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Suitability of dried DNA for long-range PCR amplification and HLA typing by next-generation sequencing

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ABSTRACT

Storage and stable shipment of genomic DNA are of great concern to laboratories that may need to perform testing off archived samples. There are some dry-state storage methods that are available that have the potential to provide a way to store samples at room temperature for long periods of time as well as offer a means to ship DNA to other facilities without the same safety concerns that come with shipping liquid samples. The recovered DNA should be of sufficient integrity such that downstream applications can be performed without concern of the sample quality. This work describes sample properties between two methods of DNA storage, dried (room temperature) and traditional (−80 °C). DNA was evaluated for purity, fragment length, and the ability to generate HLA typing using next-generation sequencing.

1. Introduction

Within clinical genetics laboratories, genomic DNA (gDNA) represents a vital resource that many laboratories store indefinitely for reflex testing or research projects years after collection. Storing DNA samples at −80 °C is standard practice in most laboratories. However, continual storage of samples at −80 °C can be difficult not only for the physical space of the freezers but also the cost of maintaining them. Dry-state storage is more efficient for transport and storage of samples for extended periods.

Clinical assays using dried DNA have been used with a variety of technologies including whole-genome microarray analysis, quantitative PCR (qPCR), gel electrophoresis, amplified fragment length polymorphism (AFLP), short tandem repeat (STR) profiling, Sanger and next-generation sequencing (NGS) to generate high-quality results [1–4]. With respect to HLA typing, the majority is performed using either freshly isolated DNA or stored frozen DNA. Previous studies using room temperature (RT) stored DNA have performed HLA typing via bead array, Sanger, and NGS [1,2]. While Noble et al. [2] demonstrated successful HLA typing using dried DNA with targeted amplification of HLA gene exons by NGS, the majority of commercial HLA typing assays for NGS have adopted long-range PCR as the amplification method [5–7]. The utility of dried DNA with long-range PCR amplification has not been studied for HLA typing. The drawback of using long-range PCR for amplification is amplicons requiring long gDNA fragments tend to fail more often [6,8]. Therefore, it is important to understand the impact of various DNA storage conditions on HLA typing when long-range PCR is used as the amplification methodology.

Taken together, the utility of dried DNA with long-range PCR and its impact on DNA purity have yet to be proven. This study evaluates the effect of storing DNA dried at RT on DNA purity and integrity (fragment length). These important characteristics of DNA are evaluated in the context of generating high-quality HLA typing.

2. Materials and methods

2.1. DNA extraction

gDNA was extracted from 20 peripheral blood samples using Promega (Madison, WI) Maxwell 16 Blood DNA Purification kit, approximately 500 µL of buffy coat from ACD tubes was used for input DNA. Samples were stored at −80 °C for 7 days.

2.2. Drying of genomic DNA

Stored samples were removed from −80 °C and approximately 5 µg of gDNA (calculated using fluorescent-based quantification) were brought up to 150 µL of volume with molecular grade diH$_2$O. A portion

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Abbreviations: gDNA, genomic DNA; qPCR, quantitative PCR; AFLP, amplified fragment length polymorphism; STR, short tandem repeat; NGS, next generation sequencing; DIN, DNA integrity number; DOC, depth of coverage

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of the originally isolated gDNA remained for future testing and comparison at −80 °C.

The diluted portion of gDNA was transferred into the GenTegra-DNA 0.5 mL screw cap tube (GenTegra LLC, Pleasanton, CA) and was mixed to solubilize the DNA with the stabilizing solution. Tubes were dried in a fume hood for 3 days at room temperature (RT). Once drying was completed the tubes were capped and left at RT for 53 days. Samples were then rehydrated with 150 µL of molecular grade diH_2O. DNA recovery was carried out per manufacturer's instructions.

2.3. Measurement of gDNA purity

gDNA purity was measured using a UV spectrophotometer (NanoDrop, ThermoFisher) according to the manufacturer's instructions. Each DNA storage condition was blanked using the appropriate eluate. The ratio of 260/280 was used to determine the amount of protein contamination of each gDNA sample while the 260/230 ratio was used to determine the amount of salt contamination. The manufacturer (GenTegra LLC, GenTegra DNA product) states their chemistry has an absorbance of 230 nm thus interfering with 230 nm readings. Per the product insert, a correction factor was applied based on the volume of DNA added to the matrix. The correction factor for 150 µL of DNA (the volume added during rehydration) added to the GenTegra-DNA chemistry tube is 6 when the eluate is diH_2O. For example, if the raw 260/230 ratio is 0.31, to apply the correction factor simply multiply by 6. Therefore the adjusted 260/230 ratio is 0.31*6 or 1.86. See Supplemental Table 1 for the raw and corrected 260/230 values. The coefficient of variation (CV) for the 260/280 and 260/230 ratios were determined by running the same specimen (liquid and dried) for three consecutive days. The 260/280 CVs were 2.2% and 2.8% for liquid and dried samples, respectively. The 260/230 CVs were 1.6% and 7.0% for liquid and dried samples, respectively.

2.4. Quantification

Isolated gDNA was quantified using a QuBit 2.0 fluorometer (Thermofisher, Carlsbad, CA) dsDNA BR assay and UV spectroscopy from the NanoDrop according to manufacturer's instructions as previously described [8,9]. Total DNA amount was determined using sample volume and QuBit or NanoDrop determined concentration.

2.5. Measurement of DNA fragmentation

Fragmentation of gDNA was determined using Agilent (Santa Clara, CA) TapeStation 4200 genomic DNA assay following the manufacturer's instructions. The TapeStation plots DNA fragment size (bp) against the sample intensity (FU) and generates a DNA Integrity Number (DIN). gDNA fragment sizes from 200 to 60,000 bp can be analyzed. The DIN is on a scale of 1–10, where 10 represents highly intact DNA [10].

2.6. HLA genotyping and data analysis

Isolated gDNA was HLA genotyped using methodology previously described [8,9]. HLA locus-specific amplicons were generated using TruSight HLA v2 assay (Illumina, San Diego, CA). HLA amplicons were then prepared for sequencing following the manufacturer's instructions and loaded onto a MiSeq for paired-end sequencing (2 × 150 bp). HLA genotyping analysis was performed using Assign for TruSight HLA version 2.1 with IMGT/HLA database 3.26.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism (v7.04). Paired sample analysis was performed when applicable and DNA sample characteristics were analyzed using Wilcoxon matched-pairs signed rank test was used (Prism) for statistical analysis when possible with the nonparametric test. When comparing between HLA loci or when the paired analysis was unwarranted, multiple t-tests were performed and each HLA locus was analyzed individually without assuming a consistent variation between loci. Technical variation was comparable between DNA sources (unpublished observation) except for 260/280 ratios (see Section 4). P values of less than 0.05 were considered significant.

3. Results

3.1. DNA quality characteristics

In order for dried DNA to be a viable alternative to traditional stored DNA, there must be sufficient DNA recovered. Additionally, there must be adequate DNA quality to enable successful completion of downstream applications. To evaluate DNA recovery and purity, dried DNA was reconstituted and the amount of DNA recovered was determined using fluorescent and UV-based quantitation methods (see Section 2). The initial DNA input averaged 5363.4 ng (± 634.2 ng) (UV-based) and samples had an average loss of 1.1% (± 12.7%) on recovery. Comparatively, fluorescent-based DNA determination had an average initial DNA input of 5003.5 ng (± 6.9 ng) and demonstrated a significant loss of an average of 40.5% (± 10.9%) (Fig. 1A). All samples demonstrated lower recovery using fluorescent-based DNA detection compared to only half by UV-based. Comparison of the 260/280 ratio between the liquid and dried samples showed no significant difference with an average of 1.90 (± 0.04) and 1.87 (± 0.23), respectively (Fig. 1B). However, the 260/230 ratio was significantly ($P = 0.005$) different between the liquid and dried stored DNA averaging 1.75 (± 0.26) and 2.12 (± 0.28), respectively (Fig. 1C). The average DNA fragment length was significantly ($P = < 0.0001$) longer for liquid DNA (DIN = 8.4, ± 0.46) compared to dried DNA (DIN = 7.8, ± 0.54) (Fig. 1D and E). There were no observable differences between dried and liquid samples when comparing gel electrophoresis amplification patterns (data not shown). Therefore, dried samples had sufficient DNA quality and quantity recovered for downstream applications such as NGS.

3.2. HLA genotyping metrics

HLA typing via NGS has specific requirements in order to achieve successful long-range PCR amplification. The dried DNA, even though it did have a significant recovery loss, does still yield enough DNA to proceed to HLA typing. HLA typing from the dried DNA should not be significantly impacted given the DNA characteristics found in Fig. 1. The HLA typing by NGS was 100% concordant (121/121 unique alleles, 357/357 total alleles) (Table 1) between the two sample types for *HLA-A, -B, -C, -DRB1/3/4/5, -DQA1, -DQB1, -DPA1, -DPB1* (all loci tested). The mean Q30 was 97.3 (± 0.4) from the dried DNA compared to 95.3 (± 0.6) from the liquid DNA with all loci showing a higher Q30 on the dried tubes (Fig. 2A). The average depth of coverage (DOC) for liquid DNA (all loci, 240.4, ± 10.8) was not significantly different from the dried tubes (all loci, 234.7, ± 9.9) (Fig. 2B). The average allele balance was not significantly different between the dried (44.1%, ± 2.6) and liquid DNA (43.5%, ± 2.6) (Fig. 2C). Average PCR crossover is shown in Fig. 2D and significantly varied depending on HLA locus (ranging from 0.2% to 10.2%). *HLA-A* had the most PCR crossover (10.2% dried and 9.6% liquid), while *HLA-DQA1* had the least (0.2%, both sample sources). When averaging all loci together the PCR crossover rates between liquid and dried DNA were quite similar at 3.4% (± 2.8%) and 3.5% (± 2.8%) respectively.

Overall, liquid DNA performed better on average DOC (240.4 vs 234.7) and PCR crossover (3.4% vs 3.5%) while dried DNA had a better allele balance (44.1 vs 43.5) and mean Q30 (97.3 vs 95.3). Exceptions to these trends are noted below. The average DOC for liquid DNA was consistently higher than the dried DNA except for *HLA-DQB1* (228.5 vs

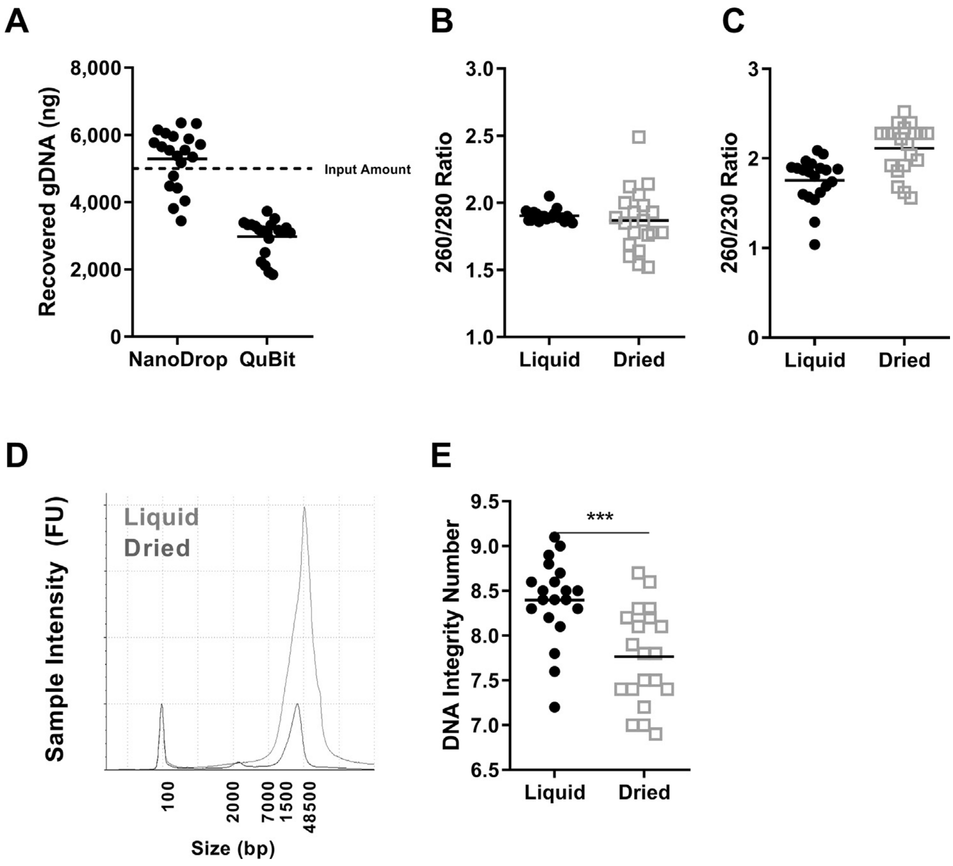

Fig. 1. Properties of dried and liquid stored genomic DNA. (A) Twenty samples were split for genomic DNA storage either as liquid or dried DNA. A total of 5,000 ng was input for storage. The recovery of the DNA after storage was determined by UV (NanoDrop) or fluorescent (QuBit) detection. Dotted line indicates input amount. The 260/280 ratio (B) and 260/230 (C) for the twenty samples were determined from NanoDrop. The level of genomic DNA fragmentation was determined using TapeStation 4200 gDNA assay and fragmentation quantitated by DNA integrity number. (D) Representative gDNA tracings for liquid and dried stored samples. (E) The individual DNA integrity numbers. Bars indicated mean of each condition. ***, $P < 0.001$.

227.9) and *HLA-DRB5* (243 vs 240, n = 1). The average allele balance for the HLA loci are within one percentage point except for *HLA-DPA1*, *-DQB1*, and *-DRB1*. Dried samples had allele balance percentages closer to the ideal 50:50 ratio than the liquid samples on *HLA-DPA1* (45.8% vs 44.2%) and *HLA-DQB1* (41.4% vs 37.9%), while on *HLA-DRB1* locus the liquid DNA was higher than dried (41.7%. vs 39.8%). Only *HLA-C* and *-DPB1* had PCR crossovers that differed by greater than 1% for each sample type. Dried samples had an average PCR crossover of 6.7%

Table 1
HLA alleles identified in the study. –; represents ambiguity at that resolution. XX; represents novel allele identified.

	HLA locus A*	B*	C*	DPA1*	DPB1*	DQA1*	DQB1*	DRB1*	DRB3*	DRB4*	DRB5*	TOTAL
COUNT	15	26	17	5	14	11	10	16	3	3	1	121
	01:01:01:01	07:02:01	01:02:01	01:03:01:03	01:01:01	01:01:01:–	02:01:01	01:01:01	01:01:02:–	01:01:01:01	02:02	
	02:01:01:01	08:01:01:–	02:02:02:01	02:01:01:–	02:01:02	01:02:01:–	02:02:01:01	03:01:01:–	02:02:01:–	01:03:01:–		
	02:05:01	13:02:01	03:03:01:01	02:02:02	03:01:01	01:05:01	02:10	04:01:01:–	03:01:03	01:03:01:02N		
	03:01:01:01	14:01:01	03:04:01:02	03:01	04:01:01:–	02:01:01:–	03:01:01:–	04:03:01				
	11:01:01:–	15:01:01:–	04:01:01:01	04:01	04:02:01:02	03:01:01	03:02:01:–	04:04:01				
	23:01:01	15:09	05:01:01:02		05:01:01	03:02	03:03:02:–	04:05:01				
	24:02:01:01	15:11:01	06:02:01:01		06:01:01	03:03:01:–	04:02:01	07:01:01:–				
	26:01:01:01	18:01:01:02	06:06		105:01	05:01:01:02	05:01:01:–	09:01:02				
	29:02:01:02	27:05:02	07:01:01:01		11:01:01	05:05:01:–	06:02:01:–	11:01:02				
	30:01:01	27:06	07:02:01:01		13:01P	05:11	06:04:01	11:03:01				
	30:02:01:–	35:01:01:–	07:04:01:01		14:01:01	06:01:01		11:11:01				
	31:01:02:01	35:03:01:–	07:112		17:01			12:01P				
	36:01	40:01:02	08:02:01:02		18:XX			12:02:01				
	68:02:01:–	40:02:01	08:04:01		296:01			13:02:01				
	74:01:01	44:02:01:–	12:03:01:01					13:03:01				
		44:03:01:–	15:02:01:01					16:02:01:–				
		44:18/45:01	16:01:01:01									
		45:01:01										
		49:01:01										
		50:01/49:01										
		51:01:01:–										
		53:01:01										
		56:01:01:–										
		57:01:01										
		58:01:01:–										
		81:01										

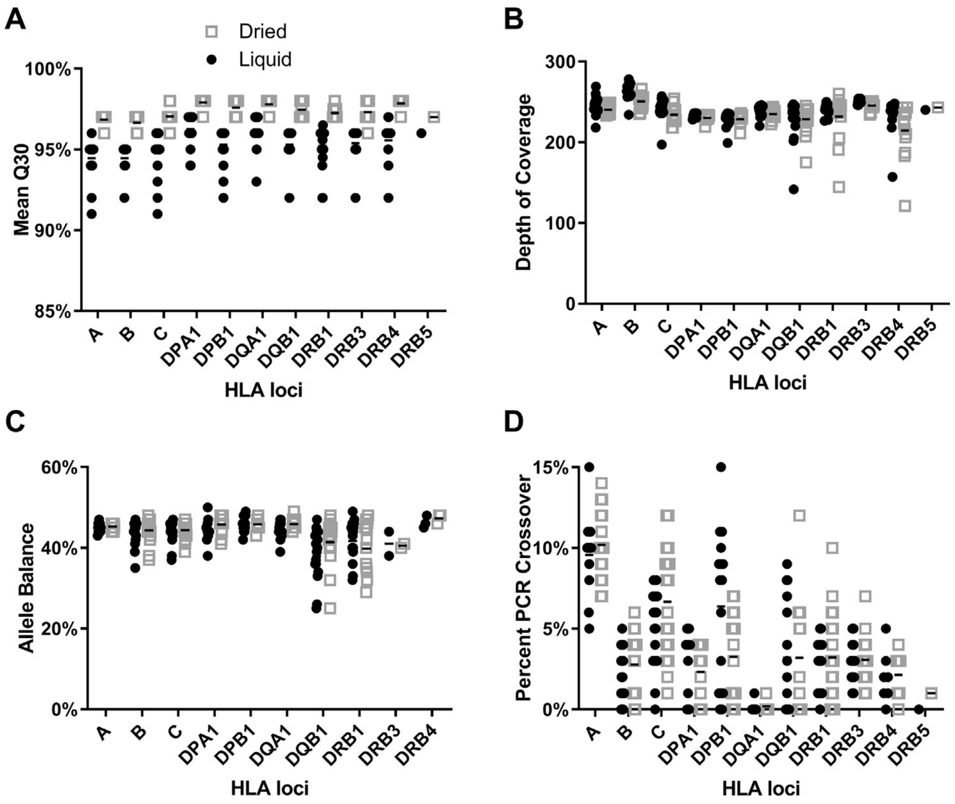

Fig. 2. Next-generation sequencing (NGS) data characteristics for dried and liquid stored samples. Following NGS the mean Q30 (A), depth of coverage (B), allele balance (C), and PCR crossover (D) were determined using Assign for TruSight HLA. Liquid stored samples (filled circles) and dried (open squares). No significant differences were identified between the two storage conditions.

(± 3.4%) for *HLA-C* compared to 5.1% (± 2.5%) for liquid stored samples, while *HLA-DPB1* dried average is 3.3% (± 2.5%) and liquid having an average of 6.4% (± 4.3%) (Fig. 2D). The differences of these metrics show no effect to the HLA genotyping.

4. Discussion

Determining the needs of the laboratory and the DNA specifications required should impact the decision making regarding the use of dried DNA. The data presented here shows that dried storage of DNA is a viable option for laboratories that will utilize long-range PCR for their downstream HLA typing assays. The characteristics between the liquid and dried DNA (Fig. 1) show no significant difference in the 260/280 ratio (Fig. 1B) but increased 260/230 ratio (Fig. 1C) as well as significantly different in DNA recovery (Fig. 1A) and DNA fragment length (Fig. 1E). However, there was significant variation between samples for 260/280 ratio (Fig. 1B). The significance of this variation may indicate increased susceptibility to PCR inhibition by protein contaminates on dried samples particularly within difficult to HLA type samples (i.e. buccal swabs).

The recovery loss was up to 40.5%; however, the yield was still within an acceptable range for NGS testing utilizing long-range PCR. Previous studies noted that recovered DNA from umbilical cord [1] and whole blood (EDTA tubes) [2] was sufficient for downstream HLA typing, however, no data was presented indicating the percent recovery. There is a large difference observed between the UV spectroscopy and fluorescence-based recovery of gDNA (Fig. 1A). Fluorescence-based methods only measure dsDNA, meaning contaminating ssDNA, RNA, salts, and protein have the potential to affect the UV spectroscopy reading, as demonstrated by previous studies [11,12]. When calculating the mass needed for assays, using a fluorescence-based method will provide a more accurate measure of high-quality DNA. DNA fragment length is a good predictor of a samples HLA typing success [8]. There was a significant drop in DNA fragment length of the dried samples; however, the average DIN (7.8) is within the range of long DNA fragments [8,10]. Importantly, there was consistent PCR amplification between the sample types indicating.

Liquid DNA performed better on DOC and PCR crossover while dried DNA had a better allele balance and mean Q30, however, none demonstrated a significant difference (Fig. 2D). There were some outliers to these trends. Allele balance was more even on liquid DNA for *HLA-DRB1*. The DOC of dried samples was better on *HLA-DQB1* and *-DRB5* than liquid samples. Even though PCR crossover was not consistently better for one source over another there were greater differences on *HLA-DPB1* where dried DNA performed better and *HLA-C* where liquid DNA was better. *HLA-DRB1/3/4/5* is a multiplexed amplification which some researchers noted in other NGS assays is more susceptible to mispriming [13]. The differences in the allele balance on *HLA-DRB1* and DOC on *HLA-DRB5* may be an indication of such susceptibility. Our previous work, as well as other publications, indicate that *HLA-DPA1, -DPB1*, and *-DQB1* are the most difficult HLA loci to genotype [8,14–16]. This is attributed in part to the size of those amplicons in the NGS assay. Since the variation of the NGS metrics is primarily in these loci, our data indicates that these loci are more susceptible to fluctuation in sequencing quality resulting from the sample source.

Previous studies which analyzed the impact of extreme temperature variations on the dry-state storage samples from umbilical cord blood and EDTA tubes were able to achieve accurate HLA typing [1,2]. Our data show that dried storage of gDNA is effective and stable for use with long-range DNA amplification methods such as HLA typing. The integrity of the DNA was maintained which allowed for the success of long-range PCR amplification. The results indicate that RT stored dried DNA may be suitable for long-term storage of gDNA and that the recovered DNA is suitable for HLA typing. The variations between the two storage types did not hinder any laboratory applications. A limitation of the current study is that uses gDNA isolated from peripheral blood and other gDNA sources were not evaluated (i.e. buccal swab, saliva, tissue). Long-term storage (i.e. years) and suitability for HLA typing were not evaluated in this study (see Section 2). Additionally, other

NGS HLA typing assays rely on long-range PCR amplicons or targeted exon amplification were not evaluated. Additional testing may also be beneficial in order to provide more insight into dried DNA storage for other HLA applications such as real-time PCR.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2018.12.002.

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