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Research Article

Development and Validation of A Spectrofluorimetric Determination of Calf Thymus DNA Using a Terbium-Danofloxacin Probe

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ABSTRACT

Background: Analysis of biomolecules is required in many biomedical research areas. A spectrofluorimetric method is proposed for determination of calf thymus DNA (ctDNA) based on the fluorescence enhancement of terbium-danofloxacin (Tb^{3+}-Dano) in the presence of ctDNA.

Methods: A probe with maximum excitation and emission wavelengths of 347 nm and 545 nm, respectively, was developed. The enhanced fluorescence intensity of Tb^{3+}-Dano system was proportional to the concentration of ctDNA. The effective factors and the optimum conditions for the determination of ctDNA were studied. Under the optimum conditions of [Tris buffer]= 0.01 mol L^{-1} (pH 7.8), [Tb^{3+}]= 1×10^{-5} mol L^{-1} and [Dano]= 5×10^{-5} mol L^{-1}, the maximum response was achieved. The developed method was evaluated in terms of accuracy, precision and limit of detection.

Results: The linear concentration range for quantification of ctDNA was 36-3289 ng mL^{-1} and the detection limit (S/N=3) was 8 ng mL^{-1}. The concentration of DNA extracted from *Escherichia coli* as an extracted sample was also determined using the developed probe. The concentration of DNA in extracted sample was determined using UV assay and developed method, the results were satisfactory.

Conclusion: The proposed method is a simple, practical and relatively interference free method to follow up the concentrations of ctDNA.

Introduction

Nucleic acids have an important function in the life process. Therefore the quantitative determination of micro amounts of nucleic acids is of utmost importance in the life science.[1,2] Various methods for the determination of nucleic acids have been reported so far, including spectrophotometery,[3] which use the special absorption of nucleic acids at 260 nm to calculate the concentration directly, chemiluminescence,[4] light scattering,[5,6] electrochemical,[7,8] chromatographic[9] and fluorimetric methods.[10,11] It is well known that the fluorescence intensity of DNA itself is very weak, so the direct use of the fluorescence properties to investigate their biological properties is limited.[12] The specific strong fluorescence of lanthanides as a result of an efficient intra molecular energy transfer from the excited triplet state of the antenna ligand to the emitting electronic level of the lanthanide ion possesses luminescence characteristics of narrow spectral width, long luminescence lifetime, large Stock's shifts (> 200 nm) and µs-luminescence decay times.[13,14] Tb^{3+} ions for their resonance energy levels overlap with ultraviolet light, often used as the fluorescence probes to determine several classes of organic compounds because of the high fluorescence quantum efficiency of their chelates.[13]

There are many luminescence probes for the determination of DNA. Luminescence probes including organic dyes,[15,16] metal complex compounds[17] and lanthanide ions[18] are used for improving sensitivity in determining nucleic acids. Because of the luminescence characteristics of rare earth ions such as narrow spectral width, long luminescence lifetime, large stocks shift and strong binding with biological molecules, they are widely used as fluorescent probes to study DNA. In particular, attentions have been directed towards two rare earth cations, Tb^{3+} and Eu^{3+}. In recent years, the coordination complexes of metal ions especially rare earth ions as a probe to study DNA have attracted much attention.[19-25]

Here we employed the fluorescence of terbium-danofloxacin (Tb^{3+}-Dano) complex as a probe for the determination of calf thymus DNA (ctDNA).

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Danofloxacin (Dano) is one of the third generations of quinolone antibiotics. Dano could form coordination complex with terbium, which emitted fluorescence characteristic of terbium.[26] Interestingly the fluorescence intensity of the Tb^{3+}-Dano system is greatly enhanced when ctDNA was added. Under optimal conditions the enhanced fluorescence intensity is proportional to the concentration of ctDNA. It was found that the fluorescence probe of the Tb^{3+}-Dano complex had a high sensitivity and wide range for determination of ctDNA at nanogram levels. In addition, the proposed fluorescence probe was inexpensive and the reagents were easily obtainable and environment friendly. The Tb^{3+}-Dano system has good stability and solubility in water and do not require the addition of luminescence enhancers such as micelles. In this work we used Tb^{3+}-Dano as a fluorescence probe for determination of ctDNA in standard and extracted sample from *Escherichia coli*.

Materials and methods

Analytical-grade ethanol, methanol, 2-propanol, hydrochloric acid (HCl), acetonitrile, phenol, acetic acid, tris-[hydroxymethyl] aminomethan (Tris), ethylendiamintetraacetic acid (EDTA), agar and sodium hydroxide were obtained from Merck company (Darmstadt, Germany), terbium(III) chloride hexahydrate ($TbCl_3.6H_2O$) from Acros organics company (Geel, Belgum), Dano powder from Jamedat Afagh pharmaceutical company (Semnan, Iran), ctDNA from Sigma Aldrich company (Milwaukee, WI, USA), Lactose Broth from HIMEDIA (Mumbai, India), agarose from invitrogen origin (Waltham, Massachusetts, USA), RNase A, proteinase K, DNA ladder, loading dye from Fermentase (St. Leon-Rot, Germany), *E. coli* from Pasteur Institute (Tehran, Iran), double distilled water prepared using the Millipore-Q-Puls water purification system (Millipore, Bedford, MA, USA) were used in this work.

Commercially prepared ctDNA were dissolved in 0.05 mol L^{-1} sodium chloride solution as stock solutions. Working solutions of ctDNA were prepared by appropriate dilution with water. The stock and working solutions were stored at 4˚C. A 1×10^{-2} mol L^{-1} terbium(III) solution was prepared by dissolving the appropriate amount of $TbCl_3.6H_2O$ powder in double distilled water and stored in a polyethylene container to avoid memory effects of terbium adsorbed to glass vessels. A 1×10^{-2} stock solution of Dano was prepared in double distilled water. Working solutions of terbium and Dano were prepared by dilution with water. 0.01 mol L^{-1} Tris-hydrochloric acid (Tris-HCl) buffer solutions were prepared by dissolving the 1.21 g of Tris-base in 100 mL water, adjusting the pH with HCl solution. Tris-EDTA solution was prepared by mixing 10 mL of 0.1 mol L^{-1} Tris-HCl solution (pH=8.0) with 10 mL of 0.01 mol L^{-1} EDTA-NaOH solution (pH=8.0) to the final volume of 100 mL in water. Lysis buffer solution was prepared by mixing the 9.340 mL of Tris-EDTA solution and 600 µL of sodium dodecyl sulphate (SDS 10 % v/v) and 60 µL of proteinase K solution. The Tris-Acetic acid-EDTA (T.A.E) solution was prepared by mixing 50 mL of 1 mol L^{-1} EDTA-NaOH solution (pH=8.0) and 40 mL of 4 mol L^{-1} Tris solution and 5.710 mL of acetic acid glacial at the final volume of 100 mL in water.

Apparatus

All fluorescence spectra were recorded using a Jasco FP-750 spectrofluorimeter (Kyoto, Japan) equipped with a 150 w xenon lamp using a 1 cm quartz cell. The excitation and emission monochromator bandwidths were 10 nm and 5 nm, respectively. All measurements were performed at 25±0.1˚C controlled using a Peltier thermostated cell holder Jasco (Kyoto, Japan). The UV absorption were performed with, Du-650 spectrophotometer Beckman (Fullerton, USA) equipped with 1 cm quartz cell. The pH of solutions was measured with Metrohm 654 pH meter (Herisau, Switzerland). The gel electrophoresis was performed by minipac electrophoresis APELEX (Paris, France) equipped with a transluminator imaging instrument VILBERLOURMAT (Paris, France) for imaging of gel.

Commercial DNA sample preparation

Standard stock solution of ctDNA was prepared by dissolving commercially purchased ctDNA in 0.05 mol L^{-1} sodium chloride solution and stored at 4°C to use within 2 days.

Experimental procedure

To a 10 mL test tube, reagents were added in the following order (in the absence and presence of ctDNA): 1 mL of 1×10^{-4} mol L^{-1} Tb^{3+} solution, 1 mL of 5×10^{-4} mol L^{-1} Dano solution, ctDNA with appropriate concentration and 1 mL of 0.1 mol L^{-1} Tris-HCl buffer (pH 7.8) (Tb^{3+}- Dano-ctDNA-Tris.HCl buffer). The volume of this mixture was diluted to 10 mL with double distilled water and after 3 min, the luminescence intensity (F) was measured in 1 cm quartz cell with an excitation wavelength of λ_{ex}= 347 nm and an emission wavelength of λ_{em}= 545 nm. All measurements were performed at 25 ˚C. The enhanced fluorescence intensities of Tb^{3+}-Dano by ctDNA were represented as $\Delta F\% = 100\ (F-F_0)/F_0$ where F and F_0 are the fluorescence intensity of the probe (Tb^{3+}-Dano) with and without ctDNA, respectively.

DNA extraction from E. coli

Total DNA from *E. coli* bacteria was extracted and used as another DNA sample as follow:

The fresh overnight culture of *E. coli* was centrifuged at 14000 rpm for 2 minutes and the cell pellets were re-suspended in 600 µL lysis buffer solution. Then the samples were incubated in water at 56˚C for 3 hours to completely lyse the cells followed by cooling down to the room temperature. 3 µL of RNase solution was

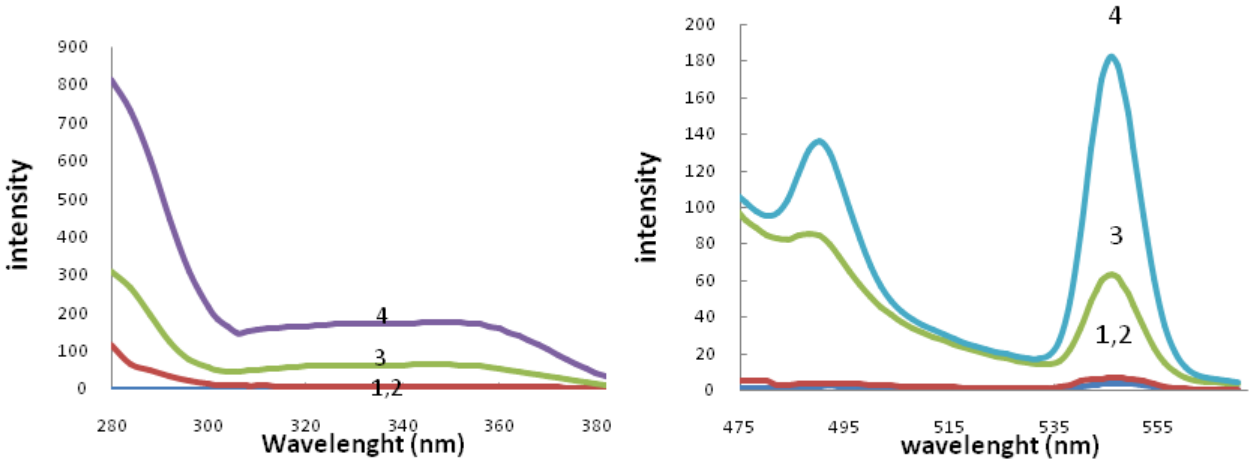
added to each cell lysate and the tubes inverted 2-5 times to mix and accordingly they were incubated at 37 ˚C for 30 minutes to digest RNA. 200 µL of phenol:chloroform mixture (1:1) was added to the RNase-treated cell lysate and incubated in an ice/water slurry for 5 minutes. Then the samples were centrifuged at 14000 rpm for 3 minutes and the supernatant containing the DNA were transferred to new tubes and 600 µL 2-propanol was added to each tube followed by their gentle mixing by inverting for 5 minutes. Consequently the samples were centrifuged at 14000 rpm for 2 minutes, the supernatant was poured off and the pellet was washed using ethanol by centrifugation at 14000 rpm for 2 minutes. Finally the pellets were air-dried for 10-15 minutes and dissolved in 100 µL of Tris-EDTA solution and kept at 4°C for further analysis.

After extraction, DNA sample was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and then photographed using transluminator imaging instrument to assess the quality of extracted DNA.[27] To the extracted DNA concentration was determined using measurement of absorption of extracted sample at 260 nm.

Results and discussion

Fluorescence spectra

Fluorescence emission and excitation spectra of Tb^{3+}, Tb^{3+}-ctDNA, Tb^{3+}-Dano and Tb^{3+}-Dano-ctDNA are shown in Figure 1. Tb^{3+} and Tb^{3+}-ctDNA did not show the characteristic fluorescence spectrum. Tb^{3+}-Dano complex displayed an excitation peak at 347 nm and two emission peaks at around 490 nm and 545 nm which corresponded to the transmission $^5D_4$ level of terbium to the $^7F_6$ and $^7F_5$ levels, respectively. Since the emission intensity at 545 nm was stronger than that at 490 nm therefore, the excitation and emission peaks were set at 347 nm and 545 nm, respectively. The fluorescence of the Tb^{3+}-Dano-ctDNA was similar to that of Tb^{3+}-Dano. However the fluorescence intensity of Tb^{3+}-Dano was enhanced by ctDNA which indicates that there was an interaction between the nucleic acids and Tb^{3+}-Dano. The complementary experiments showed the enhanced fluorescence intensity was proportional to the concentration of ctDNA.



a) Excitation spectra b) Emission spectra

Figure 1. Excitation and emission spectra. (a): Excitation spectra (b): Emission spectra. (1) Tb^{3+}, (2) Tb^{3+}-ctDNA, (3) Tb^{3+}-Dano, (4) Tb^{3+}-Dano-ctDNA.

Optimization of experimental conditions

Effect of pH

The pH of the solution had a great influence on the both the formation of the Tb^{3+}-Dano complex and the capability of ctDNA to bind the complex. Fluorescence intensity of series of 0.01 mol L^{-1} Tris buffer solution with the pH range of 5.5-8.5 were measured at $\lambda_{ex}/\lambda_{em}$=347nm/545nm (Figure 2). Experimental results indicated that the maximum fluorescence intensity of system was reached the peak at the pH 7.8, Therefore pH 7.8 was selected for further research. The Tris-HCl was used as a buffer for adjusting the pH of solutions because of its good characteristics such as not quenching the fluorescence of probe and good stability and having the wide pH range.[28,29]

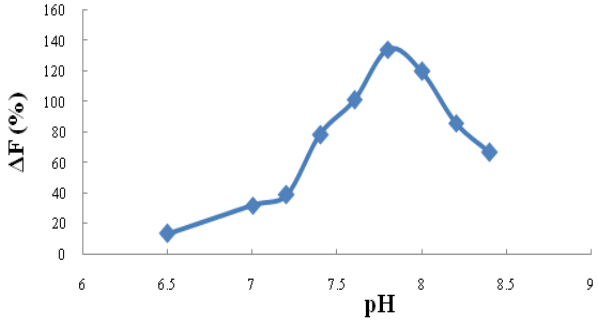


Figure 2. Effect of pH on the enhanced fluorescence intensity (ΔF%). Experimental condition: [Tb^{3+}]: 1×10^{-5} mol L^{-1}; [Dano]: 5×10^{-5} mol L^{-1}; [ctDNA]: 0.657 µg mL^{-1}.

Effect of concentration of buffer

The influence of Tris buffer (pH 7.8) concentration on enhanced fluorescence intensity of Tb^{3+}-Dano system (ΔF%) was studied in a range of 0.002 mol L^{-1} to 0.02 mol L^{-1}, while the value of Tb^{3+}, Dano, ctDNA were kept constant at 10^{-5} mol L^{-1}, 5×10^{-5} mol L^{-1} , 0.657 µg mL^{-1}, respectively . The coordination of Tb^{3+} ions by Tris prevents the OH groups of water molecules from surrounding the terbium ions and reduces the complexation of Dano. In lower concentrations of Tris, the buffer could not coordinate terbium ions completely and the fluorescence intensity decreased. The results indicated that the ΔF% of the probe in the presence of the analyte at buffer concentration of 0.01 mol L^{-1} was maximum. 1 mL of 0.1 mol L^{-1} Tris-HCl buffer solution in 10 mL mixture was the optimum buffer volume.

Effect of Tb^{3+} concentration

Effect of Tb^{3+} concentration on the enhanced fluorescence intensity (ΔF%) of Tb^{3+}-Dano-ctDNA system was studied at the constant concentration of Dano, ctDNA and Tris buffer (pH 7.8) at 5×10^{-5} mol L^{-1}, 0.657 µg mL^{-1} and 0.01 mol L^{-1}, respectively. The results indicated the enhanced fluorescence intensity (ΔF%) of probe reached the maximum when the concentration of Tb^{3+} was 1×10^{-5} mol L^{-1}. 1×10^{-5} mol L^{-1} was selected as optimum concentration of Tb^{3+}.

Effect of Dano concentration

The influence of Dano concentration on the fluorescence intensities was studied. It was found that the enhanced fluorescence intensity of Tb^{3+}–Dano–ctDNA system increased with the increasing of the Dano concentration and reached a maximum when the concentration of Dano was 5×10^{-5} mol L^{-1}. 5×10^{-5} mol L^{-1} was used as optimum concentration of Dano for further study.

Effect of reaction time

Under the optimum conditions, the effect of time on the fluorescence intensity was investigated. The results showed that the fluorescence intensity was stable at 60 min after all the reagents were added. It showed that the formation of complex between Tb^{3+} and Dano and ctDNA was fast. All measurements were performed 3 min after the addition of all reagents.

Effect of temperature

Another parameter influencing the fluorescence intensity was temperature. The effect of temperature on the enhanced fluorescence intensity was investigated. It was found that the fluorescence of the probe in the presence of the analyte at temperature between 25˚C - 35˚C was constant, and 25 °C was selected for further study.

Effect of addition order of reagents

The experiments showed that addition order of reagents could affect on the fluorescence intensity of probe. To study this, series of solutions were prepared with different addition sequence and the fluorescence intensities were measured. It was found that there was negligible difference among ΔF% amounts of additional sequences. Thus, the addition order of Tb^{3+}-Dano-ctDNA-Tris was selected as optimum addition order.

Effect of surfactant

The effect of the addition of various surfactants such as SDBS, SDS, Triton X-100, Tween 80 and Tween 20 on the fluorescence intensity was investigated. The experiments indicated that the ΔF% without surfactants were better than the ΔF% in the presence of surfactants.

Solvent effect

At the optimum conditions the effect of organic solvents such as methanol, ethanol, 2-prppanol and acetonitrile in the range of 0-60 % (v/v) was studied (Figure 3). The results indicated that increasing the volume of organic solvents decreased the fluorescence intensity (%ΔF).

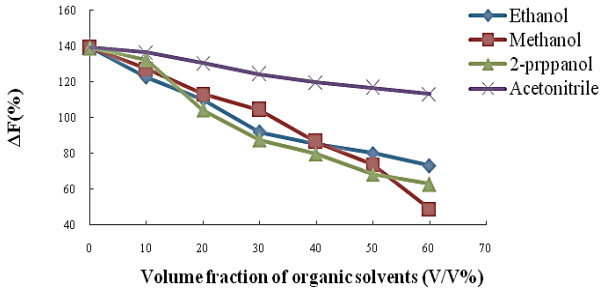


Figure 3. Solvent effect on the enhanced fluorescence intensity (ΔF %). Experimental condition: [Tb^{3+}]: 1×10^{-5} mol L^{-1}; [Dano]: 5×10^{-5} mol L^{-1}; [ctDNA]: 0.657 µg mL^{-1}; pH=7.8.

Interference studies

Under the optimum conditions, the interferences of coexisting substances including $Na^{+}(Cl^{-})$, $Fe^{3+}(Cl^{-})$, $Fe^{2+}(Cl^{-})$, $K^{+}(Cl^{-})$, $NH_4^{+}(Cl^{-})$, $Zn^{2+}(SO_4^{2-})$, $Ca^{2+}(Cl^{-})$, $Al^{3+}(Cl^{-})$, $Mg^{2+}(Cl^{-})$, $Cu^{2+}(Cl^{-})$, phosphate, glycine, sacarose, lactose and human serum albumin on the enhanced fluorescence intensity were tested. The results showed that human serum albumin, phosphate, $Al^{3+}(Cl^{-})$, $Fe^{3+}(Cl^{-})$ and $Fe^{2+}(Cl^{-})$ have considerable interference with the enhanced fluorescence intensities. However, as the results indicate, after the treatment of samples, intererference free determinations were possible.

Analytical application and assay validation

Calibration curve and detection limit

The calibration graph for ctDNA was obtained under the optimum condition. There were a good liner relationship between the increased fluorescence intensities and the concentration of ctDNA in the wide range of 36-3289 ng mL^{-1}. The detection limit (S/N=3)

was 8 ng mL^{-1}. The regression equation was $\Delta F\%=2.40+0.187C$ (ng mL^{-1}) and the correlation coefficient was 0.999.

Precision and accuracy

Table 1 shows the results of intra assay precision and accuracy of calibration standards. All of the relative standard deviation percentages (RSD%) were below 5% for standard samples. Inter and intra assay precision along with accuracy for quality control samples are listed in Table 2.

Table 1. Intra assay precision and accuracy of calibration standards.

Nominal concentration of ctDNA (ng mL^{-1}) (N=3)	Found concentration of ctDNA using developed method (ng mL^{-1}) (N=3)	Precision (RSD %)	Accuracy (RE %)
148	143	2.75	-3.28
246	238	3.51	-3.31
986	980	1.92	-0.59
1973	2009	2.08	1.83
2467	2527	2.38	2.44

Table 2. Assay precision and accuracy of quality control samples.

Nominal Concentration of ctDNA (ng mL^{-1})	Intra-assay precision (RSD %) (N=5)	Precision (RSD %)	Accuracy (RE %)
246	3.72	4.93	-2.25
986	1.24	3.61	-1.59
2467	1.78	4.89	4.09

Stability

The stability studies showed that the ΔF% of Tb^{3+}-Dano-ctDNA system was stable for 60 min at room temperature after addition of all reagents. Also stability at refrigerator temperature for three days cycle was studied. The results showed that after one day, decrease in the concentration of analyte was highest but in the second and third days the deviation was less. The stability results are summarized in Table 3.

Table 3. Stability at refrigerator temperature (4 ºC) for three day cycles.

Concentration of ctDNA (ng mL^{-1})	After one day at 4 ˚C		After two days at 4 ˚C		After three days at 4 ˚C	
	Concentration found (ng m L^{-1})	Accuracy (RE%)	Concentration found (ng m L^{-1})	Accuracy (RE%)	Concentration found (ng mL^{-1})	Accuracy (RE%)
246	223	-9.34	220	-10.57	217	-11.79
986	902	-8.52	892	-9.53	885	-10.24
2467	2308	-6.44	2289	-7.21	2273	-7.68

Determination of extracted sample

The DNA of *E. coli* bacteria was extracted (DNA extraction process was explained in DNA extraction from *E. coli* section) and It's concentration of 187.75 µg mL^{-1} was measured in the extracted sample using the spectrophotometer assay as standard method. The absorption of sample was measured in 260 nm and the concentration calculated using: (sample absorption at 260 nm) × 50 ppm (30). The sample was diluted to achieve desired concentration of DNA. Then the concentrations of diluted DNA were determined using developed spectrofluorimetric method. The results are shown in Table 4. The RSD% values less than 15% were achieved for the samples which is acceptable for biologic samples according to the FDA guidelines (31).

Table 4. Determination of DNA in extracted sample form *E. coli* bacteria.

Concentration of DNA using a UV method(ng mL^{-1})	Found Concentration Using the Developed Method (ng mL^{-1})	RSD%) (N=3)	Recovery %
92	93	6.52	101.1
375	377	9.97	100.5

Conclusion

In this paper a new fluorimetric method for determination of ctDNA is proposed. Under the optimum conditions the fluorescence intensity of system Tb^{3+}-Dano can be enhanced in the presence of ctDNA and there are a good linear relationship between

the increased fluorescence intensities and the concentration of ctDNA in the wide range of 36 ng mL^{-1} to 3289 ng mL^{-1}. The detection limit (S/N=3) and the quantification limit were 8 ng mL^{-1} and 26 ng mL^{-1}, respectively. This method was applied for the determination of ctDNA. In addition, the method had satisfactory results for the determination of DNA in an extracted sample from *E. coli* bacteria. The validation experiments showed that this method has a good sensitivity and stability. Table 5 shows a comparison of figures of merit for the proposed method with other previously published methods. The overall finding reveal that the proposed method could be employed as a sensitive alternative method in determination of DNA.

Table 5. Analytical characteristics of available methods for determination of DNA.

Method	Linear Range	LOD	Analyte	Ref
Spectrophotometric method	4 - 100 µg mL^{-1}	-	DNA extracted from animal tissues	3
Chemiluminescence method (flow injection analysis)	2×10^{-6} - 0.2 µg mL^{-1}	3.5×10^{-8} µg mL^{-1}	ctDNA	4
Resonance Light-Scattering method	(Tb^{3+}–quercetin–ctDNA) 0.03 - 2 µg mL^{-1}	0.012 µg mL^{-1}	ctDNA	5
	(Eu^{3+}–quercetin–ctDNA) 0.05 - 2.5 µg mL^{-1}	0.018 µg mL^{-1}	ctDNA	
Electrochemical method	1×10^{-4} - 5×10^{-7} g mL^{-1}	9.5×10^{-8} g mL^{-1}	ctDNA	7
chromatographic method	10 - 206 µg mL^{-1}	0.28 µg mL^{-1}	supercoiled plasmid DNA	9
Spectroflourimetric method	0.05 - 3 µg mL^{-1}	0.03 µg mL^{-1}	ctDNA	21
Developed method	36 - 3289 ng mL^{-1}	8 ng mL^{-1}	ctDNA	This study

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Conflict of interests

The authors claims that there is no conflict of interest.

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