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Whole genome amplification of buccal cytobrush DNA collected for molecular epidemiology studies

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Abstract

When cytobrush buccal cell samples have been collected as a genomic DNA (gDNA) source for an epidemiological study, whole genome amplification (WGA) can be critical to maintain sufficient DNA for genotyping. We evaluated REPLI-g™ WGA using gDNA from two paired cytobrushes (cytobush 'A' kept in a cell lysis buffer, and 'B' dried and kept at room temperature for 3 days, and frozen until DNA extraction) in a pilot study ($n=21$), and from 144 samples collected by mail in a breast cancer study. WGA success was assessed as the per cent completion/concordance of STR/SNP genotypes. Locus amplification bias was assessed using quantitative PCR of 23 human loci. The pilot study showed >98% completion but low genotype concordance between cytobrush wgaDNA and paired blood gDNA (82% and 84% for cytobrushes A and B, respectively). Substantial amplification bias was observed with significantly lower human gDNA amplification from cytobrush B than A. Using cytobrush gDNA samples from the breast cancer study ($n=20$), an independent laboratory demonstrated that increasing template gDNA to the REPLI-g reaction improved genotype performance for 49 SNPs; however, average completion and concordance remained below 90%. To reduce genotype misclassification when cytobrush wgaDNA is used, inclusion of paired gDNA/wgaDNA and/or duplicate wgaDNA samples is critical to monitor data quality.

Keywords: *Whole genome amplification, multiple displacement amplification, genetic susceptibility, molecular epidemiology, genotyping, TaqMan® assay*

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Introduction

Many epidemiological studies use buccal cells obtained from mouthwash or buccal cytobrushes as a genomic DNA (gDNA) source; however human gDNA yields and quality are lower than that provided by blood cells and yields vary widely between subjects (Garcia-Closas et al. 2001). To overcome these limitations, whole genome amplification (WGA) can be used to generate amplified DNA (wgaDNA) and maintain a usable DNA supply (Hughes et al. 2005). Recently, multiple displacement amplification (MDA) WGA (Dean et al. 2002) has become popular because the reaction is scalable, can amplify gDNA directly from cell lysates (potentially eliminating DNA extraction), and it performs better than other methods such as degenerate oligonucleotide-primed (DOP) and primer preamplification (PEP) PCR (Dean et al. 2002).

The authors, and others, have shown that MDA wgaDNA from mouthwash gDNA samples is adequate for genotype analysis (Dean et al. 2002, Hosono et al. 2003, Bergen et al. 2005); however, it is unclear whether MDA can be successfully used with gDNA from cytobrush-derived buccal cell samples because the overall yields and quality can be lower than mouthwash samples (Garcia-Closas et al. 2001). In this study, we selected the MDA-REPLI-g™ method because in our previous studies of MDA wgaDNA derived from mouthwash, buffy coat, and lymphocyte samples, REPLI-g resulted in higher yields of wgaDNA, and greater genotype completion and concordance than the two other methods evaluated (Bergen et al. 2005). In the same laboratory we also examined the performance of REPLI-g using 40 cytobrush samples that were self-collected in the field and 84 cell lysates (40 matched to cytobrush samples, 44 unmatched) to determine whether the DNA extraction step could be eliminated prior to genotyping. After unblinding and analysing the results from projects 1 and 2, we conducted additional experiments (project 3) in an independent laboratory to assess whether the genotyping completion and concordance for MDA-REPLI-g DNA could be improved by increasing the amount of template gDNA in the WGA reaction.

Materials and methods

Study populations, specimen collection

Samples were collected from two study populations (Table I). Three projects were conducted in two laboratories. In a Division of Cancer Epidemiology and Genetics (DCEG) pilot study (project 1), volunteers (15 female, 6 male) from the National Cancer Institute (NCI), aged 42–65 years, were recruited to obtain paired blood and buccal cytobrush samples. Buffy coat samples were isolated from blood samples and stored frozen at −70/80°C until DNA extraction as described (Bergen et al. 2005). Two cytobrush samples were obtained from each volunteer using a standard NCI protocol (Garcia-Closas et al. 2001) by brushing the right and left inner cheeks ten times (one brush per side). One cytobrush was immediately dipped in a 1.5 ml tube containing 300 µl of Gentra® cell lysis solution and kept at room temperature until DNA extraction (cytobrush A). The other cytobrush was first dried and kept at room temperature for 3 days (imitating transport time in a study using self-collection by mail) and stored frozen at −70/80°C until DNA extraction. DNA was isolated from the buffy coat and cytobrush B using phenol-chloroform extraction as previously

Table I. Study populations and study overview.

Project and study population	Analysis laboratory	Source of gDNA	gDNA template (ng)	DNA quantification method	Amplification bias	STR genotyping	SNP genotyping
Project 1 –NCI Pilot (21 subjects)	Molecular Staging	Buffy coat Cytobrush A[a] Cytobrush B[b]	10	PicoGreen® pre- and post-WGA	Q-PCR assays at 23 loci	9 STRs sex determination	None
Project 2–US Breast Cancer Study (84 subjects)	Molecular Staging	Cytobrush[c] ($n = 40$)	2	PicoGreen pre- and post-WGA			Q-PCR assays at 3 loci
		Cytobrush cell lysates ($n = 84$; 40 from the same subjects as cytobrush DNA and 44 unmatched subject samples)	Not determined[d]	PicoGreen post-WGA			
Project 3–US Breast Cancer Study (20 subjects)	NCI Core Genotyping Facility	Cytobrush[c]	10, 25, 50, 100	PicoGreen, Q-PCR			49 TaqMan SNPs

[a]Cytobrushes were placed in cell lysis buffer immediately after collection and kept at room temperature until DNA isolation using a Gentra DNA extraction kit.
[b]Cytobrushes were kept at room temperature for 3 days and subsequently frozen until DNA isolation using an organic (phenol-chloroform) extraction.
[c]Cytobrushes were self-collected by study participants and mailed at room temperature to processing laboratory where they were frozen until DNA isolation using an organic (phenol-chloroform) extraction.
[d]Cell lysate was not quantified prior to WGA.

described (Bergen et al. 2005). DNA from cytobrush A was extracted using a Gentra Puregene DNA Purification kit (Gentra, Plymouth, MN, USA).

We also used cytobrush specimens collected from patients enrolled in a US breast cancer case–control study (projects 2 and 3) conducted in 1998–2001 among residents of Wisconsin, western Massachusetts, and New Hampshire, aged 20–74 years. Women were mailed a sample collection kit containing two sterile cytobrushes in sealed plastic tubes (Cyto-Pack Cytosoft Brush, Medical Packing Corp., Camarillo, CA, USA), instructions for collection, and a pre-paid return envelope. Women first brushed their teeth. After 10–15 min they brushed the inside of their right and left cheeks each for at least 30 s using one cytobrush per cheek. Then cytobrushes were returned to the plastic tubes and mailed directly to the extraction laboratory. Upon arrival, the brush was separated from the handle, placed in a cryovial and frozen at −70/80°C until DNA isolation. Extraction was performed using phenol-chloroform extraction as previously described (Garcia-Closas et al. 2001). The NCI IRB approved both studies and all participants provided written informed consent.

Molecular assays

A commercial laboratory (Molecular Staging) performed assays for projects 1 and 2, and the NCI Core Genotyping Facility (CGF) performed assays for project 3 (Table I). All samples were coded and analysed blindly in both laboratories.

For project 1, a standard protocol for MDA-REPLI-g (April 2003 protocol) was performed using gDNA samples from the pilot study. MDA-REPLI-g was scaled to a 500 μl volume as described previously to maximise wgaDNA yields (Dean et al. 2002, Hosono et al. 2003). Double-stranded DNA mass was estimated before and after WGA using the PicoGreen® assay (Molecular Probes, Eugene, OR, USA) (Table I). Genome coverage and variation in specific loci amplification (amplification bias) was assessed using quantitative polymerase chain reaction (Q-PCR) for 23 human loci, one locus per chromosome as described previously (Dean et al. 2002, Hosono et al. 2003). An algorithm based on Q-PCR assays at two of these loci (WIAF 1004 and 699) was used to estimate the percentage human DNA and predict the suitability of wgaDNA for genotyping (Yan et al. 2004). Nine single tandem repeat (STR) loci and the AMEL locus were genotyped using the AmpF*l*STR® Profiler Plus ID PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA).

In project 2, Q-PCR genotyping of three loci (WIAF 525, WIAF 1004, WIAF699) was performed on MDA-REPLI-g WGA from 40 cytobrush DNA samples and 84 cell lysates (40 cytobrush paired and 44 unpaired) from the US breast cancer study. Scatter plots of the fluorescent signals were evaluated for allele scatter as previously described (Alsmadi et al. 2003).

In project 3, performed at the NCI-CGF, gDNA from cytobrushes from 20 aditional women enrolled in the US breast cancer study was quantified using PicoGreen and Q-PCR as previously described (Haque et al. 2003, Bergen et al. 2005). Increasing amounts of cytobrush gDNA (10, 25, 50 and 100 ng, determined using Q-PCR) were used as template in a 100 μl volume MDA-REPLI-g reaction (July 2003 protocol). WgaDNA was quantified, the mass of single-stranded (ss) DNA and double-stranded (ds) DNA in each wgaDNA sample was estimated and differences in yield distributions were evaluated as described (Bergen et al. 2005).

As template DNA, 2.5 ng of dsDNA (for both gDNA and wgaDNA determined by PicoGreen) was used to determine 15 STR loci and the AMEL X/Y gene in the AmpFISTR® Identifiler™ assay (Applied Biosystems Inc., Foster City, CA, USA), while 5 ng of gDNA and wgaDNA, (as determined by PicoGreen), were used as template for each of the 49 TaqMan® SNP genotyping assays as previously described (Haque et al. 2003, Bergen et al. 2005). All samples were genotyped in duplicate and all genotyping calls determined independently.

Statistical analyses

In project 1, DNA yield differences between paired samples were tested using the Wilcoxon sign-rank test. Also in project 1, human MDA-REPLI-g wgaDNA yield from cytobrushes estimated using Q-PCR assays in 23 loci was compared to wgaDNA yield from paired buffy coat samples using a sign test for paired observations. p Values were adjusted for multiple comparisons using the false discovery rate (FDR) procedure (Benjamini & Hochberg 1995). In projects 1 and 3 the per cent completion was calculated by dividing the total number of successful genotypes by the total number of attempted genotypes. The per cent concordance was calculated by dividing the total number of genotypes concordant with genotypes from paired buffy coat gDNA by the total number of pairs with genotype calls. In project 3, increases in genotype completion and concordance rate with increases in cytobrush DNA input were tested using test for trend and analysis of variance. Analyses were conducted using STATA 8.1 (College Station, TX, USA) and SAS 8.2 (Cary, NC, USA).

Results

Project 1: evaluation of WGA cytobrush genomic DNA in a pilot study

Median wgaDNA yields for MDA-REPLI-g from 10 ng of white blood cell gDNA (in a 500 μl reaction) was 77.5 μg (range 67.3–91.9 μg). Median wgaDNA yields from cytobrushes A and B were significantly ($p<0.05$) higher than for white blood cells (85.1 μg (range 72.3–104.3 μg) and 95.8 μg (range 71.4–127.1 μg), respectively). Genomic and wgaDNA was also evaluated using genotyping per cent completion and concordance (with genotypes from white blood cell gDNA as reference) for a panel of nine STR loci and the AMEL locus. For the gDNA samples, genotype completion was low for cytobrush A and B (68% and 64%, respectively), but concordance was high (99% and 100%, respectively). Completion was distributed evenly across specific samples and assays. In contrast, after WGA, the completion improved for cytobrush A and B samples (98% and 99%, respectively), but the concordance compared with white blood cell gDNA was lower, 82% and 84%, respectively (data not shown). An algorithm based on Q-PCR of WIAF 1004 and WIAF 622 (Yan et al. 2004) predicted that 100% of wgaDNA samples from white blood cell DNA, 95% of wgaDNA from cytobrush A, and 50% of wgaDNA from cytobrush B would be usable for genotyping assays (data not shown).

Human wgaDNA yields were estimated for MDA-REPLI-g wgaDNA for blood and both types of cytobrushes using Q-PCR assays for 23 human loci (Figure 1). We observed considerable locus over- and under-representation for all wgaDNA types, which resulted in large differences in human wgaDNA yield estimates. Variability was

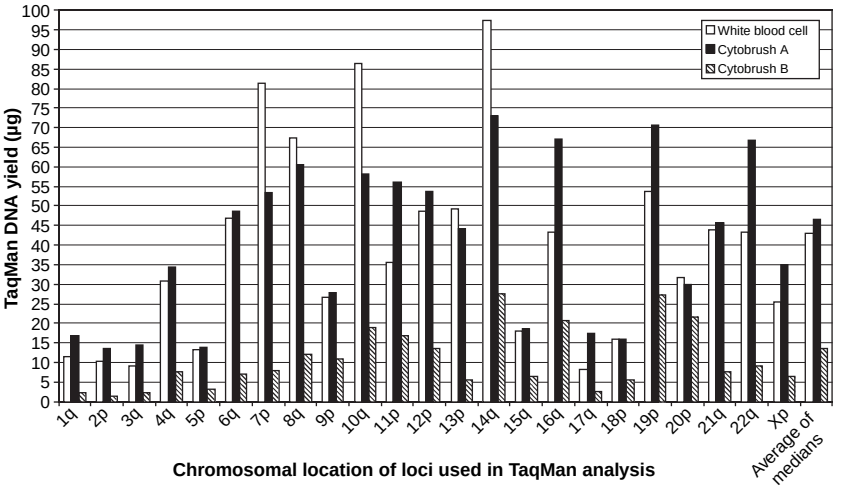

Figure 1. Median wgaDNA yield after MDA-REPLI-g™ from an NCI pilot study of 21 volunteers, estimated using 23 real-time quantitative PCR assays with loci from each of the 22 human autosomes and AMEL (work conducted at Molecular Staging).

also observed between the different DNA sources within the same locus. Median yields for cytobrush A wgaDNA were not significantly different than those for white blood cell gDNA for any of the 23 loci (FDR-adjusted p values ranging from 0.17 to >0.99). In contrast, yields from nine of 23 assays were significantly lower for cytobrush B compared to white blood cell DNA (FDR-adjusted $p < 0.02$).

Project 2: MDA-WGA using cytobrush cell lysates and gDNA in the US breast cancer study

The performance of MDA-REPLI-g WGA directly from 40 cytobrush gDNA samples and 84 cell lysate (40 paired to cytobrush gDNA samples, 44 unpaired) was compared. MDA-REPLI-g successfully amplified all specimens. However, the total wgaDNA yield from 29/84 lysates was very low (<0.02 µg µl^{-1} in 500 µl). Q-PCR genotyping of three SNPs (WIAF 525, WIAF 1004 and WIAF 622) was performed on both types of cytobrush wgaDNA samples and scatterplots of the fluorescent signals were compared. Normally, data points from samples corresponding to a particular genotype appear as a distinct tightly clustered scatter of plots, allowing for genotype determination of data points within each cluster. Allele scatter, caused by unequal amplification of one allele compared to another can make assignment of genotypes difficult because data points, especially presumptive heterozygotes, fall between and/or outside of clusters and result in undetermined genotypes. Results for WAIF1004 are presented in Figure 2A,B. We observed extensive allele scatter for each SNP analysed due to heterozygote dropout for both cytobrush gDNA samples (Figure 2A) and cell lysate (Figure 2B) samples. An algorithm based on WIAF 1004 and WIAF 622 (Yan et al. 2004) predicted that only 60% of wgaDNA samples from either cell lysates (50/84 samples) or cytobush gDNA (24/40 samples) would be usable for genotyping assays (data not shown).

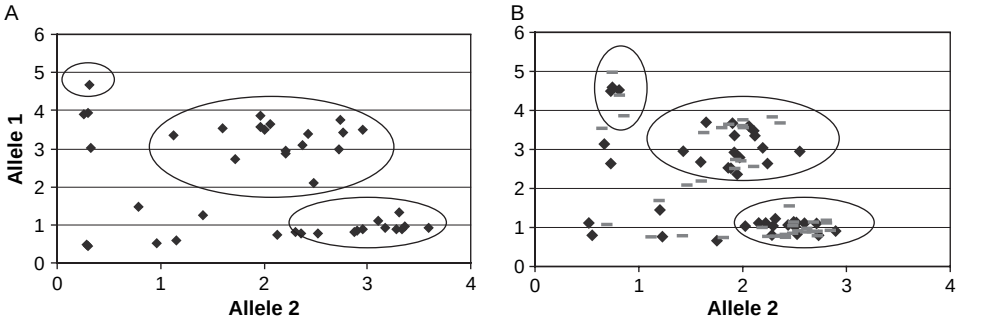

Figure 2. Real-time PCR analyses of the WIAF-1004 SNP using wgaDNA from (A) 40 matching WGA cytobrush DNA samples and (B) 84 cytobrush buccal cell lysate samples. ◆, 40 matched to cytobrush DNA samples from subjects in (A); –, 44 unmatched samples (all work conducted at Molecular Staging).

Project 3: Increasing gDNA template improved WGA performance

Experiments were conducted at the NCI-CGF laboratory to evaluate whether MDA-REPLI-g wgaDNA yields and genotyping performance would improve for cytobrush samples collected in the US breast cancer study by increasing template gDNA. When the amount of template gDNA added to a 100 µl MDA-REPLI-g amplification reaction was increased from 10 to 50 ng, the median DNA yield increased substantially from 17 µg (range 9–32 µg) to 30 µg (range 17–55 µg) ($p < 0.0001$) (Table II). Increasing the amount of template to 100 ng, increased the wgaDNA yield to 32 µg (range 11–47 µg). Increasing the amount of template gDNA used in the WGA reaction from 10 to 100 ng resulted in significant increases in genotyping performance for both completion (from 43% to 82%; p trend < 0.0001) and concordance (from 74 to 89%; p trend 0.002). However, for all gDNA input levels including 100 ng of gDNA, genotyping performance of wgaDNA achieved only 82% completion and 89% concordance, compared with 97% completion and 100% concordance for gDNA from cytobrushes (Table II). Other measures also differed significantly between wgaDNA generated from 100 ng of cytobrush gDNA and unamplified cytobrush gDNA. For example, the number of WGA samples that failed

Table II. Total wgaDNA yield and average per cent completion and concordance for 49 SNPs, using increasing amounts of buccal cytobrush gDNA from 20 individuals in an MDA-REPLI-g™ reaction.

Amount of gDNA template (ng) in WGA reaction	Median wgaDNA yield (µg) (range)[b]	p Value	Average% SNPs[a] completed (range)[d]	Average% concordance (range)[d]
gDNA prior to WGA	NA	NA	97%	100%
10	16.5 (9.3–32.4)	reference	43%[c] (10–87%)	74%[c] (59–88%)
25	18.3 (9.9–30.2)	0.23	65%[c] (41–91%)	77%[c] (58–97%)
50	29.9 (16.9–54.6)	<0.0001	75%[c] (30–93%)	85%[c] (68–99%)
100	32.2 (11.3–47.3)	<0.0001	82% (56–94%)	89% (73–98%)

[a]Number of attempted genotyes was 1960 in each row (49 SNPs analysed in duplicate using samples from 20 individuals).
[b]Significant increase in double-stranded DNA with increasing input gDNA, $p < 0.0001$, trend test.
[c]Significantly reduced genotype completion and concordance compared to genomic cytobrush, $p < 0.05$.
[d]Significant increases in genotype completion and concordance with increases in cytobrush DNA template added to the WGA reaction, trend test $p < 0.0001$ and 0.002, respectively.

PCR amplification and/or those with undetermined genotypes was respectively ten and four times greater than when gDNA was used (data not shown).

Discussion

Similar to previous studies, we found that MDA-REPLI-g amplification far exceeded that of either PEP or iPEP for gDNA collected from white blood cell or cytobrush samples (Dean et al. 2002) and that it can be used to amplify gDNA from blood and cytobrush collected buccal cell DNA for genotyping studies (Alsmadi et al. 2003, Hosono et al. 2003, Pask et al. 2004, Shao et al. 2004, Yan et al. 2004, Bergen et al. 2005). Our findings also indicated that wgaDNA obtained from MDA-REPLI-g amplification of gDNA from cytobrushes could introduce genotype misclassification to epidemiological studies. Concordance, using cytobrush wgaDNA remained below 90% even after increasing the amount of input gDNA to 100 ng.

In previous studies, wgaDNA obtained by MDA amplification of gDNA samples extracted from blood, mouthwash, or cell lines has reported >98% concordance for similar genotyping assays (Hosono et al. 2003, Shao et al. 2004, Yan et al. 2004, Bergen et al. 2005). The MDA-REPLI-g concordances for an STR panel observed in this study were lower than those previously observed using mouthwash samples from this same pilot study that were analysed using the AmpFISTR Plus ID PCR kit (range from 93% to 96% depending on the height of allele used to determine genotype calls) (Bergen et al. 2005). This study showed that genotyping performance of mouthwash-derived samples could be substantially improved by increasing the amount of DNA template added to the WGA reaction. SNP analysis showed high (99%) concordance rates for 49 TaqMan assays and demonstrated that mouthwash derived DNA samples can be MDA-REPLI-g amplified and attain high completion and concordance levels for genotyping studies.

The poorer performance of MDA with buccal cytobrush DNA in this study is probably the result of the lower quantity and quality of gDNA obtained from these samples (Garcia-Closas et al. 2001). This finding is corroborated by the fact that adding additional DNA template to the MDA reaction improved overall performance. Cytobrush samples in the US breast cancer study were self-collected using a standard NCI collection protocol and mailed at room temperature before processing (Garcia-Closas et al. 2001). gDNA from these samples was previously shown to be of lower quality than that obtained from mouthwash samples from the same study population (Garcia-Closas et al. 2001), suggesting that DNA degradation occurs during transport. Compared to studies conducted by other groups (Alsmadi et al. 2003, Hosono et al. 2003, Pask et al. 2004, Shao et al. 2004, Yan et al. 2004), we analysed a large number of buccal cell cytobrush samples that were collected in the field rather than a laboratory or clinic. Therefore, the results presented here may be more relevant to epidemiologists or field investigators than those obtained in a laboratory for methods development (Hosono et al. 2003, Yan et al. 2004). In all three projects, standard NCI protocols for sample collection and DNA extraction have yielded high levels of suitable DNA for genotyping (Garcia-Closas et al. 2001). MDA-REPLI-g is unlikely to be responsible for the low concordance rates for two reasons. First, all laboratory analyses were performed by company experts that were blinded as to the type of DNA they were analysing. Second, previous studies conducted at CGF had previously found that MDA-REPLI-g produced the highest

wgaDNA yields, and genotype completion and concordance rates for mouthwash buccal cell, buffy coat, and lymphoblast genomic DNA than two other methods tested (Bergen et al. 2005).

Q-PCR of 23 loci in 22 human autosomes and the X chromosome using MDA-REPLI-g wgaDNA from blood and cytobrush-collected buccal cells demonstrated substantial variation in locus representation. Amplification bias makes the use of Q-PCR assays to measure concentration of human wgaDNA less reliable since the estimated concentration and yield vary widely depending on the target loci selected. Previous studies of amplified DNA have suggested that loci representation was more uniform that that observed here and also could be explained by the use of high-quality (less variable) template gDNA for methods development.

In conclusion, when cytobrush wgaDNA is used, we strongly recommend inclusion of paired gDNA/wgaDNA samples and/or duplicate wgaDNA samples (when gDNA yields are limited) to monitor data quality and detect genotype misclassification in molecular epidemiological studies. Alternative PCR-based WGA technologies are being evaluated that may improve PCR fidelity when amplifying low-yield/degraded samples but they too have limitations (Tanabe et al. 2003, Wang et al. 2004). To reduce genotype misclassification risk and failure (which will ultimately reduce study power), these findings stress the importance of using protocols that are convenient, but also maximise the quality and quantity of DNA collected

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