A TBS-380 Mini-Fluorometer method for Protein Quantitation using NanoOrange™

1. INTRODUCTION

Fluorometric quantitation of proteins in solution using the Turner BioSystems TBS-380 Mini-Fluorometer and Molecular Probes' NanoOrange™ Protein Quantitation Kit offers a combination of convenience and sensitivity. Protein concentrations as low as 100 ng/mL can be measured. This level of sensitivity is superior to spectrophotometric techniques such as the Bradford assay (1 µg/mL), the Lowry assay (1 µg/mL), or 280-nm absorbance (50 µg/mL).[1-3] The NanoOrange™ assay also shows less protein-to-protein variability than the Bradford assay.

To perform a protein assay, the protein sample is simply added to the NanoOrange™ reagent in a specialized diluent and this mixture is heated at 95° C for ten minutes. Fluorescence can be measured as soon as the mixture has cooled to room temperature. Alternatively, samples can be read up to six hours after preparation with no loss in sensitivity, as long as samples are protected from light. The NanoOrange™ reagent is virtually non-fluorescent in aqueous solution, becoming strongly fluorescent at about 570–590 nm upon interaction with proteins, when excited at about 470–490 nm. Detection of the fluorescence using the TBS-380 fluorometer allows protein concentrations from 100 ng/mL to 10 µg/mL to be measured relative to a standard curve (Figures 1 and 2).

2. MATERIALS REQUIRED

- TBS-380 Mini-Fluorometer (P/N 3800-003).
- 10x10 mm Polystyrene cuvettes (P/N 7000-957)
- NanoOrange™ Protein Quantitation Kit, by Molecular Probes, Inc., catalog number N-6666.

  The kit contains:
  1. 1.0 mL NanoOrange protein quantitation reagent (500X concentrate),
  2. 50 mL NanoOrange protein quantitation diluent (10X concentrate),
  3. 0.5 mL bovine serum albumin (BSA) standard (2 mg/mL).

  The kit contents are sufficient for 200 assays using a 2 mL volume in a standard cuvette.

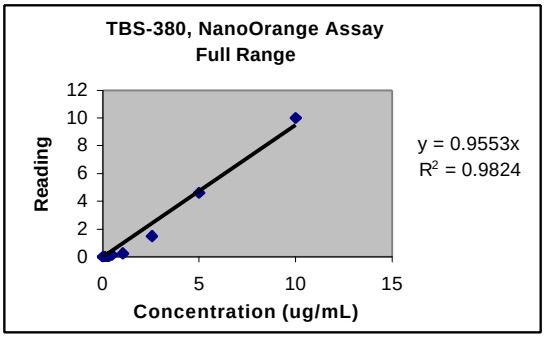

Figure 1.

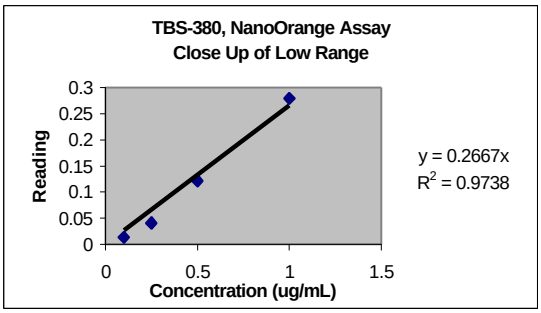

Figure 2.

Figure 1 and 2. Full-range calibration plot and close up of low range plot for bovine serum albumin (BSA) using the TBS-380 Mini-Fluorometer and the NanoOrange™ Protein Quantitation Kit.

3. EXPERIMENTAL PROTOCOL

3.1 Reagent Preparation

Dilute the concentrated NanoOrange™ protein quantitation diluent 10-fold in distilled water. For each assay using 10x10 mm cuvette, 2.5 mL of 1X protein quantitation diluent will be required. Just prior to running the experiment, dilute the NanoOrange™ protein quantitation reagent 250-fold into the 1X protein quantitation diluent to make 2X NanoOrange working solution. For example, to

prepare 25 mL of 2X NanoOrange working solution (enough for 20 assays in 10x10 mm cuvette), first prepare the 1X diluent by mixing 2.5 mL of the 10X diluent stock with 22.5 mL of distilled water; next add 100 µL of the NanoOrange reagent and mix thoroughly. Protect the 2X NanoOrange working solution from photodegradation by storing it in an opaque bottle, covering it with foil or placing it in the dark.

For best results, the working solution should be used within a few hours of its preparation.

3.2 Protein Standard Curve

A standard curve should be generated for converting sample fluorescence into protein concentration. Ideally, the protein type used for the standard curve should be the same as that which is used in the experiment; however, as with other protein assays, bovine serum albumin (BSA) serves as a convenient reference standard. The NanoOrange™ Kit includes a 2 mg/mL sample of BSA that can be used to prepare a standard curve. To serve as an effective control, the protein solution used to prepare the standard curve should also contain levels of contaminants similar to those present in the unknown samples[A]. The reference standard curve is used not only to convert fluorescence to protein concentration, but also to control for any day-to-day readout variation of the fluorometer. The standard curve may be generated to cover the full assay range, 0 to 10 µg/mL, or to cover a selected range.

3.2.1 Prepare a 20 µg/mL solution of BSA by diluting 60 µL of the BSA standard into 5.94 mL of the 1X protein quantitation diluent prepared in section 3.1.

3.2.2 Dilute the 20µg/mL BSA solution to make a series of BSA standard solution at 2X final concentration as described in Table 1. Mix equal volume of the 2X NanoOrange working solution with each 2X BSA standard solution.

Concentration of the 2X BSA standard solution (µg/mL)	Final BSA Concentration (µg/mL)
20	10
10	5
5	2.5
2	1
1	0.5
0.5	0.25
0.2	0.1
0	Blank

Table 1. Preparation of a standard curve using BSA

3.2.3 Incubate samples at 90°C to 96°C for 10 minutes, protected from light. After heating, cool to room temperature for 20 minutes, protected from light.

3.2.4 After cooling, transfer at least 2.0 mL[B] of the sample to a polystyrene cuvette or 50 µL of the sample to a Minicell cuvette, and measure the fluorescence using the *TBS-380* fluorometer. Set-up the fluorometer as per instructions in the User's manual. Power up the instrument by pressing the [ON/OFF] button. Use the [A/B] button to toggle to the "Blue" channel. Press [STD VAL] to program in the concentration of your calibration standard. Use the up and down arrows to change the concentration value. When ready, press the [CAL] button to start the calibration. The TBS-380 Mini-Fluorometer's screens will lead you through the calibration process.

3.2.5 Measure the fluorescence of the remaining standards. Use the data from the standards to generate a standard curve of fluorescence versus protein concentration. (Figures 1 and 2).

3.3 SAMPLE ANALYSIS

3.3.1 Dilute unknow protein samples to a desired volume in 1X protein quantitation diluent (prepared in section 3.1). Mix equal volume of the sample with the 2X NanoOrange working solution. You may wish to use two or three different dilution factors for a given sample. Higher dilution factors will diminish levels of contaminants[A]; however, extremely small

sample volumes should be avoided, as they are difficult to pipette accurately.

3.3.2 Incubate samples at 90°C to 96°C for 10 minutes, protected from light. After heating, cool to room temperature for 20 minutes, protected from light.

3.3.3 After cooling, transfer 2.0 mL[B] of the sample to a 10x10 mm cuvette or 50 µL to a Minicell cuvette and measure the fluorescence using the same

instrument parameters as used in generating the standard curve (Section 3.2.4).

3.3.4 Determine the protein concentration of the sample from the standard curve generated in Section 3.2.5.

4. PATENTS AND TRADEMARKS

The NanoOrange™ Protein Quantitation Reagent is the subject of patent applications filed by Molecular Probes, Inc. and is not available for commercial uses without a specific agreement from Molecular Probes, Inc. NanoOrange is a trademark of Molecular Probes, Inc. Triton is a registered trademark of Rohm & Haas, Inc. Tween is a registered trademark of ICI Americas, Inc.

5. REFERENCES

1. Anal Biochem 72, 248 (1976)
2. J Biol Chem 193, 265 (1951)
3. Scopes, R.K., Protein Purification, Principles and Practice, 2nd Edition, Springer-Verlag (1987).

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[A] Various compounds known to contaminate protein preparations, including salts, detergents and reducing agents may interfere with the NanoOrange™ protein quantitation assay. Protein standard and blank samples should be prepared in solutions that match the composition of the unknown samples as closely as possible. The maximum tolerable concentrations for avoiding appreciable interference are approximately 10 mM for salts (including ammonium sulfate), 100 mM for reducing agents (DTT and β-mercaptoethanol) and 0.01% (w/v) for SDS. For other detergents (Tween®-20 and Triton® X-100), the tolerance level is lower (0.001% (w/v)). See Molecular Probes' product information sheet MP6666 for further details.

[B] Pipetting and sample handling are the largest sources of experimental error in the assay. Accurate volume measurements are essential when making up and transferring samples.