analytica

MDPI

Article

Performance of Spectrophotometric and Fluorometric DNA Quantification Methods

Brigitte Bruijns [1,2,*,†,‡], Tina Hoekema [3], Lisa Oomens [4], Roald Tiggelaar [5] and Han Gardeniers [1]

[1] Mesoscale Chemical Systems, MESA$^+$ Institute, University of Twente, Drienerlolaan 5, 7500 AE Enschede, The Netherlands
[2] Life Sciences, Engineering & Design, Saxion University of Applied Sciences, M. H. Tromplaan 28, 7513 AB Enschede, The Netherlands
[3] BIOS Lab on a Chip Group, MESA$^+$ Institute, University of Twente, Drienerlolaan 5, 7500 AE Enschede, The Netherlands
[4] VyCAP BV, Abraham Rademakerstraat 41, 7425 PG Deventer, The Netherlands
[5] NanoLab Cleanroom, MESA$^+$ Institute, University of Twente, Drienerlolaan 5, 7500 AE Enschede, The Netherlands
* Correspondence: b.b.bruijns@saxion.nl
† Current affiliation: Technologies for Criminal Investigations, Saxion University of Applied Sciences, M.H. Tromplaan 28, 7513 AB Enschede, The Netherlands.
‡ Current affiliation: Politieacademie, Arnhemseweg 348, 7334 AC Apeldoorn, The Netherlands.

Abstract: Accurate DNA quantification is a highly important method within molecular biology. Methods widely used to quantify DNA are UV spectrometry and fluorometry. In this research, seven different DNA samples and one blank (MilliQ ultrapure water) were quantified by three analysts using one spectrophotometric (i.e., a NanoDrop instrument) and three fluorometric (i.e., the AccuGreen High Sensitivity kit, the AccuClear Ultra High Sensitivity kit, and the Qubit dsDNA HS Assay kit) methods. An analysis of variance (ANOVA) scheme was used to determine the influence of the analyst, the method, and the combination of analyst and method, on DNA quantification. For most samples, the measured DNA concentration was close to or slightly above the concentration of 10 ng/µL as specified by the supplier. Results obtained by the three analysts were equal. However, it was found that, compared to the fluorometric kits, the used spectrophotometric instrument in the case of fish DNA samples tends to overestimate the DNA concentration. Therefore, if sufficient sample volume is available, a combination of a spectrophotometric and a fluorometric method is recommended for obtaining data on the purity and the dsDNA concentration of a sample.

Keywords: DNA quantification; absorbance; fluorescence

Citation: Bruijns, B.; Hoekema, T.; Oomens, L.; Tiggelaar, R.; Gardeniers, H. Performance of Spectrophotometric and Fluorometric DNA Quantification Methods. *Analytica* **2022**, *3*, 371–384. https://doi.org/10.3390/analytica3030025

Academic Editor: Marcello Locatelli

Received: 26 August 2022
Accepted: 9 September 2022
Published: 16 September 2022

1. Introduction

Quantification of the exact amount of dsDNA in a sample is very important within a wide variety of molecular biology applications [1–3]. In order to avoid wasting samples in cases where only a limited amount of sample is available, which is often the case in, e.g., forensic and clinical settings, a reliable quantification method is required. Several methods are available on the market which can handle mass-limited samples, each with their own benefits and limitations.

The NanoDrop is a spectrophotometric instrument that measures the absorption of light at 260 nm to determine the amount of DNA in a sample. ssDNA, dsDNA, and RNA absorb at this wavelength, and therefore this method cannot discriminate between these types of nucleic acids. To obtain an indication of the purity of a sample, the 260/280 nm and the 260/230 nm ratios are determined. A ratio of 1.7–2.0 for 260/280 nm is acceptable (pure DNA has a ratio of 1.8), whereas a lower value can be caused by protein or phenol contamination. When RNA (or ssDNA) is present in a sample, this results in a higher ratio. For the 260/230 nm ratio, a value of more than 1.5 is indicative for a DNA sample of good quality. Since the NanoDrop determines the absorbed light at 260 nm, it tends to give

higher values for the measured concentration than methods that are dsDNA-specific (e.g., Qubit) [1,3,4]. The expected accuracy and reproducibility are 2% and about 2 ng/µL for samples below 100 ng/µL, respectively [5].

Fluorometric methods are widely used for DNA quantification. These kits contain an intercalating dye, such as PicoGreen, that binds in between the DNA strands of dsDNA. The fluorescent signal that is measured is related to the DNA concentration. The Qubit fluorometer, in combination with the Qubit High Sensitivity quantification kit, can be used for sample concentrations of 10 pg/µL till 100 ng/µL [6]. The AccuGreen quantitation kit is a recently developed dsDNA quantification method that can be used with a fluorometer, such as the Qubit fluorometer. According to the manufacturer, this kit is suitable for samples in the range of 0.1–10 ng/µL [7]; however, detailed information about this kit is not yet available in the literature. These two kits, based on fluorescent intercalating dyes, cannot give an indication of the purity of a sample. The AccuClear Ultra High Sensitivity kit with seven standards contains a green fluorescent dye (468/507 nm) that is compatible with fluorescence microplate readers. Samples between 0.03 and 250 ng are within the linear range of this kit [8]. Additionally, for this kit, only data provided by the supplier are available and—similar to the other two investigated kits—this kit also cannot determine the purity of the DNA sample.

UV absorbance spectroscopy (e.g., with a NanoDrop instrument) has been compared previously to several other DNA quantification methods (e.g., Qubit, SYBR Green, and PicoGreen dye staining). Haque et al. concluded that spectrophotometric DNA quantification was the most concordant and precise method in comparison with the PicoGreen assay and a real-time quantitative genomic PCR assay [9]. Simbolo et al. showed, for two DNA samples with a known concentration, that the NanoDrop and Qubit overestimated and underestimated the DNA concentration, respectively [10]. Nielsen et al. encountered higher DNA concentrations than expected based on the manufacturers information with, among other methods, UV spectroscopy and SYBR Green dye staining [2]. Nakayama et al. compared the Qubit with the NanoDrop and qPCR and also concluded that the Qubit, depending on the method for DNA extraction and dilution (e.g., salt concentration and denatured DNA), tends to underestimate the amount of DNA [11]. Additionally, He et al. measured significantly lower concentrations of DNA with the broad-range Qubit assay compared to the absorbance value. The AccuGreen assay and the high-sensitivity Qubit assay gave concentrations that were comparable to the spectrophotometric measurements [12]. The level of fragmentation does not influence spectrophotometric measurements, but this method has the lowest sensitivity when compared to PicoGreen and qPCR. The accuracy of PicoGreen and qPCR is influenced by fragmented DNA according to Sedlackova et al. [13]. Hussing et al. compared various quantification methods, among which were spectrophotometry with a NanoDrop instrument and fluorometry with a Qubit system. For all samples tested, e.g., adapter-dimer-rich, fragmented, and PCR-inhibited libraries, the NanoDrop gave much higher concentration values compared to the Qubit measurements, of which the latter were comparable with the quantification results from electrophoresis-based methods [14]. Additionally, high-molecular-weight DNA is difficult to quantify due to the complexity of the sample [12]. Li et al. compared PicoGreen with the diphenylamine reaction method and UV absorbance and concluded that the latter is the best method for measuring impurities. PicoGreen performed best with degraded DNA samples, and in the case of contaminants, diphenylamine would be the method of choice [15]. The Qubit and the NanoDrop were used by Masago et al. to determine the RNA and DNA concentration of samples extracted from lung cancer patients. They concluded that the absolute DNA concentration determined with the NanoDrop was higher than that found with the QuBit. The concentration of RNA, however, showed no significant difference between the Qubit and the NanoDrop measurements [16]. Quantification is also important when analyzing circulating cell-free tumor DNA (cfDNA). Ponti et al. compared the NanoDrop and both the ssDNA and dsDNA kit for the Qubit with cfDNA samples. The ssDNA kit gave the highest average value, 23.08 ng/µL, while the NanoDrop and dsDNA kit gave average values of 8.48 ng/µL and 4.32 ng/µL, respectively. Additionally, qPCR was performed,

which gave a much lower average value of only 0.39 ng/µL of cfDNA. Ponti et al. advised to use both the NanoDrop and the Qubit ssDNA kit in sequential combination in order to have a cost-effective solution for cfDNA quantification and only to use qPCR in the case of discordant values [17]. Khetan et al. found that for concentrations below 2.71 ng/µL, the NanoDrop was neither precise nor accurate. They recommend to use a fluorometric method for the quantification of cfDNA in blood samples, such as the Qubit [18]. In conclusion, the literature indicates that it can occur that spectrophotometric methods (slightly) overestimate the DNA concentration in comparison with fluorometric methods.

In this research, seven different DNA samples (four control samples from the tested kits and three in-house available DNA samples) and MilliQ ultrapure water as negative control were analyzed with one spectrophotometric method and three fluorometric methods. For the spectrophotometric analysis, a NanoDrop instrument was used, and for the fluorometric methods, the AccuGreen High Sensitivity kit, the AccuClear Ultra High Sensitivity kit, and the Qubit dsDNA High Sensitivity Assay kit were used. The goal of this research is, besides comparing the quantification methods as was performed in the above described literature, to determine the influence of the factor analyst. Therefore, all samples and methods were tested by three analysts to determine the variance between persons.

2. Materials and Methods

2.1. Materials

The Qubit® dsDNA HS Assay kit, including a 10 ng/µL standard DNA sample (Q) (λ dsDNA), was purchased from Thermo Fisher Scientific, Nieuwegein, NL, USA. The AccuClear Ultra High Sensitivity kit and the AccuGreen High Sensitivity kit (gift from Biotium), including 10 ng/µL standard DNA samples (AC and AG) (calf thymus dsDNA) in both kits, were obtained from Biotium. TaqMan® Control Genomic DNA (TM) (human, male, 10 ng/µL) was purchased from Applied Biosystems®. The TaqMan and AccuClear vials contained a limited amount of DNA, and to ensure that the same DNA sample was used in all the methods, a stock solution was made of 1 ng/µL TaqMan DNA (AccuGreen, AccuClear, and Qubit experiment) and AccuClear DNA (AccuGreen and Qubit experiment) prior to the experiments. All analysts used the same 1 ng/µL stock solution. Additionally, several other DNA samples were tested: 10 ng/µL salmon DNA (S) (D1626, Sigma-Aldrich, Zwijndrecht, NL, USA), 10 ng/µL herring DNA (H) (74782, Sigma-Aldrich), and 10 ng/µL DNA from Jurkat cells (J).

2.2. Spectrophotometric DNA Quantification

Measurements with the NanoDrop Instrument

For the NanoDrop measurements, a Nanodrop 2000c instrument (Thermo Fisher Scientific) was used. MilliQ ultrapure water (M) was used as blank measurement, and absorption at 340 nm was used as baseline. After each measurement, the pedestals were wiped with a clean wipe (KIMTEX). A total of 1.5 µL of each DNA sample was measured in triplo by all three analysts.

2.3. Fluorometric DNA Quantifcation

2.3.1. Measurement with the AccuGreen High Sensitivity Kit

The protocol of the manufacturer was used for the measurements with the AccuGreen High Sensitivity kit. Each DNA sample was measured in triplo by all three analysts with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

2.3.2. Measurement with the AccuClear Ultra High Sensitivity Kit

The protocol of the manufacturer was used for the measurements with the AccuClear Ultra High Sensitivity kit. Each DNA sample was measured in triplo by all three analysts in a Corning 96 flat bottom black polystyrene microplate with a Tecan M200 PRO multimode reader, operated by Tecan I-control software.

2.3.3. Measurements with the Qubit dsDNA HS Assay Kit

The protocol of the manufacturer was used for the measurements with the Qubit dsDNA HS Assay kit. Each DNA sample was measured in triplo by all three analysts with a Qubit 2.0 Fluorometer.

2.4. Statistical Analysis

To determine whether there are significant differences per method, one-way ANOVA was conducted ($\alpha = 0.05$). To check if the factors 'analyst', 'method', or 'analyst × method' were significant, ANOVA with repeated measures with two within-subjects factors was used, while taking sphericity into account. The Excel add-in "Real Statistics Using Excel" was used to carry out the ANOVA analyses ($\alpha = 0.05$) [19].

3. Results

3.1. Spectrophotometric DNA Quantification

Measurements with the NanoDrop Instrument

Almost all DNA samples measured with the NanoDrop showed 260/280 nm ratios above 2.0. This suggests a contribution of single-strand nucleic acids (ssDNA or RNA) in the solution. Only the fish samples, salmon and herring DNA, gave values of 1.7–2.0 for the 260/280 nm ratio. These fish DNA samples also showed a 260/230 nm ratio above 1.5, while the other samples gave values well below 0.5.

The measured concentrations of the DNA samples are depicted in Figure 1 and can also be found in Table 1. Most of the DNA samples gave a value of 10 ng/µL ± 2 ng/µL, which is within the specifications of this method.

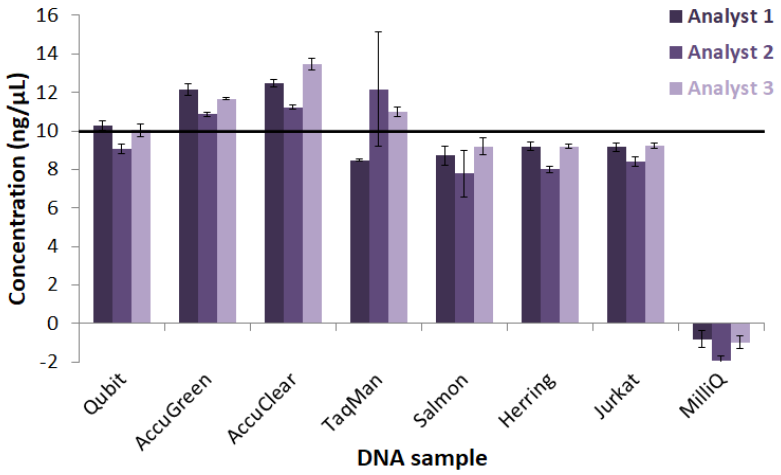

Figure 1. DNA concentrations of the samples as measured by the three analysts with the NanoDrop.

Table 1. DNA concentrations (in ng/µL) of the samples as measured by the three analysts with all four quantification methods.

	Spectrophotometric Nanodrop			Fluorometric AccuGreen			AccuClear			Qubit		
Sample	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Qubit (Q)	10.3 ± 0.3	9.1 ± 0.2	10.0 ± 0.3	11.3 ± 0.4	10.9 ± 0.1	9.8 ± 0.2	9.8 ± 0.1	9.6 ± 3.7	10.4 ± 1.0	10.0 ± 0.3	10.5 ± 0.1	10.1 ± 0.1
AccuGreen (AG)	12.1 ± 0.3	10.9 ± 0.1	11.7 ± 0.1	10.2 ± 0.3 [a]	10.0 ± 0.7	9.8 ± 0.4	10.4 ± 0.4	7.9 ± 2.2	10.6 ± 0.6	9.9 ± 0.5	9.9 ± 0.3	10.4 ± 0.3
AccuClear (AC)	12.5 ± 0.2	11.2 ± 0.1	13.5 ± 0.3	5.5 ± 1.4	10.2 ± 0.2	10.1 ± 0.6	10.2 [b]	8.4 ± 3.6	10.8 ± 0.8	9.9 ± 0.4	10.4 ± 0.2	9.8 ± 0.6
TaqMan (TM)	8.5 ± 0.1	12.2 ± 3.0	11.0 ± 0.3	9.1 ± 0.9	8.0 ± 0.3	7.9 ± 0.6	8.7 ± 5.6	8.0 ± 1.8	7.0 ± 0.4	6.9 ± 0.3	7.3 ± 0.3	7.2 ± 0.1
Salmon (S)	8.7 ± 0.5	7.8 ± 1.2	9.2 ± 0.5	1.2 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	0.5 ± 0.0	1.0 ± 0.1	0.6 ± 0.0	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.0
Herring (H)	9.2 ± 0.2	8.0 ± 0.2	9.2 ± 0.1	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	1.0 ± 0.2	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
Jurkat (J)	9.2 ± 0.2	8.4 ± 0.3	9.2 ± 0.2	10.8 ± 0.2	9.7 ± 0.3	9.3 ± 0.4	9.7 ± 0.8	10.2 ± 2.1	10.1 ± 0.3	9.9 ± 0.2	10.0 ± 0.1	10.5 ± 0.3
MilliQ (M)	−0.8 ± 0.4	−1.9 ± 0.3	−1.0 ± 0.3	≤0.05	≤0.05	≤0.05	−0.3 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	≤0.05	≤0.05	≤0.05

[a] n = 2. [b] n = 1.

3.2. Fluorometric DNA Quantification

3.2.1. Measurement with the AccuGreen High Sensitivity Kit

The measured concentrations of the DNA samples with the AccuGreen High Sensitivity kit are depicted in Figure 2 and can also be found in Table 1. The Qubit cannot measure values below 0.50 ng/mL, so this is displayed as 0 in Figure 2. This was the case for all the MilliQ ultrapure water (negative control) samples. Additionally, values above 600 ng/mL give a notification error ("fluorescence signal too high") which implies that no further quantification can be performed. This happened for two samples (once for the Qubit control and once for the AccuGreen control) of analyst 1, which means the original sample had a concentration above 12 ng/µL (these values were not included in the averaged data given in Figure 2).

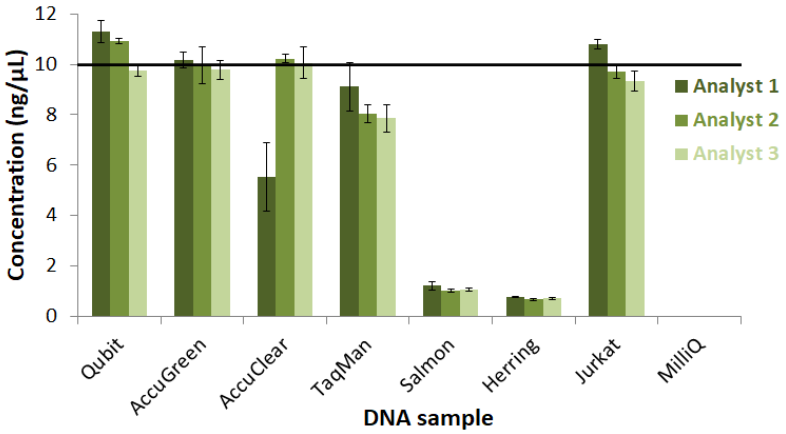

Figure 2. DNA concentrations of the samples as measured by the three analysts with the AccuGreen High Sensitivity kit.

3.2.2. Measurement with the AccuClear Ultra High Sensitivity Kit

The measured concentrations of the DNA samples with the AccuClear Ultra High Sensitivity kit are depicted in Figure 3 and can also be found in Table 1. Seven DNA standards are provided with the kit in order to generate a standard curve, by averaging the triplicate value for each sample. The equation of the trend line of this standard curve is used to calculate the amount of unknown DNA in each well. The AccuClear DNA sample was only quantified once by analyst 1, as can be seen in Table 1, due to a pipetting mistake. One well contained a double amount of DNA sample, and one well received no sample at all; a mistake that became clear from the fluorescence measurements.

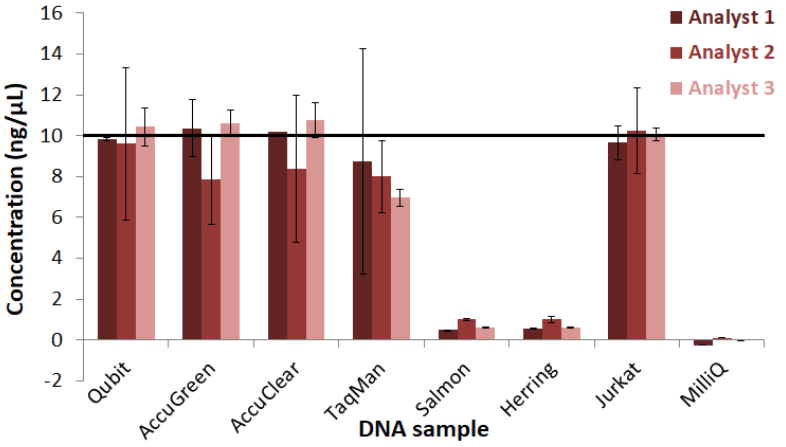

Figure 3. DNA concentrations of the samples as measured by the three analysts with the AccuClear Ultra High Sensitivity kit.

3.2.3. Measurements with the Qubit dsDNA HS Assay Kit

The measured concentrations of the DNA samples with the Qubit dsDNA HS Assay kit are depicted in Figure 4 and can also be found in Table 1. Similarly to the AccuClear Ultra High Sensitivity kit, values below 0.50 ng/mL are displayed as 0 in Figure 4. This was the case for all the MilliQ ultrapure water (negative control) samples.

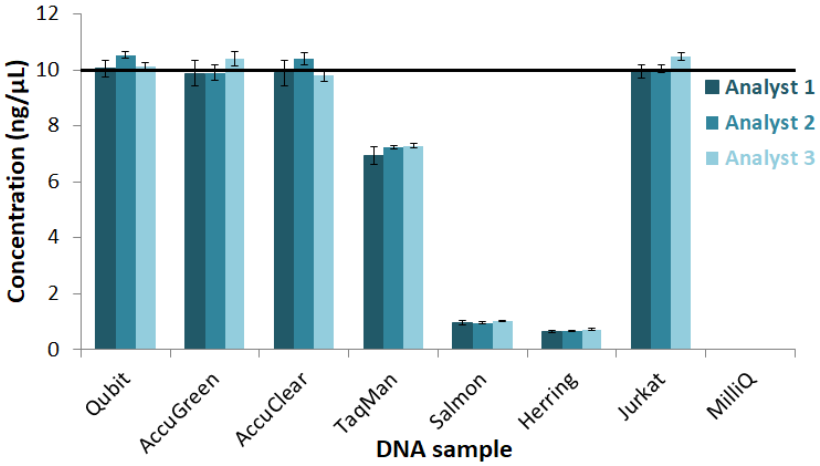

Figure 4. DNA concentrations of the samples as measured by the three analysts with the Qubit dsDNA HS Assay kit.

3.2.4. Statistical Analysis

By using one-way ANOVA, with the values given in Table 1, it turned out that the factor 'analyst' did not result in significant differences (sample concentrations) for each method (Tables A1–A4). To determine the influence of the factors 'method' and 'analyst × method', an ANOVA with repeated measures was performed. The two-factor ANOVA with repeated measures with two within-subjects factors showed that the factors 'analyst' and 'analyst × method' did not show a significant difference. In contrast, the factor 'method' did show a significant difference ($p \leq 0.05$) (Table A5). The differences in the mean values are also depicted in Figure 5. Upon comparison with the fluorometric DNA quantification methods, the spectrophotometric method using the NanoDrop instrument overestimated the DNA concentrations, as can be observed in Figure 5. This can be explained by the measured DNA concentration of the fish samples, which was, on average, 8.7 ng/µL for the spectrophotometric method and 0.8 ng/µL for the fluorometric methods.

To check if the variances of the differences between all factors are equal, sphericity must be determined. In the case that the variances of the differences between all combinations of related groups are equal, sphericity must be taken into account, which is the case when epsilon is equal to 1. The factor 'analyst' shows an epsilon of (close to) 1 for both the Greenhouse–Geisser (GG) and the Huynh–Feldt (HF) epsilon. However, the factors 'method' and 'analyst × method' show epsilon values far below 1 (Table A6). Using the corrected values, the factor 'method' is not significantly different (Table A7) [19].

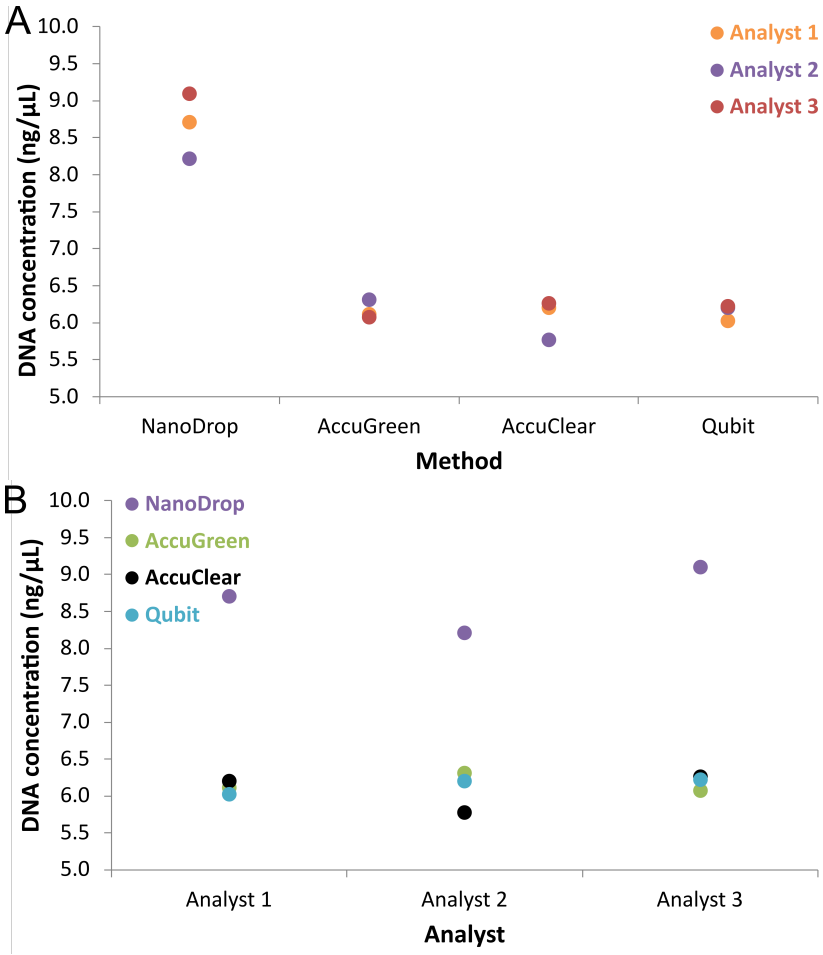

Figure 5. Comparison of means for interaction: (**A**) The measured DNA concentration versus the method for each analyst; (**B**) the measured DNA concentration versus the analyst for each method.

4. Discussion

Measuring the DNA concentration of a sample with a spectrophotometer has several advantages. The method is fast, no additional reagents are required, no calibration is needed (besides measuring the blank), and the sample can be reused. Whereas with a standard spectrophotometer relatively large volumes are needed for cuvette measurements (in the order of milliliters), the NanoDrop instrument does not require cuvettes, and even volumes as low as 1 µL can be used. The main drawback of this spectrophotometric DNA quantification method is its nonspecificity: all compounds that absorb at 260 nm will contribute to a measurement, and no distinction between dsDNA, ssDNA, and RNA can be made. Apparently, the fish DNA samples contain a substantial amount of unknown specimen that is not dsDNA (according to the investigated fluorometric methods) that exhibits absorption at 260 nm.

Fluorometric methods used to measure the concentration are dsDNA-specific. These methods require more sample preparation steps, since the fluorescent dye (and additional buffer) must be added to each sample. It is also mandatory to create a standard curve with the fluorometer (as is the case for the AccuGreen High Sensitivity kit and the Qubit dsDNA HS Assay kit), or afterwards with software (e.g., Excel, as is the case for the AccuClear Ultra High Sensitivity kit). Preferentially, a new standard curve is made before each new set of measurements (Qubit readings) or per well plate (AccuClear). For Qubit readings, only two standards, 0 and 10 ng/µL, are available within the kit, which makes the standard curve a bit questionable. When the quality of one of the standards is compromised (e.g., contamination or pipette error), the curve is not trustworthy anymore, possibly going unnoticed by the analyst. The AccuClear Ultra High Sensitivity kit has seven standards

included, measured in triplo, which makes this standard curve more reliable. The Qubit dsDNA HS Assay kit requires an incubation time of 2 min, and the AccuGreen High Sensitivity kit prescribes an incubation time of at least 2 min. Therefore, these latter fluorometric methods are relatively time-consuming in the case of a large amount of samples. With the AccuClear Ultra High Sensitivity kit, a whole 96-well plate (including reference samples) can be read at once. This makes this method more suitable for larger amounts of samples.

Although the fish samples, salmon and herring DNA, showed the best results in terms of purity, these samples did not contain 10 ng/µL according to the fluorometric methods. This is striking, since the sample does contain a DNA concentration of 10 ng/µL based on weighing (original sample is in solid state and must be diluted by the analyst to the appropriate concentration) and performed spectrophotometric measurements. With UV spectroscopy, all sources of nucleic acids, single- and double-stranded, are measured, while the fluorometric methods are dsDNA specific. Apparently, these fish samples do not contain the amount of dsDNA that is expected based on weight. Carvalho et al. used salmon sperm DNA samples as it turned out that the λDNA standard was not representative for fragmented DNA. The low-molecular-weight salmon sperm DNA is less purified and more fragmented. They measured dsDNA concentrations for the salmon sperm DNA, which were only around 10% of the expected concentration [20], which is in accordance with the results of this research. He et al. suggest to use a nucleic acid standard that matches the samples that are being measured [12].

For a spectrophotometric reading using the NanoDrop instrument, a sample volume of 1 µL is sufficient, while the fluorometric methods require 10 µL (AccuGreen and AccuClear) or 1–20 µL (Qubit). However, the fluorometric methods have a lower detection limit in comparison with methods based on absorbance, and therefore a more diluted sample can be used. The drawback of the fluorometric methods is that the purity, the 260/280 nm and 260/230 nm ratios, of the sample cannot be determined. Therefore, a combination of the NanoDrop (or another spectrophotometric method) in combination with a fluorometric method is recommended, in agreement with the suggestion of Simbolo et al. [10].

For all fluorometry-analyzed DNA samples, the expected concentration of 10 ng/µL was measured, since calibration standards of the the Qubit High Sensitivity quantification kit, the AccuGreen quantitation kit, and one of the standards of the AccuClear Ultra High Sensitivity kit contained this concentration. Additionally, this concentration falls within the range of measurable concentrations as indicated by the suppliers of the kits.

Some DNA quantification measurements showed pretty large standard deviations ($\geq$0.5 ng/µL), so it is recommended to perform all measurements in triplo. Additionally, the used DNA quantification methods are nonspecific to the species. Moreover, it should be mentioned that, in contrast to commercial DNA samples, real-life/case samples (such as a buccal swab or a bone sample) might contain DNA from multiple biological sources. Therefore, in areas such as forensic genetics, human-specific DNA quantification methods are used (e.g., qPCR). In fact, it is noted that such real-life/case samples can (negatively) affect spectrometric as well as fluorometric DNA quantification.

Since epsilon is lower than 0.70 for the factor 'method', an MANOVA might be used, instead of an ANOVA. However, this is not recommended for this sample size (eight samples), which is lower than k (the number of levels of the repeated measures factor) + 10 [19,21].

5. Conclusions

A total of four different DNA quantification methods were investigated by three analysts for seven DNA samples and one blank. Based on the conducted ANOVA, it can be concluded that the factors 'analyst' and 'analyst × method' do not result in significant differences in sample concentration. In contrast, the factor 'method' does show a significant difference; in the case of fish samples, the applied spectrophotometric method overestimated the DNA concentration in comparison to the fluorometric methods used. This can be explained by the measured DNA concentration of the fish (herring and salmon DNA) samples, which was, on average, 8.7 ng/µL and 0.8 ng/µL for the spectrophotometric method and fluorometric methods, respectively. Presumably, these DNA samples contain a substantial amount of material that exhibits absorption at 260 nm, which is not dsDNA according to the fluorometric methods. Except for these fish samples, the measured samples show a concentration around 10 ng/µL, as is expected based on the information of the supplier. The fluorometric methods (the AccuGreen High Sensitivity kit, the AccuClear Ultra High Sensitivity kit, and the Qubit dsDNA HS Assay kit) do not show a significant difference among the samples or analysts. To conclude, in order to achieve information on the purity and the dsDNA concentration of a sample, a combination of a spectrophotometric and a fluorometric method is recommended, provided that enough sample volume is available.

Author Contributions: Conceptualization, B.B. and H.G.; methodology, B.B. and H.G.; validation, B.B., T.H., and L.O.; writing—original draft preparation, B.B.; writing—review and editing, B.B. and R.T.; supervision, R.T. and H.G.; project administration, H.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ANOVA	Analysis of variance
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleic acid
ssDNA	Single-stranded DNA
UV	Ultraviolet

Appendix A. ANOVA Analysis

Table A1. One-way ANOVA for the NanoDrop.

DATASET			**NanoDrop**			
			1	2	3	
		Qubit	10.3	9.1	10.0	
		AccuGreen	12.1	10.9	11.7	
		AccuClear	12.5	11.2	13.5	
		TaqMan	8.5	12.2	11.0	
		Salmon	8.7	7.8	9.2	
		Herring	9.2	8.0	9.2	
		Jurkat	9.2	8.4	9.2	
		MilliQ	−0.8	−1.9	−1.0	
ANOVA: one-way						
DESCRIPTION						
Groups	Count	Sum	Mean	Variance		
1	8	69.7	8.7125	17.04982143		
2	8	65.7	8.2125	19.33553571		
3	8	72.8	9.1	18.94		
ANOVA						
Sources	SS	df	MS	F	*p* value	F crit
Between groups	3.1675	2	1.58375	0.085878343	0.918026473	3.466800112
Within groups	387.2775	21	18.44178571			
Total	390.445	23				
			F < F crit: No significant difference.			

Table A2. One-way ANOVA for the AccuGreen High Sensitivity kit.

DATASET			**AccuGreen**			
			1	2	3	
		Qubit	11.3	10.9	9.8	
		AccuGreen	10.2	10.0	9.8	
		AccuClear	5.5	10.2	10.1	
		TaqMan	9.1	8.0	7.9	
		Salmon	1.2	1.0	1.0	
		Herring	0.8	0.7	0.7	
		Jurkat	10.8	9.7	9.3	
		MilliQ	0.0	0.0	0.0	
ANOVA: one-way						
DESCRIPTION						
Groups	Count	Sum	Mean	Variance		
1	8	48.9	6.1125	23.51553571		
2	8	50.5	6.3125	23.37839286		
3	8	48.6	6.075	21.31928571		
ANOVA						
Sources	SS	df	MS	F	*p* value	F crit
Between groups	0.260833	2	0.130416667	0.005735692	0.994282283	3.466800112
Within groups	477.4925	21	22.7377381			
Total	477.7533	23				
			F < F crit: No significant difference.			

Table A3. One-way ANOVA for the AccuClear Ultra High Sensitivity kit.

DATASET			**AccuClear**			
			1	2	3	
		Qubit	9.8	9.6	10.4	
		AccuGreen	10.4	7.9	10.6	
		AccuClear	10.2	8.4	10.8	
		TaqMan	8.7	8.0	7.0	
		Salmon	0.5	1.0	0.6	
		Herring	0.6	1.0	0.6	
		Jurkat	9.7	10.2	10.1	
		MilliQ	−0.3	0.1	0.0	
ANOVA: one-way						
DESCRIPTION						
Groups	Count	Sum	Mean	Variance		
1	8	49.6	6.2	24.45714		
2	8	46.2	5.775	18.33929		
3	8	50.1	6.2625	25.01982		
ANOVA						
Sources	SS	df	MS	F	*p* value	F crit
Between groups	1.125833	2	0.562917	0.024902	0.975434	3.4668
Within groups	474.7138	21	22.60542			
Total	475.8396	23				
			F < F crit: No significant difference.			

Table A4. One-way ANOVA for the Qubit dsDNA HS Assay kit.

DATASET			**Qubit**			
			1	2	3	
		Qubit	10.0	10.5	10.1	
		AccuGreen	9.9	9.9	10.4	
		AccuClear	9.9	10.4	9.8	
		TaqMan	6.9	7.2	7.3	
		Salmon	1.0	1.0	1.0	
		Herring	0.6	0.6	0.7	
		Jurkat	9.9	10.0	10.5	
		MilliQ	0.0	0.0	0.0	
ANOVA: one-way						
DESCRIPTION						
Groups	Count	Sum	Mean	Variance		
1	8	48.2	6.025	21.79928571		
2	8	49.6	6.2	23.15714286		
3	8	49.8	6.225	23.03357143		
ANOVA						
Sources	SS	df	MS	F	*p* value	F crit
Between groups	0.19	2	0.095	0.004191793	0.995817813	3.466800112
Within groups	475.93	21	22.66333333			
Total	476.12	23				
			F < F crit: No significant difference.			

Table A5. Two-factor ANOVA with repeated measures with two within-subjects factors.

	NanoDrop			**AccuGreen**			**AccuClear**			**Qubit**			
	1	2	3	1	2	3	1	2	3	1	2	3	Sample
Qubit	10.3	9.1	10.0	11.3	10.9	9.8	9.8	9.6	10.4	10.0	10.5	10.1	10.15
AccuGreen	12.1	10.9	11.7	10.2	10.0	9.8	10.4	7.9	10.6	9.9	9.9	10.4	10.31666667
AccuClear	12.5	11.2	13.5	5.5	10.2	10.1	10.2	8.4	10.8	9.9	10.4	9.8	10.20833333
TaqMan	8.5	12.2	11.0	9.1	8.0	7.9	8.7	8.0	7.0	6.9	7.2	7.3	8.483333333
Salmon	8.7	7.8	9.2	1.2	1.0	1.0	0.5	1.0	0.6	1.0	1.0	1.0	2.833333333
Herring	9.2	8.0	9.2	0.8	0.7	0.7	0.6	1.0	0.6	0.6	0.6	0.7	2.725
Jurkat	9.2	8.4	9.2	10.8	9.7	9.3	9.7	10.2	10.1	9.9	10.0	10.5	9.75
MilliQ	−0.8	−1.9	−1.0	0.0	0.0	0.0	−0.3	0.1	0.0	0.0	0.0	0.0	−0.325
	8.7125	8.2125	9.1	6.1125	6.3125	6.075	6.2	5.775	6.2625	6.025	6.2	6.225	6.767708333

	Method				**Analyst**		
	NanoDrop	AccuGreen	AccuClear	Qubit	1	2	3
Qubit	9.8	10.7	9.9	10.2	10.4	10.0	10.1
AccuGreen	11.56666667	10.0	9.6	10.1	10.7	9.7	10.6
AccuClear	12.4	8.6	9.8	10.0	9.5	10.1	11.1
TaqMan	10.56666667	8.3	7.9	7.1	8.3	8.9	8.3
Salmon	8.566666667	1.1	0.7	1.0	2.9	2.7	3.0
Herring	8.8	0.7	0.7	0.6	2.8	2.6	2.8
Jurkat	8.933333333	9.9	10.0	10.1	9.9	9.6	9.8
MilliQ	−1.233333333	0.0	−0.1	0.0	−0.3	−0.5	−0.3
	8.675	6.166666667	6.079166667	6.15	6.8	6.6	6.9

WORKING TABLE

	a	b	m	n
	4	3	8	96

	count	SS	df	MS			
Total	1	1936.669896	95	20.3859989			
A (Method)	24	116.5119792	3	38.83732639			
B (Analyst)	32	1.352708333	2	0.676354167			
C (Sample)	12	1558.075729	7	222.582247			
AB Bet	8	121.2561458	11	11.02328598			
A × B		3.391458333	6	0.565243056			
AC Bet	3	1893.703229	31	61.08720094			
A × C		219.1155208	21	10.43407242			
BC Bet	4	1566.972396	23	68.1292346			
B × C		7.543958333	14	0.538854167			
A × B × C		30.67854167	42	0.730441468			
ANOVA							
		SS	df	MS	F	*p*-value	Fcrit
A (Method)		116.5119792	3	38.83732639	3.722164	0.027287	3.072467
A × C		219.1155208	21	10.43407242			
B (Analyst)		1.352708333	2	0.676354167	1.255171	0.315213	3.738892
B × C		7.543958333	14	0.538854167			
A × B		3.391458333	6	0.565243056	0.773838	0.594905	2.323994
A × B × C		30.67854167	42	0.730441468			
C (Sample)		1558.075729	7	222.582247			
	Method		**F > F crit: Significant difference.**				
	Analyst		**F < F crit: No significant difference.**				
	Method × Analyst		**F < F crit: No significant difference.**				

Table A6. Covariance matrices taking sphericity into account.

COVARIANCE MATRIX			**ANALYST × METHOD**											
ND1	ND2	ND3	AG1	AG2	AG3	AC1	AC2	AC3	Q1	Q2	Q3			
17.05	16.74	17.52	11	13.31	12.88	13.63	10.94	13.86	12.94	13.32	13.18	13.87		
16.74	19.34	18.66	13.07	14.31	13.98	15.19	12.43	14.2	13.41	13.86	13.82	14.92		
17.52	18.66	18.94	11.35	13.86	13.55	14.48	11.65	14.12	13.22	13.68	13.48	14.54		
11	13.07	11.35	23.52	21.7	20.47	22.11	19.69	21.83	20.74	21.26	21.61	19.03		
13.31	14.31	13.86	21.7	23.38	22.28	23.77	20.33	24.04	22.48	23.21	23.06	20.48		
12.88	13.98	13.55	20.47	22.28	21.32	22.79	19.35	22.97	21.46	22.13	22.02	19.6		
13.63	15.19	14.48	22.11	23.77	22.79	24.46	20.73	24.46	22.86	23.56	23.52	20.96		
10.94	12.43	11.65	19.69	20.33	19.35	20.73	18.34	20.74	19.54	20.16	20.15	17.84		
13.86	14.2	14.12	21.83	24.04	22.97	24.46	20.74	25.02	23.32	24.03	23.92	21.04		
12.94	13.41	13.22	20.74	22.48	21.46	22.86	19.54	23.32	21.8	22.45	22.38	19.72		
13.32	13.86	13.68	21.26	23.21	22.13	23.56	20.16	24.03	22.45	23.16	23.02	20.32		
13.18	13.82	13.48	21.61	23.06	22.02	23.52	20.15	23.92	22.38	23.02	23.03	20.27		
13.87	14.92	14.54	19.03	20.48	19.6	20.96	17.84	21.04	19.72	20.32	20.27	18.55		
7.867	6.503	7.663	−3.34	−2.48	−2.03	−2.65	−2.21	−2.5	−2.1	−2.32	−2.4			
6.503	8.048	7.744	−2.33	−2.54	−1.98	−2.14	−1.78	−3.21	−2.67	−2.83	−2.82			
7.663	7.744	8.401	−3.67	−2.61	−2.05	−2.48	−2.18	−2.91	−2.49	−2.63	−2.78			
−3.34	−2.33	−3.67	4.005	0.743	0.386	0.663	1.37	0.302	0.544	0.462	0.867			
−2.48	−2.54	−2.61	0.743	0.973	0.75	0.876	0.565	1.065	0.835	0.958	0.866			
−2.03	−1.98	−2.05	0.386	0.75	0.668	0.774	0.458	0.873	0.687	0.762	0.704			
−2.65	−2.14	−2.48	0.663	0.876	0.774	1.08	0.475	1.005	0.728	0.825	0.837		#A	4
−2.21	−1.78	−2.18	1.37	0.565	0.458	0.475	1.212	0.41	0.535	0.551	0.594		#B	3
−2.5	−3.21	−2.91	0.302	1.065	0.873	1.005	0.41	1.483	1.111	1.214	1.157		GG numerator	1351
−2.1	−2.67	−2.49	0.544	0.835	0.687	0.728	0.535	1.111	0.913	0.963	0.947		GG denominator	5674
−2.32	−2.83	−2.63	0.462	0.958	0.762	0.825	0.551	1.214	0.963	1.064	0.983		GG epsilon	0.238
−2.4	−2.82	−2.78	0.867	0.866	0.704	0.837	0.594	1.157	0.947	0.983	1.05			
30.45	4.017	1.476	0.613	0.137	0.047	0.026								

Table A6. *Cont.*

COVARIANCE MATRIX	METHODS					COVARIANCE MATRIX	ANALYST			
	NanoDrop	AccuGreen	AccuClear	Qubit			Analyst 1	Analyst 2	Analyst 3	
NanoDrop	17.90722222	13.04	13.39	13.43		Analyst 1	18.33678571	18.2	18.65	
AccuGreen	13.0368254	21.9	21.87	22		Analyst 2	18.19821429	18.3	18.67	
AccuClear	13.39146825	21.87	22.19	22.34		Analyst 3	18.64674107	18.67	19.28	
Qubit	13.43444444	22	22.34	22.63			18.39391369	18.39	18.86	means
	14.44249008	19.7	19.95	20.1	means		18.33678571	18.3	19.28	variance
	17.90722222	21.9	22.19	22.63	variance					
EPSILON	METHODS					EPSILON	ANALYST			
# Groups	4					# Groups	3			
Means of var	21.15703869					Means of var	18.63832961			
Matrix mean	18.54852					Matrix mean	18.54852			
SS matrix	5787.246376					SS matrix	3097.357059			
SS row means	1398.750839					SS row means	1032.291337			
GG numerator	108.8698673					GG numerator	0.072590953			
GG denominator	306.0045449					GG denominator	0.075163632			
GG epsilon	0.355778596					GG epsilon	0.965772291			
# Subjects	8					# Subjects	8			
# Groups	4					# Groups	3			
GG epsilon	0.355778596					GG epsilon	0.965772291			
HF numerator	6.538686298					HF numerator	13.45235666			
HF denominator	17.79799264					HF denominator	10.13691083			
HF epsilon	0.367383358					HF epsilon	1			
Lower bound	0.333333333					Lower bound	0.5			

Table A7. ANOVA with repeated measures corrected for sphericity.

ANOVA							
Sources of Variation		SS	df	MS	F	*p*-value	F
A (Method)	Sphericity	116.5	3	38.83732639	3.722163775	0.027287316	3.072466986
	GG	116.5	1.067	109.1615034	3.722163775	0.095021447	5.591447851
	HF	116.5	1.102	105.7133524	3.722163775	0.095021447	5.591447851
	Lower Bound	116.5	1	116.5119792	3.722163775	0.095021447	5.591447851
A × C (Error)	Sphericity	219.1	21	10.43407242			
	GG	219.1	7.471	29.32743157			
	HF	219.1	7.715	28.40104811			
	Lower Bound	219.1	7	31.30221726			
B (Analyst)	Sphericity	1.353	2	0.676354167	1.255171081	0.315212719	3.738891832
	GG	1.353	1.932	0.700324676	1.255171081	0.282838366	4.667192732
	HF	1.353	2	0.676354167	1.255171081	0.315212719	3.738891832
	Lower Bound	1.353	1	1.352708333	1.255171081	0.299527953	5.591447851
B × C (Error)	Sphericity	7.544	14	0.538854167			
	GG	7.544	13.52	0.55795157			
	HF	7.544	14	0.538854167			
	Lower Bound	7.544	7	1.077708333			
A × B	Sphericity	3.391	6	0.565243056	0.773837576	0.594904763	2.323993797
	Lower Bound	3.391	1	3.391458333	0.773837576	0.408214928	5.591447851
A × B × C (Error)	Sphericity	30.68	42	0.730441468			
	Lower Bound	30.68	7	4.38264881			
C (Sample)		1558	7	222.582247			
Total		1937	95	20.3859989			

References

1. Nicklas, J.; Buel, E. Quantification of DNA in forensic samples. *Anal. Bioanal. Chem.* **2003**, *376*, 1160–1167. [CrossRef] [PubMed]
2. Nielsen, K.; Smidt Mogensen, H.; Hedman, J.; Niederstätter, H.; Parson, W.; Morling, N. Comparison of five DNA quantification methods. *Forensic Sci. Int. Genet.* **2008**, *2*, 226–230. [CrossRef] [PubMed]
3. Li, Z.; Zhang, P.; Yang, B.; Liu, J.; Xi, H.; Zhang, D.; Yamaguchi, Y. High throughput DNA concentration determination system based on fluorescence technology. *Sens. Actuators B Chem.* **2021**, *328*, 128904. [CrossRef]
4. O'Neill, M.; McPartlin, J.; Arthure, K.; Riedel, S.; McMillan, N. Comparison of the TLDA with the Nanodrop and the reference Qubit system. *J. Phys. Conf. Ser.* **2011**, *307*, 012047. [CrossRef]
5. Thermo Fisher Scientific. NanoDrop 2000c UV-Vis Spectrophotometer. Available online: http://www.nanodrop.com/Productnd2000coverview.aspx (accessed on 16 November 2020).
6. Thermo Fisher Scientific. Qubit Fluorometric Quantitation. Available online: http://www.thermofisher.com/nl/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit.html# (accessed on 16 November 2020).
7. Biotium. AccuGreen High Sensitivity dsDNA Quantitation Kit . Available online: http://biotium.com/product/accugreen-high-sensitivity-dsdna-quantitation-kit/ (accessed on 6 November 2020).
8. Biotium. AccuClear Ultra High Sensitivity dsDNA Quantitation Kit with 7 DNA Standards. Available online: http://biotium.com/product/accuclear-ultra-high-sensitivity-dsdna-quantitation-kit-with-7-dna-standards/ (accessed on 6 November 2020).

9. Haque, K.; Pfeiffer, R.; Beerman, M.; Struewing, J.; Chanock, S.; Bergen, A. Performance of high-throughput DNA quantification methods. *BMC Biotechnol.* **2003**, *3*, 20. [CrossRef] [PubMed]
10. Simbolo, M.; Gottardi, M.; Corbo, V.; Fassan, M.; Mafficini, A.; Malpeli, G.; Lawlor, R.; Scarpa, A. DNA qualification workflow for next generation sequencing of histopathological samples. *PLoS ONE* **2013**, *8*, e62692. [CrossRef] [PubMed]
11. Nakayama, Y.; Yamaguchi, H.; Einaga, N.; Esumi, M. Pitfalls of DNA quantification using DNA-binding fluorescent dyes and suggested solutions. *PLoS ONE* **2016**, *11*, e0150528.
12. He, H.J.; Stein, E.; DeRose, P.; Cole, K. Limitations of methods for measuring the concentration of human genomic DNA and oligonucleotide samples. *Biotechniques* **2018**, *64*, 59–68. [CrossRef] [PubMed]
13. Sedlackova, T.; Repiska, G.; Celec, P.; Szemes, T.; Minarik, G. Fragmentation of DNA affects the accuracy of the DNA quantitation by the commonly used methods. *Biol. Proced. Online* **2013**, *15*, 5. [CrossRef] [PubMed]
14. Hussing, C.; Kampmann, M.L.; Mogensen, H.S.; Børsting, C.; Morling, N. Quantification of massively parallel sequencing libraries—A comparative study of eight methods. *Sci. Rep.* **2018**, *8*, 1110. [CrossRef] [PubMed]
15. Li, X.; Wu, Y.; Zhang, L.; Cao, Y.; Li, Y.; Li, J.; Zhu, L.; Wu, G. Comparison of three common DNA concentration measurement methods. *Anal. Biochem.* **2014**, *451*, 18–24. [CrossRef] [PubMed]
16. Masago, K.; Fujita, S.; Oya, Y.; Takahashi, Y.; Matsushita, H.; Sasaki, E.; Kuroda, H. Comparison between Fluorimetry (Qubit) and Spectrophotometry (NanoDrop) in the Quantification of DNA and RNA Extracted from Frozen and FFPE Tissues from Lung Cancer Patients: A Real-World Use of Genomic Tests. *Medicina* **2021**, *57*, 1375. [CrossRef] [PubMed]
17. Ponti, G.; Maccaferri, M.; Manfredini, M.; Kaleci, S.; Mandrioli, M.; Pellacani, G.; Ozben, T.; Depenni, R.; Bianchi, G.; Pirola, G.M.; et al. The value of fluorimetry (Qubit) and spectrophotometry (NanoDrop) in the quantification of cell-free DNA (cfDNA) in malignant melanoma and prostate cancer patients. *Clin. Chim. Acta* **2018**, *479*, 14–19. [CrossRef] [PubMed]
18. Khetan, D.; Gupta, N.; Chaudhary, R.; Shukla, J.S. Comparison of UV spectrometry and fluorometry-based methods for quantification of cell-free DNA in red cell components. *Asian J. Transfus. Sci.* **2019**, *13*, 95. [CrossRef] [PubMed]
19. Zaiontz, C. Real Statistics Using Excel. Available online: http://www.realstatistics.com (accessed on 5 July 2020).
20. Carvalho, J.; Negrinho, R.; Azinheiro, S.; Garrido-Maestu, A.; Barros-Velázquez, J.; Prado, M. Novel approach for accurate minute DNA quantification on microvolumetric solutions. *Microchem. J.* **2018**, *138*, 540–549. [CrossRef]
21. O'Brien, R.; Kaiser, M. MANOVA method for analyzing repeated measures designs: An extensive primer. *Psychol. Bull.* **1985**, *97*, 316. [CrossRef] [PubMed]