Interaction with DNA – Guidelines, with a particular focus on fluorescence titrations

AUTHORS: Members of WORKING GROUP 2 (WG2 - Studies on strong and/or multifunctional ligands, macromolecules, polyelectrolytes) TASK GROUP 2 (TG2 – DNA BINDING)*

1 - INTRODUCTION

Within the wider aim of the NECTAR project and network, working group two (WG2) is devoted to studies on strong and/or multifunctional ligands, macromolecules, polyelectrolytes. The task group 2 of WG2 (WG2-TG2) is devoted to DNA. WG2-TG2 identified a target system for studies on DNA binding to be used as validation standard. WG2-TG2 started by concentrating on the dye/DNA intercalation process, as the one producing the higher signal changes and, therefore, the most suitable for validation purposes. A target ("golden standard") system was chosen based on reactants' stability and cost, availability and ease of handling. The different research groups performed tests for the target system. We will focus here on guidelines on how to perform experiments. The aim is the development and testing of a recommended procedure. Some of the suggestions expressed in this document are intended as general, however, this document is focused on and is especially devised for **fluorescence titrations.**

To pursue the aim above, the members of WG2-TG2 decided to first carry out an in-depth analysis of the literature data, in order to identify the best golden standard for fluorescence titrations to obtain the DNA binding constant of a tested molecule. The idea is that we need a very well known, cheap fluorescent material which may be easily purchased in high purity and which is stable in solution (aggregation or aquation reactions should be avoided). Toxicity should be the lowest possible (even if intercalators will never be totally harmless). Among the variety of possible binding modes, intercalation is chosen because it is both a process of high biomedical interest and that, from the experimental point of view, is usually the one which produces the higher signal changes. As for the literature review, it was agreed to do data collection only on natural DNA, and not on expensive and too peculiar engineered sequences. Thus, **calf thymus DNA was selected**.

Different families of compounds were considered (cyanine dyes, porphyrins, Schiff-bases, ruthenium complexes) which, however, showed several weak points such as strong aggregation, low purity, few literature data and presence of enantiomeric mixtures. Overall, the most suitable candidate seems Ethidium Bromide (EB). Cyanine dyes and EB share most of the items we look for in a golden standard: commercial availability, reasonable price, considerable literature on DNA-dye interaction, strong affinity and suitable spectroscopic behavior. However, while toxicity is the major weakness of EB, aggregation and low-purity availability are the drawbacks for cyanine dyes. Therefore, **EB was selected** and an in-depth survey of data related to EB/natural DNA studies was done. Moreover, several members of the WG2 have personal experience with EB binding studies.

**Italy: T. Biver, F. Binacchi, University of Pisa; G. Barone, A. Terenzi, University of Palermo. Hungary: E.A. Enyedy, O. Dömötör, University of Szeged. Portugal: N. Basílio, NOVA University of Lisbon; I. Correia, N. Ribeiro, Instituto Superior Tecnico, University of Lisbon; I. Cavaco, University of Algarve. Spain: N. Busto, University of Burgos; E. Garcia-España, J. Gonzalez, University of Valencia. France: J. Hamacek, CNRS Orleans*

2 – LITERATURE RESULTS: PARAMETERS TO CAREFULLY CHECK OR NOT

On the basis of a wide literature on EB/DNA systems (see **ANNEX A**), it can be commented that the **salt content (ionic strength) of the medium dramatically affects the EB/DNA interaction**. Therefore, a careful choice of a buffer is needed to perform reliable experiments. On the other hand, **temperature** variation in the 23-37 °C range does not seem to produce dramatic changes on the binding constant (K). However, temperature-controlled devices are obviously needed if high accuracy of the K values is requested. The **pH is** a **non-critical** parameter: between pH equal to 3 and 11 the adduct formation between EB and DNA base pairs is not considerably disturbed. Below pH = 3 and above pH = 11 a definite breakdown in fluorescence was observed most probably due to the denaturation of DNA. The same holds for type of natural DNA and GC/AT content of DNA: **no difference in binding affinity was found between DNA types prepared from different species** (*Proteus vulgaris* 37% GC; *E. coli*/50% GC; calf thymus/42% GC). No considerable deviation was observed between binding constants determined for DNA of different origins (calf thymus, *E. coli*, bacteriophage T2, *Micrococcus lisodeikticus* – *see for instance the papers by M.J. Waring*). Meyer – Almes F.J. and Porschke D. have studied EB-DNA interaction by a kinetic approach (stopped flow and T-jump) and reported **similar equilibrium constants for of DNA different lengths** (200, 500 and 4228 bp).

According to the conditions that were chosen in our work for the proposal of a protocol for titrations (BUFFER = 0.1 M KCl, 0.01M Hepes, pH 7.4, 25.0°C), literature data collected in ANNEX A suggests that logK is close to 5.4 and DNA bp/EB ratio is 2.5.

3 – EXPERIMENTAL ASPECTS

In this section we collect observations and tips on the two reactants involved: calf thymus DNA and ethidium bromide. Purity of the reagents and comments on the linearity range of their optical response are taken into account. Some comments on the molar extinction coefficients to be used is present here; however, for a detailed description on the preparation and evaluation of DNA or EB concentrations please refer to the protocol – Chapter 4.

3.1 General remarks on purity Purity of all reactants and system components (buffer) needs to be carefully checked. In principle, no matter how minor is the impurity: if it has a striking reactivity or non-negligible contribution to the overall signal it will anyway interfere with the experiment. It may be stated that purity lower than 95% should be avoided whereas HPLC purified – reagent grade species may be sufficiently robust.

3.2 General remarks on the spectrophotometric evaluation of the molar concentration In the absence of sufficiently pure reagents to be used as analytical standards, the molar concentration of a solution can be spectroscopically obtained by using the Lambert and Beer's law ($A_\lambda = \varepsilon_\lambda \ell C$, where A_λ is the absorbance at the wavelength λ, ε_λ the molar extinction coefficient in this solvent at λ, ℓ the optical path in cm, and C the molar concentration). First step is thus the registration of the absorbance spectrum of the solution. Note that this is more tricky than it appears at first sight: (i) scan rate needs to be not too fast, to avoid spectrum distortions (maximum 120 nm/min); (ii) blank recording and subtraction are needed in the absence of double beam instruments – careful autozero tests are needed for double beam instruments; (iii) absorbance reads and strictly those used to evaluate concentrations need to be in the 0.2 – 0.8 range for optimal response/bias minimisation; (iv) data corresponding to the wavelength of a maximum would better be used, again, to minimise errors. Given that it may occur that (iii) is not fulfilled using the stock solution

we want to quantify, a dilution may be needed. Very high dilution factors should be avoided as they produce high propagation of errors. A 1:10 dilution may be the right one in many cases as a good compromise between fulfilment of (iii) and error control. Note that fixed volume volumetric pipettors are more accurate than variable volume ones.

3.3 DNA purity and sonication Within our work, we have considered that DNA purity may be an uncertainty factor, which may bias the binding constants evaluation. The 260nm/280nm ratio is a common parameter to check DNA purification level (absence of proteins): it should be higher than 1.8. All the research groups experimentally involved in WG2-TG2 have checked the 260nm/280nm values of their Calf Thymus DNA, which is not only acceptable (> 1.8), but highly reproducible (1.89 ± 0.1). Note: the provider was the same for all groups (Sigma). On the whole, based on these observations and on a statistical study on the obtained values for the binding parameters (these will be the object of another WG2-TG2 document), it turned out that **DNA purity is not a main bias source**.

As for sonication, it has to be highlighted that polynucleotides may be sonicated to reduce the length to such an extent that the polymer information/geometry is not lost (a few hundred base pairs), but such that high winding is avoided. This is particularly true for those groups which will merge thermodynamic studies to kinetics ones as too long polymers produce kinetic artefacts. Among WG2-TG2 participants, Pisa and Burgos usually sonicate their DNA samples, whilst other group members do not. The procedure for sonication by the two research groups is quite the same. However, according to the literature findings of Porschke's group [F.J. Meyer-Almes and D. Porschke, Biochemistry, 32, 4246, 1993], and also on the basis of the reproducibility of the experimental responses and binding isotherms obtained by the different groups, it may be concluded that **DNA sonication is not a bias source in spectrofluorometric titrations, and the results are independent of sonication**.

3.4 EB purity **Ethidium Bromide** (**EB**, 3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide, $C_{21}H_{20}BrN_3$, MW 394.31, CAS Number: 1239-45-8) is sold both as a powder (95% HPLC grade) or as a solution. Solid should be preferred for the ease to prepare solutions at different concentrations in different buffers. Stock solutions of EB (in the mM range, obtained by weight) should be kept in the fridge (4°C) and in the dark (for instance with aluminium wrap). Under these conditions the solutions may be stable for months. However, the exact concentration of the solution needs to be checked prior to use by recording an absorbance spectrum of one dilution of the stock. **Table 1 shows that the molar extinction coefficient at 480 nm for EB in aqueous buffers is (5.7 ± 0.1)×10^3 M^{-1} cm^{-1} (error 2%).** The EB absorbance spectra collected in the different laboratories, starting from different EB batches, turned out to be superimposable. It can therefore be concluded that **EB purity is not a main bias source**.

Table 1. Values for the molar extinction coefficient (ε, M $^{-1}$ cm^{-1}) of EB at 480 nm in different aqueous buffers.

BUFFER	ε	Reference
7 mM Na_2HPO_4, 2 mM NaH_2PO_4, 180 mM NaCl, 1 mM Na_2EDTA, pH 7.0 -7.1	5600	W. Mueller and D. M. Crothers, Eur. J. Biochem, 54, 267 , 1975
6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, 1 mM NaCac pH 7.2	5850	J.L. Bresloff and D. M. Crothers, J. Mol. Biol, 95, 103, 1975
32 mM Na_2HPO_4, 10 mM KH_2PO_4, 1 mM EDTA, pH 7.45	5860	G. Baldini and G. Varani, Biopolymers, 25, 2187, 1985
0.1 M NaCl	5680	S.A. Winkle etal, NAR, 10, 8211, 1982
0.1 M $NaClO_4$, 10 mM NaCac, 0.25 mM EDTA, pH 7.0	5700	F.J. Meyer-Almes and D. Porschke, Biochemistry, 32, 4246, 1993

Note that EB is a hazardous species (toxic and carcinogenic GHS06,GHS08; hazard statements H302 - H330 - H341; precautionary statements P201 - P202 - P260 - P264 - P301 + P312 - P304 + P340 + P310). Nevertheless, it has been chosen for its low cost, wide commercial availability, and wide use in many types of chemical/biological laboratories, which are organised to correctly handle and dispose it.

3.5 Tests for linearity ranges Reliable quantitation of binding requires that the optical responses of the free and bound drug/dye (in this case EB) forms are linear over the range of concentrations used in the assay. Thus, the Lambert & Beer law has to be obeyed and the extinction coefficients or fluorescence optical factors must be invariant for a given species. Non-linear concentration dependencies of absorbance and fluorescence may result from polymerisation, aggregation or simple precipitation. In general, quantitative analysis of the binding is not recommended for situations in which linear concentration dependence cannot be ensured. This needs therefore to be carefully checked, both for the absorbance and fluorescence responses of the species analysed and under the experimental conditions chosen. Note that, **in the case of fluorescence, inner filter effects may also be at play**, which will produce non-linear dependencies even in the presence of a stable monomer. A general and useful rule to avoid non-linear dependencies due to inner-filter is to check that Abs < 0.05 at the sum of excitation and emission wavelengths. **Our protocol (Chapter 4) will propose reactants' concentrations such that inner filter corrections can be neglected.** WG2-TG2 has done an inter-laboratory exercise where the suppliers for EB and linearity check for both absorbance and fluorescence signals were done. **On the whole, all research groups agree that the molar extinction coefficient at 480 nm for EB in an aqueous buffer agrees with the literature value of $(5.7 \pm 0.1)\times10^3$ M^{-1} cm^{-1}, which should be used from now on. Also, all research groups involved agree that the absorbance response in the analysed linearity range is obeyed until 7×10^{-5} M. Finally, it is homogenously found that the linearity of the signal response on concentration for fluorescence emission holds only in the 0 – 1.7×10^{-5} M range.** An inhomogeneity on the position of the fluorescence emission signals is found between the different research groups (at excitation 510 nm, the maximum emission of free EB ranges from 600 to 630 nm, the maximum emission of DNA-bound EB in the 590-610 nm range). However, it is concluded that this 20 nm shift is a technical bias, which often afflicts different spectrofluorometric apparatus. The shift biases both the unbound and bound spectra, resulting in a signal downshift, upon Calf Thymus DNA binding, that is quite reproducible. On the whole, we expect that, given that any data treatment uses signal **variation** upon titrant addition, these shifts due to different apparatuses, would not afflict significantly the binding parameters.

4 – THE PROTOCOL FOR TITRATIONS

On the basis of what was discussed above, the following protocol is prepared and proposed to allow also a non-expert researcher to practice a correctly designed fluorescence titration.

Recipe for EB – Calf Thymus DNA (CT-DNA) fluorometric titrations

0. Buffer preparation

Prepare the following buffer: 10 mM HEPES buffer with 0.1 M KCl in water, pH = 7.40

Possible recipe:
- weight on an analytical balance:
- m(HEPES) = 0.6566 g; M_r = 328.30
- m(KCl) = 1.491 g M_r = 74.55
- - dissolve them in ca. 180 mL milliQ or ultra-pure water in a beaker
- - calibrate a pH meter (preferably do 3 point calibration in the pH range of pH 4-10)
- - set the solution to pH= 7.4 by the addition of aqueous KOH solution
- - transfer the buffer solution to a 200.0 mL volumetric flask, wash the beaker and collect the washings in the flask and fill the flask to the nominal volume with water.

1. Preparation of stock solutions, dilution of working solutions and determination of their concentration

1.a) ca. 1 mM EB in water

CAUTION: EB is highly toxic, carcinogenic and teratogenic compound. Keep the necessary precautions listed in the available Safety Data Sheet (e.g. link) Treat all solutions containing EB as special waste, handle it in accordance with the regulations.

- weight on an analytical balance the EB
- dissolve it in ultra-pure water
- This solution is stable for 1-3 years if stored in the freezer at -20 °C

TIP: measure the weight of the closed empty (plastic) vial in advance, then dose some EB in the vial under the fumehood, close it, and measure its weight again.

Possible recipe:
7.89 mg EB in 20.00 mL water (M_r = 394.29)

Preparation of 100 µM and 20 µM EB working solutions in buffer

- For 100 µM: make a tenfold dilution of the EB stock solution with the buffer used for the experiments
- No matter how you do it, in the next step the actual concentration of this working solution will be determined by UV-vis spectrophotometry
- For 20 µM: make an accurate 5-fold dilution from the 100 µM working solution with the buffer. Preferably use a volumetric flask.

Concentration of the 100 µM EB working solution

- ε(480 nm) = 5740 $M^{-1}cm^{-1}$ in phosphate buffer (pH = 7.0-7.5) [1]
  i. Measure the UV-vis spectrum of the buffer (background)
  ii. Measure the UV-Vis spectrum of the 100 µM working solution (l = 1 cm)
  iii. subtract the background absorbance spectrum and calculate the EB concentration of both working solutions

NOTE1: in the case of a double beam spectrophotometer an autozero procedure, done when both measuring and reference cells contain the buffer, may automatically subtract

the background. Once the autozero done, measure a spectrum to control the autozero correctness. A UV-vis spectrum of the baseline should be repeated at the end of the work, to check for baseline drift or contaminations. Concentration measurements should be done in triplicate to enhance precision.

1.b.) 1 mg/mL CT-DNA in buffer

- weight on an analytical balance the CT-DNA
- dissolve it in buffer
- solubilize, keeping it for 3-5 days in fridge
- filter it on cotton wool

Possible recipe:
2 mg CT-DNA in 2.00 mL buffer
Usually it results in a 0.4-0.5 mM stock solution.

NOTE2: DNA in solution can be degraded by DNases, which are generally present in our environment. Degradation can be minimalized by the use of sterile devices and solutions. Fortunately, the effect of DNA chain length on the EB intercalation is not considerable.

Concentration of the CT-DNA stock solution

- ε(260 nm) = 13200 $M^{-1}cm^{-1}$ (in base pairs, bp) at pH = 7-7.5 [2]
  i. Place 2.00 mL of buffer in a 1.0 cm quartz cuvette and measure its UV-vis spectrum
  ii. Add 0.200 mL of the CT-DNA stock solution and measure the UV-vis spectrum again
  iii. subtract the background absorbance and calculate the concentration expressed in base pairs in the measured sample.
  iv. Repeat steps i.-iii twice and calculate the mean concentration of the stock solution

NOTE3: See **NOTE1**, which holds also here

NOTE4: Calculate the ratio $A_{260nm}/A_{280\ nm}$, which should be near 2.0. Typically it is ≥1.8. Lower values indicate the presence of non-negligible protein impurities in the DNA stock solution.

Preparation of 50 uM and 200 uM ct-DNA working solutions

- dilute the DNA stock with buffer depending on the obtained concentration of the stock solution.
- dilutions are preferably done in volumetric flasks.

TIP: In order to be more accurate, measure also the absorbance of the diluted solutions. For the 200 uM CT-DNA solution: 0.5 mL of working solution in 1.5 mL buffer (*l* = 1cm) and for the 50 uM solution: direct measurement of the solution (*l* = 1cm)

2. Sample preparation and titration

Instrument settings:

λ_{EX} = 510 nm λ_{EM} = 530 - 750 nm

slit widths: chose according to your instrument's performance*

***** Be aware of the linear intensity range of your instrument! In order to get good data quality the choice of proper slit widths is essential. For this reason, it is recommended to prepare a sample containing 2 µM EtBr and 50 µM CT-DNA and optimize the instrument slit widths to this sample. For many instruments, the linear response will be lost for EB concentrations higher than 1.7×10^{-5} M.

Use thermostatation for the fluorimeter's cell holder: T = 25.0 °C

2.a) EB titrated by CT-DNA

Sample: 2 μM EB in buffer

NOTE5: The concentration indicated above is a rough evaluation. The exact EB concentration needs to be obtained by the previous steps/calculations. The same holds for CT-DNA concentrations below.

Titrant: (1) 50 μM CT-DNA working solution
for 0-5 CT-DNA-to-EB ratios,
collect 8-10 spectra in this concentration range
(2) 200 μM CT-DNA working solution
for 5-30 CT-DNA-to-EB ratios,
collect 8-10 spectra in this concentration range

Possible recipe:
- Measure 2.00 mL buffer in a 1×1 cm fluorometric cell, and measure its emission spectrum (fluorescence intensity must be near zero over the whole emission range)
- Add 40.0 μL of 100 μM EB working solution, and measure the emission spectrum
- Start the titration with the 50 μM CT-DNA working solution: add 20 μLs of it to the sample and measure the emission spectrum after each addition. Go up to 10 addition steps (10×20μL of 50 μM CT-DNA added).
- Switch to 200 μM CT-DNA working solution, add 20-20-20 μLs, then 40-40-40 μLs, finally 100-100-100-100 μLs of working solution to the EB sample and measure the emission spectrum after each addition. (Σ: 580 μL of 200 μM CT-DNA added)

NOTE6: Do not forget to homogenize carefully the sample after each addition! However, do it gently and do not shake it too much: it may produce bubbles, which interfere with the light beam.

NOTE7: Since the intercalation reaction is very fast, there is no real need to wait between one addition and the following one. However, and in particular if temperature is very different from room temperature, it may be decided to wait a short time (for instance 30s) to let the cell reach the temperature of the compartment.

2.b) CT-DNA titrated by EB

Sample: 1 μM CT-DNA in buffer

Titrant: (1) 20 μM EB working solution
for 0-2 EB-to-CT-DNA ratios,
collect 10-12 spectra in this concentration range
(2) 100 μM EB working solution
for 2-10 EB-to-CT-DNA ratios,
collect 6-8 spectra in this concentration range

See **NOTE6-7**, they hold also here! …and in 2.c)!

Possible recipe:
Possible recipe:
- Measure 2.00 mL buffer in a 1×1 cm fluorometric cell, and measure its emission spectrum (fluorescence intensity must be near zero over the whole emission range)
- Add 40.0 µL of 50 µM CT-DNA working solution, and measure the emission and absorption spectra
- Start the titration with 20 µM EB working solution: add 20-20-… µLs of the EB solution to the sample, and measure the emission spectrum after each addition. Go up to 10 addition steps (10×20µL of 20 µM EB added)
- Switch to 100 µM EB working solution: add 20-20-20 µLs, then 40-40-40 µLs of working solution to the sample and measure the emission spectrum after each addition. (Σ: 180µL 100 µM EB added)

2.c) EB calibration

Sample: buffer

Titrant: 100 µM EB working solution
for 0-10 µM EB concentrations,
collect 5-8 spectra in this concentration range

Possible recipe:
- Measure 2.00 mL buffer in a 1×1 cm fluorometric cell, and measure its emission and absorption spectra (fluorescence intensity and UV-Vis absorbance must be near zero over the whole detection range)
- Titrate by adding a total of 100 µM EB working solution: add 40-40-… µLs of it to the buffer and measure the emission and absorption spectra after each addition. Go up to 5 addition steps (5×40µL of 100 µM EB added)

References for the protocol

[1] W. Mueller and D. M. Crothers, Eur. J. Biochem, 54, 267 -277 , 1975
J.B. Le Pecq and C. Paoletti, J.Mol.Biol, 27, 87-106, 1967
G. Baldini and G. Varani, Biopolymers, 25, 2187-2208, 1985
B.N. Amas and D.T. Dubin, J. Mol, Biol, 235, 769, 1960

[2] S.R. Gallagher, Quantitaion of DNA and RNA with absorption and fluorescence spectroscopy, in: Current Protocols in Molecular Biology, F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Greene and Wiley-Interscience, New York (1994).

Calculations

EB titrated by CT-DNA

	working solutions													
		EB	DNA1	DNA2										
	c =	100	50	200	uM									
	volumes in uL				single added volumes of DNA			moles expressed in nanomoles (nmol)				concentrations		DNA/EB
Nr	buffer	EB	DNA1 sum	DNA2 sum	DNA1	DNA2	V sum	n EB	n DNA1	n DNA2	n DNA sum	c EB / uM	c DNA / uM	ratio
1	2000	40	0	0	0		2040	4000	0	0	0	1.96	0.00	0.00
2	2000	40	20	0	20		2060	4000	1000	0	1000	1.94	0.49	0.25
3	2000	40	40	0	20		2080	4000	2000	0	2000	1.92	0.96	0.50
4	2000	40	60	0	20		2100	4000	3000	0	3000	1.90	1.43	0.75
5	2000	40	80	0	20		2120	4000	4000	0	4000	1.89	1.89	1.00
6	2000	40	100	0	20		2140	4000	5000	0	5000	1.87	2.34	1.25
7	2000	40	120	0	20		2160	4000	6000	0	6000	1.85	2.78	1.50
8	2000	40	140	0	20		2180	4000	7000	0	7000	1.83	3.21	1.75
9	2000	40	160	0	20		2200	4000	8000	0	8000	1.82	3.64	2.00
10	2000	40	180	0	20		2220	4000	9000	0	9000	1.80	4.05	2.25
11	2000	40	200	0	20		2240	4000	10000	0	10000	1.79	4.46	2.50
12	2000	40	200	20		20	2260	4000	10000	4000	14000	1.77	6.19	3.50
13	2000	40	200	40		20	2280	4000	10000	8000	18000	1.75	7.89	4.50
14	2000	40	200	60		20	2300	4000	10000	12000	22000	1.74	9.57	5.50
15	2000	40	200	100		40	2340	4000	10000	20000	30000	1.71	12.82	7.50
16	2000	40	200	140		40	2380	4000	10000	28000	38000	1.68	15.97	9.50
17	2000	40	200	180		40	2420	4000	10000	36000	46000	1.65	19.01	11.50
18	2000	40	200	280		100	2520	4000	10000	56000	66000	1.59	26.19	16.50
19	2000	40	200	380		100	2620	4000	10000	76000	86000	1.53	32.82	21.50
20	2000	40	200	480		100	2720	4000	10000	96000	106000	1.47	38.97	26.50
21	2000	40	200	580		100	2820	4000	10000	116000	126000	1.42	44.68	31.50

CT-DNA titrated by EB

	working solutions														
		DNA	EB1	EB2											
	c =	50	20	100	uM										
	volumes in uL				single added volumes of EB			moles expressed in nanomoles (nmol)				concentrations		EB/DNA	estimted' abs coming from EB at
Nr	buffer	DNA	EB1 sum	EB2 sum	EB1	EB2	V sum	n DNA	n EB1	n EB2	n EB sum	c EB / uM	c DNA / uM	ratio	λmax 480 nm*
1	2000	40	0	0	0		2040	2000	0	0	0	0.00	0.98	0.00	0.000
2	2000	40	20	0	20		2060	2000	400	0	400	0.19	0.97	0.20	0.001
3	2000	40	40	0	20		2080	2000	800	0	800	0.38	0.96	0.40	0.002
4	2000	40	60	0	20		2100	2000	1200	0	1200	0.57	0.95	0.60	0.003
5	2000	40	80	0	20		2120	2000	1600	0	1600	0.75	0.94	0.80	0.004
6	2000	40	100	0	20		2140	2000	2000	0	2000	0.93	0.93	1.00	0.005
7	2000	40	120	0	20		2160	2000	2400	0	2400	1.11	0.93	1.20	0.006
8	2000	40	140	0	20		2180	2000	2800	0	2800	1.28	0.92	1.40	0.007
9	2000	40	160	0	20		2200	2000	3200	0	3200	1.45	0.91	1.60	0.008
10	2000	40	180	0	20		2220	2000	3600	0	3600	1.62	0.90	1.80	0.009
11	2000	40	200	0	20		2240	2000	4000	0	4000	1.79	0.89	2.00	0.010
12	2000	40	200	20		20	2260	2000	4000	2000	6000	2.65	0.88	3.00	0.015
13	2000	40	200	40		20	2280	2000	4000	4000	8000	3.51	0.88	4.00	0.020
14	2000	40	200	60		20	2300	2000	4000	6000	10000	4.35	0.87	5.00	0.025
15	2000	40	200	100		40	2340	2000	4000	10000	14000	5.98	0.85	7.00	0.034
16	2000	40	200	140		40	2380	2000	4000	14000	18000	7.56	0.84	9.00	0.043
17	2000	40	200	180		40	2420	2000	4000	18000	22000	9.09	0.83	11.00	0.052

ANNEX A

Useful links to some literature on EB/DNA

1965_WAR	https://doi.org/10.1016/S0022-2836(65)80096-1
1966_WAR	https://doi.org/10.1016/0005-2787(66)90305-4
1967_LEP_PAO	https://doi.org/10.1016/0022-2836(67)90353-1
1968_BAU_VIN	https://doi.org/10.1016/0022-2836(68)90286-6
1971_PAO_LEP	https://doi.org/10.1016/0022-2836(71)90282-8
1972_POH_JOV	https://doi.org/10.1073/pnas.69.12.3805
1975_KRU_WIT	https://doi.org/10.1002/bip.1975.360140114
1977_OLM_KEA	https://doi.org/10.1021/bi00635a022
1978_REI_KRU	https://doi.org/10.1021/bi00616a001
1978_BAG_FAL	https://doi.org/10.1093/nar/5.1.161
1981_BAG_DEN	https://doi.org/10.1021/jm00134a009
1981_GRA_CHA	https://doi.org/10.1021/bi00510a026
1985_WIL_KRI	https://doi.org/10.1002/bip.360241008
1986_ATH_BEA	https://doi.org/10.1021/j100401a051
1992_NOR	https://doi.org/10.1021/j100193a073
1993_MEY_POR	https://doi.org/10.1021/bi00067a012
1994_HEL_GRE	https://doi.org/10.1016/0301-4622(93)E0101-A
1998_BYR_MEL	https://doi.org/10.1016/S0301-4622(97)00091-4
1999_TAN_HUA	https://onlinelibrary.wiley.com/doi/abs/10.1002/%28SICI%291521-4109%28199911%2911%3A16%3C1185%3A%3AAID-ELAN1185%3E3.0.CO%3B2-%23
2007_NAF_SAB	https://doi.org/10.1016/j.molstruc.2006.05.004
2010_PIO_WAS	https://doi.org/10.2478/s11535-009-0077-2
2017_DOM_ALM	https://doi.org/10.1016/j.jinorgbio.2016.12.008