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Influence of pre-analytical procedures on genomic DNA integrity in blood samples: The SPIDIA experience

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ABSTRACT

Background: DNA integrity is a critical part of the definition of genomic DNA (gDNA) quality and can influence downstream molecular applications. Pre-analytical variables as sample storage and DNA extraction methods can influence the quality and quantity of isolated DNA and affect molecular test performances. The aim of this paper is to investigate the role of blood sample storage and DNA extraction procedures on gDNA integrity and gDNA fragmentation impact on a molecular test.
Methods: 157 DNA samples deriving from the Pan European 1st SPIDIA DNA External Quality Assessment (EQA), aimed to investigate the influence of blood storage on gDNA quality and quantity, have been analyzed by Pulsed Field Gel Electrophoresis and ImageJ imaging software. 157 DNA samples derived from the Pan European 1st SPIDIA DNA External Quality Assessment (EQA), which aimed to investigate the influence of blood storage on gDNA quality and quantity, have been analyzed by Pulsed Field Gel Electrophoresis and ImageJ imaging software.
Results/conclusions: Our results demonstrate that blood sample storage and DNA extraction procedures influence gDNA integrity and that the same blood sample which underwent a long range multiplex PCR based analytical test can provide different results if the adopted pre-analytical procedures are not standardized.

1. Introduction

Molecular biology-based procedures have opened new perspectives in diagnosis, prognosis and therapeutic treatments, but the level of standardization of these technologies is often lower than in other areas of laboratory medicine, in particular, despite the increasing number of publication related to the development of standard operating procedures for biobanking [1,2] the effects of pre-analytical factors on analytical performances of molecular test are still poorly investigated [3].

Pre-analytical variables such as storage (time and temperature) and DNA extraction methods may influence the quality and quantity [4,5] of isolated DNA and can affect analytical test performances.

Genomic DNA (gDNA) integrity plays a critical role for the definition of DNA quality and can influence downstream molecular applications, such as comparative genomic hybridization (CGH) [6,7] or whole genome sequencing approach [8,9]. Several factors can affect the gDNA integrity, mainly represented by pre-analytical procedures as sample DNA storage [1], repeated freeze-thawing [10], retention to the tubes [11], evaporation and/or denaturation [12]. Additional factors as humidity, temperature, persistence of nucleases and other chemical agents as well as other suboptimal conditions that may occur during transportation and during DNA extraction could also compromise the DNA integrity [13,14].

Overall gDNA quality is also determined by short- or long-term blood or tissue storage; the whole blood (or tissue) preservation [4] and the consequent DNA integrity status can deeply modify and induce mistakes when the aim of the experiment is to estimate the DNA integrity itself [15,16].

In tissue samples different reagents and procedures for tissue preservation (i.e. tissue fixatives, processing protocols, and storage conditions, pH) can affect the quality and quantity of DNA [17,18]. Regarding blood samples, the procedures and the reagents of pre-analytical phase affecting the integrity of the isolated gDNA, in

Abbreviations: gDNA, genomic DNA; HMW, High Molecular Weight; SPIDIA, Standardization and improvement of generic Pre-analytical tools and procedures for In vitro DIAgnostic; EQA, External Quality Assessment; PFGE, Pulsed Field Gel Electrophoresis; TCR, T cell receptor.

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particular High Molecular Weight (HMW) DNA, are not completely investigated.

SPIDIA (www.spidia.eu), a four year integrated project funded by the European Commission, was aimed to develop quality guidelines and tools for in vitro molecular diagnostics and standardize the pre-analytical process with the net result of improving the stabilization and handling of biological samples. An essential part of this project was the implementation of External Quality Assessment schemes (EQAs) for the collection, transport and processing of blood samples for RNA [19] and DNA-based analyses.

We have already reported the description and the results of 1st SPIDIA DNA EQA [20], a large, Pan European trial, involving 197 laboratories from 29 countries. The aim of this trial was to investigate the influence of blood storage on gDNA quality and quantity. To carry out the External Quality Assessment, the SPIDIA facility, Florence, Italy, sent the same blood sample to all participants for processing. In this EQA, no time or temperature limitations and extraction method procedures were imposed. Purified DNA from each of the participating laboratories was then shipped back to the SPIDIA facility. DNA quality parameters performed at the SPIDIA facility included UV spectrophotometric analysis of DNA purity and yield, analysis of PCR inhibition, a qPCR assay for DNA quantification and the DNA integrity by Pulsed Field Gel Electrophoresis (PFGE).

The aim of this paper is to deeply investigate the role of gDNA fragmentation measured in the 1st SPIDIA DNA EQA samples on some considered pre-analytical factors and its influence on the results of an analytical multiplex PCR test based on semi-quantitative long range PCRs to detect and characterize the repertoire recombination of human T lymphocyte receptor [21,22].

2. Material and methods

2.1. 1st SPIDIA DNA EQA

2.1.1. Scheme design

Details of the 1st SPIDIA DNA EQA are reported by Malentacchi et al. [20]. Blood was collected from two consented, adult donors (tested negative for HIV, HBV and HCV) after approval by the Institutional Committee of Azienda Ospedaliero-Universitaria Careggi (Florence, Italy). The donors signed a written informed consent. Briefly, 350 ml of blood was collected from each donor using a classical phlebotomy procedure in a CPDA-containing blood collection bag. After collection, blood was pooled in a sterilized flask, gently stirred, and immediately aliquoted into 2 ml Polypropylene tubes (BioClass). The shipping was performed by an international courier the day after the blood collection, and the blood-containing tubes were stored at 4 °C until packaging and shipment. Shipping boxes contained a frozen soft gel ice pack to maintain cooled conditions during shipping. The participating laboratories extracted DNA following their standard procedure with no restrictions concerning sample storage temperature or time. The participants recorded detailed information about storage conditions and procedure used during the DNA extraction phase in the on-line Results form. As concerns the temperature of blood storage, the majority of the participants (83.4%) stored blood sample at 4 °C before DNA extraction.

After DNA extraction, they sent back DNA samples to the SPIDIA facility where samples were stored at −20 °C until analysis. Ninety-four percent (94%) of the laboratories sent a DNA sample at 4 °C.

2.1.2. Evaluation of gDNA integrity

In order to test the HMW integrity, DNA samples have been analyzed by (PFGE) [20].

Briefly, 800 ng of DNA was analyzed using a 1% agarose gel (Ultra Pure Agarose, Invitrogen), 0.5× TBE buffer (45 mM Tris, 45 mM Borate, 2.5 mM EDTA) and a CHEF DRII system (BioRad). Low Range PFG Marker (2.03–194 kb; New England Biolabs) was used as a DNA size ladder marker. Electrophoresis was performed for 16 h at 10 °C with 6 V/cm and a switch time of 1–12 s. The gel was stained for 30 min using 0.5 μg/ml ethidium bromide solution and destained for 1–2 h in distilled water. Documentation was performed using the EASY Win32 system (Herolab).

The analysis of DNA integrity has been performed by analyzing PFGE gel with the ImageJ imaging software (www.rsbweb.nih.gov/ij), as described by Malentacchi et al. [20]. The ImageJ peak corresponding to the size of DNA fragments that are most represented in the sample, was used for the evaluation of gDNA integrity.

2.2. Time course experiment

At the SPIDIA facility, DNA extraction was performed in a subset of blood samples from the collection blood bag used for the External Quality Assessment immediately after the blood draw (Time 0, T0) and after 1, 3 and 7 days (T1, T3, T7, respectively) storing blood at 4 °C by three different methods: EZ1 DNA Blood 350 μl kit (Qiagen); QIAamp DNA Blood Mini kit (Qiagen); and Gentra Pure Gene Blood kit (Qiagen), following manufacturer's instruction.

2.3. gDNA integrity on the downstream analytical test

2.3.1. Combinatorial recombination analysis: multiplex PCR

Repertoire recombination of human T lymphocyte receptor has been performed in a subset of 26 SPIDIA DNA samples using DNALymphoCheck™ test (ImmunID Technologies, Grenoble, France) based on a long range multiplex PCR. DNALymphoCheck™ is a test, selected to evaluate gDNA integrity impact on its downstream analytical process examining sample material from the human body. PCR products were generated using iProof GC rich Master Mix (BioRad). The cycling condition followed a touchdown PCR cycle protocol according to manufacturer's instruction. In order to perform semi-quantitative analysis, PCR reactions were stopped at the exponential step of the PCR. In order to normalize DNA quantity in each reaction the actin gene was amplified in the same PCR run.

The multiplex PCR was performed using an upstream primer specific for a given V family of gene segment and a downstream primer shared by J gene segment. This assay allowed the simultaneous detection of several V–J rearrangements in the same reaction. The multiplex PCR allowed to perform twenty-three different reactions covering the possible recombination that could be clustered in the following rearrangement characterized by different amplicon lengths as follows: J2.7 (250–490 bp), J2.6 (700 bp), J2.5 (820 bp), J2.4 (950 bp), J2.3 (1100 bp), J2.2 (1380 bp), J2.1 (1500 bp), J1.6 (2600 bp), J1.5 (3100 bp), J1.4 (3300 bp), J1.3 (4000 bp), and J1.2/J1.1 (4500 bp). All V–J PCR amplification products were separated according to their size by high resolution electrophoresis separation on a Lapchip® GX (PerkinElmer, USA) using a microfluidic technology. PCR signals were detected and analyzed using in-house developed software (ImmunID Technologies) that generates an overall evaluation of percentage of observed T cell receptor (TCR) gene recombination (% TCR).

2.4. Statistical analysis

2.4.1. 1st SPIDIA DNA EQA — lab performance evaluation

Details of the adopted statistical procedure are reported by Malentacchi et al. [20]. Briefly, a two-step statistical procedure based on a distribution-free approach was adopted in order to process the quality parameter variables. Specifically, for the ImageJ peak (kb) data the 5th and 20th bootstrap centiles were adopted to identify the one-sided Action Limit (AL) and Warning Limit (WL), respectively. According to these limits the performance of each participant (lab-performance) was classified as (i) *out of control* (*high fragmented*), if the ImageJ peak value was below the one-sided AL (black dots), (ii) *warning* (*intermediate fragmented*), if the value is between the one-sided WL

and AL (gray dots) or (iii) *in control* (*low fragmented*), if the value exceeds the one-sided WL (white dots).

2.4.2. 1st SPIDIA DNA EQA — relationship between the gDNA integrity and pre-analytical factors

The relationship between extraction method (categorized as bead-based, column-based and precipitation-based), blood sample storage (defined as the time interval between blood collection at the SPIDIA facility and DNA extraction by each participant laboratory and categorized as ≤6 days, 6–10 days, ≥10 days), and gDNA integrity was investigated by using a non-parametric approach (Kruskal–Wallis Test) [23]. To account for multiple comparisons, a Bonferroni correction p-value was computed.

2.4.3. Influence on gDNA integrity on the downstream analytical test

The relationship between the number of successful PCRs and the amplicon length was investigated by means of the non-parametric Kruskal–Wallis Test [23]. The influence of gDNA integrity on the DNALymphoCheck™ test results was assessed, in terms of correlation, by the Spearman correlation coefficient (ρ_s) and its corresponding 95% confidence interval (CI) computed according to the bias-corrected and accelerated (BCa) bootstrap method (95% CI_{BCa}) [24].

All statistical analyses were performed using SAS software v. 9.2 (SAS Institute).

3. Results

3.1. Evaluation of HMW DNA integrity

3.1.1. Evaluation of High Molecular Weight DNA integrity by PFGE

One hundred and fifty seven (157) SPIDIA DNA samples have been analyzed by PFGE: Fig. 1 shows PFGE image obtained in a subset of 25 SPIDIA DNA samples (panel B). HMW DNA integrity showed a high variability probably reflecting the influence of some pre-analytical factors, such as DNA extraction procedures and/or time-interval from blood collection (from 2 to 40 days) to DNA isolation, in particular when compared to the PFGE image obtained from the T0 sample extracted by Gentra Pure Gene Blood kit (Qiagen) in the time course experiment (panel A).

3.1.2. Evaluation of High Molecular Weight DNA integrity by ImageJ software

157 SPIDIA DNA samples have been analyzed using ImageJ software in order to obtain numerical data from gel electrophoresis pictures. The procedure has been already reported by Malentacchi et al. [20]. The distribution of the ImageJ peak (kb) in the SPIDIA DNA samples is reported in Fig. 2 (median 20.319, Interquartile range [IQR] = 4.346). In Fig. 2 the Action Limit (AL, corresponding to 14.15 kb) and the Warning Limit (WL, corresponding to 17.83 kb) are also indicated.

3.2. Relationship between gDNA integrity and pre-analytical factors

3.2.1. Influence of extraction method on HMW DNA integrity

By using the ImageJ peak (kb) as a measure of gDNA integrity, a statistically significant difference was observed depending on the method used for the DNA isolation (Kruskal–Wallis p-value = 0.006). By performing all the pair-wise comparisons between extraction methods, bead-based vs column-based resulted the only comparison statistically significant after the Bonferroni correction (Bonferroni p-value = 0.012).

On the basis of the distribution of the ImageJ peak within each method reported in Fig. 3, we observed that samples extracted by precipitation methods showed the higher median value (median: 22.470) and the narrowest distribution (IQR: 3.626) in comparison to magnetic beads (median: 21.988, IQR: 4.258) and column-based methods (median: 19.850, IQR: 4.406).

3.2.2. Influence of blood time storage on HMW DNA integrity

As concerns the blood sample storage duration, defined as the time interval between blood collection and DNA extraction, we observed a statistically significant trend (Kruskal–Wallis p-value < 0.001) of gDNA integrity. In details, there is a relevant discrepancy between values obtained in sample extracted within 6 days from blood collection compared to those obtained in the samples extracted between 6 and 10 days (Bonferroni p-value < 0.001) and after 10 days (Bonferroni p-value = 0.005). In particular, as reported in Fig. 4, the median value of the ImageJ peak is the highest (median: 22.484, IQR: 2.682) in blood samples stored up to 6 days.

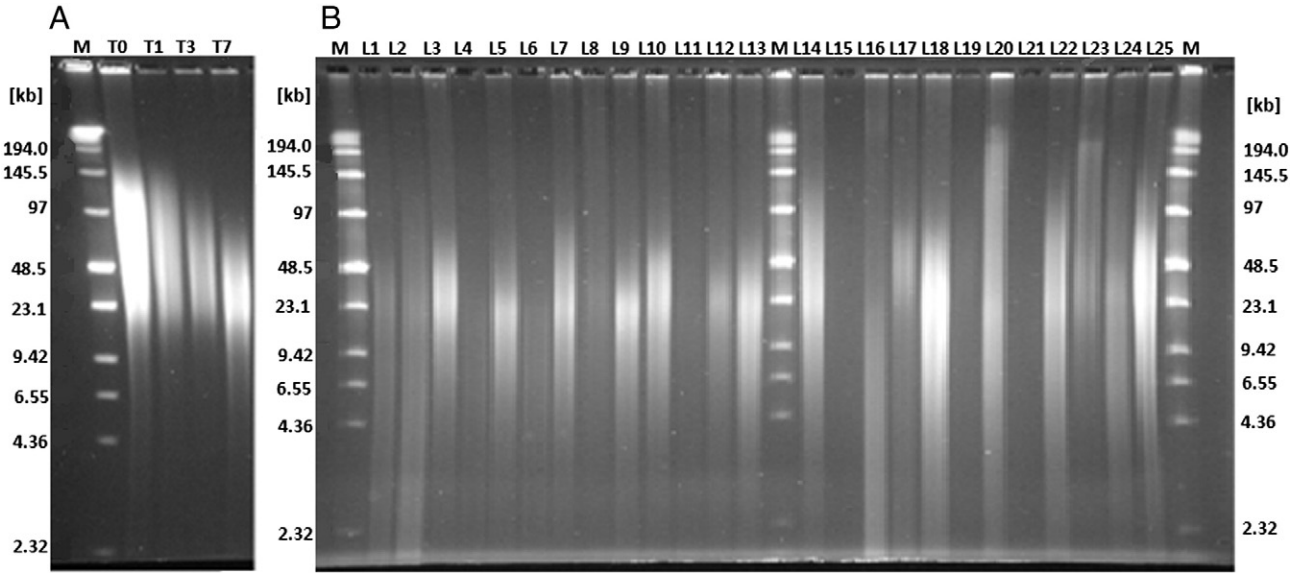
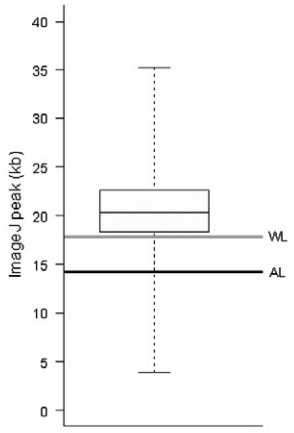


Fig. 1. HMW DNA integrity evaluated by Pulsed Field Gel Electrophoresis (PFGE). (A) Time course: samples have been extracted by Gentra Pure Gene Blood kit (Qiagen) at different time points. M = ladder marker; T0 = DNA extracted immediately after blood draw; T1 = DNA extracted 1 day; T3 = DNA extracted 3 days; T7 = DNA extracted 7 days after blood collection. (B) A subset of 25 SPIDIA-DNA samples. M = ladder marker, L1 to L25 = 1st SPIDIA DNA EQA samples.



	n	min	25th centile	median	75th centile	max	IQR*
ImageJ peak (kb)	157	3.905	18.305	20.319	22.651	35.220	4.346

* IQR: Interquartile range

Fig. 2. Distribution of ImageJ peak in the 1st SPIDIA DNA EQA samples. The box indicates the 25th and 75th centiles of the ImageJ peak (kb) distribution. The horizontal line inside the box indicates the median, and the whiskers indicate the extreme measured values. The black horizontal line represents the Action Limit (AL) that corresponds to the 5th bootstrap centile of the outlier-free distribution, whereas the gray horizontal line represents the Warning Limit (WL) which corresponds to the 20th bootstrap centile of the same distribution. The table reports the descriptive statistics.

3.3. Influence of gDNA integrity on the downstream analytical test

3.3.1. Influence of High Molecular Weight DNA on multiplex PCR assay (long range PCR-based)

To investigate the role of HMW DNA integrity on the long range PCR amplification results we performed the multiplex PCR to determine the T cell receptor gene recombination on a subset of 26 SPIDIA-DNA samples.

We analyzed the number of positive PCRs obtained for each 12 amplicon length clusters. As explained, each amplicon length cluster was obtained by a specific multiplex PCR procedure. The number of successful PCRs significantly changes according to the amplicon length (Kruskal–Wallis p-value < 0.001, Fig. 5). For explorative purpose, the distribution of the number of successful PCRs for each amplicon length according to the lab-performance judgment is also depicted. High fragmented samples (peak classified as out of control in the lab-performance evaluation — black dots) tend to have a lower number of PCRs compared to those classified as in control/warning (white and gray dots), particularly in the clusters of high amplicon length (from 3100 bp to 4500 bp) showing the impact of fragmentation on the long range PCR amplification but not on small length PCR amplicons.

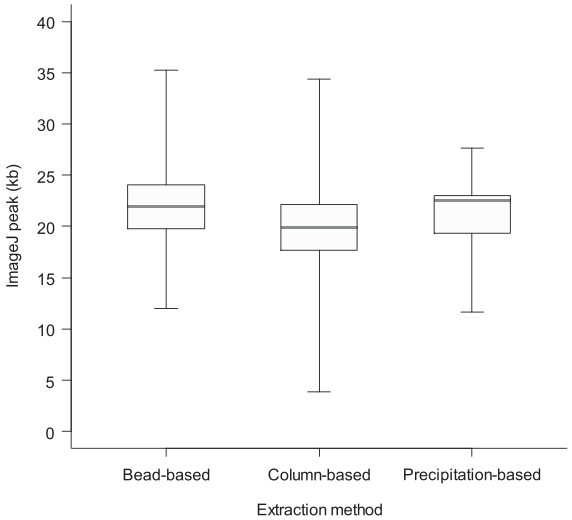
3.3.2. Influence of High Molecular Weight DNA on an analytical test results

Fig. 6 shows the pattern of correlation between the ImageJ peak and % TCR on a subset of 26 samples of SPIDIA-DNA EQA. An overall positive correlation between ImageJ peak and % TCR ($\rho_s = 0.447$, 95% CI_{BCa}: 0.06–0.765) was observed. Of note, the out of control samples are those characterized by lowest values for both ImageJ peak and % TCR. On the contrary, samples with high ImageJ peak (i.e. in control) have a high % TCR.

4. Discussion

Obtaining high-quality DNA of an appropriate size distribution is a fundamental aspect to many technology platforms used in genomic analysis. Several analytical methods, included the most recent high-throughput NGS technologies, rely on pre-analytical procedures that provide sufficient yields of targeted, high-quality nucleic acids. NGS quality control requisites are rather stringent and require extracted High Molecular Weight DNA samples, including no evidence of contamination from protein, RNA or phenol [25].

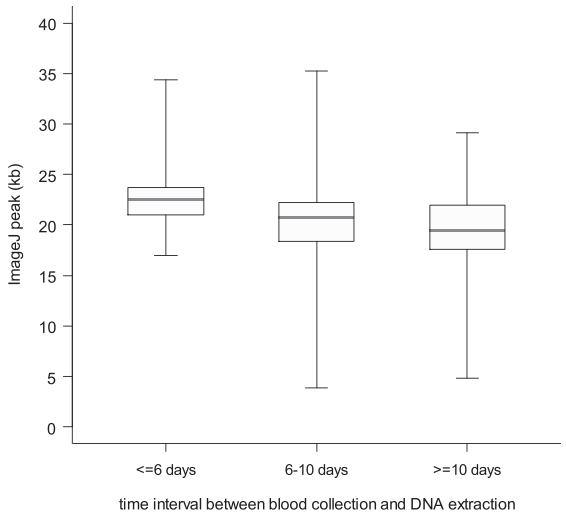
Pre-analytical variables, including storage temperature and time and DNA extraction methods, may affect the quality of isolated DNA for molecular application [2] and thus affecting analytical results [26].



	n	min	25th centile	median	75th centile	max	IQR*	p-value†
Bead-based	33	11.955	19.816	21.988	24.074	35.220	4.258	
Column-based	80	3.905	17.692	19.850	22.098	34.334	4.406	0.006
Precipitation-based	20	11.608	19.348	22.470	22.975	27.630	3.626	

*IQR: Interquartile range
† p-value of the Kruskal-Wallis test

Fig. 3. Distribution of ImageJ peak in the 1st SPIDIA DNA EQA samples according to the extraction method. Each box indicates the 25th and 75th centiles of the ImageJ peak (kb) distribution according to the extraction method used for the DNA isolation. The horizontal line inside the boxes indicates the median, and the whiskers indicate the extreme measured values. The table reports the descriptive statistics.



	n	min	25th centile	median	75th centile	max	IQR*	p-value†
≤6 days	33	16.995	21.001	22.484	23.683	34.334	2.682	
6-10 days	55	3.905	18.305	20.707	22.317	35.220	4.012	<0.001
≥10 days	45	4.835	17.615	19.426	21.988	29.147	4.373	

*IQR: Interquartile range
† p-value of the Kruskal-Wallis test

Fig. 4. Distribution of ImageJ peak in the 1st SPIDIA DNA EQA samples according to the blood sample storage duration. Each box indicates the 25th and 75th centiles of the ImageJ peak (kb) distribution according to the blood sample storage duration (time interval from blood collection and DNA extraction). The horizontal line inside the boxes indicates the median, and the whiskers indicate the extreme measured values. The table reports the descriptive statistics.

An important part of the SPIDIA project, a four-year integrated project granted by the European Commission, aimed at developing quality guidelines for molecular in vitro diagnostics and to standardize the pre-analytical process in related procedures, was the implementation of External Quality Assessment schemes (EQAs) for the collection, transport and processing of blood samples for RNA and DNA-based analyses [19,20]. On the basis of the results obtained in the SPIDIA DNA EQA [20] , we investigated the role of pre-analytical phase on gDNA integrity.

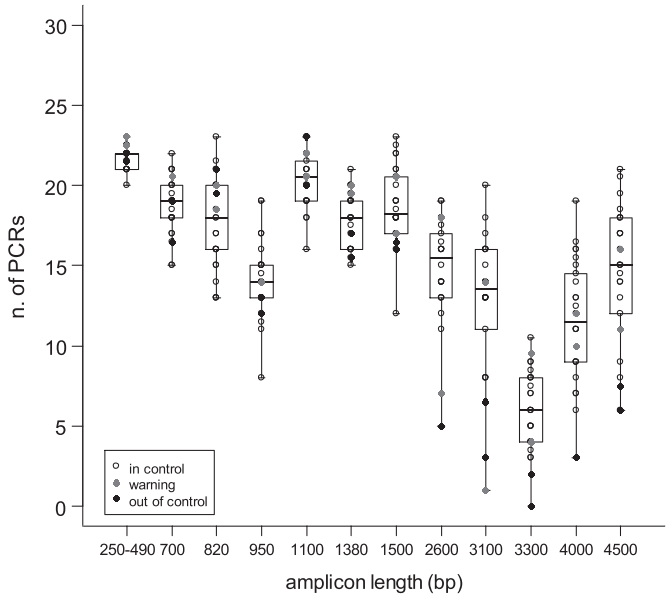


Fig. 5. Distribution of the number of PCRs according to the amplicon length (bp). Each box indicates the 25th and 75th centiles of the number of PCRs according to the amplicon length (bp) on the subset of 26 SPIDIA DNA samples. The horizontal line inside the boxes indicates the median, and the whiskers indicate the extreme measured values. Each individual value is represented by a dot according to the lab-specific performance judgment.

DNA integrity can be evaluated using a gel electrophoresis technique: 0.8% agarose gel gives a general evaluation of DNA molecular size (kb) and the DNA electrophoretic pattern (i.e. presence of apoptotic electrophoretic pattern) whereas PFGE is a technique used for the separation of large (from kb to Mb) DNA molecules by applying to a gel matrix an electric field that periodically changes direction. The results of both these techniques are usually evaluated by experts by a "by-eyes" analysis.

In the SPIDA DNA EQA, we performed the analysis of the DNA integrity by using the PFGE gels with the ImageJ imaging software, in order to transform the experimental data (PFGE image) on a numerical scale. The ImageJ peak corresponds to the size of DNA fragments that are most represented in the sample [20].

On the basis of the PFGE results (Fig. 1) and of the ImageJ peak distribution (Fig. 2) of the SPIDIA DNA EQA samples, we observed that the same blood sample, that had been sent by the SPIDIA facility to the SPIDA participants for gDNA isolation, showed a high variability in

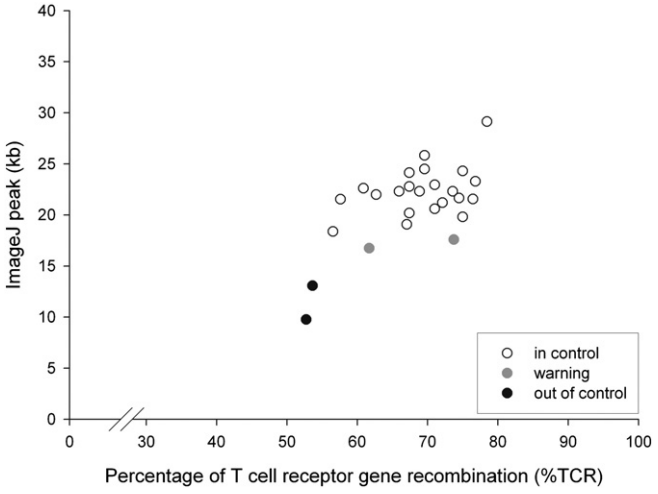


Fig. 6. Scatter plot of ImageJ peak (kb) and % of T cell receptor gene recombination. Each individual value is represented by a dot according to the lab-specific performance judgment.

terms of gDNA fragmentation, depending on pre-analytical procedures (blood storage and DNA extraction methods).

The gDNA isolation procedure seems to play a role on gDNA integrity as a statistically significant difference was observed by comparing the ImageJ peak distribution among the extraction procedures used by SPIDIA participants. The role of the extraction method on gDNA integrity has been also confirmed in an internal time course experiment in which high DNA integrity has been obtained by salting out precipitation based procedure in comparison to magnetic beads and column-based DNA isolation methods (data not shown). As concerns the participating laboratories (Fig. 3), the distribution of samples extracted by precipitation methods (almost all were salting-out precipitation procedures) showed a higher median value ImageJ peak and a lower variability in comparison to magnetic beads and column-based procedures ("column based", a category that includes mostly silica-based membrane columns) even if it was not statistically different from that of the other two methods.

The results reported in Fig. 3 have been necessarily categorized in groups that include different commercial kits with very different features. If HMW DNA is mandatory for the downstream analytical tests (i.e. CGH), the use of dedicated kits that isolate HMW DNA (commercially available in the market) should be used.

The blood storage conditions also play a role on gDNA integrity. In particular the influence of the storage duration has been investigated. The participant laboratories that extracted gDNA within 6 days from blood collection, obtained a higher DNA size in comparison to the DNA samples isolated after 6 days (Fig. 4). No differences have been observed by analyzing the DNA yield within and after 6 days from blood collection (data not shown). Six days could represent a large time interval in an ordinary molecular laboratory routine, but SPIDIA participants were asked to isolate DNA without any temperature or time limitation and, moreover, the delay from the blood collection was also partially due to some logistical aspects of the External Quality Assessment (i.e. shipment and delivery of blood sample). The influence of the storage duration on gDNA fragmentation has been observed also in the SPIDIA time course experiment, in which, having extracted the gDNA by precipitation procedure at different time point from the collection, a slight fragmentation seemed to appear since one day after blood collection with a strong fragmentation after 7 days (Fig. 1 panel A).

Finally we have evaluated the influence of gDNA integrity on a downstream analytical test. The downstream long range multiplex PCR test has been selected for this purpose as it is based on twenty-three different multiplex PCR reactions, of different amplicon lengths, covering the possible rearrangements. This assay was used for further analysis on a subset of 26 SPIDIA DNA samples.

Fig. 5 shows the analysis of gDNA fragmentation on the long range PCR amplification of V–J cluster ranging from 299 bp to 4500 bp. As expected, short amplicons were not influenced by the DNA integrity (same number of successful PCRs independently from lab performance evaluation). For longer amplicons (higher than 1500 bp), high fragmented samples (peak classified as out of control in the lab-performance — black dots) tend to systematically have a lower number of successful PCRs compared to those classified as in control (high gDNA integrity).

Our results demonstrated the influence of HMW gDNA on the used analytical test results, based on long range multiplex PCRs. ImageJ peaks and the percentage of T cell receptor gene recombination (% TCR) showed an overall positive correlation (Fig. 6); the out of control samples were characterized by lowest values for both ImageJ peak and % TCR. On the contrary, samples with high ImageJ peak (in control lab performance evaluation) had a high % TCR. These results show that the same blood sample (used in the SPIDIA EQA), which underwent an analytical test based on long range multiplex PCRs, can provide different results if the adopted pre-analytical procedures (blood storage, DNA extraction) are not standardized. It is therefore a prerequisite for future and current molecular based diagnostic assays such as NGS technologies, to develop standards, guidelines and new stabilization technologies that can limit errors in the pre-analytical phase.

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