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Research Article

Gold nanoparticle biosensors, A novel aplication in gene transformation and expression

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ABSTRACT
The conventional techniques of PCR, Southern blot, Northern blot, in situ hybridization, and RNase protection assay have long been used to investigate transformation and expression of genes, but most of them are time-consuming and have relatively low sensitivity.In recent years, the tendency toward using biosensors has been expanding significantly. Hence in this study, Zabol mildew melons were used as the model plant to introduce new DNA and RNA-based biosensors for confirmation of gene transformation and expression. First, the melon plants were grown *in vivo* and GUS reporter gene was transferred to the plant using *Agrobacterium*. In order to analyze GUS gene transfer and expression, probes were designed based on DNA, RNA and cDNA of GUS gene sequence. Finally, using the designed probes, gene transformation and expression analysis were performed using a probe attached to gold nanoparticles to observe color variation of gold nanoparticles in presence of the target molecules. Hybridization of the probes with target molecules was evaluated at a wavelength of 400 to 700 nm, so that maximum change was observed in the wavelength range of 550 to 650 nm. The results of this study showed that using detectors attached to gold nanoparticles was a more rapid, specific, and economic way to detect than the biochemical and molecular techniques. These tests can be carried out with a little accuracy and initial optimization at research centers using the least facilities so there will be no need for special equipment.

Keywords: gene transformation, gene expression, *GUS* gene, PCR, RT-PCR, gold nanoparticles

INTRODUCTION
Gene transfer to plants and production of transgenic plants follows various significant objectives such as improved yield and quality, increased resistance to pests, diseases, so on. Transferring access to a convenient, fast, easy and efficient way to confirm gene transfer and expression to plants is one of the aims of genetic scientists(Kumar et al., 2003; Fischer et al., 2004).Application of PCR based methodsto detect gene transfer, pattern of gene expression and identifying of rare RNA versions has changed the sensitivity of the methods used for gene transformation and expression. Although these methods are widelyused, most of them are time-consuming with relatively low sensitivity making detection of biomolecule difficult or impossible. In recent years, a great tendency toward using tests which are based on DNA has expanded. Thus, DNA-recognition biosensors have been created which can facilitate DNA identification. This type of biosensors can be used in detecting DNA, gene analysis, rapid identification of pathogenic agents and criminal cases. These DNA-oriented identification systems work based on hybridization of a target DNA with its complementary probe that can be performed in

solution or on a solid surface. This method can also be used for DNA sequencing (Vo-Dinh and Cullum, 2000). During the processes to design DNA biosensors which work chiefly on hybridization, it is essential to consider two issues of sensitivity and electability of the tools, i.e., the tools must be able to detect low concentrations of DNA and identify point-to-point mutation in DNA. Full compliance of target sequence with the probe results in a stable two- strand DNA, whereas if there are one or more errors, it will decrease the DNA two-strand stability leading to changes in the signal (Liu *et al*., 2005). Biosensors also can be design using RNA sequence and apply to detect target RNA.

Nano-biotechnology is one of the most promising areas of science and nano-technology. This technology is emerging in various fields of science, including chemistry, biology and material science (Narayanan and Sakthivel, 2011). A variety of nanoparticles have been used in biological researches. Among nanoparticles, colloidial gold nanoparticles have been utilized for centuries due to the specific chemical, physical and optical characteristics with respect to size, shape and surface charge (Azzazy and Mansour, 2009; Sanvicens and Marco, 2008) and the vibrant colors produced by their interaction with visible light. An identified light with a longer wavelength of nanoparticles creates dipole fluctuations in Au, which in turn leads to electron transfer from one orbit to the surface of the nanoparticles (Huang and El-Sayed, 2010). In case of a particular frequency, the magnitude of fluctuations reaches its maximum limit. This frequency is known as the surface Plasmon resonance (SPR) andisresponsible forchanging the color ofthe colloidalAu (Baptista*et al*., 2008; Huang *et al*., 2007).When gold nanoparticles are sedimented, they will have something called Plasmon–Plasmon response that results in color variation of Au solution from red topurple (Hussain et al., 2013). The very minute size of nanoparticles makes them able to react to different biological molecules (Azzazy*et al*., 2006; Jain, 2005). Gold nanoparticles can be used as a scaffold, which has a high surface to volume ratio, therefore they can react simultaneously with 100 small molecules or 30 oligonucleotide strands (Russ Algar*et al*., 2009).

So far, the most common technique in analyzing gene transfer and expression were Southern and northern blot, however, there are some fundamental problems to use these methods. There is no report on application of Gold nanoparticles probe to confirm gene transformation and expression, hence this study was conducted to develop a valuable and new GNP based detection method.

MATERIALS AND METHODS

Synthesis of gold nanoparticles

Gold nanoparticles were synthesized using sodium citrate (Turkevich*et al*., 1951; Frens, 1973). In order toevaluate the morphologyof the synthesized gold nanoparticles, two methods were used: electronmicroscopy of transmissionand analysis of absorptionspectrumof gold nanoparticlesusinga spectrophotometer. The generated gold nanoparticles were kept in the dark and at room temperature.

Preparation of putative transformed plants

Putative transformed plants were obtained by insertion of GUS reporter gene to *CicerArietinum* through xylem vessels using protocol described by Nouri *et al.,* (2013). First, Cicerseed were grown in pots for 5 days and plantlets with 10cm in length were used for inoculation. To prepare *Agrobacterium* solution one of the single colonies of *Agrobacterium tumefaciens* harboring pCAMBIA1305.2 plasmid was cultured in liquid LB medium and kept in a shaking incubator at a temperature of 28 ° C with 160 rpm overnight. Finally the plantlets were inoculated with the *Agrobacterium* suspension and control plants inoculated with distilled water.

Confirmation of *GUS* reporter gene transfer

DNA extraction from leaves

DNA extraction was done using protocol of Dellaporta*et al*.,(1983). Briefly, 0.2 g of leaves

were grounded in liquid nitrogen and transferred to a 1.5 ml sterile tube. Next, 600 ml extraction buffer was added to the tube and was placed for 15 minutes in a 65° C water bath. 200 ml 5M potassium acetate and 300 ml phenol and 300 ml chloroform were added to the tube. The tube was centrifuged for 10 minutes with 13000 rpm. The top phase was carefully transferred to a new 1.5 ml tube, 700 ml of cold isopropanol was added, and it was centrifuged for 10 minutes at the previous round. Ethanol was discarded and 30 ml 70% ethanol was added to the sediment. Then, it was centrifuged for 2 minutes with 10000 rpm. ethanol was discarded and the tube was placed with the door open at room temperature to evaporate the remaining ethanol. In the final step, 30 ml of TE buffer was added to the deposit (DNA) was added and it was kept at -20 °C until use**.**

Confirmation of *GUS* reporter gene transfer using PCR

Primers were design based on the sequence of GUS gene using Oligo7 bioinformatics software. The primer sequences were: 5-´GTCGTGATCGACCAGACTCC-3´ (forward primer) and 5´-GCTCACCCACGAAGTTCTCA-3´ (reverse primer). The oligonucleotides were synthesized by Bioneer (South Korea). PCR reactions was performed in 15 µL final volume containing 2 µL of the template DNA (100 ng/µL), 8µL of Taq Master Mix RED (Ampliqon) and 0.5µL of each primer (0.3 pmol/µL). The applied conditions was: 94˚C for 4min, 94˚C for 40s, 57.8 to 65˚C (according to Table 1) for 30s and 30s at 72˚C for 30 cycles, with a final 5min extension at 72˚C, and all the PCRs were performed in the Thermal Cycler (Eppendorf, Germany). Finally the amplified products were run on 1% agarose gel electrophoresis and visualized with UV transilluminator.

Confirmation of *GUS* reporter gene transfer using gold nanoparticle probe

Confirmation of the *GUS* reporter gene transfer was done using gold nanoparticle probe. First, 2µL of the extractedDNA was mixed with phosphate buffer (0.01 M) and5 µL of each probe and 0.25 MNaCl were first added to the tube resulted from the additional reaction mixture, then they were vortexed and spinned. The tube was placed for 5 minutes at a temperature of 94°C to strain DNA. Then, be hybridized between the probe and DNA and after cooling at room temperature, 120 μl of gold nanoparticles was added to it. After cooling at room temperature, 120 $\mu l$of gold particles is added to create stronger connections between the DNA and the probe. As soon as the change in the color of its absorption is observed, it is read by a spectrophotometer.

Confirmation of *GUS* reporter gene expression

RNA extraction from leaves using RNX-Plus method

Extraction of RNA was performed using leaves and RNX-Plus method. 100 mg of leaves were grounded in liquid nitrogen and transferred to 1.5ml eppendorf tube under laminar, then 1000 µL RNX was added.

The solution was pipetted several times and placed at room temperature for 5 minutes in order to separate nucleoprotein complexes. 200 µL chloroform was also added under the hood and it was shacked vigorously for 15 s, then, it was put on ice or at 4° C for 5 min followed by centrifugation at 4°C with 12000g for 10 min. Supernatant was transferred to a new sterile tube and 500 µL isopropanol was added and incubated on ice or at 4°C for 10 min.

The tube was again centrifuged with 12000 g at 4°C for 10 min. At this stage, RNA sediment is seen at the bottom of the tube as a yellow white pellet. The supernatant is discarded and the precipitate is washed with 1000 µLof 75% ethanol (water-ethanol must be DEPC).

Followed by centrifugation with 7500g at 4° C for 5 min. Then, ethanol is discarded and the remained is left for 10- 15 minat room temperature let the pellet. 50µL DEPC is added to the pellet and was tapped with a finger for more 5 min. Finally, it is placed in 60° C for 10 min and the resulted RNA is kept at -20°C until use.

Reverse transcription reaction

RT-PCR was performed by the 2-step RT-PCR Kit (Product Code: RTPL 12) For avolume of 20microlitersofreaction, it is possible to use 1pgto 2microgramsoftotalRNAor10pgto 500ngofpoly-A. We incorporated the materials according to the following table(Table2).

Confirmation of GUS gene expression using RT-PCR

For performing RT-PCR, first, the mRNA sequence of the desirable GUS and actin genes were achieved from NCBI site (National center for biotechnology information). Then, after considering them, the desirable primer sequences were designed using Oligo7 software (Table 3). Next, IDT site was used to check the quality of the designed primers. Finally, NCBI site was used to confirm the specificity of the designed primers of the Primer Blast.

Table 3: sequence of primers designed for *GUS* genes and actin

gene	primer	Primer sequences(5'-3')	Length Of primer	Tm	GC%	segment Size (bp)
GUS	F	GTCGTGATCGACCAGACTCC	20	62/5	60	601
	R	GCTCACCCACGAAGTTCTCA	20	60/5	55	
ACTIN	F	GTCGACCTCGTCATACTGGT	20	57	55	200
	R	GAGGATGTTCTTCGGGAGCA	20	56	55	

Confirmation of GUS reporting gene expression using gold nanoparticleprobe

GUS gene sequence is identified using the NCBI database with the accession number AF354046.1(Figure 1)and the probes are designed using its exonregions(Table4). The designed probes were put beside each other head-to-tail. These two detectors will create an open oligonucleotide fragment with a length of40. The probability thatthis40- open is randomly hybridized in genomes of other bacteria, pathogens or other organisms is null indicating the specificity of this method for approving transfer of the GUS reporting gene.

***GUS* reporter gene sequence**

GUS reporter gene sequence was obtained from the NCBI database with the access no. AF354046.1. the area used in designing primers and their sequences are shown in Figure 4.

1321 GTCTTGCGGATCGCGAGGGTCTGGTCGTGATCGACGAGACTCCGGCAGTTGGCGTGCACC
1381 TCAACTTCATGGCCACCACGGGACTCGGCGAAGGCAGCGAGCGCGTCAGTACCTGGGAGA
1441 AGATTCGGACGTTTGAGCACCATCAAGACGTTCTCCGTGAACTGGTGTCTCGTGACAAGA
1501 ACCATCCAAGCGTCGTGATGTGGAGCATCGCCAACGAGGCGGCGACTGAGGAAGAGGGCG
1561 CGTACGAGTACTTCAAGCCGTTGGTGGAGCTGACCAAGGAACTCGACCCACAGAAGCGTC
1621 CGGTCACGATCGTGCTGTTTGTGATGGCTACCCCGGAGACGGACAAAGTCGCCGAACTGA
1681 TTGACGTCATCGCGCTCAATCGCTATAACGGATGGTACTTCGATGGCGGTGATCTCGAAG
1741 CGGCCAAAGTCCATCTCCGCCAGGAATTTCACGCGTGGAACAAGCGTTGCCCAGGAAAGC
1801 CGATCATGATCACTGAGTACGGCGCAGACACCGTTGCGGGCTTTCACGACATTGATCCAG
1861 TGATGTTCACCGAGGAATATCAAGTCGAGTACTACCAGGCGAACCACGTCGTGTTCGATG
1921 AGTTTGAGAACTTCGTGGGTGAGCAAGCGTGGAACTTCGCGGACTTCGCGACCTCTCAGG

Figure 4: shows a part of the *GUS* gene exon at the junction of Primers (red) and probe (blue).

Cucumismelo mRNA for actin, partial cds

GenBank: AB033599.1

GenBankGraphics

>gi|6097868|dbj|AB033599.1| Cucumismelo mRNA for actin, partial cds

GGTGACGATGCTCCTAGGGCTGTGTTCCCCAGTATTGTTGGTCGACCTCGTCATACTGGTGCTATGGTTG
GGATGGGCCAAAAAGATGCCTATGTTGGTGATGAAGCTCAGTCCAAAAGAGGTATTCTTACCTTGAAATA
TCCCATTGAACATGGAATTGTCAGTAACTGGGATGACATGGAAAAGATTTGGCATCACACCTTCTACAAT
GAGCTTCGTGTTGCTCCCGAAGAACATCCTCTTCTTCTTACTGAAGCACCACTCAACCCCAAGGCTAACA
GGGAAAAGATGACTGAAATCATGTTTGAAACCTTCAATGTTCCTGCCATGTATGTTGCAATCCAGGCCGT
TCTATCTCTCTATGCCAGT

Reagents	Final Volume: 16 µL
ddH_2O	4 µL
Primer: F (10 pmol/µL)	1 µL
Primer: R (10 pmol/µL)	1 µL
Master Mix	8 µL
Template DNA	40 ng

PCR program			
Steps	Temperature (°C)	time	Number of cycle
Initial denaturing	94	5 min	1
Denaturing	94	60 second	30
Annealing	54-62	50 second	
Extension	72	70 second	
Final extension	72	7 min	1

Table 4: sequence of designed binders for connecting the target RNA and cDNA

Sequence	probe
TGG AGC ATC GCC AAC GAG GC	FC
GGC GAC TGA GGA AGA GGG CG	RC
GCC TCG TTG GCG ATG CTC CA	FR
CGC CCT CTT CCT CAG TCG GC	RR

In order to confirm the expression of GUS gene in melon plant using gold nanoparticle probe is taking advantage of the method used for detection of telomeric DNA introduced in 2009 by Qi.

However, it coincided with changes in the amount and concentration of the used materials and buffers.

Moreover, simple probes (Unmodify) were also used in this research.

Using gold nanoparticles probe to confirm the expression of GUS gene at RNA level

1. In order to confirm the expression of GUS reporting gene in melon plant, the following protocol was used.

2.First, 2µl RNA was mixed and vertexed with 10 µlphosphate buffer 0/02 M

3.Then,5mlofeach probe and some NaCl 0.25 M were first addedto the tubes resulted from the additional reaction mixture, then they were vortexed and spinned.

4. The tube was taken for 25minutes at a temperature of 65C to be hybridized between the probe and RNA and after cooling at room temperature, 120 ml of gold nanoparticles was added to it. Finally, after observing the desired color variation, its an sorption rate was read by spectrophotometer.

Using gold nanoparticle probe to confirm the expression of GUS reporting gene in cDNA level

1- In order to confirm the expression of GUS reporting gene in melon plant, the following protocol was used.
2- 2µlcDNA was mixed and vertexed with 10µlphosphate buffer0/02 M
3- 5mlofeach probe and some NaCl 0.25 M were first addedto the tubes resulted from the additional reaction mixture, then they were vortexedandspinned.
4- The tube was placed for 5minutes at a temperature of 94C to strain cDNA. Then, be hybridized between the probe and RNA and after cooling at room temperature, 120 ml of gold nanoparticles was added to it. Finally, after observing the desired color variation, its ansorption rate was read by spectrophotometer.
5- After cooling at room temperature, 120 micro liters of gold particles is added to create stronger connections between the cDNA and the probe. As soon as the change in the color of its absorption is observed, it is read by a spectrophotometer.

DNA extraction

To determine the concentration of extracted DNA, the amount of light absorption at 260 nm was measured. Wavelength absorption (A_{260}) which is usually equal to 1 is equivalent to 50 µg of DNA in the two monofilaments per ml. In order to determine the purity of extracted DNA, the ratio of light absorption to its length at a wavelength of 260 nm to 280 nm was used. In a pure sample DNA, the absorption ratio A280 / A260, is equal to 1.8 to 2. The lower value indicates a protein contamination while rates higher than 2 stands for large amounts of RNA in the samples. Moreover, in case of A260 / A230, if it is more than 2, it will indicate phenol contamination in the sample. The results of extracting the *Cucumismelo* DNA represent the extracting boiling method. The ratio of absorption for the extracted samples in the boiling method is %2. Agarose gel was also used to check the quality of the extracted DNA. Presence of a band indicates the safety and quality of DNA (Figure 1).

Fig. 2: Results of DNA extracted from *Cucumismelo*. 1- Molecular marker,100 bp, 2- Total DNA

PCR amplification of the*GUS* reporter gene

PCR of temperature gradient for *GUS* gene was set at temperatures of 54° C, 56° C and 59 ° C . PCR results for the *GUS* reporter gene. clearly states that at all temperatures, the band is clear and transparent (Figure 3).

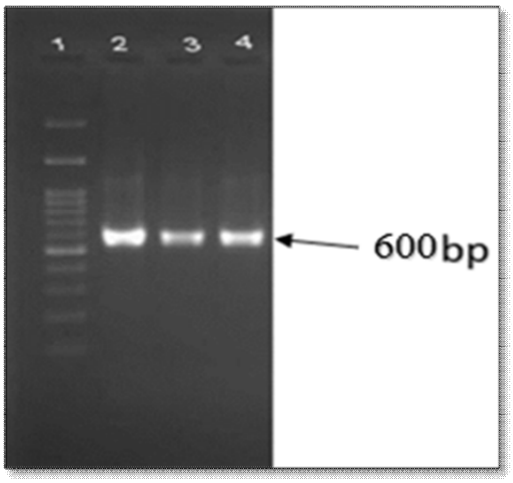

Figure 3- PCR results of temperature gradient for *GUS* reporter gene. 1- molecular marker 100 bp, 2- PCR product for *GUS* reporter gene at a temperature of 54 ° C. 3- PCR product at 56 ° C for *GUS* reporter gene. 4- PCR to reporter gene *GUS* at a temperature of 59 ° C

Next, the PCR results for transfer *GUS* reporter gene. The 600 bp band, were confirmed approving presence of plasmids in bacterium. In addition, no band was detected in plasmid free bacteria **(**Figure 4**).**

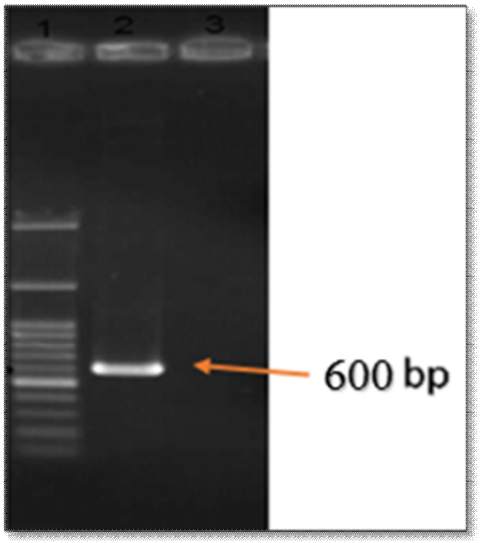

Figure 4. PCR results for *GUS* reporter gene. 1- 100 bp molecular marker, 2- PCR product for *Agrobacterium* containing pCAMBIA1305.2 plasmid (positive control), 3- PCR product for *Agrobacterium* without pCAMBIA1305.2 plasmid (negative control)

Transfer *GUS* reporter gene to *Cucumismelo* plant using gold nanoparticle probes

The results of aggregation of gold nanoparticles and changes of their color or wavelength in presence of a probe for *GUS* gene and genomic DNA are in accordance with Figure 5. Its indicating, DNA probe's coupling with genomic DNA.

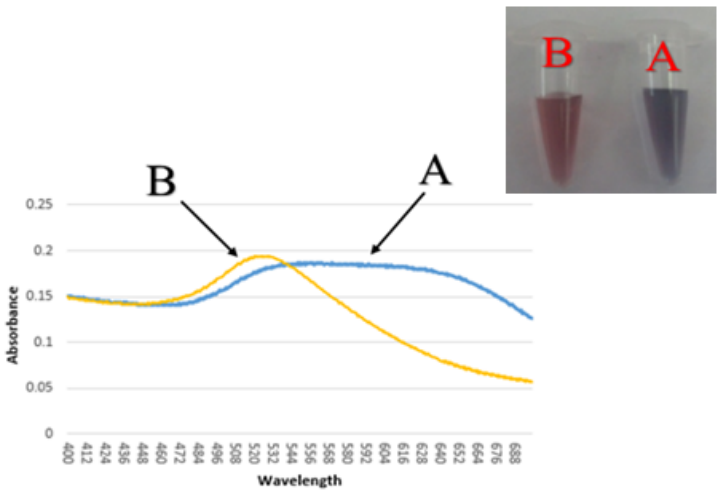

Figure 5. changes in wavelength and colorimetric resulting from single- bind probe for *Gus* gene to target sequence in A- positive control sample compared to B- negative control.

RNA extraction results using agarose gel

As it was explained in chapter 3, Total RNA is extracted. The figure below shows an example of the extracted Total RNA (Figure 2). Over 85% of total cellular RNA is composed of rRNA or ribosomal RNA. Hence, in the process of extracting Total RNA, it is essential to consider the two strands, 18s rRNA and 28S rRNA. Moreover, due to lack of mRNA and tRNA, they are displayed as stretching or weak strands in the gel. So, with presence of the two strands, 28srRNAand 18srRNA in each extraction, it is possible to be ascertained of its accuracy.

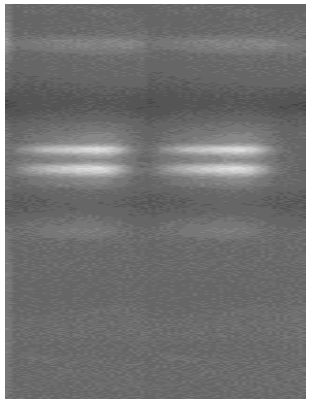

Figure 2: Results of RNA extraction

The results of determining RNA concentration and quality by spectrophotometry

After RNA extraction, its purity rate and light absorption were selected at a wavelength of 260 nm to 280 nm. In addition, in order to determine its concentration, the absorbance was considered as 260 nm. The absorption ratio for all samples was between 1.5 and 1.8 and an average RNA concentration of 230 micrograms per ml was also estimated.

RT-PCR analysis

RT-PCR analysis for the controlled melon plant indicated a 200bp band for actin gene; however, for melon plants infected with Agrobacterium, a 600bp band of GUS reporter gene was confirmed (Figure 3).

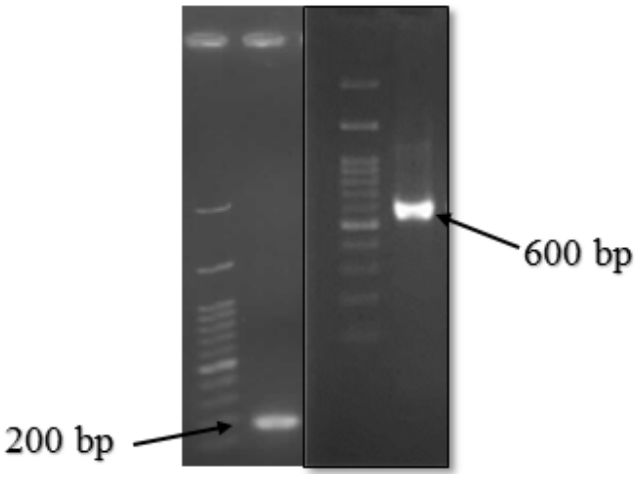

Figure 3: results of RT-PCR for melon plant (control)
Figure A indicates a 200 bp band for actin gene, whereas for melon plants infected with Agrobacterium, a 600bp band of GUS reporter gene was confirmed.

Analysis of synthesized gold nanoparticles using an electron microscope

Synthesized gold nanoparticles are the result of regeneration of gold salt with trisodium citrate with regenerative properties. The taken Images indicate spheric and uniform state of the synthesized gold nanoparticles (about 20 nm) (Figure 4).Figure 4 shows the image of gold nanoparticles taken by TEM.

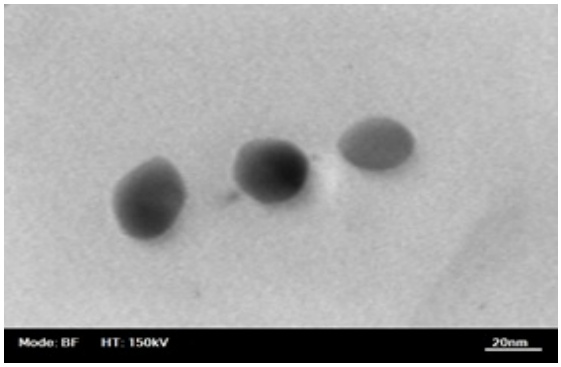

Figure 4: Image of gold nanoparticles synthesized by trisodium citrate taken by TEM with an enlargement of 140000, As is evident in the picture, all synthesized gold nanoparticles are spherical, yet no assembly is observed.

Absorption spectrum of gold nanoparticle synthesis

The maximum absorption (OD) of nanoparticles synthesized after viewing the cherry-red color was achieved with a spectrophotometer at a wavelength of 528 nm (5).

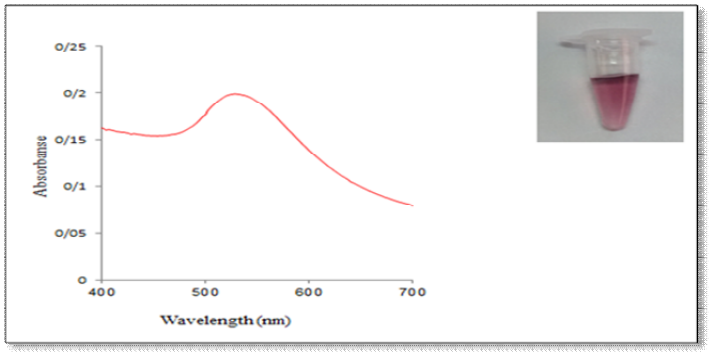

Figure 5: absorption spectrum of gold nanoparticles. As shown in the picture, the maximum absorption of gold nanoparticles occurred at the wavelength of 528 nm.

Results obtained from using Gold nanoparticle probe to confirm GUS reporting gene expression at RNA level

The results of the aggregation of gold nanoparticles and changes the wavelength and their color in presence of the probe and RNA were in accordance with Figure 6 indicating connection of the oligo nucleotide changed into RNA.

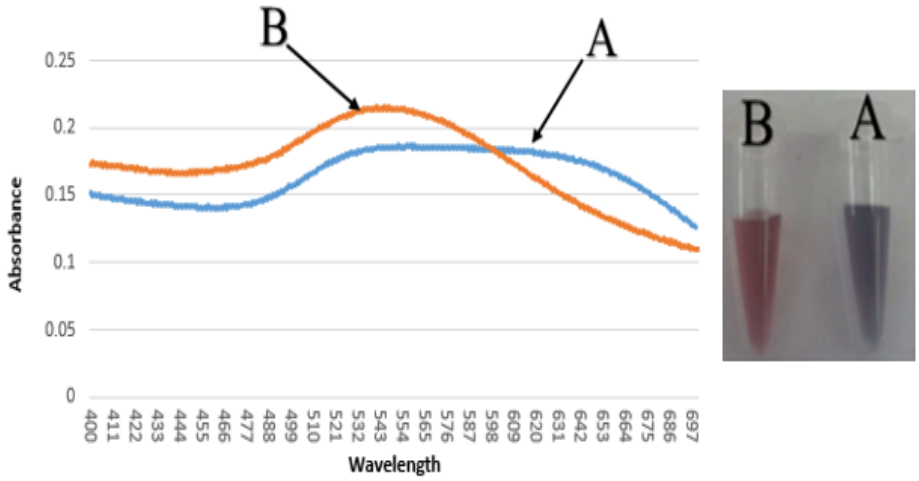

Figure 6: wavelength changes and colorimetric resulting from probe connection to the target sequence in RNA (A) compared to the negative control (B).

The color change from red to purple in the reaction medium is an indicative of the presence of the target sequence in this medium.

In addition, an increase in wavelength from 530 nm (for negative controlled sample) in comparison with 560 nm (for positive controlled sample) is a positive proof of the above- listed claims.

Results of using gold nanoparticle probes to confirm the expression of GUS reporting gene at cDNA level

The result of the aggregation of gold nanoparticles and changes in their wavelength and color in presence of the probe and cDNA (Figure 7) indicates oligo connection of the nucleotide changed into cDNA.

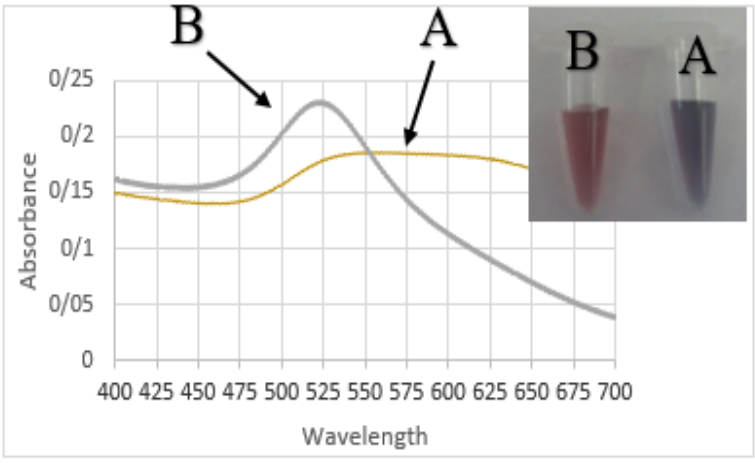

Figure 7: wavelength changes and spectrum resulting from probe connection to the cDNA sequence (A) compared to the negative control (B).

The color change from red to purple in the reaction medium is an indicative of the presence of the target sequence in this medium. In addition, an increase in wavelength where the maximum absorption takes place (from 530 nm to 550 nm) confirmed the above- mentioned claims.

DISCUSSION

The conventional methods of Northern blots, in situ hybridization, and RNase Protection assay have all been used for years to study the expression of genes. Although these methods are used widely, most of them are time- consuming and have relatively low sensitivity, so it has made the detection of small RNA versions difficult or impossible. PCR development as a method for detecting the pattern of gene expression and recognizing rare RNA versions has changed the sensitivity of the methods of gene expression. One of these common methods is RT-PCR (Weis *et al.*, 1992). Fundamental problems such as the time needed to check the products and the possibility of contamination with the previous PCR products while working with large numbers of samples can be felt during PCR.

Yet, current advances in fluorescence energy transfer systems have eliminated these problems. They have also provided the opportunity for quantitative assessment of the finished products as well as their kinetic safely in real time (Weis *et al.*, 1992). The results of using agarose gel, which are achieved at the end of the experiment, are very time- consuming. The results, which are obtained based on the separation of the bands as well as the size, are in most cases inaccurate. Moreover, the results of these samples vary greatly. On the other hand, gels may not be able to show such changes in PCR product Real Time PCR sensitivity has enough sensitivity for detection of these changes. Agarose gel is very poor in judgment and cannot distinguish the differences which are less than 10 times.

In contrast, Real Time PCR can detect changes in the doubles. It is a sensitive and reproducible technique in high yields which represents precise changes in terms of relative quantity of the gel or even small amount (Bustin, 2005). Because of its high adaptability, tracking by Syber Green is often suggested in quantitative systems (Rasmussen *et al*, 1998; Schmittgen*et al*, 2000; Vandesompele, 2002).Nevertheless,for accurate interpretation of data, normalization is more useful.

The principles of this technique resemble those of other techniques such as PCR. Yet, Real Time PCR has some advantages compared to other conventional methods: 1- The product rate is traceable at every cycle, whereas in traditional methods, the product is identified after the end of the reaction and the electrophoresis .2- it is possible to review and analyze several different copies of a tube. 3- Its sensitivity and dynamic range is 1000 times more than the traditionalPCR.4- This technique can be used for determining quantitative value and estimating the initial model precisely.

Despite these advantages, one can refer to its inability to estimate the size of the amplified product and its high cost. Chemical process used in the Real-Time PCR system provides the opportunity to distinguish PCR products during the early phases of PCR reaction. This has many advantages compared with the conventional PCR methods. The conventional methods use agarose gels to identify PCR products in the terminal phase or the final point of the reaction. Real-Time PCR methods are 10000 to 100000 times and 1000 times more sensitive than RNase protection assay (Wong, 2005) and dot blot hybridization methods, respectively. They are even capable of detecting only one copy of a particular copy (Gentle *et al*., 2001). In addition, Real-Time PCR can distinguish between mRNAs and very close sequences.

This method requires much less amounts of DNA samples and, if appropriate equipment, it can produce relatively high amounts of reaction products. The most important disadvantage of Real-Time PCR is its high expenses for equipment and required materials. In addition, because of its relatively high sensitivity, performing perfect

design test and acquiring full understanding of the normal procedures are required for proper and accurate conclusions (Tichopad*et al*., 2003). In comparison with other procedures such as biochemical and molecular detection, or PCR methods, modern methods of nano-biosensors have greater sensitivity and specificity. Designing primers in molecular methods like multiplex PCR sign can suffer from some faults such as proliferation of non-specific deficiencies as well as unwanted bands, low efficiency of DNA replication in the selective pattern or absence of PCR product, and nucleotide mutations that results from errors in connection of neucloids to each other. However, due to the optical properties, using biosensors and nano-biosensors can remove this limitation. For detectors attached to gold nanoparticles, it is possible to design two oligonucleotide simultaneously as a unique detector.

After identifying the position, it increases more than 40specifitiesto nearly100percent. It is also probable that identical points in the other genome, even with a different base pair, get closer to zero, because it uses gold nanoparticles to illustrate even a single nucleotide along with its color variation and absorption spectrum.Taking advantage of detectors attached to gold nanoparticles is a more specific and rapid way to detect than the biochemical and molecular techniques. It can also be achieved spending lower costs.

Time required to identify the target genome expressed in this study is less than 1 hour, which is less than time needed in the traditional methods and even PCR. There is no need for toxic substances such as ethidium bromide. Due to using RNA, total step of the fragment reproduction is removed by PCR. These tests can be performed with a little attention and initial optimization as well as minimal facilities in research centers. Moreover, they do not require special equipment. Using gold nanoparticles can optimize factors such as temperature, size, shape, and salt concentration. Controlling the size and shape of gold nanoparticles by sodium citrate concentration can act as an inhibitor. The common principle is based on the ability of mono-strand primers and fixing them by van der Waals links and electrostatic N forces at the level of gold nanoparticles and their resistance to deposition in presence of some electrolytes such as NaCl. Negative loads of citrate ions on the surface of gold nanoparticles in the solution inhibit gold nanoparticles'deposition. Nevertheless, in the presence of salt solution, they undergo changes and precipitation. Likewise, in presence of target DNA and after hybridization reaction, the primers connect to their complements in case of salt solution and phosphate buffer leading to sediment and its color changes from red to blue.

Due to their optical properties, whenever gold nanoparticles are placed side by side and become dense, they change color. Using the wavelength measurement tools such as Nanodrop and spectrophotometer, they become visible. Color changes can also be seen with eyes, but there is no clear- cut definition of colorchange, it cannot be certainly used for identification. High temperature itself can causese dimentation and change the color of gold nanoparticles. Among the advantages of the diagnostic method used in this study, the absence of gold nanoparticles at high temperatures as well as hybridization detection with RNA target is spatially unimpeded.

The benefits of the diagnostic methods used in this study are: removal of the PCR process and using total RNA, removal of toxic and expensive materials such as Ethidium bromide and agarose, lack of need for cost-based laboratory equipment and fluorescent markers, reduced diagnostic costs in every reaction compared with other diagnostic methods, early detection (in less than 60 minutes), and application of testing in any research laboratory. In fact, there is a similarity between the results of using gold nanoparticle-based detection method and the results in most published papers and the other results observed in the field (Shawky*et al*., 2010; Ali *et al*., 2012; Qi *et al*. 2009, Khalil et al., *et al*., 2014). In case of

using RNA and cDNA as the target used in gold nanoparticles, it is necessary to remind that although using cDNA seems more comfortable mainly because if its higher resistance, the results will show that it will not only make dealing with RNA difficult but it is also more convenient than Cdna, because, firstly, for the synthesis of cDNA, it needs a kit. Moreover, in addition to cDNA which is a single string and can somewhat cover nanoparticles, the RNA which is used as a template for cDNA synthesis breaks down after the construction of cDNA, mainly due to the activity of RnaseH. So, the resulting fragments will act in the form of probe to cover the surface of the nanoparticles and thus, to reduce the amount of color change.

To solve this problem, the following actions are recommended:

Using nano with higher concentration

Since a specific amount of RNA is used for cDNA synthesis, it is possible to see a desired color variation while increasing the concentration of nanoparticles.

Optimizing with RNA

It is possible to provide the required RNA amount for making cDNA synthesis and expose it to RnaseH based on the time needed for this synthesis and optimize it with nanoparticles and then did the rest.

Using GSP instead of using OligodT

While using GSP, only cDNA of a specific gene should be synthesized and only RNA of a specific gene RNA should be degraded. This greatly reduces RNA pollution.

Using detectors attached to gold nanoparticles for detecting biochemical and molecular techniques has unique specificity and high speed and lower costs. The time required to identify the target genome, as expressed in the protocol discussed in this study, is less than 1 hour which is less than that of the conventional methods and even PCR.

There is no need for toxic substances such as ethidium bromide. Moreover, due to the use of genome, the amplification process of the desired fragment is removed by PCR. These tests can be performed with little care and initial optimization in research centers with the least possible facilities, since they do not need any special equipment.

In those affairs such as gene expression, good quality of RNA extraction is considered a critical point. In fact, the hardest and the most important part are extracting RNA with proper quality, because the rest of the process includes using kits and devices that are usually not problematic. However, it was inferred in this study that RNA extraction with high quality is not necessary. In fact, the required results can be achieved if the target fragment attached to the probe is safe. In other words, it is possible to extract RNA using even the simplest methods. Another problem in the use of techniques such as RT-PCR and Real time is that there are false positive results, which result from DNA contamination. However, although gold nanoparticles cannot be problematic since the probes connected to the target are transferred directly to oiling temperature, so it will not meet any 94° C temperature even in case of DNA contamination.

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