sample is not in accordance with the protocol of the mini kit method. DNA isolation protocols using appropriate kits usually use fewer samples weighing 0.1 g. This is suspected to be due to too many samples and relatively short incubation

protocols, resulting in *less efficient buffer* lysis of the sample and degrading the cell wall so that DNA cannot escape from the cell nucleus. Shahzadi, *et al*, (2010) stated that the use of a larger amount of sample weight is inefficient without resulting in a high concentration of DNA. The value of the DNA concentration using a relatively low kit cannot be further tested to the next analysis such as amplification, electrophoresis and PCR because the value of the DNA concentration will indicate the amount of the DNA isolation result of the organism sample (Mulyani *et al.*, 2011).

DNA Purity of *Ph. amabilis* Using the CTAB Method

High DNA purity is indicated by the amount of DNA that is not contaminated in the isolated DNA sample. DNA containing purine bases and pyrimidines will absorb UV light. Double DNA bands can absorb UV light at 260 nm wavelengths, while contaminants such as proteins or phenols are absorbed at light lengths at 280 nm wavelengths (Aras, 2003). The purity value of 1.8-2.0 of the

number has met the requirements required in molecular analysis (Sambrook, *et all*, 1989) The results of the quantitative DNA purity test have important factors that affect the level of DNA purity such as the type of chemical (PVP, phenol, and NaCl), the concentration of chemicals, and the treatment in cold conditions to prevent the action of the endonuclease restriction enzyme. The following are the results of the analysis of the purity value test of the DNA isolation of the *Ph.amabilis* orchid root presented in Table 3.

Table 3. Effect of Sample Weight Treatment and Incubation Duration on DNA Purity of *Ph. amabilis* Root.

Treatment	Deuteronomy	Purity (1.8-2.0 ng/ul)	Average (ng/ul)
0,25 g 30 minute	1	2.0	1.9
	2	1.8	
	3	2.0	
0,25 g 60 minute	1	2.1	2.1
	2	2.1	
	3	2.1	
0,5 g 30 minute	1	2.0	1.9
	2	2.0	
	3	1.9	
0,5 g 60 minute	1	2.0	2.0
	2	2.0	
	3	2.0	
0,75 g 30 minute	1	2.1	1.9
	2	1.8	
	3	1.9	
0,75 g 60 minute	1	1.9	1.6
	2	1.2	
	3	1.8	

Description : The concentration value was calculated from the DNA isolation of the root of the *Ph. amabilis* orchid at wavelengths of 260 nm and 280 nm.

Based on test data, the effect of sample weight and incubation period of *Phalaenopsis amabilis* moon orchid root on the quantitative results of DNA purity (Table 3). Overall, it shows an average value of DNA purity ranging from 1.6 to 2.0. The results of DNA isolation with a ratio of 1.8 to 2.0 are DNA that has a high purity value and is not contaminated by RNase, proteins, ethanol and other secondary metabolite compounds.

DNA Purity of *Ph. amabilis* Moon Orchid Root Using Mini Kit Method

The results of the research data on modification of the length of incubation and the weight of the

Treatments that have good purity are shown in the treatment (sample weight 0.5g incubation period 60 minutes), (sample weight 0.25 g incubation period 30 minutes), (sample weight 0.75 g incubation duration 60 minutes). Results showing purity values below or even above from a ratio of 1.8 to 2.0 were shown at the treatment (sample weight 0.75 g incubation time 60 minutes and sample weight 0.25 g incubation time 60 minutes).

root of the root of the *Ph. amabilis* orchid using the mini kit method are presented in Table 4

Table 4. DNA purity of *the root of Ph. amabilis* using the mini kit method

Treatment	Purity		Average
	U1	U2	
0,25 g 10 minute	-0,7	-0,2	-0,9
0,5 g 10 minute	0,4	0,3	0,35
0,75 g 10 minute	-0,6	-0,4	-1

Description : The concentration value was calculated from the DNA isolation of *the root of Ph. amabilis* orchids at wavelengths of 260 nm and 280 nm.

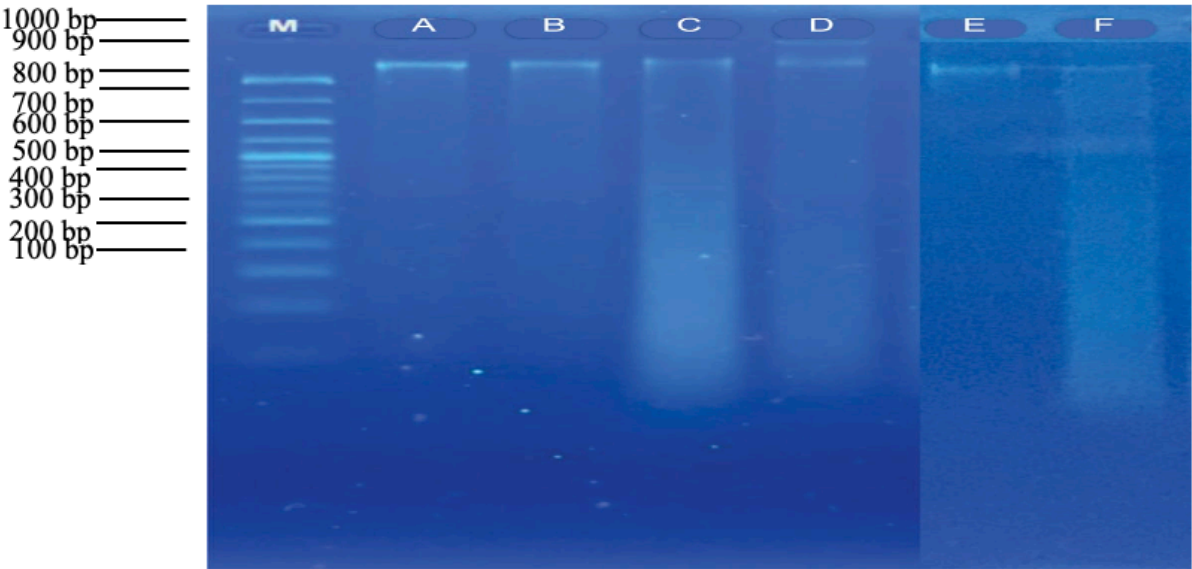
The data in table 4 shows the value of DNA purity using the DNA isolation protocol using a relatively low average purity value, which ranges from (-1-0.35) table 4. The purity of DNA is obtained from the ratio of absorbance A260/280, the value (-) shows that at the value of A260 nm which is the maximum value of DNA that can absorb light has a value smaller than the value of A280 nm. This shows that there is still contamination such as proteins, phenols, polysaccharides and other

secondary metabolites, this is suspected to be due to several factors, namely, the use of too many samples results in the reduction of *buffer wash to* purify DNA so that it cannot remove debris/cell wall residue and is less than optimal in denaturing proteins and polysaccharides. Another factor is that there are still purification solutions such as W1 and W2 solutions that are incorporated into the DNA pellets, thereby lowering the quality of DNA purity.

DNA Visualization Using the CTAB Method

The results of DNA visualization of electrophoresis gel from the root of *Ph. amabilis*

moon orchid using the CTAB method are presented in Figure 6.



Description : M: Marker 1000bp, A: 0.25g 30 minutes, B: 0.25g 60 minutes, C: 0.5g 30 minutes, D: 0.5g 60 minutes, E: 0.75g 30 minutes, F: 0.75g 60 minutes

Figure 1. Results of DNA Electrophoresis of *Ph. amabilis* Using the CTAB Method

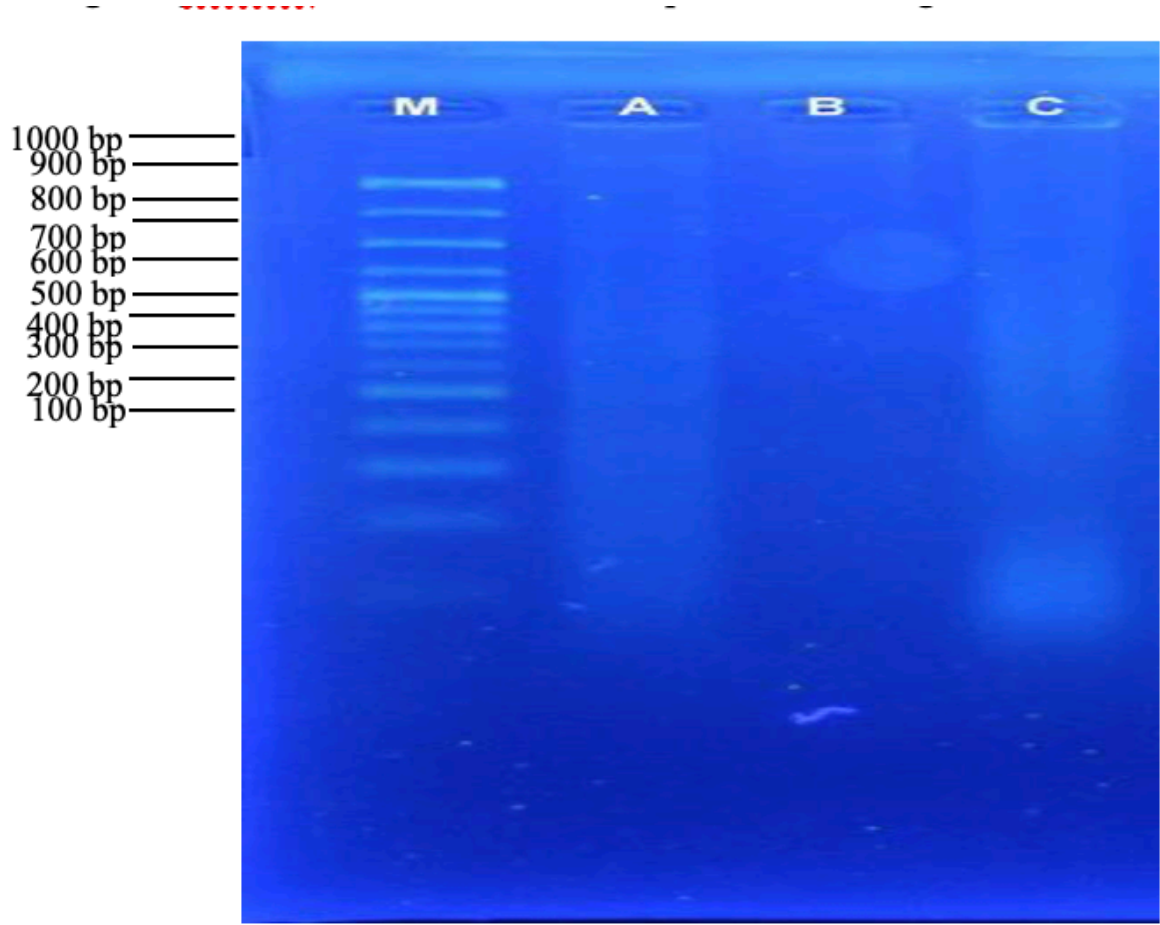
The best electrophoresis visualization results (Figure 1.) were shown at the sample weight treatment of 0.75 g of sample weight for a 30-minute incubation period (E). This is shown by the results of observation with UV light that there is a DNA band that is quite thick and clear. According to (Irmawati, 2003) states that a thick and single DNA band (not spreading) shows a high concentration and total DNA extracted in an uncut condition, while a DNA band that appears to be spread shows a bond between DNA molecules that is broken during the extraction process. In the treatment 0.25 g incubation time 30 minutes (A),

sample weight 0.25 g incubation time 60 minutes (B), sample weight 0.5 g 30 minutes (C), sample weight 0.5 g incubation time 60 minutes (D) there was a DNA band. However, the DNA ribbon that was obtained from the electrophoresis was still *smear*d. The presence of a *smear* indicates that there are molecules other than DNA that are isolated so that *smear* appears under the DNA band such as solutions that are carried during the DNA isolation process or the total DNA is cut into smaller parts, this can result in a *smear* on the DNA band being tested (Sholihah, 2014).

DNA Visualization Using the Geneaid Isolation Kit Method

Data Results of DNA visualization of electrophoresis gel from orchid roots *Ph. amabilis*

using the geneaid isolation kit method presented in Figure 7.



Description: M: Marker 1000bp; A: 0.25 g treatment and 10 minutes incubation time; B: 0.50 g treatment and 10 minutes incubation time; C: 0.75 g treatment and 10 minutes incubation time

Figure 2. Electrophoresis Results of *Ph. amabilis* DNA Using the Mini Kit Method

Based on the visualization results of the *Ph.amabilis root DNA electrophoresis gel* visualization presented in figure 2, it shows that from the entire treatment sample, namely (A) 0.25 g 10 minutes, (B) 0.5 g 10 minutes, (C) 0.75 g 10 minutes shows the results of the visualization without DNA bands and only smears are visible. This is because the concentration and purity values of the entire sample are relatively very low so that it can be identified that there is no isolated total DNA. In addition, it can be caused by the results that the DNA quality test produced is classified as impure. According to Mulyani, *et al* (2011), the appearance of *smears* can be caused by the presence of remnants of solution that are still carried during the DNA isolation process.

CONCLUSION

The results of DNA isolation of *Ph. amabilis* orchid root using the CTAB method with a sample weight of 0.75g, an incubation period of 30 minutes produced the best DNA quality, which had a concentration value of 384 ng/μl with a

Therefore, it is necessary to optimize the DNA isolation protocol from *the root of Ph.amabilis* at each stage of the isolation process, namely, cell licitization, DNA extraction and DNA purification. The optimal stages of DNA isolation will get good quality and quantity of DNA from the isolation so that it can be used as a molecular analysis.

Based on data on the influence of sample weight, incubation duration and DNA isolation method of *Ph.amabilis root*, at 0.75g treatment, 30 minutes incubation time using the CTAB method produces better concentration, purity and visualization of DNA, namely with a concentration value of 384 ng/μl, purity of 1.9 and produces thick and clear DNA bands. It can be identified that the isolated DNA is intact or not cut into pieces.

purity of 1.9. The method of DNA isolation of *the root of Ph. amabilis* using *the Geneaid isolation kit* requires isolation optimization with a smaller sample count

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