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Development and validation of an HPLC-UV method for purity determination of DNA

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Abstract

Determination of the concentration and the purity of DNAs is crucial for measuring the DNA copy number since it will influence further DNA analysis such as digital PCR (dPCR) and next-generation sequencing (NGS). Precise and scientifically validated DNA measurements empower healthcare professionals and authorities to deliver reliable outcomes. DNA agarose gel electrophoresis is commonly used to check DNA purity; however, its resolution is limited. In this study, a quantitative HPLC-UV measurement method to separate DNAs was established as an alternative to both DNA electrophoresis and spectrophotometric techniques. The method was fully validated to separate DNAs ranging between 75 and 20,000 base pairs. DNA mixtures were prepared gravimetrically. Chromatographic separations were conducted on a TSKgel DNA-NPR column with dimensions of 2.5µm, 4.6mm ID x 7.5cm, using a flow rate of 0.75 mL/min. The uncertainty of the method was assessed following the guidelines provided by EURACHEM/CITAC. The method demonstrated linearity for the 200 bp DNA fragment within the range of 0.4 ng to 800 ng DNA, with a high regression coefficient of R^2=0.999. The Limit of Detection (LOD) for the 200 bp DNA fragment was determined to be 1 ng, while the Limit of Quantitation (LOQ) was found to be 3 ng. The recovery percentages for the 1%, 5%, and 10% impurities of the 150 bp DNA in 200 bp DNA fragments were measured at 101.8%, 97.4%, and 99.5%, respectively. The method established can be used in the value assignment stages of the reference materials which are required for SI traceable DNA measurements.

Key words: Purity, DNAs, high performance liquid chromatography

Introduction

DNA analysis plays a crucial role in molecular biology research due to the significance of DNA molecules in carrying the genetic information necessary for the functioning of various organisms and certain viruses. Several diseases such as cancer, trauma, stroke and myocardial infarction induce DNA damage and the amount of cell free DNA (cfDNA) increases in blood stream [1,2] or feces [3]. The diagnosis of these diseases relies on the characterization and quantification of these markers. The most recent applications of nucleic acid (NA) characterization and quantification methods include Next Generation Sequencing (NGC) and PCR enumeration strategies mainly digital PCR (dPCR). The accurate results of these methods rely on the purity and the integrity of the NAs. Contamination of NAs with other NAs as a result of NA extraction protocols may result in inaccurate measurement of target DNA concentration. Determination of the concentration and the purity of DNAs is crucial for measuring the DNA copy number, since it will influence further DNA analysis. In addition, accurate determination of DNA concentration is highly critical for special applications in molecular biology such as array comparative genomic hybridization (aCGH) [4].

The total amount of DNA can be measured easily by spectrophotometric methods (A260/280 and A260/230 ratio) [5] and by fluorescent intercalating dyes such as PicoGreen dsDNA kit [2]. However, DNA integrity and purity is very critical in characterization and quantification studies. Agarose gel electrophoresis [6] is commonly used to check DNA integrity and DNA purity [7]. However, these methods require

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time-consuming manual processes such as gel preparation and sample loading also has a low resolution separation [7]. The resolution of these methods may not be sufficient if the size of the impurity is very close to the size of the DNA of interest. Capillary gel electrophoresis (CGE) [8] and capillary array electrophoresis (CAE) [9] have been suggested as techniques to enhance efficiency, reduce separation time, improve resolution, and increase yield in DNA analysis. However, the loading and replacement of the polymeric sieving matrices used in these techniques pose new challenges after each run. High performance liquid chromatography (HPLC) has also been employed for DNA analysis in free solution with various modes such as ion exchange chromatography [10], ion pair reversed phase chromatography [11,12], size exclusion chromatography [13-15], slalom chromatography [16,17] and hydrodynamic chromatography (HDC) [18] with a coupled UV detector (HPLC-UV). These approaches are potential alternatives for gel electrophoresis but their resolution could not compete with gel electrophoresis. Significant progress in free solution DNA separations using chromatography techniques is well summarized in two recent articles [7,19] that review the most recent and advanced developments. Continuous improvement in instrumentation and column packs has provided methods for the analysis and purification of both synthetic materials and compounds from biological fluids.

The aim of this study is to establish an HPLC-UV methodology for DNA purity characterization as well as to bring current practice up to the standards of traceability and uncertainty evaluation required for higher order traceability of DNA purity. The evaluation of the DNA purity can also be used as a biomarker in clinical research [20,21]. Most chromatography suppliers have columns and application notes for oligonucleotide analysis. However, in reality good, analytical methods exists up to 25 nucleotides and the analytical method becomes less optimal as the length of oligonucleotide increases [22]. Therefore, it is important to develop a novel and sensitive method to investigate structural integrity and impurity of DNA for longer oligonucleotides, longer than 50 nucleotide. In this study, the purity of DNA was evaluated with an HPLC-UV technique. An HPLC-UV method was developed and validated to determine DNA purity using in house DNA fragments.

Material and Methods

Chemicals and materials

TRIS was obtained from Merck KGaA (Darmstadt, Germany). HPLC Columns; TSKgel DNA-NPR, 2.5µm, 4.6mm IDx0.5cm and TSKgel DNA-NPR, 2.5µm, 4.6mm IDx7.5cm were purchased from Tosoh Bioscience LLC (Tokyo, JAPAN). In-house DNA fragments of 100, 150 and 200 bp were produced by PCR. After PCR, DNA fragments were purified using High Pure product purification kit. 10 mM Tris, 0.1 mM EDTA, pH 8.5 was used as elution buffer. The integrity and size of the PCR amplifications were initially determined by 2% DNA agarose gel stained with ethidium bromide electrophoresis and they were visualized under UV light (Figure 1).

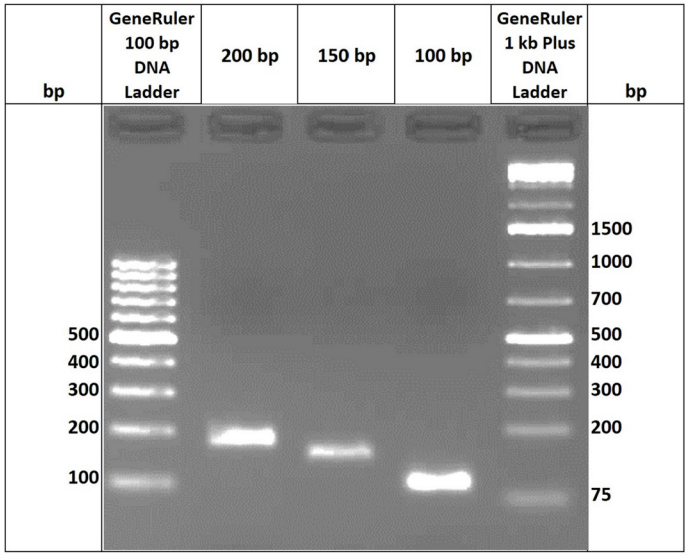

Figure 1. Agarose gel electrophoresis of 100, 150 and 200 bp DNA fragments amplified by PCR analysis

DNA synthesis and concentration determination

Quant-iT Picogreen dsDNA reagent (Thermo Fischer Scientific, USA) was used to evaluate the amount of total quantitation of double-stranded DNA (dsDNA) in DNA samples according to the manufacturer's instructions. Briefly, Quant-iT™ DNA HS reagent was diluted to 1:200 in Quant-iT™ DNA HS buffer and 190 µL of the solution was loaded in each well and 10 µL of DNA standards (0 ng/µL, 3 ng/µL, 6 ng/µL, 13 ng/µL, 25 ng/µL, 50 ng/µL and 100 ng/µL) or samples were added. Subsequently, the microplate was shielded from light and left to incubate for a duration of 1 hour at ambient temperature. Following the incubation period, the fluorescence signal was assessed at 530±20 nm, with excitation carried out at 485±20 nm, utilizing a microplate reader (Beckman DTX880, USA). The quantity of DNA was determined by referencing a standard curve, which was generated by plotting the known quantities of DNA against the corresponding fluorescence signals.

Preparation procedure of DNA mixtures

Mixtures of 1%, 5% and 10% of 150 bp DNA in 200 bp DNA were prepared gravimetrically by diluting them with 25 mM Tris HCl pH 8. The concentration of each DNA fragment was determined with the Quant-it kit before sample preparation. 2.5 ng, 12.5 ng, and 25.0 ng of 150 bp DNA were added to 250 ng of 200 bp DNA fragments, respectively, to prepare 1%, 5%, and 10% of 150 bp DNA in 200bp DNA mix solutions. Gravimetric correspondence of 1%, 5% and 10% of 150 bp DNA to 200 bp DNA fragment was calculated as 1.06%, 4.50% and 8.72%, and 1% 100 bp DNA fragment to 200 bp DNA fragment ratio was calculated as 0.59% w/w. For the evaluation of precision, control samples were prepared for repeated measurements over 3 days with 5 measurements in each day.

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Chromatography

The chromatographic separations were conducted using an HPLC-UV system from the Hitachi LaChrom Elite series. The separations were carried out on a TSKgel DNA-NPR column with dimensions of 4.6mm IDx7.5cm, packed with 2.5µm particles. A guard column, TSKgel DNA-NPR with dimensions of 4.6mm IDx0.5cm and packed with 2.5µm particles, was also used. The flow rate of the mobile phase was set at 0.75 mL/min. For the chromatographic separation, a binary gradient was employed with the mobile phase consisting of two solutions: 25 mM Tris HCl pH 8 (A) and 25 mM Tris HCl pH 8.6 (B). The gradient started at 0% eluent B and increased linearly to 100% eluent B over 35 minutes. Subsequently, it returned to the initial condition within 7 minutes, followed by a 7-minute equilibration period. To minimize carryover, the auto sampler syringe and the injection valve were sequentially washed with a mixture of isopropyl alcohol (IPA) and water in a 50:50 volume ratio. The sample injection volume was 20 µL. Detection was performed at UV 260 nm. Data evaluation was performed with Hitachi EZChrome Elite Software. The following parameters were set for the integration of chromatograms and calculations of the peak areas; "Integration off" from 28 min to 50 min.

Validation

The standard curve was created by plotting the peak area ratios of the 200 bp DNA against the corresponding concentrations of the 200 bp DNA in the calibration graph. To assess the linearity of the calibration graph, regression analysis was performed at various concentrations of the 200 bp DNA, including 6, 13, 25, 50, 100, 200, 400, and 800 ng. To determine recovery, the analyses of 150bp/200 bp mixtures were performed and the ratios of the peak areas of 150bp/200 bp were calculated and compared with quantities obtained with Quant-iT Picogreen dsDNA reagent analyses. The assay's coefficients of variation for intra-day and inter-day analysis were determined by analyzing five samples at each concentration on the same day and on three different days, respectively.

Results

Chromatographic separation

The goal of this study was to develop an HPLC-UV test for analysis of relative DNA impurity in a situation where the size of the impurity is very similar to the size of the relevant DNA. We aimed to develop a method applicable in cases where the resolution of DNA agarose gel electrophoresis and spectrophotometric (A260/280 and A260/230 ratio) methods is not sufficient. In order to test the resolution of separation, 150 bp DNA was spiked into 200 bp DNA gravimetrically at 1%, 5% and 10% (w/w) and it was used as a model for impurity analysis. The TSKgel DNA-NPR column packed with non-porous, hydrophilic polymer beads was chosen for further studies since it produced narrow and symmetrical peaks. The final selective HPLC mobile phase consisting of 25 mM Tris HCl pH 8 (A) and 25 mM Tris HCl pH 8.6 (B) was used for the separation of peaks. A linear gradient was applied starting from 0 min with 0% to 100% eluent B in 35 min. Results of the separation of 150 bp DNA and 200 bp DNA are presented in Figure 2.

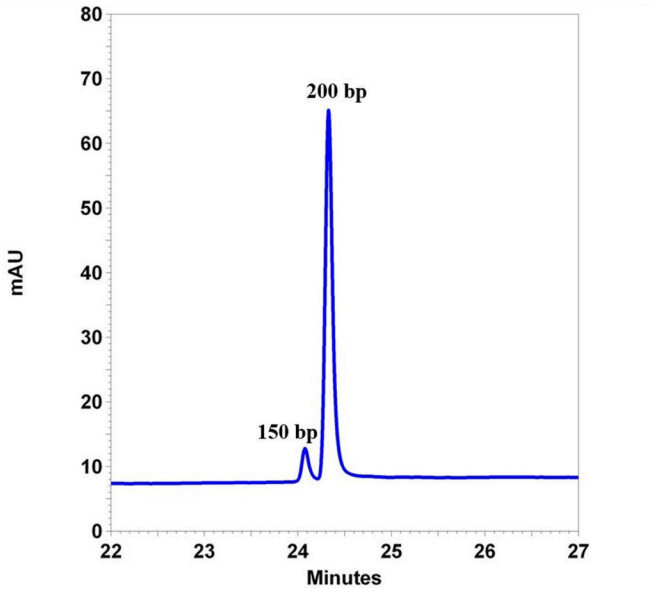

Figure 2. HPLC UV chromatogram of 150 bp DNA spiked in 200 bp DNA (10% w/w)

In addition, the DNA ladders of 1kb Plus (Fermentas) and 100 bp (Fermentas) were also injected in order to test the ability of the developed method to separate the DNA fragments of sizes lower than 300 bp (Figure 3). 1kb Plus DNA Ladder and 100 bp DNA Ladders were run with HPLC separately and two chromatograms were overlapped, 100 bp ladder and 1kb Plus ladder were colored with red and blue, respectively (Figure 3). The results have shown sufficient peak resolutions for the DNA fragment sizes below 300 bp and above 2000 bp (Figure 3).

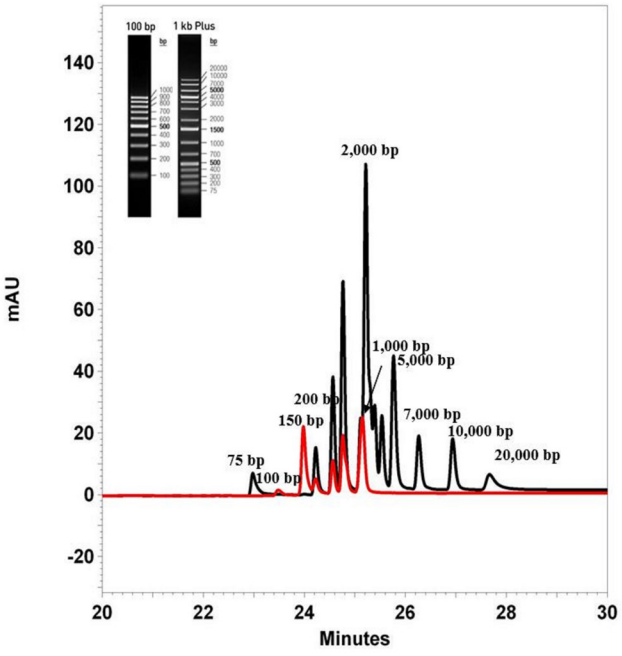

Figure 3. HPLC UV Chromatograms of 1kb Plus DNA Ladder (blue line) and 100 bp DNA Ladder (red line) which were loaded separately

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Purity assay

The purity assay was conducted in relation to main DNA content, in other words, the content of DNA impurities was calculated referring to the reference DNA peak. The approach is based on the assumption that the extinction coefficient of the main peak and the impurities are the same. The advantage of the approach is that the impurity can be regarded as the X% of the reference peak regardless of its initial concentration, thus, the assay does not need any other standard material for relative quantification. Results should be stated that as value expressed in relation to the reference DNA content of the sample.

Method validation

Linear range

The method validation process involved the use of in-house produced DNA fragments measuring 100, 150, and 200 bp in length. To establish the linearity of the detector response, a graph was constructed by plotting the "quantity" (amount) of the 200 bp DNA against the corresponding "peak area." The quantities of the 200 bp DNA used for the plot were 3, 6, 12, 25, 50, 100, 200, 400, and 800 ng. The EzChrom Elite Hitachi software program was utilized to automatically integrate the peak areas. The total DNA quantity was calculated with the Quant-it DNA kit measurements. The test method was found to be linear for 200 bp in the range of 3 ng to 800 ng DNA with the regression coefficient of 0.9999 as shown by the equations presented in Table 1. The limit of detection (LOD) for the 200 bp DNA fragment was determined using the calculation method defined by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [23]. The LOD was calculated as 3 times the standard deviation of the intercept (Sa) divided by the slope of the calibration curve (b). The LOD value obtained for the 200 bp DNA fragment was found to be 1 ng. Similarly, the limit of quantification (LOQ), which is calculated as 10 times Sa divided by b, was also determined, and the LOQ for the 200 bp DNA fragment was found to be 3 ng.

Table 1. Linear range of measurement for estimation of 200bp DNA by HPLC

Parameters	200 bp DNA
Optimum range (ng)	3–800
Regression equation for the peak area of DNA vs quantity in ng	$A_{200bp\ DNA}$=6042.13$C_{200bp\ DNA}$+4906.4
Correlation Coefficient	0.999
Limit of quantification, LOQ (ng)	3
Limit of detection, LOD (ng)	1

Accuracy

According to the ICH Topic Q 2 (R1) Guide titled "Validation of Analytical Procedures: Text and Methodology", accuracy is a measure of both precision and trueness in test results. The evaluation of repeatability, which is the precision within a single day, should include a minimum of 9 determinations that cover the specified range of the procedure. This typically involves analyzing three concentrations with three replicates each. Intermediate precision, on the other hand, assesses variations across different days. To evaluate the precision of the measurement system, the method's repeatability and intermediate precision were assessed. Repeatability was determined by analyzing DNA quality control solutions with replicates of five on the same day (intra-day analysis), while intermediate precision was evaluated by analyzing replicates of five on three different days (inter-day analysis). These analyses allowed for the assessment of assay variance and the overall precision of the method. Four quality control (QC) samples containing different concentrations of impurities were prepared; The 150 bp fragment was added into 200 bp DNA fragment in ratios of 1%, 5% and 10%, and 1% 100 bp DNA fragment was added into 200 bp DNA fragment. Five replicates of each sample were analyzed each day with HPLC-UV. According to the method validation criteria, the repeatability (intra-run) and intermediate precision (inter-run), acceptance standards should be less than 10% RSD (Relative Standard Deviation). $RSD_{Repeatability}$ (1) and $RSD_{intermediate\ precision}$ (2) were calculated with the formulas given below, where MS_{within} is within group mean square, $MS_{between}$ is between group mean square and the W_{mean} is the arithmetic average of all measurements. Table 2 shows that the obtained repeatability and intermediate precision values for all the QC levels were below 10% RSD. These values met the acceptance requirements, implying that the current procedure has enough precision.

$$RSD_{Repeatability} = \frac{\sqrt{MS_{within}}}{W_{mean}} x\ 100 \quad (1)$$

$$RSD_{IntermediatePrecision} = \frac{\sqrt{MS_{between} - MS_{within}}}{W_{mean}} x\ 100 \quad (2)$$

Table 2. The precision results of different DNA additions

	150 bp/200 bp			100 bp/200 bp
	1%	5%	10%	1%
Repeatability (n=5)				
Mean±SD	1.1±0.02	4.20±0.10	8.63±0.15	0.58±0.04
RSD %	1.90	1.73	1.33	6.58
Intermediate precision (n=15)				
Mean±SD	1.03±0.02	4.35±0.06	8.88±0.10	0.57±0.03
RSD %	6.38	6.77	5.53	5.26

The trueness assessment was performed with the recovery evaluated by the ratio of HPLC results to the by Quant-it Kit result. Four QC samples were prepared; The 150 bp fragment was added in 1%, 5% and 10% to 200 bp DNA fragment, and 1% 100 bp DNA fragment was added into 200 bp DNA fragment.

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The mixtures were analyzed with HPLC-UV. Recovery of 150 bp DNA in 200 bp DNA was evaluated for three different days. The obtained recovery data for 200 bp DNA was 101.7%, 97.5% and 99.5% for 1%, 5% and 10% ratios of 150 bp/200 bp DNA, respectively. The recovery for 100 bp in 200 bp DNA was calculated as 97.7%. Accordingly, % recovery values were calculated and presented in Table 3. Those values met the recovery acceptability requirements, showing that the current approach was adequate in terms of trueness.

Table 3. The trueness results of different DNA mixtures

	150 bp/200 bp			100 bp/200 bp
	1%	5%	10%	1%
% Trueness (n=5)				
Mean, day1	100.1	93.3	98.9	97.4
Mean, day2	99.5	99.3	99.9	99.2
Mean, day3	105.7	99.7	99.6	96.4
Mean±SD	101.8±3.4	97.4±3.4	99.5±1.3	97.7±6.1

Carryover test

In the current method, a solution composed of isopropanol and water in a 50/50 ratio (v/v) was utilized to thoroughly clean the syringe and injection port. This cleaning procedure was performed multiple times before and after each injection. Under these wash conditions, the signal observed at the retention time of 200 bp DNA (area below the peak) was less than 1% compared to that found in the LOD after injection of three blank samples.

Uncertainty evaluation

Measurement uncertainty is a useful measure for demonstrating the quality and particularly the fitness for purpose of analysis results. It is a measure of the confidence that can be placed in the analytical results and is an essential part of any quantitative analysis. The evaluation of uncertainty regarding an analytical result is now widely accepted. Without a measurement uncertainty the statement of an analytical result cannot be considered complete. The uncertainty of the method in this study was assessed following the guidelines outlined in the third edition of the EURACHEM/CITAC Guide CG 4, titled "Quantifying Uncertainty in Analytical Measurement." [24-26]. Sources of uncertainty arising from working steps such as pipetting, dilution, balances and volumetric measuring devices are covered by the reproducibility, intermediate precision and recovery uncertainty. The uncertainty sources identified for the current method include the following defined parameters: uncertainty of repeatability (3), uncertainty of intermediate precision (4), uncertainty of trueness) (5 & 6) standard uncertainty (7) given below. Rm is an estimate of the mean method recovery obtained from method comparison. Expanded uncertainty was calculated by considering a coverage factor of 2 for a level of confidence of approximately 95%. Breakdown of the uncertainty budget is presented in Table 4.

$$u_{rep} = \sqrt{MS_{withingroup}} \quad (3)$$

$$u_{int\,Pre} = \sqrt{\frac{MS_{within} - MS_{between}}{n_{repeat}}} \quad (4)$$

$$R_m = \frac{Ratio_{Obs.}}{Ratio_{Dif.Method}} \times 100 \quad (5)$$

$$u_{Truness} = \frac{\frac{STD_{R_m}}{\sqrt{n_{rep}}}}{Mean_{R_m}} \quad (6)$$

$$U_{combined} = k\sqrt{\frac{u_{rep}^2}{n_{rep}} + \frac{u_{intPre}^2}{n_{days}} + u_{truness}^2 + u_{balance}^2} \quad (7)$$

Table 4. Breakdown of the uncertainty budget

	150 bp/200bp			100 bp/200bp
	1%	5%	10%	1%
Mean	1.025	4.352	8.882	0.568
Uncertainty components				
Repeatability (u_{rep})	0.020	0.075	0.118	0.037
Intermediate precision (u_{intPre})	0.029	0.132	0.068	0.021
Trueness; ($u_{Truness}$)	0.009	0.039	0.030	0.009
Balance	0.000	0.000	0.000	0.000
k	2.000	2.000	2.000	2.000
Uncertainty, u_c	0.021	0.092	0.073	0.023
Expanded uncertainty, U	0.042	0.184	0.145	0.045

Discussion

The development and implementation of highly efficient HPLC methods are essential for enhancing DNA characterization and quantification techniques, such as Next Generation Sequencing (NGS) and PCR counting strategies. These well-developed HPLC methods play a crucial role in facilitating process improvements and advancements in DNA analysis. In this study, an HPLC-UV methodology for DNA purity characterization was developed and validated. The method was sufficient to resolve DNA fragment sizes below 300 bp and above 2000 bp. At present, the NanoDrop spectrophotometer is widely utilized for estimating DNA concentration and purity through absorbance measurements. However, it is worth noting that, to our knowledge, this methodology has not undergone the recommended validation process according to international standards, similar to the validation conducted for HPLC methods. Consequently, the uncertainty associated with the measurements obtained using the NanoDrop spectrophotometer has not been established. A recent methodological validation study of the use of the NanoDrop Spectrophotometer for DNA concentration reported a precision of ≤2% CV and a percent recovery of 100%±5% [27]. Although

these parameters are similar to the parameters developed by the HPLC-UV method, the purity of each oligonucleotide can be measured separately due to the separation feature of the HPLC method. The evaluation of uncertainty in the developed HPLC-UV methodology is crucial for ensuring higher-order traceability of DNA purity. Accurate and reliable measurements of DNA are essential in various fields of life sciences, such as food safety, medicine, and environmental monitoring. Despite the significant importance of accurate and reliable DNA measurements, many users lack awareness regarding the global metrological infrastructure established to support these measurements. Accurate and metrologically traceable DNA measurements enable decision makers such as healthcare professionals and authorities to provide reliable results. Certified reference materials and reference methods for DNAs are required for establishing a metrological traceability chain. The production of certified reference materials in this field has just begun, and CRMs and calibrants in this field are almost non-existent. In this study, a quantitative impurity analysis method has been developed that can be used in the value assignment stages of the reference materials which are required for SI traceable DNA measurements.

Conclusion

In conclusion, the development and validation of the HPLC-UV methodology for DNA purity characterization offers a substantial leap forward in accurate DNA analysis, complementing techniques like NGS and PCR. The HPLC method's capability to provide both accurate DNA concentration and individual oligonucleotide purity measurements underscores its importance. The study underscores the significance of establishing a robust metrological infrastructure through reference methods to ensure accurate and reliable DNA measurements, thereby enabling informed decision-making and advancing the fields of life sciences.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical Approval
Ethical committee approval is not required as the study does not involve clinical samples and the used DNAs are synthetic.

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