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Internal Validation of a DNA Quantification Method using the Quantifiler® Human DNA Quantification Kit and the 7500 Real-Time PCR System with the Hid Real-Time PCR Analysis Software V 1.1 at the Biology Department of the Central Forensic Laboratory of the Police

Summary

The aim of the present study was the internal validation of a DNA quantification method employing Quantifiler® Human DNA Quantification Kit from Applied Biosystems, used for the quantification of human total DNA, coupled with the 7500 Real-Time PCR System and the HID Real-Time PCR Analysis Software v1.1, performed at the Biology Department of the Central Forensic Laboratory of the Police. The selection of parameters relevant to the laboratory's routine casework was made based on the ENFSI DNA Working Group recommendations included in the document *Recommended Minimum Criteria for the Validation of Various Aspects of the DNA Profiling Process*. The assessment regarded: sensitivity, linearity, range covered by the method and precision, including intralaboratory repeatability and reproducibility. Moreover, an attempt was made to determine whether continuation of genetic analyses is rational if the 7500 Real-Time system reads are negative, i.e. no nuclear DNA is detected, while the internal positive control (IPC) results are correct. Based on conducted validation experiments, it was found that the extent to which the validated method met the predetermined acceptance criteria is satisfactory, taking into account the specificity of the tests conducted in our laboratory. However, any negative indications of 7500 Real-Time PCR system, suggesting no presence of nuclear DNA along with correct results obtained for the IPC, should be interpreted very carefully. Apparently, it is not rational to discontinue further analyses of such samples due to the availability of very sensitive new DNA amplification kits which often allow obtaining a full DNA profile. To sum up, the validation of the DNA quantification method employing the Quantifiler® Human DNA Quantification Kit coupled with the 7500 Real-Time PCR System and the HID Real-Time PCR Analysis Software V1.1 was successful, therefore the method could be implemented and routinely used for the quantification of human total DNA in biological samples subject to forensic analyses conducted at the Biology Department of the Central Forensic Laboratory of the Police.

Keywords internal validation, quantification of DNA, Real-Time PCR, Quantifiler® Human DNA Quantification Kit

Introduction

Methods of molecular genetics are used in a variety of studies in the field of, e.g., ecology, evolution of organisms, oncology, forensics. Information carried by molecular markers (inherited variable alterations of DNA, RNA and proteins) allow either to draw conclusions regarding population genetics, analysis of parenthood and relationship, or to identify of individuals. Commonly used molecular biology techniques are characterized by, for instance, applicability for multiple organisms (from viruses, bacteria through humans), accuracy of the obtained results and short time of analysis. Moreover, only traces of biological material are required. Researchers in laboratories around the world constantly develop new methods intended to improve workflows and reduce the time spent on the analyses. This way, reliable information is obtained at reduced time and material consumption, as well as improved cost-effectiveness. Modern forensics follows these new trends, taking into account the increasing efficiency and throughput of the processes, along with their cost reduction. Current investigation techniques are based on advanced scientific methods, especially in the field of biology, which improves the efficiency of crime perpetrators' identification. On the crime scene,

crime scene investigators collect trace evidence, including biological traces. Biological material that arrives in forensic biology laboratories very often contains highly degraded DNA and high levels of impurities, which may result in both small quantity and poor quality of usable DNA. The correct quantitative and qualitative assessment of DNA samples affects the final results of STR analysis. It is therefore a very important step in the analysis of biological evidence to reliably determine the quantity and quality of human DNA by assessing the potential presence of inhibitors in the analyzed sample.

Initially, quantitative and qualitative DNA analysis was performed using electrophoresis on low-concentration agarose gel, where DNA was stained with ethidium bromide, a mutagenic DNA-intercalator, or other fluorescent dyes, such as SYBR Gold, SYBR Safe or Gel Red, which fluoresce under UV light. Another method was "Slot-Blot" hybridization with a probe specific for human DNA, which was imprecise and required a significant amount of effort. Subsequently, a non-specific method of fluorimetric DNA quantification using the Fluoroskan Ascent instrument was implemented. The method was based on an indirect calculation of DNA quantity by measuring the fluorescence of a dye intercalating into the double strand of the nucleic acid. Taking into account that in practice, biological traces are often highly degraded, "difficult" archival material that, apart from human DNA, also contains e.g. bacterial DNA, fluorimetric DNA quantification was not reliable. The method is non-specific for human DNA, thus the results also included DNA of other origin. Another negative aspect of this method is the type of the dye used (PicoGreen), which is a very potent mutagen due to its physicochemical (intercalating) properties. Another commonly used method is the spectrophotometric measurement of absorbance at the wavelengths of 230, 260 and 280 nm. The obtained absorbance values are automatically converted by the instrument into concentration, as well as the purity from protein and organic contaminants. NanoDrop is one of the devices that employ this technique, used for the spectrophotometric analysis of the concentration (quantity) and purity (quality) of the isolated material (DNA and RNA). The range of concentrations at which the spectrophotometric quantification is reliable is approximately 10–3,000 ng/μL, which makes this method imprecise or insufficiently sensitive in relation to forensic tests and the quantities needed for STR analysis.

Only one of the latest molecular biology techniques, Real-Time PCR, has enabled precise identification and quantification of the product. By using fluorescent techniques, it is possible to monitor product quantity in each cycle of PCR conducted. The greatest advantage of the method is its high sensitivity, as well as the speed and the efficiency of the reaction. A disadvantage of Real-Time PCR is the possibility of interruption of the reaction by certain chemical compounds called inhibitors, often naturally occurring in the tested biological samples (e.g., hemoglobin, urea, humic acids and fabric dyes), as well as by organic solvents (e.g. phenol, chloroform, alcohol) and salts (e.g. chloride) used in the extraction of nucleic acids. However, this apparently negative characteristic may bring benefits to forensic analyses, providing valuable information on the quality of the tested sample that might help to optimize the amplification by removing PCR inhibitors.

Applied Biosystems has met the demands of forensic laboratories which routinely deal with difficult-to-manage evidence, such as biological samples, and has created three new laboratory kits: Quantifiler® Human DNA Quantification Kit, Quantifiler® Y Human Male DNA Quantification Kit and Quantifiler® Duo DNA Quantification Kit, which, together with the 7500 Real-Time PCR System and the Hid Real-Time PCR Analysis Software V 1.1, are used for DNA quantification in identification tests conducted in forensic laboratories (Fig.1 The instrument 7500 Real-Time PCR System and Fig. 2 The inside of the instrument 7500 Real-Time PCR System; see Polish version).

In accordance with the guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDAM) and recommendations of the standard PN-EN ISO/IEC 17025:2005 an internal validation was performed, before the routine use of new testing method in laboratories of the Biology Department of the Central Forensic Laboratory of the Police [1, 2]. Trained laboratory personnel conducted a number of experiments in order to verify the effectiveness of the new testing method in the analyses carried out by the laboratory [3, 4].

Aim of the study

The main aim of the presented study was the internal validation of a DNA quantification method employing the Quantifiler® Human DNA Quantification Kit from Applied Biosystems, used for quantification of human total DNA, coupled with the 7500 Real-Time PCR System and the HID Real-Time PCR Analysis Software v1.1, performed at the Biology Department of the Central Forensic Laboratory of the Police.

Moreover, an attempt was made to determine whether performing further genetic analyses is rational when the 7500 Real-Time system readings are negative, i.e. no nuclear DNA is detected, while the internal positive control (IPC) results are correct. The experiment was important from the perspective of reagent consumption.

Materials and methods

In the conducted experiments, the *pooled human male genomic DNA* standard of known profile and baseline concentration [200 ng/μL], provided with the Quantifiler Human DNA Quantification Kit, was used.

Quantitative and qualitative DNA analysis was performed via Real-Time PCR using the Quantifiler® Human DNA Quantification Kit from Applied Biosystems coupled with the 7500 Real-Time PCR System and the HID Real-Time PCR Analysis Software v1.1.

Samples were amplified via multiplex PCR on the GeneAmp PCR System 9700 thermal cycler from Applied Biosystems, using kit reagents: AmpFISTR NGM PCR and AmpFISTR Y-filer PCR from Applied Biosystems and ESI 17 Power Plex from Promega.

PCR products were separated using the ABI PRISM 3130XL capillary DNA sequencer from Applied Biosystems coupled with data collection software. The separation was run in the 10 × Genetic Analyzer Buffer containing EDTA. The products were separated in 36-cm capillaries filled with the denaturing medium POP4TM from Applied Biosystems. Result analysis was carried out using the GeneMapper ID-X 1.1 expert software from Applied Biosystems.

Description of the validated method

The validated method is specific for human DNA due to the use of the TaqMan MGB probe. The used oligonucleotides contain a fluorescent reporter dye, FAM (hybridizing with the target human DNA) or VIC (hybridizing with the internal positive control), at the 5' terminus and a fluorescence quencher at the 3' terminus. After binding to a complementary sequence, the probe is degraded during the elongation step by the AmpliTaq Gold polymerase, endowed with 5'-exonuclease activity, which causes the fluorochrome to separate from the quencher. This leads to fluorescent light emission. The initial quantity of DNA in the sample is measured based on the increase in fluorescence. The stronger the fluorescence, the higher the number of copies of DNA, which is monitored in each amplification cycle. After a certain number of cycles, the level of fluorescence exceeds a predefined CT threshold indicating that the reaction kinetics has entered the exponential phase of product generation. The CT value is used for the calculation of the quantity of DNA present in the mixture at the onset of the reaction. To this end, a series of DNA standard dilutions is prepared, and the program calculates the CT values of standard quantification, which subsequently are used to draw the standard curve. After the amplification, the CT values of the tested samples are extrapolated onto the curve, which provides information on DNA concentration in the sample.

The Hid Real-Time PCR Analysis Software V1.1 generates a regression line by computing the best fitting data of standard quantity. The formula that describes the CT value is (1):

$$CT = m\,[\log (Qty)] + b \quad (1),$$

where:
m – slope,
b – Y-intercept,
Qty – initial quantity of DNA.

The resulting data are analyzed according to the following values:

- R2 defined as the degree of match between the standard curve regression line and the individual quantification results of tested samples. The value 1.00 indicates a perfect match between the regression curve and the value data points. R2 > 0.99 indicates a very close match between the standard curve and quantification values of tested samples. If R2 < 0.98, the correctness of microplate settings (e.g., values of standard quantity entered during the creation of document for the plate, correctness of dilution of the standards) should be checked, followed by error elimination and repeated analysis.
- Curve slope, indicating the efficiency of amplification for the experiment. It is important to note that slope values differ for each set of reagents: Quantifiler Human, Quantifiler Y, Quantifiler Duo, and are presented in Table 1;
- Y-intercept indicating the expected CT value for a sample of the quantity = 1 (e.g., 1 ng/μL).

CT values of the internal positive control (IPC) are measured to check the quality and correctness of amplification, as well as the presence of inhibitors in the tested sample [5].

Description of the validated kit

The Quantifiler® Human DNA Quantification Kit from Applied Biosystems is used for the quantification of human total DNA. The kit amplifies a fragment of genomic DNA, hTERT (*human telomerase reverse transcriptase gene*), of 62 bp. The kit includes: mix of primers, reaction mix, DNA standard (*pooled human male genomic DNA*, male DNA sequence of a known profile). The mix of primers contains: pairs of synthesized primers, internal positive control (IPC) and two probes labeled with the reporter dyes FAM and VIC. The internal positive control (IPC) indicates a correct amplification. Based on the value of IPC CT, it can be inferred if the sample actually does not contain DNA or contains an inhibitor. The probe with the FAM dye hybridizes with target human DNA, while the probe with VIC dye hybridizes with the internal positive control. The reaction mix contains: AmpliTaq Gold polymerase, dNTP, passive ROX dye and buffer.

Table 1

Slop values specific for each reagent kits recommended by the manufacturer

Kit	Recommended slope values	Średnia wartość slope
Quantifiler Human	–2.9 do –3.3	–3.1
Quantifiler Y	–3.0 do –3.6	–3.3
Quantifiler Duo	–3.0 do –3.6	–3.3

In order to minimize the chance of differences in the tests results, kits with the same serial number were used for validation tests.

Results

The selection of parameters relevant for routine tests was made based on the ENFSI DNA Working Group recommendations included in the document "Recommended Minimum Criteria for the Validation of Various Aspects of the DNA Profiling Process". The assessed parameters included: sensitivity, linearity, range covered by the method and precision, including intralaboratory repeatability and reproducibility [2]. The obtained results are discussed below.

Sensitivity tests

Sensitivity tests were carried out to determine the range of human DNA concentrations that are reliably measured by the 7500 Real-Time PCR system with the HID Real-Time PCR Analysis Software v1.1 using the Quantifiler® Human DNA Quantification Kit. In the validation experiment, 10 dilutions of the DNA standard at the initial concentration of 200 ng/μL were prepared. Dilutions A to E were measured three times, while dilutions F to J were measured six times. A diagram of the DNA standard dilutions is presented in Table 2. The adopted acceptance criterion for sensitivity was the range of concentrations recommended by the manufacturer, i.e. 23 pg/μL to 50 ng/μL [7, 8].

The experiment indicated that obtained results of human DNA quantification within the range of 11.5 pg/μL to 75 ng/μL are similar to the quantities of tested DNA. Analysis based on the median values led to the conclusion that the scatter of the results is relatively small, indicating that obtained values are similar. At 5.7 pg/μL and below, indications of the instrument are not repeatable. Summary of the results is shown in Table 3 and Figure 3 (The sensivity of the method for the range of dilutions/samples A-D; see Polish version) and Figure 4 (The sensivity of the method for the range of dilutions/samples E-J; see Polish version)

Based on the sensitivity tests, it was found that the range of sensitivity of the method experimentally determined at the Central Forensic Laboratory of the Police is between 11.5 pg/μL and 75 ng/μL DNA and meets the current criteria for acceptance.

Linearity tests

The tested method of DNA quantification is characterized by a linear correlation between the initial quantity of DNA template and CT value, i.e. the cycle in which product generation enters the exponential phase. The CT value of the breakpoint cycle depends on the reaction kinetics and initial DNA concentration. Usually, the CT value indicates the moment in which product generation enters the exponential phase.

In order to determine the linearity of the method, the researchers used the above results obtained in the experiment aimed to establish the sensitivity range. The linearity of the method was assessed using linear correlation coefficient R. The linear correlation coefficient R is a measure of a linear correlation between two variables (e.g., DNA concentration and CT). The closer the linear correlation coefficient R

Table 2

Dilution scheme of DNA standard at a concentration of 200 ng/μl

Dilution	Obtainde DNA concentration [ng/μl]	Dilution scheme	No. of repetitions
dil. A	75	75 μl **std.** (200 ng/μl) + 125 μl H_2O_{dd}	3x
dil. B	20	20 μl **std.1** (50 ng/μl) + 30 μl H_2O_{dd}	3x
dil. C	5	10 μl **dil. B** (20 ng/μl) + 30 μl H_2O_{dd}	3x
dil. D	1	10 μl **dil. C** (5 ng/μl) + 40 μl H_2O_{dd}	3x
dil. E	0.1	10 μl **dil. D** (1 ng/μl) + 90 μl H_2O_{dd}	3x
dil. F	0.023	50 μl **std. 7** (0,068 ng/μl) + 100 μl H_2O_{dd}	6x
dil. G	0.0115	100 μl **dil. H** (0,023 ng/μl) + 100 μl H_2O_{dd}	6x
dil. H	0.00575	100 μl **dil. I** (0,0115 ng/μl) + 100 μl H_2O_{dd}	6x
dil. I	0.00288	100 μl **dil. J** (0,00575 ng/μl) + 100 μl H_2O_{dd}	6x
dil. J	0.00144	100 μl **dil. K** (0,00288 ng/μl) + 100 μl H_2O_{dd}	6x

Table 3

Sensivity of method – mediane calculated concentration of DNA and desired concentration of DNA in the analyzed samples

Sample	DNA [ng/μl] concentration used	Obtained DNA [ng/μl] concentration	Mediane calculated of DNA [ng/μl]	CT	Mediane CT	CT IPC	Mediana CT IPC
A	75	57.66867	58.4976	22.794	22.77	28.459	28.25
A		58.49761		22.773		28.250	
A		59.71556		22.744		28.114	
B	20	17.37836	19.1131	24.506	24.37	27.077	27.08
B		19.11313		24.370		27.077	
B		19.66435		24.329		27.131	
C	5	5.07792	5.0123	26.261	26.28	27.127	27.13
C		4.97904		26.290		27.182	
C		5.01229		26.280		27.090	
D	1	1.04971	1.0021	28.511	28.58	27.363	27.48
D		1.00210		28.578		27.481	
D		0.91189		28.712		27.637	
E	0.1	0.09604	0.0960	31.924	31.92	27.658	27.60
E		0.08260		32.140		27.596	
E		0.09685		31.912		27.404	
F	0.023	0.02024	0.0230	34.146	33.97	27.580	27.38
F		0.02049		34.129		27.437	
F		0.02550		33.817		27.348	
F		0.02736		33.716		27.306	
F		0.00810		35.454		27.403	
F		0.03360		33.423		27.296	
G	0.0115	0.01149	0.0094	34.954	35.23	27.335	27.39
G		0.00535		36.047		27.341	
G		0.00972		35.193		27.338	
G		0.00917		35.276		27.448	
G		0.01447		34.626		27.479	
G		0.00706		35.649		27.575	
H	0.00575	0.00487	0.0019	36.180	37.51	27.490	27.36
H		0.00122		38.158		27.379	
H		0.00121		38.162		27.392	
H		0.00192		37.512		27.268	
H				Undetermined		27.331	
H		0.00967		35.201		27.229	
I	0.00288	0.00283	0.0027	36.953	37.02	27.332	27.34
I		0.00258		37.086		27.258	
I				Undetermined		27.266	
I		0.00129		38.077		27.355	
I		0.00615		35.846		27.442	
I				Undetermined		27.520	
J	0.00144		0.0023	Undetermined	37.22	27.447	27.33
J				Undetermined		27.457	
J		0.00227		37.272		27.358	
J		0.00243		37.171		27.292	
J				Undetermined		27.213	
J				Undetermined		27.112	

Table 4
Values of correlation coefficient R and nature / character of the linear relationship

Values of corelation coefficient R	Linear relationship
< 0.2	no linear relationship
0.2–0.4	weak relationship
0.4–0.7	moderate relationship
0.7–0.9	quite strong relationship
>0.9	very quite strong relationship

gets to 1, the stronger the linear correlation is. Table 4 presents the criteria for linear correlation coefficient assessment.

The adopted acceptance criterion was correlation coefficient R > 0.9, which would indicate a very strong linear correlation between the initial quantity of DNA template and the CT value.

The results of linearity test obtained experimentally are presented graphically in Figure 5 (The linearity test of the method; see Polish version). The graph shows the area with a linear correlation between median calculated DNA concentration and median CT value.

For the full range of DNA concentrations, samples A to J, the linear correlation coefficient **R** obtained in the experiments was: 0.999997. In view of the above, it should be noted that the target acceptance criterion for the linearity of the method was met within the full range of concentrations.

Range of measurement

Based on a comprehensive analysis of the results of sensitivity and linearity tests, the range of correct measurement, i.e. the range of values between the maximum and minimum DNA concentration determined correctly in the sample by 7500 Real-Time PCR system, was established. It was found that in the laboratory of the Biology Department, correctly measured DNA concentrations ranged from 11.5 pg/μL to 75 ng/μL.

Precision

Precision is the degree of consistency between single test results or the scatter of the results obtained by using tested method for multiple repeats of independent measurements of the same sample. The precision of validated method was established by studying the repeatability of obtained results and establishing within-laboratory reproducibility. Precision can be measured using standard deviation, relative standard deviation or coefficient of variation (CV).

In conducted experiments, the adopted measure of precision was the coefficient of variation (CV), which was the ratio between standard deviation (SD) and mean calculated DNA concentration (X), expressed as a percentage. The formula that describes the coefficient of variation is (2):

$$CV = \frac{SD}{X} * 100\ \% \quad (2),$$

where:
SD – standard deviation,
X – mean calculated DNA concentration, where $X \neq 0$.

The coefficient of variation indicates the variability of results, allowing the determination of the relative measure of their scatter. A result is considered precise when its CV value does not exceed 15% and for samples of low concentrations, the CV value can be up to 20%, which indicates good readings performed by the instrument [6, 7].

Repeatability estimates the degree of consistency of DNA concentration measurements performed at short intervals by the same analyst under the same conditions (the same measurement device, reagents and laboratory). Repeatability tests were carried out twice with a three-day interval by one analyst and each of the 6 dilutions of known DNA concentration was analyzed 6 times, thus 12 results per dilution were obtained. The results were analyzed and mean DNA concentration and standard deviation were calculated. The results of the repeatability tests are presented in Table 5 and Figure 6 (The repeatability of the method for the range of dilutions/samples B–D; see Polish version) and Figure 7 (The repeatability of the method for the range of dilutions/samples E–G; see Polish version). Figure 8 shows the repeatability – dependence of the average CT values on DNA concentration in the analyzed samples/relationship between the average CT values and DNA concentration in the analyzed samples and Figure 9 shows the repeatability – average CT values of internal control IPC in the analyzed samples obtained using Quantifiler® Human DNA Quantification Kit.

The adopted acceptance criterion for repeatability was the CV value which should not exceed 20%. It was observed that validated method met the acceptance criterion for repeatability for samples with the concentrations ranging from 0.1 to 20 ng/μL (samples E to B).

Table 5
The repeatability expressed by values of variation coefficient (CV)

Sample n = 12	DNA [ng/µl] concentration used	Value of variation coefficient (CV) [%]
B	20	**7**
C	5	**8**
D	1	**5**
E	0.1	**20**
F	0.023	41
G	0.0115	36

Reproducibility is the precision of results or variability of the results obtained in multiple tests of the same sample with the same method by different analysts with different knowledge and experience at different times, therefore under different conditions. Reproducibility tests were carried out at three-day intervals. Two different analysts conducted 6 analyses per each of 6 dilutions (dilutions B to G) of known DNA concentration, thus 12 results per dilution were obtained. The obtained results were analyzed, calculating mean DNA concentration and standard deviation. The results of reproducibility tests are presented in Table 6 and Figure 10 (Reproducibility of the method for the range of dilutions/samples B–D; see Polish version) and Figure 11 (Reproducibility of the method for the range of dilutions/samples E–G); see Polish version).

The adopted acceptance criterion for reproducibility was CV value which should not exceed 20%. The results that met this criterion were considered to be acceptable. It was observed that the validated method met the acceptance criterion for within-laboratory reproducibility for samples with DNA concentrations ranging from 0.1 to 20 ng/μL (samples E to B).

It is important to note that a method used in a forensic laboratory is expected to meet the criterion of precision, thus determining this parameter in validation tests is essential. The comprehensive analysis of obtained repeatability and reproducibility results supports the conclusion that validated method of DNA quantification is precise for DNA concentrations ranging from 0.1 to 20 ng/μL. A summary of obtained repeatability and reproducibility results for the method is shown in Table 7.

Table 6

Reproducibility expressed by values of variation coefficient (CV)

Próbka n = 12	DNA [ng/μl] concentration used	Value of variation coefficient (CV) [%]
B	20	**7**
C	5	**8**
D	1	**11**
E	0.1	**13**
F	0.023	21
G	0.0115	23

The validity of further analysis of STR markers when no nuclear DNA is detected in the sample

As part of the within-laboratory validation of the DNA quantification method employing the Quantifiler® Human DNA Quantification Kit coupled with the 7500 Real-Time PCR System and the HID Real-Time PCR Analysis Software V.1.1, an experiment was conducted, whose aim was to establish whether it is practical to continue genetic analyses when the results obtained with 7500 Real-Time PCR System are negative, i.e. no presence of nuclear DNA is detected, while IPC results are correct. The experiment was important from the perspective of reagent consumption.

Further analysis of STR markers was performed with the DNA standard of 0.0057 ng/μL and lower concentrations (dilutions H to J), for which negative results were indicated by the instrument. The amplification employed three currently available commercial kits: AmpFISTR NGM PCR and AmpFISTR Y-filer PCR from Applied Biosystems and ESI 17 Power Plex from Promega. The results of the experiment are presented in Table 8.

Amplification using the **AmpFISTR NGM PCR** kit from *Applied Biosystems.* Samples of 5.75 pg/μL: partial profiles obtained, loss of single alleles observed (sample H). Samples of 2.88 pg/μL and 1.44 pg/μL: single loci obtained (samples I and J).

Amplification using the **Power Plex ESI 17** kit from Promega. Samples of 5.75 pg/μL: full STR profiles obtained. Only in one sample, a loss of one locus/allele was observed (sample H). Samples of 2.88 pg/μL: full profile obtained in one sample, loss of two to four loci observed in two samples (sample I). Samples of 1.44 pg/μL: full profile obtained in one sample, loss of two to four loci observed in two samples (sample J).

Amplification using the **AmpFISTR Y-filer PCR** kit from *Applied Biosystems.* Samples of 5.75 pg/μL: full

Table 7

Precision – parallel results of repeatability and reproducibility

Próbka	DNA [ng/μl] concentration used	REPEATABILITY of value of variation coefficient (CV) [%]	REPEATABILITY of value of variation coefficient(CV) [%]	PRECISION
B	20	**7**	**7**	**Precise method**
C	5	**8**	**8**	
D	1	**5**	**11**	
E	0.1	**20**	**13**	
F	0.023	**41**	**21**	Method of low precision
G	0.0115	**36**	**23**	

Table 8

Comparison of amplification results

Próbka	Concentration used DNA [ng/μl]	Obtained concentration DNA [ng/μl]	AmpFISTR NGM PCR	ESI 17 Power Plex	AmpFISTR Y-filer PCR
H	0.00575	0.00315	amel, 12/15 loci	amel, 15/16 loci	**full profile**
H			amel, 13/15 loci	**full profile**	15/16 loci
H			amel, 14/15 loci	**full profile**	**full profile**
I	0.00288	0.00215	3/15 loci	amel, 12/16 loci	11/16 loci
I			5/15 loci	**full profile**	9/16 loci
I			4/15 loci	amel, 14/16 loci	9/16 loci
J	0.00144	0.00078	4/15 loci	amel, 13/16 loci	4/16 loci
J			1/15 loci	amel, 10/16 loci	7/16 loci
J			1/15 loci	amel, 11/16 loci	12/16 loci

profiles obtained in two samples, partial profile obtained in one sample (sample H). Samples of 2.88 pg/μL and below: partial profiles obtained (samples I and J).

The presented results clearly indicate that, although the 7500 Real-Time PCR System did not detect nuclear DNA in the samples and gave negative readings, discontinuation of STR marker analysis in these samples was not appropriate. The tests should be continued in such cases. Examples of the results in the form of electrophoregrams are presented in the figures (Figures 12–16; Fig. 12 The DNA profile for sample **H** [5,75 pg/μl] marked using NGM System; Fig. 13. The DNA profile for sample **H** [5,75 pg/μl] marked using **ESI 17 Power Plex** System; Fig. 14 The DNA profile for sample **I** [2,88 pg/μl] marked using **ESI 17 Power Plex** System; Fig. 15. The DNA profile for sample **J** [1,44 pg/μl] marked using **ESI 17 Power Plex** System; Fig. 16. The DNA profile for sample **H** [5,75 pg/μl] marked using **Y-filer PCR** System; see Polish version).

Conclusions

Based on the conducted validation tests of the DNA quantification method using the Quantifiler® Human DNA Quantification Kit and the 7500 Real-Time PCR System with the HID Real-Time PCR Analysis Software V.1.1, the following conclusions were drawn:

The validated method met the acceptance criterion for sensitivity for samples with DNA concentrations ranging from 11.5 to 75 ng/μL. A significant discrepancy of the results was observed for samples with a low DNA concentration. The above could be due to, e.g., variations in amplification efficiency, owing in turn to a too low quantity of DNA template in the tested samples, as well as the possibility of manual errors committed by analyst during the preparation of dilutions and sample transfer (pipetting errors). Taking into account that the DNA quantities recommended for STR marker analysis with each DNA amplification kit fall within the range of 0.5 to 2.0 ng/μL, the range of sensitivity of the method of DNA quantification in biological samples is satisfactory in relation to the tests conducted in our laboratory. In case of DNA samples at the concentration of 0.1 ng/μL or below, it is necessary to add the maximum recommended volume of DNA extract to the amplification reaction to obtain the best possible results of STR marker analysis.

The validated method met the acceptance criterion for linearity for samples with DNA concentrations ranging from 1.44 to 75 ng/μL.

Based on the test results of the sensitivity and linearity of method, the range of correct measurement of the method was established to fall within the range of 11.5 do 75 ng/μL.

Methods used in a forensic laboratory are expected to meet the criterion of precision, thus determining this parameter in validation tests is essential. The comprehensive analysis of obtained repeatability and interlaboratory reproducibility results supports the conclusion that the validated method of DNA quantification is precise for DNA concentrations ranging from 0.1 to 20 ng/μL.

The results obtained with samples for which negative readings of the 7500 Real-Time PCR System suggested no presence of nuclear DNA, while correct results were obtained for the IPC, confirmed the necessity to perform further analyses of STR markers. It is not rational to discontinue further analyses of samples with such negative readings from the instrument, as using very sensitive new DNA amplification kits often allows obtaining a full DNA profile.

Source

Figs.: 1–16: authors

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