A SIMPLE, RAPID, EFFICIENT AND LOW COST METHOD FOR MINIPREP DNA FROM DIFFERENT SOURCES

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ABSTRACT

DNA extraction and purification are routine processes in most plant genetic transformation laboratories. Although there are different commercial kits that allow accurate DNA purification, the total cost of buying multiple sets of these kits can be spectacular. We use spin column and laboratory solutions to develop a simple method of DNA purification that can meet different research needs. This method is used to extract DNA from the leaves of Brassica and genetically modified plants and also bacteria; extract plasmid DNA from *E. coli* or *Agrobacterium tumefaciens*; purify the DNA fragments of PCR products and the resulting fragments of digestion with restriction endonuclease. DNA concentration of optical density (OD) value was calculated at 260 nm wavelength and OD260 / OD280 ratios were used to determine DNA quality. The quantity and quality of DNA obtained by this method was similar to that of isolated DNA using commercial Kits. In comparison, it has been shown that this method allows obtaining DNA from different sources with similar quantity and purity and low costs.

Key words: DNA purification, Brassica plant, *Escherichia coli*, *Agrobacterium tumefaciens*, plasmid DNA, quantity and quality of DNA.

1. Introduction

DNA extraction is required for a variety of molecular biology applications (**Tan and Yiap, 2009; Thatcher, 2015**). Research in plant genetic transformation involves genomic and plasmid DNA extraction from plant tissues, *E. coli* and *A. tumefaciens* cells, as well as purification of PCR products. Genomic DNA often needs to be extracted from plant tissues to isolate the target gene using polymerase chain reaction (PCR). Many commercial kits are available, whereas the sensitivity of PCR detection has been shown to be different for various DNA kits (**Yoshikawa *et al*., 2011).**

One of the major problems commonly associated with DNA is that it does not give reproducible PCR results (**Lodhi *et al*., 1994; Sharma *et al*., 2002**). The purification of PCR product for this gene has facilitated constructs the recombinant plasmid and DNA sequencing. For screening the transformed Agrobacterium colonies, plasmid DNA was isolated from several transformants bacteria. In order to test that T_0 plants carrying target gene in

their tissue, total genomic DNA was isolated from T_0 plant tissues and subjected for PCR analysis.

Transgenic plants are usually generated using an *A. tumefaciens*-mediated transformation procedure, where the bacteria carry an engineered binary plasmid harboring the gene of interest for integration into the plant genome. It is important to isolate the binary plasmid from *A. tumefaciens* cells to verify the correct construct prior to plant transformation. However, the extraction of the binary plasmid from *A. tumefaciens* is very difficult because Ti-plasmid is large and low in copy number in Agrobacterium and the resistance of the bacteria strain to cell lysis (**Chen *et al*., 2003**). To solve these problems, the isolated DNA is usually re-transformed into *E. coli* to propagate before subsequent restriction digestion verification (**Wise *et al*., 2006**).

Good quality high molecular weight intact DNA is a pre-requisite for various molecular biology experiments. DNA extraction kits are available, but the use of commercially available kits, which have a high cost per sample ratio (**Kang *et al*., 2004; Ahmed *et al*., 2009; Li *et al*., 2010**). Although, each kit is only designed for single-purpose DNA extraction and the cost of purchasing multiple kits can represent a significant research cost. Thus, there is a strong need for a simple and inexpensive protocol that could be adapted to the extraction and purification of DNA from diverse sources.

In an attempt to develop a simple DNA extraction and purification methods that could meet diverse research needs, we describe easy procedures using lab made simple solutions and silica membrane spin column without the above-mentioned limitations and efficient in terms of time and cost.

2. Materials and Methods

2.1. Plant materials

The accession numbers PI 649105 and PI 271442 of rapeseed *B. juncea* (L.) Czern was kindly provided by the North Central Regional Plant Introduction Station, USDA, USA. The cultivar Serw 4 of spring oilseed rape *B. napus* (L.) was kindly provided by the Agricultural Research Centre, Giza, Egypt. The transgenic cultivar Serw 4 of *B. napus* (L.) was achieved through the Ph. D. thesis studies **(Eid *et al*., 2018)**.

2.2. Microorganism materials

Escherichia coli DH5α, JM 109 and TOP10, *A. tumefaciens* LBA4404 and the plant expression vector (pCAMBIA1301) were obtained from Prof. Dr. Maria Mercedes Bonfill Baldrich, Unitat de Fisiologia Vegetal, Facultat de Farmàcia, Universitat de Barcelona, Spain. The RBC T&A cloning vector was obtained from Real Biotech.

2.3. DNA extraction and purification from multiple sources

Plasmid DNA and genomic DNA extraction from plant tissues, *E. coli* and *A. tumefaciens* cells, and as well as purification of PCR products and restriction endonuclease digests using a protocol described by **Li *et al*. (2010),** where

the silica membrane spin column was used instead of the silica matrix with a some modifications.

I. Genomic DNA extraction procedure

Genomic DNA was extracted from accession numbers PI 649105 and PI 271442 of rapeseed *B. juncea*, *B. napus* cultivar Serw 4, transgenic Serw 4, *E. coli* (DH5α, JM 109 and TOP10) and *A. tumefaciens* (LBA4404) as follows:

1. A 0.2 gm plant grained tissue was added to 2 ml microfuge tube.
2. Pellet the cells of 2ml *E. coli* overnight culture (OD_{600} = 2.0) growing at 37 ºC by centrifugation at 13000 rpm for 30 sec.
3. Pellet the cells of 2ml *A. tumefaciens* (OD_{600} = 1-1.5) growing on LB medium (g/l^{-1} tryptone,10; yeast extract, 5; and NaCl, 10) with Kanamycin (50µg/ml) and Rifampicin (100µg/ml) at 28 ºC.
4. Each of the grained plant tissue, *E. coli* pellet and *A. tumefaciens* pellet were resuspended in 200 µl from resuspension solution (50mM Tris-HCl, pH7.5, 10mM EDTA, 10µg/ml RNase), then 30 µl 10% SDS were added and tubes were inverted several times before incubation at 70°C for 60 min for plant sample and 70°C for 20 min for bacteria .
5. Two handred fifty µl phenol:chloroform:isoamyl alcohol (25:24:1) was added and tubes were vortexed vigorously for 30 sec before incubated on ice for 2 min.
6. The tubes were centrifuged at 13000 rpm for 5 min at 4 °C.
7. The upper phase (400µl) was transferred to new tubes contained 500 µl NaI (6M) and mixed well.
8. The mix was transferred to spin column and incubated for 2-3 min before centrifugation at 13000 rpm for 1 min then the column was washed 2 times by washing solution (50% ethanol, 10mM Tris-HCl, pH7.5, 100mM NaCl, 1mM EDTA).
9. Fourty µl of 10mM Tris-HCl was used to collect DNA from column by incubation for 2 min and centrifugation for 1.5 min at 13000 rpm.
10. DNA stored at -20 °C until using in PCR amplification.

II- Plasmid DNA extraction from transformed E. coli and A. tumefaciens procedure:

The DNA of RBC A&T cloning vector and pCAMBIA 1301 exepression vector were extracted from *E. coli* DH5α and *A. tumefaciens* LBA4404, respectively as follows:

1. Pellet the cells of 2ml *E. coli* overnight culture (OD_{600} = 2.0) growing on LB medium with ampicillin (100 µg/ml) at 37 ºC by centrifugation at 13000 rpm for 30 sec.
2. Pellet the cells of 2ml *A. tumefaciens* overnight culture (OD_{600} = 1-1.5) growing on LB medium with Kanamycin (50µg/ml) and Rifampicin (100µg/ml) at 28 ºC by centrifugation at 13000 rpm for 30 sec.

3. Pellet was resuspended in 100 µl of resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 g/ml RNase A by brief vortexing.
4. One handred µl of alkaline lysis solution (0.2 mM NaOH, 1% SDS) was added and tubes were inverted several times and incubated for 2 min at room temperature then 100 µl from neutralization solution (1.32 M KOAc, pH 4.8) was added and tubes were inverted for a few times.
5. After centrifugation at 13000 for 5 min the supernatant was transferred to new tubes containing 500 µl NaI and mixed well.
6. The mix was transferred to spin column and incubated for 2-3 min before centrifugation at 13000rpm for 1 min.
7. Then the column was washed 2 times by washing solution (50% ethanol, 10mM Tris-Hcl, pH7.5, 100mM NaCl, 1mM EDTA).
8. Fourty µl of elution buffer 10mM Tris-HCl was used to collect DNA from column by incubation for 2 min and centrifugation for 1.5 min at 13000 rpm.
9. The molecular weight marker (XLarge DNA Ladder 0.250-25 kb) was also added along with plasmids in agarose gel electrophoresis.

III- Purification of PCR products and restriction endonuclease digests procedure

The PCR products amplified from PI 649105 and PI 271442 and each of PCR products digested with BamH1 and HindIII restriction endonucleases individually were purified as follows:
1. Add 150 µl NaI (6M) Solution to 40 µl PCR product and mix well by inverting the tube.
2. Add 75 µl NaI (6M) Solution to 20 µl restriction endonuclease digests and mix well by inverting the tube.
3. The mix was transferred to spin column and incubated for 2-3 min before centrifugation at 13000 rpm for 1 min then the column was washed 2 times by washing solution (50% ethanol, 10Mm Tris-Hcl, Ph7.5, 100Mm NaCl, 1Mm EDTA).
4. Twenty µl of 10mM Tris-HCl was used to collect DNA from column by incubation for 2 min and centrifugation for 1.5 min at 13000 rpm.

2.4. Determination of DNA concentration and purity

Absorbance measurements made on a IMPLEN Pearl Nanophotometer at 260 nm and 280 nm. The ratio of absorbance at 260 nm and 280 nm and DNA concentration (ng/µl) were calculated. Then calculate DNA Yield (µg/sample) by multiplying total volume of elution buffer (µl) in the concentration.

2.5. Agarose gel electrophoresis for quality determination of genomic DNA

Genomic DNA was checked by electrophoresis using 0.8% agarose gel in 1x TAE buffer as follows:

1. A 0.8 g or 1.2 g agarose was weighed and mixed with a 100 ml of 1x TAE buffer for genomic DNA or PCR products and plasmids respectively.
2. The agarose was melted in microwave and allowed to be warm (hand-hot) before adding 3 µl of ethidium bromide (10mg/ml) and mixed thoroughly.
3. The melted agarose was poured into a gel casting tray fitted with acrylic comb and allowed to solidify.
4. A seven µl of DNA sample was mixed with 3 µl of 6x loading dye and loaded into wells.
5. Four ul of molecular weight marker (1kb DNA ladder) was also added along with PCR product to indicate the base pair length.
6. Electrophoresis was run at a constant 60 volts for 2 hours.
7. The DNA was visualized by using transilluminator.

2.6 Buffers and Solutions

- **Antibiotics Stock:**
  - Ampicillin (100mg/ml): dissolve100mg in 1ml distilled water
  - Kanamycin 50mg/ml: dissolve 50 mg in 1 ml distilled water
  - Rifampicin 100 mg/ml dissolve100mg in 1ml distilled water
- **1% Sodium dodecyl sulphate (SDS)**

1g SDS is dissolved in 9ml of deionized water. Solution is heat to 65°C to dissolve. The pH is adjusted to 7.2 by adding a few drops of concentrated HCl. The volume is brought to 1000ml with water.

- **TE buffer (pH 8.0)**

10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) was mixed.

- **1M EDTA (pH 8.0)**

186.1g EDTA are added to 800ml of distilled water. Solution is stirred vigorously and the pH is adjusted to 8.0 with NaOH pellets (~ 20g). Volume is completed to 1000 ml with distilled water. Solution is dispensed into aliquots and sterilized by autoclaving.

- **resuspension solution:**

1ml from EDTA 100mM and 1 ml from Tris HCL 50 mM Ph7.5 and 8 ml d.d water.

- **Alkaline lysis solution:**

1ml from NaOH 2N and 1ml SDS 10% and up to 10 ml with sterile distilled water.

- **Neutralization solution:**

6 ml from 1.32M potassium acetate and 1.15 glacial acetic acid and 2.85 ml d.d water.

- **NaI (6M):**

Dissolve 9 gm from NaI in 6 ml double distilled water then up to 10 ml with water.

- **washing solution:**

1 ml EDTA 100mM and 2ml Tris Hcl 50 mM and 3ml water that have 0.06 g sod.chloride up to 10 ml with ETOH absolute.

- **50X TAE**

242g Tris base is dissolved in distilled water, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH 8.0) are added. Volume is adjusted to 1000ml with distilled water.

- **1M Tris-HCl (pH 7.2/pH 8.0)**

121.1g Tris base is dissolved in 800ml of distilled water. The desired pH is obtained by adding concentrated HCl. The volume of the solution is brought to 1000ml with distilled water.

- **Ethidium bromide stock solution (10mg/ml)**

0.1 g Ethidium bromide is dissolved in 10ml of distilled water by stirring on a magnetic stirrer to dissolve the dye completely. Solution is transferred to a dark bottle and stored at room temperature.

- **Gel-loading dye (6X)**

Two ml of 10X TAE, 6ml of glycerol is mixed in a falcon tube and the volume is adjusted to 20ml with sterile distilled water. Bromophenol blue is added until the adequate color is obtained.

3. Results and Discussion

Total genomic and plasmid DNA extracted with our method showed intact and sharp fragments and no degradation appear (Figures 1 and 2). The absorbance at 260nm and 280nm, A260/A280 and DNA Yield (µg/sample) are given in Table (1). The absorbance at 260nm is used to calculate the concentration of DNA (ng/µl) then calculate DNA Yield (µg/sample) by multiplying total volume of elution buffer (40 µl) in the concentration. The ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) is used to assess the purity of DNA.

Nucleic acids have absorbance maxima at 260 nm, as a guideline, the A_{260}/A_{230} is best if greater than 1.5. The ratio of this absorbance maximum to the absorbance at 280 nm has been used as a measure of purity in DNA extractions. A 260/280 ratio of 1.7 to 2.0 is generally accepted as "pure" DNA. The purity of the extracted DNA varied as determined by the A260/A280 ratio and was generally of high purity. The ratio ranged from 1.745 to 1.972, 1.75 to 1.92 and 1.861 to 1.867 in plants, bacteria and plasmids, respectively (Table 1). **Rogers and Bendich, 1994** reported that low DNA quality due to contaminants (polyphenols and other secondary compounds), which affect restriction endonuclease, polymerase and/or ligase activity, and premature DNA degradation.

Table (1) show the DNA yield (µg/sample) with an overall mean of 20.76 µg on plant followed by plasmids (4.19 µg), bacteria (2.7 µg). Transgenic Serw 4 of *B. napus* had a higher overall yield (27.80 µg) than other plants. Also,

DNA yield of pCAMBIA 1301 vector was highest (6 µg) than RBC A&T vector (2.38 µg). It is even more difficult to manipulate the plasmids *in vitro* due to the low plasmid copy number and unstable target insert and thus lost when cells are stressed.

Genomic DNA extraction from plant and bacteria can be completed in 70-100 minutes and 30-60 minutes, respectively, however plasmid DNA extraction thorough 15-30 minutes.

The extracted DNA tested for molecular use by performing the polymerase chain reaction (PCR) and restriction analysis, both of which were successful due to the absence of contaminants. PCR procedure requires high quality DNA for amplification with reproducible results. The numerous DNA isolation kits are good option for analyzing large number of samples, but these make the cost per sample very high also (**Kang *et al*., 2004; Ahmed *et al*., 2009**). Extraction of highly purified genomic DNA from plant tissues is a difficult task due in part to their rigid cell wall composed of large amounts of complex carbohydrates (**Hattori *et al*., 1987**). Contamination by polysaccharides has been reported as the most common problem affecting plant DNA purity (**Murray and Thompson, 1980; Demeke and Adams, 1992; Varma *et al*., 2007; Tamari and Hinkley 2016)**. Some classes of polysaccharides reduce the activity of polymerases, ligases, and restriction endonucleases (**Furokawa and Bhavadna, 1983; Shioda and Marakami-Muofushi, 1987; Do and Adams, 1991; Fang *et al*., 1992**). PCR is based on the efficient action of a thermostable polymerase such as Thermus aquaticus (Taq) DNA polymerase (**Arnheim and Erlich, 1992**). Several factors present in plant DNA preparations, which inhibit Taq polymerase activity have been already reported (**Gelfand and White, 1990**). Therefore, false negative polymorphic bands have been observed in PCR-based fingerprinting as a result of contamination by polysaccharides and/or other DNA-binding substances, which may confound the interpretation of genetic differences between individual samples (**Gelfand and White, 1990**).

Table (2) show the recovery of DNA fragments from PCR mixtures and restriction endonuclease digests. Direct purification method of PCR product in 15-minute and gives up to 88-95% recoveries in the range of 0.7 kb to 1.2 kb. However, purification of PCR product digested with the first restriction endonuclease recover 60 % and the second digestion recover 54-58%.

The purified PCR product and restriction endonuclase digests so obtained was used for DNA sequencing (Figure 3) and plant gene transfer. Genomic DNA was extracted from young leaves of putative transgenic Brassica plants and was used for PCR amplification using specific primer pairs to confirm the success of genetic transfromation (Figure 4).

Conclusion

This method is simple, reliable, efficient and cheaper to obtain high quality genomic DNA from Brassica plants, *E. coli*, Agrobacterium and plasmid DNA. Genomic DNA obtained can be used for PCR amplification and efficient purification of DNA fragments ideal for use in molecular biology procedures such as sequencing, restriction endonuclease digestion, ligation, cloning, *E. coli* and Agrobacterium transformation and plant gene transfer.

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Table 1: Absorbance values and DNA yield of genomic and plasmid extracted from different sources

Sample	A_{260}	A_{280}	A_{260}/A_{280}	DNA Conc ng/µl	DNA Yield µg/sample
Genomic DNA					
PI 649105 of *B. juncea* (Plant)	0.178	0.102	1.745	0.468	18.72
PI 271442 of *B. juncea* (Plant)	0.174	0.099	1.758	0.435	17.40
Serw 4 of *B. napus* (Plant)	0.191	0.105	1.819	0.478	19.12
Transgenic Serw 4 of *B. napus* (Plant)	0.278	0.141	1.972	0.695	27.80
***E. coli* DH5α (Bacteria)**	0.014	0.008	1.750	0.071	2.84
***E. coli* JM109 (Bacteria)**	0.166	0.092	1.804	0.064	2.56
Top 10 (*E. coli* (Bacteria)	0.018	0.010	1.800	0.063	2.52
LBA4404 *A. tumefaciens* (Bacteria)	0.144	0.075	1.920	0.072	2.88
Plasmid DNA					
pCAMBIA 1301 vector (high copy number)	0.28	0.15	1.867	0.15	6.00
RBC A&T vector (low copy number)	0.121	0.065	1.861	0.059	2.38

Table 2: DNA purification from PCR products and PCR product digested with restriction endonucleases.

Sample	A_{260}	A_{280}	A_{260}/A_{280}	DNA Conc. ng/µl
PCR product amplified from PI 649105 (1)	0.084	0.047	1.787	148 (100%)
PCR product amplified from PI 271442 (2)	0.091	0.049	1.857	150 (100%)
PCR product purified from (1) (3)	0.059	0.031	1.903	141 (95%)
PCR product purified from (2) (4)	0.034	0.02	1.7	133 (88%)
PCR product (3) digested with BamH1 (5)	0.046	0.013	1.769	85 (60%)
PCR product (4) digested with BamH1 (6)	0.099	0.053	1.868	81 (60%)
PCR product (5) digested with Hind III	0.059	0.032	1.843	50 (58%)
PCR product (6) digested with Hind III	0.099	0.051	1.941	44 (54%)

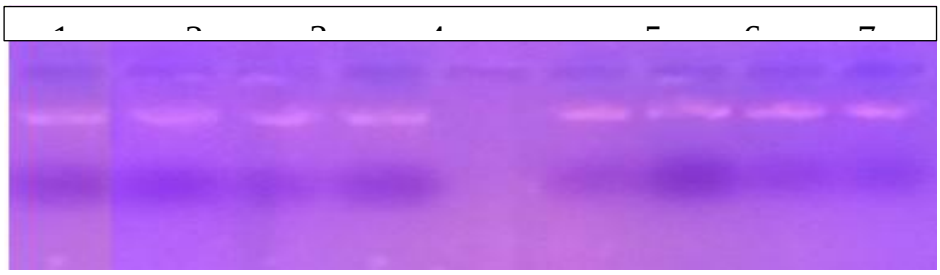

Figure 1: Genomic DNA extracted from Brassica plant, *E. coli* and *A.tumefaciens*.

1: *B. juncea* accession number PI 649105
2: *B. juncea* accession number PI 271442
3: *B. napus* cultivar Serw 4
4: *B. napus* cultivar Serw 4 transgenic.
5: *E. coli* (DH5α)
6: *E. coli* JM 109)
7: *E. coli* TOP10
8: *A. tumefaciens* LBA4404

Figure 2: Plasmid DNA extracted from *A. tumefaciens* and *E. coli*.
1: 1kb DNA ladder Marker
2: pCAMBIA 1301extracted from *A. tumefaciens*
3: RBC A&T plasmid extracted from *E. coli*
4: 1kb DNA ladder Marke

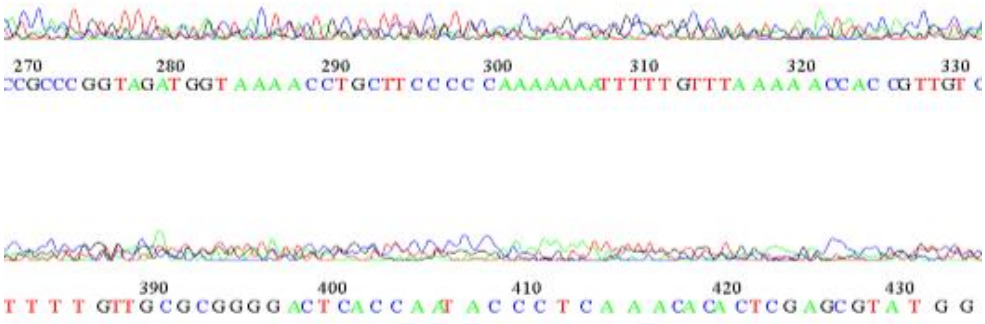

Figure 3: DNA sequencing of *SHAT* 2 gene from B. juncea

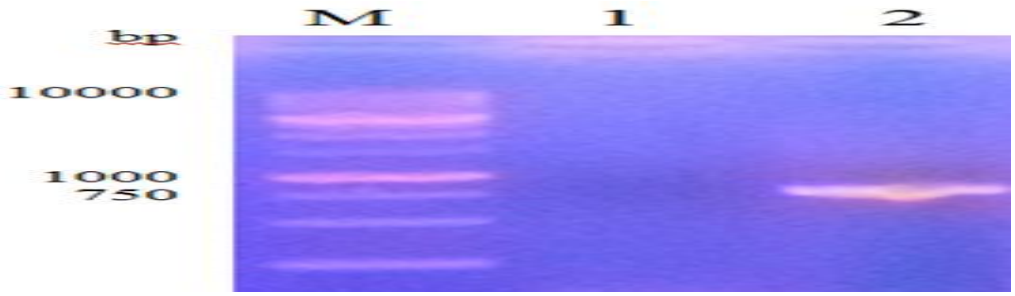

Figure 4: PCR amplification of *SHAT* 2 gene from transgenic *B. napus* Serw 4 cultivar
M: DNA marker, 1 kb DNA ladder
1: Serw 4 cultivar
2: Serw 4 transgenic (T_0)

طريقة بسيطة وسريعة وفعالة ومنخفضة التكلفة لاستخلاص الحمض النووي بكميات صغيرة من مصادر مختلفة

زكى أحمد الفقى*؛ أحمد عبد الفتاح محمد يسن؛ ندى فتحى حميدة؛ جهاد ربيع عبد الجواد عيد
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الملخص العربى

استخلاص الحمض النووي وتنقيته هي عمليات روتينية في معظم مختبرات التحول الوراثي النباتي. على الرغم من وجود مجموعات تجارية مختلفة تسمح بتنقية دقيقة للحمض النووي ، فإن التكلفة الإجمالية لشراء مجموعات متعددة من هذه المجموعات يمكن أن تكون مذهلة. نحن استخدمنا Spin column ومحاليل مجهزة فى المعمل لتطوير طريقة بسيطة لتنقية الحمض النووي والتي يمكن أن تلبي احتياجات البحث المختلفة. وهذه الطريقة تستخدم لاستخراج الحمض النووي من أوراق الكانولا والنباتات المعدلة وراثيا وأيضا من البكتيريا؛ استخراج الحمض النووي البلازميدي من *E. coli* أو *A. tumefaciens*؛ تنقية شظايا الحمض النووي من نواتج ال PCR والقطع الناتجة من الهضم بانزيمات Restriction Endonuclease. وتم استخدام هذه الطريقة لاستخراج الحمض النووي من أوراق الكانولا و النباتات المعدلة وراثيا وأيضا البكتيريا؛ استخراج DNA البلازميد من *E. coli* أو*A. tumefaciens* ؛ تنقية الحمض النووي من نواتج ال PCR وشظايا الهضم الناتجة من المعاملة بال Restriction endonuclease . تم حساب تركيز DNA من الكثافة الضوئية (OD)على طول موجة ٢٦٠ نانومتر ، واستخدمت النسبة OD_{260} / OD_{280} لتحديد جودة الحمض النووي. كانت كمية ونوعية الحمض النووي الذي تم الحصول عليه بهذه الطريقة مماثلة لنوع الحمض النووي المعزول باستخدام ال kits التجارية. بالمقارنة ، فقد تبين أن هذه الطريقة تسمح بالحصول على الحمض النووي من مصادر مختلفة بكمية ونقاوة متشابهين وبتكاليف منخفضة.

الكلمات الدالة: تنقية الحمض النووي ، نبات الكانولا *A. tumefaciens* ، *E. Coli*, الحمض النووي البلازميدي ، كمية ونوعية الحمض النووي.