



EXPERIMENT 3

QUANTITATIVE ESTIMATION OF DNA AND RNA BY UV SPECTROPHOTOMETRY

Structure

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3.1 INTRODUCTION

Gel quantification is a technique to get an estimation of quantity of DNA bands in agarose gels. This technique basically involves the visual observation of an unknown quantity of DNA to known quantity of DNA on an agarose gel. The gel is stained with ethidium bromide (EtBr) and photographed in gel documentation system.

1 nanogram (ng)
= 10^{-9} gram

Nucleic acids absorb ultraviolet light at 260 nm. More is the light absorbed by the DNA/RNA sample, higher is the nucleic acid concentration of the sample.

As per Lambert Beer's law,

$$\text{Absorbance} = \epsilon cl$$

Where ϵ is molar absorptivity constant

c is concentration in moles/L or M (molarity)

l is length of path traversed by the light through the sample or indirectly the cuvette width or 1 cm.

Extinction coefficient of single stranded RNA is $0.025 \mu\text{g}^{-1} \text{ml}^{-1} \text{cm}^{-1}$ and for double stranded DNA it is $0.020 \mu\text{g}^{-1} \text{ml}^{-1} \text{cm}^{-1}$. Thus, optical density of 1 corresponds to 50 $\mu\text{g}/\text{ml}$ DNA and 40 $\mu\text{g}/\text{ml}$ RNA.

Estimation of DNA and RNA by UV spectrophotometry is a non-destructive way of estimation with sensitivity of 5 ng/ μL . This is advantageous over DPA estimation of DNA and Orcinol method of estimating RNA. Various newer methods are available nowadays that are even more sensitive. One such method is estimation by Nanodrop spectrophotometer whose sensitivity is 2 ng/ μL .

Importance of A_{260} to A_{280} ratio: Assessment of purity

To assess the purity of nucleic acids, absorption of DNA/RNA sample is measured at different wavelengths. Ratio of A_{260} to A_{280} measurements indicates nucleic acid purity. If the nucleic acid sample is not pure and has protein contaminant, this ratio gets reduced since proteins absorb at 280 nm. Dirty cuvettes and/or particulate material in solution give absorbance at 325 nm. Presence of phenol in nucleic acid preparation absorb at 230 nm.

Purity check using A_{260} to A_{280} ratio

A_{260}/A_{280} ratio is a good indicator of presence of proteins. This ratio tells us the percentage of nucleic acids in a sample. For pure DNA, this ratio is 1.7-2.0. Ratios lower than 1.7 show presence of contaminants (protein/phenol).

DNA purity can be estimated using this equation:

DNA purity (A_{260}/A_{280}) = (A_{260} reading – A_{230} reading) ÷ (A_{280} reading – A_{230} reading)

where A_{230} is subtracted to negate absorbance due to turbidity.

Table 3.1: Assessment of purity of nucleic acid sample.

% Nucleic acid	OD ₂₆₀ /OD ₂₈₀	% Nucleic acid	OD ₂₆₀ /OD ₂₈₀	% Nucleic acid	OD ₂₆₀ /OD ₂₈₀
0	0.57	35	1.78	70	1.94
5	1.06	40	1.81	75	1.95
10	1.32	45	1.84	80	1.97
15	1.48	50	1.87	85	1.98
20	1.59	55	1.89	90	1.98
25	1.67	60	1.91	95	1.99
30	1.73	65	1.93	100	2.0

Source : <http://www.csus.edu/indiv/r/rogersa/quant3.pdf>

Expected Learning Outcomes

After studying and performing this experiment, you should be able to:

- ❖ learn how to determine concentration of DNA and RNA by UV absorption;
- ❖ understand what A_{260}/A_{280} ratio signifies; and
- ❖ describe how to assess purity of DNA.

3.2 PRINCIPLE

Nucleic acids absorb UV light at 260 nm. UV-Visible spectrophotometric based estimation of DNA and RNA is a non-destructive way of determination of concentration of nucleic acid sample. It also tells us about the purity of the nucleic acid. Abs_{260}/Abs_{280} of 0.57 indicates 0% nucleic acid, while a ratio of 2.0 indicates pure nucleic acid. A ratio of 1.7-2.0 represents a good quality DNA for further applications.

3.3 MATERIALS REQUIRED

Stock solutions

2 mg/ml DNA: Weigh 10 mg of DNA and to it add 5 ml 10mM Tris pH 8.0.

2 mg/ml RNA: Weigh 10 mg of RNA and to it add 5 ml 10mM Tris pH 8.0.

Working solutions

50 µg/ml of DNA in 10mM Tris pH 8.0: Take 0.25 ml of 2 mg/ml DNA stock and to it add 9.75 ml of 10 mM Tris pH 8.0

40 µg/ml of RNA in 10mM Tris pH 8.0: Take 0.2 ml of 2 mg/ml RNA stock and to it add 9.8 ml of 10 mM Tris pH 8.0

3.4 PROTOCOL

1. Take a series of seven test tubes. And label them as mentioned in protocol table (Table 3.1).
2. To each test tube, add the DNA working solution and 10mM Tris as mentioned in the protocol Table 3.1.
3. Take absorbance at 260 nm in double beam spectrophotometer and record observations in Table 3.2.

Table 3.2: Protocol table for DNA (Working solution: 50 µg/ml)

S. No	Volume of working solution of DNA (ml)	Volume of 10 mM Tris (ml)
B (Blank)	0.0	1.0
1	0.2	0.8
2	0.4	0.6
3	0.6	0.4
4	0.8	0.2
5	1.0	0.0
U (Unknown)	1.0 of unknown	0.0

4. Repeat steps (1) to (3) but now taking RNA working solution and follow protocol Table 3.3. Record observations of A_{260} for RNA in Table 3.4.

Table 3.3: Observation for DNA Sample

Tube No.	Amount of DNA (in μg)	A_{260}
B		
1		
2		
3		
4		
5		
U	-	

5. Using values in Table 3.2, plot a graph between concentration of DNA on x-axis and A_{260} on y-axis. Determine the concentration of unknown from the graph.
6. Repeat the same for values in Table 3.4, to determine concentration of unknown RNA sample.

Table 3.4: Protocol for RNA (Working solution: 40 $\mu\text{g}/\text{ml}$)

S.No	Volume of working solution of RNA (ml)	Volume of 10 mM Tris (ml)
Blank	0.0	1.0
1	0.2	0.8
2	0.4	0.6
3	0.6	0.4
4	0.8	0.2
5	1.0	0.0
U	1.0 of unknown	0.0

Table 3.5: Observation for RNA

Tube No.	Amount of RNA (in μg)	A_{260}
B		
1		
2		
3		
4		
5		
U	-	

3.5 PRECAUTIONS

1. Handle quartz cuvettes carefully. They are costly and fragile.
2. Switch on the double beam spectrophotometer for initialization 15 minutes prior to the conduct of the experiment.
3. Always hold the cuvette from the opaque side.
4. Do not forget to set the blank by using the blank tube.

3.6 SUMMARY

Different concentrations of DNA and RNA were prepared in a buffer and a standard curve was plotted by taking their absorbance at 260 nm. Concentration of unknown DNA sample was then calculated from the graph.

3.7 SELF ASSESSMENT QUESTIONS

1. Define Lambert Beer's Law.
2. What is molar absorptivity?
3. Explain extinction coefficient.
4. What is the significance of A_{260}/A_{280} ratio?