

## Use of whole genome amplification to rescue DNA from plasma samples

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While DNA of good quality and sufficient amount can be obtained easily from whole blood, buccal swabs, surgical specimens, or cell lines, these DNA-rich sources are not always available. This is particularly the case in studies for which biological specimens were collected when genotyping assays were not widely available. In those studies, serum or plasma is often the only source of DNA. Newly developed whole genome amplification (WGA) methods, based on  $\varphi$ 29 polymerase, may play a significant role in recovering DNA in such instances. We tested a total of 528 plasma samples kept in storage at -40°C for approximately 10 years for 8 single nucleotide polymorphisms (SNPs) using the 5' exonuclease (*TaqMan*<sup>®</sup>) assay. These specimens yielded undetectable levels of DNA following extraction with an affinity column but produced an average 52.7 µg (standard deviation of 31.2 µg) of DNA when column-extracted DNA was used as a template for WGA. This increased the genotyping success rate from 54% to 93%. There were only 3 disagreements out of 364 paired genotyping results for pre- and post-WGA DNAs, indicating an error rate of 0.82%. These results are encouraging for expanding the use of poor DNA resources in genotyping studies.

### INTRODUCTION

Genotyping of single nucleotide polymorphisms (SNPs) has recently become a major activity in biomedical research (1) and promises to contribute significantly to our knowledge of disease susceptibility and response to pharmacological treatment.

While DNA of sufficient quantity and good quality, suitable for genotyping, can be obtained easily from whole blood, buccal swabs, surgical specimens, or cell lines, these DNA-rich sources are not always available. This is particularly the case in studies for which biological specimens were collected when genotyping was not widely available. Typical examples are epidemiologic studies on cardiovascular disease and cancer that were initiated in the 1950s–1980s (2–5). In those studies, serum or plasma, which are poor sources of DNA, are the only available ones, while buffy coat samples, which are more suitable for DNA extraction, have not been kept. In these situations, whole genome ampli-

fication (WGA) may play a significant role in recovering DNA.

Protocols for WGA have been in use for over 10 years. Typically, old WGA methods were based on *Taq* DNA polymerase and highly nonspecific PCR. These approaches included degenerate oligonucleotide-primed PCR (DOP-PCR) (6,7) and primer extension PCR (8). However, these methods are known to generate nonspecific amplification artifacts (7), provide incomplete coverage of the genome, with allelic imbalance, which may make genotyping impossible (9,10) and produce short products (<3 kb) that may be suitable for most genotyping techniques but preclude other applications, such as sequencing (6).

Recently, a new method for WGA has been developed. Multiple-displacement amplification (MDA) was originally developed to amplify large circular DNA templates such as plasmids and bacterial artificial chromosome (BAC) DNAs (11), but it has also been adapted for the amplification of large linear templates, such as human chromosomes. It uses a

highly processive DNA polymerase from virus  $\varphi$ 29 and random primers in an isothermal amplification reaction (10). This method is based on strand-displacement synthesis (12,13) and generates DNA products estimated to be >10 kb in length. In particular, it has been tested for a variety of genotyping techniques, such as SNP and microsatellite analysis (14–16), restriction fragment length polymorphism (RFLP), and comparative genome hybridization (CGH) (10,13).

While the previous studies employing MDA have been shown to substantially overcome the limitations of the older WGA methods, they have been performed on DNA obtained from high-quality sources, such as cell lines or whole blood samples. In this study, we tried to validate the usefulness of MDA in rescuing DNA samples obtained from a series of plasma samples collected over 10 years ago. We set up a protocol to obtain minute amounts of genomic DNA from the plasma samples and then amplified this DNA using MDA. We estimated the performance of our protocol by comparing the results of SNP genotyping on the same DNA samples before and after WGA.

### MATERIALS AND METHODS

#### Biological Samples

Plasma samples used for this study were part of a chemoprevention trial performed in the State of Tachira, Venezuela (17). Samples were collected on heparin between July 1991 and February 1995. After fractioning, plasma samples were shipped to the International Agency for Research on Cancer (Lyon, France) and kept frozen at -40°C until use. Five hundred twenty-eight samples, of the 2200 in storage, were used for the present study. The local Institutional Review Board cleared the use of biological samples.

#### DNA Extraction

The initial step of DNA extraction was based on the use of an affinity column suited for processing various body fluids, including serum and

**Table 1. Probes and Primers Used for SNP Genotyping**

Gene	SNP <sup>a</sup>	PCR Primers	TaqMan Probes <sup>b</sup>	Chemistry
<i>IL1B</i>	rs1143627	5'-CCCCCTTCCTTAACTGATTGTG-3' 5'-AGGTTTGGTATCTGCCAGTTCTC-3'	5'-VIC-TTTGAAAGCTATAAAAACAG-3' 5'-FAM-TTTGAAAGCCATAAAA-3'	MGB
<i>IL12A</i>	rs2243151	5'-TTATTGCATGGTTAGTTTTCACTTTT-3' 5'-GGAGCATTGAATGATTGGTT-3'	5'-VIC-AGCATACTACACTACTTG-3' 5'-FAM-AGCATACTACACAACCT-3'	MGB
<i>IL12A</i>	rs2133310	5'-GGGACCTAATTAACTGTGTTATTGTGAA-3' 5'-AGCGGGTGTCTGTGTTCTG-3'	5'-VIC-AGTCCAACACCTAG-3' 5'-FAM-TGCCAGCACCTAGT-3'	MGB
<i>GH1</i>	rs2665802	5'-ACTTTGAGAGCTGTGTTAGAGAAC-3' 5'-TTCACGAGGGAAATGAAGAATAAG-3'	5'-HEX-TAG+CAG+ACC+AGG+CCCTG-3' 5'-FAM-TAG+CAG+TCC+AGG+CCCTG-3'	LNA
<i>GH1</i>	rs2005172	5'-CATTAGCACAAAGCCCCGTAGT-3' 5'-CCTTTTATACCTGGCCCCCTTC-3'	5'-HEX-TCCCCTGTGTTGACCCCACCTGTTT-3' 5'-FAM-TCCCCTGTGTTGCCCCCACCTGTTT-3'	Unmodified DNA
<i>IL4RA</i>	rs1801275	5'-CCTGCTCCACCGCATGTA-3' 5'-GAAATGTCCCTCCAGCATGGG-3'	5'-VIC-TGGCTATCAGGAGTTT-3' 5'-FAM-TGGCTATCAGGAGTT-3'	MGB
<i>CCL2</i>	rs1024611	5'-GGGAGGGCATCTTCTTGAC-3' 5'-GGTGAAGGGTATGAATCAGAAAAGA-3'	5'-VIC-ACAGCTATCACTTC-3' 5'-FAM-AGACAGCTGTCACTT-3'	MGB
<i>AR</i>	rs1337082	5'-CCTGGGCCATAGATTCATTGATG-3' 5'-CTGCTACTTACCAAGCTTGATCT-3'	5'-VIC-CCAAGGTTAGAAAGGTTA-3' 5'-FAM-CAAGGTTAGGAAGGTTA-3'	MGB

SNP, single nucleotide polymorphism; LNA, locked nucleic acid; MGB, minor groove binder.  
<sup>a</sup>Polymorphisms are identified by their dbSNP accession number. dbSNP can be accessed at [www.ncbi.nlm.nih.gov/SNP](http://www.ncbi.nlm.nih.gov/SNP).  
<sup>b</sup>The fluorescent dye attached to each probe is indicated. For LNA probes, a plus symbol (+) shows chemically modified bases.

plasma (QIAamp® DNA Blood Mini Kit; Qiagen, Hilden, Germany), which has previously been used successfully to purify minute amounts of DNA from plasma (18,19). Plasma samples (200 µL) were mixed with 200 µL lysis buffer from the kit and incubated at 56°C for 10 min. Two hundred microliters of ethanol were then added, and the mixture was applied to the spin column. After centrifugation at 6500×g rpm for 1 min, the column was washed twice with 500 µL washing buffer from the kit and centrifuged at 6500×g for 1 min. Finally, the DNA was eluted with 100 µL pure water and centrifugation at 6500×g for 1 min. Material eluted from each spin column was dried by heating at 65°C and resuspended in 10 µL TE (Tris-EDTA) buffer.

### Whole Genome Amplification

WGA was performed according to an MDA protocol based on φ29 polymerase (REPLI-g®; Qiagen). An aliquot of 2.5 µL from each sample was used for the WGA reaction and mixed with 2.5 µL freshly prepared denaturation solution (0.05 M KOH, 0.005 M EDTA). After incubation at room temperature for 3 min, 5 µL freshly diluted neutralization buffer (as provided by the manufacturer) were added. φ29 DNA polymerase was

then added, as per the manufacturer's protocol, and the volume was brought to 40 µL with pure water. The WGA reactions were incubated at 30°C in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) for 16 h, and the enzyme was inactivated by heating at 65°C for 3 min.

DNA was then quantified with the PicoGreen® dsDNA Quantification Reagent Kit (Molecular Probes, Leiden, The Netherlands). PicoGreen specifically binds to double-stranded DNA (dsDNA) and, after excitation at 485 nm, the complex dsDNA/PicoGreen fluorescence is detected at 538 nm. A calibration curve ranging between 500 and 4000 ng/µL using DNA at a known concentration provided in the kit was included in each experiment. One microliter of WGA-DNA was diluted 1/5000 in a 200-µL reaction mixture containing PicoGreen (1:400). After 5 min of incubation, the reaction was excited, and the fluorescence was recorded with a GENios™ fluorimeter (Tecan, Mannedorf, Switzerland). WGA DNA concentrations were estimated using the calibration curve.

### Genotyping

DNA samples obtained before and after WGA were genotyped by the 5' exonuclease (TaqMan®) assay (20) on

8 SNPs located in 6 different genes, mapping to 5 different chromosomes. The primers and probes used for TaqMan genotyping are listed in Table 1. Primers and probes were manufactured by either Applied Biosystems or Proligo (Boulder, CO, USA). Each SNP was tested on a number of samples ranging from 88 to 182. The reaction mixture included 1 µL from the DNA aliquot for the pre-WGA samples or 10 ng post-WGA DNA, 5 pmol of each primer, 1 pmol of each probe, and 4 µL 2x TaqMan Universal PCR Master Mix (Applied Biosystems) in a final volume of 8 µL. The thermal cycling was performed in 96-well plates and included 40 cycles of 30 s at 95°C followed by 60 s at 60°C. PCR plates were read on an ABI PRISM® 7900HT instrument (Applied Biosystems). All readings were performed independently by two operators, and only concordant calls were kept. All samples that did not give a reliable result in the first round of genotyping were resubmitted to up to two additional rounds of genotyping. Data points that were still not filled after this procedure were left blank.

### RESULTS AND DISCUSSION

We set up a method that uses WGA to obtain large amounts of genomic

**Table 2. Comparison of Genotyping Results from Pre- and Post-WGA DNA**

Gene	SNP	Pre-WGA		Post-WGA		Sample Pairs Available for Comparison	Number of Discrepancies
		Samples Tested	Genotypes Successfully Scored	Samples Tested	Genotypes Successfully Scored		
<i>IL1B</i>	rs1143627	182	86	182	163	81	1
<i>IL12A</i>	rs2243151	88	85	88	85	82	0
<i>IL12A</i>	rs2133310	88	35	88	83	33	0
<i>GH1</i>	rs2665802	88	28	88	82	27	1
<i>GH1</i>	rs2005172	88	9	88	85	8	0
<i>IL4RA</i>	rs1801275	88	41	88	85	41	0
<i>CCL2</i>	rs1024611	94	77	94	82	68	0
<i>AR</i>	rs1337082	94	79	94	91	76	1
<b>Total</b>		<b>528<sup>a</sup></b>	<b>440</b>	<b>528<sup>a</sup></b>	<b>756</b>	<b>364</b>	<b>3</b>

WGA, whole genome amplification; SNP, single nucleotide polymorphism.

<sup>a</sup>The total does not correspond to the sum of the above cells because several samples have been tested for more than one SNP.

DNA, suitable for SNP genotyping, from minute amounts of DNA extracted from archival plasma samples.

DNA can be extracted from serum or plasma, but only in very small amounts. Serum and plasma are considered to be equivalent sources of DNA. While we have worked on plasma samples, we are confident that our results can be applied to DNA extracted from serum as well. The quality of serum/plasma-extracted DNA can often be low as well because this DNA is probably freely floating in the blood and therefore more susceptible to degradation. In previous investigations, serum samples from healthy individuals yielded an average of 13 ng DNA/mL of starting material (whole blood), ranging from 0 to 100 ng/mL (19), which is much lower than what can be extracted from whole blood or buffy coat.

When we attempted to measure DNA concentration after extraction with an affinity column, it was undetectable in most samples. We performed WGA on all DNAs extracted from plasma and obtained high yields, as expected (average, 52.7 µg; standard deviation, 31.2 µg).

Given the low amount (and possibly low quality) of pre-WGA DNAs, we wanted to estimate the faithfulness of WGA. We speculated that partially degraded DNA might provide poor templates of WGA and lead to the generation of artifacts. We therefore genotyped eight SNPs located in six different genes on pre- and post-WGA

DNAs and compared the results. We tested genotyping assays routinely used in our laboratory, representing three slightly different chemistries; that is, unmodified DNA (i.e., allele-specific probes were synthesized without further chemical modification other than standard labeling with fluorescent dyes and quencher), minor groove binder (MGB) (21), and locked nucleic acid (LNA) (22). MGB and LNA are very different chemistries, but they serve the same purpose of stabilizing perfectly matched duplexes with DNA targets, thus allowing the design of shorter probes with higher specificity in allelic discrimination. The assay for SNP rs2005172 was synthesized with unmodified probes, the assay for SNP rs2665802 was synthesized with LNA chemistry, and the other assays were synthesized with MGB chemistry.

Results of genotyping are listed in Table 2, and representative examples of TaqMan graphs are presented in Figure 1. We obtained interpretable results for 440 of 810 (54%) genotyping reactions performed on the pre-WGA samples. In practice, pre-WGA samples in this study were unsuitable for genotyping. Samples had been collected over 10 years ago, and it would not have been feasible to contact study participants to obtain new blood samples. On the other hand, we obtained 756 of 810 (93%) genotyping reactions performed on the post-WGA samples.

Figure 1 shows that genotyping performed on pre-WGA samples

resulted in graphs that were difficult to interpret, with weaker signals and much more dispersed clusters of points, which led to very high no-call rates. For SNPs rs2005172 and rs2665802, it was not even possible to distinguish the heterozygotes from one of the homozygotes group in the pre-WGA genotyping results, although the three groups were clearly identifiable in the genotyping results obtained from the same subjects after WGA (Figure 1, A and B). These two assays were also the only ones not synthesized with MGB chemistry, and they correspond to SNPs with rather high GC content in the surrounding sequence.

Three hundred sixty-four genotypes could be confidently scored both on the pre- and post-WGA samples. We observed three discrepancies between the two series of genotypes. These discrepancies are probably artifacts generated by WGA. Based on our results, we estimated the genotyping error rate due to WGA at 0.82% on samples obtained from plasma. The discrepancies consisted of two heterozygotes in pre-WGA scored as homozygotes in post-WGA [one each for SNPs rs1143627 (visible in Figure 1C) and rs1337082] and a homozygote for the common allele in pre-WGA scored as heterozygote (SNP rs2665802). Apparent allelic loss, which we observed in two cases out of three, is the artifact most frequently expected of WGA (15) and is derived

from differential amplification of one allele over the other.

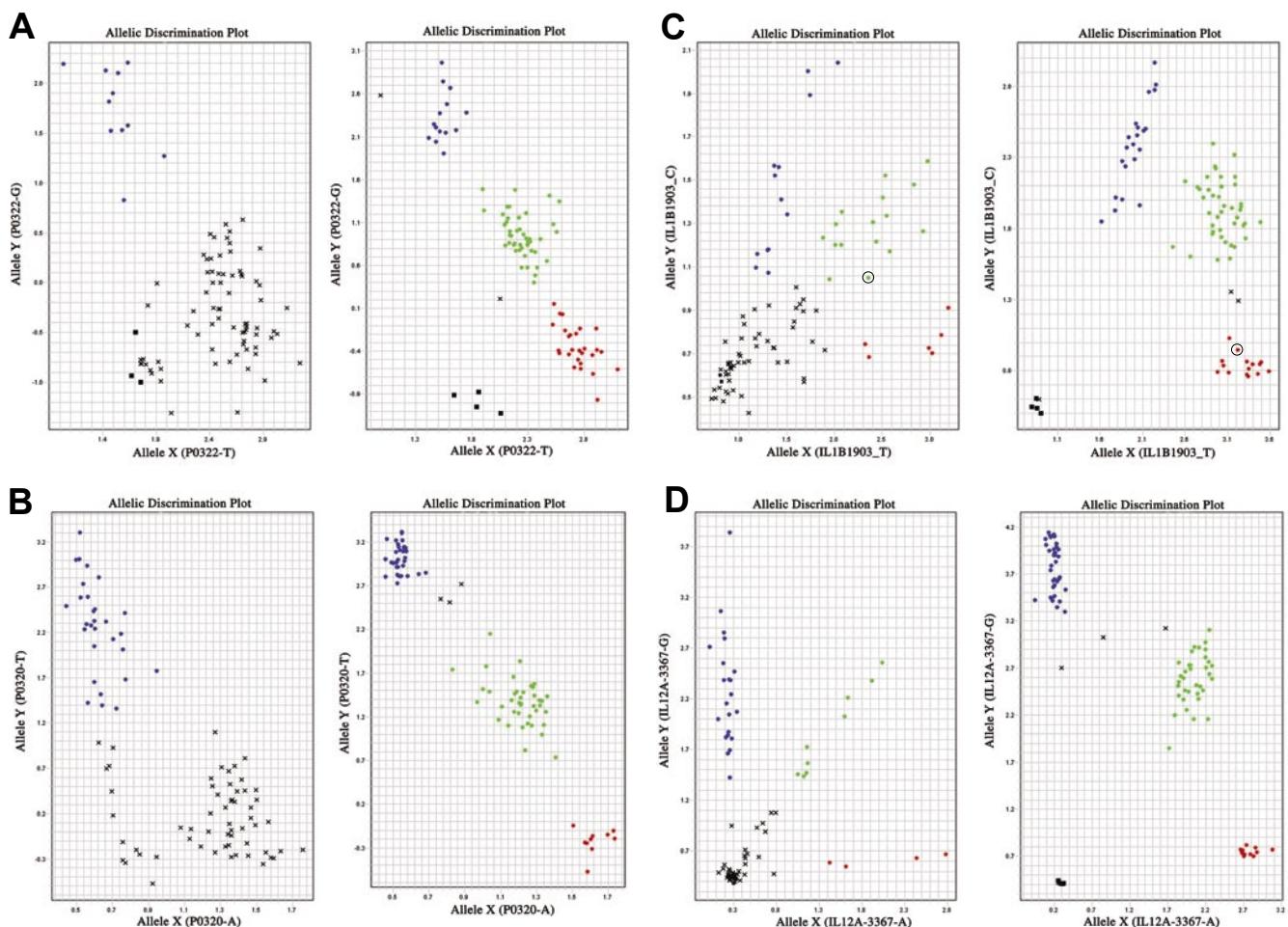
A previous large-scale study found that the discrepancy rate between genotypes obtained on pre- and post-WGA was 0.14% (16). Samples used in that study were obtained from cell lines. We showed that performing WGA on DNA samples extracted from plasma results in a comparable error rate.

A recent study (23), focused on the comparison of DNA from plasma and formalin-fixed paraffin-embedded hepatic tissue, included an exper-

iment whereby three plasma DNA samples were subjected to WGA, and genotyping on pre- and post-WGA samples was compared. The WGA protocol proposed in that study was similar to ours, although the column used for DNA extraction was different. Both studies suggest that DNA in archival plasma is structurally intact, even when stored under suboptimal conditions for several years.

It can be speculated that the error rate we observed was underestimated because it might be based on a subset of plasma samples with DNA of sufficient

quantity and quality to be genotyped prior to WGA. If this were the case, many of the genotypes obtained from post-WGA samples where pre-WGA genotyping failed could just be artifacts. On the other hand, the distribution of genotypes obtained on post-WGA DNAs was in Hardy-Weinberg equilibrium for all SNPs (data not shown). This would not be expected if a large proportion of genotypes were the result of artifacts introduced by WGA. In addition, genotypes could be successfully read for at least one SNP in nearly all of the pre-WGA samples.



**Figure 1. Results from different TaqMan SNP genotyping assays.** In each panel, the left-hand graph was obtained using pre-WGA DNAs, and the right-hand graph was obtained using the same DNAs after WGA. Blue dots represent samples that were called allele 1 homozygotes, green dots are heterozygotes, red dots are homozygotes for allele 2, black squares are no DNA template controls, and black crosses are low or no amplification samples. (A) Genotyping results of SNP rs2005172. Allele-specific probes for this assay were synthesized without further chemical modification other than standard labeling with fluorescent dyes and quencher. In the graph of pre-WGA genotypes, only the homozygotes for allele 1 could be called. Clusters of heterozygotes and homozygotes for allele 2 could not be distinguished. (B) Genotyping results of SNP rs2665802. Allele-specific probes for this assay were labeled with standard fluorescent dyes and quencher, and several bases were modified with LNA chemistry. In the graph of pre-WGA genotypes, only the homozygotes for allele 1 could be called. Clusters of heterozygotes and homozygotes for allele 2 could not be distinguished. (C) Genotyping results of SNP rs1143627. Allele-specific probes for this assay were labeled with standard fluorescent dyes and quencher and modified with MGB chemistry. The circled sample was read as heterozygote in the pre-WGA plate and homozygote for allele 2 in the post-WGA plate. (D) Genotyping results of SNP rs2133310. Allele-specific probes for this assay were labeled with standard fluorescent dyes and quencher and modified with MGB chemistry. SNP, single nucleotide polymorphism; WGA, whole genome amplification; LNA, locked nucleic acid; MGB, minor groove binder.

We therefore concluded that the high failure rate of genotyping in pre-WGA DNAs extracted with the affinity column was essentially due to very low yield of DNA, but not to low quality.

Although the direct application of WGA to plasma/serum specimens is a possible choice, it would be prohibitively expensive due to the large amount of enzyme required. Our protocol, which is based on DNA extraction with the use of an affinity column prior to WGA, yields DNAs of good quality and quantity, with only a modest increase in labor. This offers great opportunities to expand the use of previously collected samples for genotyping analyses.

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## COMPETING INTERESTS STATEMENT

*The authors declare no competing interests.*

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