NanoOrange® Protein Quantitation Kit

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage	Stability
NanoOrange® protein quantitation reagent (Component A)	1 mL	500X in DMSO	• ≤2–6˚C • DO NOT FREEZE	When stored as directed the kit is stable for at least 6 months.
BSA standard (Component B)	500 µL	2 mg/mL in water, contains 2 mM sodium azide		
NanoOrange® protein quantitation diluent	50 mL	10X, contains 2 mM sodium azide	• 18–25˚C • DO NOT REFRIGERATE	
Number of assays: 200 assays based on 2.5 mL volumes of which 2.0 mL is transferred to a cuvette for fluorescence determination. If the assay volume is reduced by 10-fold, there is sufficient material for 2,000 assays using 200 µL volumes for detection in a fluorescence microplate reader.				
Approximate fluorescence excitation and emission maxima: 470/570 nm for NanoOrange® reagent.				

Introduction

Molecular Probes' NanoOrange® Protein Quantitation Kit represents a technological breakthrough for the quantitation of proteins in solution, combining ease of use and superb sensitivity. The unique chemistry of the NanoOrange® reagent allows for accurate detection of proteins in solution at concentrations between 10 ng/mL and 10 µg/mL (Figure 1). This level of sensitivity is considerably better than that achieved with the BCA method, Bradford assay, Lowry assay, or absorption at 280 nm (Table 2).[1-4] 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 diluted NanoOrange® reagent, 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 nonfluorescent in aqueous solution, but upon interaction with proteins undergoes a dramatic fluorescence enhancement. When bound to proteins in the diluent provided, the NanoOrange® protein quantitation reagent has a broad excitation peak centered at about 470 nm and a broad emission peak centered at about 570 nm (Figure 2); the reagent is suitable for use with a variety of instruments, including standard fluorometers, minifluorometers, or fluorescence microplate readers. With a fluorometer or minifluorometer, the NanoOrange® assay can detect from 10 ng protein per mL to 10 µg protein per mL using a single dye concentration; with fluorescence-based microplate readers, the assay is reliable from 100 ng/mL to 10 µg/mL.

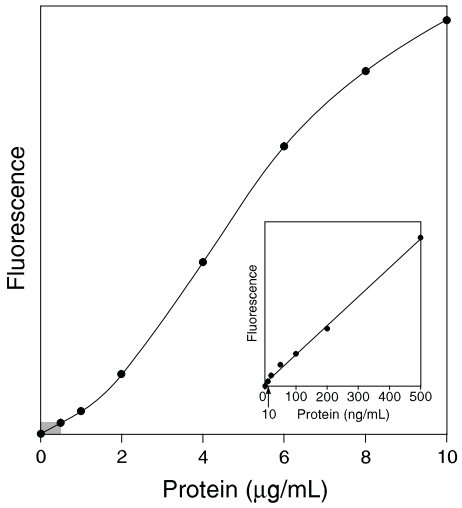

Figure 1. Quantitative analysis of bovine serum albumin (BSA) using the NanoOrange® Protein Quantitation Kit. The inset corresponds to the shaded area in the lower left corner of the plot (0 to 500 ng protein per mL) and illustrates the detection limit of 10 ng/mL. Fluorescence measurements were carried out on a fluorometer using excitation/emission wavelengths of 485/590 nm.

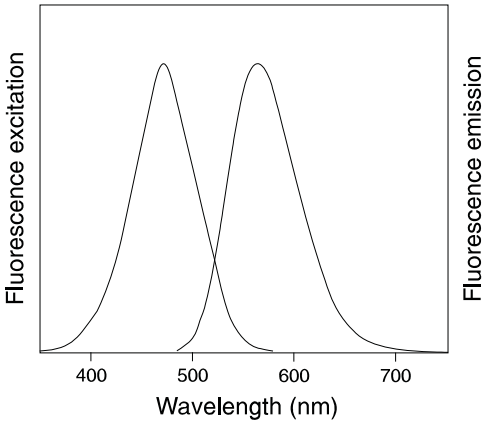

Figure 2. The normalized fluorescence excitation and emission spectra of the NanoOrange® reagent in the presence of protein quantitation diluent and 150 µg/mL BSA.

Table 2. Comparison of methods for quantitating proteins in solution.

Assay Method	Useful Range	Comments
NanoOrange® assay	10 ng/mL to 10 µg/mL	• Samples can be read up to six hours later without any loss in sensitivity • Low protein-to-protein signal variability • Detection not influenced by reducing agents or nucleic acids
BCA method[1]	0.5 µg/mL to 1.5 mg/mL	• Samples must be read within 10 minutes • Not compatible with reducing agents
Bradford assay[2]	1 µg/mL to 1.5 mg/mL	• Proteins precipitate over time • High protein-to-protein signal variability • Not compatible with detergents
Lowry assay[3]	1 µg/mL to 1.5 mg/mL	• Lengthy, multistep procedure • Not compatible with detergents, carbohydrates or reducing agents
Absorbance at 280 nm[4]	50 µg/mL to 2 mg/mL	• High protein-to-protein signal variability • Detection influenced by nucleic acids and other contaminants

Before You Begin

Handling the NanoOrange® Reagent

Allow the NanoOrange® reagent vial to warm to room temperature before opening and then briefly centrifuge in a microcentrifuge to deposit the DMSO solution at the bottom of the vial. For convenient short-term storage (up to one week), the NanoOrange® reagent may be kept at room temperature, protected from light. Store the NanoOrange® protein quantitation diluent at room temperature. At cooler temperatures, some of the components may precipitate.

Caution

We must caution that no data are available addressing the toxicity of the NanoOrange® reagent. The DMSO stock solution should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. Exercise appropriate care and judgment when using this reagent, and dispose of the stain in compliance with all pertaining local regulations.

Experimental Protocol

This section describes the protocol for 2 mL assays. For 200 µL microplate assays, adjust all volumes accordingly.

Reagent Preparation

1.1 Prepare 1X protein quantitation diluent: Mix the concentrated NanoOrange® protein quantitation diluent and dilute 10-fold in distilled water. For each assay, 2.5 mL of 1X protein quantitation diluent will be required.

1.2 Prepare 1X NanoOrange® reagent working solution: Dilute the NanoOrange® protein quantitation reagent (Component A) 500-fold into the 1X protein quantitation diluent from step 1.1.

For example, to prepare 50 mL of 1X NanoOrange® working solution (enough for 20 assays), first prepare the 1X diluent by mixing 5 mL of the 10X diluent stock with 45 mL of distilled water; next add 100 µL of the NanoOrange® reagent and mix thoroughly. Protect the 1X NanoOrange® working solution from light to prevent photodegradation of the NanoOrange® dye. For best results, use the working solution within a few hours of its preparation.

Notes About Heating Solutions in Microplates

This assay requires heating the sample to 90°C for 10 minutes to denature the protein. When samples are heated in a microplate, coever the plate must with a plastic cover or heat-resistant film to prevent evaporation. During the heating and cooling steps, water may condense on the cover, unless a special heater cover is used. To achieve accurate results, collect these water droplets by centrifuging the microplate briefly in a centrifuge. Alternatively, samples may be processed in microcentrifuge tubes, centrifuged in a microcentrifuge, and then transferred to microplate wells for reading. If transferring samples, we recommend transferring only 200 µL of the 250 µL sample, as it is difficult to retrieve all 250 µL using a pipette.

Protein Standard Curve

The reference standard curve is used to convert fluorescence to µg/mL protein, and also to control for variation between fluorometers and for day-to-day variation in the performance of a single fluorometer.

Ideally, the protein type used for the standard curve should be the same as the protein that 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 (Component B) that can be used to prepare a standard curve.

The standard curve may be generated to cover the full assay range, 0–10 µg/mL, or to cover a selected range. This section describes how to generate a simple standard curve with points corresponding to 0, 1, 3, 6, and 10 µg BSA per mL. If desired, serial dilutions can be made to create additional standards ranging from 0.01 to 0.6 µg/mL, to fill out the standard curve in the low range.

2.1 Prepare a 10 µg/mL solution of BSA: Dilute the 2 mg/mL BSA standard (Component B) 1:200 into the 1X NanoOrange® working solution. For example, dilute 30 µL of BSA standard into 5.97 mL of 1X NanoOrange® working solution (prepared in step 1.2).

2.2 Dilute the 10 µg/mL BSA solution to make 0, 1, 3, 6, and 10 µg/mL standards, as described in Table 3.

2.3 If desired, prepare 0.1, 0.3, and 0.6 µg/mL standards, as described in Table 3, by diluting a

Table 3. Protocol for preparing a standard curve using BSA.

Volume (µL) of BSA Solution*	Volume of 1X NanoOrange® Working Solution	Final BSA Concentration
0 mL	2.50 mL	0 µg/mL
2.50 mL of 10 µg/mL	0 mL	10 µg/mL
1.50 mL of 10 µg/mL	1.00 mL	6 µg/mL
0.75 mL of 10 µg/mL	1.75 mL	3 µg/mL
0.25 mL of 10 µg/mL	2.25 mL	1 µg/mL
1.50 mL of 1 µg/mL	1.00 mL	0.6 µg/mL
0.75 mL of 1 µg/mL	1.75 mL	0.3 µg/mL
0.25 mL of 1 µg/mL	2.25 mL	0.1 µg/mL
1.50 mL of 0.1 µg/mL	1.00 mL	0.06 µg/mL
0.75 mL of 0.1 µg/mL	1.75 mL	0.03 µg/mL
0.25 mL of 0.1 µg/mL	2.25 mL	0.01 µg/mL

* The BSA solutions must be made up in 1X NanoOrange® working solution, as described in the text.

1 µg/mL BSA solution. Prepare the 1 µg/mL BSA solution by diluting 300 µL of 10 µg/mL BSA (made in step 2.2) into 2.70 mL of 1X NanoOrange® working solution.

2.4 If desired, prepare 0.01, 0.03, and 0.06 µg/mL standards, as described in Table 3, by diluting a 0.1 µg/mL BSA solution. Prepare the 0.1 µg/mL BSA solution by diluting 300 µL of 1 µg/mL BSA (made in step 2.3) into 2.70 mL of 1X NanoOrange® working solution.

2.5 Incubate samples at 90°C to 96°C for 10 minutes, **protected from light**.

2.6 Cool to room temperature for 20 minutes, **protected from light**.

2.7 **Measure the fluorescence:** Transfer 2.0 mL of the sample to a standard disposable fluorescence cuvette and measure the fluorescence using a fluorometer equipped with filters or settings capable of allowing excitation at about 485 nm and capturing the emission at about 590 nm. To ensure that the sample readings remain in the detection range of the fluorometer, set the instrument's gain so that the 10 µg/mL sample yields a fluorescence intensity near the maximum. To minimize photobleaching effects, keep the time for fluorescence measurement as short as possible and constant for all samples.

2.8 **Generate a standard curve:** Subtract the fluorescence value of the reagent blank (0 µg/mL) from that of each sample. Use these corrected values to generate a standard curve of fluorescence versus protein concentration (for example, see Figure 1).

Sample Analysis

3.1 Dilute the experimental protein solution in 1X NanoOrange® working solution (prepared in step 1.2) to achieve a final volume of 2.5 mL. It is best if the sample volume is no more than ~4% of the total volume. If higher volumes must be used, we recommend performing a standard curve using similar volumes. You may wish to use two or three different dilution factors for a given sample. Higher dilution factors may dilute contaminants to acceptable levels (see Table 4 for contaminant tolerance limits in the final assay solution); however, avoid extremely small sample volumes as they are difficult to pipet accurately.

3.2 Incubate samples at 90°C to 96°C for 10 minutes, **protected from light**.

3.3 Cool to room temperature for at least 20 minutes, **protected from light**.

3.4 **Measure the fluorescence:** Transfer 2.0 mL of the sample to a standard disposable fluorescence cuvette and measure the fluorescence using instrument parameters identical to those used in

Table 4. Tolerance levels for contaminants in the NanoOrange® protein quantitation assay.

Contaminating Compound	Maximum Tolerable Concentration*
DTT	100 mM
β-mercaptoethanol	100 mM
urea	1 M
sodium chloride	20 mM
potassium chloride	20 mM
magnesium chloride	1 mM
calcium chloride	1 mM
zinc chloride	0.5 mM
sodium acetate	20 mM
sodium phosphate	20 mM
ammonium sulfate	10 mM
HEPES	10 mM
sodium azide	10 mM
EDTA	5 mM
sodium hydroxide	10 mM
hydrochloric acid	10 mM
ascorbic acid	10 mM
sucrose	10 mM
glycerol	10%
PEG	1%
DNA	100 ng/mL
amino acids	10 µg/mL
Tween® 20	below 0.001%
SDS	0.01%
Triton® X-100	0.001%

* Compounds present in the final assay solution at or below the indicated concentration do not appreciably interfere with the NanoOrange® protein quantitation assay. Whenever feasible, the blank and protein standards should be prepared in a solution closely matching that of the experimental samples.

generating the standard curve (step 2.6). To minimize photobleaching effects, keep the time for fluorescence measurement as short as possible and the same as that used for the standard curve.

3.5 Determine the protein concentration: Subtract the fluorescence value of the reagent blank from that of the sample and use the standard curve generated in step 2.7 to determine the protein concentration of the sample.

References

1. Anal Biochem 150, 76 (1985); **2.** Anal Biochem 72, 248 (1976); **3.** J Biol Chem 193, 265 (1951); **4.** Scopes, R.K., *Protein Purification, Principles and Practice, 2nd Edition*, Springer-Verlag (1987).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat. no.	Product Name	Unit Size
N6666	NanoOrange® Protein Quantitation Kit *200-2000 assays*	1 kit

Contact Information

Molecular Probes, Inc.
29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:
6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
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Technical Service:
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Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probestech@invitrogen.com

Invitrogen European Headquarters
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

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