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**ARTICLE**

# Development of a method for DNA extraction from oil palm leaves and the effects of pH and ionic strength on nucleic acid quantification

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Abbreviations used: BSA, bovine serum albumin; CTAB, cetyltrimethylammonium bromide; DIECA, Diethyldithiocarbamic acid; IRD, infrared fluorescent dye; LiCl, lithium chloride; MATAB, Mixed Alkyl,trimethylammonium bromide; PEG, polyethylene glycol; PVP, polyvinylpyrrolidone

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**ABSTRACT** Oil palm (*Elaeis guineensis* Jacq.) has benefited little from molecular marker technologies although the long generation time and the unpredictable variations due to allogamy make it a high priority for improving breeding efficiency. Progress would require an efficient, low cost and high-throughput deoxyribonucleic acid (DNA) extraction method adapted for oil palm. A method for genomic DNA extraction from oil palm leaves that is simple and cost-effective was developed without the use of liquid nitrogen. $A_{260/280}$ ratios between 1.987 and 2.078 were obtained. Furthermore, we could demonstrate that nucleic acid concentration and yield were inversely proportional to the pH and ionic strength of the solvent. The measured values varied significantly between 572 ng/µl and 496 ng/µl. DNA extracted with this method is stable and can be reproducibly amplified by PCR.

**Keywords:** DNA, liquid nitrogen, oil palm, pH, DNA yield

## INTRODUCTION

The oil palm (*Elaeis guineensis* Jacq.) belonging to the family Arecaceae [1], a diploid oil-producing crop with a genomic size of 1.8 Gb [2], is one of the most important oil-bearing crops in the world. It is a large feather palm having a solitary columnar stem, short internodes, and short spines on both the leaf bases and within the fruit bunches [3]. It has irregular sets of leaflets on the leaf, which gives the palm its characteristic appearance. The palm is monoecious with male or female inflorescence, but hermaphroditic inflorescences sometimes develop in the axils of the leaves [3]. The fruit, which is borne on the large compact bunch, is called a drupe [1]. Distinguishing the different types of oil palms has been controversial. These attempts have been unsatisfactory since in the wild state, each palm represents a hybrid with respect to some of its traits [1]. Oil palm is classified based on the fruit type and fruit form. It has three fruit forms: *Dura, Pisifera* and *Tenera* (hybrid fruit form) and different fruit types namely, virescens, albescens, nigrescens and poissoni [1]. Oil palm has benefited immensely from conventional breeding program in Nigeria. This has solely been made possible through the dedicated breeding program put in place by the Nigerian Institute for Oil Palm Research (NIFOR). The progress made so far has been very limited for two reasons: (1) the long generation time and (2) the outcrossing nature of the crop. With the emergence of deoxyribonucleic acid (DNA) marker technologies, scientists see the possibility that significant success can be achieved if markers are extensively applied by oil palm breeders as exemplified by the cloning of the shell thickness gene [4]. Exploration of DNA marker technologies, combining the knowledge from research in molecular genetics and genomics, offers great possibilities to oil palm breeding [5]. With DNA marker technologies, the underlying genetic basis of phenotypic traits can be studied independent of environmental influences. However, critical to the adoption, application and the domestication of these technologies is an effective DNA extraction method that is less complex than the methods that have been previously applied to extract DNA from palms [6-10]. The basic principles underlying DNA extraction procedures are not very complicated, but the growing numbers of DNA extraction procedures indicate that it is not always simple and the published protocols are not necessarily reproducible for all species [11,12]. The objective of this study was to develop a method for DNA extraction from oil palm leaves that is cost effective and adaptable to low budget laboratories.

## MATERIALS AND METHODS

### Development of protocol for the extraction of total nucleic acids from oil palm

A protocol for the extraction of total cellular nucleic acids from leaf tissue of oil palm was embarked upon without the use of liquid nitrogen.

### Reagents:

- ✓ Extraction buffer [100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl, 5 % SDS]
- ✓ 5 M potassium acetate (3 M with respect to potassium and 5 M with respect to acetate)
- ✓ Phenol-chloroform-isoamylalcohol (25:24:1)
- ✓ Absolute ethanol
- ✓ 70% ethanol
- ✓ TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)]

### DNA extraction procedure

1. Between 0.15 g to 0.2 g of disease-free leaf tissue was obtained and put into a mortar. The tissue was cut into smaller pieces with a pair of scissors and 800 µl of DNA extraction buffer was added. The tissue was ground quickly until buffer turned dark green
2. The homogenate (buffer and ground tissue) was transferred into 1.5 ml Eppendorf tube. Extra 200 µl of DNA extraction buffer was added, centrifuged for 2 min at 10,000 g (4°C), and the supernatant was collected into a new Eppendorf tube and labeled
3. 200 µl of 5 M potassium acetate was added and vortexed.
4. Equal volume of phenol-chloroform-isoamylalcohol (25:24:1) was added, vortexed, centrifuged for 5 min at 10,000 g (4°C), and the supernatant was carefully transferred into a new Eppendorf tube without disturbing the interface between the upper and the lower phases
5. 800 µl of absolute ethanol was added. Eppendorf tube was gently inverted for the content to mix well, centrifuged for 5 min at 10,000 g (4ºC) and supernatant was discarded
6. Nucleic acids were washed twice with 70% ethanol and air dried at ambient temperature
7. Nucleic acids were resuspended in 200 µl of TE and stored at −20ºC.

### Determination of purity and quantity of extracted nucleic acids

To determine the purity and quantity of extracted nucleic acids, four different solvents (sterile distilled water, deionized water, diluted TE buffer (1:2 with distilled water) and TE buffer) were used. The quality of the extracted nucleic acids was also verified by separation on 0.8% to 1% agarose gel stained with ethidium bromide. The nucleic acid concentrations (ng/µl) and the optical density (OD) ratio $A_{260/280}$ were calculated by measuring the OD at wavelength of 260 nm and 280 nm using a spectrophotometer (Genesys 10uv Scanning, Thermo Electron Corporation, USA: model Genesys 10-S).

### PCR analysis of extracted DNA

To determine the usability of the extracted DNA for PCR, primer combinations were designed from three simple sequence repeats (SSR) sequences mEgCIR3275, mEgCIR3533 and mEgCIR3557 [10] using the online program Primer3 (http://frodo.wi.mit.edu/):

mEgCIR3275M13for 5'-TTTCCCAGTCACGACGTTGGTGGAAGCTTTTTGTCTGC-3'and mEgCIR3275rev 5'-ATTGAAGAGGGCAGGGTTTT-3

mEgCIR3533M13for 5'-TTTCCCAGTCACGACGTTACGGTCTATGGCTCTGTCGT-3'and mEgCIR3533rev 5'-ACATGAGGAAAGCGCTAGGA-3'

mEgCIR3557M13for 5'-TTTCCCAGTCACGACGTTCATTGCCATTCCCTTCAAGT-3'and mEgCIR3557rev 5'-TCCCCTCTGTTCACTCAAGC-3'.

Primers were labeled using M13 tailing procedure (shown in bold) [13].To visualize the PCR products, anIRD-800(=DY-781) labeled M13 primer was added to each SSR primer combination. All unlabeled primers were ordered from Life Technologies GmbH, Darmstadt, Germany, the M13-IRD800 (=DY-781) labeled primers were from Biomers, Ulm, Germany.

The PCR amplification was performed in a GeneAmp® PCR System 2700 (Applied Biosystems) thermocycler. For the PCR, 4 µl of DNA (50 ng/µl) was mixed with 11 µl master mix. The master mix contained 0.3 µl dNTPs (10 mM), 8.7 µl $H_2O$, 1.5 µl 10 × PCR buffer, 0.15 µl M13-IRD800 primer (5 pmol/µl) for labeling, 0.05 µl FIREPol DNA polymerase (5 U/µl, Solis Biodyne), 0.15 µl forward primer (5 pmol/µl) and 0.15 µl reverse primer (5 pmol/µl).

### Statistical analyses

Statistical analysis was carried out using GenStat version 8.1. Duncan Multiple Range Test (DMRT) was used for all pairwise comparison of means at 95% probability.

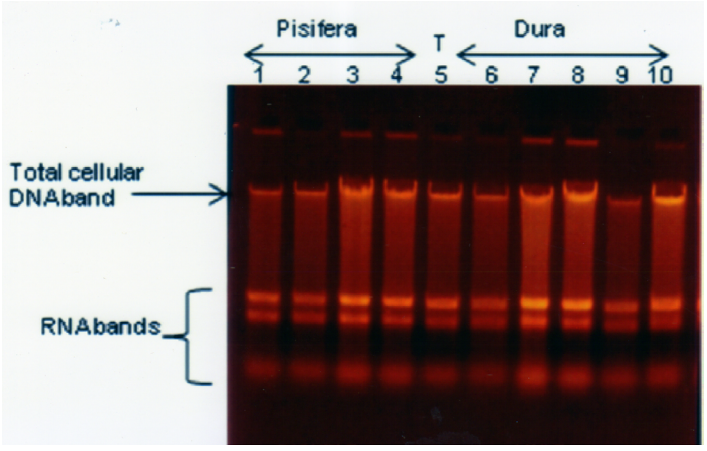


**Figure 1. Total DNA extracted from oil palm leaves with the described protocol.** Using the developed protocol, DNA was isolated from 10 palm trees comprising four *pisifera*, one *tenera* (T) and five *dura* palms. Eight microliters (8 µl) of each sample were loaded on a 0.8% agarose gel and the gel was stained with ethidium bromide.

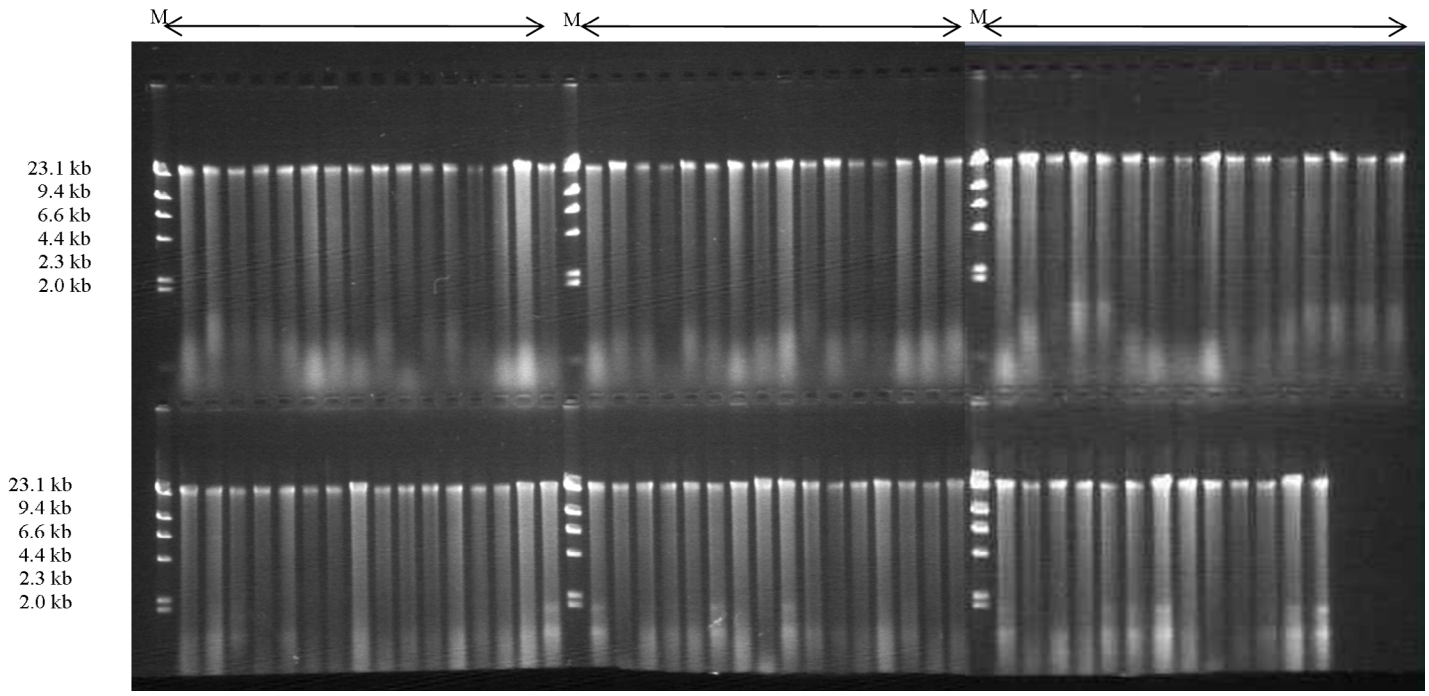


**Figure 2. Total DNA from oil palm leaves of 93 trees extracted with the protocol.** Lambda DNA digested with HindIII (New England Biolabs) was used as marker. Five microliters of each sample were loaded on a 1% agarose gel and the gel was stained with ethidium bromide.

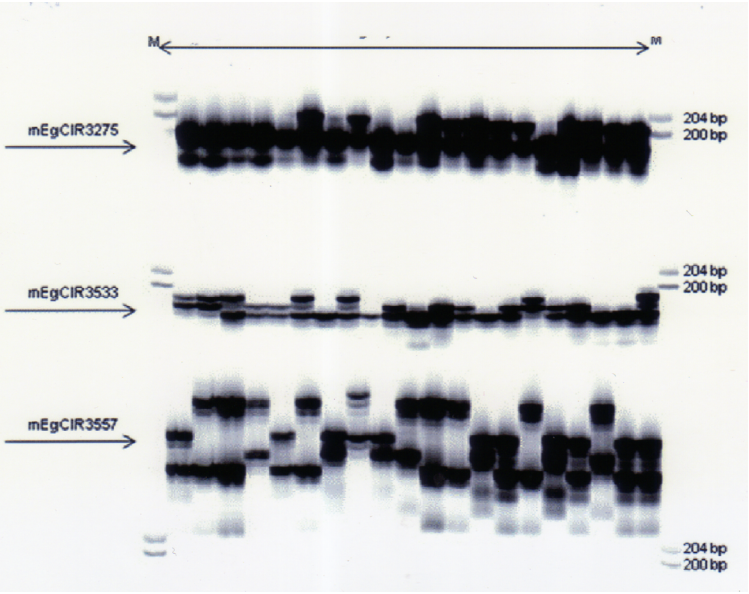


**Figure 3. PCR products of DNA extracted from 21 palm trees of a segregating $F_1$ population (Tenera x Deli Dura) separated on 6% polyacrylamide gels using the DNA Analyzer Model 4300 (LI-COR Biosciences).** The IRDye®800 Sizing Standard 50–700 bp (LI-COR Biosciences) was used as marker. Two bands (204 bp and 200 bp) of this marker are shown as reference. For each PCR reaction 200 ng of DNA were used and 0.8 µl of the PCR reaction (diluted 1:2 with loading buffer) was pipetted into each well. The expected product sizes of the SSR markers were 192 bp for mEgCIR3275, 199 bp for mEgCIR3533 and 226 bp for mEgCIR3557.

## RESULTS AND DISCUSSION

In the present study, a new protocol for nucleic acid extraction from oil palm leaves was developed without the use of liquid nitrogen (**Fig. 1**). This method isolates DNA, which can be well amplified by PCR, even though the nucleic acid preparations will contain traces of RNA. No differences were observed in the total DNA extracted from the three fruit forms (*Pisifera, Dura* and *Tenera*) of oil palm *E. guineensis* in terms of size. The method reported here was also used to extract DNA from a large population of oil palm (93 trees) (**Fig. 2**). The method is considered fast as it takes only about 25 min to complete one extraction and it is considered easy as it involves only seven steps. The DNA separated on 1% agarose gel was of high quality with a major band larger than 23 kb with no visible sign of degradation by DNase. The extracted DNA samples dissolved in TE buffer were stable for more than a year, when stored at −20ºC. The minimum storage period was 90 days before PCR amplification. In some instances, extracted DNA was stored for 1 year prior to PCR amplification. The key steps in the developed DNA extraction protocol are the fast homogenization of the leaf tissue in the presence of DNA extraction buffer using mortar and pestle, and the introduction of 5 M potassium acetate before the phenol:chloroform:isoamylalcohol (25:24:1) step. Potassium acetate is known to form complexes with proteins and polysaccharides [12,14]. Proteins and polysaccharides are often the major contaminants during

DNA extraction because they form complexes with DNA and they must be eliminated to enhance the purity of the DNA [14]. Proteins are known to interfere with enzymes activities in both restriction digestions and polymerase reactions [15]. The ability of the DNA polymerase to selectively amplify the DNA extracted with the developed protocol means that the DNA is good enough for PCR reaction and also inferred that it can be used for restriction digestions. Also, the presence of residual RNA in the sample (as the method presented here extracted total cellular nucleic acids) does not present any problem to the use of the extracted DNA for molecular analyses as shown in **Figure 3**. This agrees with the findings of Murray and Thompson [15], thus eliminating the need for RNase treatment of the DNA, which would be an extra cost for a low budget laboratory. Diluted DNA solution (5 ng/µl, using double distilled sterile water) for PCR reactions, also stored at -20ºC, gave reproducible products over at least a three-month period (**Fig. 3**).

**Table 1. Comparative assessment of the different protocols used to isolate DNA from oil palms.**

| Protocols | Chemicals Required | Need for liquid nitrogen | Number of steps (time per extraction) | Work-load | Cost per sample in Nigeria in US$ |
|---|---|---|---|---|---|
| Ihase *et al*. (from this study) | Tris-HCl, EDTA, NaCl, SDS, potassium acetate, phenol, chloroform, isoamyl alcohol, ethanol | No | 7 (25 min) | Low | 33 |
| Ying *et al*.[6] | Tris-HCl, EDTA, NaCl, CTAB, PVP, sorbitol, sodium bisulfite, DIECA, ascorbic acid, sarkosyl, β-mercaptoethanol | No | 16 (2 h: 21 min) | High | 65 |
| Arif *et al*. [7] | chloroform, isoamyl alcohol, sodium acetate, isopropanol, ethanol, Trizma Base, EDTA, CTAB, NaCl, PVP, LiCl, sterile sand | No | 13 (1 h: 28 min) | High | 44 |
| Ouenzar *et al*. [8] | Tris-HCl, Mannitol, EDTA, PEG, BSA, β-mercaptoethanol, chloroform, phenol, SDS, sodium acetate, isopropanol, ethanol, RNase A, acid washed sand, glass powder | No | 23 (4 h: 30 min) | High | 74 |
| Risteruci al., Billotte *et al*. [9,10] | NaCl, Tris-HCl, EDTA, $Na_2SO_4$, PEG, MATAB, chloroform, isoamyl alcohol, isopropanol, QIAGEN genomic tip | Yes | Numerous steps (> 13 h) | High | 100 |

The comparison made between the different DNA extraction methods previously applied in palms and the new method presented in this study is shown in **Table 1**. The components of the DNA extraction buffer used in the new protocol are entirely new and in several ways different from a previous protocol used for mature leaves of oil palm [6]. Mannitol, PEG 6000, BSA and β-mercaptoethanol (to mention a few), which formed part of the extraction buffer of Ying and Zaman [6], are not part of the new extraction buffer reported here and the concentrations of Tris-HCl, EDTA and SDS are also different. The method reported here is also very different from the methods described for date palm [7,8]. Liquid nitrogen, which is not always easy to obtain in most third world laboratories, was used by the methods of Risterucci *et al*. [9] and Billotte *et al*. [10] (**Table 1**). The cost of DNA extraction per sample in Nigeria using the new method is only a third of the most expensive protocol [9,10] and considerably less expensive than any of the published methods[6,7,8] as shown in **Table 1**. Regarding the leaf sampling for DNA extractions, three statements can be made: 1) DNA extraction worked best with fresh leaf samples prior to any drying procedure, 2) the DNA extraction method is non-destructive to either mature or nursery palms, and 3) oven-dried leaf sample (at 50ºC) and very young (spear) leaves can also be used for DNA extraction.

Following the successful extraction of DNA from any sample, the next most important steps are the determination of the purity and concentration of the extracted DNA. Here, we determined the effect of different solvents (sterile distilled water, deionized water, diluted TE buffer [1:2 with distilled water] and TE buffer) on the spectrophotometric absorbance of the extracted nucleic acids. The method reported here extracted total cellular nucleic acids comprising of DNA and RNA as visible in **Figure 1**. The treatment of the nucleic acids with RNase was omitted for two reasons. First, the cost of RNase in Sub-Saharan Africa is very high and cost is one of the major factors considered in this work. Secondly, the presence of RNA did not interfere with the PCR amplification of the extracted DNA. Thus the values obtained at $A_{260}$ actually represent DNA and RNA. From our result, the nucleic acids diluted with sterile distilled water had the highest absorbance value of 0.143 at $A_{260}$ followed by that of deionized water (0.137), diluted TE buffer (0.129) and TE buffer (0.124), respectively (**Table 2**). These values were significantly different ($P < 0.05$). These differences in the mean value of $A_{260}$ using the same nucleic acid extract but different solutions for dilution may be due to the ionic strength and the pH of the solutions. This agrees with the report of Wilfinger *et al*. [16], who reported that RNA absorbance at $A_{260}$ was dependent on the pH and the ionic strength of the solution used for spectrophotometric analysis. They further stated that the pH and ionic strength of the solution can substantially influence the qualitative and quantitative determination of nucleic acids as observed in our study.

It is established that resonance structure of the pyrimidine and purine bases are responsible for the maximal absorption of nucleic acids and proteins in the 260–280 nm regions of the UV spectra [16]. Molecular biology manuals [14,18,19] report that an $A_{260/280}$ ratio of 1.8 is an indication for pure DNA preparations and an $A_{260/280}$ ratio of 2.0 for pure RNA preparations free of proteins. The absorption spectra for nucleic acid bases, nucleosides and nucleotides are reported to be strongly pH-dependent because of the degree of ionization of the bases at different pH values [17]. This information has not been adequately emphasized in molecular biology reports [16]. In our findings, $A_{260/280}$ ratio indicated the presence of DNA in the sample diluted with sterile distilled water (1.987 ± 0.029) against the other solutions, which indicated more of RNA than DNA at a value of 2.005 ± 0.010 (for deionized water), 2.087 ± 0.023 (for 1:2 diluted TE buffer) and 2.078 ± 0.006 (for

TE buffer). The values obtained for the different water solutions were significantly different from the values obtained for the TE buffers ($P < 0.05$), but were not significantly different when compared within, that is, sterile distilled water versus deionized water, and 1:2 diluted TE buffer versus TE buffer. These values increased from the different water samples to the different TE buffers and they were directly related to the increase in the pH and conductivity of the dilution solutions (**Table 2**). This finding agrees with the findings of Wilfinger *et al*. [16].

In our experiments, we observed that changes in the pH and ionic strength of the different solutions used for the determination of $A_{260/280}$ ratio of the same extracted nucleic acid sample altered the $A_{260/280}$ ratios (**Table 2**) and this agrees with the findings of Wilfinger *et al*. [16] reported for RNA preparation. Ratio greater than 2.0 is often recorded for $A_{260/280}$. This may be related to DNA degradation and measurement of free nucleotides, but this assumption is not applicable in our case because the same DNA sample that gave an $A_{260/280}$ value less than 2.0 for sterile distilled water also gave a value greater than 2.0 value for 1:2 diluted TE and TE buffers by simply altering the pH and ionic strength of the solutions. It can be stated from our results that the apparent $A_{260/280}$ ratio varies from one solvent to the other due to the pH and ionic strength of the different solutions. Proteins absorb light at 280 nm and 230 nm. Thus, $A_{260/230}$ ratio could also be used for the detection of protein contaminations, but this is rarely done because common buffers such as Tris are known to also absorb light at 230 nm as well as phenol and other organic contaminants. The widely used ratio for determination or assessing DNA purity is $A_{260/280}$, which is easy to determine. DNA absorbs light so strongly at 260 nm and it takes significant protein contamination to have a noticeable effect on the $A_{260/280}$ ratio.

We also compared the effects of pH and ionic strength on nucleic acid concentration and yield using different solvents. The highest nucleic acid yield of 460.8 µg was obtained for a sample diluted in sterile distilled water. 438.4 µg was obtained in deionized water and 413.9 µg and 396.8 µg was obtained in 1:2 diluted TE buffer and undiluted TE buffer, respectively. The mean yields were significantly different ($P < 0.05$) from each other except for 1:2 diluted TE and undiluted TE buffer (**Table 2**; column 7). Similarly, the mean nucleic acid concentration was inversely correlated with the pH and conductivity of the solvent and the values were statistically significant for all four solvent conditions ($P < 0.05$) (**Table 2**; column 6). Our results suggest the nucleic acid yield and concentration were inversely related to the pH and conductivity of the solution used. These findings are consistent with the findings from Wilfinger *et al*. [16], who reported the same observations for RNA by comparing different solvents.

**Table 2. Effect of different solutions on the yield, concentration, $A_{260}$ and $A_{260/280}$ ratio of extracted nucleic acids.**

| Nucleic acid dilution solution | Conductivity (µS/cm) | pH | $A_{260}$ | $A_{260/280}$ ratio | Concentration (ng/µl) | Yield (µg) |
|---|---|---|---|---|---|---|
| Sterile distilled water | 7.41 | 5.3 | $0.143^{a} \pm 0.002$ | $1.987^{b} \pm 0.029$ | $572.0^{a} \pm 6.9$ | $460.8^{a} \pm 6.4$ |
| Deionized water | 3.90 | 5.2 | $0.137^{b} \pm 0.001$ | $2.005^{b} \pm 0.010$ | $548.0^{b} \pm 4.6$ | $438.4^{b} \pm 3.7$ |
| 50% TE buffer | 1586 | 7.6 | $0.129^{c} \pm 0.001$ | $2.087^{a} \pm 0.023$ | $517.3^{c} \pm 5.3$ | $413.9^{c} \pm 4.3$ |
| 100% TE buffer | 2940 | 7.9 | $0.124^{d} \pm 0.001$ | $2.078^{a} \pm 0.006$ | $496.0^{d} \pm 2.3$ | $396.8^{c} \pm 1.8$ |

Results are presented as mean ± SEM. Each mean value represents three replications. Mean values within a column labeled with different superscripts are significantly different ($P < 0.05$).

In conclusion, a high throughput nucleic acid extraction method for oil palm was developed that results in amplifiable DNA and allows fast handling of large populations and is cost-effective. Separation of the nucleic acids on a 1% agarose gel showed the presence of high quality DNA with one major band larger than 23 kb with no visible signs of degradation by DNase. RNA present in the lower part of the gel that was extracted together with the DNA does not present a problem for its use in PCR reactions due to the substrate specificity of the DNA polymerase and therefore eliminates the necessity for RNase treatment. The absorbance values obtained from the spectrophotometric evaluation of the nucleic acid extractions represent total cellular nucleic acids because both DNA and RNA are known to absorb at 260 nm. The omission of an RNase treatment in the extraction process will lead to an over-estimation of these values for the DNA. For laboratories that can afford RNase and desire accurate evaluation of DNA concentration using this method, the extracted nucleic acids can further be treated with RNase following standard procedure [14]. The yields and concentrations of the extracted nucleic acids were subject to the variation in pH and ionic strength of the solvents used for the measurement of photometric absorbance. However, the accuracy of the nucleic acid measurement, even though it is only a rough estimate, is sufficient in its precision to perform PCR for qualitative evaluation of bands as required in marker-assisted selection for breeding programs.

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