Short Communication

DNA Quantification of Whole Genome Amplified Samples for Genotyping on a Multiplexed Bead Array Platform

Helen M. Hansen, Joseph L. Wiemels, Margaret Wrensch, and John K. Wiencke

Departments of Neurological Surgery and Epidemiology and Biostatistics, University of California, San Francisco, California

Abstract

Whole genome amplification (WGA) permits genotyping DNA samples of limited quantity, expanding the number of samples available for genetic epidemiology studies. WGA, however, produces various nucleic acid side products that may interfere with accurate DNA quantification and further analysis. Although quality control of whole genome amplified DNA (wgaDNA) before genotyping is essential to prevent failed or poor genotyping results, little information is available to choose the best method for wgaDNA quantification. Therefore, we quantified wgaDNA from 54 buccal or poor quality blood samples by four methods: UV absorbance, PicoGreen fluorescence calibrated with λ bacteriophage or calf thymus DNA, and absolute quantification by real-time PCR amplification of human-specific *Alu* Yd6. We then genotyped these wgaDNA samples and paired high-quality genomic DNA samples on a custom 384-plex Illumina Golden Gate Panel. Of the 54 paired samples, 39 gave high concordance (>99%), whereas 7 had moderate concordance (>90-99%) and 8 had poor concordance (≤90%) of wgaDNA and genomic DNA genotyping results. Quantitative PCR of *Alu* was the only wgaDNA quantification method to distinguish wgaDNA samples that gave high, moderate, or low concordance results (i.e., wgaDNA quantities in the high, moderate, and poor concordance groups ranged at 4.14-118.32, 0.29-2.19, and 0.01-0.27 ng/μL, respectively). Human-specific quantitative PCR is a highly useful guide for determining the suitability of wgaDNA before high-throughput single-nucleotide polymorphism analysis. (Cancer Epidemiol Biomarkers Prev 2007;16(8):1686–90)

Introduction

The advent of whole genome amplification (WGA) for samples with limited quantity DNA significantly expands the population of subjects available for genetic analyses in epidemiologic research. Although cheek swab (buccal) samples or mouthwash preparations are noninvasive and cost-effective methods for DNA sample collection from populations such as children and those who are geographically dispersed (1-5), such samples often yield limited quantities of DNA (4). For example, in one study, average human DNA yields were 1.6 μg total for two cytobrushes and 16.6 μg total for mouthwash preparations (6), as compared with the 25 μg/mL DNA routinely obtained from blood of healthy individuals. Furthermore, patients with advanced cancer or those undergoing anticancer treatments may exhibit low WBC counts that result in low yields of DNA from peripheral blood specimens. DNAs from these types of specimens are good candidates for WGA (7, 8). After WGA, accurate methods are needed to estimate the yield of whole genome amplified DNA (wgaDNA) from each subject before large-scale single-nucleotide polymorphism genotyping. This is necessary because both multiple displacement amplification and Omniplex WGA methods have been shown to generate a significant amount of artefactual side product (8, 9) as shown by no template controls (NTC) that show a mixture of ssDNA and dsDNA ranging from 0.4 to 21.0 μg of total product (8). These products are detected by UV absorbance and fluorescent DNA quantification commonly used to assess WGA sample quality (8-10) and cannot be distinguished from genomic amplification by these physical methods. Alternative to physical DNA quantification methods are PCR-based assays that specifically amplify only human DNA sequences. Here, we applied a real-time quantitative PCR method that targets Yd6 *Alu* insertion sequences that are both highly specific for human DNA and exquisitely sensitive. The Yd6 subfamily contains 97 members of *Alu* distributed throughout the genome. We have found the limit of detection to be 0.060 pg DNA for genomic DNA (gDNA) and 7.78 pg for wgaDNA samples, when assayed on the ABI 7900. In our study of lung cancer, we applied Omniplex WGA to a variety of gDNA samples before genotyping on a custom Illumina Golden Gate panel (Illumina, Inc.) containing 384 candidate and ancestry-specific single-nucleotide polymorphisms. Here, four methods for measuring the wgaDNA concentrations of genotyped specimens are evaluated to determine which were appropriate for wgaDNA quantification in terms of predicting quality and usefulness of the wgaDNA for genotyping on a multiplexed platform.

Materials and Methods

Biospecimens. Subjects were recruited as part of the San Francisco Bay Area Minority Lung Cancer Study from 1998 to 2003 (11). Biospecimens were obtained with informed consent using a protocol approved by the University of California San Francisco Institutional Review Board. Blood samples from 46 participants served as a reference for genotyping results obtained using wgaDNA from the same individuals. Blood specimens were collected in heparinized vacutainer tubes and stored at −80°C for 6 to 8 years. DNA was isolated by an automated phenol-chloroform method (Autogene 3000). Where samples failed to meet the 50 ng/μL minimum DNA

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Requests for reprints: John K. Wiencke, Division of Neuroepidemiology, Department of Neurological Surgery, University of California, Suite AC-34, 1 Irving Street, San Francisco, CA 94143-0441. Phone: 415-476-9323; Fax: 415-502-7411. E-mail: john.wiencke@ucsf.edu

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concentration recommended for Illumina genotyping after repeated isolation attempts, multiple DNA aliquots were combined, reprecipitated, and rehydrated in a 100-μL volume according to Puregene DNA Concentration Protocol. Buccal swabs (Medical Packaging Corp.) were obtained at the time of interview by the participant under the direction of the interviewer/phlebotomist. DNA was recovered from swabs using a common sodium hydroxide extraction protocol adapted from Richards et al. (12).

Samples were later re-extracted on the Autogen 3000 automated DNA extraction system (Autogen, Inc.) to remove salts and proteins present after the initial NaOH extraction (6). The resultant 100-μL samples were SpeedVac concentrated (Thermo Electron Corp.) to 15-μL volumes, and DNA concentrations measured by DNA fluorescence. Buccal DNA concentrations averaged 0.5 ± 0.6 ng/μL (SD).

Whole Genome Amplification. Omniplex WGA, which amplifies template after a fragmentation step, was used in this study because the buccal samples were treated with a NaOH extraction shown to decrease the yield of high molecular weight DNA (6). DNA samples derived from whole blood were normalized to 1.5 ng/μL, for 15 ng total DNA input. Buccal-derived DNA was amplified at the highest total gDNA input attainable (an average of 5.64 ng total gDNA input). Four NTCs were also amplified with 10-μL 1× Tris-EDTA in place of DNA. WGA was done according to the Omniplex protocol (Sigma-Aldrich Corp.). Post-amplification wgaDNA products were cleaned with a Montage PCR_{96} filter plate (Millipore), producing final volumes of 75 μL.

DNA Quantification

UV Absorbance and DNA Fluorescence. Absorbance at 260 nm (A_{260}) was measured for each wgaDNA sample using the NanoDrop (ND-1000) spectrophotometer (NanoDrop Technologies). Sample concentration was calculated by the NanoDrop nucleic acid application module using Beer's law, and assuming 50 ng cm/mL absorbance for dsDNA, A_{260}/A_{280} ratios averaged 1.79 ± 0.12 SD. PicoGreen DNA quantification reagent (Invitrogen) is a fluorescent dye that preferentially detects dsDNA over ssDNA and RNA in solution and is accurate in the presence of common DNA contaminants. It has excitation/emission wavelengths at 502/523 nm and a linear quantitation range extending from 25 pg/mL to 1,000 ng/mL (13). DNAs from two different genomic sources, λ phage and calf thymus, were used to create standard curves for alternate fluorescent quantification protocols. Lambda phage gDNA standard (Invitrogen) was diluted according to the Illumina Custom Genotyping Service Guidelines to create an eight-point λ phage standard curve from 0 to 75 ng/μL. Calf thymus gDNA (Invitrogen) was diluted to form an eight-point curve ranging from 0 to 100 ng/μL. Reactions for both protocols were prepared as follows: PicoGreen dsDNA reagent (Invitrogen) was diluted 1:200 in 1× Tris-EDTA buffer and 198 μL were aliquoted into each well of a 96-well plate. Two microliters of DNA sample were aliquoted per well for curve, controls, and unknowns. Samples were mixed and allowed to develop 5 min in darkness before their fluorescence was measured with the FLx800 Multi-detection microplate reader (BioTek Instruments, Inc.).

Real-time quantitative PCR. To create a measure of human amplifiable DNA present in our wgaDNA samples, an *Alu* element–based assay was slightly modified from Walker et al. (14) who showed the Intra-Yd6–based PCR assay to be specific for human DNA with a linear quantitation range of 0.1 to 100 ng. Primer Express software v2.0 (Applied Biosystems) was used to create dual-labeled probes and primers. The Yd6 forward primer 5′-GAGATCGAGACCAC/GGTGAAA3-3′ spans a Yd subfamily–specific deletion. The Yd6 reverse primer 5′-TCCGGGTTCACGCCATT-3′ includes a polymorphism specific to the Yd6 subfamily. The probe, labeled with 5′ FAM and 3′ Blackhole Quencher 1 (MWG-Biotech, Inc.), is 5′-AGCTACTCGGGAGGCTGAGGCAGGA-3′. Quantitative PCR reactions were done on an ABI 7900 as follows: 50-μL reactions were prepared using 25 μL of 2× Universal PCR Master Mix, 5 μL of 9.0 μmol/L forward primer, 5 μL of 9.0 μmol/L reverse primer, 5 μL of 2.5 μmol/L probe, 5 μL of water, and 5 μL of DNA. Reactions were then pipetted into a 384-well optical reaction plate in four 10-μL replicates. WgaDNA sample concentrations were calculated from a four-point human gDNA curve ranging from 0.1 to 100 ng/μL (PE Applied Biosystems Ceph 1347-02, part #403062). Samples were heated to 95°C for 10 min, cycled 40 times at 95°C for 15 s, and at 60°C for 60 s. Results were plotted, after baseline correction, using the Standard Curve Plot included in the ABI 7900 Absolute Quantification software. The resulting curve is log linear over the 0.1 to 100 ng/μL range, with an r^2 of 0.995. The limit of detection was calculated (15) using the average fluorescence of 12 gDNA blanks and 72 wgaDNA blanks.

Genotyping. Samples (5 μL) were genotyped according to the manufacturer's protocol at the University of California Davis Genome Center on an Illumina BeadStation 500G Golden Gate genotyping platform using a custom panel (GS0006747-OPA) of 384 candidate and ancestry informative single-nucleotide polymorphisms. All 54 wgaDNA samples (20 amplified from concentrated whole blood derived DNA, 8 from whole blood DNA, and 26 from buccal DNA) were genotyped alongside unamplified gDNA from the same individuals.

Analysis. Two outcome measures were considered: genotype concordance and pass fail status. We calculated the concordance of wgaDNA genotype calls with those obtained from unamplified gDNA. Genotypes were assigned in separate cluster files using Beadstudio v2.0 Genotyping software, GenCall threshold 0.25. Genotypes with GenCall scores >0.25 were called. WgaDNA and gDNA sample genotypes were compared using GTS Reports software v5.1.2.0. Genotype concordance for wgaDNA and gDNA sample pairs was calculated from the total number of matching genotypes divided by the total number of genotype pairs passing quality control. A priori criteria for high-quality genotyping included

Table 1. DNA concentrations of wgaNTCs

WgaNTC*	DNA quantification method				Illumina genotyping
	Quantitative PCR *Alu* Yd6 (ng/μL)	UV spectrophotometry (ng/μL)	Fluorescence λ curve (ng/μL)	Fluorescence calf thymus curve (ng/μL)	Sample call frequency†
NTC 1	<0.008	42.3	26.6	52.9	0.969
NTC 2	<0.008	39.8	19.7	39.1	0.938
NTC 3	<0.008	34.5	19.5	40.0	0.940
NTC 4	<0.008	38.9	22.3	44.1	0.909

*NTC samples were WGA amplified with 10 μL of 1× Tris-EDTA in place of DNA alongside wgaDNA samples.
†See Analysis.

Table 2. WgaDNA concentrations and genotyping outcomes

WGA sample name	DNA quantification method				Illumina quality control metrics		
	Quantitative PCR *Alu* Yd6 (ng/μL)	UV spectrophotometry (ng/μL)	Fluorescence λ curve (ng/μL)	Fluorescence calf thymus curve (ng/μL)	WGA sample call frequency*	Sample pair call frequency †	Genotype concordance ‡ (%)
High quality, >99% genotyping concordance							
36-B	68.58	90.0	16.1	33.2	0.9844	0.9609	100.00
32-B	42.18	73.6	25.2	50.6	0.9870	0.9531	100.00
21-B	61.49	83.7	44.2	97.0	0.9766	0.9427	99.86
30-B	4.14	75.6	45.3	89.6	0.9844	0.9531	99.73
2-W	107.94	101.5	26.4	50.0	0.9818	0.9766	99.60
12-W	88.92	96.7	29.9	61.3	0.9792	0.9740	99.60
39-W	81.65	91.6	36.5	78.0	0.9792	0.9714	99.60
1-W	76.01	94.0	41.3	69.9	0.9792	0.9714	99.60
45-W	50.16	72.5	35.4	79.2	0.9792	0.9766	99.60
23-W	43.75	88.3	52.5	88.8	0.9792	0.9740	99.60
6-B	25.64	98.1	20.1	40.5	0.9818	0.9792	99.60
17-B	48.84	88.4	44.1	82.4	0.9844	0.9479	99.59
43-B	15.16	65.2	34.9	53.4	0.9818	0.9635	99.59
37-W	111.62	108.6	21.5	47.7	0.9792	0.9740	99.47
34-W	101.34	100.0	27.4	56.2	0.9818	0.9740	99.47
4-W	91.47	97.5	24.6	48.8	0.9844	0.9766	99.47
25-W	89.21	83.5	24.6	73.1	0.9818	0.9766	99.47
8-W	80.86	86.0	36.5	63.6	0.9792	0.9740	99.47
32-W	65.60	79.4	32.0	79.4	0.9766	0.9740	99.47
19-B	41.20	74.1	13.6	39.1	0.9870	0.9844	99.47
46-B	5.09	61.0	43.0	71.8	0.9792	0.9740	99.47
3-W	106.57	101.4	29.8	54.7	0.9792	0.9714	99.46
13-W	85.95	106.6	33.2	67.3	0.9766	0.9714	99.46
10-W	61.71	89.0	31.9	59.5	0.9766	0.9714	99.46
43-W	93.13	90.4	26.7	54.6	0.9818	0.9792	99.34
36-W	118.32	113.1	22.7	52.6	0.9766	0.9740	99.33
17-W	101.36	112.7	27.0	54.9	0.9844	0.9688	99.33
30-W	99.23	106.2	27.7	69.5	0.9792	0.9766	99.33
41-W	93.55	87.0	30.0	52.3	0.9792	0.9766	99.33
11-W	90.95	103.9	29.7	62.0	0.9818	0.9766	99.33
5-W	65.93	91.9	28.1	63.3	0.9792	0.9740	99.33
7-W	60.98	88.9	35.8	73.9	0.9792	0.9714	99.33
40-B	9.09	70.0	21.6	34.8	0.9792	0.9766	99.33
21-W	94.86	103.5	22.1	48.9	0.9792	0.9740	99.20
26-W	82.11	83.7	30.6	77.7	0.9870	0.9818	99.20
9-W	75.63	93.1	47.9	84.5	0.9740	0.9714	99.20
20-W	82.23	93.0	40.6	74.6	0.9740	0.9688	99.19
37-B	8.27	77.1	53.0	68.4	0.9844	0.9583	99.18
14-W	59.12	90.2	35.7	68.3	0.9792	0.9766	98.93
Average	68.97	90.0	32.0	63.5	0.9805	0.9710	99.46
SD	31.78	12.8	9.5	15.6	0.0033	0.0090	0.22
Range	4.14-118.32	61.0-113.1	13.6-53.0	33.2-97.0	0.9740-0.9870	0.9427-0.9844	98.93-100.00
Moderate quality, >90-99% concordance							
29-B	1.25	69.0	40.2	87.6	0.9818	0.9792	98.27
45-B	2.19	44.0	35.9	86.4	0.9635	0.9323	97.77
15-B	0.29	58.6	46.6	83.2	0.9740	0.9688	97.31
42-B	0.75	70.4	44.3	83.2	0.9792	0.9766	97.07
18-B	0.65	66.5	54.0	154.8	0.9870	0.9844	96.83
33-B	1.67	64.7	44.7	99.3	0.9740	0.9714	96.65
31-B	1.29	50.2	46.6	113.0	0.9766	0.9714	96.11
Average	1.15	60.5	44.6	101.1	0.9766	0.9692	97.14
SD	0.65	10.0	5.7	26.0	0.0074	0.0171	0.72
Range	0.29-2.19	44.0-70.4	35.9-54.0	83.2-154.8	0.9635-0.9870	0.9323-0.9844	96.11-98.27
Poor quality, <90% concordance							
27-B§	0.01	53.1	40.6	84.2	0.8385	0.8333	76.09
16-B§	0.24	54.3	50.6	86.0	0.8724	0.8698	75.15
24-B§	0.27	62.1	52.6	139.0	0.8932	0.8828	74.04
44-B§	0.19	61.1	50.1	130.8	0.8542	0.8490	71.01
38-B§	0.04	65.7	42.0	92.3	0.8333	0.8281	68.08
35-B§	0.08	59.8	47.3	141.4	0.7917	0.7865	67.72
28-B§	0.02	62.3	47.2	97.2	0.8568	0.8516	67.28
22-B§	0.26	57.7	48.6	102.3	0.8021	0.7969	65.03
Average	0.14	59.5	47.4	109.2	0.8428	0.8373	70.55
SD	0.11	4.3	4.2	24.0	0.0341	0.0333	4.13
Range	0.01-0.27	53.1-65.7	40.6-52.6	84.2-141.4	0.7917-0.8932	0.7865-0.8828	65.03-76.09

Abbreviations: B, buccal swab wgaDNA; W, whole blood wgaDNA.

*Genotypes with GenCall score <0.25 were not included in the analysis (GenCall threshold as defined in Illumina BeadStation 500G System Manual Rev. B, p. 4-8: ''. . .a value between 0 and 1 that indicates the confidence in that call. A higher score indicates a higher confidence in the call. The score is a function of the quality of the genotype clusters. . . and the position of that specific DNA sample relative to its corresponding cluster'').

†Genotype pairs were evaluated where both genotypes had a GenCall score >0.25.

‡(Total number of matching genotypes / total number of genotype pairs passing quality control) × 100.

§Failed sample [samples with a sample call frequency >0.969 were designated as passed, those with <0.969 (less than an unamplified blank) as failed].

paired gDNA-wgaDNA genotype concordance values of >99%, moderate quality of 90% to 99%, and poor quality of <90% concordance. Additionally, failed samples were defined by call frequencies equal or less than our NTCs (i.e., ≤0.969). Sample pair call frequencies were calculated from the total number of genotype pairs evaluated divided by the total number of genotype pairs tested. Pearson's correlation coefficients were used to assess the association of DNA concentration measurements with one another.

Results/Discussion

Table 1 illustrates the problem addressed in this report. High concentrations of nucleic acid products are detected by physical measures such as UV absorbance and DNA fluorescence following WGA despite the absence of any added genomic DNA. The nature of these products is unknown; however, *Alu* Yd6 quantitative PCR results indicated that there was no human DNA contamination in our NTCs. In addition, the genotype calling software clustered fluorescence output from blanks and assigned them reasonably good sample quality scores. WgaNTCs genotyped with wgaDNA on the 384 single-nucleotide polymorphism panel produced genotypes with GenCall >0.25 for an average of 361 loci tested; sample call frequencies ranged from 0.909 to 0.969. Our results are consistent with those of previous studies (8, 16) showing both Omniplex and multiple displacement amplification WGA methods produce significant amounts of measurable WGA side product in NTCs that produce genotype calls with current software applications.

Table 2 summarizes our results comparing wgaDNA quantification by different methods with genotype quality on the Illumina bead array platform. The rows are sorted in descending order of the quality score (concordance) into the three nonoverlapping groups of samples: 39 high quality, 7 moderate, and 8 poor quality. Buccal wgaDNAs comprised both the moderate-quality and poor-quality sample groups. All eight samples that failed the call frequency threshold of 0.969 made up the poor-quality low genotype concordance group.

Next, we compared the DNA concentration measurements among each of these groups. WgaDNA concentrations measured by the four protocols differed considerably. Quantitative PCR sample concentrations were the most accurate predictor of genotype concordance and revealed three distinct concentration ranges for the three different sample qualities (Fig. 1). High-quality samples had human DNA concentrations as low as 4.14 ng/μL, showing the robustness of the Illumina genotyping platform, whereas quantitative PCR indicated that human DNA concentrations among poor quality samples were <0.27 ng/μL. When measured by UV spectrophotometry, the concentrations of high-quality (61.0-113.1 ng/μL), moderate quality (44.0-70.4 ng/μL), and poor quality (53.1-65.7 ng/μL) samples overlapped considerably (Fig. 1), suggesting an inability to distinguish between the presence of human amplifiable DNA and WGA side products. UV absorbance concentrations were highly correlated with human DNA measured by quantitative PCR ($r = 0.90$, $P < 5.29e{-}17$), but UV absorbance concentrations were high even among failed samples whereas quantitative PCR concentrations were much lower in poor-performing samples.

When measured by DNA fluorescence, the concentrations of high-quality, moderate-quality, and poor-quality sample groups also showed a significant degree of overlap. Additionally, samples with high concentrations of DNA, as measured by DNA fluorescence, failed more often than samples with low concentrations (Fig. 1). Similar results were observed regardless of the source of DNA standard (i.e., λ versus calf thymus DNA). A negative correlation was shown between DNA concentrations measured by DNA fluorescence and concentrations of amplifiable human DNA measured by quantitative PCR ($r = -0.64$, $P < 2.7e{-}07$ and $r = -0.59$, $P < 2.98e{-}06$, respectively). Possible factors responsible for these results include the sizes and ssDNA/dsDNA composition of Omniplex WGA products (8, 16, 17). The calf thymus and λ phage DNA fluorescence protocols displayed a systematic difference from one another, with calf thymus concentrations, on average, 2-fold greater than λ phage concentrations (Table 2). Georgiou and Papapostolou (17) recently reported decreases in PicoGreen fluorescence of as much as 70% in samples where the size of the template was <23 kb. Gel electrophoresis of our two standards showed that the λ phage DNA was larger than the 25-kb limit of mobility, whereas the calf thymus standard was <500 bp in length. The fluorescence of the calf thymus standard curve would therefore be reduced and concentrations estimated from the curve would be higher than those estimated from the λ curve.

Previous studies have indicated the importance of providing sufficient quantities of input DNA for the WGA reaction to avoid allelic drop out (16, 18). The size of input DNA could

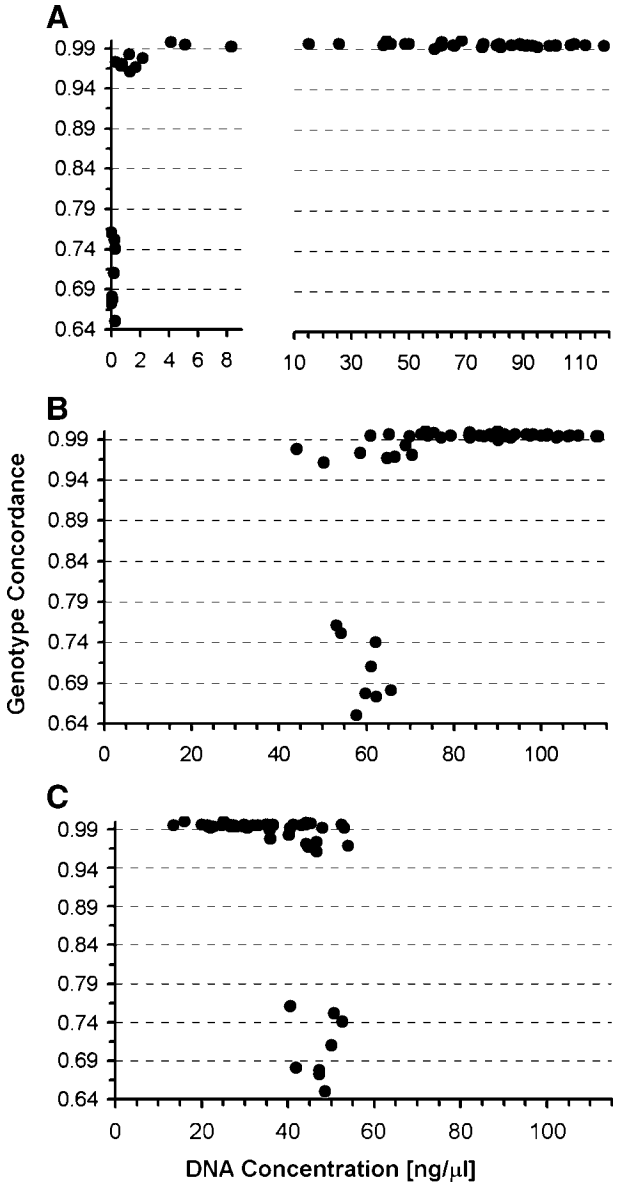

Figure 1. Genotype quality by wgaDNA concentration measured with human-specific *Alu* Yd6 quantitative PCR (**A**), UV absorbance (**B**), and DNA fluorescence relative to a λ phage curve (**C**).

also be important; however, because of the limited quantity of buccal DNA available for this study, the effect of gDNA size on the success of the WGA reaction could not be investigated here. Our results indicate that following WGA, it is prudent to use quantitative PCR to judge whether the amplification was successful in achieving sufficient amplifiable DNA for the genotyping phase. Based on these findings, our strategy for future quality control of wgaDNA is to set a minimum acceptable DNA concentration by genotyping a panel of wgaDNA samples with decreasing quantitative PCR DNA concentrations alongside paired gDNA samples to find the lowest wgaDNA concentration that produces acceptable genotype reproducibility. Different multiplex panels and platforms may require different quantitative PCR thresholds to achieve a desired level of genotyping quality. Each laboratory should establish its own set of quality controls. However, our finding that UV absorbance and DNA fluorescence are of limited value as predictors of WGA efficiency should be generalizable.

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