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THE EFFICIENCY OF DNA AMPLIFICATION IN PCR TUBE COATED WITH NANOPARTICLES

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บทคัดย่อ

การเพิ่มจำนวนของดีเอ็นเอภายในหลอดทดลองโดยการเติมสารอนุภาคระดับนาโนเมตรในปฏิกิริยาเป็นวิธีหนึ่งที่พบว่ามีประสิทธิภาพในการเพิ่มจำนวนของดีเอ็นเอของงานวิจัยในปัจจุบัน สำหรับในงานวิจัยนี้อนุภาคระดับนาโนเมตรของอะลูมิเนียม ทอง เงิน และไททาเนียมไดออกไซด์ มาเคลือบบนพื้นผิวทั้งด้านในและด้านนอกของหลอดพีซีอาร์โดยกระทำภายใต้เงื่อนไขเดียวกับการปลูกฟิล์มบางของไอระเหยด้วยเทคนิคลำอิเล็กตรอนในสุญญากาศของวัสดุบนฐานกระจกที่ค่าความหนาของฟิล์ม 10 และ 50 นาโนเมตรตามลำดับ หลอดพีซีอาร์ที่ถูกเคลือบด้วยอนุภาคนาโนที่ความหนาต่างๆ ถูกนำมาใช้ในการศึกษาการประสิทธิภาพการเพิ่มจำนวนของดีเอ็นเอที่มีขนาดสั้น (376 คู่เบสของโปรโมเตอร์ CaMV 35S) และดีเอ็นเอที่มีขนาดยาว (2393 คู่เบสของโปรโมเตอร์ CaMV 35S ร่วมกับของยีน *GUS*) เปรียบเทียบผลกับหลอดควบคุม ภายใต้สภาวะของการเกิดพีซีอาร์ที่มีการเติมแมกนีเซียมคลอไรด์เข้มข้น 2, 4 และ 6 มิลลิโมลาร์ พบว่าการเพิ่มจำนวนของดีเอ็นเอเป้าหมายที่มีขนาดสายสั้นและสายยาวภายในหลอดพีซีอาร์ที่ถูกเคลือบด้วยอนุภาคนาโนบริเวณผิวด้านนอกของหลอดทุกชนิดแสดงผลเหมือนกับหลอดควบคุม ยกเว้นในหลอดทดลองที่ถูกเคลือบด้วยทองและไททาเนียมไดออกไซด์ ไม่พบการเพิ่มจำนวนดีเอ็นเอดีเอ็นเป้าหมายที่มีขนาดสายยาว ในหลอดที่เคลือบด้วยอะลูมิเนียมที่ภายในหลอดแสดงผลการเพิ่มจำนวนดีเอ็นเอเหมือนกับหลอดที่ไม่ได้เคลือบ หลอดมีความหนาของฟิล์ม 10 นาโนเมตรของเงินส่งผลต่อการเพิ่มจำนวนดีเอ็นเป้าหมายที่มีขนาดสายสั้นแต่ลดจำนวนดีเอ็นเป้าหมายที่มีขนาดสายยาวภายใต้สภาวะพีซีอาร์ที่เติมแมกนีเซียมคลอไรด์เข้มข้น 4 มิลลิโมลาร์ สภาวะการทำพีซีอาร์ซึ่งเพิ่มแมกนีเซียมคลอไรด์เข้มข้นถึง 6 มิลลิโมลาร์ สามารถเพิ่มจำนวนดีเอ็นของดีเอ็นเอเป้าหมายที่มีขนาดสายยาว ในขณะที่หลอดที่เคลือบด้วยฟิล์มเงินและไททาเนียมไดออกไซด์ภายในยับยั้งการเพิ่มจำนวนดีเอ็นเอภายใต้ทุกสภาวะของการทดสอบ

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ABSTRACT

The DNA amplification *in vitro* by adding various nanoparticles in polymerase chain reaction (PCR) is one of the methods which have recently been explored to enhance the amplification efficiency. In this research, aluminum (Al), gold (Au), silver (Ag) and titanium dioxide (TiO_2) nanoparticles were deposited inside or outside a surface of the PCR tubes using the same conditions of electron beam evaporating on glass substrates at 10 and 50 nm film thicknesses. The PCR tube deposited with each nanoparticles at difference thicknesses were used for the DNA amplification efficiency of short fragment (CaMV35s promoter: 376 bp) and long fragment (CaMV35S promoter combining with *GUS* gene: 2393 bp) in comparing to the control tube. Under the PCR condition adding 2, 4 and 6 mM $MgCl_2$, DNA amplifications of short and long fragments in tube coated outside with all types and thickness of nanoparticles showed a similar result with control tube. Except in those in the tubes coated with 10 nm thickness of Au and TiO_2, the amplification results show a loss of the long DNA target. The Al-coated inside tubes showed the similar effects in DNA amplification to the non-coated control. Ten nanometer thickness Ag-coated inside tubes affected increase of short DNA target but reducing long DNA target under PCR condition adding 4 mM $MgCl_2$. The PCR condition which increased $MgCl_2$ to 6 mM could enhance DNA amplification of long target fragments. While the tubes deposited inside with Ag and TiO_2 films inhibit DNA amplification under all test conditions.

Keywords : PCR, nanoparticles, DNA amplification, thin films-deposited technique.

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TABLE OF CONTENTS

	Page
Abstract in Thai	I
Abstract in English	III
Acknowledgement	IV
Table of contents	V
List of tables	VIII
List of figures	IX
Chapter 1 Introduction	1
1.1 Statement and significance of the problems	1
1.2 Goal and objectives	2
1.3 Scope of the study	2
1.4 Expected results	3
Chapter 2 Literature review	4
2.1 Structure of Deoxyribonucleic acid	4
2.2 Melting of double-stranded DNA	4
2.2.1 Effect of ionic strength	5
2.2.2 Effect of base composition	6
2.2.3 Effect of pH	6
2.2.4 Reliance on DNA chain length	7
2.2.5 Reliance on DNA concentration	7
2.3 Detection of genetically modified organisms (GMPSs) by PCR DNA based amplification method	8
2.4 Enhance DNA amplification	10
2.5 Nanoparticle interactions	13
2.5.1 Gold nanoparticles	14
2.5.2 TiO_2 nanoparticles	14
2.6 Thin films deposition by evaporation technique	15
2.6.1 Electron beam evaporation	16
2.6.2 Biological thin films applications	16

TABLE OF CONTENTS (CONTINUED)

	Page
Chapter 3 Research methodology	19
3.1 Materials	19
3.1.1 Nanoparticles	19
3.1.2 Nucleic acid	19
3.2 Reagents and laboratory apparatus	19
3.3 Instruments	19
3.4 Methods	20
3.4.1 Tube deposition with nanoparticles	20
3.4.1.1 Preparation for depositing nanoparticle	20
3.4.1.2 Thin film deposition	20
3.4.2 Condition of transgene amplifications	24
3.4.3 Transgene amplifications in PCR tubes coated with material thin films	26
3.4.4 Thin film characterization	26
Chapter 4 Research methodology	28
4.1 Effect of different types of nanoparticles deposited... PCR tube	28
4.2 Effect of PCR condition on the transgene fragments... Amplification	30
4.3 The effect of the different nanoparticles coated PCR tubes on DNA fragment amplification	31
4.3.1 The effect of nanoparticles and $MgCl_2$..... concentration on the amplification of short DNA..... fragments	31
4.3.2 The effect of nanoparticles and $MgCl_2$..... concentration on the amplification of long DNA fragment	33
4.4 Characterization of the deposited nanoparticles..... on PCR tube surfaces	37

TABLE OF CONTENTS (CONTINUED)

	Page
Chapter 5 Discussion.....	42
5.1 The effect of the different types of nanoparticles deposited PCR tubes on DNA fragment amplification	42
5.1 .1 DNA amplifications using tubes coated outside with different nanoparticles	42
5.1 .2 DNA amplifications using tubes coated inside with different nanoparticles	42
5.2 Characterization of the deposited nanoparticles on PCR tube surfaces	44
References.....	46
Author biography.....	53

LIST OF TABLES

Table		Page
3.1	The evaporation conditions of thin film deposition	25
3.2	Primer sequences for amplification of CaMV 35s promoter and CaMV 35S promoter combining with *GUS* gene	25
3.3	The amplifying condition of short and long transgenes fragments using conventional PCR tube	26
3.4	The amplifying condition of short and long transgenes fragments using tube coated wirh material thin films	26

LIST OF FIGURES

Figure		Page
2.1	Covalent backbone structure of DNA	5
2.2	Diagrammatic representation of the binary vector pCAMBIA 1301	9
2.3	DNA amplification using polymerase chain reaction	10
2.4	Optical detection with gold nanoparticles as fluorescent quencher	12
2.5	Scanometric DNA assay	12
2.6	An illustration of a typical electron-beam evaporation system	17
2.7	Diagram represent the outline research methodology	21
3.2	Materials for thin films deposition	22
3.3	Electron beam evaporator	22
3.4	The electron beam evaporator systems in vacuum chamber	23
3.5	The PCR tubes on the sample substrate holder	23
3.6	Thin film tube deposition with nanoparticle	24
3.7	Atomic Force Microscope	27
4.1	Tube coated with TiO_2 and Al	28
4.2	Tube coated with Ag	29
4.3	Tube coated with Au	29
4.4	The PCR product of amplified short DNA fragments at 60, 62, and 64°C annealing temperature with 2-6 mM $MgCl_2$ concentration	30
4.5	The PCR product of amplified long DNA fragments at 66 and 68 °C annealing temperature with 2-6 mM $MgCl_2$ concentration	31
4.6	The affectation of Al films on short fragment amplification at 62 °C annealing temperature	32
4.7	The affectation of Ag films on short fragment amplification at 62 °C annealing temperature	33
4.8	The affectation of 10 nm films thickness Au films on short fragment amplification at 62 °C annealing temperature	34
4.9	The affectation of TiO_2 films on long fragment amplification at 68 °C annealing temperature	34

LIST OF FIGURES

Figure		Page
4.10	The affectation of Al films on long fragment amplification at 68 °C annealing temperature	35
4.11	The affectation of Ag films on long fragment amplification at 68 °C annealing temperature	36
4.12	The affectation of Au films on long fragment amplification at 68 °C Annealing temperature	37
4.13	AFM image of TiO2 thin films grown on glass with thickness of 10 nm	38
4.14	AFM images of the morphologies and phase contrast of outside-PCR tube ; conventional tube and TiO_2	38
4.15	AFM images of the morphologies and phase contrast of inside-PCR tube ; conventional tube and TiO_2	39
4.16	AFM images of the morphologies and phase contrast of Al nanoparticles grains grown on glass substrate	40
4.17	AFM images of the morphologies and phase contrast of outside-PCR tube ; conventional tube and Al	40
4.18	AFM images of the morphologies and phase contrast of inside-PCR tube ; conventional tube and Al	41

CHAPTER 1
INTRODUCTION

1.1 Statement and significance of the problems

The technique of polymerase chain reaction (PCR) has become an extensive and well-developed tools of molecular biology, clinical DNA diagnostics and the developing device of biosensor (Sambrook and Russell, 2001; Weissensteiner *et al.*, 2003). This technique can exponentially amplify target DNA or RNA and generate a large number of DNA copies in a short period of time. Its applications, however, is often frustrated by inadequate yield of the target DNA sequence and the accompanying amplification of undesired nonspecific bands (Newton and Graham, 1994; Roux, 1995)

Miscellaneous techniques have been developed to improve PCR specificity for rapid heating-cooling response (Nagai *et al.*, 2001). Many studies indicated the diversity of agents has been added to PCR reaction for enhancing specificity of PCR product. Nanotechnology is rapidly progressing in life science, thus many researchers have been studying and solving vital issues in molecular biology using nanotechnology. Recently, it has been reported that gold nanoparticles significantly improved the yields of PCR reaction under fast cycling condition. These nanoparticles have been used in order to elude nonspecific amplification even at low annealing temperatures (Li *et al.*, 2005a; Li *et al.*, 2005b). The previous researches presented that single-walled carbon nanotubes and carbon nanopowders could increase the yields of PCR and improve the efficient amplification for long PCR reactions, respectively (Daxiang *et al.*, 2004; Zhang *et al.*, 2007).

In recently, the genetically modified plant (GMP)-derived products have been increased in many countries. The regulation involved in identification and labeling of GMP products was urged. Analytical methods have been developed in order to screening of GMP product. To verify GMPS product contaminant in cereals and foodstuff, nanoparticles thin films tube were used for increasing sensitivity of transgene fragment amplification.

In this study, PCR tube were deposited with nanoparticles thin films and used for a study of the enhancing amplification efficiency of transgene fragments (Short DNA fragment, CaMV35S promoter; 376 base pairs and long DNA fragment, CaMV35S promoter combining with *GUS* gene; 2393 base pairs). The relationship between nanoparticles and the concentration of magnesium chloride ($MgCl_2$) on DNA fragment amplifications will be investigated. This technique may be benefit to increase efficiency for screening transgene in product derived GMPs.

1.2 Goal and objectives

The goal and objectives of this research are as follows:

1. To produce PCR tube deposited with different type of nanoparticles.
2. To study the suitable condition for PCR amplification of the transgene fragments.
3. To compare the different types of nanoparticles on PCR tubes which are suitable for DNA fragment amplification.
4. To study the effects of depositing positions (inside and outside PCR tubes) and thickness of each type nanoparticle on DNA fragment amplification.
5. To study the interaction between nanoparticles and $MgCl_2$ concentration affected on the amplification of short and long DNA fragments.
6. To characterize the deposited nanoparticles on PCR tube surfaces using atomic force microscope.

1.3 Scope of the study

In this study, each type of nanoparticles (aluminum (Al), gold (Au), silver (Ag) and titanium dioxide (TiO_2)) will be deposited on a surface of the PCR tubes and glass substrates using the electron beam evaporator. The outside and inside of PCR tubes will be deposited with materials vapor using the same condition of 10 and 50 nanometers film thicknesses glass substrates. The tube coated with different nanoparticle thicknesses will be used for the short fragment (CaMV35S promoter: 376 bp) and long fragment (CaMV35S promoter combining with *GUS* gene: 2393 bp) of DNA amplification in comparing with the non deposited tube (control). The changes in an annealing temperature of each primer pair will be adjusted for the suitable DNA

amplification. The relationship between nanoparticles and the concentration of $MgCl_2$ on DNA fragment amplifications will be investigated.

1.4 Expected results

The expected results from this research are as follows:

1. PCR tube coated with different types of nanoparticles.
2. The suitable condition for PCR amplification of transgene fragments.
3. The identification of type of nanoparticles on PCR tubes which are suitable for DNA fragment amplification.
4. The result of the effects of depositing positions (inside and outside PCR tubes) and thickness of each type nanoparticle on DNA fragment amplification.
5. The interaction between nanoparticles and $MgCl_2$ concentration affected on the amplification of short and long DNA fragments.
6. The characterizations of PCR tube surfaces.

CHAPTER 2
LITERATURE REVIEW

2.1 Structure of deoxyribonucleic acid

Deoxyribonucleic acid (DNA), a polyelectrolyte in which the monomeric unit is the nucleotide, is a nucleic acid responsible for the storage of biological information. Nucleotides have three characteristic components: a nitrogen containing base, a pentose, and a phosphate group. The nitrogenous bases are derivatives of two components, pyrimidine and purine. DNA contains two major purine bases, adenine (A) and guanine (G), and two major pyrimidine base, cytosine (C) and thymine (T). Pyrimidines are planar molecules and purines are nearly planar. This geometry and the fact of bases are hydrophobic and relatively insoluble in water. The hydrophobic stacking interaction also involves van der Waals and dipole-dipole interaction between the bases. The stacking helps minimizing the contact with water and the important modes of interactions between bases in nucleic acids. The other is the hydrogen bond that is formed between the bases and allows for a complementary association of two strands of nucleic acid. Besides the successive nucleotides are covalently bonded through phosphate group bridges by a phosphodiester linkage. The covalent bonds of nucleic acids consist of alternating phosphate and pentose residues, while the nitrogenous base can be visualized as side groups connected to the backbone at regular intervals. The covalent backbone structure of DNA is shown in figure 2.1. The backbone is hydrophilic, phosphate groups have a very low pK_a, close to one, and are completely ionized and negatively charged at pH 7 (Cantor and Schimmet, 1980).

2.2 Melting of double-stranded DNA

The DNA molecules in their native state conformation certainly change in the solution conditions lead to the melting or denaturation of the DNA molecules, that is, a loss of the secondary structure. DNA melting is the mechanism of separating the two strands of a double-stranded DNA molecule into two single

strands. The denaturation of the nucleic acids destroys in the part the close interaction between the stacked bases, which leads to an increase in the absorption called the hyperchromic effect. The transition is thermally induced and the temperature at the midpoint of the transition is called the melting temperature, T_m (Doty *et al.*, 1959; Marmur and Doty, 1962). The melting temperature is sensitive to the environment of DNA chains such as ionic strength, pH, DNA concentration, strand length, and base sequence.

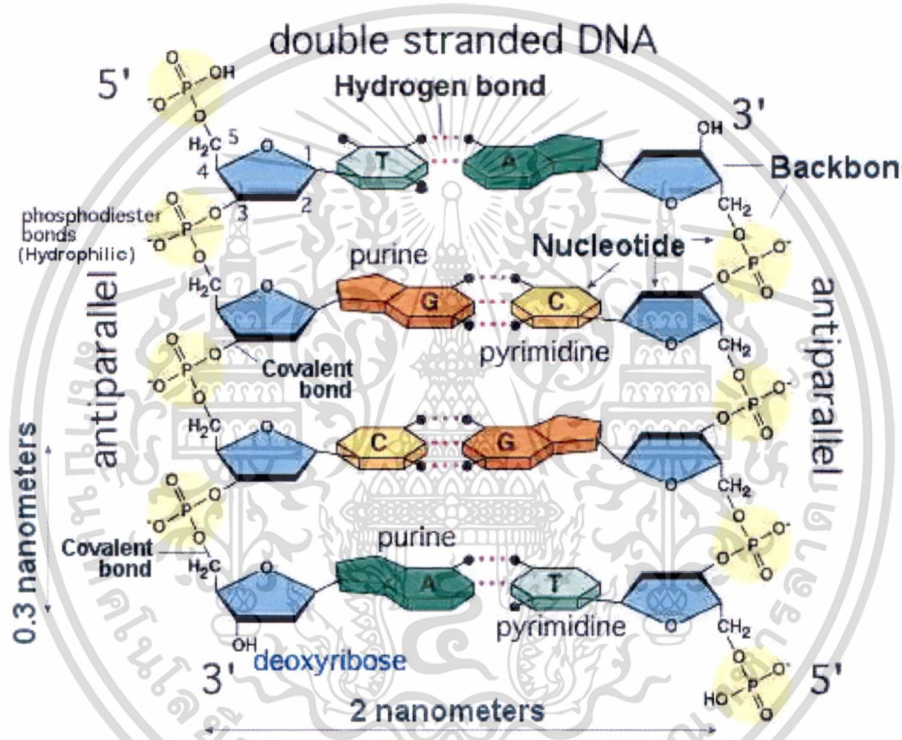

Figure 2.1. Covalent backbone structure of DNA

Source: http://academic.brooklyn.cuny.edu

2.2.1 Effect of ionic strength

Many studies indicated the ionic strength controlling the stability of the DNA molecules in solution (Clausen-Schaumann *et al.*, 2000 ; Conwell *et al.*, 2003 ; Viasnoff *et al.*, 2006). The melting temperature increases with the ionic strength of the solution. According to the study of Shchidkraut and Lifson (1965), the T_m has a

linear dependence with the logarithm of the concentration of salt, and that the slope is basically independent on the base composition. The dependence of the T_m on the salt concentration is related to the electrolyte can stabilize the double helix by screening the electrostatic repulsions between the phosphate groups. Thus double-stranded DNA molecules are more stable when the concentrations of monovalent salt are increased.

2.2.2 Effect of base composition

The change in the melting temperature with the base composition is due to the G-C base pair has three hydrogen bonds, requiring more energy to dissociate than an A-T base pair with only two hydrogen bonds. By this approach, DNA molecules with higher amounts of G-C base pairs give higher melting temperatures for the same concentration of salt. Furthermore the nucleic acid molecules with the same base composition but a different sequence have different helix-coil transition (Wells *et al.*, 1970 and Borer *et al.*, 1974). Moreover, Lohman and Bujalowski (1994) have examined the ability of the *Escherichia coli* single-stranded DNA binding protein (SSB) tetramer to form its different binding modes over a range of salts concentrations and base composition indicates that both electrostatic and nonelectrostatic factors contribute to the negative cooperativity associated with single-stranded DNA binding to the SSB tetramer.

2.2.3 Effect of pH

As a result of the protonation and deprotonation of the bases under acidic and alkaline condition, which is non-monotonic in action of the T_m of DNA with pH increasing (Privalov and Ptitsyn, 1969 ; Lando *et al.*, 1994). In alkaline conditions, some of bases (U, T, and G) will become deprotonated, which destabilizes the formation of the double helix and lowers the melting temperature (Cantor and Schimmel, 1980). Besides at the pH below 4.5 G, C, and A become protonated, which disequilibrates the double helix and lowers the T_m (Lando *et al.*, 1994).

However, for low pH the protonation process can be more complicated, since both polyA and polyC can form double-stranded protonated helical structure. Likewise, it has been observed that the T_m of both the acid form of polyC and polyA increase with the decrease of the ionic strength (Cantor and Schimmel, 1980).

2.2.4 Reliance on DNA chain length

The reliance of the T_m on the chain length has been studied for a homologous series of oligonucleotide duplexes of the kind $A_nU_n.A_nU_n$. According to the investigated, the T_m of the duplex increased with the length of the chain up to certain chain length (Martin *et al*, 1971). However, few experimental studies have been performed that it is difficult to control the base-pair composition and sequences of large molecules during the polymerase chain reaction (Ririe *et al*, 1997).

2.2.5 Reliance on DNA concentration

The dependence of the T_m on DNA concentration was observed by Inman and Jordan (1960) when studying denatured of DNA for solutions of low ionic strengths. In 1971, Martin and co-workers was demonstrated the T_m of systematic oligonucleotide complexes on strand concentration. More recently Korolev and co-authors (1994) have shown a linear dependence of the T_m on the logarithm of DNA concentration for different DNA salt. Recent Monte Carlo simulations have demonstrated the melting of the simple DNA model, and it was distinguished that the counterions are more closely bound to the DNA chains in more concentration regimes (Skepö *et al*, 2007).

2.3 Detection of genetically modified plants (GMPs) by PCR

Genetically modified plants (GMPs) or transgenic plants are typically performed by output traits as an increased content of precious components such as amino acids, fatty acids, vitamins, *etc.*, improving availability of nutrients or a lower concentration of undesirable substances. (ILSI, 2004). The diversity of GMPs and derived products are constantly increasing on the market (James *et al*, 2009). Meanwhile, the detection is becoming challenge in various levels of specificity, multiplexing, speed, and cost-efficiency. Based on legal requirements in the European Union (EU), the enforcement laboratories have primarily focused on testing for compliance with labeling requirements, responding to these rapid alerts and emergency measures has required. As the information on analyzes GMP detection, it is proposed to differentiate GMPSs into several classes according to the information

available on the analytical target, DNA sequences, present in each GMP. There are presently two main types of GMP detection methods in use, those that target the genetic material subject to DNA modification and those that target the resulting protein product.

Methods protein based techniques are the antibody recognition such as transgene product specific strip test, and Enzyme linked immunosorbent assays (ELISA). There are widely used for rapid screening of plant materials from the field or the harvest, but these methods are usually not suitable for detection of GMPs in processed products due to the degradation of the target molecules. On the other hand, the methods targeting DNA are more time consuming but offer potentially all required levels of specificity and ability to quantify the target. However, there is a general consensus in DNA-based GMP methodologies for detection and identification because of their superior specificity of a detection method is linked with its targets (European Commission, 2004 ; European Network of GMPS Laboratories, 2008 ; Holst-Jensen *et al.*, 2003 ; Holst-Jensen *et al.*, 2006). Therefore, the degree of homology between the targets of a detection method and the corresponding DNA sequence or protein in the GMPs is very important.

DNA based amplification methods

At present, s screening are based on the DNA-based detection of the *35S promoter* in the cauliflower mosaic virus (CaMV), the CaMV 35S terminator (T-*35S*), the *Agrobacterium tumefaciens* nopaline synthase terminator (T-*nos*) sequence, and in some case, the *NptII* terminator (Lipp *et al.*, 1999 ; Trapmann *et al.*, 2002). The amplification assay is estimated exogenous β-glucuronidase (GUS) and hygromycin phosphortransferase gene copy numbers in transgenic GMPS plants. The reporter gene GUS driven by CaMV 35S promoter and *nos* poly-A terminator sequences along with a selectable marker gene *h*ygromycin *p*hospho*t*ransferase (*hpt*) under the control CaMV 35S promoter and poly-A terminator were located in the binary expression vector. This vector is based on pCAMBIA1301 which kanamycin (*kan*R) resistant for the bacterial selection marker. The express gene was cloned and inserted into the expression vector pCAMBIA1301. The recombinant gene was transformed into the *Agrobacterium tumefaciens*-mediated, and expressed in GMPS plant. The binary vector of pCAMBIA1301 is represented in figures 2.2.

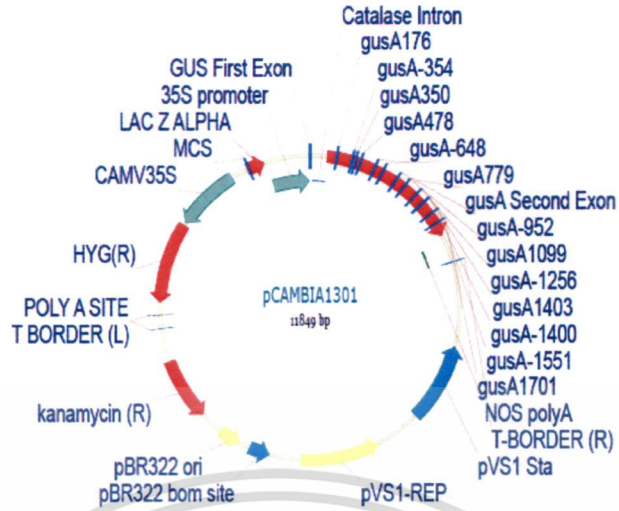

Figure 2.2 Diagrammatic representation of the binary vector pCAMBIA 1301

Source : http://www.cambia.org

For GMP products screening, the commonly used for rapid detection is the polymer chain reaction (PCR) technique. PCR is amplification technique involve denaturation of the double stranded nucleic acid followed by annealing of a short oligonucleotide or primer. Two short pieces of synthetic DNA or called primers are needed, each complementary to one end of the DNA to be multiplied. The first primer matches the start and the coding strand of the DNA to be multiplied, while the second primer matches the end and the non-coding strands of the DNA to be multiplied. The second step involves binding of the two primers to their complementary strands, respectively. The third step involves making two perfect copies of the original double stranded DNA molecule by adding the right nucleotides to the end of each primer, using the complementary strands as templates. Once the cycle is completed, it can be repeated, and for each cycle the number of copies is doubled, resulting in an exponential amplification. After 20 cycles the copy number is approximately 1 million times higher than at the beginning of the first cycle (figure 2.3)

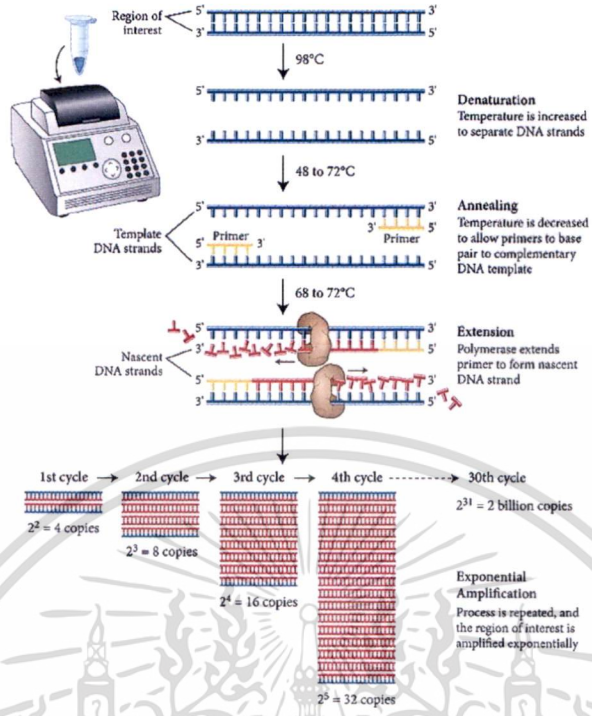

Figure 2.3 DNA amplification using polymerase chain reaction
Source : https://www.neb.com.

2.4 Enhance DNA amplification

The PCR is one of the most important methods in target DNA amplification. This method has become an extensive and well-developed tool of *in vitro* amplification technique for the target DNA sequence and pathogen detection (Sambrook and Russell, 2001 ; Weissensteiner *et al.*, 2003). Specificity and yield of nucleic acid amplification are important features for a reliable of this technique. These amplification features are controlled by many factors such as primer design, a nucleotide sequence of the template DNA, denaturation/annealing/extension times and temperatures, enzyme type, primer-dimer occurrence and buffer content of the PCR reaction (Chen and Janes, 2002 ; Brownie *et al.*, 1997).

It has been found that various organic additives significantly improve yields in GC-rich template DNA, including dimethylsulfoxide (DMSO) (Winship, 1989), glycerol, polyethylene glycol (Varadaraj and Skinner, 1994 ; Baskaran *et al.*, 1996),

betaine (Henke *et al.*, 1997), amides (Chakrabarti and Schutt, 2001) and single-stranded DNA-binding proteins (SSBs) (Olszewski *et al.*, 2005). Moreover, the dependence of $MgCl_2$ under appropriate concentration additives can increase DNA polymerase stability on the amplification of DNA fragment. $MgCl_2$ is a cofactor for Taq polymerase enzyme and also it helps in addition of correct deoxyribonucleotide triphosphate (dNTPs) complementary to the sequence in newly synthesizing strand by binding to dNTPs. Hence it is important to maintain a balanced concentration both in the reaction mixture for effective amplification (Innis *et al.*, 1990 ; Mc. Pherson *et al.*, 2000 ; Chen and Janes, 2002 ; Weissensteiner *et al.*, 2003).

At present, applications of nanotechnology in molecular biology are trying to extend the limits of contemporary molecular biology techniques. Nanotechnology and DNA-nanoparticle coordinate systems have emerged as novel and extremely powerful tools for DNA assay (Li *et al.*, 2005a ; Li *et al.*, 2005b. ; Daxiang *et al.*, 2004 ; Condon, 2006 ; Zhang *et al.*, 2007). Various types of nanoparticles were investigated but distinctive attendance was brought upon metal nanoparticles. Since metal nanoparticles also increase in out-put signal and sensitivity can be achieved through amplify signal by automettallography (figure 2.4) (Storhoff *et al.*, 2004b ; Tansil and Gao, 2006). In addition to increase the detection capability of point mutation in genetic lesions, and chip-based hybridization have been used the properties of gold nanoparticles (AuNPs) to enhance signals that can be easily detected (figure 2.5) (Li *et al.*, 2006 ; Nam *et al.*, 2002; Taton *et al.*, 2000; Thaxton *et al.*, 2006). AuNPs have widely applications in molecular biology and have been reported to improve the yield of PCR reaction under fast cycling condition (Nagai *et al.*, 2001). Besides silver nanoparticles conjugated to oligonucleotides have recently emerged as powerful tools for the detection of target DNA sequences, and have been used in the design of colorimetric assays based on aggregation induced by sequence-specific hybridization (Chen *et al.*, 2004; Liu *et al.*, 2005; Lee *et al.*, 2007). Additionally, the addition of silver nanoparticles in PCR amplification can significantly remain long PCR specificity after repeated rounds of cycles (Wang *et al.*, 2007). Furthermore, functionalized tetrapods ZnO nanostructures were also shown to increase the PCR product (Nie *et al.*, 2007). Daxiang and teams and Zhang and coworkers proposed that single-walled carbon nanotubes and carbon nanopowder could increase the yield of PCR and could improve the amplification efficiency for

long PCR reactions, respectively (Daxiang *et al.*, 2004 ; Zhang *et al.*, 2007). Moreover, the impact of titanium dioxide (TiO_2) nanoparticles on DNA synthesis was investigated *in vitro* (Shiqiang *et al.*, 2008). Further, the yields of PCR reactions were gradually decreased when the concentrations of various nanoparticles were exceeded (Binh *et al.*, 2008).

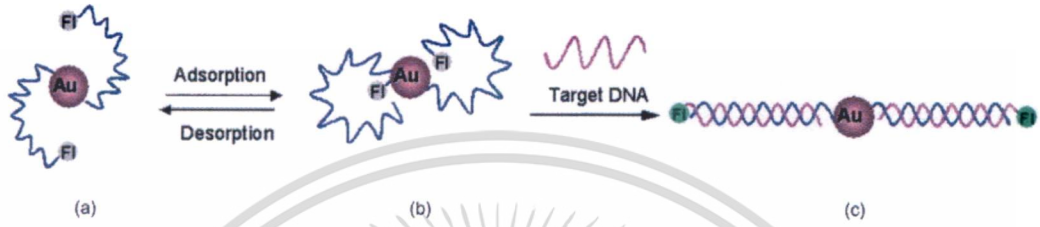

Figure 2.4. Optical detection with Au nanoparticles as fluorescent quenchers. (a, b) owing to the conformation, the fluorescence labels are in close to the Au nanoparticles and their signals are quenched. (c) hybridization, the rigid double-helical DNA molecules 'open up' their conformation and fluorescence is restored.

Source: Tansil and Gao (2006)

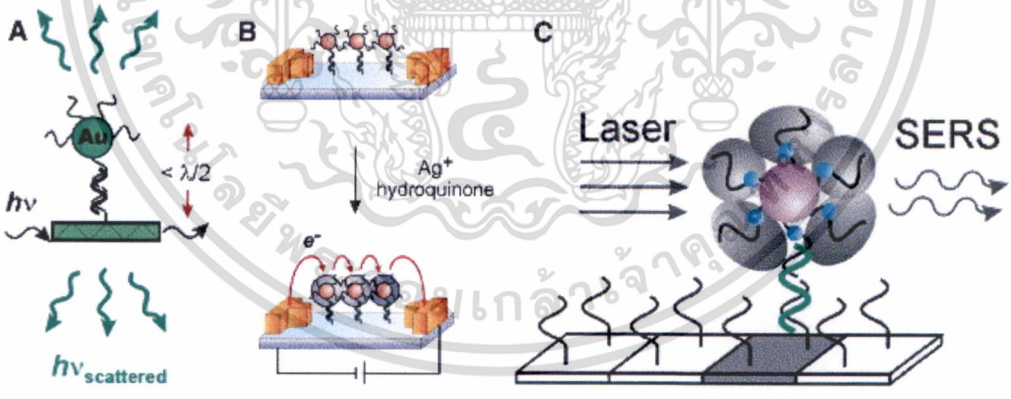

Figure 2.5. Scanometric DNA assay (a) immobilization of capture Au nanoparticles probes on electrode. (b) hybridization with target DNA, labeled detection probe and amplification by reductive deposition of Ag. (C) surface-enhanced Raman spectroscopy followed by scanometric detection.

Source: Thaxton *et al.* (2006)

An alternative to enhance the PCR amplification efficiency is to provide a physical barrier to prevent aggregation. Therefore, the amplify PCR enhancement can be achieved by coating metal and metal oxide nanoparticles, each material of Au, Ag, TiO_2, and Al nanoparticles, on substrate or micro-tubes, efficient of DNA amplification can be verified.

2.5 Nanoparticle interactions

The interactions of nanoparticles with biosystems are multidimensional. Nanoparticles come in a plenty of shapes, sizes, and surface, and can be decorated with a variety of functionality. The surface functionalization of nanoparticles is one method for conforming the properties of particles to fit targeted applications. The surface modification of nanoparticles is typically tasks to improve mechanical and chemical performance at the surface, modify the capability to assemble the particles to target desired chemical, physical or biological environments, and enhance the soluble nanoparticles in various solutions (Alejandro and Marta, 2010). The chemical functionality at the surface of the nanoparticle affects the interactions of nanoparticles with biosystems (Parag *et al.,* 2009). The surface chemistry of a nanoparticle effects on the structure and function such as the protein adsorbtion which change in the secondary structure of both bovine serum albumin and fibrinogen on hydrophobic silica spheres than on hydrophilic (Roach *et al.,* 2006). Moreover, the demonstrate control over both protein structure and function by tailoring the surface chemistry of nanoparticles, with three levels of interaction: no binding, inhibition with denaturation, and inhibition with retention of structure (Hong, *et al.,* 2004). The effect of this protein corona may have special significance for nanoparticles, due to the increased importance of surface effects for particles of this size. Several studies have shown that biological responses to nanoparticles tend to scale with surface area rather than mass. The surface areas shrink much more slowly than their volumes, causing nanoscale materials to have greater surface-to-volume ratios than larger particles. A larger surface-to-volume ratio also implies more proteins will bind a nanoparticle than a particle of larger size (Alejandro and Marta, 2010).

Besides, the interaction of biological systems with inorganic materials is of interest in biomolecules adsorption modulates the formation of biofilms onto surfaces. The dissolution or precipitation processes, reconstruction of the surface, adsorption of ions, small molecules and macromolecules, and redox reactions may occur when an inorganic material meets an extremely complex. Large differences in chemical composition, ionic strength and acidity of biological fluids exist depending on tissues, cellular compartments and nanoparticle types (Nel. *et al.,* 2009 ; Ivana *et al.,* 2011).

2.5.1 Gold nanoparticles

Gold nanoparticles are the most stable metal nanoparticles such stability and surface functionalization. The use of gold expands over a wide range of applications, including chemical/biological sensors, cytological staining, and electronics. Due to their high surface energy, gold nanoparticles are extremely sensitive to environmental conditions. Maeda and colleagues reported that color change occurred even between a single DNA-modified probe nanoparticles and target DNA. The color change arose vary rapidly in their framework, compared to the crosslink method. A mismatch in the forefront of the probe anchored on the gold nanoparticles was sensitive to hybridization in their system (Maeda *et al.,* 2003 ; Maeda *et al.,* 2005). The modified single stranded DNA was absorbed onto the gold nanoparticles surface to form a lying-down structure, leading to a nonspecific adsorption of the probe DNA. This adsorption was attributed to the interaction between the particle surface and one of the four bases constituting the probe DNA, thus giving rise to particle aggregation (Storhoff *et al.,* 2002). The interparticle attraction force is van der Waals force, which is responsible for the particles aggregation. The two major repulsion forces that contribute to Au stabilization are electrostatic and steric repulsion forces. DNA–Au conjugates are a promising system for the development of ultrasensitive biosensors. The dimensions of nanoscale structures are well matched with those of biomolecules, which in principle should allow smaller collections of analysts to be monitored.

2.5.2 TiO_2 nanoparticles

Titanium surface, when in contact with air is very rapidly covered by a thin native oxide layer, and incorporate the stable titanium oxide of TiO_2, TiO and Ti_2O_3. The biomolecules which are adsorbed onto the surface will make direct contact with the oxide and, under physiological condition, the TiO_2 surface will have a negative net charge (Ellingsen, J.E. 1991 ; Kasemo, B. 1983). The study structural information relation relating to protein layers adsorbed onto TiO_2 is used collagen, which is the most important protein and is extensively adsorbed onto TiO_2 surfaces. Köppen and team have been report a direct interaction between the amino acids of the collagen helix and the TiO_2 surface. The only for collagen without charged amino acids in the side chain was observed. These modeling results showed that the side chains formed the high number of connections to the surface atoms of the TiO_2. The ammonium group interected with two adjacent oxygen atoms on the surface, while one hydrogen of the NH^+_3 group formed a stable hydrogen bond with the OH on the surface, and two other hydrogen atoms formed unstable hydrogen bonds with one hydroxyl group on the TiO_2 surface (Köppen *et al.*, 2002).

2.6 Thin films deposition by evaporation technique

Thin film technology has expanded into an efficient technique used to fabricate many products. Currently, this development goes the explosion of scientific and technological break-through in microelectronics, fabricating medical devices, process monitoring in food and environmental testing, and nanotechnology. Applications include vary large scale integrated, electronic packing, optical films and devices, sensors and devices, as well as protective and decorative coating. The more important thin film processes are based on liquid phase chemical techniques, gas phase chemical processes, glow discharge processes and evaporation methods. In this research, the electron-beam evaporation technique, whereas one to achieve technique for some typical coating matallic thin films, is employed to fabricate thin films on PCR tube surface.

2.6.1 Electron beam evaporation

The electron beam evaporation is the method of evaporation and condenses as films to decorative and functional metallic on solid substrate surface. A wide verity of materials including refractory metals, low vapor pressure metal, and several different materials can be deposited without breaking the vacuum (Harsha, 2006). The representative of electron beam evaporation system is shown figure 2.6. In the evaporation process, an electron beam is used to heat the source material and cause evaporation. A block of the source material placed in the crucible a filament below the crucible to be deposited is heated to the point where it starts to boil and evaporate. Then it is allowed to condense on the substrate. This process takes place inside a vacuum chamber, enabling the molecules to evaporate freely in the chamber, where they then condense on all surfaces. The energy range is classified in range of 0.1-1.0 eV per vaporized atoms. Typically, vacuum evaporation occurs in the gas pressure range of 10^{-3} mTorr to 10^{-6} mTorr, depending on gaseous contamination in the deposition chamber. Besides, the deposition rate is monitored by quartz crystal in situ real-time deposition, in which it is often used to determine the film growth rate, deposition time, and film thickness. A noticeable advantage of electron beam evaporation over thermal evaporation is the possibility to add a larger amount of energy into the source material. This yields a higher density film with an increased adhesion to the substrate. Because the electron beam only heats the source material and not the entire crucible, a lower degree of contamination from the crucible will be present than in the case of thermal evaporation (Mahan, 2000).

2.6.2 Biological thin films applications

For many decades, thin films down to monolayer thicknesses have been of significant attention, with their great potential and increasing numbers of applications. Both, organic and inorganic materials may deposit in thin and ultrathin film forms on an extensive variety of substrate materials and forms. The thin films surface properties of natural and factitious material are determinants for a range of biocompatibility, biointegration, and functionality. These properties are crucial not

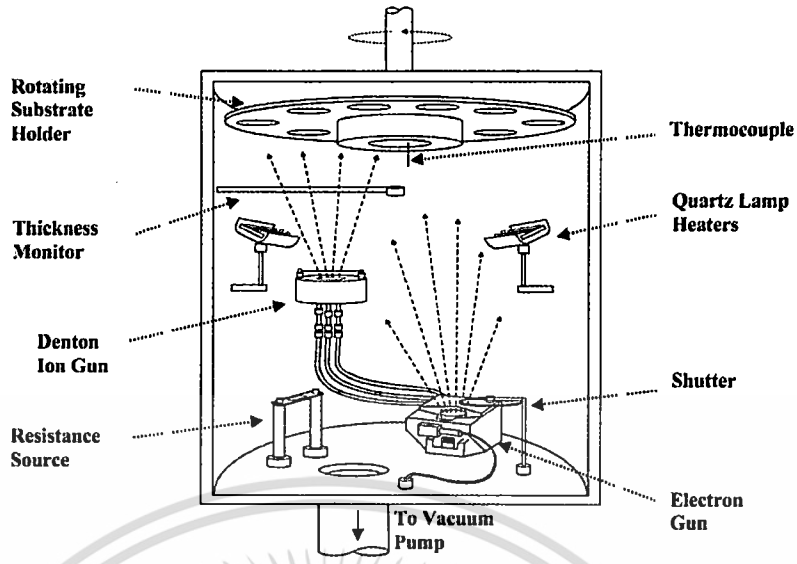

Figure 2.6 An illustration of a typical electron-beam evaporation system.
Source : Mattox (2010)

only for biomedical devices and implants, but also for sensors in various formats, and other applications (Kasemo, 2002 ; Kumar, 2006). In general, whenever an object comes into contact with a biological system, such as DNA, protein or with correspondingly derived fluids (e.g., PCR reaction), the processes that occur at newly formed interface determine whether that object will fulfill its function or will fail. These newly formed interfaces, where the initial contact between material and biological system occurs, are frequently referred to as a biointerfaces. Thin structured polymeric films currently form the focal point for development of advanced materials and conformed interfaces for applications such as biosensors and biologically oriented research involving biointerfaces (Castner and Ratner, 2002 ; Yoshida *et al.,* 2006). In the case of biosensor, the thin films surface roughness and chemical composition may determine the success of the catheter-based *in situ* sensors that can be used to quantify glucose levels in blood, or *ex situ* sensors that address the presence and abundance of particular antibodies (Franchina *et al.,* 1999 ; Seurynck-Servoss *et al.,* 2007). In this respect, the suppression of nonspecific protein adsorption is a key element. The interaction between the analytes in solution and receptors immobilized on a sensor surface can depend crucially on the orientation of the receptor (Malmqvist and Karlsson, 1997 ; Frank and Seamus, 2005). In addition to

thin films surface, the topographical structures and control of the substrate modulus are important in determining PCR reaction on surfaces. This combination of factors and determinants predestines biopolymers (e.g., macromolecular materials) for the fabrication of functional biointerfaces. Biopolymer can be structured conveniently on length scales that cross many orders of magnitude. Biopolymers also possess hierarchical structures, with many levels of different complexities. Here, new approaches, which include the fabrication of reactive surfaces on polypropylene tube by metallic thin films, using electron beam physical vapor deposition, will be used.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Materials

1. Nanoparticles: aluminum (Al), gold (Au), silver (Ag), and titanium dioxide (TiO_2)
2. Nucleic acid: pCAMBIA 1301, and primers (BioDesign Co., Ltd.)

3.2 Reagents and laboratory apparatus

1. Taq DNA polymerase kit is supplied with 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 100 U Taq DNA polymerase, and 50 mM $MgCl_2$ (Invitrogen)
2. 10 mM Deoxynucleotide Solution Mix (dNTP) (NEB)
3. Tris/Borate/EDTA buffer (TBE)
4. Agarose gel electrophoresis
5. Isopropanol (Analytical grade) (BDH)
6. Ethanol (Analytical grade) (BDH)
7. Laboratory apparatuses, including
   - 0.2 mL PCR tube flat cap (Avant)
   - Glass wares
   - Glass slides
   - Micropipettes
   - Forceps

3.3 Instruments

1. Electron beam evaporator (EVT1 auto 306, EDWARDS)
2. PCR instrument (Mastercycle® Ep, Eppendorf)
3. Gel documentation (InGenius, Syngene)
4. Autoclave (Tommy ES-315, Japan)
5. High Speed refrigerated micro-Centrifuge (Tommy MX-305, Japan)
6. Ultrasonic bath (Elmasonic, Germany)
7. Atomic Force Microscope (Seiko,SPA 400, Japan)

3.4 Method

The diagram of research methodology represented in figure 3.1.

3.4.1 Tube deposition with nanoparticles

The PCR tube coated were deposited by electron beam evaporator with different types of nanoparticles and use for studying DNA amplification in various aspects.

3.4.1.1 Preparation for depositing nanoparticle

The sample chambers including inside equipment and the substrates was cleaned before the deposition process. Small parts of equipment or glass slides were put in a beaker of the NaOH cleaning solution, ultrasonicated for up to 15 min and rinsed with distilled water. Large parts will be swabbed down with lint-free cloth, and soaked in the cleaning solution and then rinsed under running distilled water. Consequently, rinse several times after the last cleaning in the best quality deionized water, and then ultrasonic rinsing in methanol and isopropanol, respectively. After that, small parts and glass slide were the air dried and evaporated on clean hot plate. The 0.2 mL PCR tube was sterilized by autoclave at 121 $^{\circ}$C for 15 min.

3.4.1.2 Thin film deposition

Figure 3.2 shows the materials (Al, Au, Ag, and TiO_2) for thin film deposition. These materials were grown inside and outside PCR tubes substrate by the electron beam evaporator (Figure 3.3). Figure 3.4 shows the electron beam evaporator systems in vacuum chamber. The position of PCR tubes on the sample substrate holder during the electron beam deposition was shown in figure 3.5. The tube before and after thin films deposition with nanoparticle process are illustrated in figure 3.6a and b.

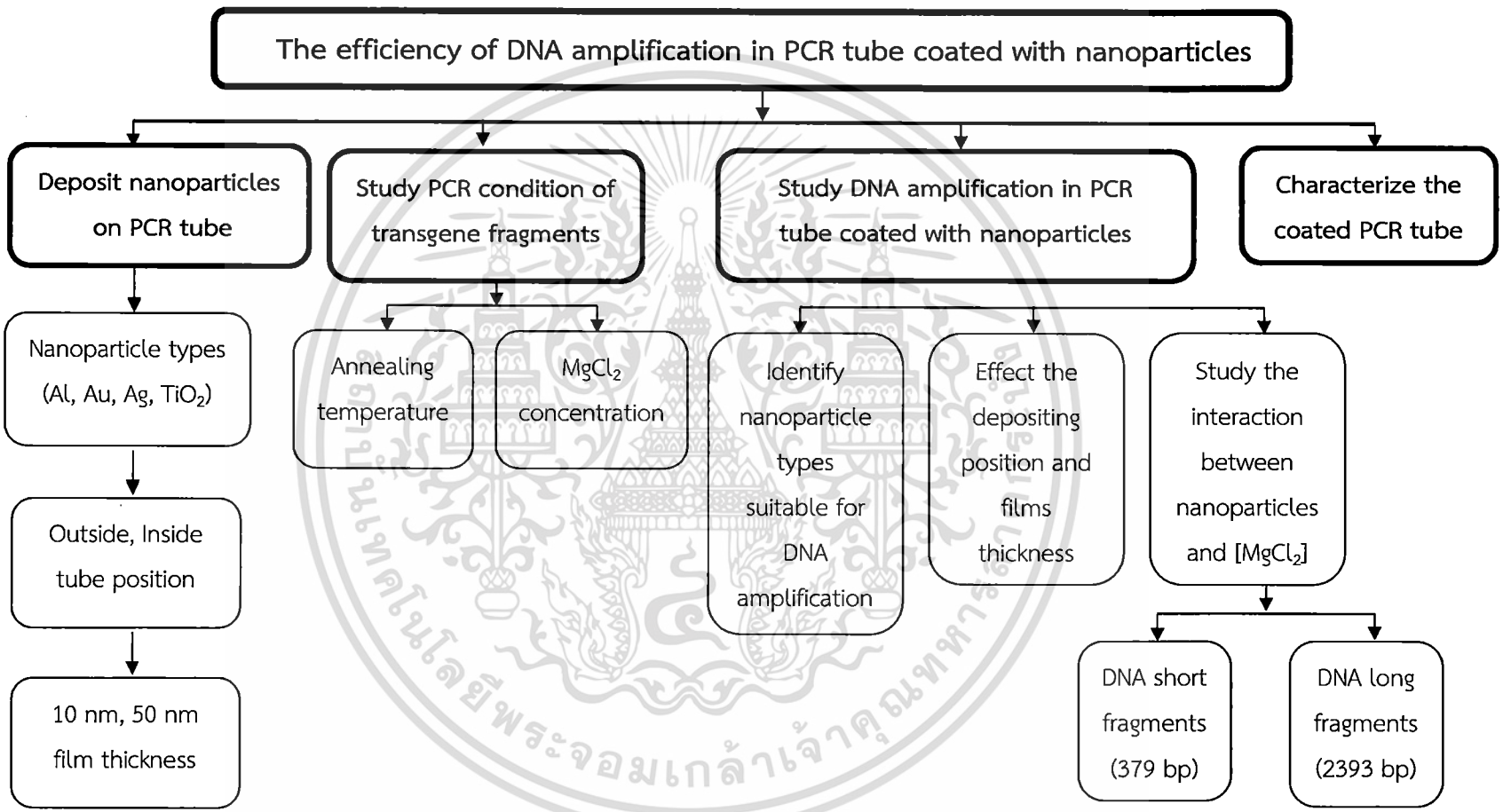

Figure 3.1 Diagram represent the outline of research methodology.

(a) (b) (c) (d)

Figure 3.2 Materials for thin films deposition (a) titania tablets, (b) aluminium tablets, (c) silver pellets, and (d) gold pellets.

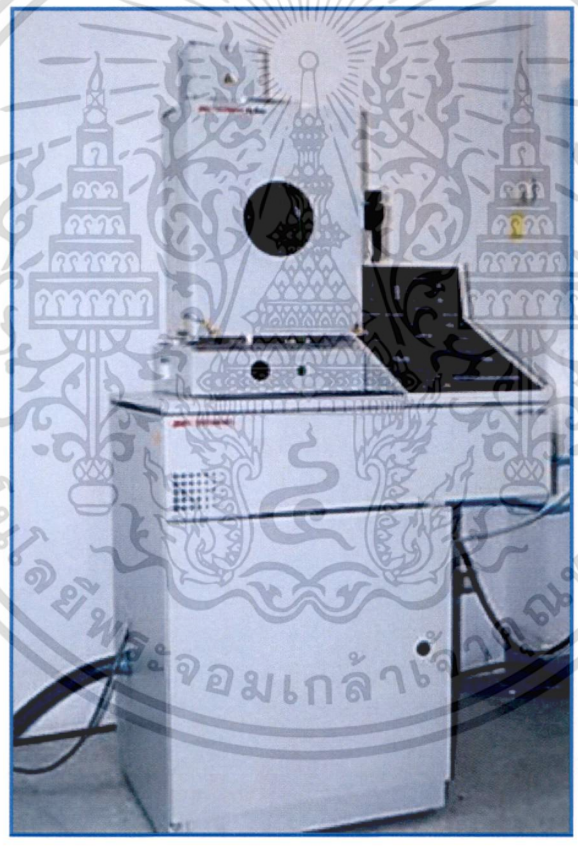

Figure 3.3 Electron beam evaporator (EVT1 auto 306, EDWARDS).

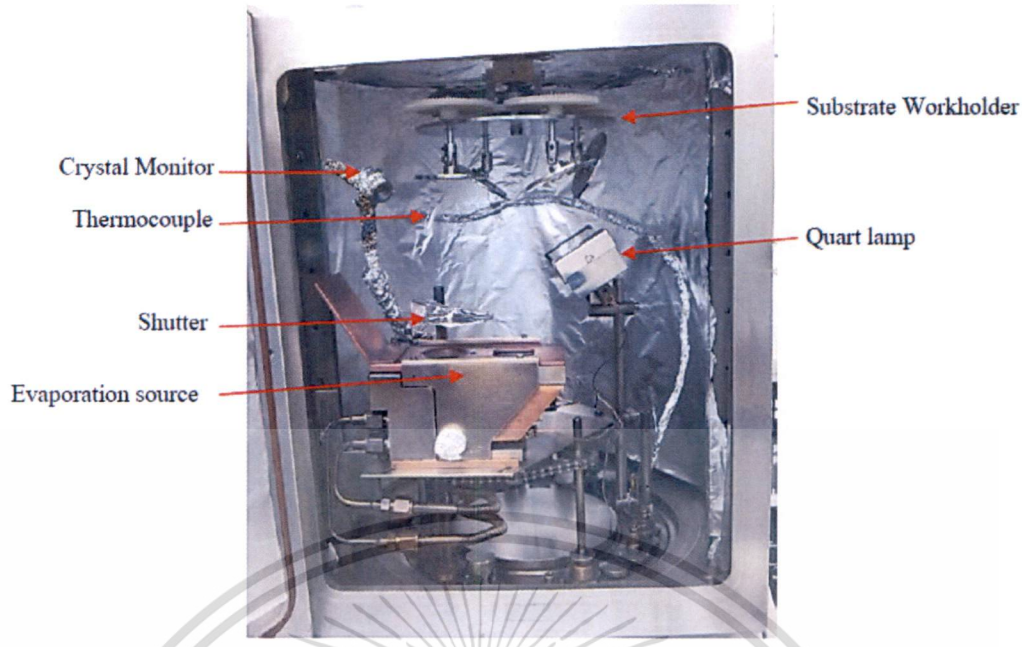

Figure 3.4 The electron beam evaporator systems in vacuum chamber.

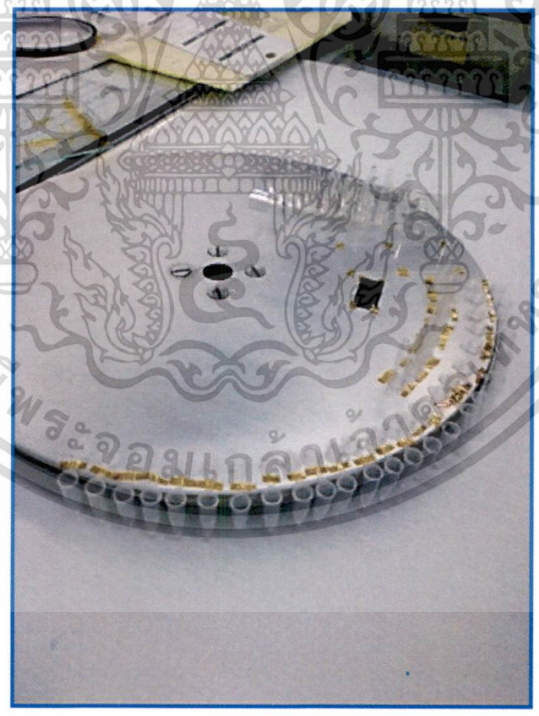

Figure 3.5 The PCR tubes on the sample substrate holder.

(a) (b)

Figure 3.6 Thin films tube deposition with nanoparticle (a) before deposition process, and (b) after deposition process.

Each material had adjusted the evaporation condition. The sample holder substrate was kept at 60 °C. The base pressure of the deposition chamber was approximately 10^{-6} mbar, while the pressure during the evaporation process was in the range between 10^{-6} to 10^{-5} mbar. The deposition rate was adjusted according to the density and melting point temperature of each material. The electron-beam current, the base pressure and the temperature of each the deposited-thicknesses of material films were controlled until the materials vapor depositing on surface of glass substrate at 10, 50, and 100 nm thicknesses (table 3.1). PCR tubes were coated with each material under the same condition of material vapor on glass substrate at different thickness.

3.4.2 Condition of transgene amplifications

PCR amplification was subsequently performed in 50 µl of a reaction mix containing 1.5U of *Taq* DNA polymerase (Invitrogen), 5 µl of 10x reaction buffers, 0.2 mM of each dNTP, 0.2 µM of each gene-specific primer pair of short fragment (CaMV 35S promoter : 379 bp) and long fragment (CaMV 35S promoter combining with GUS gene : 2393 bp) as present in table 3.2. pCAMBIA 1301 plasmid was served as template. The $MgCl_2$ concentration in the PCR reaction was adjusted in 2, 4, and 6 mM. A Mastercycle EPgradient (Eppendrof) was used with an initial denaturation step of 94°C for 3 min. Then, the reaction was followed by 3 step-cycles. The PCR condition for each fragment was performed using the following protocol in table 3.3

Table 3.1 The evaporation conditions of thin film deposition.

Material	Density (g/cm^3)	Deposition time (min)	Fine-Base pressure (mbar)	I-Beam (mA)	I-Filament (mA)	Deposition rate (nm/sec)
Al	2.70	10	$5.0x10^{-6}$	159	1500	0.02
		39	$1.8x10^{-5}$	232	1590	0.01
		57	$3.2x10^{-6}$	250	1627	0.01
Ag	10.50	10	$1.6x10^{-6}$	82	1364	1.00
		50	$3.4x10^{-6}$	104	1407	1.00
		100	$4.2x10^{-6}$	135	1529	1.00
Au	13.92	170	$1.2x10^{-5}$	211	1591	0.001
TiO_2	4.26	7	$3.2x10^{-5}$	36	1256	0.03
		22	$8.0x10^{-6}$	56	1331	0.04
		30	$3.2 x10^{-6}$	59	1378	0.05

Table 3.2. Primer sequences for amplification of CaMV 35S promoter and CaMV 35S promoter combining with *GUS* gene.

Fragment		Primer sequences	size (bp)
CaMV 35S promoter	Forward	5' ATCAAAGATACAGTCTCAGAAGACC 3'	379
	Reverse	5' CTCTCCAAATGAAATGAACTTCCTT 3'	
CaMV 35S promoter combining with *GUS* gene	Forward	5' ATCAAAGATACAGTCTCAGAAGACC 3'	2393
	Reverse	5' ACCGAAGTTCATGCCAGTCCAGC 3'	

Table 3.3. The amplifying condition of short and long transgenes fragments using conventional PCR tube.

Fragment	Denaturing	Annealing	Extension
Short fragment	95 °C/45sec	60, 62, 64 °C/50 sec	72 °C/1.0 min
Long fragment	95 °C/45sec	66, 68 °C/50 sec	72 °C/2.5 min

PCR amplification was performed for 35 cycles, followed by a final extension step of 72 °C for 3 min. The PCR products were separated on a 1.0 % agarose gel containing ethidium bromide in Tris/Borate/EDTA buffer (TBE) and visualized under UV light.

3.4.3 Transgene amplifications in PCR tubes coated with material thin films

The PCR tubes-deposited differently with materials vapor on the surfaces were used for DNA amplification in comparison to conventional PCR tube. The effects of deposited condition on the thickness formation of material thin films on PCR tube were investigated. The short and long DNA fragments (379 and 2393 base pairs, respectively) were separately amplified in the reactions with difference in the $MgCl_2$ concentration. The PCR amplification was operated in 50 µl of a reaction mix Tthe reaction was followed by three step-cycles. The PCR condition for each fragment was performed using the following protocol in table 3.4.

Table 3.4. The amplifying condition of short and long transgenes fragments using tube coated with material thin films

Fragment	Denaturing	Annealing	Extension
Short fragment	95 °C/45sec	62 °C/50 sec	72 °C/1.0 min
Long fragment	95 °C/45sec	68 °C/50 sec	72 °C/2.5 min

3.4.4 Thin film characterization

The surface morphologies of the particles grains were analyzed by the atomic force microscope (AFM) (DFM mode, SPA-400, SEIKO, Japan) as shown in figure 3.5. A dynamic force microscopy, the one of non-contact mode of atomic force

microscope was employed to image the morphology of the TiO_2 and Al films. Scanning was carried out with a silicon cantilever with a nominal resonant frequency 200 kHz and force constant of 5.8 N/m. Scan area was performed 5 μm with scan rate 1 Hz.

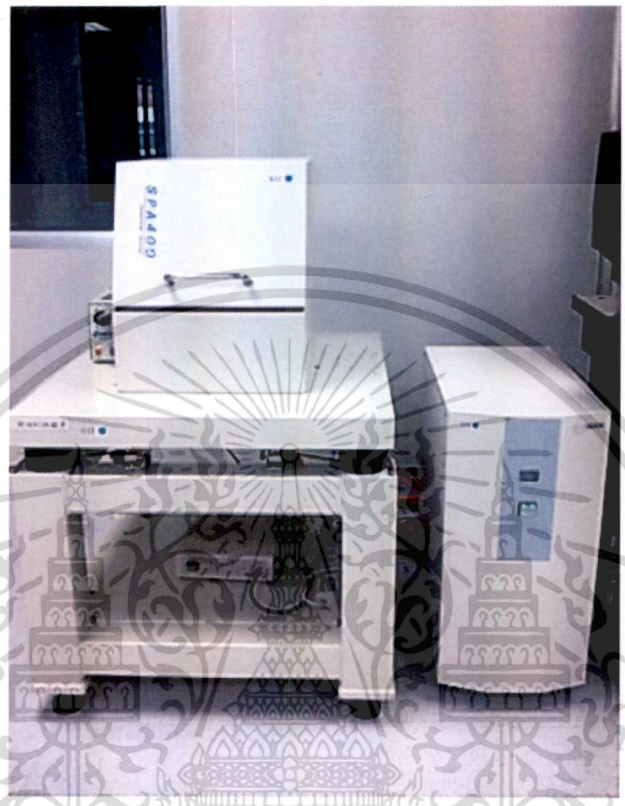

Figure 3.7 Atomic Force Microscope (Seiko,SPA 400, Japan).

CHAPTER 4

RESULTS

4.1 Effect of different types of nanoparticles deposited PCR tube

The different types of nanoparticles (TiO_2, Al, Ag, and Au) were deposited on the inside and outside PCR tube surfaces by the electron beam evaporator. TiO_2-tubes and Al-tubes coated with various thicknesses were shown in figures 4.1 (a) and 4.1 (b), respectively. The color of individual tubes coated with both TiO_2 and Al are nearly clear as same as with conventional PCR tube.

Figure 4.1 Tube coated with TiO_2 (a) and Al (b) 10, 50, 100 nm indicated tube were deposited with vapor material using the same condition with depositing on surface of glass thickness at 10, 50, 100 nm, respectively. IN, OUT indicated the vapor material depositing inside or outside tube.

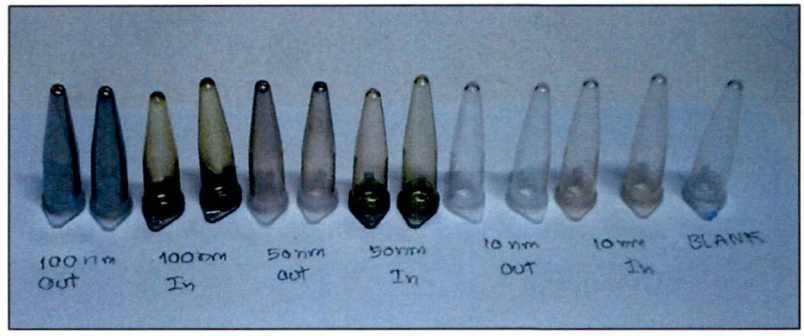

Figure 4.2 Tube coated with Ag 10, 50, 100 nm indicated tube were deposited with vapor material using the same condition with depositing on surface of glass thickness at 10, 50, 100 nm, respectively. IN, OUT indicated the vapor material depositing inside or outside tube.

Figure 4.3 Tube coated with Au 10, 50, 100 nm indicated tube were deposited with vapor material using the same condition with depositing on surface of glass thickness at 10, 50, 100 nm, respectively. IN, OUT indicated the vapor material depositing inside or outside tube.

The PCR tube coated with Ag particles showed pale to intensive colors depending on the film thickness (figure 4.2). At each film thickness, the Ag deposited on in or out tubes surfaces presented the varied color rank of particles accretion. Quality and type of materials had effect on the films color of tube. Figure 4.3 shows tube coated with Au. The result implies that the color change of tube depends on the material types, films thickness, and the position of particles decorated on the tube surface.

4.2 Effect of PCR condition on the transgene fragments amplification

Transgene fragments of CaMV 35S promoter (short fragment) and CaMV 35S promoter combining with GUS gene (long fragment) was amplified in 50 µl of a PCR reaction mixing, and pCAMBIA 1301 plasmid was served as template. To study the suitable amplification condition for transgene fragments, the annealing temperature of gene-specific primer pair and $MgCl_2$ concentration were adjusted in PCR conditions. In the PCR, the short fragment amplification (379 bp) was performed, in three annealing temperatures (60, 62, and 64°C). While the amplification of long fragments (2393 bp) was operated in two annealing temperatures (66 and 68°C). In the parallel experiment with the adjusted condition of annealing temperatures, the various concentrations of $MgCl_2$ (2 - 6 mM) in the PCR condition were examined. The amplified PCR products were analysis using agarose electrophoresis techniques. The amplification results of 379 bp fragment were showed in Figure 4.4.

In the 379 bp amplification, non specific products were higher when annealing temperature increased. On the contrary, increasing $MgCl_2$ concentration affected on reducing non specific products but increasing target DNA fragment.

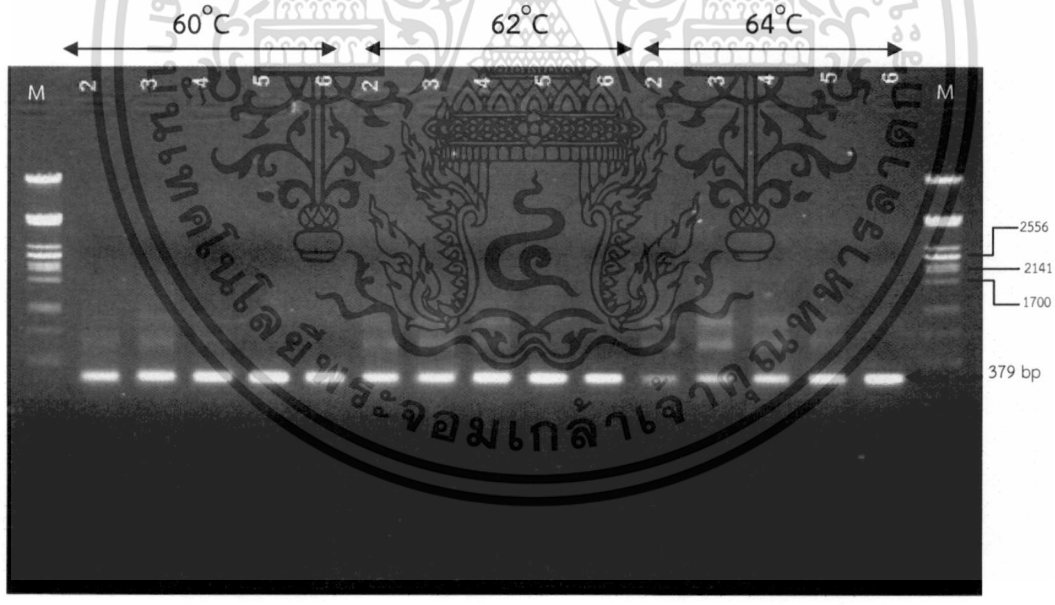

Figure 4.4 The PCR product of amplified short DNA fragments at 60, 62, and 64°C annealing temperature with 2-6 mM $MgCl_2$ concentration. Lane M is markers.

The PCR amplification of long DNA fragments in reaction adding various $MgCl_2$ concentrations at 66°C and 68°C annealing temperature presented the similar pattern of PCR the amplification of short DNA fragments. Non-specific bands were observed in the reaction containing 2, 3 and 4 mM $MgCl_2$ (Figure. 4.5). In this experiment, the annealing temperature at 68°C were selected for studying effect of DNA amplification in tube coated with nanoparticle thin films.

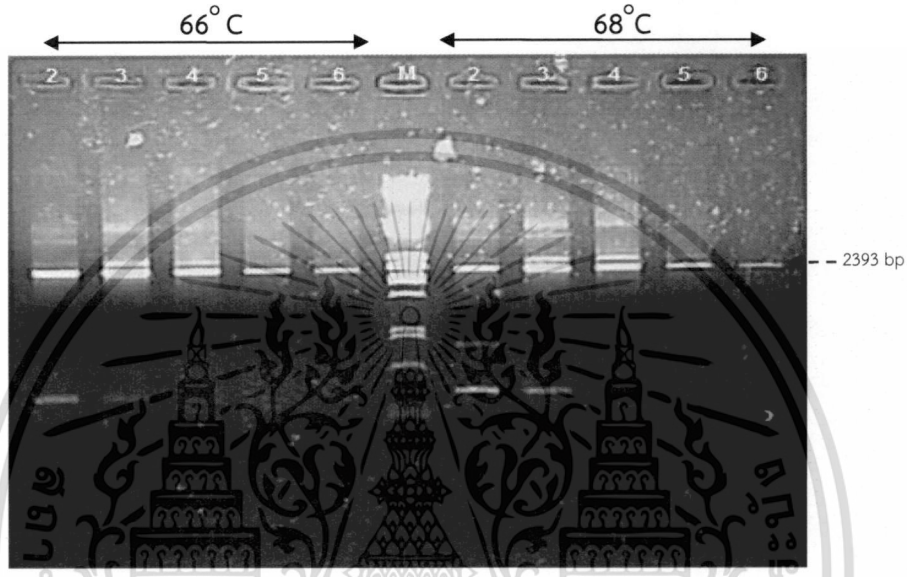

Figure 4.5 The PCR product of amplified long DNA fragments at 66 and 68 °C annealing temperature with 2-6 mM $MgCl_2$ concentration. Lane M is markers.

4.3 The effect of the different nanoparticles coated PCR tubes on DNA fragment amplification

4.3.1 The effect of nanoparticles and $MgCl_2$ concentration on the amplification of short DNA fragments

The short DNA fragment was amplified in the reactions with difference in the $MgCl_2$ concentrations (2, 4, 6 mM). In conventional PCR tube, the increase of $MgCl_2$ concentration in PCR reaction effected on the enhancing target band of small DNA, and also the decreasing upper band of non-specific fragments. PCR tube

coated with Al at outside surface did not have the effect on PCR amplification of small fragments. However, the reactions performed in PCR tube coated with 50 nm thickness Al inside surface show the enhancing specificity of small target amplification at 2, 4 mM $MgCl_2$ concentration in the reaction (figure 4.6).

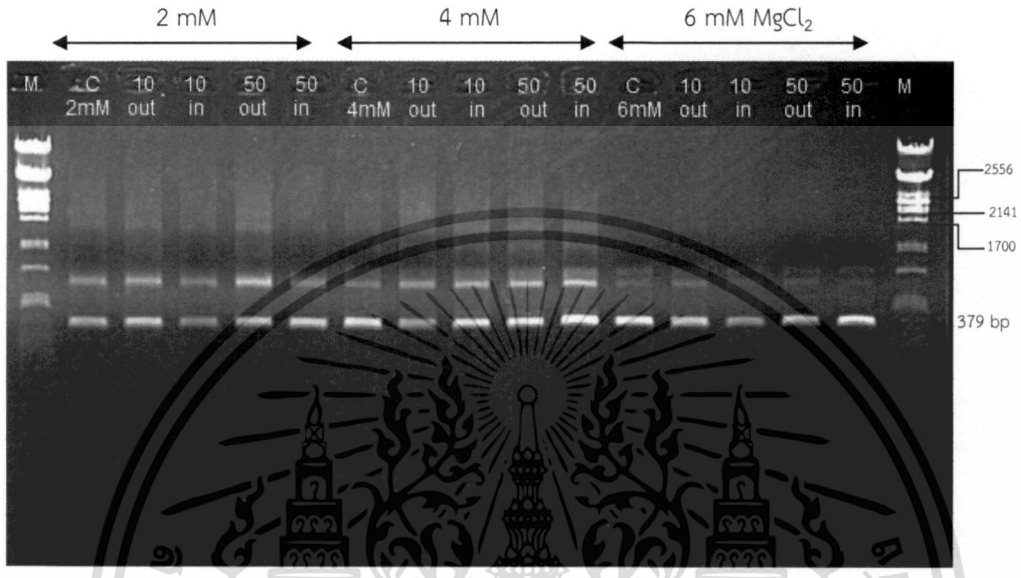

Figure 4.6 The affectation of Al films coated PCR tube on short fragment amplification at 62 °C annealing temperature. Lane M is markers, C is PCR reaction in conventional tube. Lane 2-6 contains 2 mM $MgCl_2$. Lane 7-11 contains 4 mM $MgCl_2$ and Lane 12-16 contains. At 10 and 50 nm thickness of Al films on the surface of PCR tube. Out and in indicated Al films decorated outside and inside PCR tube.

The Ag films grown inside tube affected on the intensity band of short fragment amplification (figure 4.7). In the PCR reaction with 4 mM $MgCl_2$ concentration, Ag films deposited outside tube show similar band with those in normal tube. The short fragment amplification of target DNA was enhanced in PCR tube deposited inside with 10 nm Ag films, while increasing films thicknesses interrupted the DNA amplification. Tube deposited outside with Au films has no effect on the amplification of short fragment.

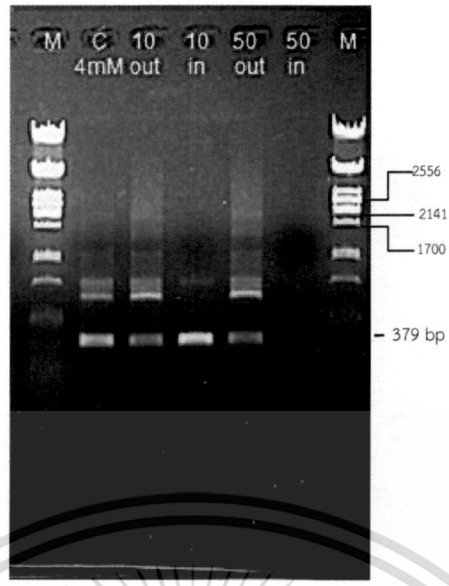

Figure 4.7 The affectation of Ag films coated PCR tube on short fragment amplification at 62 °C annealing temperature. Lane M is markers, C is PCR reaction in conventional tube. Lane 2-6 contains 4 mM $MgCl_2$. At 10 and 50 nm films thicknesses of Ag films on the surface of PCR tube. Out and in indicated Ag films decorated outside and inside PCR tube.

However, the reactions performed in PCR tube coated with Au thin film at inside surface show the inhibition of small target amplification at all tested $MgCl_2$ concentration in the reaction as shown in figure 4.8.

4.3.2 The effect of nanoparticles and $MgCl_2$ concentration on the amplification of long DNA fragment

The PCR tube deposited with TiO_2 at outside surface were used for DNA amplification. These results presented a similar band of DNA fragment with fragment amplifying in normal tube, excepting in the reaction in PCR tube deposited with 50 nm TiO_2 films adding 4 mM $MgCl_2$, band of DNA fragment amplification was invisible (figure 4.9). The TiO_2 films inside PCR tube can inhibit DNA amplification in all tested conditions of $MgCl_2$ concentration.

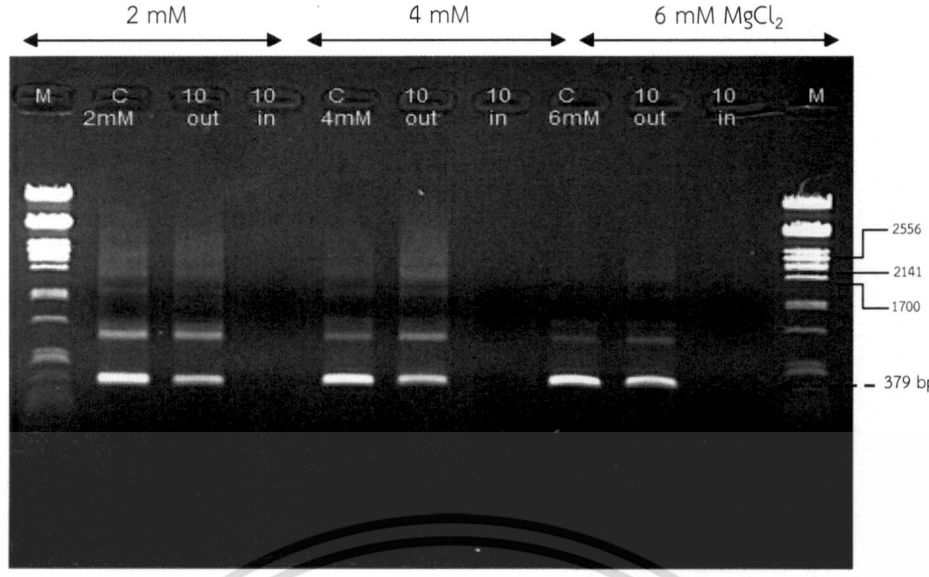

Figure 4.8 The affectation of 10 nm films thickness Au films coated PCR tube on short fragment amplification at 62 °C annealing temperature. Lane M is markers, C is PCR reaction in conventional tube. Lane 2-4 contains 2 mM $MgCl_2$. Lane 5-7 contains 4 mM $MgCl_2$. Lane 8-10 contains. Out and in indicated Au films decorated outside and inside PCR tube.

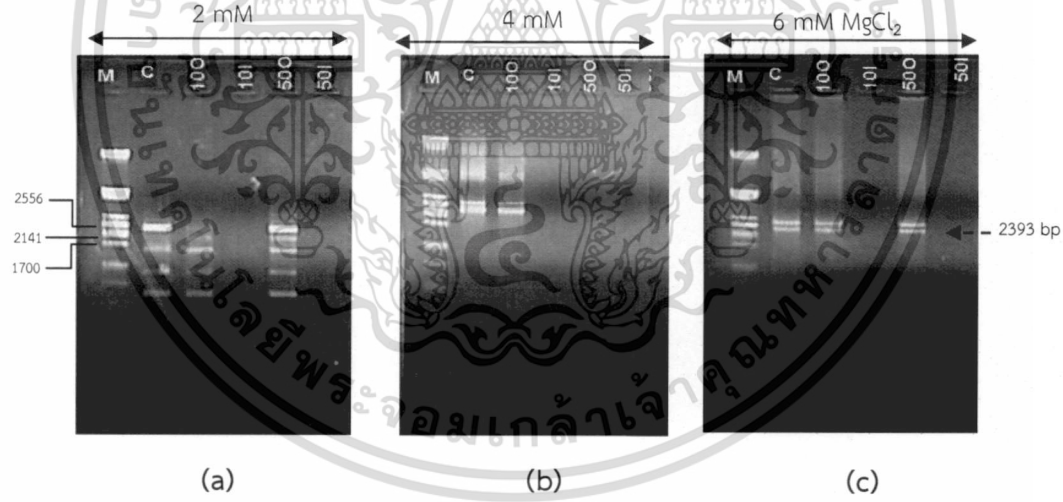

Figure 4.9 The affectation of TiO_2 films coated PCR tube on long fragment amplification at 68 °C annealing temperature. Lane M is markers, C is PCR reaction in conventional tube. With the concentration of 2 mM $MgCl_2$ (a), 4 mM $MgCl_2$ (b), and 6 mM $MgCl_2$ (c). 10 and 50 nm films thickness of TiO_2 films on the surface of PCR tube. O indicated materials thin films grown outside tube and I indicated materials thin films grown inside tube.

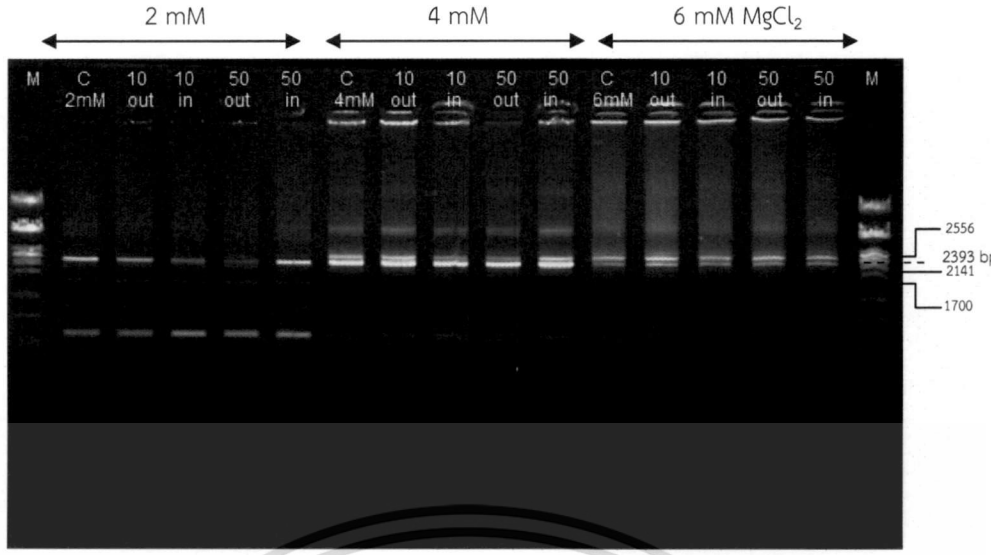

Figure 4.10 The affectation of Al films coated PCR tube on long fragment amplification at 68 °C annealing temperature. Lane M is markers, C is PCR reaction in conventional tube. Lane 2-6 contains 2 mM $MgCl_2$. Lane 7-11 contains 4 mM $MgCl_2$ and lane 12-16 contains 6 mM $MgCl_2$. 10 and 50 nm films thickness of Al on the surface of PCR tube. Out and in indicated Al films decorated outside and inside PCR tube.

An effectiveness of amplified 2393 bp in PCR tubes deposited with Al was shown in Figure 4.10. In the normal reaction of 2393 bp amplification, PCR reaction with 2 mM $MgCl_2$ concentration amplified non specific small fragments more than that with 4 mM $MgCl_2$ concentration while PCR reaction with 6 mM $MgCl_2$ concentration increased non specific large fragments. DNA products amplified at 4 and 6 mM $MgCl_2$ in all PCR tubes deposited with Al presents a similar band with those in normal tube. DNA products amplified by using 2 mM $MgCl_2$ concentration in the reaction show the reducing intensity band of specific fragments and the increasing intensity band of small non-specific fragments in the Al deposited inside and outside tube compared with the control tube. In tubes deposited inside with 50 nm films thickness, the reaction with 2 and 4 mM $MgCl_2$ presents slightly higher band intensity compare to the control tube.

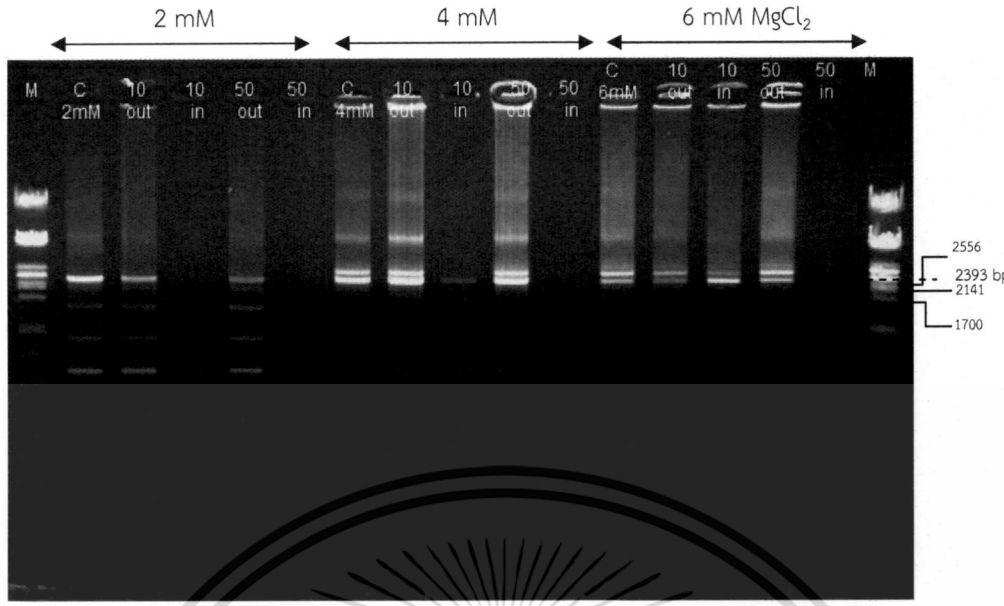

Figure 4.11 The affectation of Ag films coated PCR tube on long fragment amplification at 68 °C annealing temperature. Lane M is markers, C is PCR reaction in conventional tube. Lane 2-6 contains 2 mM $MgCl_2$. Lane 7-11 contains 4 mM $MgCl_2$ and lane 12-16 contains 6 mM $MgCl_2$. 10 and 50 nm films thickness of Ag films on the surface of PCR tube. Out and in indicated Ag films decorated outside and inside PCR tube.

Besides, the affection of PCR product amplified using a pair of 2393 bp fragment primers with the 2, 4 and 6 mM $MgCl_2$ concentration in the Ag deposited inside and outside tube compared with the control tube as shown in figure 4.11. DNA products amplified within reaction containing all $MgCl_2$ concentration in Ag deposited outside tube show similar band with those in normal tube.

While the tube deposited inside with 50 nm Ag thin films was inhibited of DNA amplification under condition adding 2, 4, and 6 mM $MgCl_2$ (figure 4.11). Figure 4.12 shows a result of the large fragments amplification in Au deposited tube. DNA amplification in tube deposited outside with gold presented the similar band with control tube. The reaction with 2 mM $MgCl_2$ concentration occurred in tube deposited outside with 10 nm thickness, show the lost target fragment. DNA amplification in tube deposited inside with 10 nm Au thin films was also inhibited under all testing $MgCl_2$ conditions.

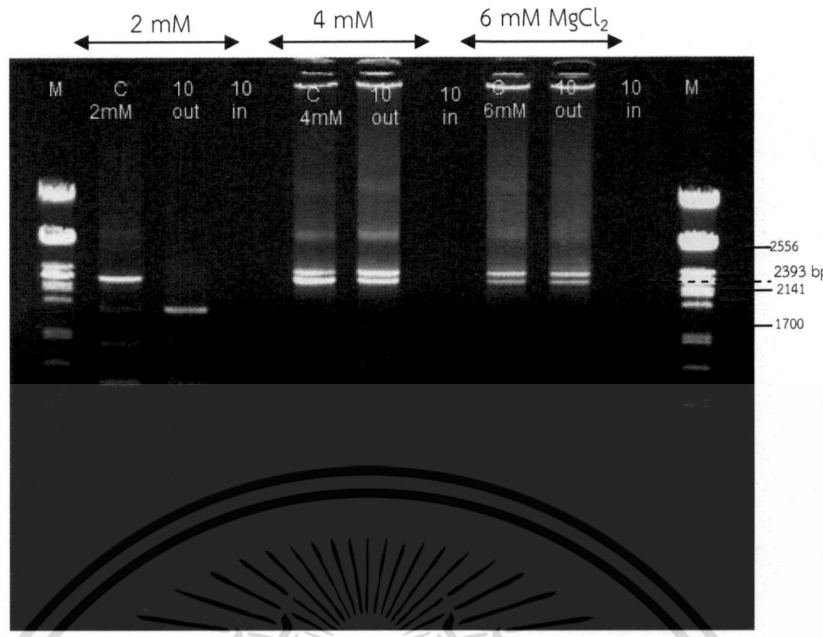

Figure 4.12 The affectation of Au films coated PCR tube on long fragment amplification at 68 °C annealing temperature. Lane M is markers, C is PCR reaction in conventional tube. Lane 2-3 contains 2 mM $MgCl_2$. Lane 4-6 contains 4 mM $MgCl_2$ and lane 7-9 contains 6 mM $MgCl_2$. 10 nm films thickness of Au films on the surface of PCR tube. Out and in indicated Au films decorated outside and inside PCR tube.

From these comparative studies, these result implied that the types of nanoparticles thin films, the thickness and the position of thin films were grown on the PCR tubes, and $MgCl_2$ concentration played in the role of quality and quantity in the amplification of DNA fragments.

4.4 Characterization of the deposited nanoparticles on PCR tube surfaces

To investigate nanoparticles thin film on the surface of flat glass substrate and PCR tube, AFM was used. All images were obtained under room temperature condition. The AFM image shows that the surface of glass coating 10 nm thin films of TiO_2 is dense with non-homogenous grain (figure 4.13). Surface characterization of

TiO_2 thin films varying in thicknesses at outside and inside PCR tube revealed different surface topography and phase contrast as shown in figure 4.14 and 4.15.

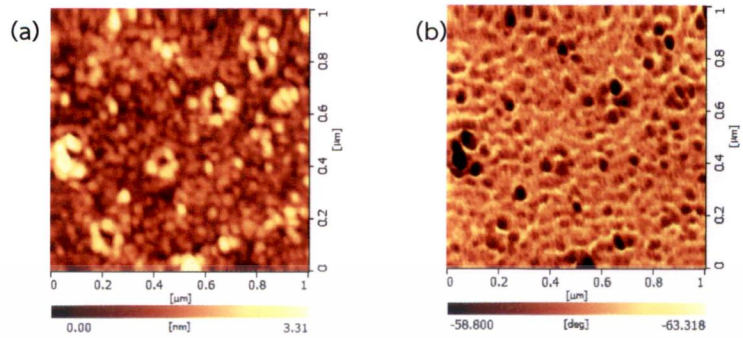

Figure 4.13 AFM image of TiO2 thin films grown on glass with thickness of 10 nm morphology image (a) and phase contrast image (b).

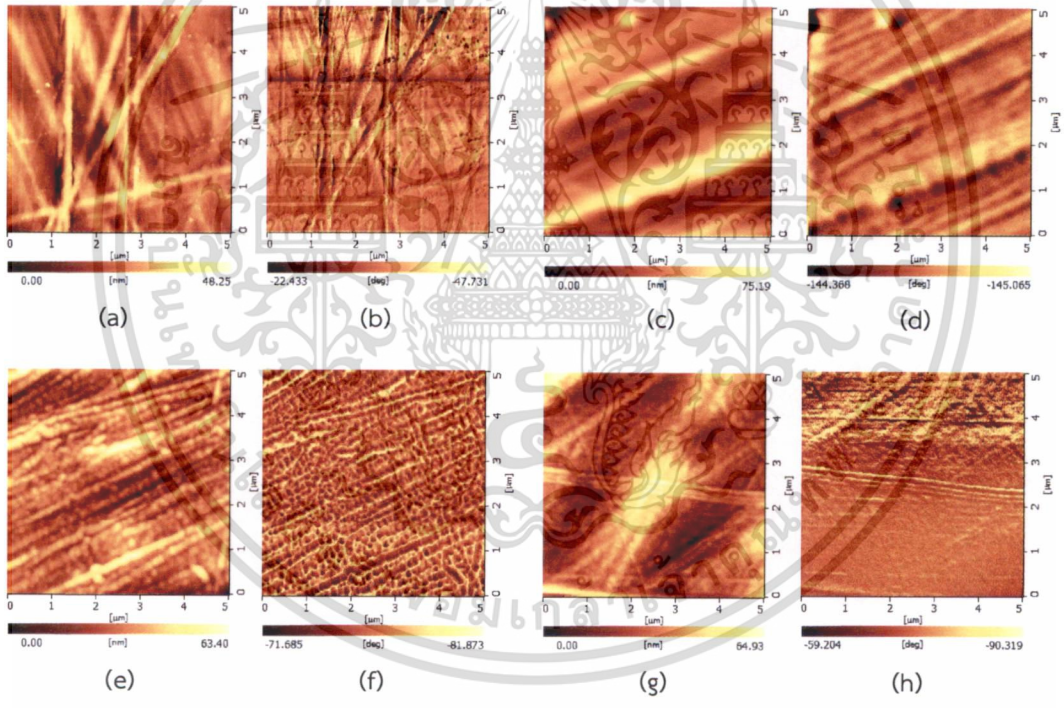

Figure 4.14 AFM images of the morphologies and phase contrast of outside-PCR tube. Conventional PCR tube (a), (b), and TiO_2 thin films grown on the outside-PCR tube using the same condition as glass with a thickness of 10 nm (c), (d), 50 nm (e),(f), and 100 nm (g), (h).

Point probe silicon cantilever tip used in non-contacting mode by the accompanying software to determine the surface roughness of investigated surfaces. The roughness of each sample was evaluated on two scanned areas of 5μm x 5μm each. The surfaces of PCR tube using 10 nm films thickness of glass condition were covered with a grain layer less than those using 50 and 100 nm thickness of glass conditions. Figure 4.14a and b show the AFM analysis of outside-conventional tube surface roughness appeared in groove and pits on plastic surface. The contrast image revealed that the plastic specimen exhibited rather low surface roughness in contrast to TiO_2 films coated outside-tube (figure 4.14f and h). Likewise, the roughness surface inside-tube presented in groove and pits on plastic surface (figure 4.15a and b). The contrast image showed that the particle grains increases with increasing thickness. The surface is decorated with particles grains to simulate the **surface roughness** inside-tube (figure 4.15d, f and h).

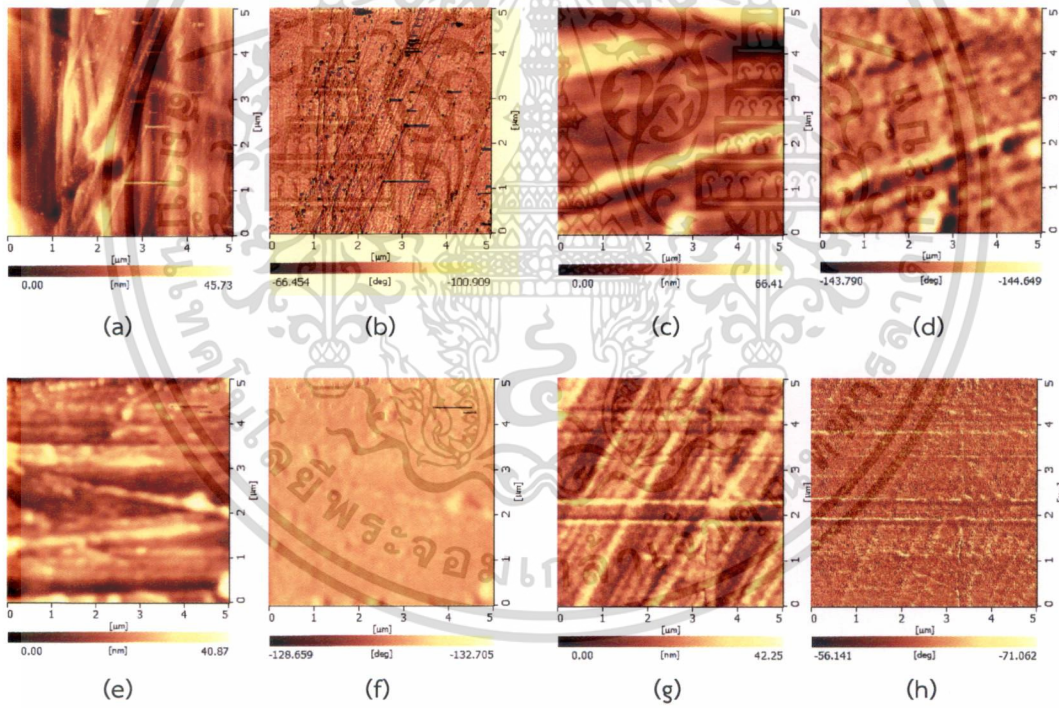

Figure 4.15 AFM images of the morphologies and phase contrast of inside-PCR tube. Conventional PCR tube (a), (b), and TiO_2 thin films grown on the inside-PCR tube using the same condition as glass with a thickness of 10 nm (c), (d), 50 nm (e), (f), and 100 nm (g), (h).

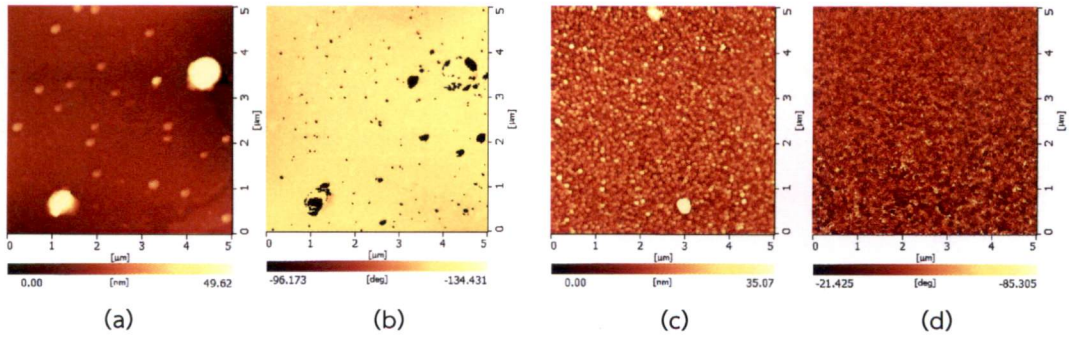

Figure 4.16 AFM images of the morphologies and phase contrast of Al nanoparticles grains grown on glass substrate with thin film thicknesses of 10 nm (a) and (b), 50 nm (c) and (d).

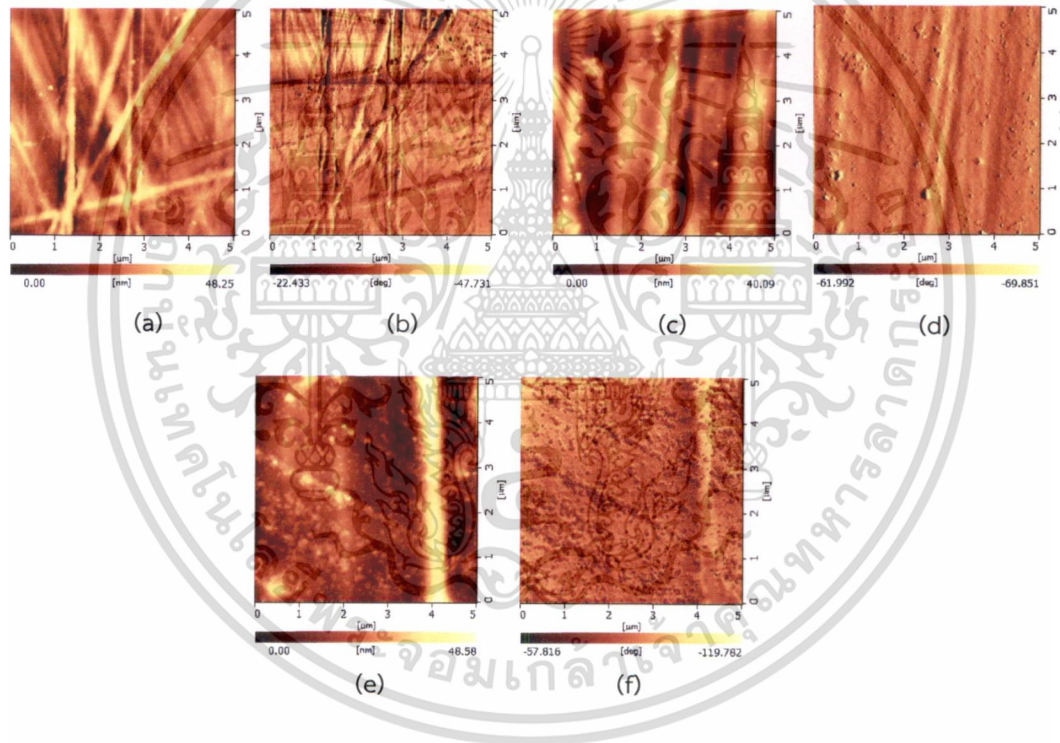

Figure 4.17 AFM images of the morphologies and phase contrast of outside-PCR tube. Conventional PCR tube (a), (b), and Al grains grown on the outside-PCR tube thin film thicknesses of 10 nm (c), (d), and 50 nm (e), (f).

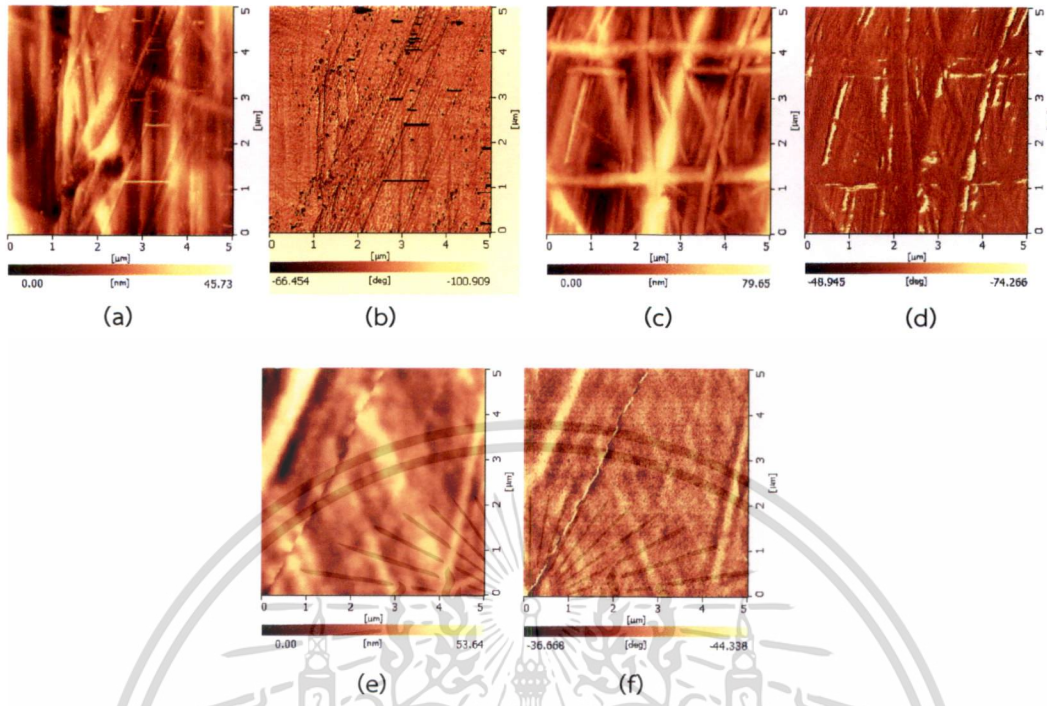

Figure 4.18 AFM images of the morphologies and phase contrast of inside-PCR tube. Conventional PCR tube (a), (b), and Al grains grown on the outside-PCR tube thin film thicknesses of 10 nm (c), (d), and 50 nm (e), (f).

Moreover, the effects of films thickness on the formation of Al films on PCR tube were investigated. Figure 4.16 shows AFM images of the Al films morphologies on the surface of glass substrate. The micrograph shows that the surface of glass deposited with Al for 10 nm was scanty with Al nanoparticle grains (figure. 4.16a), whereas the surface of glass deposited with Al for 50 nm presented a plenty of Al nanoparticle grains (figure 4.16b). The same conditions with Al deposited glass were performed outside and inside surface of PCR tubes. The surfaces of PCR tubes deposited with Al for 10 and 50 nm film thicknesses were analyzed by AFM. The surface characterization of Al grain varying in thin film thicknesses at outside and inside PCR tube showed different surface topography (figure 4.17 and figure 4.18). The surfaces of PCR tube using 10 nm of Al were uncovered with a grains layer, while 50 nm thicknesses were presented a thin layer of grains.

CHAPTER 5
DISCUSSSION

5.1 The effect of the different types of nanoparticles deposited PCR tubes on DNA fragment amplification

The various types of nanoparticles thin films grown on the outside and inside of PCR tube positions at difference thicknesses presented the significant quality and quantity of DNA products compared with conventional PCR tube.

5.1.1 DNA amplifications using tubes coated outside with different nanoparticles

The amplifications of short DNA fragments in tubes coated outside with four types of nanoparticles had similar results with control tube under all condition studies of $MgCl_2$ adding concentration. The amplifications of long DNA fragments in these tubes depended on nanoparticle types and $MgCl_2$ concentration in PCR. Under PCR condition adding 2 mM $MgCl_2$, long DNA fragments in tubes coated outside with 10 and 50 nm thicknesses of Al and Ag had similar results with control tube, while those in tubes coated with 10 nm thicknesses of Au and TiO_2 showed a loss of the long DNA target. In the PCR condition adding 4 and 6 mM $MgCl_2$, DNA amplifications in tube coated outside with all types and thicknesses of nanoparticles showed similar results with control tube.

5.1.2 DNA amplifications using tubes coated inside with different nanoparticles

After using Al coated tubes (10 and 50 nm) in DNA amplification of short and long fragments, the result shows similarity to that of the control tube under all tested $MgCl_2$ conditions. 10 nm thickness Ag inside PCR tube affected increase of short DNA target but reducing long DNA target under PCR condition adding 4 mM $MgCl_2$. The PCR condition which increased $MgCl_2$ to 6 mM could enhance DNA amplification of long target fragments. While the tube deposited inside with Au and TiO_2 films inhibited DNA amplification under conditions adding 2, 4, and 6 mM $MgCl_2$.

Hence, the positions at difference thicknesses had the effect on long DNA fragments amplification. In addition, the normal reactions of long DNA fragments were amplified in the reactions with difference in the $MgCl_2$ concentrations present PCR reaction. In 2 mM $MgCl_2$ concentration, the normal reactions amplified nonspecific small fragments more than that with 4 mM $MgCl_2$ concentration, while PCR reaction with 6 mM $MgCl_2$ concentration increased nonspecific large fragments. The increasing of $MgCl_2$ concentration is essential role to play in DNA polymerase activity in PCR reaction. Therefore, the surface charge distribution effect of nanoparticle types may interrupt DNA polymerase activity. Functional groups of DNA adsorbed onto TiO_2 particles have been reported (Shiqiang *et al.*, 2008). Moreover the interaction between TiO_2 thin film and polymerase can cause a reduction in the effective polymerase concentration. In addition to the surface interactions between particles and biomolecules in PCR reaction and the variety of nanoparticles thin films were grown on inside the PCR tube should effect on the PCR yields. Mixing PCR product with cationic amphiphiles results in the spontaneous formation of mixed complexes that contain both DNA and amphiphiles. Colloids with charged biopolymers such as proteins and nucleic acids are more complex systems than classical ionic or non-ionic stabilizers because of the unique inter- and intra-biomolecular interactions such as hydrogen bonding and hydrophobic interaction. Whether colloids are stabilized or undergo aggregation depends on the net potential of interparticle attraction and repulsion forces. The interparticle attraction force is van der Waals force, which is responsible for the particles aggregation. The two major repulsion forces that contribute to Au stabilization are electrostatic and steric repulsion forces. Therefore, the conformation of DNA (such as folded and unfolded structures) on nanoparticle films surface, which directly contributes to the surface charge distribution, could be a key factor determining their relative PCR stability.

In recently, Haikuo and team have developed a highly selective nanoparticle PCR strategy by employing gold nanoparticles. In the presence of appropriate concentrations of gold nanoparticles, PCR amplification can be optimized with respect to both yields and specificity. However, the presence of excess gold nanoparticles (1.0 nm) effectively inhibited the PCR reaction (Haikuo *et al.*, 2005). In addition, Shiqiang and colleague have reported the impact of size and concentration of TiO_2 nanoparticles on DNA synthesis. The presence of excess

TiO_2 nanoparticles (>600 µg/mL) effectively inhibited the DNA synthesis. 500 µg/mL with TiO_2 concentration, the particle size impact of TiO_2 showed the significant DNA synthesis inhibition in TiO_2 nanoparticles (Shiqiang *et al.*, 2008). Therefore, the addition of appropriate concentrations and size of nanoparticles can be optimized with respect to both PCR yields and specificity. In this study, the particles decoratede on the surface of tube can not be adjusted concentrations of nanoparticles. Therefore, the effectively inhibited in the PCR product may effect on excess nanoparticles concentration.

5.2 Characterization of the deposited nanoparticles on PCR tube surfaces

Once the atoms or molecules are deposited, the film nucleates on the substrate and grows by a number of processes. Microstructure and composition of the film can be modified by bombardment of the growing film by ions from the vapor phase, resulting in re-condensation of the film atosms and enhanced surface mobility of the atoms in the near surface and surface of the film. The demonstration nanoparticles thin film on the surface of flat glass substrate coating 10 nm thin films is dense with non-homogenous grain. Surface characterization of TiO_2 thin film varying in thicknesses at outside and inside PCR tubes revealed different surface. From the PCR tube characterization, the morphology and phase contrast of tube show the rough surface, which are distinctive deposition process in random fluctuations results of form a rough morphology. The surfaces of PCR tube using 10 nm films thickness of glass condition were covered with a grain layer less than those using 50 and 100 nm thicknesses of glass conditions.

Besides, the AFM image shows that the surface of glass deposited with 10 nm thickness of Al was scanty with Al nanoparticle grains, whereas the surface of glass deposited with 50 nm thickness of Al presented a plenty of Al nanoparticle grains. The same conditions as Al deposited glass were performed outside and inside surfaces of PCR tubes. The surface characterization of Al grain varying in thin film thickness on outside and inside PCR tube showed different surface topography. The surfaces of PCR tube coated with 10nm thickness of Al were uncovered with a grains layer, while that of 50 nm thickness presented a thin layer of grains.

The particle may then deposit on the surface at a different location, or it may bounce around the surface more before it settles, which leads to a smoothing effect.

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2. O. Ketchar, S. Porntheeraphat, J. Nukeaw, K. Sompornpailin, **"Al Nanoparticles deposited on PCR tube enhance DNA amplification at suitable $MgCl_2$ concentration"**, *The 8th International Symposium on Biocontrol and Biotechnology,* October 2010, Pattaya, Thailand (Poster Presentation Award)

Al Nanoparticles Deposited on PCR Tube Enhance DNA Amplification at Suitable $MgCl_2$ Concentration

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Abstract

Aluminum, the one of biocompatible nanoparticles, were deposited on a surface of the polymerase chain reaction (PCR) tube by the electron beam evaporator at the deposition rate of 0.01 nm/sec. The base pressure of the deposition chamber was 10^{-6} mbar, substrate temperature was kept at 60°C, electron-beam current was controlled at 160 mA and 230 mA. PCR tube depositing was performed for 20 and 85 min. The AFM results showed the positive relationship between depositing time and quantity of Al grain accumulated on PCR tube surface. PCR tubes deposited with Al vapor were used to amplify small (376 bp) and large (2393 bp) fragments using 2, 4 and 6 mM $MgCl_2$ in reactions comparing to the control tube. In tubes deposited inside with 85 min Al vapor, the PCR reaction using 2 and 4 mM $MgCl_2$ presents slightly higher band intensity of large fragments compare to the control tube and also the increase of band specificity of small fragments. Al grain inside surface of PCR tube should have the improving effects on the reaction of short and long fragment amplification at a suitable $MgCl_2$ concentration.

Keywords: PCR, Aluminum, Nanoparticles, DNA amplification, Biocompatible.

1. Introduction

The technique of polymerase chain reaction (PCR) has become a prevalent and well-developed tool of molecular biology, clinical DNA diagnostics and the developing device of

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biosensor [1-2]. This technique can exponentially amplify target DNA or RNA and generate a large number of DNA copies in a short period of time. Specificity and yield of nucleic acid amplification are important features for a reliable of this technique. These amplification features are controlled by many factors such as primer design, a nucleotide sequence of the template DNA, denaturation/annealing/extension times and temperatures, enzyme type, primer-dimer occurrence and buffer content of the PCR reaction [3-4]. Miscellaneous techniques have been developed to improve PCR specificity for rapid heating-cooling response [5]. A diversity of agents has been added to PCR reaction for enhancing specificity of PCR product. It has been found that various organic additives significantly improve yields in GC-rich template DNA, including dimethylsulfoxide (DMSO), glycerol, polyethylene glycol, betaine, amides and single-stranded DNA-binding proteins (SSBs) [6-11]. Recently, It has been reported that Au nanoparticles (AuNPs) significantly improved the yields of PCR reaction under fast cycling condition. These nanoparticles have been used in order to elude nonspecific amplification even at low annealing temperatures [12-13]. The previous researches presented that single-walled carbon nanotubes and carbon nanopowders could increase the yields of PCR and improve the efficient amplification for long PCR reactions, respectively [14-15]. The yields of PCR reactions were gradually decreased when the concentrations of various nanoparticles were exceeded [15-18].

However, the reactions between metallic films on PCR tube and DNA fragment amplification have still not been reported yet. In the present work, we investigated the effect of aluminum (Al) grains on the PCR tube surface at the different of the deposited time on DNA amplification.

2. Materials and Methods

Thin film deposition

The Al grains were grown inside and outside PCR tubes substrate by the electron beam evaporator (EVT1 auto 306, EDWARDS, USA). Al films were deposited at a rate of about at 0.01 nm/sec, divided film thickness by deposition time 20 and 85 min, respectively. The deposited-thicknesses were also performed under the same condition of Al vapor on glass substrates in order to measure thicknesses by spectroscopic ellipsometry technique. The base pressure of the deposition chamber was approximately 10^{-6} mbar, while the pressure during the evaporation process was in the range between 10^{-5} to 10^{-4} mbar, electron-beam current of 20 and 85 min of Al films deposition time was controlled at 160 mA and 230 mA, respectively. The sample holder substrate was kept at 60 °C. After preparation, the films were kept into the chamber at vacuum for about 1 hour. This was made to be sure that the film temperature be uniform so that no thermal stress will appear when taken out from the chamber.

Thin film characterization

The surface morphologies of the Al nanoparticles grains were analyzed by the atomic force microscope (AFM) (DFM mode, SPA-400, SEIKO, Japan). A·dynamic force microscopy, the one of non-contact mode of atomic force microscope was employed to image the morphology of the Al films. Scanning was carried out with a silicon cantilever with a nominal resonant frequency 200 kHz and force constant of 5.8 N/m. Scan area is 5 μm with scan rate 1 Hz.

DNA amplification

PCR amplifications were carried in 50 μl reaction volumes containing 1.5U of Taq DNA polymerase (Invitrogen, USA), 5 μl of 10x reaction buffers, 0.2 mM of each dNTP, 0.2 μM of each primer. pCAMBIA 1301 plasmid was served as template. The $MgCl_2$ concentration in the PCR reaction was verified at 2, 4 and 6 mM. PCR condition for each fragment was performed

using the following protocol in table 2. 3 μL of PCR products were analyzed by using 1 % agarose gel electrophoresis in Tris/Borate/EDTA buffer (TBE).

Table 1. The DNA fragment size and primers of the experiments

Size (bp)	Forward primer	Reverse primer
376	TGCGAAGGATAGTGGGATTGTGC	GGATTGATGTGAACATGGTGGAG
2393	TGCGAAGGATAGTGGGATTGTGC	ACCGAAGTTCATGCCAGTCCAGC

Table 2. The PCR conditions of 376 and 2393 bp fragments

Size (bp)	cycles	denaturing	Annealing	Extension
376	35	95 °C/ 45sec	68 °C/50 sec	72 °C/1 min
2393	35	95 °C/ 45sec	62 °C/40 sec	72 °C/2 min

3. Results and Discussion

Al films PCR tube characterization

All images were obtained at a room temperature condition. The effects of time deposited condition on the formation of Al films on PCR tube were investigated. Figure 1 shows AFM images of the Al films morphologies on the surface of glass substrate. The glass surface deposited with Al vapor for 20 and 85 min presents 10 nm and 50 nm films thickness respectively. The micrograph shows that the surface of glass deposited with Al vapor for 20 min was scanty with Al nanoparticle grains (Fig. 1a), whereas the surface of glass deposited with Al vapor for 85 min presented a plenty of Al nanoparticle grains (Fig. 1b). The same conditions with Al vapor deposited of glass were performed outside and inside surface of PCR tubes. The surfaces of PCR tubes deposited with Al vapor for 20 and 85 min were analyzed by AFM. The surface characterization of Al grain varying in depositing time at outside and inside PCR tube showed different surface topography. (Fig. 2 and Fig. 3) The surfaces of PCR tube using 20 min of Al vapor deposited time were uncovered with a grains layer, while 85 min thicknesses were presented a thin of grains layer.

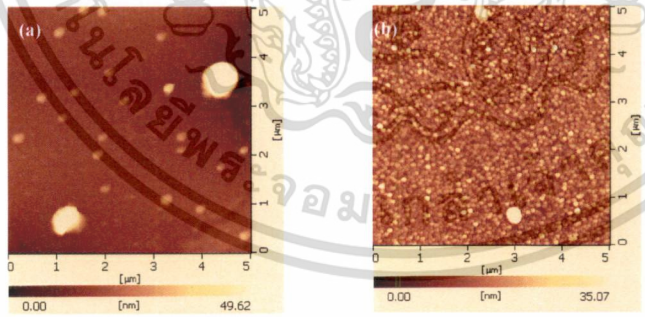

Figure 1. AFM images of Al nanoparticles grains grown on glass substrate with deposited time of (a) 20 min, and (b) 85 min.

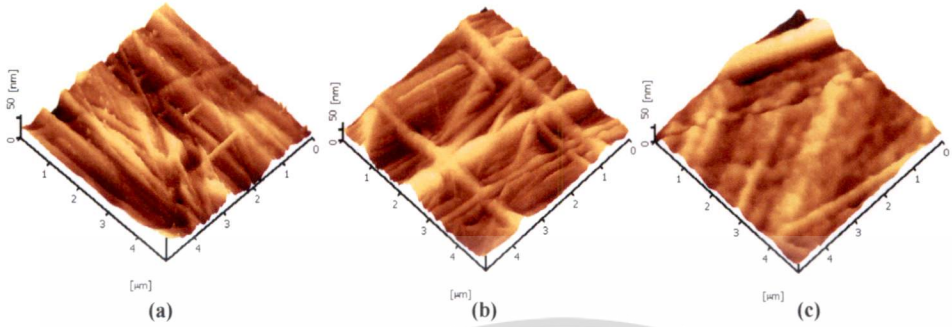

Figure 2. AFM images of the morphologies of inside-PCR tube (a) conventional PCR tube, and Al grains grown on the inside-PCR tube using the deposited time of (b) 20 min, and (c) 85 min.

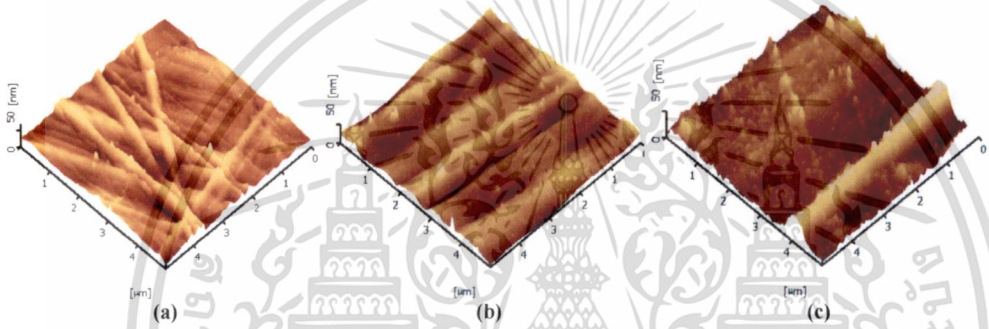

Figure 3. AFM images of the morphologies of outside-PCR tube (a) conventional PCR tube, and Al grains grown on the outside-PCR tube using the deposited time of (b) 20 min, and (c) 85 min.

Effect of $MgCl_2$ concentration

The PCR tubes differently deposited with Al vapor on the surfaces were used for DNA amplification comparing with conventional PCR tube. Two sizes of DNA fragments (2393 and 379 bp) were separately amplified in the reactions with difference in the $MgCl_2$ concentrations (2, 4, 6 mM). Equal volume of products derived from each PCR reaction were loaded on agarose gel and detected under UV light. These results present in Figure 4 and 5.

In the normal reaction of 2393 bp amplification, PCR reaction with 2 mM $MgCl_2$ concentration amplified non specific small fragments more than that with 4 mM $MgCl_2$ concentration while PCR reaction with 6 mM $MgCl_2$ concentration increased non specific large fragments. DNA products amplified at 4 and 6 mM $MgCl_2$ in all PCR tubes deposited with Al presents a similar band with those in normal tube. DNA products amplified by using 2 mM $MgCl_2$ concentration in the reaction show the reducing intensity band of specific fragments (2393 bp) and the increasing intensity band of small non-specific fragments in the Al deposited inside and outside tube comparing with the control tube. Especially the reaction occurred in tube deposited outside with 85 min Al vapor; large specific fragments were greatly reduced. In tubes deposited inside with 85 min Al vapor, the reaction with 2 and 4 mM $MgCl_2$ presents slightly higher band intensity compare to the control tube.

Figure 5 shows the PCR product amplified using a pair of 376 bp fragment primers with the 2, 4 and 6 mM $MgCl_2$ concentration. In conventional PCR tube, the increase of $MgCl_2$ concentration in PCR reaction effected on the enhancing target band of small DNA, and also the

decreasing upper band of non-specific fragments. PCR tube coated with Al at outside surface did not have the effect on PCR amplification of small fragments. The reactions performed in PCR tube coated with Al vapor for 85 min at inside surface show the enhancing specificity of small target amplification at 2, 4 mM $MgCl_2$ concentration in the reaction. The Al grains on PCR tube surface may reduce heat transfer between the tube contacting area and the PCR block and effect on reaction temperature of long fragment amplification. Although various reports presented that excess concentration of nanoparticles such as Au, TiO_2 adsorbed the components in PCR reaction and reduced DNA amplification. Al grain inside surface of PCR tube should have the improving effects on the reaction of short and long fragment amplification at a suitable $MgCl_2$ concentration.

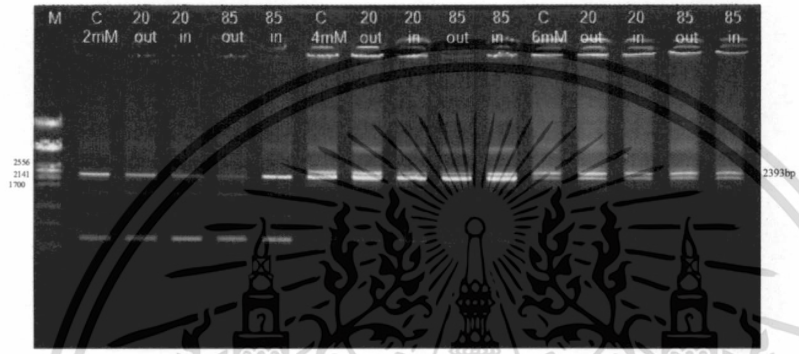

Figure 4. The affectation of Al grains on long fragment amplification at 68 °C annealing temperature. Lane M is for markers, C is for PCR reaction in conventional tube. Lane 2-6 contains 2 mM $MgCl_2$. Lane 7-11 contains 4 mM $MgCl_2$, and lane 12-16 contains 6 mM $MgCl_2$. 20 and 85 is the deposited time of Al grains on the surface of PCR tube. Out and In is Al grains decorated outside and inside PCR tube.

Figure 5. The affectation of Al films on short fragment amplification at 62 °C annealing temperature. Lane M is for markers, C is for PCR reaction in conventional tube. Lane 2-6 contains 2 mM $MgCl_2$. Lane 7-11 contains 4 mM $MgCl_2$, and lane 12-16 contains 6 mM $MgCl_2$. 20 and 85 is the deposited time of Al grains on the surface of PCR tube. Out and In is Al grains decorated outside and inside PCR tube.

4. Conclusions

Al vapor deposited on a surface of the PCR tube by the electron beam at different times presents the different in quality and quantity of Al grains. The quality and quantity of grains at outside surface of PCR tube may involve in heat transfer of surface contacting and effect on DNA amplification in long DNA fragments. Al grain inside surface of PCR tube should have the improving effects on the reaction of short and long fragment amplification at a suitable $MgCl_2$ concentration.

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Characterization and DNA Amplification Effect of Titanium Dioxide Thin Films on PCR Tube

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Abstract

TiO_2 thin films were deposited on a surface of the polymerase chain reaction (PCR) tube by using the electron beam evaporator at the deposition rate of 0.05 nm/sec. The base pressure of the deposition chamber was 1.5 x 10^{-6} mbar, while the pressure during the evaporation process was 4.5 x 10^{-5} mbar, electron-beam current was controlled at 50 mA and substrate temperature was kept at 60°C. PCR tube depositing was performed under the same conditions as 10, 50 and 100 nm film thickness of glass substrates. The SEM images with EDX analysis and AFM results showed the positive relationship between thickness and Ti particle accumulation on PCR tube surface. DNA fragments amplified form PCR tube with TiO_2 thin films outside had the similar result with normal PCR tube while the DNA amplification using PCR tube with TiO_2 thin films inside were decreased at all annealing temperatures. Increase in thickness of TiO_2 thin films in PCR tube can effects the decreasing yield of target DNA in PCR reaction. At 70°C annealing condition, a non specific of the small DNA fragments were increased in PCR reaction. When these reactions performed in TiO_2 thin films inside PCR tube, smaller fragments were increased more than that in the control tube. Surface interactions between TiO_2 particles and biomolecule in PCR reaction should affect the PCR yield.

Background

The polymerase chain reaction (PCR) is one of the most important methods in molecular biology. This method is an *in vitro* amplification technique for the target DNA sequence [1]. Au nanoparticles (NPs) have been reported to improve the yield of PCR reaction under fast cycling condition [2]. Functionalized tetrapods ZnO nanostructures were also shown to increase the PCR product [3]. Cui and teams and Zhizhou and coworkers proposed that single-walled carbon nanotubes and carbon nanopowder could increase the yield of PCR and could improve the amplification efficiency for long PCR reactions, respectively [4-5].

Recently, the impact of TiO_2 nanoparticles on DNA synthesis was investigated *in vitro* [6]. Functional properties of titanium dioxide (TiO_2) are influenced by many factors such as crystallinity, particle size, surface morphology, and preparation method. However, the reaction between TiO_2 thin film on PCR tube and DNA fragment amplifications has still not been reported. We investigated the effect of TiO_2 thin film grown on the PCR tube substrate at different thicknesses on the amplification of DNA fragment.

Materials and Methods

Thin film deposition

The TiO_2 thin films were grown inside and outside of PCR tube substrates by the electron beam evaporator (EVT1 auto 306, EDWARDS, USA) at the deposited rate of 0.05 nm/sec. The TiO_2 thin films were also deposited on glass substrates in order to measure thickness by spectroscopic ellipsometry technique. The base pressure of the deposition chamber was 1.5 x 10^{-6} mbar, while the pressure during the evaporation process was 4.5 x 10^{-5} mbar, electron-beam current was controlled at 50 mA and substrate temperature was kept at 60°C. The same as glass substrates deposited conditions varied from 10, 50 and 100 nm were performed with PCR tubes. These tubes were used for further analysis

Thin film characterization

The surface morphologies of the TiO_2 thin films were analyzed using the variable chamber pressure-scanning electron microscope (VP-SEM) (S-3400N, Hitachi, Japan) and Energy dispersive X-ray spectroscopy (EDX) was used to detect Titanium particles of the surface. The surface morphologies of the TiO_2 thin films were also

analyzed by the atomic force microscope (AFM) (DFM mode, SPA-400, SEIKO, Japan).

DNA amplification

A pair of primers (0.2 μM of each) was used to amplify the fragments of 35S promoter combining with *GUS* gene (2393-bp). Primer sequences used in experiments were as follows: Forward primer, 5'-ATCAAAGAT ACAGTCTCA GAA GACC-3' and reverse primer, 5'-ACCGAAG TTCATGCCAGTCCAGC-3'. The PCR reaction was carried out in a 50 μl reaction volume containing 5 μl of 10 x reaction buffers, 0.2 mM dNTPs and 1.5 U of Taq DNA polymerase (RBC Bioscience). pCAMBIA 1301 plasmid was served as the DNA template. PCR was performed with the Eppendorf PCR system (Eppendorf, U.S.), using the following protocol: 3 min at 94 °C; 40 cycles of 45 s denaturing at 95 °C, 40 s primer annealing varied at 65 °C, 67.5 °C, and 70 °C, 1.5 min extension at 72 °C; and finally 2 min at 72 °C. 2 μL of PCR products were analyzed by using 1 % agarose gel electrophoresis in Tris/Borate/EDTA buffer (TBE) and visualized under an ultraviolet lamp.

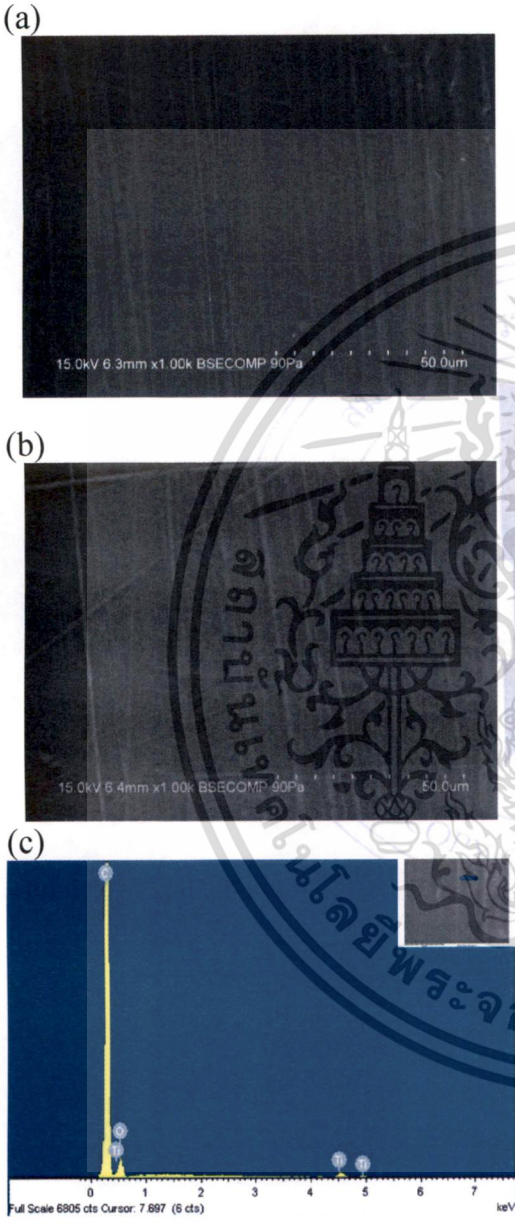


Figure 1 VP-SEM image of (a) PCR tube at a magnification of X1000, (b) TiO_2 thin films were grown on the PCR tube in 100 nm by VP-SEM/EDX at a magnification of X1000, and (c) then EDX analysis of the thin film, the voltage for energy dispersive analysis was 15 kV, 90Pa, and the time of x-ray collection 60s.

Results and Discussion

TiO_2 thin films characterization

The processing conditions affecting the formation of TiO_2 thin film on PCR tube were investigated. Figure 1 shows VP-SEM images (at a magnification of X1000) of surface on normal PCR tube (a) and PCR tube coated with the TiO_2 thin film using the same condition as 100 nm film thickness on glass (b). EDX analysis of PCR tube surfaces clearly showed a titanium peak that came from TiO_2 in Fig. 1c. The titanium signal was evidently seen on the both surfaces of tube. The spectrum from titanium particle was quantified to give a composition (in weight percentage).

Using the conditions of 10, 50 and 100 nm film thickness of glass, the average weight percentages of Ti outside surfaces of PCR tube were 0.416±0.07, 0.75±0.08 and 1.08±0.06 respectively. The average weight percentages of Ti using the same condition of 50 and 100 nm film thickness inside-PCR tubes were 0.64±0.03 and 0.83±0.05 respectively. There could not investigate Ti inside surfaces of PCR tube with EDX technique when the condition of 10 nm thickness of TiO_2 thin film on glass was used.

Quantitative of Ti shows the relationship with the thickness of standard glass deposited conditions. The average weight percentages of Ti deposited at the inner surface of PCR tube less than

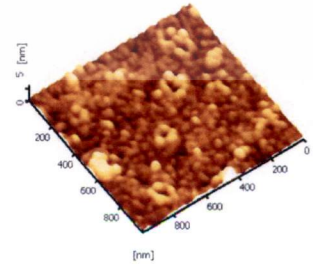
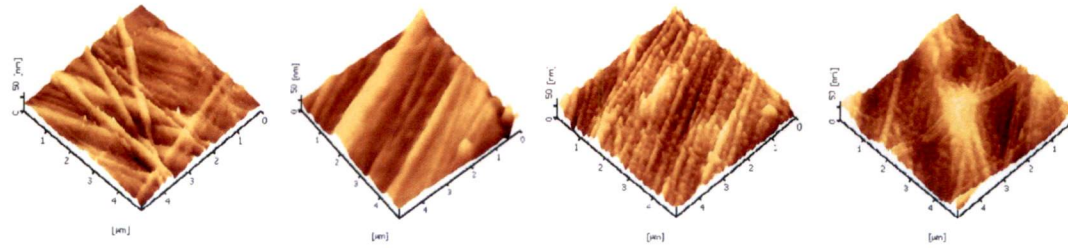
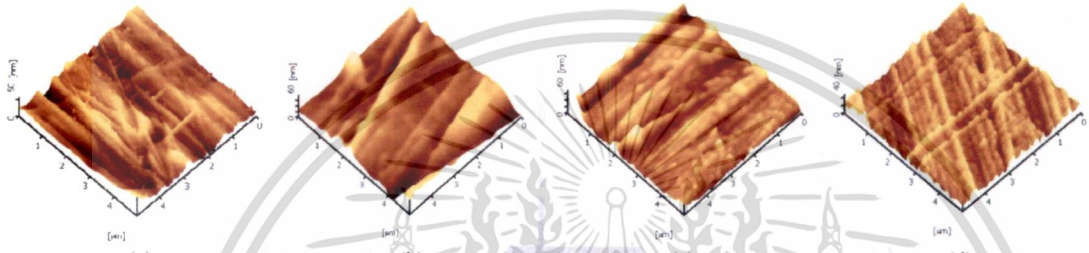


Figure 2 AFM images of TiO_2 thin films grown on glass with a thickness of 10 nm.



(a) (b) (c) (d)

Figure 3 AFM images of the morphologies of outside-PCR tube (a) conventional PCR tube, and TiO_2 thin films grown on the outside-PCR tube using the same condition as glass with a thickness of (b) 10 nm, (c) 50 nm, and (d) 100 nm.



(a) (b) (c) (d)

Figure 4 AFM images of the morphologies of inside-PCR tube (a) conventional PCR tube, and TiO_2 thin films grown on the inside-PCR tube using the same condition as glass with a thickness of (b) 10 nm, (c) 50 nm, and (d) 100 nm.

those of the outer surface of PCR tube because of the tube shape affected on thin film deposition.

Atomic Force Microscope Dynamic Force Mode

To investigate TiO_2 thin film on the surface of flat glass substrate and PCR tube, AFM was used. The micrograph shows that the surface of glass coating 10 nm thin films is dense with non homogenous grain. (Fig. 2) Surface characterization of TiO_2 thin film varying in thicknesses at outside and inside PCR tube revealed different surface topography (Fig. 3 and Fig. 4). The surfaces of PCR tube using 10 nm film thickness of glass condition were covered with a grain layer less than those using 50 and 100 nm thickness of glass conditions.

DNA amplification

The thin film of TiO_2 grown on the surface inside and outside of PCR tubes at various thicknesses presented the difference quality and quantity in DNA products as shown in Fig. 5. In this study, 2393-bp DNA fragments were amplified by using three different annealing temperatures. At 65 °C and 67.5 °C annealing temperature, DNA fragments on agarose gel showed the similar in all different tube conditions. In both annealing temperatures, PCR tube, outside coated with all film thickness conditions, had a similar pattern of PCR products comparing to the control while PCR tubes, inside coated with film thickness at 50 and 100 nm of glass conditions were not found the target fragment.

When PCR condition performed at 70 °C annealing temperature in normal tube, the intensity of target DNA band was reduced when compared with PCR condition at 65 °C and 67.5 °C annealing temperature. At 70 °C annealing condition, this temperature may be higher than primer annealing temperature, thus a non specific of the small DNA fragments were increased. In all conditions of annealing temperature, the increases of TiO_2 film thickness inside PCR tube decreased the amplification of DNA while the increases of TiO_2 films thickness outside PCR tube were not different comparing to the control. TiO_2 thin films on the inside surface of PCR tube can inhibit DNA synthesis by the adsorption of biomolecules in the reaction onto the thin film surface of TiO_2. Functional groups of DNA adsorbed onto TiO_2 particles have been reported [6]. Moreover the interaction between TiO_2 thin film and polymerase can cause a reduction in the effective polymerase concentration.

Conclusion

The different thickness in TiO_2 thin film deposited conditions by the electron beam evaporator effects on the quality and quantity of TiO_2 on surface of PCR tube. The SEM images with EDX analysis and AFM results showed the

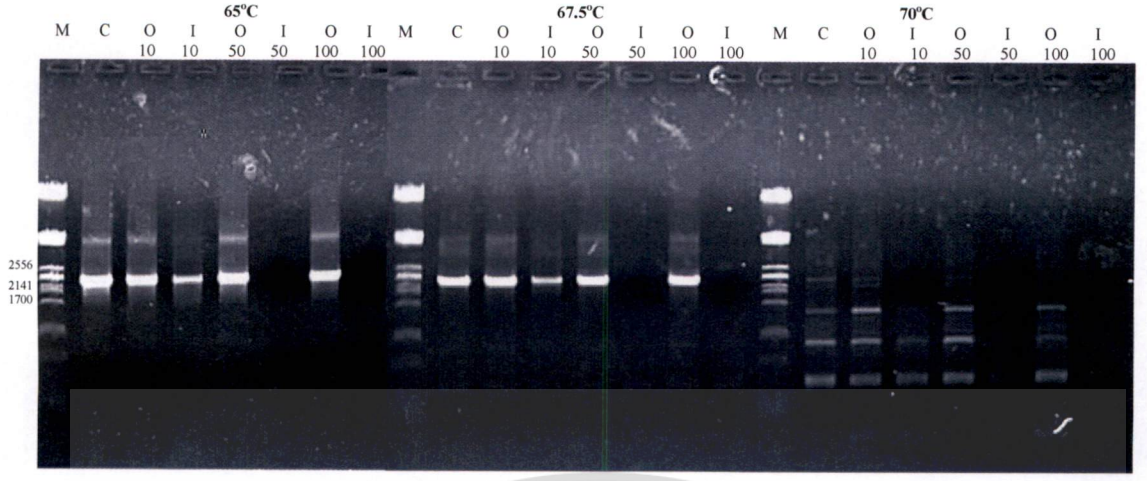


Figure 5 The affectation of TiO_2 thin films on the DNA amplification. Lane M is for markers, C is for PCR reaction in conventional tube, I is for TiO_2 thin films grown inside tube and O is TiO_2 thin films grown outside tube.

increasing accumulation of Ti particles on PCR tube surface after using higher thickness condition of films. Increase thickness of TiO_2 thin film inside PCR tube directly decreased the DNA amplification while TiO_2 thin film outside PCR tube was not. Thus the surface interactions between particles and biomolecule in PCR reaction should affect the PCR yield.

Acknowledgements

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