Direct Amplification from Various Biospecimens Using a Novel PCR Buffer System

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Abbreviations

apo E: apolipoprotein E

AS-PCR: allele-specific PCR

BSA: bovine serum albumin

Col: Columbia

DMSO: dimethyl sulfoxide

EDTA: N,N,N’,N’-ethylenediaminetetaacetic acid

gp32: T4 gene 32 protein

HIV-1: human immunodeficiency virus type-1

IEF: isoelectric focusing

Ler: Lansberg erecta

PCR: polymerase chain reaction

RFLP: restriction fragment length polymorphism

SDS: sodium dodecylsulfate

SNP: single nucleotide polymorphism

SSCP: single-stranded conformational polymorphism

T-ARMS: tetra-primer amplification refractory mutation system

Abstracts

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Polymerase chain reaction (PCR) using DNA from various biospecimens, such as blood, plant, tissue, etc, is a valuable tool in the field of molecular biology and medical diagnostics. However, DNA isolation from biospecimens is a laborious and sample-consuming step, and hampers the automation of PCR for large-scale studies. And attempt to PCR from biospecimens without DNA isolation is not achieved, since numerous endogenous and exogenous constituents may inhibit PCR. We used a novel buffer system, 'AnyDirect' that conserves the enzymatic activity of DNA polymerases for effective use in direct PCR from biospecimens under various conditions

Using AnyDirect, DNA amplification was achieved from whole blood with a variety of thermostable DNA polymerases. Amplification occurred regardless of target size (up to 1.7 kb), presence of various known PCR inhibitors, and high target GC content. Importantly, low copy number DNA targets were effectively amplified

from whole blood.

The downstream procedures from direct PCR using AnyDirect were examined with various apolipoprotein E (apo E) genotyping methods such as sequencing, SNaPshot assay, PCR- restriction fragment length polymorphism (RFLP), and allele-specific (AS)-PCR. In this study, we have also developed a new genotyping method for apo E. Our multiplex tetra-primer amplification refractory mutation system (multiplex T-ARMS) PCR was performed in a single reaction tube and gave definitive electropherograms at each genotypes. Using AnyDirect solution, multiplex T-ARMS PCR for apo E genotyping from whole blood is a simple, rapid, and accurate method that requires only a single PCR reaction without DNA extraction and any another treatments or expensive instrumentation.

The extraction of PCR-compatible genomic DNA from higher plants requires complicated and tedious work because plant cells have rigid cell walls and contain various endogenous PCR inhibitors such as polyphenolic compounds. Therefore, we developed a simple lysis system that can give rise to an appropriate template for direct PCR with AnyDirect solution, making the direct amplification of DNA fragments from plant leaves possible.

Our experimental procedure provides a simple, convenient, non-hazardous, non-expensive, and rapid process for DNA amplification from various biospecimens. And our AnyDirect solution allows direct PCR from whole blood and may facilitate detection of genetic diseases or infections by eliminating the time and effort for DNA extraction. The use of AnyDirect could facilitate the development of high-throughput PCR for large-scale diagnostic screening or investigation of various medical conditions. And this method may be applicable for use with lab-on-a-chip

and bio-MEMS technology.

Keywords: AnyDirect; PCR; Direct PCR; Thermostable DNA Polymerase; Whole Blood; Arabidopsis; Apolipoprotein E; SNP; Sequencing; Multiplex T-ARMS-PCR; AS-PCR; PCR-RFLP; SNaPshot

I. A novel buffer system, AnyDirect, can improve polymerase chain reaction from whole blood without DNA isolation

1. Introduction

Nucleic acid amplification is a powerful and useful molecular biological method in the field of *in vitro* diagnostics. PCR using whole blood as the source of a template can be applied to identify polymorphisms and genes underlying genetic diseases, or to detect microbial infections such as the hepatitis B virus. Until recently, highly purified template DNA has been required for gene amplification, since biospecimens and whole blood contain numerous inhibitors of PCR, such as hemin (Akane *et al.*, 1994), immunoglobin G (Abu Al-Soud *et al.*, 2000), bile salts, other unknown inhibitors (Abu Al-Soud and Radstrom, 2001), and anticoagulants (Satsangi *et al.*, 1994). These agents suppress PCR by decreasing the activity of DNA polymerases. Therefore, the amplification of template DNA that is not purified from whole blood or biospecimens is inefficient, and it is difficult to obtain valid results.

Many researchers have attempted to develop direct PCR protocols that do not require DNA isolation for the following reasons: 1) time savings, 2) convenience, 3) avoidance of infection in sample handlers, 4) prevention of loss of trace samples in the DNA purification step, and 5) potential automation for large-scale diagnosis.

The simple salting out procedure was the first attempt at rapid DNA purification (Miller *et al.*, 1988), and pretreatment of whole blood with heat (Mercier *et al.*, 1990), microwave (Cheyrou *et al.*, 1991), or formamide (Panaccio *et al.*, 1993) has been attempted to obtain target amplicons. However, these methods failed to generate successful results compared to PCR with isolated DNA. Other methods involve

alterations in salt and $MgCl_2$ concentrations (Burckhardt, 1994), the addition of single-strand DNA binding proteins such as T4 gene 32 protein (gp32) (Kreader, 1996), and replacement of Taq DNA polymerase with Tth DNA polymerase (Panaccio *et al.*, 1993). Al-Soud et al. (Abu Al-Soud and Radstrom, 2000) reported that specific molecules such as betaine, gp32, bovine serum albumin (BSA), and proteinase inhibitors facilitate nucleic acid amplification, even in the presence of PCR blockers. Unfortunately, these methods are limited with respect to the amount of whole blood required, and significant variations have been observed in the data obtained. A simple microchip device for DNA extraction has recently been developed, but its method also requires a cell lysis step with proteinase K, and its recovery of extracted DNA from whole blood was only 27~40% (Nakagawa *et al.*, 2005).

Recently, Ampdirect solution (Shimadzu, Kyoto, Japan) was developed to facilitate PCR without DNA isolation. This reagent is capable of suppressing inhibition of PCR by biological substances, and consequently, DNA from human whole blood (Nishimura *et al.*, 2000; Nishimura *et al.*, 2002) and transgenic mice blood (Nishimura *et al.*, 2002) has been successfully amplified. However, the solution was not effective when used with chemically modified Taq DNA polymerases such as AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), since their activities were not recovered (Nishimura *et al.*, 2000). Moreover, GC-rich regions could not be amplified (Nishimura *et al.*, 2000). The issue of whether this buffer is applicable to other thermostable DNA polymerases remains to be established.

To efficiently perform direct PCR without DNA purification, we have developed

a novel reaction buffer, 'AnyDirect' solution (BioQuest, Seoul, Korea), and tested its efficacy in various amplification conditions. Notably, the solution suppressed strong PCR inhibitors such as hemin, sodium dodecylsulfate (SDS) (Gelfand and White, 1990), and N,N,N',N'-ethylenediaminetetaacetic acid (EDTA). Additionally, we investigated amplification reactions using various amounts of whole blood, GC-rich regions, various thermostable DNA polymerases, and low copy number templates in the presence of AnyDirect.

2. Materials and Methods

2.1. Human whole blood and genomic DNA

Human whole blood was obtained from the Korean Red Cross Blood Center (Seoul, Korea). Human genomic DNA was purchased from Promega (Madison, WI, USA).

2.2. Amplification of p53, retinoblastoma, and apolipoprotein E Genes

PCR procedures were performed according to standard protocols, with 0.2 mM of each dNTP, 0.5 μM of each primer, 1.5 U of thermostable DNA polymerase, and whole blood or human genomic DNA as a template in a total volume of 50 μL. The primers employed are described in Table 1, and reaction buffers were supplied by the respective manufacturers (Applied Biosystems: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$) or AnyDirect solution. For PCR of GC-rich regions, betaine (Sigma, St. Louis, MO, USA) was added at concentrations of 0~2.5 M. The following conditions were employed: incubation for 5 min at 94 °C, denaturation at 94 °C for 30

s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min for 40 cycles, and a final extension step at 72 °C for 7 min. Amplicons were resolved by 2% agarose gel electrophoresis, and stained with ethidium bromide (0.5 mg/L). Reactions were incubated with hot-start DNA polymerases such as AmpliTaq Gold, FastStart Taq (Roche Applied Science, Mannheim, Germany), and HotStarTaq DNA polymerase for 15 min at 95 °C to recover enzyme activity prior to PCR.

2.3. Tolerance tests

PCR was performed according to standard protocols, using human genomic DNA (10 ng) as the template with or without inhibitors in a 50 μL reaction volume. For amplification, 1.5 U of AmpliTaq DNA polymerase (Applied Biosystems) and 0.5 μM of primers (p53f1 and p53r1) were employed. General PCR reaction buffer (Applied Biosystems) or AnyDirect solution was used, and inhibitors were added at the following concentrations: 0~3 mM hemin (Fluka Chemie GmbH, Buches, Switzerland), 0~0.1 % SDS (Sigma), or 0~2 mM EDTA (Sigma). Products were amplified as described above.

2.4. Amplification of p53 with various thermostable DNA polymerases

Amplification of the p53 gene was performed with 0.2 mM of each dNTP, 0.5 μM of each primer (p53f1 and p53r2), 1.5 U of various thermostable DNA polymerases, and 1 μL of heparinized blood as the source of the template in a total volume of 50 μL, and reaction buffers were supplied by the respective manufacturers or AnyDirect solution. The following DNA polymerases were examined in our

experiments: 1) enzymes from other thermophilic organisms [Pfu (Stratagene, La Jolla, CA, USA), Pwo (Roche Applied Science) and Tth DNA polymerase (Roche Applied Science)], 2) Taq DNA polymerases purchased from Applied Biosystems, Qiagen, Roche Applied Science, Invitrogen (California, USA) and Promega, 3) Blended DNA polymerase [EX Taq DNA polymerase (Takara Bio, Shiga, Japan)], 4) Chemically modified Taq DNA polymerase [AmpliTaq Gold, HotStarTaq, and FastStart Taq DNA polymerase], and 5) temperature-dependent hot-start Taq DNA polymerase [HotMaster Taq DNA polymerase (Eppendorf AG, Hamburg, Germany)]. Products were amplified as described above.

2.5. Amplification of human immunodeficiency virus type-1 (HIV-1) plasmid DNA

Low copy HIV-1 DNA (Birch *et al.*, 1996) was amplified from plasmid DNA that contained the entire genome of the HIVZ6 isolate (Hart *et al.*, 1990) (GeneAmplimer HIV-1 control reagents from Applied Biosystems). For these experiments, 2.5 U of AmpliTaq Gold DNA polymerase and various copies of HIV-1 plasmid DNA in 1 μL heparinized blood or 20 copies of HIV-1 plasmid DNA in different volumes of heparinized blood were added, along with SK145 and SK431 primers (0.5 μM each), 0.2 mM dNTP mixture, 2.5 mM $MgCl_2$, and AnyDirect solution in a total volume of 50 μL. HIV-1 amplification was initiated at 95 °C for 15 min, followed by 50 cycles of denaturation at 94 °C for 30 s, annealing and extension at 60 °C for 1 min, and a final extension step at 60 °C for 10 min. Amplicons were resolved by 2% agarose gel electrophoresis treated with ethidium bromide (0.5 mg/L).

3. Results

3.1. AnyDirect can improve PCR amplification in the presence of PCR inhibitors.

It is known that hemin, SDS, and EDTA inhibit PCR amplification of DNA (Abu Al-Soud and Radstrom, 2001; Akane *et al.*, 1994; Gelfand and White, 1990). In order to test whether AnyDirect can suppress the negative effect of these inhibitors, PCR was performed using genomic DNA and AnyDirect or commercially available general PCR buffer (Figure 1). Compared to general PCR buffer, AnyDirect was able to amplify the DNA in the presence of various concentrations of inhibitors. The AnyDirect solution showed tolerance against up to 0.75 mM hemin and 0.025 % SDS (Figure 1A), whereas the general, commercially available PCR reaction buffers failed to work in the presence of low concentrations of both inhibitors (0.1875 mM of hemin and 0.006 % of SDS) (Figure 1B). AnyDirect solution and general PCR buffer were comparable in term of the effect of EDTA (0.5 mM, Figure 1), although the yield of the PCR product was severely reduced with general buffer, but not with AnyDirect. These data show that AnyDirect can suppress the inhibitory effect of hemin, SDS, and EDTA on PCR amplification from genomic DNA.

3.2. AnyDirect can support amplification of GC-rich region as well as target sequencing unto 1.7kb from anticoagulant treated whole blood.

To determine whether AnyDirect solution can effectively enhance PCR from whole blood without DNA isolation, we examined the effects of this reagent on p53

amplification from varying volumes of anticoagulant-treated blood. Human genomic DNA and general PCR reaction buffer were employed as positive controls for PCR. Amplicons for p53 were obtained using a maximum volume of 10 μL of EDTA-blood, 5 μL of citrated blood, and 2.5 μL of heparinized blood in a total reaction volume of 50 μL (Figure 2).

To determine the effects of AnyDirect solution on the size of the PCR product, we designed seven p53-specific primers (Table 1). PCR products of various sizes, ranging from 146 bp to 1,702 bp, were obtained (Figure 3). Amplification from human genomic DNA with general PCR reaction buffer produced artifactual smears, with considerably weaker (lanes 2 and 6) or nonspecific (lanes 4 and 8) bands. However, except for lanes 2 and 6, a single PCR product with expected size was amplified from whole blood samples in the presence of AnyDirect solution. We cannot fully explain the reasons that we were able to perform effective amplification from whole blood compared to genomic DNA, but it is possible that the initial heating step contributes to the amplification process. Specifically, the initial annealing step is performed at elevated temperatures following the reduction of nonspecific product formation. Thus, AnyDirect solution may mimic hot-start effects in PCR, leading to effective target amplification.

Templates with high GC content are difficult to amplify, due to strong bond stability between GC base pairs and formation of secondary structures within each strand before annealing between specific template sites and primers (Chevet *et al.*, 1995; Henke *et al.*, 1997; Sun *et al.*, 1993). High GC content generally induces inefficient annealing and incomplete extension in PCR. Negative effects of GC-rich

templates on PCR can be suppressed with additives such as dimethyl sulfoxide (DMSO) (Sun *et al.*, 1993), tetramethylammonium chloride (Chevet *et al.*, 1995), or betaine (Henke *et al.*, 1997). In fact, it is possible to amplify a gene with up to 75% GC content in the presence of betaine in a PCR solution. As expected, AnyDirect solution successfully stimulated the amplifications of GC-rich DNA sequences, retinoblastoma genes (Bookstein *et al.*, 1990), and apolipoprotein E genes (Wu *et al.*, 2000) from heparinized blood, even in the presence of over 1 M betaine (Figure 4). The above data show that AnyDirect is suitable for the amplification of GC-rich genes as well as large target DNA sequences from whole blood without isolation of the DNA.

3.3. AnyDirect can support PCR amplification by various thermostable DNA polymerases and support amplification of HIV-1 DNA from whole blood.

Numerous thermostable DNA polymerases, originally isolated from thermophilic organisms, are now commercially available. These polymerases have been mutated and modified to further improve function or to attain new functions. Diverse buffer systems for PCR have also been developed by many manufacturers, although some companies do not divulge the compositions of their specific reaction buffers. As shown in Figure 5, various thermostable DNA polymerases successfully facilitate direct amplification of p53 target genes from 1 μL of whole blood in the presence of AnyDirect solution (Figure 5, even number lanes). However, these DNA polymerases failed to amplify the p53 gene with reaction buffers supplied from manufacturers when 1 μL of whole blood was employed to obtain a PCR template (Figure 5, odd

number lanes). Our results clearly show that AnyDirect solution is an innovative reagent that stimulates thermostable DNA polymerase activity to amplify DNA directly from whole blood.

For *in vitro* diagnostic application of PCR to detect infectious microorganisms, it is necessary to perform efficient gene amplification of templates with low copy numbers (Kellogg *et al.*, 1994). Hot-start PCR with DNA purified from whole blood is generally applied for the identification of infectious diseases. Thus, direct amplification of low copy number templates in whole blood with AnyDirect solution would be quite advantageous. To test this possibility, we mixed various copy numbers of HIV-1 plasmid DNA with heparinized blood, and attempted to amplify these genes with AmpliTaq Gold DNA polymerase in AnyDirect solution. As few as ten copies of HIV-1 plasmid in 1 μL heparinized blood as the PCR template were amplified to produce 142 bp amplicons (Figure 6). Next, we examined the effects of the amount of heparinized blood on amplification of a low copy number of HIV-1 DNA. In these experiments, 20 copies of HIV-1 plasmid DNA were successfully amplified using 3 μL heparinized blood, whereas the yield of the PCR product significantly decreased when 4 μL of heparinized blood was used. The data indicate that AnyDirect solution is suitable for amplifying low copy number templates in whole blood, although high amounts of heparin inhibit the PCR, as shown in Figure 2 (Heparin blood 5 μL).

4. Discussion

Numerous compounds, including as hemin (Akane *et al.*, 1994), bile salts, immunoglobin G (Abu Al-Soud *et al.*, 2000), proteinase and DNase, anticoagulants

(including EDTA (Abu Al-Soud and Radstrom, 2001) and heparin (Abu Al-Soud and Radstrom, 2001; Satsangi *et al.*, 1994)), and detergents for cell lysis (including SDS (Gelfand and White, 1990)), inhibit PCR. Failure to eliminate these compounds in the DNA purification step contributes to poor PCR results with purified DNA. Using our novel buffer system (AnyDirect), higher tolerance was observed against hemin and SDS compared to commercially available buffers (Figure 1). AnyDirect solution was successfully applied for the amplification of target genes from whole blood samples (Figure 2 and 3), such as those with GC-rich regions (Figure 4) or low copy number (Figure 6). Along with Taq DNA polymerases provided by several manufacturers, AnyDirect is effective for use with thermostable DNA polymerases isolated from other thermophiles, and modified or mutated Taq DNA polymerase (Figure 5).

Direct PCR from biospecimens or whole blood will abolish a number of the existing problems of DNA purification, including longer reaction times, high cost, exposure to toxic compounds, danger of infection, loss of trace samples, and difficulty of control in mass samples. Additionally, it is possible to perform direct amplification from low copy number DNA in whole blood with a hot-start enzyme, AmpliTaq Gold DNA polymerase (Figure 6). Another significant advantage of direct PCR is the hot-start effect, as described in Figure 3. Thus, AnyDirect can be applied in the detection of infectious microorganisms through *in vitro* diagnosis. Our data collectively indicate that direct PCR with AnyDirect may generate high-throughput results for large-scale diagnosis (Csako, 2006) or investigation of various medical conditions (Hiratsuka *et al.*, 2006); this method may be applicable for use with lab-on-a-chip technology (Kricka, 2001).

II. Apolipoprotein E genotyping from whole blood can be achieved with direct sequencing, SNaPshot multiplex, PCR-RFLP, AS-PCR, and multiplex T-ARMS PCR

1. Introduction

Human apolipoprotein E (apo E), a component of lipoproteins, is a 34 kDa glycoprotein synthesized mainly in the liver that plays a central role in lipid metabolism and transportation (Mahley, 1988). The apo E gene is polymorphic with three common alleles, designated as ε2 , ε3, and ε4 (Das *et al.*, 1985). These genes encode three apo E protein isoforms, E2 (Cys112/Cys158), E3 (Cys112/Arg158) and E4 (Arg112/Arg158), that differ by cysteine-arginine interchanges at sites 112 and 158 in the polypeptide chain (Table 4) (Emi *et al.*, 1988; Utermann *et al.*, 1980). The common apo E isoforms exhibit variations in structure and function and are involved in several pathological processes (Mahley and Rall, Jr., 2000). The familiar type III hyperlipoproteinemia may progress from the apo E2/E2 isoform (Breslow *et al.*, 1982), while the apo E4/E4 isoform is associated with high cholesterol levels, coronary artery disease (Menzel *et al.*, 1983), and Alzheimer's disease (Mayeux *et al.*, 1998; Roses *et al.*, 1994). Thus, various analytical methods have been developed to identify apo E polymorphisms.

The phenotyping method, isoelectric focusing, is based on the charge difference (pI) of apo E polypeptides and is conducted with human plasma and serum (Cartier and Sassolas, 1992). However, isoelectric focusing is a very complicated procedure that requires considerable expertise. On the other hand, the genotyping method to identify genetic differences in the apo E genomic sequence (single nucleotide

polymorphism, SNP) is a simpler and more accurate method than isoelectric focusing. One of the early methods of genotyping, PCR restriction fragment length polymorphism (RFLP) analysis (Hixson and Vernier, 1990; Wu *et al.*, 2000), has several disadvantages such as the requirement for restriction endonuclease, the time-consuming process, and the use of acrylamide gel to identify the results. Other genotyping methods include real-time PCR analysis (Nauck *et al.*, 2000), mass spectrometry (Ghebranious *et al.*, 2005; Srinivasan *et al.*, 1998), and single base extension genotyping technology (SNaPshot) (Ben Avi *et al.*, 2004). However, the cost of these methods is high because they each require expensive reagents or instruments. Only allele-specific PCR (AS-PCR) (Donohoe *et al.*, 1999) does not require post-PCR treatment or instrumentation, but two reactions must still be performed to amplify the two different alleles of a single SNP.

Recently, T-ARMS PCR involving a single reaction at a single SNP was developed (Hersberger *et al.*, 2000; Liu *et al.*, 1997; Ye *et al.*, 2001), and Piccioli et al. reported that a single T-ARMS PCR procedure was sufficient for the detection of two different mutations in the MUTYH gene (Piccioli *et al.*, 2006). However, because the two SNPs of apo E gene are only 138 nucleotides apart (GenBank M10065) unlike the MUTYH mutations, which are 646~1,247 nucleotides apart, Piccioli's method is not particularly attractive for apo E genotyping. Therefore, we have designed a new multiplex T-ARMS PCR procedure that is performed in a single-tube reaction (Table4 and Figure 7), using specific T-ARMS primers (Table 3 and Figure 8).

These methods for apo E genotyping are used currently and gave a faithful result, but researcher must extract clean DNA from biospecimens to obtain accurate

genotyping results. If one does not obtain pure DNA from samples or have only the trace sample not to isolate DNA, you do not obtain accurate result whether or not your method are excellent. We have developed a novel cocktail solution, AnyDirect solution that is efficiently performed direct PCR without DNA purification. This solution is capable of suppressing inhibition of PCR by strong PCR inhibitors and is effective of GC-rich region, various thermostable DNA polymerases, and low copy number templates in PCR (Chapter I).

Because direct PCR with AnyDirect is DNA amplification without DNA isolation, the removal of inconvenience of carrying out DNA isolation such as time-consuming, labor-intensive, high-cost, use of hazard materials, loss of target DNA, and contamination, is provided more simple, convenient, and rapid method. Thus, we attempted to use AnyDirect for apo E genotyping with direct sequencing, mini-sequencing, PCR-RFLP, AS-PCR, and new developed multiplex T-ARMS PCR.

2. Materials and Methods

2.1. Human whole blood and genomic DNA

Human whole blood was obtained from the Korean Red Cross Blood Center. Genomic DNA was extracted from 0.2 mL of human whole bloods by QIAamp DNA Blood mini kit (Qiagen).

2.2. Direct sequencing from genomic DNA and whole blood

PCR reaction was performed with 20 ng genomic DNA, 0.3 μM of FO and RO primers (Table 3), 0.2 mM of dNTP mixture, 0.75 mM of betaine, 1.5 mM of $MgCl_2$,

1.5 U of HotStarTaq DNA polymerase and its reaction buffer (Qiagen) in total 25 μL reactions. But in blood, we used 0.5 μL of EDTA-blood and AnyDirect. The amplification was carried out with an enzyme activation step for 15 min at 95 °C, and followed by 40 cycles of 30 s at 94 °C for denaturation, 30 s at 66 °C for annealing and 60 s at 72 °C for extension, and then final extension for 7 min at 72 °C. This amplicon of 514 bp was identified with 2 % agarose and then 20 μL amplicon was diafiltrated with 80 μL dH_2O using Centricon YM-100 membrane (Millipore, Billerica, MA, USA).

The sequencing was performed with 3 μL of retentate, 8 μL of BigDye Terminator v3.1, (Applied Biosystems) and 0.2 μM of the same PCR primer in a 20 μL reaction. The sequencing reaction was 25 cycles of 96 °C for 10 s, and 60 °C for 4 min. After sequencing reaction, the unused reactive components were removed with ethanol precipitation. Sequencing data were obtained from ABI 3100 Genetic Analyzer and analyzed by Sequencer 3.1.1. Software (Applied Biosystems).

2.3. Apo E genotyping by minisequencing (SNaPshot multiplex) from DNA and whole blood

PCR reaction was performed with the same method with direct sequencing reaction as above. The amplicon of 514 bp was identified with 2 % agarose and then carried out enzymatic clean-up with ExoSAP-IT (USB, Cleveland, Ohio, USA). Five μL amplicon was incubated with 2 U of ExoSAP-IT from 45 min at 37 °C, followed by 15 min at 80 °C. The SNaPshot reaction was performed with 1 μL of cleaning amplicon, 5 μL of SNaPshot multiplex mix containing fluorescent labeled dideoxy-

NTPs (Applied biosystems), and 0.2 μM of SP-112 or SP-158 primers (Table 3) in 10 μL reaction. The minisequencing reaction was 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. After reaction the unincorporated fluorescent labeled dideoxy-NTPs were removed by treating with 1 U of shrimp alkaline phosphatase (Fermentas, Hanover, MD, USA) and incubating at 37 °C for 30 min, followed at 85 °C for 15 min for enzyme inactivation. To analyze this SNaPshot product with ABI 3100 Genetic Analyzer, 1 μL of reaction product with 9 μL of formamide (Sigma) was loaded, run using POP4 polymer (Applied Biosystems) and analyzed using the Genescan (version3.1) software (Applied Biosystems).

2.4. Apo E genotyping by PCR-RFLP from DNA and whole blood

ApoE genotyping with PCR-RFLP was performed PCR and followed treatment with HhaI restriction endonuclease (NEB, Ipswich, MA, USA) (Wu *et al.*, 2000). PCR was performed with 100 ng genomic DNA or 1μL EDTA-blood, 0.5 μM of apoEf1 and apoEr1 primers (Table 1), 200 μM of dNTP mixture, 1 M of betaine, 2 mM of $MgCl_2$, 2.5 U of Taq DNA polymerase (BioQuest), and reaction buffer or AnyDirect in 50 μL reaction. The amplification was initiated at 95 °C for 2 min, followed by 45 cycles of 95 °C for 30 s, 68 °C for 30 s and 72 °C for 30 s, and then final extension at 72 °C for 7 min in human DNA. But in EDTA-blood, the amplification was 95 °C for 10 min, followed by 50 cycles of 95 °C for 30 s, 68 °C for 30 s and 72 °C for 60 s, and then final extension at 72 °C for 7 min. The resulting PCR products (20 μL) were treated with 5 U HhaI for 3 hours. The restriction fragments were identified with 12.5 % discontinuous polyacrylamide gel electrophoresis (Haas *et al.*, 1994), and

stained with ethidium bromide (0.5 mg/L).

2.5. Apo E genotyping by AS-PCR from DNA and whole blood

ApoE genotyping with AS-PCR (Donohoe *et al.*, 1999) was performed with specific primers Cys primers (Cys112 and Cys158) primers, Arg primers (Arg112 and Arg158) and common reverse primer, AS-R (Table 3). The PCR protocol was similar to that described above except that 20 ng genomic DNA or 0.5 μL EDTA-blood, 0.4 μM of Cys primers or Arg primers, and 0.4 μM AS-R primer were used in 25 μL reaction. PCR was initiated denaturation at 95 ℃ for 2 min, amplified by 35 cycles of 95 ℃ for 30 s, 62 ℃ for 30 s and 72 ℃ for 30 s, and followed final extension at 72 ℃ for 7 min in DNA, but in blood, initiated denaturation at 94 ℃ for 10 min, amplified by 40 cycles of 95 ℃ for 30 s, 62 ℃ for 30 s and 72 ℃ for 60 s, and followed final extension at 72℃ for 7min. Amplicons were resolved by 2 % agarose gel electrophoresis treated with ethidium bromide (0.5mg/L).

2.6. Apo E genotyping by T-ARMS PCR and multiplex T-ARMS-PCR from DNA and whole blood

PCR reactions were performed with 20 ng of human genomic DNA, 8 % DMSO, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mixture, 0.5 U of HotStarTaq DNA polymerase, and its reaction buffer (Qiagen) in 20 μL PCR reaction. But in whole blood, 0.5 μL EDTA-blood, 0.75 M betaine, 2.0 mM $MgCl_2$, 0.2 mM of dNTP mixture, 0.5 U of HotStarTaq DNA polymerase, and AnyDirect in 20 μL PCR reaction. In T-ARMS PCR, each of four primers (Table 3) was used for each allele site (0.08 μM FO, , 0.4

μM RO, 1.0 μM FI-1 and 1.0 μM RI-1 at codon 112, and 0.08 μM FO, , 0.4 μM RO, 0.4 μM FI-2 and 1.0 μM RI-2 at codon 158). However, in multiplex T-ARMS PCR, all six primers (0.08 μM FO, 0.4 μM RO, 1.0 μM FI-1, 1.0 μM RI-1, 0.4 μM FI-2, and 1.0 μM RI-2) were used in a single reaction tube. The amplification was carried out with an enzyme activation step for 15 min at 95 ℃, and followed by 35 or 40 cycles (30 s at 95 °C, 30 s at 64 °C, and 30 s at 72 °C), and then final extension for 7 min at 72 °C in DNA or whole blood, respectively. The amplicons were separated with 2 % agarose II (Amresco, Ohio, USA) gel electrophoresis containing ethidium bromide (0.5 mg/L), and identified with UV.

3. Results

3.1. ApoE genotyping can be achieved from whole blood by direct sequencing and minisequencing.

Direct sequencing for genotyping is gave an accurate result and used in basic data. For apo E genotyping, direct sequencing from several DNA samples was specific five apo E genotypes, such as ε3/ε3, ε4/ε4, ε2/ε3, ε2/ε4, and ε3/ε4. And the natural bloods of these DNA samples were identified with direct sequencing using AnyDirect solution. Specially, ε2/ε4 genotype is contained each two mutations of two SNP at codon 112 and 158. Figure 9 was showed that direct sequencing from ε2/ε4 genotype DNA and EDTA-blood is the same results.

Minisequencing (SNaPshot) analysis is a good method for SNP identification. We identified five apo E genotypes from genomic DNA and EDTA-blood that have conformed to direct sequencing as above. Three genotypes, ε2/ε4, ε2/ε3, and ε3/ε4

were showed in Figure 10. The ε2/ε4, ε2/ε3, and ε3/ε4 genotypes were showed CT/CT, TT/CT, and CT/CC at codon 112/158, respectively. Using AnyDirect solution, EDTA-blood was the same result as genomic DNA. Particularly, because PCR clean-up was completed with enzymatic clean-up system ExoSAP-IT, our AnyDirect system was also working at other nucleic acid modifying enzymes, such as exonuclease I and shrimp alkaline phosphatase.

3.2. Apo E genotyping can be achieved from whole blood by PCR-RFLP and AS-PCR.

In PCR-RFLP, the restriction endonuclease, HhaI, cleaved specific sites (Figure 11) was resulting in a specific restriction pattern for each genotype (Figure 12). Thus, apo E genotypes are determined by the patterns of these fragment from electropherogram obtained from PCR-RFLP [ε3/ε3 (91, 48, and 35 bp), ε4/ε4 (72, 48, and 35bp), ε2/ε3 (91, 83, 48, and 35 bp), ε2/ε4 (91, 83, 72, 48, and 35 bp), and ε3/ε4 (91, 72, 48, and 35 bp)]. Two of five samples, ε2/ε3 and ε2/ε4, were weaker band at 48and 35 bp than other polymorphisms. The reason is that ε2 allele is not cleaved at codon 158 and shows 83 bp fragment, but other alleles are cleaved at codon 158 and produce 48 and 35 bp. As an identical reason, ε4 allele is cleaved at codon 112 and produces 72 and 19 bp, but others are not cleaved. Therefore, both ε2/ε4 and ε3/ε4 polymorphisms are weaker band at 72 bp than ε4/ε4 polymorphism. But, both DNA and whole blood are showed specific restriction patterns according to each genotype (Figure 12). The amplification product with whole blood and AnyDirect solution is practicable to perform restriction endonuclease.

AS-PCR was performed with allele specific primers, Cys primers (Figure 13, lane A) containing Cys112 and Cys158 primers, or Arg primers (lane B) containing Arg112 and Arg158 primers. AS-PCR also yields allele-specific amplicons of 588 bp and 451 bp at codons 112 and 158, respectively (Figure 13). The ε3/ε3 genotype that contains Cys at codon 112 and Arg at codon 158 was produced 588 bp at Cys primers and 451 bp at Arg primers. The ε4/ε4 genotype contains Arg at both codon 112 and 158, thus was produced 588 and 451 bp at Arg primers but not Cys primers. The heterozygote ε2/ε3, Cys at codon 112 but both Cys and Arg at codon 158, was produced 588 and 451 bp at Cys primers and 451 bp at Arg primers. However, the ε3/ε4 genotype that contains Cys and Arg at codon 112 and Arg at codon 158 was produced 588 bp at Cys primers and 588 and 451 bp at Arg primers. Because the ε2/ε4 genotype is both Cys and Arg at both codon 112 and 158, this genotype was produced 588 and 451 bp amplicon at both Cys and Arg primers together. We obtained the accurate results from each genotype blood in AnyDirect solution in AS-PCR.

3.3. ApoE genotyping can be achieved from whole blood by multiplex T-ARMS PCR.

Our specialized primers were redesigned from primer sequences obtained from Ye's internet site (http://cedar.genetics.soton.ac.uk/public_html/primer1.html) and adapted to the new, multiplex T-ARMS PCR method. Two common outer primers (FO and RO) flank and amplify the non-allele-specific common amplicon (514bp), and each of two sets of allele-specific inner primers (FI-1 and RI-1 at codon 112, and FI-2 and RI-2 at codon 158) produces allele specific amplicons (Figure 14). At codon 112,

the ε2 and ε3 alleles contain **T**GC and produce an allele-specific amplicon with the FO and RI-1 primers (114 bp). However, the ε4 allele contains **C**GC and is amplified by the FI-1 and RO primers to produce the ε4-specific amplicon (443 bp). At the other polymorphism site, codon 158, the ε2 allele contains **T**GC and produces an amplicon with the FO and RI-2 primers (252bp), whereas the ε3 and ε4 alleles, which contain **C**GC, produce an amplicon with the FI-2 and RO primers (307bp) (Table 4).

T-ARMS PCR and multiplex T-ARMS PCR from samples of five different genotypes (ε3/ ε3, ε4/ ε4, ε2/ ε3, ε2/ ε4, and ε3/ ε4) that were confirmed by DNA sequencing gave the expected results (Figure 14). T-ARMS PCR at each apo E allele produced one non-specific 514 bp amplicon with the common outer primers and one or two allele-specific amplicons with allele-specific primers (Figure 14, lanes 1-10). In multiplex T-ARMS PCR with six primers in a single tube, we obtained a mixture of the allele-specific amplicons obtained from T-ARMS PCR (Figure 14, lanes 11~15). The multiplex T-ARMS PCR method was subsequently applied to 14 additional human genomic DNA samples (Figure 15, lane 6~19) for apo E genotyping and compared with the DNA used previously (Figure 15, lane 1~5). According to the apo E genotype of each sample, we obtained amplicons of the same size as those described above from these specific PCR products.

In whole blood, we performed direct multiplex T-ARMS PCR with each EDTA-blood which is correlated with used DNA. Using AnyDirect solution, multiplex T-ARMS PCR from 19 whole blood samples was gave the accurate result that is comparable to the result obtained from DNA (Figure 16). Thus, AnyDirect solution could be feasible to carry out direct multiplex T-ARMS PCR from whole blood that

was combined with multiplex and allelic discrimination.

4. Discussion

The downstream procedures of PCR are the important works and make use of practical application in fields of molecular biology and molecular diagnosis. We tested major procedures such as sequencing, PCR-RFLP, AS-PCR, and ARMS-PCR for apo E genotyping from DNA and whole blood. Five genotypes (ε3/ ε3, ε4/ ε4, ε2/ ε3, ε2/ ε4, and ε3/ ε4) of six apo E genotypes were identified with direct sequencing from DNA. And the corresponding whole bloods of these DNA samples were the same results from direct PCR using AnyDirect and sequencing (Figure 9). In SNaPshot assay, we used with enzymatic clean-up system ExoSAP-IT, and were obtained the identical results with direct sequencing from DNA and blood together (Figure 10). Thus, we should suggest that direct PCR from blood with AnyDirect is applicable to sequencing, and other nucleic acid modifying enzymes was usable in AnyDirect system.

PCR-RFLP and AS-PCR are the most common methods in genotyping, and are widely used, recently. AnyDirect system was amplified from whole blood and followed to digest with restriction endonuclease at recognition sites in PCR-RFLP without any treatment (Figure 12). And the amplification from whole blood using AnyDirect solution keeps the power of allelic discrimination in AS-PCR (Figure 13). Thus, aop E genotyping with PCR-RFLP and AS-PCR from whole blood using AnyDirect solution can give a rapid, simple, and accurate result without DNA purification.

Because our multiplex T-ARMS PCR method involves both T-ARMS PCR and multiplex PCR, this method does not require other treatment steps or expensive instrumentation, and can be carried out with one PCR in a single tube. The running time of apo E genotyping with multiplex T-ARMS PCR was within approximately 3 hours, including extraction of DNA from blood, amplification of the apo E gene with multiplex T-ARMS PCR, and identification of the genotypes with agarose gel. Thus, our multiplex T-ARMS PCR marks the first attempt to identify apo E genotype in a single PCR reaction without any special treatment or equipment (Figure 15). We confirmed that this is a simple, sensitive, rapid and inexpensive method for detecting apo E genotype. Furthermore using AnyDirect, direct multiplex T-ARMS PCR from whole blood was gave the discrimination of apo E genotype without isolation of the DNA (Figure 16). It will be reduced total time to obtain the results of apo E genotype from whole blood removing DNA isolation step. Specially, direct multiplex T-ARMS PCR without DNA isolation may be the most rapid, simple, and accurate apo E genotyping method.

And our multiplex T-ARMS PCR is readily applicable to the genotyping of other human gene mutations or of microorganisms. Moreover, it is possible to simultaneously identify several SNPs by combining our multiplex T-ARMS PCR for SNPs located near each other and T-ARMS PCR for SNPs located at longer distances through the design and use of specialized primers that produce allele-specific amplicons of different sizes. And, using fluorescence-labeled primers and capillary electrophoresis, numerous SNPs can be identified with a single PCR reaction as with forensic human identification using short tandem repeats (Butler *et al.*, 2004). We

believe that our multiplex T-ARMS PCR procedure and AnyDirect system will enable laboratories to proceed with gene mutation studies without the need for additional expensive reagents and equipment.

III. A simple and rapid gene amplification from Arabidopsis leaves using AnyDirect system

1. Introduction

PCR is a powerful and useful molecular biological method that is used in biological research and diagnosis (Florencia *et al.*, 2006; Mattarucchi *et al.*, 2005). In plants, the use of PCR has many applications in plant molecular biology, including plant genotyping, gene mapping, map-based cloning, screening for transformants, marker-assisted plant breeding, and molecular ecology. In order to obtain DNA that can be used for PCR, two processes are required. The first of these processes is cell lysis using mechanical or chemical methods, such as grinding in liquid nitrogen or using detergents and/or Proteinase K. The second of these steps is the separation of DNA from various components that may interfere with PCR using organic solvents, proteinase K, salts, or membrane filters. Thus, most methods for preparing DNA from plant tissues are complicated, time-consuming, and labor-intensive processes.

Cetyltrimethylammonium bromide (Hwang and Kim, 2000), ROSE buffer(Steiner *et al.*, 1995), and alkaline solution (Wang *et al.*, 1993; Xin *et al.*, 2003) have been used to extract PCR-compatible DNA from plants more rapidly. However, most of these methods require precipitation of genomic DNA after it is separated from other cellular components with organic solvents such as phenol or/and chloroform (Kang and Yang, 2004). In plants with high polyphenolic content, polyvinylpyrrolidone is also used to remove phenolic terpenoids that bind to DNA after cell lysis (John, 1992; Kim *et al.*, 1997).

Recently, we reported a novel solution, “AnyDirect”, which is used to perform

PCR directly from whole blood (Chapter I). This solution overcame the inhibitory effects of various chemical entities such as blood proteins or heparin, and facilitated the performance of PCR directly from whole blood without pretreatment, making use of a PCR heating step for blood cell lysis. Because plant tissue contains additional cellular components, including a cell wall, we attempted to create a condition in which simple SDS-lysate from plant tissue is subject to conventional DNA amplification by PCR using AnyDirect PCR buffer. Our procedure, which utilized AnyDirect PCR buffer, eliminated the need to perform the second process needed to obtain DNA, thereby simplifying the process of DNA amplification by PCR directly from plant tissue.

2. Materials and Methods

2.1. Growth of Arabidopsis and DNA extraction

Experiments were performed using Arabidopsis Columbia (Col-0) ecotype and Lansberg erecta (Ler) ecotype, which were obtained from the ABRC (Ohio State University, Columbus). The seedlings of Arabidopsis were grown for two weeks on Murashige-Skoog medium containing 0.8% agar under white light (WL, 4 Wm^{-2}) at 23 °C. Genomic DNA was extracted as described by Soh (Soh, 2006).

2.2. Simple lysis from Arabidopsis leaves

We cut leaves (1~2 mg/leaf) from Arabidopsis using sterilized scissors, and mixed them with 100 μL of diluted 5X Direct-N-Lyse I solution, a cocktail solution containing 1.5% SDS [100 mM Tris (pH8.0), 25 mM EDTA, 2 M NaCl, 1.5% SDS,

and 3% Tween-20] (BioQuest, Seoul, Korea). The number of cut leaves was 1, 2, 3, 4, 5, 10, or 15, and lysis solutions were diluted at 0.25X, 0.5X, 0.75X, or 1X of Direct-N-Lyse I solution. TE buffer [10 mM Tris and 1 mM EDTA (pH 7.0)] was used as the control lysis solution. Leaves were placed in lysis solution, ground with a pestle, incubated at 80 °C for 2 h, and immediately cooled on ice. The simple lysates were centrifuged at 6,000 rpm for 5 minutes, and these supernatants were used for amplification.

2.3. Amplification of DNA fragment from a genomic DNA and a simple lysate

PCR procedures were performed with 0.2 mM of each dNTP, 0.4 μM of each primer, 2 U of Taq DNA polymerase (BioQuest), and templates in a total volume of 50 μL. The PCR reaction buffers were conventional PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$] or AnyDirect PCR buffer [1.5 mM $MgCl_2$] according to the appropriate templates, DNA, or simple lysate, respectively.

In *NIT1* gene amplification, the primers used were forward primer, 5'-CCCTACATTCTACAACCATGTAGCC-3', and reverse primer, 5'-CGGAATTGATGTTTTGGACC-3', as described previously (The Arabidopsis Information Resource, TAIR). The PCR conditions included initial incubation at 94 °C for 5 min, followed 40 cycles [denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min], and a final extension step at 72 °C for 7 min. However, primers for the *MOD1* marker were derived from the Cereon small insertion/deletion (In/Del) polymorphism database (http://www.arabidopsis.org/browse/Cereon/index.jsp); forward primer, 5'-

GATTCCCACTTATCAACCATCTCC-3', and reverse primer, 5'-CGAGTATTACAAACCAAGCTTCAG-3'. PCR was conducted in the *NIT1* PCR condition, with an annealing temperature of 57 °C. Amplicons were resolved by 2% agarose gel electrophoresis in TAE buffer and stained with ethidium bromide (0.5 mg/L).

3. Results and Discussion

We applied Direct-N-Lyse I solution for lysis of Arabidopsis (Col and Col/Ler heterozygote) leaf tissue. Five leaves of two Arabidopsis ecotypes were heat-treated with 1X Direct-N-Lyse I solution. These four simple lysate solutions were then directly used as the sources of PCR templates for amplification of the *NIT1* gene, of which the amplicon sizes were 0.8 kb and 1.0 kb in the Col and Ler ecotypes, respectively (Figure 17). In a conventional PCR buffer system, amplification was not achieved, presumably due to the inhibitory effect of SDS (Figure 17, lanes 1~4). However, the AnyDirect PCR buffer was able to amplify the *NIT1* gene from both ground (Figure 17, lanes 5 and 6) and non-ground lysates (Figure 17, lanes 7 and 8), although the level of amplification from non-ground lysate was lower than that from the ground lysate. In contrast, the process of lysis using only SDS and heating was not sufficient for amplification of the *NIT1* gene.

Next, we attempted to determine the most effective concentration of Direct-N-Lyse I solution for performing direct PCR from plant tissue. The lysates in 0.25X, 0.5X, and 0.75X lysis solutions successfully served as the sources of templates for PCR with AnyDirect PCR buffer, while those with TE buffer did not (Figure 18,

AnyDirect PCR buffer). Under our experimental conditions, one or two microliters of leaf lysate served as better templates than over three microliters of lysate for PCR with a total volume of 50 microliters. A larger volume of over three microliters of lysate might not permit successful DNA amplification, presumably due to the increment of PCR inhibitors, especially SDS. In contrast to AnyDirect PCR buffer, the use of conventional PCR buffer did not yield amplicons from leaf lysates of Direct-N-Lyse I solution, except from that of highly diluted, 0.25X Direct-N-Lyse I solution (Figure 18, Conventional PCR buffer). Because certain plant tissues are comprised of hardened cell types, the use of a strong concentration of Direct-N-Lyse I solution (e.g. 1X) may be desirable for cell lysis. Thus, we propose coupling the use of plant lysate with Direct-N-Lyse I solution with the use of AnyDirect PCR buffer for PCR.

The number of leaves used in our simple lysis method appeared not to be a critical factor in the success of PCR. The simple lysates obtained from 1, 5, 10, and 15 leaves were compared, and were shown to be sufficient for the amplification of the *NIT1* gene (Figure 19), though the amplicon band from 1/16 μL of one leaf was weaker than other bands (Figure 19, lane 5 of 1 Leaf). Amplification of the *MOD1* marker from simple lysate was also achieved and yielded specific amplicons from Col, Ler, and Col/Ler heterozygote, respectively (Figure 20). Based on our results (Figures 19, and 20), we propose that the appropriate amount of sample in our simple lysis system should be 5~10 Arabidopsis leaves, or 5~15 mg in 100 μL solution.

We have reported that our AnyDirect PCR buffer is able to overcome the inhibitory effects of exogenous and endogenous contaminants such as SDS and hemin, and can be applied to perform direct PCR with whole blood (Chapter I). Here, we

examined whether our AnyDirect system is applicable for use with a plant system. Making use of detergent (SDS) treatment and crushing with a pestle in addition to heating at 80 °C, we exposed genomic nuclear DNA as a template for PCR. Though the resulting lysate would include significant amounts of PCR inhibitors as well as template DNA, our AnyDirect PCR buffer enabled the use of PCR to successfully amplify nuclear DNA fragments, thereby overriding the inhibitory effects of other components in leaf lysate. In summary, we have provided a simple lysis system that compatible with direct DNA amplification, and would make genomic DNA amplification a more rapid, simple, and safe procedure, facilitating large-scale genotyping experiments such as positional gene cloning in higher plants.

Table 1. Primer sequences used for direct amplification from blood.

Gene Name	Primer Name	Nucleotide Sequence
p53 gene	p53f1	5’-TGTTCACTTGTGCCCTGACT-3’
	p53f2	5’-GGCGACAGAGCGAGATTCCA-3’
	p53f3	5’-GACAAGGGTGGTTGGGAGTAGATG-3’
	p53r1	5’-GGAGGGCCACTGACAACCA-3’
	p53r2	5’-GGGTCAGCGGCAAGCAGAGG-3’
	p53r3	5’-CACAAACACGCACCTCAAAG-3’
	p53r4	5’-AGAGGAGCTGGTGTTGTTGG-3’
Apolipoprotein E gene	apoEf1	5’-GGCACGGCTGTCCAAGGA-3’
	apoEr1	5’-CTCGCGGATGGCGCTGAG-3’
Retinoblastoma gene	RBf1	5’-CAGGACAGCGGCCCGGAG-3’
	RBr1	5’-CTGCAGACGCTCCGCCGT-3’
HIV-1 plasmid DNA	SK145	5’-AGTGGGGGGACATCAAGCAGCCATGCAAAT-3’
	SK431	5’-TGCTATGTCAGTTCCCCTTGGTTCTCT-3’

Table 2. Various DNA polymerase 1X PCR reaction buffer compositions.

Thermostable DNA polymerase	Compositions					
	Tris-HCl (mM)	pH	KCl (mM)	$(NH_4)_2SO_4$ (mM)	Mg^{2+} (mM) [a]	Additives
Tth (Roche Applied Science)	10	8.9	100	0	1.5	50 mg/l BSA 0.5 ml/l Tween 20
Pfu (Stratagene)	20	8.8	10	10	2	1 ml/l Triton X100 0.1 mg/ml BSA
Pwo (Roche Applied Science)	10	8.85	25	5	2	
Taq (Qiagen)	Un [b]	8.7	Un [b]	Un [b]	1.5	
Taq (Roche Applied Science)	10	8.3	50	0	1.5	
Taq (Invitrogen)	20	8.4	50	0	1.5	
Taq (Promega)	10	9.0	50	0	1.5	1 ml/l Triton X100
EX Taq (Takara Bio)	Un [b]	Un [b]	Un [b]	Un [b]	2	
AmpliTaq Gold (Applied Biosystems)	10	8.3	50	0	1.5	
FastStart Taq (Roche Applied Science)	5	8.3	10	5	2	
HotStarTaq (Qiagen)	Un [b]	8.7	Un [b]	Un [b]	1.5	
HotMaster Taq (Eppendorf AG)	Un [b]	Un [b]	Un [b]	Un [b]	2.5	

[a] Thermostable DNA polymerases are used $MgCl_2$ as magnesium ions, but Pfu and Pwo DNA polymerase are used $MgSO_4$.

[b] Un : Unknown concentration or pH.

Table 3. PCR primers for apo E genotyping with AS-PCR, multiplex T-ARMS PCR, and sequencing.

	Primers	Sequences [a]
AS-PCR	Cys112	5'-CGCGGACATGGAGGACGTT**T**-3'
	Cys158	5'-ATGCCGATGACCTGCAGAAT**T**-3'
	Arg112	5'-CGCGGACATGGAGGACGTT**C**-3'
	Arg158	5'-ATGCCGATGACCTGCAGAAT**C**-3'
	AS-R	5'-GTTCAGTGATTGTCGCTGGGCA-3'
Multiplex T-ARMS PCR	FO	5'-ACTGACCCCGGTGGCGGAGGA-3'
	RO	5'-CAGGCGTATCTGCTGGGCCTGCTC-3'
	FI-1	5'-GCGGACATGGAGGACG**g**G**C**-3'
	RI-1	5'-CGGTACTGCACCAGGCGGCC**t**C**A**-3'
	FI-2	5'-CGATGCCGATGACCTGCAGA**c**G**C**-3'
	RI-2	5'-CCGGCCTGGTACACTGCCAG**t**C**A**-3'
SNaPshot [b]	SP-112	5'-CGGACATGGAGGACGTG-3'
	SP-158	5'-<u>TTTTTTTTTT</u>CCGATACCTGCAGAAG-3'

[a] The boldface lowercase letters are deliberate mismatch, and the boldface uppercase letters is allele-specific mismatch.

[b] The TT tails in SNaPshot are underlined.

Table 4. Amplicon size of multiplex T-ARMS PCR by apo E polymorphisms.

Genotype	1st, codon 112 [a]		2nd, codon 158 [a]		Common Amplicon (bp)
	Nt / AA [b]	Amplicon (bp)	Nt / AA [b]	Amplicon (bp)	
ε2/ ε2	**T**GC / Cys	114	**T**GC / Cys	252	514
ε3/ ε3	**T**GC / Cys	114	**C**GC / Arg	307	
ε4/ ε4	**C**GC / Arg	443	**C**GC / Arg	307	
ε2/ ε3	**T**GC / Cys	114	**T**GC / Cys	252	
			CGC / Arg	307	
ε2/ ε4	**T**GC / Cys	114	**T**GC / Cys	252	
	CGC / Arg	443	**C**GC / Arg	307	
ε3/ ε4	**T**GC / Cys	114	**C**GC / Arg	307	
	CGC / Arg	443			

[a] The mark of 1st and 2nd is order of mutation sequence in GenBank, and codon number is corresponded to each mutation.

[b] Nt / AA is nucleotide / amino acid at mutation site.

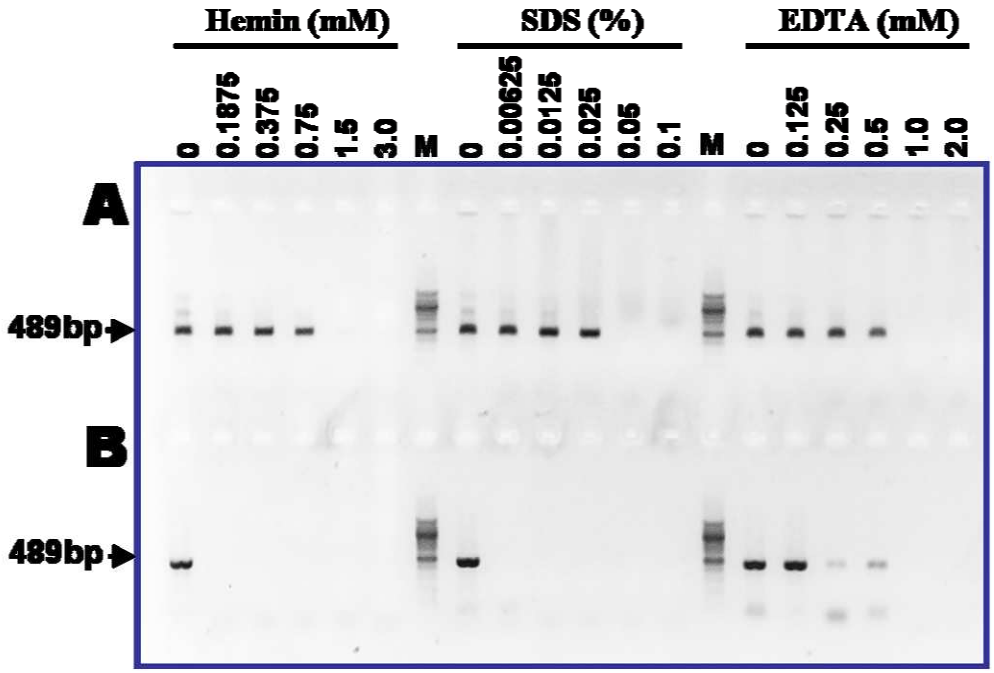

Figure 1. Effects of AnyDirect on PCR inhibitors on p53 gene amplification.

PCR was performed according to standard protocols, using human genomic DNA (10 ng) as the template with or without inhibitors in a 50 μL reaction volume. AmpliTaq DNA polymerase (1.5 U) (Applied Biosystems) and 0.5 μM primers (p53f1 and p53r1) were used for p53 gene amplification. The amplified p53 gene product was 489 bp (arrow). We employed AnyDirect solution (A) or general Taq DNA polymerase PCR reaction buffer [10 mM Tris-HCl (pH8.3), 50 mM KCl and 1.5 mM $MgCl_2$] (B). The final concentration of inhibitors (hemin, SDS, and EDTA) in the PCR is specified. Lane M: 100 bp ladder (BioQuest).

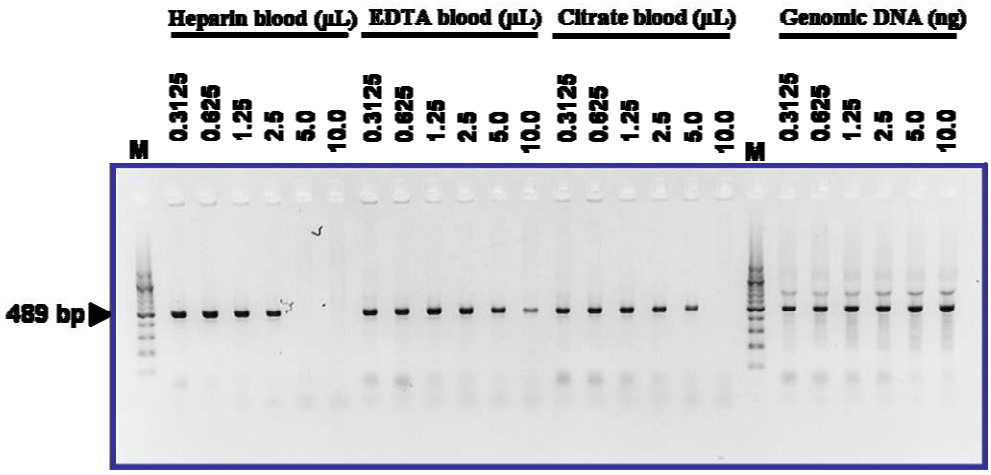

Figure 2. Effect of AnyDirect on PCR amplification of various anticoagulant treated whole blood.

PCR was performed according to standard protocols, either with human genomic DNA in general reaction buffer or whole blood in AnyDirect solution as the source of template. AmpliTaq DNA polymerase (1.5 U) and 0.5 μM of each primer were added to a total reaction volume of 50 μL. The p53 gene was amplified from EDTA, citrated, and heparinized blood samples, as well as genomic DNA. The amounts of whole blood used were 0.3, 0.6, 1.25, 2.5, 5 and 10 μL, and the human genomic DNA contents used were 0.3, 0.6, 1.25, 2.5, 5 and 10 ng, respectively.

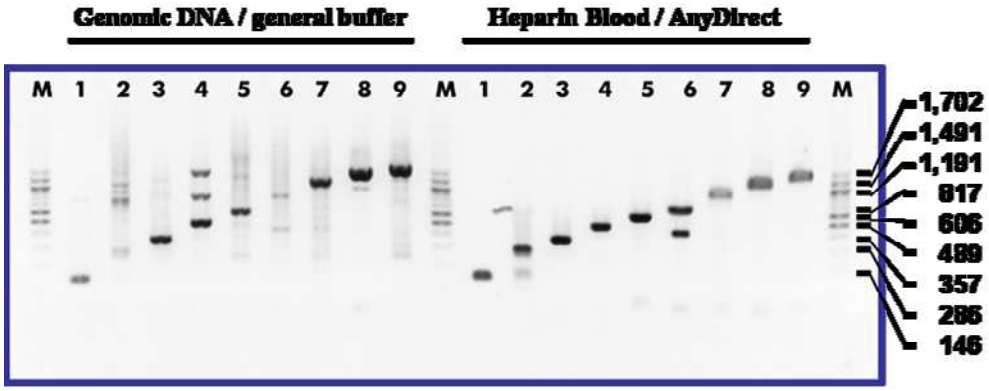

Figure 3. Effect of AnyDirect on PCR amplification of various target sizes from heparinized blood.

PCR was performed to amplify various regions of p53 gene amplification using 10 ng human genomic DNA or 1 μL of heparinized blood. The size of the PCR product and primers used in lanes 1-9: 146 bp (p53f3 & p53r2), 286 bp (p53f2 & p53r2), 357 bp (p53f3 & p53r4), 489 bp (p53f1 & p53r1), 606 bp (p53f2 & p53r3), 817 bp (p53f2 & p53r4), 1,191 bp (p53f1 & p53r2), 1,491 bp (p53f1 & p53r3), and 1,702 bp (p53f1 & p53r4).

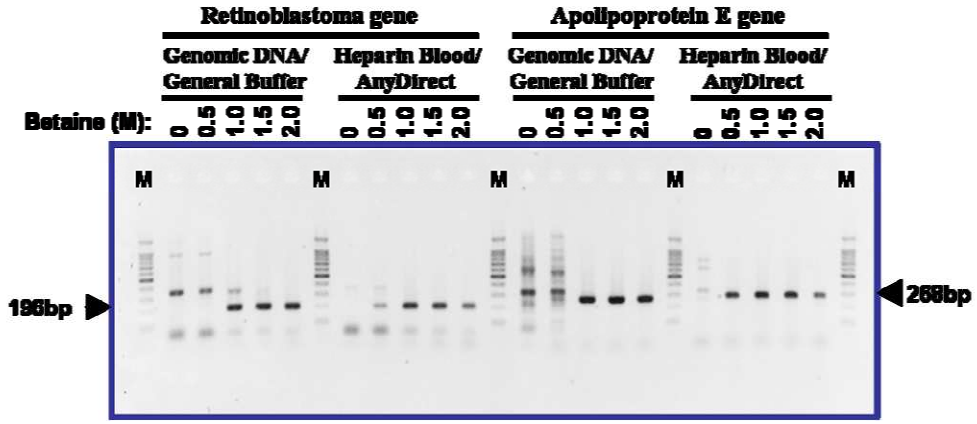

Figure 4. Effect of AnyDirect on PCR amplification of GC-rich regions from heparinized blood.

PCR was performed with human genomic DNA (10 ng) or heparinized blood (1 μL) in the presence of various amounts of betaine. The expected amplicon sizes of retinoblastoma gene (196 bp) and apolipoprotein E gene (268 bp) are indicated by arrowhead.

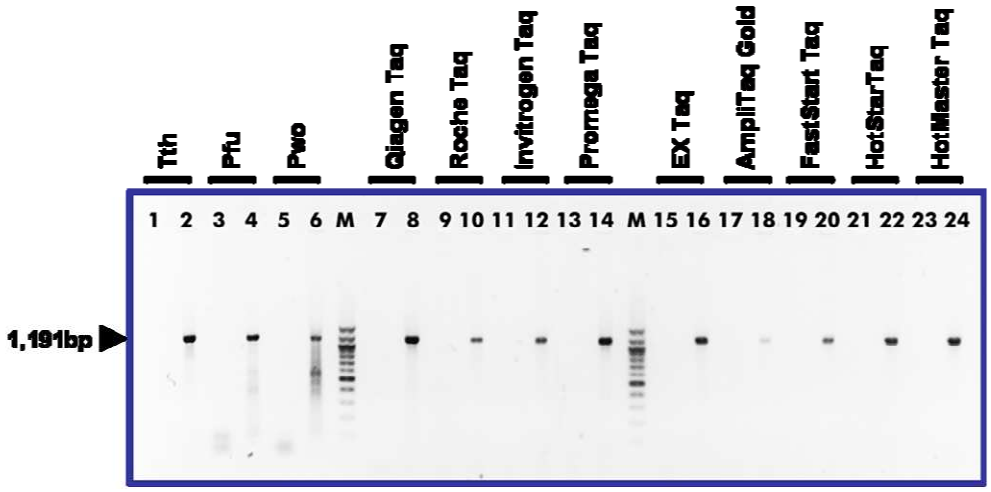

Figure 5. Effect of AnyDirect on various thermostable DNA polymerases.

PCR was performed according to standard protocols, with 1 μL of heparinized blood as the source of template DNA in AnyDirect solution (even numbers of lanes) or other PCR reaction buffers (odd numbers of lanes, Table 2) in a total reaction volume of 50 μL. The primer concentration (p53f1 and p53r2) was 0.5 μM, and twelve thermostable DNA polymerases were used (1.5 U). The expected amplicon size was 1,191 bp (arrowhead).

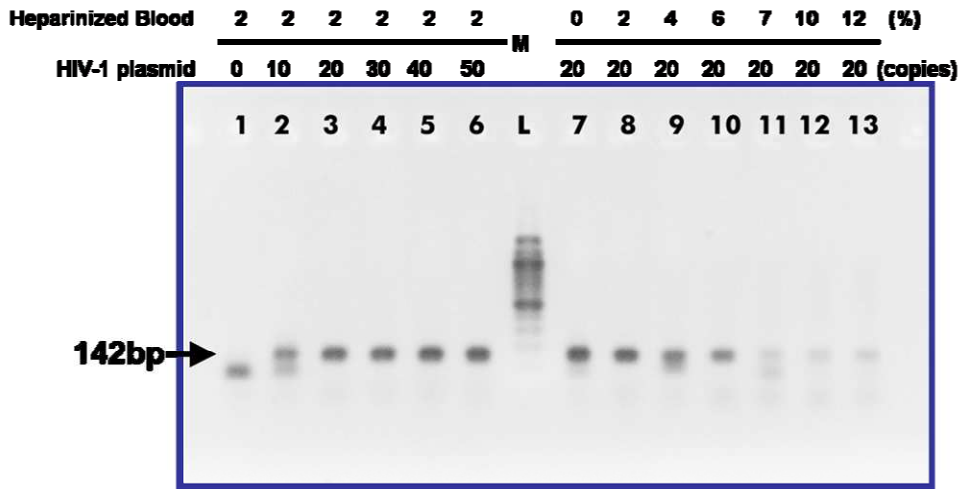

Figure 6. HIV-1 gene amplification with AmpliTaq Gold® DNA polymerase.

PCR was performed according to the procedure described in “Material and methods”. Templates comprised 0, 10, 20, 30, 40, and 50 copies of HIV-1 plasmid DNA in 1 μL heparinized blood and 0, 1, 2, 3, 4, 5, and 6 μL heparinized blood samples, each with 20 copies of HIV-1 plasmid DNA. A 142 bp amplicon product was obtained (arrowhead).

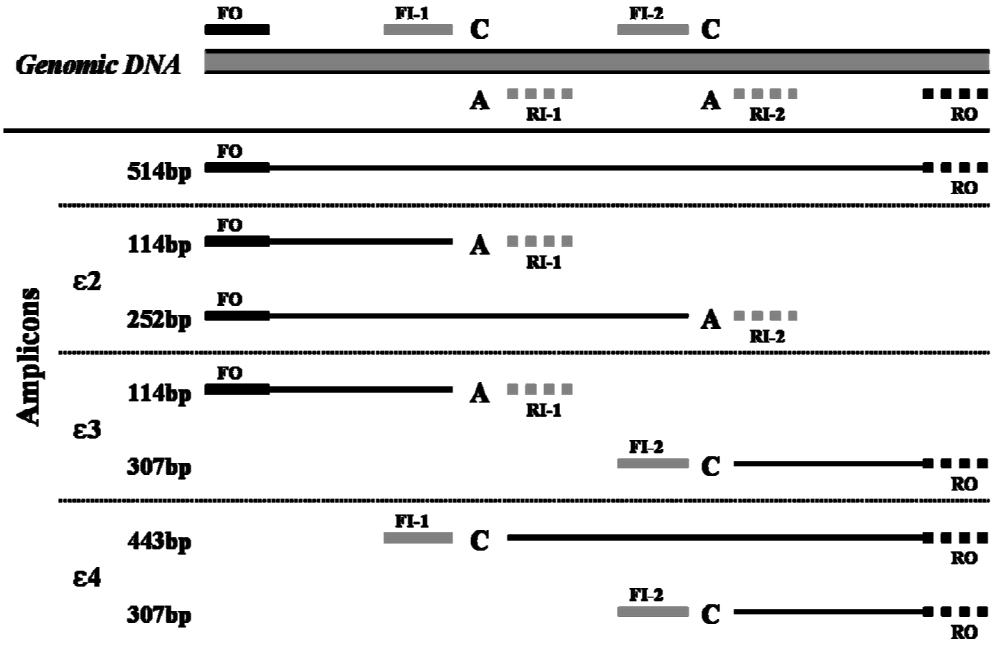

Figure 7. Diagram of multiplex T-ARMS PCR for apo E genotyping in single tube.

Outer primers (FO and RO) are amplified 514bp, and the combinations of each inner primers (FI-1, FI-2, RI-1, and RI-2) and outer primers in two SNP sites were produced specific amplicons.

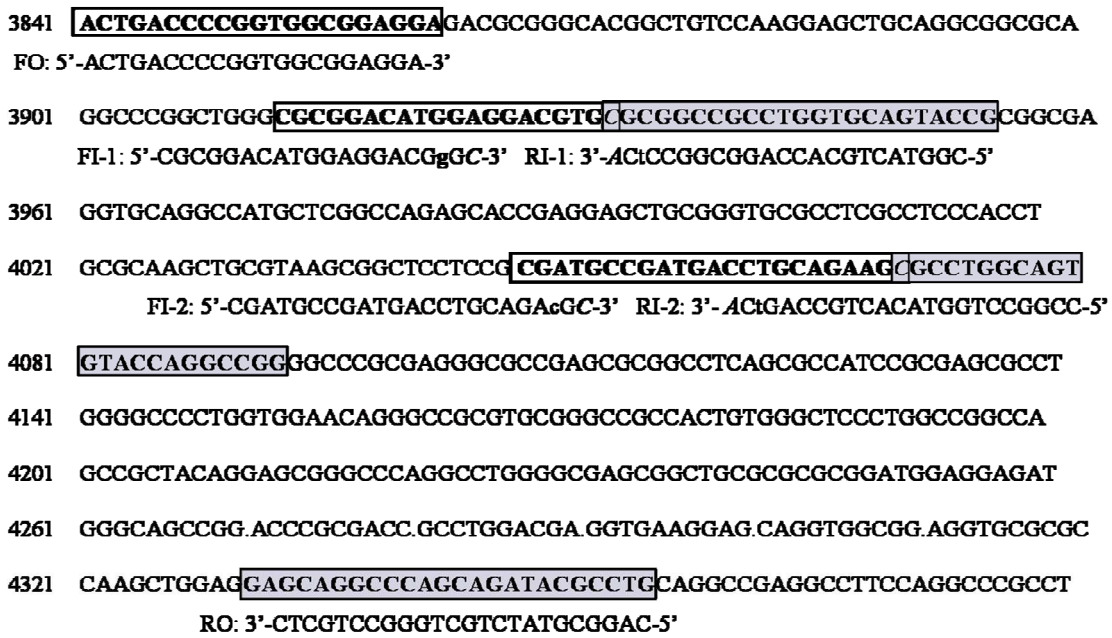

Figure 8. Apo E gene sequence (GenBank No. M10065) and multiplex T-ARMS PCR primer sequence of two SNP sites.

The boldface letters are annealing sequence with specific T-ARMS PCR primers at apo E gene (GenBank M10065), and forward and reverse primers are clear and grey boxes, respectively. The nucleotide at the mutation site is in italics. The six primers (two outer and four inner) are the same in Table 3.

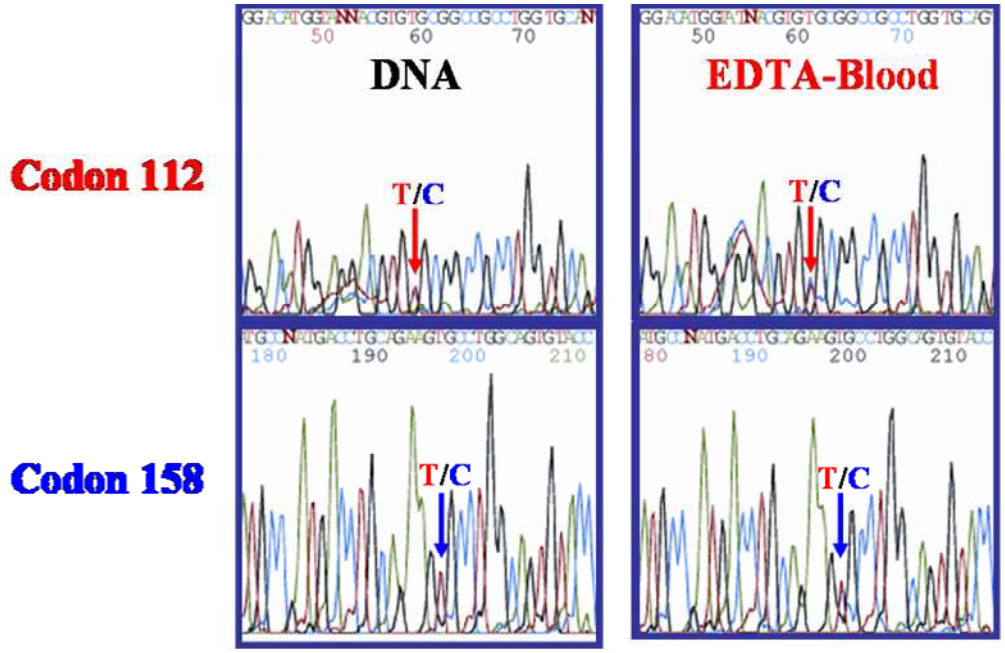

Figure 9. Direct sequencing from genomic DNA and EDTA-blood.

Apolipoprotein E, ε 2/ε 4, is identified with DNA and EDTA-blood by PCR and direct sequencing using BigDye Terminator v3.1 cycle sequencing reaction.

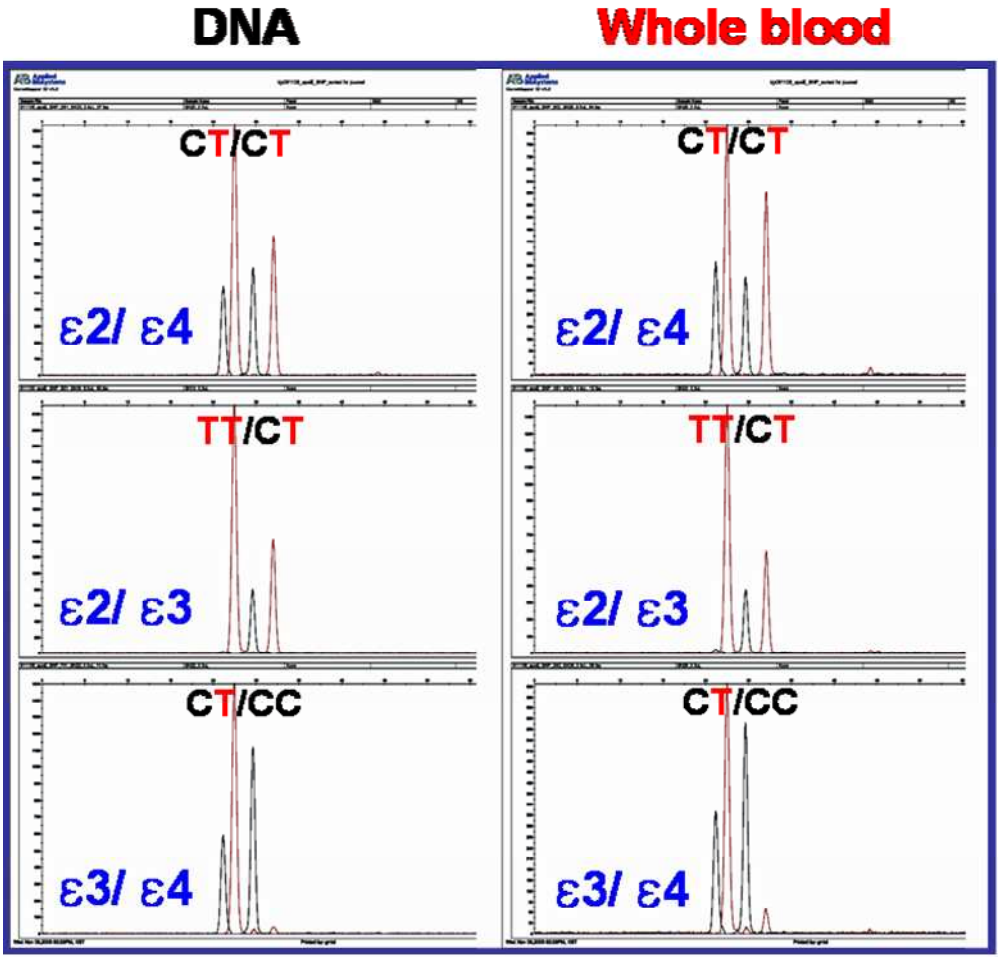

Figure 10. Apo E genotyping with minisequencing (SNaPshot) Assay from genomic DNA and EDTA-blood.

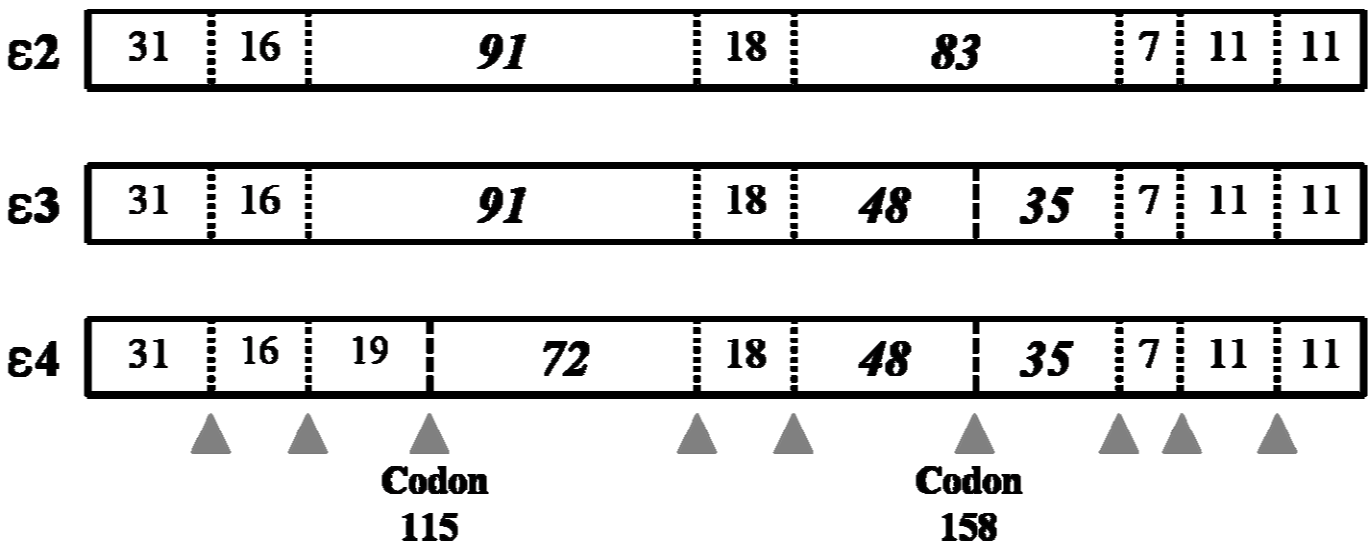

Figure 11. Diagram of restriction sites (▲) and fragment patterns of amplicon according to apo E genotypes.

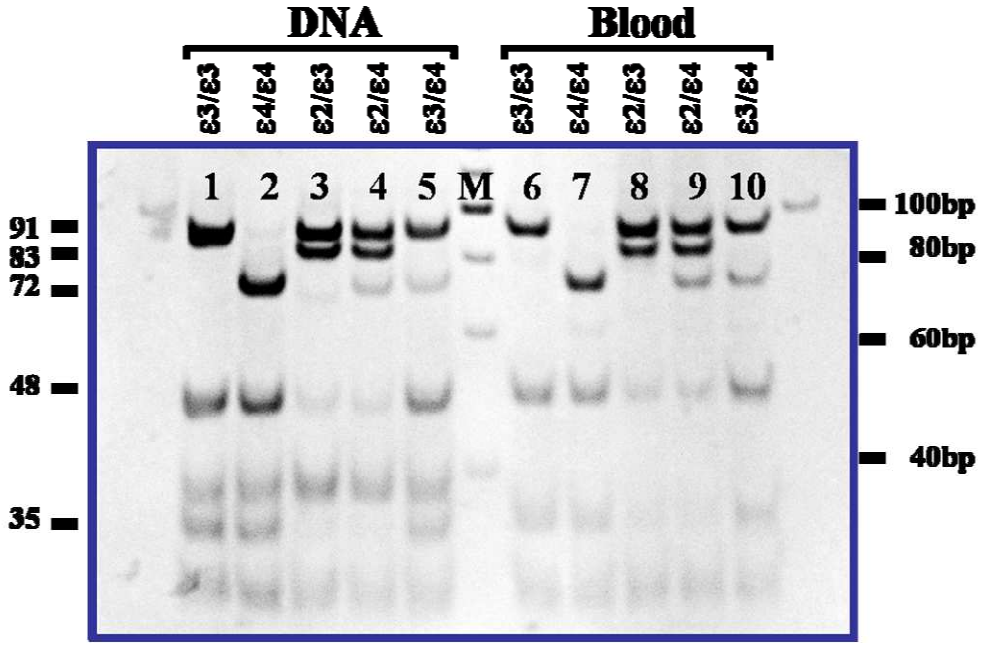

Figure 12. ApoE genotyping by PCR-RFLP from genomic DNA and EDTA-blood.

Electropherogram obtained from PCR-RFLP shows restriction fragments (91, 83, 72, 48, and 35 bp); apo E genotypes are determined by the patterns of these fragments [ε3/ε3 (91, 48, and 35 bp), ε4/ε4 (72, 48, and 35bp), ε2/ε3 (91, 83, 48, and 35 bp), ε2/ε4 (91, 83, 72, 48, and 35 bp), and ε3/ε4 (91, 72, 48, and 35 bp)]. The restriction fragments were identified with 12.5 % discontinues polyacrylamide gel electrophoresis and stained with ethidium bromide (0.5 mg/L). Lane M is Superladder-Low 20 bp ladder (ABgene, Epsom, UK).

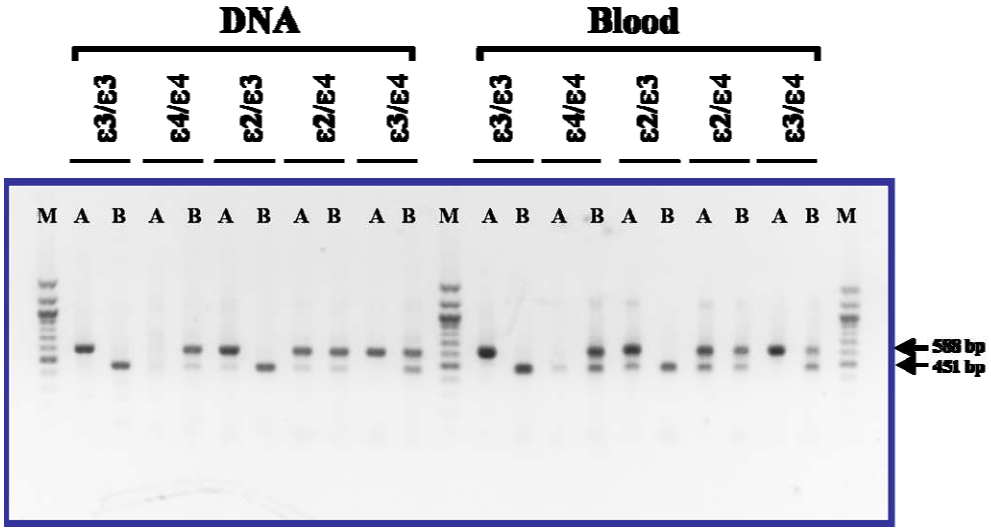

Figure 13. Apo E genotyping by AS-PCR from genomic DNA and EDTA-blood.

AS-PCR was performed with Cys primers (lane A), containing Cys112 (588 bp) and Cys158 (451 bp) primers, or Arg primers (lane B), containing Arg112 and Arg158 primers. Every apo E genotype was amplified with allele specific primers, including ε3/ε3 (Cys112 and Arg158), ε4/ε4 (Arg112 and Arg158), ε2/ε3 (Cys112, Cys158, and Arg158), ε2/ε4 (Cys112, Cys158, Arg112, and Arg158), ε3/ε4 (Cys112, Arg112, and Arg158). Amplicons were resolved by 2% agarose gel electrophoresis treated with ethidium bromide (0.5mg/L). Lane M is 100 bp ladder.

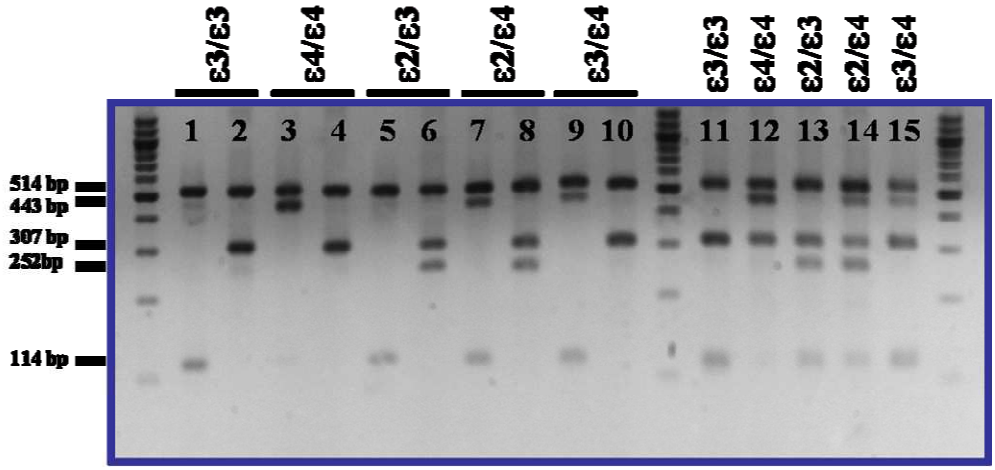

Figure 14. Electropherogram of T-ARMS and Multiplex T-ARMS PCR.

T-ARMS PCR was amplified with respective primers at separated codon 112 (lane 1, 3, 5, 7, 9) and codon 158 sites (lane 2, 4, 6, 8, 10), but multiplex T-ARMS PCR was amplified with all primers at two SNP sites together (lane 11, 12, 13, 14, 15).

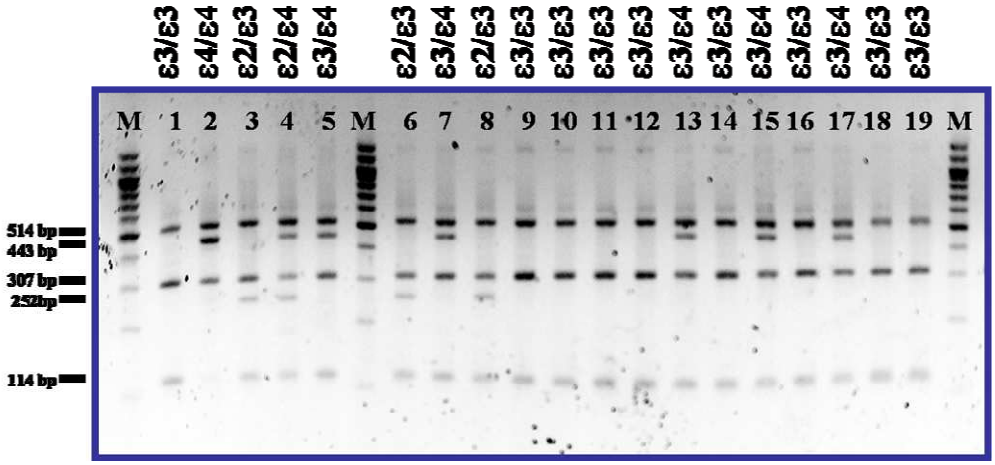

Figure 15. Apo E genotyping by multiplex T-ARMS PCR from 14 DNA samples.

Apo E genotype from previously (Figure 14) used DNA (lanes 1~5) and 14 additional DNA samples (lanes 6~19) was identified using our multiplex T-ARMS PCR as described above. Lane M: 100 bp ladder.

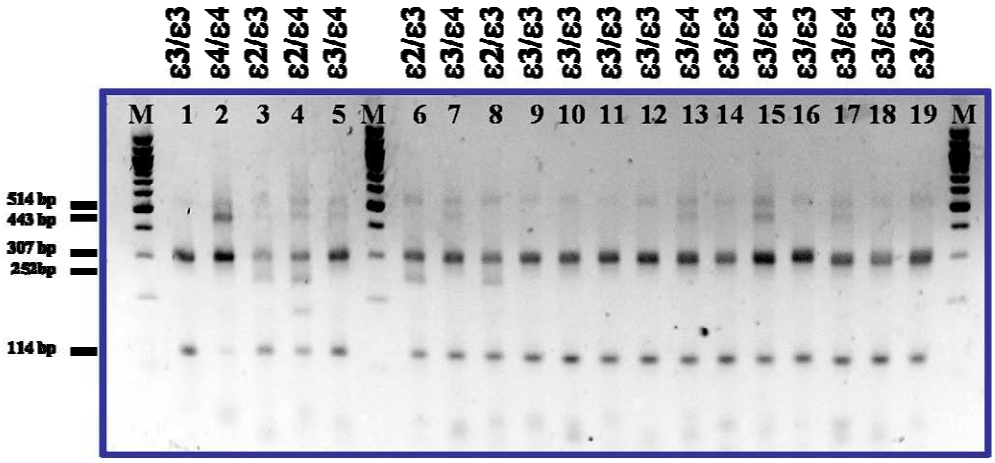

Figure 16. Apo E genotyping by multiplex T-ARMS PCR from 14 EDTA-blood samples.

Apo E genotype from 19 blood samples which are correlated with used DNA (Figure 15) was identified using AnyDirect solution and multiplex T-ARMS PCR as described above. Lane M is 100 bp ladder.

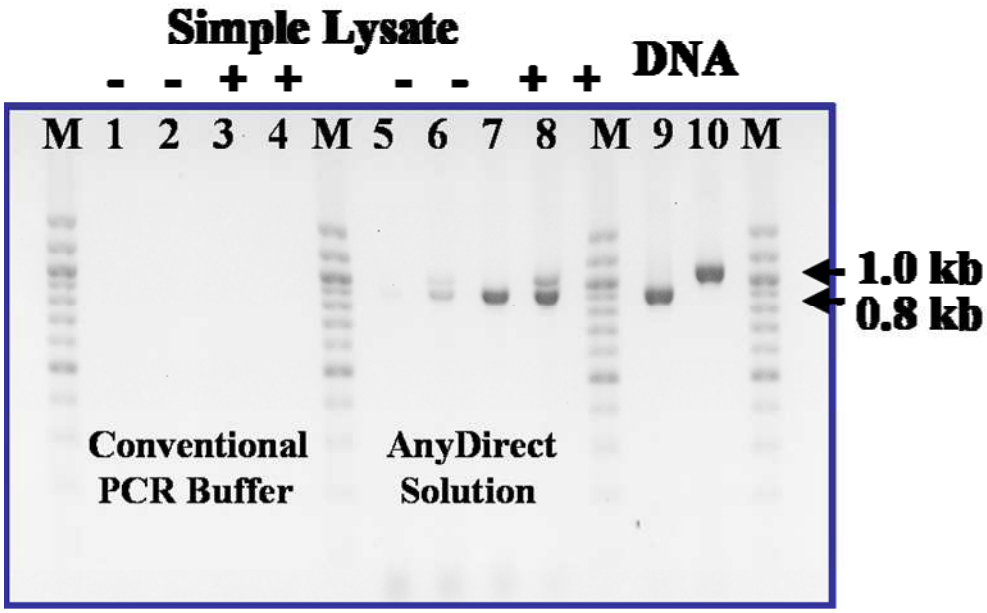

Figure 17. Electropherogram with Arabidopsis leaf lysate and purified genomic DNA.

Four leaves of Arabidopsis were mixed with 100 µL of 1X Direct-N-Lyse I solution, ground with a pestle (+) or not ground (-), followed by incubation at 80 °C for 2 hours. After heat treatment, these lysates were amplified with AnyDirect PCR buffer (lane 1~4). Amplification of genomic DNA from two ecotypes, Col (lane 5) and Ler (lane 6), was identified with amplicon sizes of 0.85 and 1.0 kb, respectively. Thus, one sample (lane 1 and 3) was a Col homozygote, and the other (lane 2 and 4) was a Col/Ler heterozygote. Lane M is 100 bp ladder.

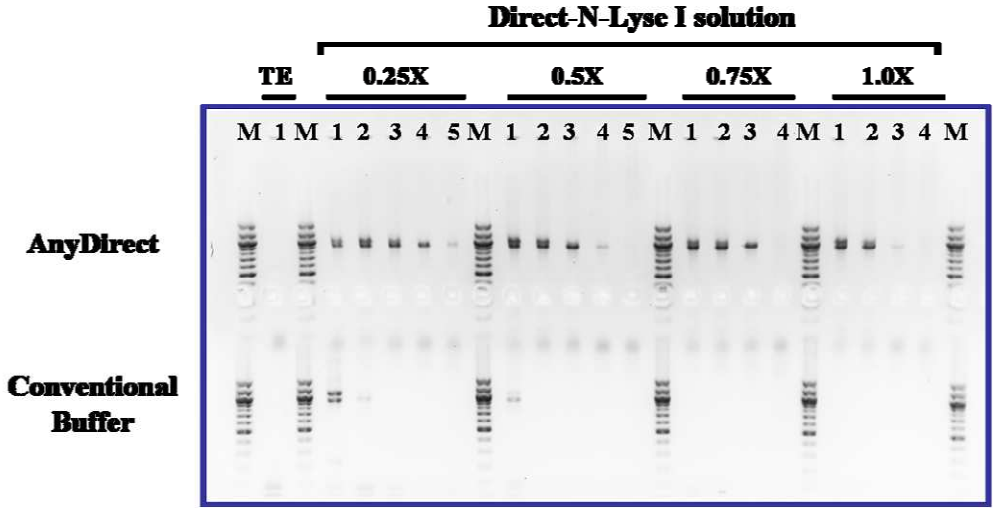

Figure 18. Electropherogram of PCR from simple lysates with diluted Direct-N-Lyse I solution.

Five leaves of Arabidopsis (Col/Ler heterozygote) in 100 μL of various dilutions of Direct-N-Lyse I solution (0.25X, 0.5X, 0.75X, and 1X) or 100 μL TE buffer were ground with a pestle and incubated at 80 °C for 2 hours. PCR was performed with AnyDirect PCR buffer (Upper) or with conventional PCR buffer (Lower) using various volumes of these lysates (1, 2, 3, 4, and 5 μL; Lane number is the volume of lysate used for PCR) in a 50 μL reaction. Other PCR conditions are the same as described above.

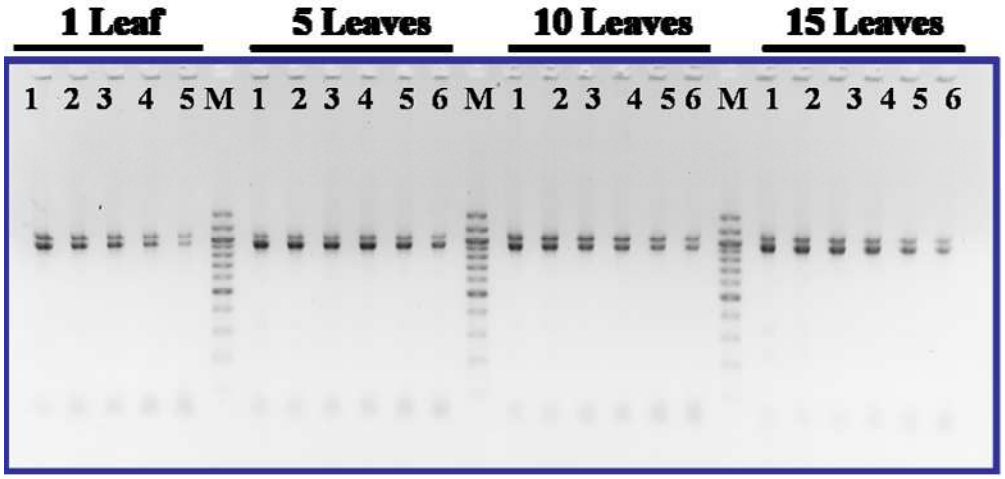

Figure 19. Electropherogram of PCR from simple lysates with amount of leaves used.

1, 5, 10 and 15 leaves were treated with 100 μL of 1X Direct-N-Lyse solution and incubated at 80 °C for 2 hours. And the lysates were diluted by two-fold serialization, 1, 1/2, 1/4, 1/8, 1/16, and 1/32 μL (lane 1, 2, 3, 4, 5, and 6), and were used as PCR templates in 50 μL PCR reaction mixtures. PCR was performed according to the above procedure, and lane M shows a 100 bp ladder.

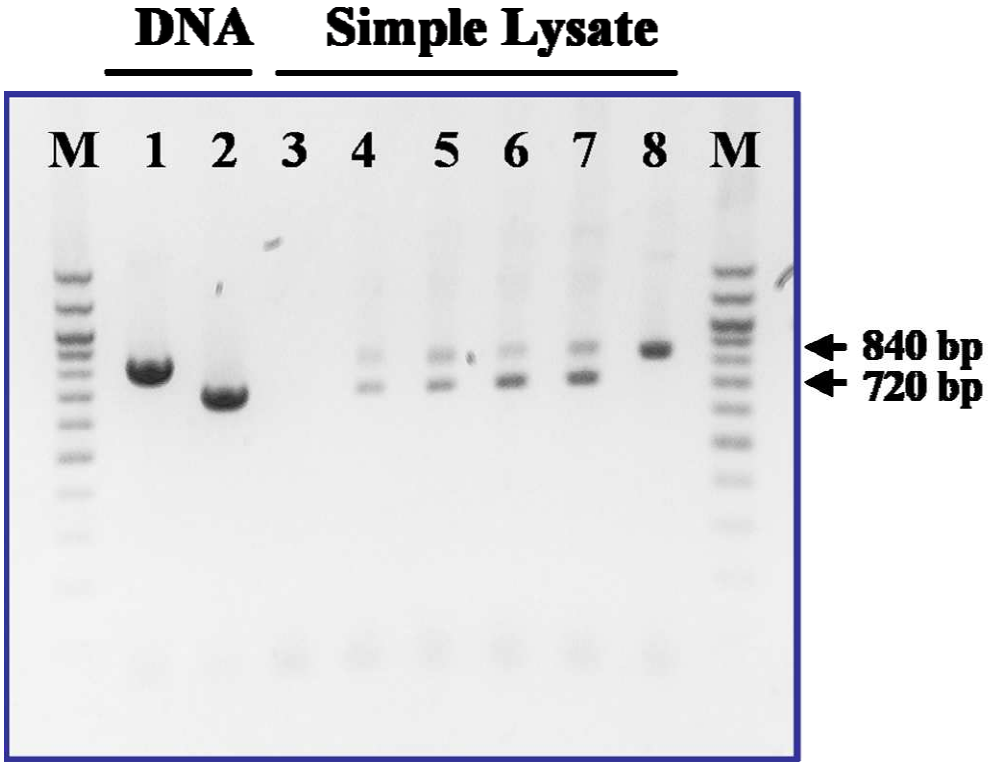

Figure 20. The MOD1 gene amplification from Arabidopsis lysates.

MOD1 (840 bp and 720 bp from Col and Ler ecotypes, respectively) gene was amplified from Arabidopsis lysate and genomic DNA. PCR was performed with DNA from Col and Ler ecotypes (lane 1 and 2), 1 μL Col/Ler heterozygote lysates in TE buffer or 0.25X, 0.5X, 0.75X, and 1X Direct-N-Lyse I solution (lane 3, 4, 5, 6, and 7), and 1 μL Col homozygote lysate in 1X Direct-N-Lyse I solution (lane 8). No amplicon was obtained from lysate in TE buffer, as seen in Figure 18. Lane M is a 100 bp ladder.

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국문요약

다양한 생체시료에서 직접 핵산 증폭에 대한 연구

<지도교수: 김 두 식>

연세대학교 대학원 생화학과

양 영 근

다양한 생체 시료에서 DNA를 증폭하는 기술인 중합효소연쇄반응(Polymerase chain reaction, PCR)은 분자 생물학과 분자 진단 등 다양한 매우 다양한 분야에서 이용되고 있는 핵산 증폭 기술이다. 그러나, PCR을 통해 유전자를 증폭하기 위해서 필요한 가장 중요한 부분은 원하는 주형 DNA를 순수하게 얻는 과정이다. 시료에서 DNA의 정제 과정 없이 바로 PCR을 진행 할 경우에는 이들 시료에 함유되어 있는 다양한 내부, 외부 성분들에 의한 PCR 활성 억제가 이루어지므로, 원하는 결과를 얻을 수가 없게 된다. 최근에 우리는 다양한 상황에서도 시료에서 DNA를 정제하지 않고 바로 유전자 증폭이 가능하도록 DNA 중합효소의 활성을 보호하는 용액 AnyDirect를 개발하였다.

여러 종류의 DNA 중합효소에서도 AnyDirect 용액을 사용할 경우 혈액에서 유전자 증폭이 가능하다. 또한, high GC를 갖는 유전자의 증폭에도 betaine 등과 같은 첨가제의 추가를 통해 유전자 증폭을 수행할

수 있다. 중요하게는 혈액 내에 적은 copy 수의 주형 DNA를 갖는 경우에도 효과적으로 증폭이 가능하다는 사실이다. 또한, AnyDirect를 이용한 직접 핵산 증폭 결과물이 다양한 추가 반응에 이용이 가능여부를 확인하기 위해 다양한 apolipoprotein E (apo E)의 genotyping 반응 [sequencing, SNaPshot assay, PCR- restriction fragment length polymorphism (RFLP) 와 allele specific (AS)-PCR 등]에 응용 하였다. 또한, 우리는 하나의 반응으로 apo E genotype을 확인할 수 있는 새로운 방법인 다중 반응 tetra-primer amplification refractory mutation system (T-ARMS) PCR법을 고안하였고, AnyDirect를 이용할 경우 혈액에서 바로 apo E genotyping이 가능하였다. 이 방법은 DNA 정제와 다른 처리과정 없이 바로 혈액을 이용하여 단 한번의 반응을 통해 apo E genotype을 확인할 수 있는 방법으로, 간편하고 빠르며, 또한 정확한 결과를 얻을 수 있는 방법이다.

식물체는 단단한 세포벽을 가지고 있으며, 또한 polyphenol 화합물들과 같은 다양한 내부 PCR 억제제들을 함유하고 있으므로, 이들에서 DNA를 정제 하는 과정은 매우 복잡하고 힘든 작업이다. 따라서, 우리는 간단한 lysis 방법의 고안을 통해 식물체에서 보다 간편하게 PCR 결과를 얻을 수 있는 방법을 설계하였다.

이와 같이 AnyDirect 용액을 이용한 다양한 실험 과정들은 여러 생체시료들을 이용하여 간단하고, 간편하며, 위험성이 적고, 경비가 절약되면, 빠른 유전자 증폭과정들로 DNA 정제 과정을 생략하므로, 유전질환이나 감염성질환을 확인하는데 매우 유용한 방법이다. 그리고

DNA 정제 과정의 생략은 다량의 시료를 손쉽게 처리할 수 있는 방법을 제공하고, lab-on-a-chip 기술등과 같이 bio-MEMS 기술과 연결될 경우 매우 유용한 기술로 발전할 수 있을 것으로 여겨진다.

핵심 단어: 애니다이렉트; 중합효소연쇄반응; 직접중합효소연쇄반응; 열안정성 중합효소; 혈액; 애기장대; 아포지단백질 E; 단일염기다형성; 시퀀싱; 다중 반응 T-ARMS PCR; AS-PCR; PCR-RFLP; SNaPshot;