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BRIEF COMMUNICATION

Use of dried blood spots for the determination of genetic variation of interleukin-10, killer immunoglobulin-like receptor and *HLA* class I genes

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Abstract

Optimal methods for using dried blood spots (DBSs) for population genetics-based studies have not been well established. Using DBS stored for 8 years from 21 pregnant South African women, we evaluated three methods of gDNA extraction with and without whole-genome amplification (WGA) to characterize immune-related genes: interleukin-10 (*IL-10*), killer immunoglobulin-like receptors (*KIRs*) and human leukocyte antigen (*HLA*) class I. We found that the QIAamp DNA mini kit yielded the highest gDNA quality ($P < 0.05$; Wilcoxon signed rank test) with sufficient yield for subsequent analyses. In contrast, we found that WGA was not reliable for sequence-specific primer polymerase chain reaction (SSP-PCR) analysis of *KIR2DL1*, *KIR2DS1*, *KIR2DL5* and *KIR2DL3* or high-resolution *HLA* genotyping using a sequence-based approach. We speculate that unequal template amplification by WGA underrepresents gene repertoires determined by sequence-based approaches.

Stored dried blood spot (DBS) samples from previously characterized cohorts can provide a useful resource for determining relationships between genetic variation and clinical outcomes. Such samples have been routinely collected for the diagnosis of human immunodeficiency virus (HIV) infection in infants in programs for the prevention of mother-to-child transmission (1–3) and for HIV-1 surveillance testing in adults (3). Previous studies have shown that stored and archived DBS samples provide a valuable resource for HIV-1 quantification (2, 4); however, the methods for characterizing host immune response-related genes in the same samples have not been well developed.

Typically low genomic DNA quality and yield from DBS samples have limited their use for extensive genetic analyses. Previously, it has been reported that the QIAamp DNA mini kit yields on average 220 ng of total gDNA from a 6-mm punch as measured by PicoGreen fluorescence (5–7); however, this yield can vary widely by the method of gDNA extraction. Although there are several commercially available kits for gDNA extraction from DBS samples, there is no universally accepted method. Single nucleotide polymorphism (SNP) analysis and complete repertoire determination of most immune response-related genes require a minimum of 10 ng of gDNA (6). Thus, sample quantity can often limit the analyses of highly polymorphic genes from DBS samples.

To address these limitations, several investigators have proposed using whole-genome amplification (WGA) to increase the amount of gDNA template for subsequent genetic analyses of highly polymorphic, immune-related genes (6, 8). The WGA method relies upon a multiple strand displacement amplification technology in which the Phi29 DNA polymerase conducts isothermal genome amplification replicating up to 100 kb without dissociating from the gDNA template (9). Previously, Singh and Spector showed that this technology

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could be used for low-resolution analysis of human leukocyte antigen (*HLA*) class I and class II alleles from DBS samples using probe-based genotyping on a Luminex 100 platform (6). Although they evaluated this method for *HLA* and chemokine receptor gene analyses, they did not assess the feasibility of using this approach for analyzing another family of immune response-related genes, the killer immunoglobulin-like receptors (*KIRs*). Allelic variation within the *KIR* locus, which is almost as polymorphic as the *HLA* class I locus, has been associated with clinical outcomes in HIV-1 disease.

In particular, Martin et al. found that particular *KIR/HLA* compound genotypes were associated with slower disease progression among chronically HIV-infected adults (10, 11). Similarly, SNP variants of other immune response-related genes, such as interleukin-10 (*IL-10*), have been associated with susceptibility to HIV-1 acquisition (12, 13). Thus, an evaluation of WGA in determining *KIR* repertoires and *IL-10* SNP variants from DBS-derived samples would provide useful information for future clinical studies and further extend the findings of Singh and Spector (6).

Recently, Chainonthee et al. tested the feasibility of using WGA of gDNA derived from DBS to characterize *KIR* and *HLA-C* genotypes by sequence-specific primer polymerase chain reaction (SSP-PCR) (8). They found that the results obtained from wgaDNA were concordant with the results obtained using unamplified gDNA. However, their analysis was limited to only 4 of the 13 described *KIR* genes and thus it remains undetermined if this technology is suitable for the analysis of complete *KIR* repertoires. They found that WGA-derived templates were effective for the characterization of *KIR2DS1*, KIR*2DL1*, *KIR3DS1* and *KIR3DL1* genes as well as particular *HLA-C* alleles. Their analysis did not include high-resolution sequence analysis of *HLA* class I genes. Although these studies suggest that WGA may offer a viable option for increasing the amount of gDNA template derived from DBS samples, a more comprehensive analysis is warranted to determine the suitability of this technology for the analysis of immune response-related genes. Here, we extend the findings of previous studies by comparing the performance of three commercially available kits for gDNA (gDNA) isolation from DBS samples and then evaluate the feasibility of using WGA for the analysis of *HLA* class I, *KIR* and *IL-10* genes.

Initially, we evaluated three different methods of genomic DNA extraction from a single punch of DBS in a pilot study to identify a robust method for subsequent genetic analyses with and without WGA (Figure 1A). We hypothesized that sufficient gDNA could be isolated from a single punch of DBS for subsequent analyses. To compare these methods, we used endpoints of total DNA yield and several measures of gDNA quality including 260/280 ratio, 260/230 ratio, the ability to amplify the internal control gene, *HLA-DRB1*, and the ability to SNP genotype two common *IL-10* polymorphisms (SNP-592 and SNP-1082). For this study, we used stored matching whole blood and DBS samples collected from 21 South African women aged 16–45 years old as a substudy of previous investigation of mother-to-child HIV-1 transmission (2, 3). These 21 women were selected based on the availability of stored matched whole blood and DBS samples. For the pilot study, a subset of DBS samples from 9 of the 21 women was selected based on the availability of at least two DBS samples collected at the same time point per individual (Figure 1A). These women were enrolled during their antenatal care visits at the clinics in Umkhanyakude or outside Durban, KwaZulu-Natal, South Africa. Written informed consent was obtained from all study participants and the research protocol for these analyses was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa, and the Internal Review Board of Massachusetts General Hospital. The DBS blood cards (S&S 903 paper card; Whatman® Schleicher & Schuell, Sigma-Aldrich, St. Louis, MO) had been stored for approximately 8 years at several different temperatures, first at −80°C for approximately 1 year, then at room temperature for 1 year and finally at −20°C for 6 years. The matching whole blood samples were stored at −80°C for approximately 8 years.

We isolated gDNA from a single punch from each DBS sample and followed manufacturers' instructions to compare the performance of three commercially available kits: the QIAamp DNA mini kit (Qiagen, Carlsbad, CA), the PrepGEM™ Storage card blood kit (Zygem, Hamilton, New Zealand) and the Nuclisens MiniMag magnetic extraction kit (Biomerieux, Boxtel, the Netherlands) (Figure 1A). A 4.8-mm DBS disk punch was used per extraction method with two replicates. Sterile filter paper cards without DBSs were punched between samples to prevent cross-contamination. The gDNA was isolated and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). In addition, gDNA quality was assessed by 260/280 ratio, 260/230 ratio and the ability to amplify *HLA-DRB1*. *HLA-DRB1* (796 bp) was amplified using forward and reverse primers: 5′-TGC CAA GTG GAG CAC CCA- 3′ and 5′-GCA TCT TGC TCT GTG CAG AT-3′, respectively, as previously described (14). To further assess gDNA quality, we performed *IL-10* genotyping by the TaqMan® SNP Genotyping Assay kit using MGB primers and probes for *IL-10* at nucleotide positions -592 A/C and -1082 A/G as per manufacturer's instructions (Applied Biosystems, Foster City, CA). We analyzed the results using the ABI PRISM® SEQUENCE DETECTION system 7000 software. Genomic DNA samples that were previously genotyped for *IL-10* were used as internal standards for both SNPs -592 A/C and -1082 A/G. For statistical analysis of the data, GRAPHPAD PRISM version 4 was used. Wilcoxon signed rank test (15) was used to compare the average gDNA yield and both 260/280 and 260/230 ratios between the three different gDNA extraction methods. McNemar's tests were performed on results for *HLA-DRB1*, *IL-10* and *HLA* class I. For the Wilcoxon signed rank and McNemar's tests, P-values

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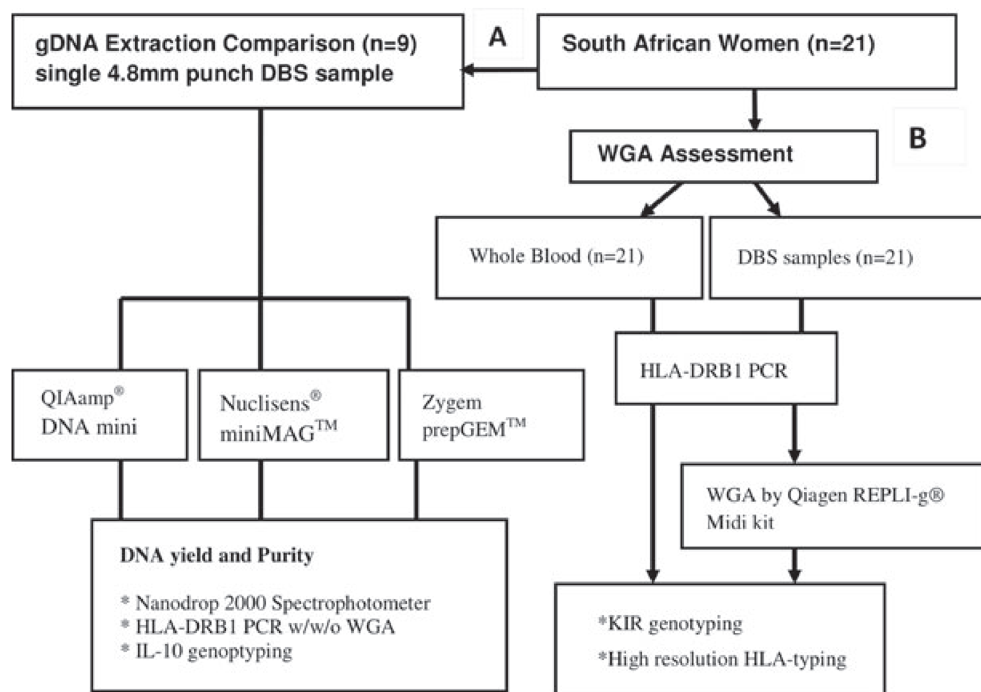

Figure 1 Overall sample and data analysis plan. (A) Comparison of gDNA extraction methods from stored dried blood spot (DBS) samples. Quality of the samples was measured by NanoDrop, *HLA-DRB1* amplification, and interleukin-10 (*IL-10*) genotyping by a single nucleotide polymorphism (SNP) genotyping kit. (B) Determination of killer immunoglobulin-like receptor (*KIR*) and human leukocyte antigen (*HLA*) class I genotypes from gDNA isolated from matched whole blood and wgaDNA from DBS ($n = 21$). In this analysis, we assessed gene repertoires with and without prior whole-genome amplification. Whole blood gDNA derived by the QIAamp blood mini kit was used as the gold standard for each individual.

were two sided and were adjusted for multiple comparisons using Holm's method (16). A P-value <0.05 was considered significant. For the detection of *KIR* genes, both sensitivity and specificity were calculated.

Among the three gDNA extraction methods that we evaluated, the QIAamp DNA mini kit was the most robust method as measured by total gDNA yield and quality (Figure 2; Table S1). The yield by this method was higher than that by the Nuclisens MiniMag approach, but lower than that by the PrepGEM method (Figure 2A). We speculate that the high total gDNA yields (average 8100 ng) using the PrepGEM method were likely due to artifactual elevation of DNA quantification, as our measurements were made by NanoDrop spectrophotometry rather than quantitative polymerase chain reaction (PCR). The PrepGEM method generates 90% single-stranded DNA (per manufacturer's protocol), which is indistinguishable from RNA by NanoDrop. Thus, artifactual elevation of total DNA yields by our method of DNA quantification cannot be excluded. Consistent with this finding, by NanoDrop we also found lower 260/280 ratios using the PrepGEM method (Figure 2B). Previous studies have shown that a 260/280 ratio of approximately 1.8–2.00 indicates pure double-stranded DNA, whereas lower values suggest less pure dsDNA (17). Unlike the other methods, the PrepGEM method lacks a purification step removing potential contaminants such as RNA, which could falsely increase DNA yield. Similarly, gDNA extracted by the PrepGEM method had on average a significantly lower 260/230 ratio than samples extracted by the QIAamp DNA mini kit ($P < 0.001$; Figure 2C). Prior studies have suggested that low 260/230 ratios are associated with possible chemical contamination (17); however, we cannot exclude a high proportion of ssDNA as a likely contributing factor to differences in measurements by NanoDrop spectrophotometry.

We found that on average approximately 550 ng of gDNA could be extracted from a single 4.8-mm punched-out DBS using the QIAamp DNA mini kit (Figure 2A). This yield was comparable to a previously reported average total yield of 220 ng from a 6-mm punched-out DBS by the same method (5–7). Previously, Martin and Carrington have established that 200 ng of good quality ($260/280 = 1.8–2.00$) gDNA is required for complete *KIR* repertoire determination by SSP-PCR (14). On the basis of our findings, we conclude that the total amount of gDNA obtained from one DBS punch extracted by the QIAamp DNA mini kit is of sufficient quality and quantity for *KIR* repertoire determination. The performance of this kit exceeded that of the other methods that we evaluated. Using a 6-mm punch, Sjoholm et al. also found that this method produced gDNA of the highest total yield compared to EZNA, Chelex 100 and alkaline lysis

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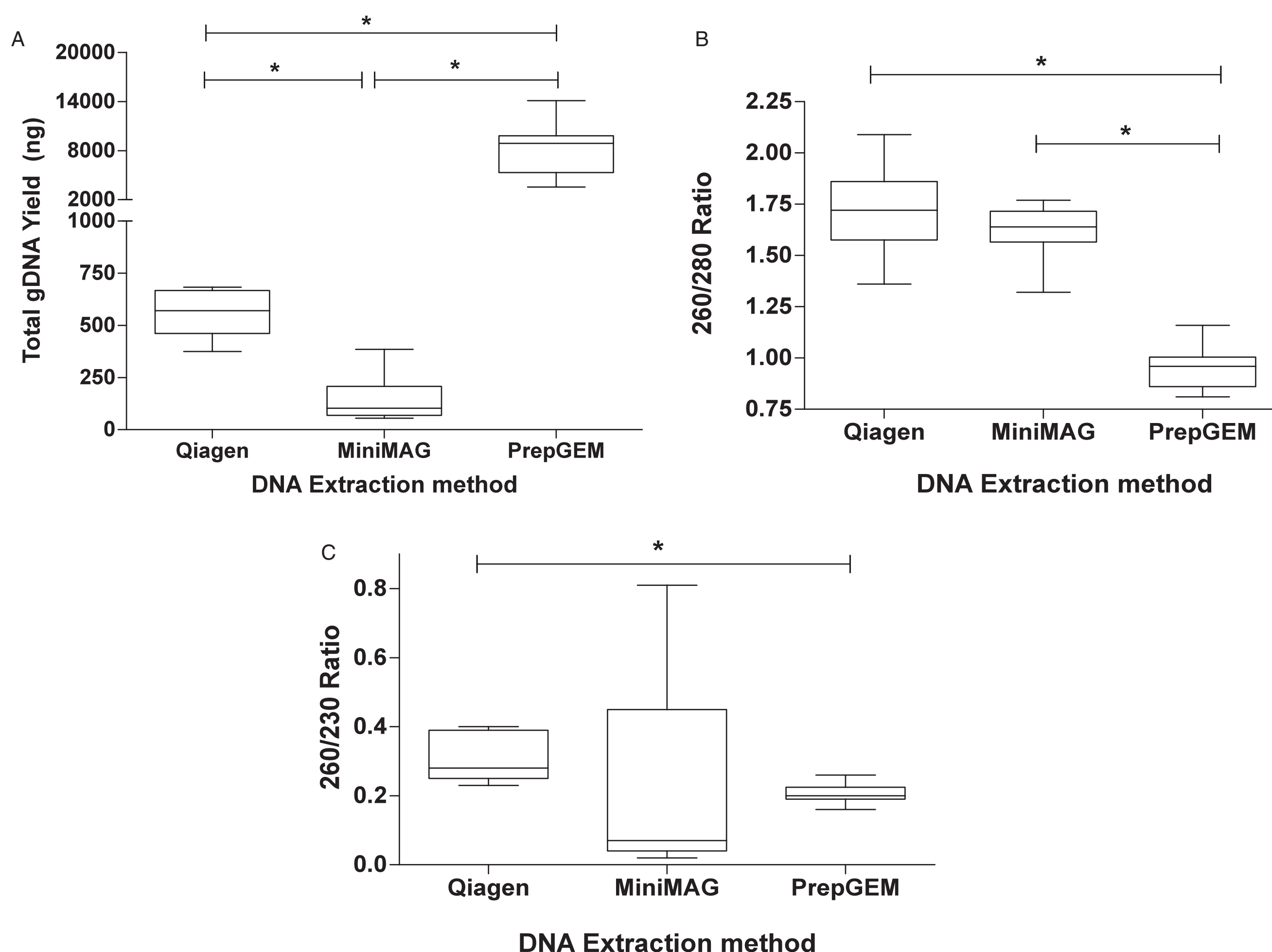

Figure 2 (A–C) The Qiagen QIAamp gDNA extraction kit yields the most optimal quality and quantity of gDNA from a 4.8-mm disk of dried blood spot (DBS) samples. Statistical comparisons between groups were made using Wilcoxon signed rank test and adjusted for multiple comparisons, where * denotes *P*-values of <0.05.

methods (5). We did not observe increased gDNA yield when one or two DBS punches were used compared to a whole DBS (data not shown). Similar findings have been reported previously by Hollegaard et al. (18). We speculate that the carrying capacity of the DNA extraction columns may limit the total gDNA yield obtained. Thus, the QIAamp DNA mini kit consistently yielded adequate total gDNA from a single 4.8-mm DBS punch.

To further assess the quality of gDNA obtained by these extraction methods, we used two independent approaches. First, we measured the ability to amplify the internal control gene, *HLA-DRB1*, and second, we genotyped for two *IL-10* SNP variants (592 and 1082) using a TaqMan® SNP Genotyping Assay kit (Applied Biosystems). Using whole blood-derived gDNA as the standard, we compared the average proportion of successful *HLA-DRB1* amplifications and correct *IL-10* SNP identifications at positions 592 A/C and 1082 A/G (in two different experiments) among gDNA isolated by the three isolation methods (Table 1). For both *HLA-DRB1* amplification and *IL-10* SNP determination, the results obtained from gDNA samples isolated using the QIAamp DNA mini kit were concordant with the results obtained using gDNA derived from whole blood. We found that the QIAamp DNA mini kit yielded gDNA with the highest frequency of successful detection of *HLA-DRB1* and *IL-10* SNP variants compared to the other methods ($P < 0.01$; Table 1). Samples extracted by either the PrepGEM approach or Nuclisens MiniMag method had low frequencies (on average 0 of 9 samples) of successful *HLA-DRB1* and *IL-10* SNP variant detection. We speculate that chemical carryover from the extraction process may interfere with subsequent analyses. This has been shown previously with gDNA extracted from animal tissues (19).

However, our results were not consistent with previous findings by Pachot et al., who reported complete concordance in *HLA-DRB1* oligotyping between DBS-MiniMag and phenol

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Table 1 Pairwise comparison of the average frequency of successful *HLA-DRB1* PCR amplification and *IL-10* SNP determination on samples isolated by three isolation methods

	Methods			
	QIAamp DNA mini	Nuclisens MiniMag	Prep-GEM	*P*-value
HLA-DRB1 W/O WGA				<0.01
Yes	9	0	0	
No	0	9	9	
HLA-DRB1 with WGA				<0.01
Yes	9	1	0	
No	0	8	9	
IL-10 SNP -592				<0.01
Yes	9	0	1	
No	0	9	8	
IL-10 SNP -1082				<0.01
Yes	7	0	1	
No	2	9	8	

HLA, human leukocyte antigen; *IL-10,* interleukin-10; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; WGA, whole-genome amplification; W/O, without. Both *HLA-DRB1* amplification and *IL-10* determination were performed on two replicates of the samples ($n = 9$). All methods were compared to the QIAamp DNA mini kit and statistical comparisons between groups were made using McNemar's test and adjusted for multiple comparisons, *P*-values <0.01 indicate significance.

chloroform extraction methods (20). One possible explanation for the difference in outcomes is the age of the DBS samples used for analysis. Here, we used DBS samples that were approximately 8 years old, whereas they used freshly prepared DBS samples. From this, we conclude that the duration of DBS storage may impact the choice of gDNA extraction method for subsequent analyses. The QIAamp DNA mini kit yielded gDNA that consistently performed well under the conditions that we evaluated; however, neither the Nuclisens MiniMag or PrepGEM methods yielded optimal results. Thus, we selected the QIAamp DNA mini kit for gDNA extractions in further work.

WGA offers the possibility of increasing the amount of template available to enable the analysis of multiple genes. Initially, to evaluate this method we compared the ability to amplify *HLA-DRB1* and identify *IL-10* SNP variants from wgaDNA and unamplified gDNA extracted by the QIAamp DNA mini kit, Nuclisens MiniMag and PrepGEM methods, respectively. Initially, we compared two WGA kits, REPLI-g (Southern Cross, Carlsbad, CA) and Genomeplex (Sigma-Aldrich), for the generation of suitable templates for *KIR* genotyping. We found that the REPLI-g kit yielded reproducible *KIR* genotyping results; however, the results obtained using the Genomeplex method were not consistent (data not shown). Thus, we selected the REPLI-g kit for WGA evaluation in this study. We used the Qiagen REPLI-g midi kit (Southern Cross) as per manufacturer's instructions to generate wgaDNA from DBS-derived gDNA templates from the same nine individuals who we used in the pilot study. Following WGA, we found that the average DNA yield and quality was similar between the samples regardless of the method used for gDNA extraction (data not shown). We were able to uniformly amplify the yield of gDNA by 600-fold. We found that WGA did not significantly enhance the frequencies of successful *HLA-DRB1* amplification. As with unamplified gDNA, the frequency of successful *HLA-DRB1* amplification was significantly higher for samples isolated using the QIAamp DNA mini kit (100%) compared to either Nuclisens MiniMag (11.1%) or PrepGEM (0%) ($P < 0.01$) (Table 1). For gDNA template extracted by either the QIAamp DNA mini kit or PrepGEM gDNA isolation methods, there was no difference in successful *HLA-DRB1* amplification before and after WGA. In comparison, WGA marginally increased the frequencies of successful *HLA-DRB1* from samples isolated by the Nuclisens MiniMag method (11% *vs* 0%) (Table 1). From this, we concluded that WGA did not significantly enhance the ability to amplify an internal control gene using the DNA extraction methods that we evaluated. However, the feasibility of applying this technology to assess the use of a single punch of DBS to determine a complete repertoire of highly polymorphic *HLA* class I and *KIR* genes remained unresolved.

To address the question of applying this technology more broadly to assess highly polymorphic genetic loci, we next evaluated WGA for generating templates for *KIR* gene repertoire determination. On the basis of our pilot study, we selected the QIAamp DNA mini kit to extract gDNA from one 4.8-mm DBS punch from each subject. We used gDNA isolated from stored DBS samples from a cohort of 21 South African adults as mentioned previously (Figure 1B). In our analysis, we compared *KIR* repertoires determined by SSP-PCR using previously established methods (14). To generate wgaDNA template, we used the Qiagen REPLI-g midi kit and compared *KIR* repertoires results with and without WGA. In this comparison, we used whole blood-derived gDNA isolated from the same individuals as the gold standard. The sensitivity and specificity of using wgaDNA in the detection of certain *KIR* genes were computed and statistical differences were determined by Wilcoxon signed rank test (15) with adjustment for multiple comparisons using Holm's method (16).

Overall, we observed that the frequencies of *KIR* genes in our cohort were consistent with previously described *KIR* frequencies for an African population (21). Notably, *KIR3DS1* was absent and *KIR2DS1* was present at a low frequency (Figure S1). Likewise, the distribution of *KIR* genotypes in our cohort was consistent with previously described *KIR* genotypes among Africans (21, 22). Our cohort consisted primarily of the Bx *KIR* genotype (Figure S1). Although our cohort was relatively small, the *KIR* gene content was representative of African populations.

In comparing *KIR* repertoires determined from wgaDNA compared to our gold standard, we found that wgaDNA was

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suitable for SSP-PCR-based genotyping of most *KIR* genes. We obtained concordant results for a majority (i.e. 11 of the 16) of the genes tested (Table 2). However, WGA-derived templates were not robust for four of the *KIR* genes. We found discordant results with *KIR2DL1*, *KIR2DL3*, *KIR2DL5* and *KIR2DS1*. These genes were present using unamplified, whole blood-derived gDNA, but absent using wgaDNA. Thus, the sensitivity for detecting these genes was lower using wgaDNA and ranged from 65% to 92% (Table 2). This difference in sensitivity was not attributed to differences in the source of the gDNA (whole blood *vs* DBS), as we found no difference between whole blood-derived gDNA and unamplified DBS-derived gDNA (data not shown). We did not find any false-positive results with either unamplified DBS-derived gDNA or wgaDNA (Table 2). Thus, our findings suggest that wgaDNA may yield false-negative results for the detection of these four *KIR* genes. Consistent with these findings, we also found similar discrepant results between wgaDNA and unamplified gDNA in an independent analysis of other stored DBS samples from an additional 120 individuals in the same cohort (data not shown). Thus, our data suggest that WGA is not a robust method for generating template for the detection of *KIR2DL1*, *KIR2DL3*, *KIR2DL5* and *KIR2DS1* ; however, it may be suitable for the analysis of other *KIR* genes.

The most likely explanation for failure to detect some of the *KIRs* is unequal gDNA template amplification by the method of WGA that we used; however, we cannot exclude the possibility of inadequate primers for detection by WGA. Previously, Chainonthee et al. also found discrepant results for the detection of *KIR2DS1*, *KIR2DL1*, *KIR3DS1* and *KIR3DL1* until they redesigned their primers for *KIR* detection (8). It is possible that some of our primers were complimentary for regions that are underrepresented in wgaDNA. Further sequence analysis of wgaDNA product would be required to resolve this matter. Our findings suggest that for highly polymorphic loci, such as *KIR*, genotyping primers may need to be designed and optimized specifically for using wgaDNA templates.

To further investigate the possibility of unequal gDNA template amplification, we next characterized *HLA* class I (*HLA-A, -B* and *-C*) alleles by high-resolution sequence-based typing (PCR-SBT) (four-digit) as described previously (23). This protocol for *HLA* class I genotyping was based on recommendations of the International Histocompatibility Working Group (www.ihwg.org). In brief, for *HLA-A*, *-B* and *-C* genes, we amplified and sequenced exons 2 and 3 using specific primers (Table S2) and established PCR conditions (Table S3). We sequenced using the BigDye Terminator v1.1 Cycle Sequencing kit and an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) with conditions established previously (Table S4). In addition, we cleaned PCR and sequencing reactions using magnetic beads and a Biomek FX Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA). Furthermore, we analyzed the *HLA* sequences using the

Table 2 False-positive, false-negative, sensitivity and the specificity of killer immunoglobulin-like receptor (*KIR*) genotyping results from whole-genome amplified gDNA derived from DBS compared to unamplified gDNA derived from whole blood (the gold standard) for 21 adult South Africans

Gene	False positives	False negatives	Sensitivity (%)	Specificity (%)
KIR2DL1	0	3	85.7	N/A
KIR2DL2	0	0	100	100
KIR2DL3	0	1	96.6	100
KIR2DL4	0	0	100	N/A
KIR2DS2	0	0	100	100
KIR2DS3	0	0	100	100
KIR2DS4	0	0	100	N/A
KIR2DS5	0	0	100	100
KIR3DL1	0	0	100	N/A
KIR3DL2	0	0	100	N/A
KIR3DS1	0	0	N/A	100
KIR3DL3	0	0	100	N/A
KIR2DL5	0	1	92.9	100
KIR2DP1	0	0	100	N/A
KIR2DS1	0	1	66.7	100
HLA-DRB1	0	0	100	N/A

DBS, dried blood spot; *HLA,* human leukocyte antigen.
Some samples did not have specificity because the gene was present in all samples therefore false negatives were not detected. N/A indicates values that were not determinable for specificity or sensitivity, either by the presence of the gene in all subjects (i.e. unable to determine false positives) or the absence of the gene in all subjects (i.e. unable to determine false negatives), respectively. Here, the detection of the *HLA-DRB1* gene has been included as a positive control and the experiments were performed in duplicate. Amplicons for the internal control primers (*HLA-DRB1*) were detected in all reactions.

ASSIGN software (Conexio Genomics, Fremantle, WA, Australia). In these assays, we used the same samples that had been used for *KIR* repertoire determination. As with *KIR*, we based our comparisons on the gold standard of unamplified whole blood-derived gDNA. With gDNA template produced by WGA, we found a significant increase in homozygosity for each of the loci evaluated and an increased frequency of failed reactions ($P < 0.01$) (Figure 3). We attributed the increase in homozygosity to allelic dropout (i.e. concordant for only one allele). Although we observed the lowest frequency of failed reactions with the *HLA-A* locus, we found that this locus had the highest frequency of homozygosity ($P < 0.01$). This underrepresentation of alleles is most likely due to unequal gDNA template amplification. This is also consistent with the increase in missing *KIR* genes that we found in our *KIR* repertoire analysis from wgaDNA. Likewise, we found discrepant results in our analysis of the *HLA-C* locus. For the wgaDNA samples, the *HLA-C* locus had the lowest frequency of concordant results with the gold standard compared to either the *HLA-A* or *HLA-B* loci ($P < 0.01$). In addition, we found that the highest frequency of failed

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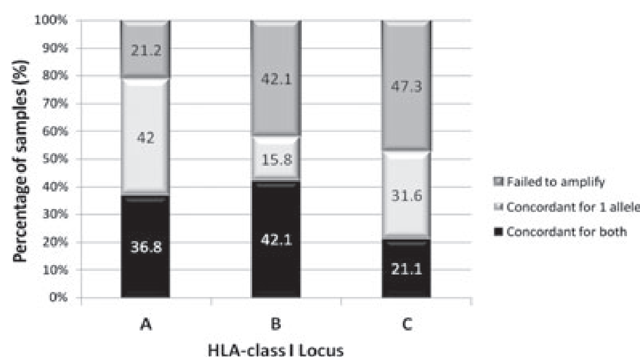

Figure 3 Comparison of the ability to perform high-resolution sequence-based human leukocyte antigen (*HLA*) genotyping from wgaDNA samples compared to unamplified samples from whole blood. Samples were either concordant for both alleles, concordant for one allele or failed to amplify *HLA* class I from wgaDNA relative to whole blood-derived DNA (gold standard).

reactions occurred in characterizing the *HLA-C* locus compared to the *HLA-A and HLA-B* loci ($P < 0.001$) (Figure 3). These results suggest that wgaDNA derived from DBS is least reliable in characterizing *HLA-C* alleles. In contrast, we obtain the most reliable results in characterizing genes in the *HLA-B* locus. In particular, we found significantly lower homozygosity ($P < 0.01$) and a significantly higher frequency of concordant results overall compared to either *HLA-A* or *-C* loci ($P < 0.01$) (Figure 3). These data suggest that wgaDNA is most accurate for characterizing *HLA-B* alleles; however, the high rate of failed reactions and missing alleles (42% and 16%, respectively) implies that this template is not suitable for high-resolution *HLA* genotype determination by sequence-based typing.

In contrast, Singh and Spector previously showed reliable low-resolution *HLA* typing using wgaDNA templates (6). Notably, they did not observe allelic dropout, increased homozygosity or failed detection. Possible explanations for our opposing findings include differences in the methods used for *HLA* genotype determination and the number of DBS samples used for DNA isolation. Here, we used high-resolution sequence-based typing, whereas they used an automated DNA typing system utilizing the Luminex® 100 platform. In the Luminex system, *HLA* genotypes are resolved by sequence-specific oligonucleotide probes (6). We speculate that sequence-specific probes are more robust in their recognition of wgaDNA compared to sequence-based approaches; however, this remains to be tested directly. In support of this conclusion, Shao et al. have previously shown that the method of genotyping impacts the outcome of PCR-based typing from wgaDNA (24). Consistent with our results, they also found that genotyping from wgaDNA for *HLA-C* locus determination yielded proportionally more failed reactions than either *HLA-A* or *-B* loci. One limitation in the interpretation of their findings is the lack of a gold standard for comparison. We have further extended their findings by including a gold standard and have confirmed increased homozygosity in the determination of *HLA* genotypes. Taken together, these findings suggest that the method of *HLA* typing may be critical for the reliable determination of high-resolution *HLA* genotypes from whole-genome amplified DNA. In this study, we found that sequence-based *HLA* typing yielded unacceptably high frequencies of failed reactions and homozygosity.

In summary, we have extended the findings of others by performing a more extensive evaluation of WGA of DBS-derived gDNA for the analysis of highly polymorphic immune response-related genes. Unlike previous studies that have either focused primarily on the characterization of *HLA* class I genes (6) or selected *KIR* genes and *HLA* alleles (8), we provide the first data on complete *KIR* and *HLA* repertoire analyses from wgaDNA derived from DBS samples. Of the gDNA extraction methods from DBS that we evaluated, we found that the QIAamp DNA mini kit was the most robust method for subsequent genetic analysis of *KIR*, *HLA* class I and *IL-10* genes. Although we found that WGA produced a template suitable for *KIR* genotyping of most *KIR* genes, it was not suitable for four commonly occurring *KIR* genes. Thus, we conclude that for the analysis of *KIR* repertoires wgaDNA should be applied selectively and not used for the analysis of all *KIR* genes. Similarly, we found that wgaDNA was not a reliable template for high-resolution *HLA* determination by sequence-based typing. Our data suggest that the downstream applications and analytical methods should determine whether WGA is suitable to increase the amount of available gDNA template. These findings have important implications for the design of genetic studies using stored DBSs and suggest that unamplified gDNA should be used when feasible for the analysis of *KIR* and *HLA* repertoires.

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Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Killer immunoglobulin-like receptor (*KIR*) genotyping results from whole-genome amplified gDNA derived from dried blood spot (DBS) compared to unamplified gDNA derived from whole blood (the gold standard) for 21 adult South Africans. Gray squares represent concordant presence, open squares represent concordant absence and black squares represent discordant results (i.e. the presence of a gene in the gold standard but not the experimental sample). Here, the detection of the *HLA-DRB1* gene has been included as a positive control and the experiments were performed in duplicates.

Table S1. Comparison of the gDNA yield and quality as per 260/280 and 260/230 ratios from dried blood spots between

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the three DNA isolation methods without correction for multiple comparisons. DNA was isolated from nine samples in duplicates using three commercially available isolation kits. Comparisons to the QIAamp DNA mini method were made by t-test, where ** denotes $P < 0.001$ and * denotes $P < 0.01$.

Table S2. Human leukocyte antigen (HLA) locus PCR (polymerase chain reaction) and sequencing primers. (A) HLA locus-specific PCR primers. (B) Secondary HLA locus-specific PCR primers were used in the event that primers in Table 1A failed. (C) Sequencing primers.

Table S3. Human leukocyte antigen (HLA) locus PCR (polymerase chain reaction) conditions and sequencing conditions. (A) HLA locus PCR reaction mix. (B) HLA locus PCR conditions with primers. (C) HLA locus PCR conditions.

Table S4. Human leukocyte antigen (HLA) sequencing conditions. (A) Sequencing reaction mix. (B) Sequencing conditions.

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