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SPIDIA-DNA: An External Quality Assessment for the pre-analytical phase of blood samples used for DNA-based analyses

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ABSTRACT

Background: The EC-funded project SPIDIA is aimed to develop evidence-based quality guidelines for the pre-analytical phase of blood samples used for DNA molecular testing. To this purpose, a survey and a pan-European External Quality Assessment (EQA) were implemented.
Methods: SPIDIA facility sent to all the participants the same blood sample to be processed without time or temperature limitation. DNA quality parameters performed at SPIDIA facility included: UV spectrophotometric analysis of DNA purity and yield, PCR interferences study by Kineret software and DNA integrity analysis by pulsed field gel electrophoresis.
Results: 197 applications have been collected from 30 European countries. A high variability of DNA fragmentation was observed whereas purity, yield and PCR interferences had a narrow distribution within laboratories. A significant difference between the RNase P single copy gene quantity obtained in the DNA samples extracted with the precipitation-based method respect to those obtained with beads and column-based methods was observed.
Conclusions: The results of this study will be the basis for implementing a second pan-European EQA and the results of both EQAs will be pooled and will provide the basis for the implementation of evidence-based guidelines for the pre-analytical phase of DNA analysis of blood samples.

1. Introduction

Molecular biology-based procedures have opened new perspectives in diagnosis, prognosis and treatments of disease, however the level of standardization of these technologies is often lower than in other areas of laboratory medicine [1–3]. For clinical laboratories performing molecular diagnostics, participation in proficiency testing/quality assessment programs (PT/EQA) is essential [4–7]. Disease-specific or methodological PT/EQA protocols are now available in genetic testing [8–11] and microbiology [12–14].

To have the greatest value, PT/EQA challenge samples should be designed to evaluate laboratory performance of the entire diagnostic workflow including the pre-analytical, analytical and post-analytical phases [15]. To meet this requirement, the PT/EQA samples should be a clinical specimen which most closely represents what is actually tested in a clinical laboratory and permits the evaluation of all phases of the testing process, including the DNA extraction step [16]. It is, however, often difficult for formal PT/EQA programs to obtain sufficient quantities of appropriate, high-quality, safe, homogeneous and stable clinical samples (e.g. a whole blood specimen) to supply all of the laboratories participating in any given PT exercise [16,17]. Consequently, most PT/EQA programs for molecular diagnostics employ highly purified extracted DNA samples rather than actual clinical specimens. In this instance, therefore, the participating laboratories cannot be evaluated for their proficiency in performing the pre-analytical phase of the analysis. Since it is known that DNA molecular characteristics can change in clinical samples during collection, transport and storage [18,19], the lack of systematic investigation of the effects of pre-analytical factors on analytical performance represents a challenge for molecular pathologists. It is therefore imperative for the proper implementation of future and current molecular diagnostic assays that standards, guidelines and new stabilization technologies are developed for pre-analytical specimen handling which yield the best possible analytical result.

SPIDIA (www.spidia.eu), a four-year integrated project funded by the European Commission, aims to develop quality guidelines and

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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 important part of this project is the implementation of quality assessment schemes (EQAs) for the collection, transport and processing of blood samples for RNA and DNA-based analysis.

Here we report the description and the results of SPIDIA DNA EQA: a large, pan- European trial, involving 197 laboratories from 30 countries. The aim of this EQA was to investigate the influence of blood collection and specimen shipment on genomic DNA quality and quantity. To date, this aspect of molecular diagnostics has been a poorly investigated [20]. To carry out the PT, the SPIDIA reference laboratory, Florence, Italy, sent the same blood sample to all participants for processing. During this phase of the study, no time or temperature limitations were imposed, but laboratories were asked to record the date of specimen processing (i.e. DNA extraction) and the extraction procedure. Purified DNA from each of the participating laboratories was then shipped back to the SPIDIA reference laboratory. DNA quality parameters performed at the SPIDIA facility included UV spectrophotometric analysis of DNA purity and yield. In addition, we further analysed the quality of DNA for PCR inhibition, performance in a qPCR assay. DNA integrity was also determined on all samples using pulsed field gel electrophoresis.

2. Materials and methods

2.1. Collection of applications

The announcement of the SPIDIA-DNA EQA was published on the EFLM web site (www.efcclm.org). Dedicated websites were created containing the description of the SPIDIA project (www.spidia.eu), the proposed protocols and the application form together with a questionnaire page (www.efcclm.org).

Details on the content of these web pages are reported as Supplemental data: the Questionnaire (Supplemental 1), the protocols describing the procedures (Supplemental 2) to follow for blood/DNA extraction/storage, and the Results forms (Supplemental 3) to record the data and information used for blood samples storage/extraction/analysis. The participants recorded detailed information about their standard procedure usually used in the laboratory practice from "blood collection to DNA analysis and storage" (Supplemental 1).

2.2. DNA1, 2 and 3 preparation, blood collection and shipment conditions

DNA was purified from a pool of 15 blood specimens collected in tubes containing EDTA as the anticoagulant (BD Vacutainer, 367864, BD). DNA was extracted using the PureGene kit (Qiagen).

After extraction, three DNA samples with different levels of yield and purity were prepared: DNA1, 65.4 ng/μl, 1.82 A260/280 nm ratio; DNA2, 17.28 ng/μl, 1.82 A260/280 nm ratio, and DNA3, 84.5 ng/μl, 1.32 A260/280 nm ratio.

Blood (350 ml) was collected from each of two consented, adult donors who tested negative for HIV, HBV and HCV. Blood was collected using classical phlebotomy procedure in 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 T334-2 s). In addition to one blood tube containing 1.2 ml of the pooled blood sample, participating laboratories received three pre-extracted DNA (DNA1, DNA2, DNA3), one empty vial to be used to send the extracted DNA (DNA4) back to the SPIDIA facility for analysis. 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.

2.3. Instructions for the participants

The participants received the box containing the three extracted DNAs, blood sample and the instruction (Supplemental 2) for performing the DNA extraction. The laboratories extracted DNA following their standard procedure under their own handling conditions with no restrictions concerning sample storage temperature or time to extraction.

2.4. Data reporting from participants

The participants recorded detailed information about the procedure used during the DNA extraction phase from whole blood. This information included date of sample arrival, date and time of DNA extraction, storage temperature and elapsed times of blood sample and extracted DNA, extraction protocol and spectrophotometric evaluation. All data were recorded in the on-line Results form (Supplemental 3).

2.5. DNA shipment and storage conditions

After DNA extraction, the participants sent the DNA sample (DNA 4) using the same shipping box in which they received the samples. All shipments were sent to the SPIDIA facility where the DNA samples were stored at −20 °C until analysis.

2.6. DNA quality parameters

The DNA quality parameters performed on DNA4 at the SPIDIA facility included a UV spectrophotometric analysis of DNA purity and yield, an analysis of DNA integrity by pulsed field gel electrophoresis interpreted using ImageJ software (www.rsbweb.nih.gov/ij), quantification of the RNase P single copy gene (Life Technologies) by qPCR, and the evaluation of PCR inhibition by Kineret software (Labonnet).

2.6.1. Spectrophotometric analysis

DNA was quantified by a NanoDrop® 1000 UV spectrophotometer (NanoDrop Technologies).

Spectrophotometric measurements were performed at 260, 280, and 320 nm. Absorption at 320 nm was used to subtract background absorption. DNA purity was calculated as absorbance ratio $R = (A_{260}–A_{320})/(A_{280}–A_{320})$ and DNA total yield as $Q = (A_{260} – A_{320}) \times 50 \times$ dilution factor $\times$ elution volume/extracted blood volume(ng/μl). Alternatively, when the reading at 320 nm was not reported by participants, the absorbance ratio was calculated as $R = A_{260}/A_{280}$ and RNA total yield as $Q = A_{260} \times 50 \times$ dilution factor $\times$ elution volume/extracted blood volume (ng/μl).

2.6.2. Analysis of DNA integrity

The analysis of the DNA integrity was performed by combining the pulsed field gel electrophoresis (PFGE) with ImageJ imaging software in order to transform the experimental data (PFGE image) into numerical form.

For Procedure used to analyzed th DNA4 integrity: the extracted DNA was analyzed by PFGE performing the following protocol. The corresponding picture was analyzed by ImageJ software in order to define the maximum, minimum and peak (DIRVs). For pulsed field gel electrophoresis, 800 ng of DNA separated on a 1% agarose gel (Ultra Pure Agarose, Invitrogen), using 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 DNA size 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. The gel image was documented with the EASY Win32 system (Herolab) and, analyzed by ImageJ software. Details of the ImageJ analysis are described in Supplemental Fig. 1.

2.6.3. Quantification of RNase P gene and evaluation of PCR interferences

qPCR was performed using TaqMan RNase P gene Detection Reagents (Life technologies). For each sample, 1 μl of DNA4 was added to 6.25 μl of 2× Universal PCR Master Mix (Life Technologies) and to 0.625 μl of TaqMan RNase P Detection Reagents and 6.25 μl of water for a final volume of 12.5 μl. The amount of each target gene was evaluated in triplicate against a standard curve generated by serial dilution (1:10) of a plasmid (from 10^7 to 10^1 pg/tube) containing the RNaseP target sequence. Samples and standards were subjected to 40 amplification cycles at 95 °C for 15 s and 60 °C for 60 s after 10 min incubation time at 95 °C in the 7900 HT fast Real-Time PCR System (Life Technolgies). After amplification, results were corrected for the blood volume used for the DNA extraction and for the elution/resuspension volume reported by the participants. The amplification kinetics obtained from the qPCR was used for the Kineret analysis in order to evaluate the amplification kinetics of each sample in respect to the other samples, which were used as "reference" value [21].

2.7. Statistical analysis and results interpretation

In the absence of known reference values for each of the factors investigated, we measured the consistency of a given participant's results against all values excluding outliers (90%) of the results provided by the other participants as previously described [22]. Briefly, a two-step statistical procedure based on a distribution-free approach was adopted in order to process the data corresponding to each of the following variables: DNA purity (DNA1, 2, 3, 4), DNA concentration (DNA1, 2, 3), DNA yield (DNA4), DIRVs (i.e. maximum, minimum and peak - DNA4), concentration of RNase P (DNA4). The aims of this procedure were to detect outliers and/or identify laboratories with issues related to performance.

The first step in the analysis involved the computation of the 95th bootstrap centile [23] of the distribution of the absolute value of the M statistic [24]. This centile was adopted as the threshold for detecting outliers. After removing the outliers from the distribution, the second step was the identification of laboratories whose performance could still be considered as outside the norm of most of the laboratories. This was done by calculating specific bootstrap centiles from the outlier-free distribution. The number of bootstrap samples used was 1000 in each step.

This procedure allowed us to calculate robust control limits (one or two sided) for the evaluation of the performance of each participant. We used the 2.5th and the 97.5th bootstrap centile to identify the lower and upper Action Limit (AL) and the 10th and 90th bootstrap centile to identify the lower and the upper Warning Limit (WL) for all the variables except for DNA yield, DIRVs and concentration of RNaseP target sequence for which higher values indicate better performance. For these latter we used the 5th and the 20th bootstrap centile to identify the one-sided AL and WL, respectively. According to these limits the performance of each participant was classified as follows:

Out of control: if the value exceeds the upper or the lower AL or if the value was below the one-sided AL.
Warning: if the value was between the upper AL and WL or between the lower AL and WL, or between the one-sided WL and AL.
In control: if the value was between the lower and the upper WL, or exceeds the one-sided WL.

The analysis and interpretation of the qPCR kinetics were performed as previously described [22].

3. Results

3.1. Applicant collection, recruitment and questionnaire information

197 applications have been collected from 30 different European countries (Fig. 1 Panel A). A description of the structure of the applicant laboratories is reported in Fig. 1, Panel B and their primary applications for DNA analyses are shown in Fig. 1, Panel C. At deadline, 183 laboratories (92.9%) had taken part in the SPIDIA DNA EQA.

Analysis of the Questionnaire (Table 1) revealed that 90% of the laboratories typically collect blood in K_2EDTA tubes, and only 10% utilize other types of blood collection tubes. The blood volume usually collected by the participating laboratories range from 2.5 to 5 ml. Forty-two per cent (42.2%) of the laboratories perform DNA extraction within 24 h of blood collection, whereas 43.3% extract DNA within 48 h of collection. Moreover, approximately 60% of the participating laboratories store blood at +4 °C prior to DNA extraction. Almost all laboratories utilize a commercial kit for DNA purification by a silica-membrane-based procedure. About 80% of the participants evaluate the DNA concentration after the extraction and almost all (94.4%) use a spectrophotometer rather than fluorescent detection dyes or other methods.

3.2. Report for the participants

At the SPIDIA facility resultant DNA samples were analyzed as described in Materials and Methods, and the results were evaluated using the statistical approach described above to produce an individual report for each participant (See Appendix A for an example of one such report). In the report, the distribution of all the data for each quality parameter was graphically displayed in a box-plot which depicted the AL and the WL together with a red dot indicating the individual value of the DNA from that particular laboratory. A red box under each graph indicated the classification of the laboratory's performance for this specific parameter. A zoom window was also displayed for each box-plot to aid in the visualization of the participant's result when the distribution of the variables was highly skewed.

3.2.1. Spectrophotometric data: Purity and concentration of pre-extracted DNAs

In Table A.1 (Appendix A), we report the spectrophotometric measurement of DNA1, 2 and 3 provided by the participants. Sections A.2 and A.3 (Appendix A) show the box-plots of the distributions of the purity and concentration of pre-extracted DNA. The median purity values are 1.86, 1.83 and 1.36 for DNA1, DNA2 and DNA3 respectively. The median DNA concentration is: 67.28 ng/μl for DNA1, 18 ng/μl for DNA2 and 86.8 ng/μl for DNA3. The DNA3 shows the highest concentration-related IQR (15.6) and the lowest IQR (0.06) in terms of purity.

3.2.2. Spectrophotometric data: Purity and quantity of DNA 4

In section B (Appendix A) we report the purity and quantity of DNA4 extracted from the blood sample sent to all participants. Tables B.1 and B.2 (Appendix A) include the spectrophotometric measurement results provided by the participants (left panel) and calculated by the SPIDIA facility (right panel) together with details of sample handling times, methods and reagents used by each participant.

Sections B.3 and B.4 (Appendix A) show the box-plots of the distributions of the DNA purity and quantity reported by the participants (left panel) and obtained by the SPIDIA facility (right panel). In particular, the median value for the purity reported by the participants is 1.81 (IQR: 0.21), whereas the median of the purity calculated by SPIDIA is 1.93 (IQR: 0.19). The median DNA concentration obtained by the participants is 19.67 ng/μl (IQR: 15.82) whereas the median value obtained by SPIDIA is 18.99 ng/μl (IQR: 13.63). Of note was the fact that both median and IQR values for DNA purity and DNA concentration calculated by the participants are very similar to those obtained by SPIDIA.

3.2.3. Integrity of DNA

This analysis was performed on DNA4 to verify DNA integrity by molecular weight. Section C.1 (Appendix A) depicts an image of the

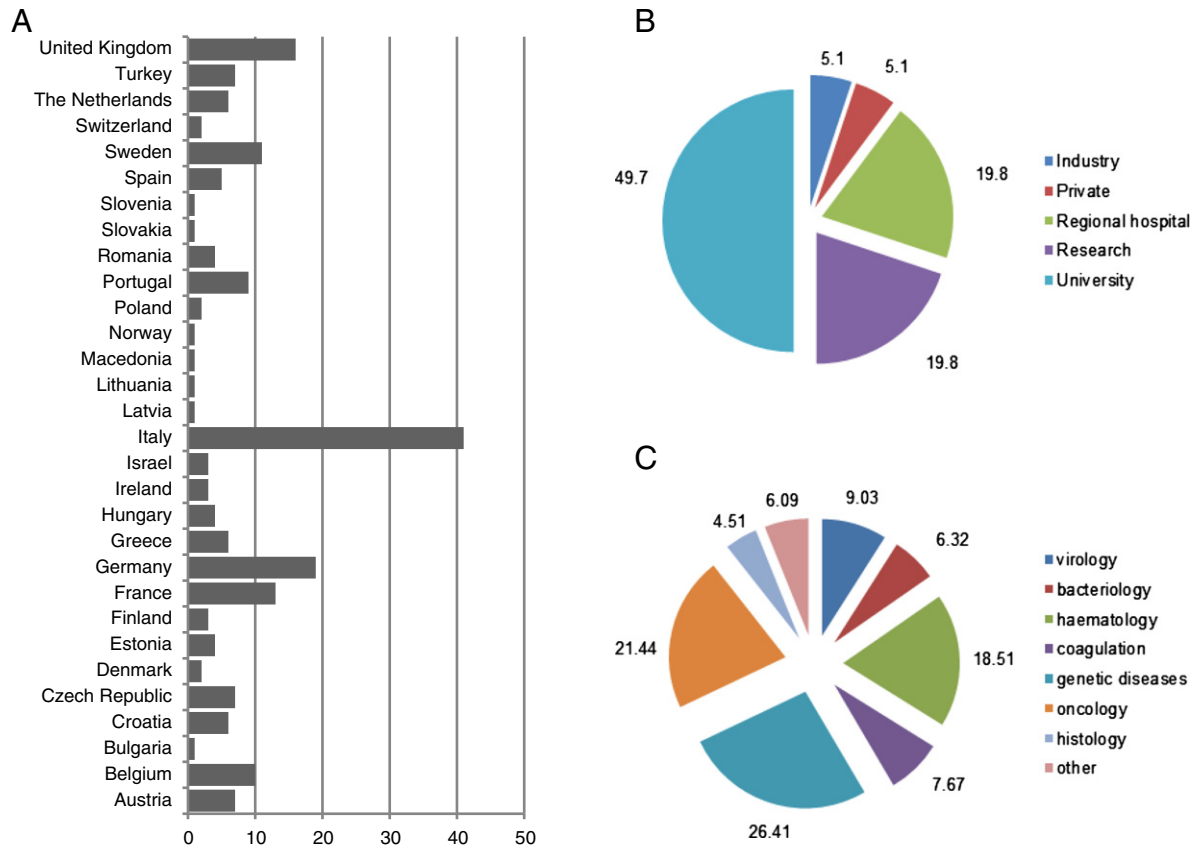

Fig. 1. Distribution of participant laboratories (n = 197) through Europe (Panel A), affiliation (Panel B) and the main area of research (Panel C).

pulsed field electrophoresis gel. In Table C.2 (Appendix A) we report each of the considered DIRVs (i.e. minimum, maximum and peak) derived from the Image J analysis of the gel image (See Materials and Methods section and Supplemental Fig 1). A graphic representation of DIRV data from all participating laboratories is shown in section C.3 (Appendix A). In particular, the median values were 2.28 kb, 80.62 kb, and 20.32 kb for minimum, maximum, and peak, respectively.

3.2.4. qPCR and kinetics analysis

Section D.1 (Appendix A) shows a graphic representation of qPCR results from all participating laboratories. The left panel shows an evaluation of the total amount of DNA4 (median 132.86 ng/μl blood) as determined by the RNase P single copy gene amplification. The right panel summarizes the evaluation of the interferences as determined by Kineret software analysis. The Kineret analysis was performed on the amplification results obtained from qPCR amplification of the RNAse P single copy gene (See Materials and methods).

3.2.5. Summary of the lab performance evaluation

Table G (Appendix A) summarizes the performance of the laboratories for DNA quality parameters. The table shows the results using three colors: green if the performance is "in control", yellow if the performance is "warning" or "weak outlier", and red if the performance is "out of control" or "strong outlier". When it was not possible to evaluate the performance due to a missing value, the word "missing" is reported in the summary table with an explanation in the "comments" column. All data are visually depicted as a radar graph with performance symbolized by a colored square (same colors as in the summary table) and the distance between the colored square and the center of the radar graph indicates the performance (the further away from the center, the worse is the performance).

3.2.6. Relationship between the DNA quantity and the extraction method

To explore the relationship between the RNase P gene quantity and the extraction methods used by participants (categorized in three categories: bead-, column- and precipitation-based method), the Kruskal–Wallis test was adopted. On the basis of this analysis, the amount of DNA extracted by using a precipitation-based method (median value of 60.996 ng/μl blood) was significantly lower in comparison to DNA extracted with either bead- (122.185 ng/μl blood) or silica membrane column-based purification procedures (164.219 ng/μl blood) (Fig. 2).

3.3. Overall performance of the laboratories participating in the RNA SPIDIA EQA program

On the basis of the DNA quality parameters and the statistical approach used in this EQA program, for 65/183 laboratories (35.5%), the performance was not critical (all parameters classified as "in control" or "warning"). For 70/183 (38.3%) of participating laboratories, only one quality parameter was "out of control and/or one or more missing data points. Two or more "out of control" quality parameters were measured for the remaining 48/183 (26.2%), including one or more missing data points (see Table 2).

4. Discussion

Molecular methods are today widely used in laboratory medicine, and tests for DNA in human genetics, hematology, molecular

Table 1
Questionnaire distribution frequencies: usual procedures used by participants laboratories (n = 108).

	N	%
1. In which tube do you usually perform blood collection?		
K_XEDTA	162	90
Na citrate	7	3.9
Li/Na heparine	6	3.3
PAX gene blood DNA	3	1.7
Other	2	1.1
2. How many milliliters of blood do you collect?		
≤2.5 ml	35	19.4
2.5 ml < x ≤ 5 ml	101	56.1
5 ml < x < 10 ml	18	10.0
≥10 ml	26	14.5
3. How long is the time interval between the blood collection and the DNA extraction?		
≤24 h	76	42.2
24 h < x ≤ 48 h	78	43.3
48 h < x < 72 h	3	1.7
≥72 h	19	10.6
Missing	4	2.2
4. At what temperature is the collected "whole blood" stored?		
Room temperature	16	8.9
+4 °C	106	58.9
−20 °C	46	25.5
−80 °C	12	6.7
5. What is the procedure for blood extraction? Do you use a kit procedure?		
No	12	6.7
Yes	168	93.3
6.The DNA extraction is based on: (on 168 laboratories performing DNA extraction by kit procedure):		
Precipitation	17	10.1
Silica membrane	79	47.0
Magnetic beads	46	27.4
Missing	26	15.5
7. Do you evaluate the concentration of extracted DNA from blood?		
No	37	20.6
Yes	143	79.4
8. What is the method? (on 143 laboratories performing the evaluation of DNA concentration):		
Fluorescent dyes	4	2.8
Spectrophotometric measurement	135	94.4
Other	3	2.1
Missing	1	0.7
9. If you use spectrophotometric measurement, specify the formula used to calculate the DNA amount: (on 135 laboratories performing the evaluation of DNA concentration by spectrophotometric measurement):		
(A260-A320) × 50 × dilution factor	20	14.7
A260 × 50 × dilution factor	65	47.8
Other	42	30.9
Missing	8	6.6
10. What kind of analysis do you usually perform on your extracted DNA from blood?		
Qualitative PCR	55	30.5
qPCR	38	21.1
Sequencing	19	10.6
Other	68	37.8
11. At what temperature do you usually store the extracted DNA from blood?		
Room temperature	1	0.5
+4 °C	64	35.6
−20 °C	103	57.2
−80 °C	12	6.7

oncology, and infectious diseases represent the fastest growing area in laboratory medicine [23]. In the last few years, an adequate quality assurance framework has been developed which is based on knowledge acquired from specific surveys [6], implementation of quality assessment and proficiency programs (www.cap.org; www.emqn.org; www.dgkl.dc; www.eurogentest.org), and by the development of method-specific guidelines [7,25].

Most of the available PT/EQA for molecular diagnostics are based on the analytical phase of the test process, however. These schemes should reflect clinically relevant challenges that incorporate all steps of the testing process, including pre- and post-analytical components.

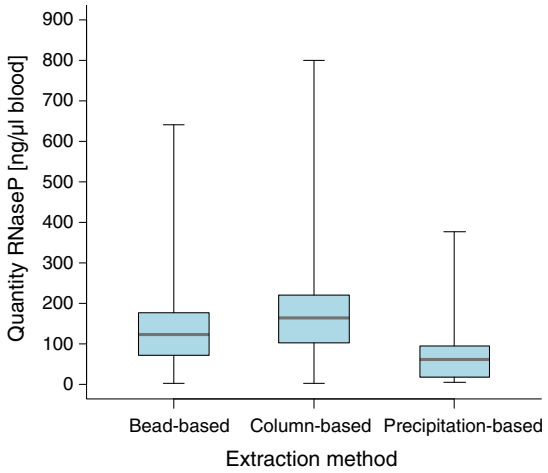

Fig. 2. Box-plot of the RNase P quantification by qPCR. RNaseP gene quantity according to extraction method categorized in bead- (N = 39), column- (N = 85) and precipitation- (N = 21) based method. The amount of DNA extracted by using a precipitation-based method was lower in comparison to DNA extracted with either bead- (Bonferroni p-value = 0.02) or column-based method (Bonferroni p-value <0.01). Each box shows the 25th and 75th percentiles, the horizontal line inside the box indicates the median, and the limits of the two whiskers correspond to minimum and maximum of the different distribution.

Here we report a description of a pan-European survey and the implementation of an External Quality Assessment program focused on the pre-analytical phase of handling and processing blood samples for DNA analysis. The survey posed questions to participating laboratories aimed at determining what laboratory policies and practices are in place for specimen handling, and respondents were requested to provide information on blood sampling and DNA extraction protocols (Table 1). A strong tendency to use commercially available extraction kits (mainly silica membrane technology) was observed. The majority of the laboratories collects blood in EDTA tubes and performs the evaluation of yield and quality of DNA by spectrophotometric UV analysis, storing the extracted DNA at −20 °C. Other aspects of sample handling and analysis protocols were more variable, such as the volume of blood collected and the time interval between blood collection and DNA extraction. The wide range of answers to these questions confirmed the need to develop specimen handling and processing guidelines for tests requiring DNA from whole blood.

Together with the survey, SPIDIA also implemented an EQA program focused on the pre-analytical phase of analyzing DNA in blood. We have

Table 2
Classification of the performance of the laboratories.

Categories	N	%
All in control or warning performance [a]	65	35.52
One out of control performance and/or one or more missing [b]	70	38.25
Two or more out of control performance with or without missing [c]	48	26.23
Total of participants laboratory	183	100.00

Description of the categories:
[a] all in control or warning performance: labs with all performances in control or warning, without missing;
[b] one out of control performance and/or one or more missing: labs with only one out of control (N = 35); labs with only one missing (N = 6) or only more than one missing (N = 18); labs with one out of control and one missing (N = 1); labs with one out of control and more than one missing (N = 10);
[c] two or more out of control performance with or without missing: labs with two out of control with at least one missing (N = 3) or without missing (N = 17); labs with more than two out of control with at least one missing (N = 8) or without missing (N = 20).

evaluated the quality of DNA extracted by the participants, and we have provided reports to each participating laboratory which describes the performance of the laboratory relative to results obtained by the majority of participants.

The distribution of pre-extracted DNAs (DNA1, DNA2, DNA3) quality and quantity results (see Appendix A; section A.2 and A.3) from participating laboratories were very close to those expected (both in samples with good and poor purity, or with high or low concentration) showing that spectrophotometric analysis of purified DNA is a well-standardized practice in the enrolled laboratories.

For DNA extracted by participants from the blood sample (DNA4) sent to all laboratories (Appendix A, section B.3, B.4), the SPIDIA laboratory determined a median value of purity close to 1.8, indicating high-quality DNA, and a median yield of 20 ng/μl. There was a very narrow distribution of DNA yields among the laboratories and only a few outliers were observed.

The range of DNA integrity of DNA4 samples as determined by pulsed field gel electrophoresis is still, somewhat objective owing to the visual nature of the results (See Appendix A, section C.1), however transformation of gel images into numerical results was productive. Using this approach, we observed a high degree of variability in DNA fragmentation from laboratory to laboratory, however, the effect of the degree of fragmentation must be further investigated using techniques such as long-range or multiplex PCR which were not within the scope of this project. In the view of a second run, we developed an ad hoc algorithm that combines the by-eye evaluation of the gel image from PFGE with the ImageJ software output.

The study of PCR interference was based on Kineret software analysis of the kinetics obtained using the RNase P single copy gene qPCR quantification of the DNA4 samples (Appendix A; section D). Only a few laboratories produced DNA which interfered in a PCR reaction.

We have then investigated if the values of the DNA quality parameters were influenced by the procedures and protocols (temperature, volumes, timing) used by the participants for DNA extraction.

A significant difference between the RNase P single copy gene quantity obtained in the DNA samples extracted with the precipitation-based method respect to those obtained with bead- and column-based methods was observed (Fig. 2). The same trend was observed when we analyzed the DNA UV quantity obtained vs. the extraction method (data not shown).

This SPIDIA DNA program was specifically designed for the evaluation of the pre-analytical phase of molecular diagnostic assays requiring DNA from whole blood and not for the evaluation of laboratory performance of a specific diagnostic test. In conducting this program, we wanted to give participating laboratories useful information concerning general DNA quality parameters and how their current policies and procedures for sample handling affected DNA quality. Regarding the performances of the participants, on the basis of the DNA quality parameters and of the statistical approach used in this EQA program, only 35% of the participants had not critical performances (see Table 2). This result seems due to both the highly variable protocol conditions and the different extraction procedures used by the participating laboratories in performing the pre-analytical phase (see Table 1) and the high number of the quality parameters investigated in this study (n = 15, see Appendix A, G. Summary). However this does not mean that the downstream results of those laboratories having critical performances on the basis of our classification criteria would be affected by their "poor" DNA quality as downstream analysis was not the target of our EQA. On the other hand this result confirms the need of dedicated Guidelines which is the final goal of the SPIDIA project.

5. Conclusions

The objective of the SPIDIA-DNA EQA program was to obtain information on pre-extracted and laboratory-extracted DNA quality when participants followed the protocols and reagents in use in their own laboratories and to correlate these results to pre-analytical variables in order to develop general guidelines for molecular in vitro diagnostics. In this study, we focused on the optimization of blood collection and shipment together with recording more detailed information on time and temperature between blood collection and DNA extraction. This protocol allowed a more in-depth analysis of the critical aspects of the pre-analytical phase. On the basis of the results of the first SPIDIA DNA EQA we will develop a second run, with more stringent pre-analytical conditions (i.e. shipping temperature, blood storage temperature) for the implementation of Evidence-Based Guidelines for the handling blood samples for cellular DNA-based analysis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2013.05.012.

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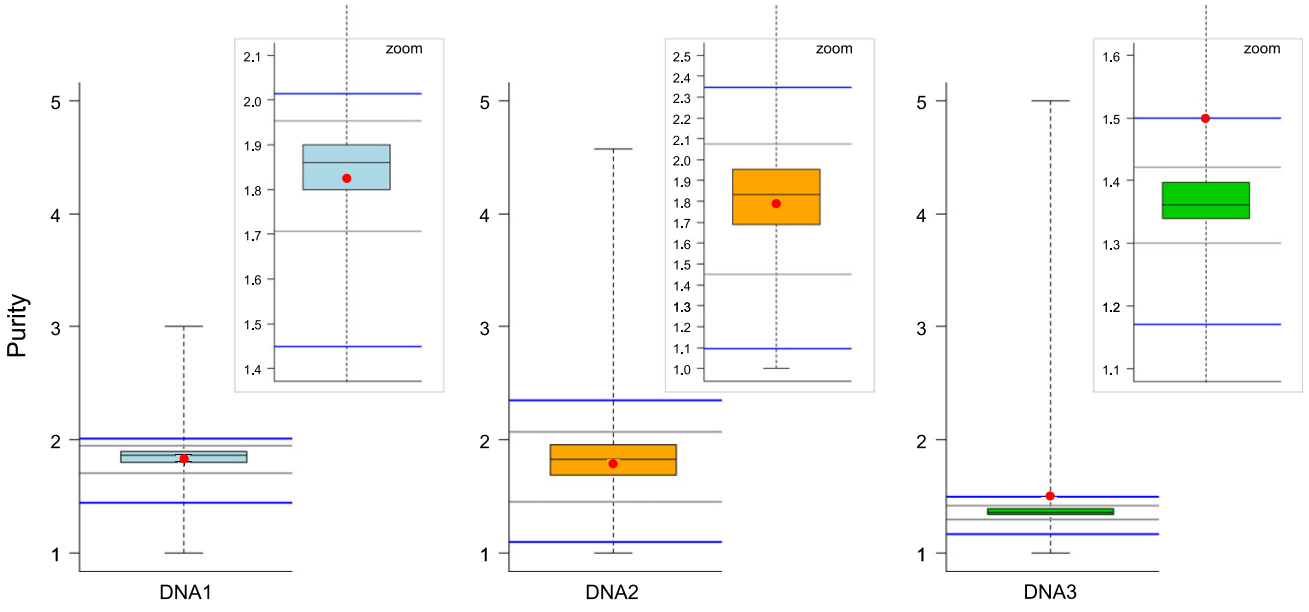
A. Purity and Concentration of DNA1, DNA2 and DNA3 (pre-extracted DNAs)

A.1. Spectrophotometric data provided by your lab

	260 nm	280 nm	320 nm	Purity	Concentration (ng/μl)	Dilution factor
DNA1	0.042	0.023	0.005	1.826	105	50
DNA2	0.034	0.019	0.003	1.789	85	50
DNA3	0.042	0.028	0.004	1.5	102.5	50

A.2. Your lab (●) versus overall distribution (N = 172) – Purity

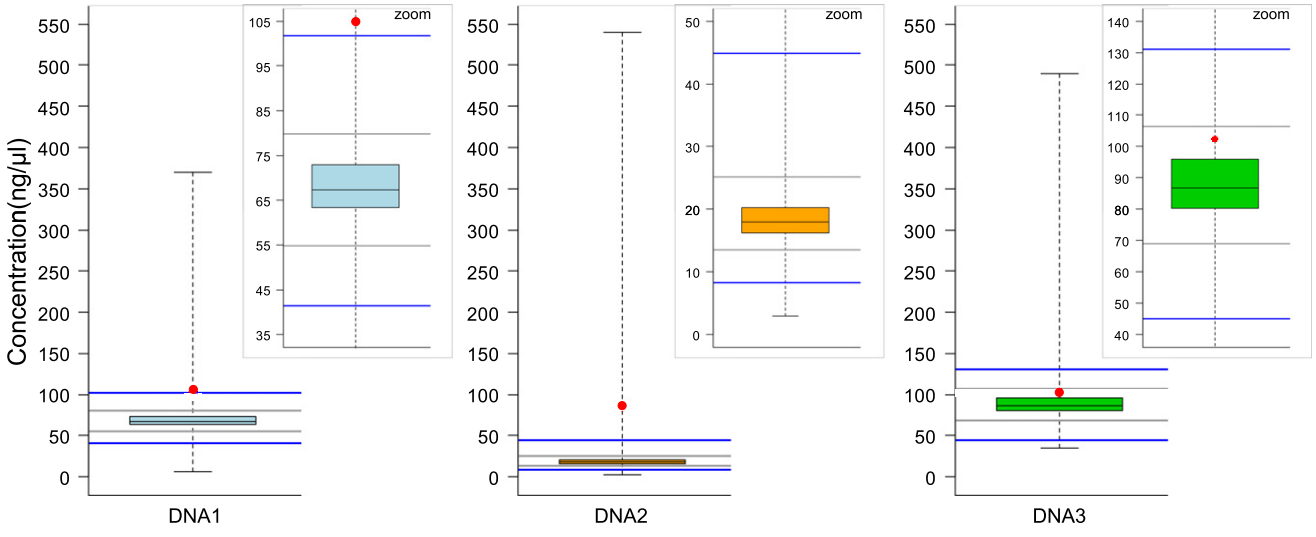
In the figures the blue lines represent the Action Limits (ALs) and the gray lines represent the Warning Limits (WLs).



DNA1	DNA2	DNA3
Lower AL=1.45; Upper AL=2.01	Lower AL=1.09; Upper AL=2.35	Lower AL=1.17; Upper AL=1.50
Lower WL=1.71; Upper WL=1.95	Lower WL=1.45; Upper WL=2.08	Lower WL=1.30; Upper WL=1.42
in control	*in control*	*out of control*

A.3. Your lab (●) vs overall distribution (N = 174) – Concentration

In the figures the blue lines represent the Action Limits (ALs) and the gray lines represent the Warning Limits (WLs)



DNA1	DNA2	DNA3
Lower AL=41.45; Upper AL=101.73	Lower AL=8.27; Upper AL=44.82	Lower AL=44.98; Upper AL=130.95
Lower WL=54.81; Upper WL=79.82	Lower WL=13.53; Upper WL=25.15	Lower WL=68.97; Upper WL=106.37
out of control	*out of control*	*in control*

B. Purity and Quantity of DNA4 (DNA extracted from blood)

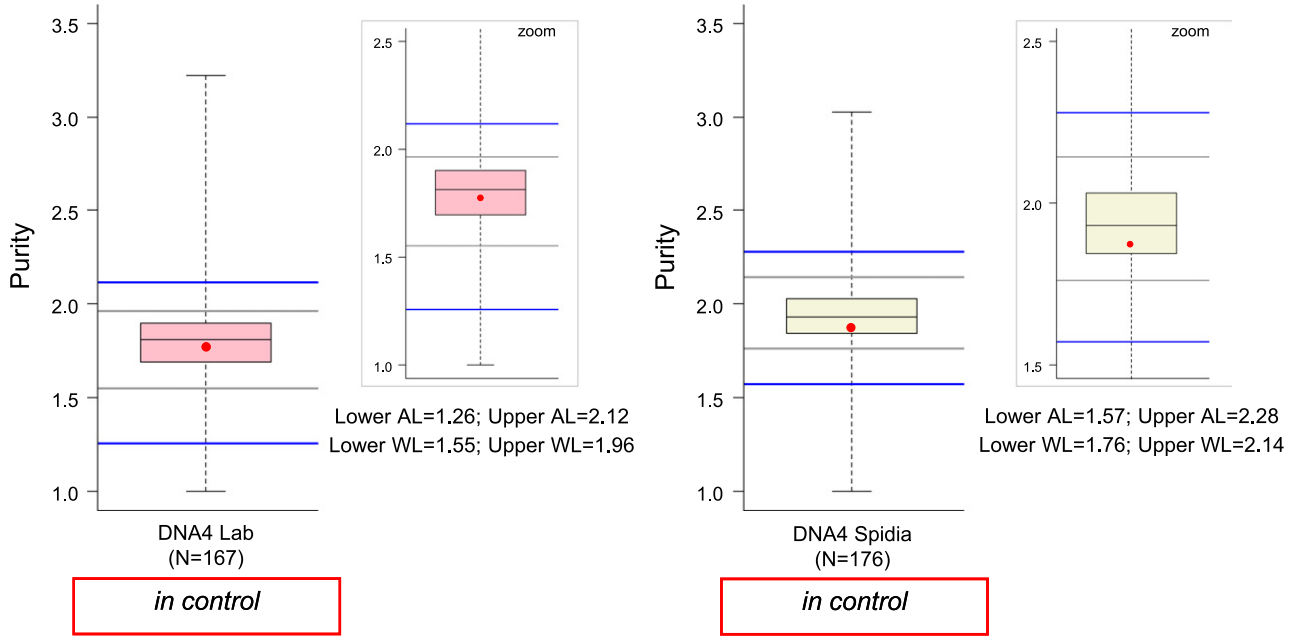
B.1. Spectrophotometric data provided by your lab and by SPIDIA lab

260nm Lab	280nm Lab	320nm Lab	Purity Lab	Quantity (ng/µl) Lab	Purity Spidia	Quantity (ng/µl) Spidia	Dilution factor	Extraction vol. (ul)	Elution vol. (ul)	Buffer
0.293	0.165	0.015	1.776	19.54	1.875	16.5	2	150	100	Elution solution 2

B.2. Additional information provided by your lab

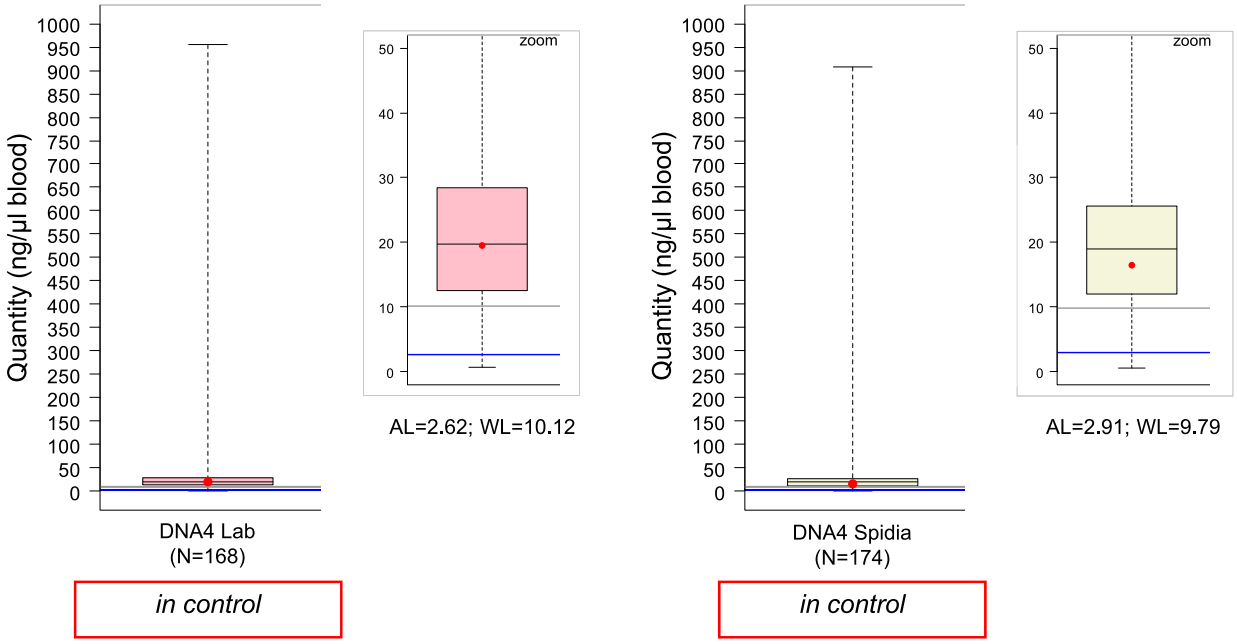
Extraction		Spectrophotometer		Temperature of DNA storage		Time interval (hours)	
producer	supplier	producer	supplier	arrival to extraction	extraction to analysis	arrival to extraction	extraction to analysis
bloodprep Chemistry	Applied Biosystems ABI PRISM 6100 Nucleic Acid Pre	biospec 1601	shimadzu	4 °C	4 °C	89.5 h	1 h

B.3. Your lab (●) versus overall distribution – Purity



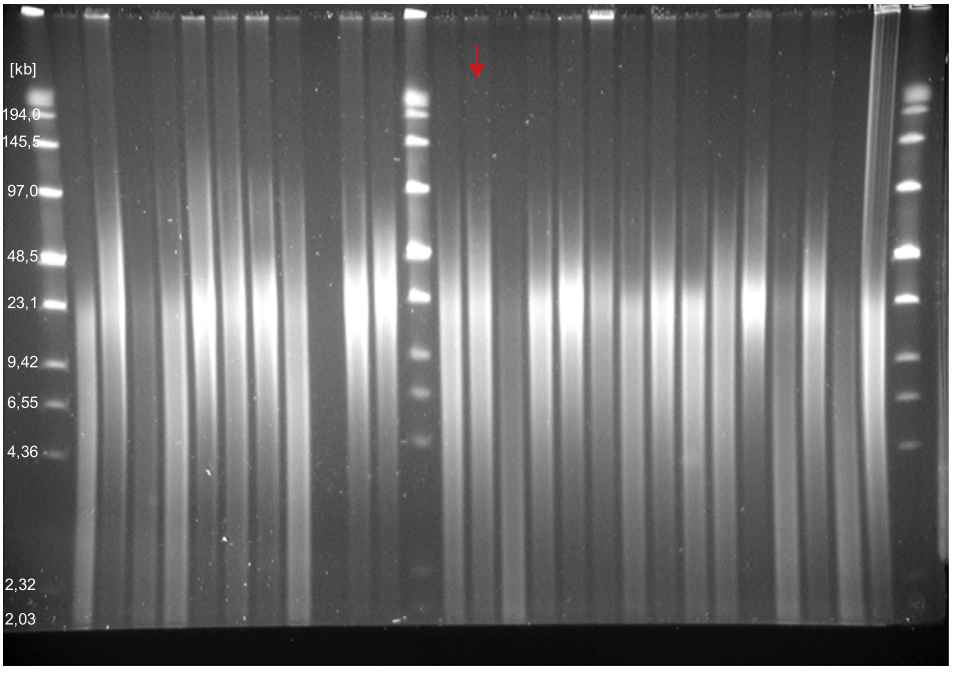
B.4. Your lab (●) versus overall distribution - Quantity

In the figures the blue line represents the Action Limit (AL) and the gray line represents the Warning Limit (WL).



C. Integrity of DNA4 (DNA extracted from blood)

C.1. Your lab (↓) - pulse field gel electrophoresis image

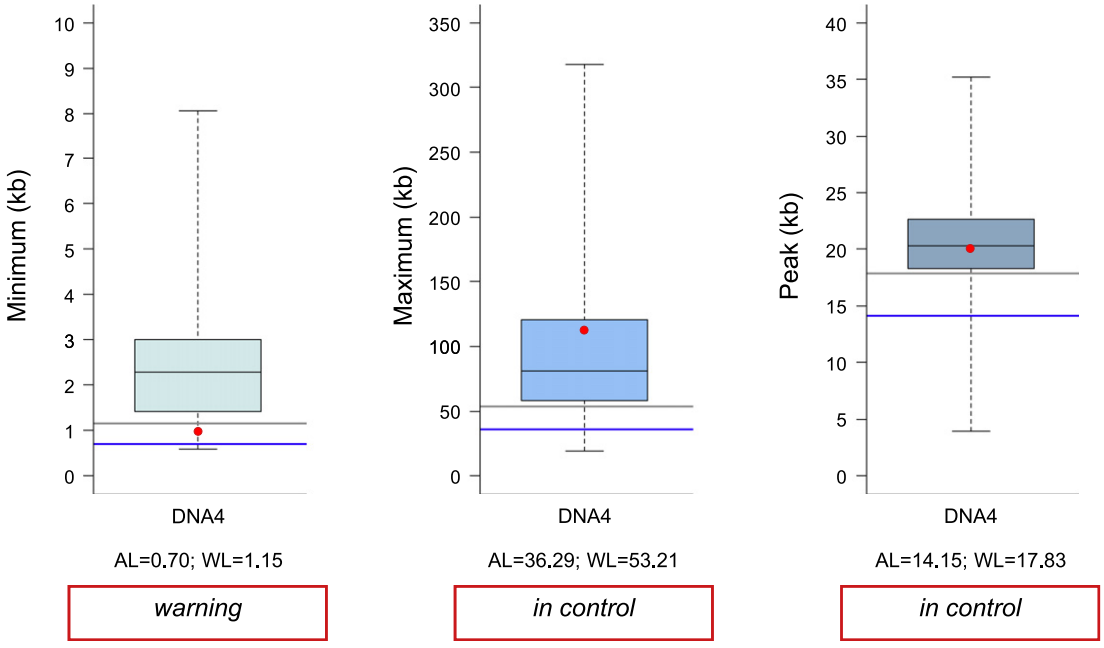


C.2. ImageJ data of your lab

Minimum (kb)	Maximum (kb)	Peak (kb)
0.967	112.105	20.012

C.3. Your lab (●) versus overall distribution (N = 157) – ImageJ data

In the figure the blue line represents the Action Limit (AL) and the gray line represents the Warning Limit (WL).

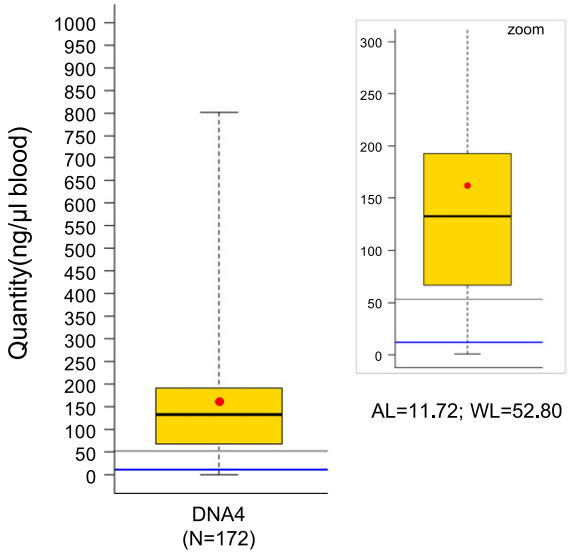


D. Quantification of RNaseP by real-time PCR on DNA4 and evaluation of interferences

D.1. Your lab (●) versus overall distribution

Quantification of RNaseP

In the figure the blue line represents the Action Limit (AL) and the gray line represents the Warning Limit (WL).

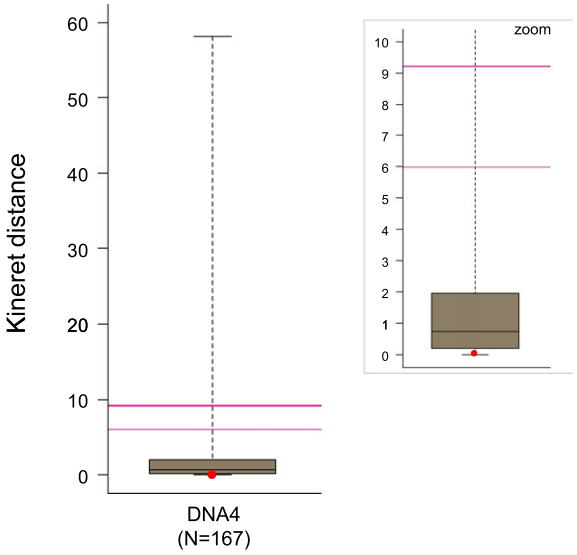


AL=11.72; WL=52.80

in control

Interferences

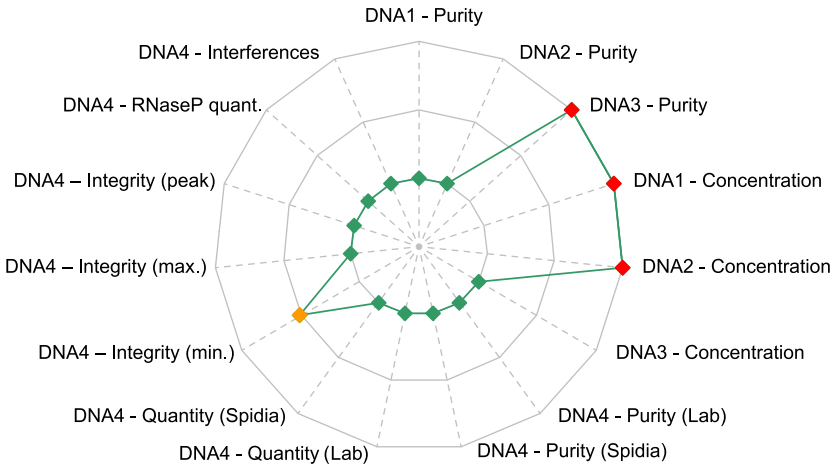
In the figure the two lines represent the two Kineret threshold for outliers identification: 5.99 (weak outlier) and 9.21 (strong outlier).



in control

G. Summary

	Performance			Missing	Comments
DNA1 – Purity	in control				
DNA2 – Purity	in control				
DNA3 – Purity			out of control		
DNA1 – Concentration			out of control		
DNA2 – Concentration			out of control		
DNA3 – Concentration	in control				
DNA4 – Purity (Lab)	in control				
DNA4 – Purity (Spidia)	in control				
DNA4 – Quantity (Lab)	in control				
DNA4 – Quantity (Spidia)	in control				
DNA4 – Integrity (min.)		warning			
DNA4 – Integrity (max.)	in control				
DNA4 – Integrity (peak)	in control				
DNA4 – RNaseP quant.	in control				
DNA4 – Interferences	in control				



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