EZQ® Phosphoprotein Quantitation Kit (E33201)

Quick Facts

Storage upon receipt:

- ≤25°C
- Protect from light

Ex/Em: 300, 555/580 nm

Number of Assays: 2000

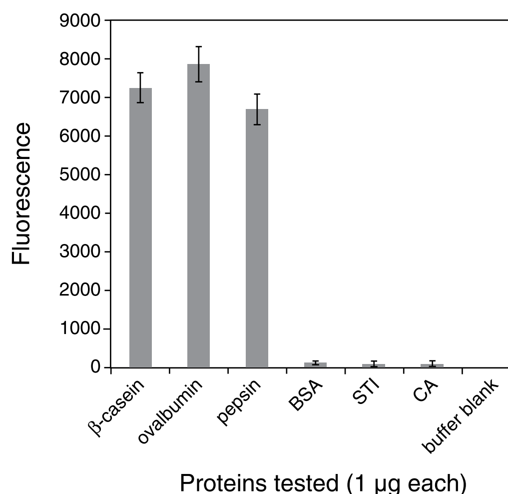

Figure 1. Highly selective staining of phosphoproteins using the EZQ® Phosphoprotein Quantitation Kit. For each of the proteins assayed, a solution of 1 µg/µL was prepared. One microliter of each solution was spotted onto the assay paper and the proteins were stained using the standard protocol. Only the phosphorylated proteins (β-casein, ovalbumin, and pepsin) show a significant level of staining with respect to the buffer blank. (For the proteins tested, BSA, STI, and CA stand for bovine serum albumin, soybean trypsin inhibitor, and carbonic anhydrase, respectively).

Introduction

The EZQ® Phosphoprotein Quantitation Kit provides a fast and simple assay for phosphoproteins in solution. The assay shows high selectivity for phosphoproteins over nonphosphorylated proteins (Figure 1). No radioactivity or antibodies are required, and sample analysis can typically be completed within 60 minutes. The phosphoprotein assay requires only 1 µL of sample, and up to 96 samples, including standards, can be assayed simultaneously. The assay is compatible with samples containing detergents, reducing agents, and urea buffers containing up to 1.0% carrier ampholytes. The kit is ideal for the analysis of protein kinase or phosphatase activities, as well as for monitoring relative phosphoprotein concentrations in chromatography or IEF fractionation samples.

In the assay, the phosphoprotein samples are spotted onto specially prepared assay paper, fixed onto the paper with methanol, and then stained with our proprietary EZQ® phosphoprotein quantitation reagent. Relative phosphate content is determined from a standard curve of ovalbumin or any standard phosphoprotein of interest. As little as 20 ng of ovalbumin, which contains two phosphate groups per molecule, can be selectively detected. The ovalbumin standard curve has an overall dynamic range of 250-fold, from 0.02–5 µg. (Figure 2). The Z-factor for the assay is in the "excellent" range at greater than 0.8, with N = 8. After phosphoprotein analysis, total protein quantitation can be easily performed on the same paper using the EZQ® Protein Quantitation Kit (R33200), for normalization of the signal to protein levels.

The EZQ® Phosphoprotein Quantitation Kit is designed for high-throughput analysis. The solid-phase format and special EZQ® 96-well microplate cassette can be used with readily available fluorescence-based detection instruments, for example:

- Fluorescence-based microplate readers, reading either from the top or bottom of the plate
- Laser-based scanning instruments, equipped with 532–560 nm lasers
- UV illuminators in combination with photographic or CCD cameras for image documentation and analysis (less sensitive)

Materials

Contents

- Component A: **EZQ® phosphoprotein quantitation reagent**, 1 L
- Component B: **EZQ® phosphoprotein destain reagent** (4X concentrate), 800 mL
- Component C: **EZQ® 96-well microplate cassette**
- Component D: **Assay paper, 25 sheets**
- Component E: **Ovalbumin standard** (MW: 44287), 2 vials, each containing 2.0 mg

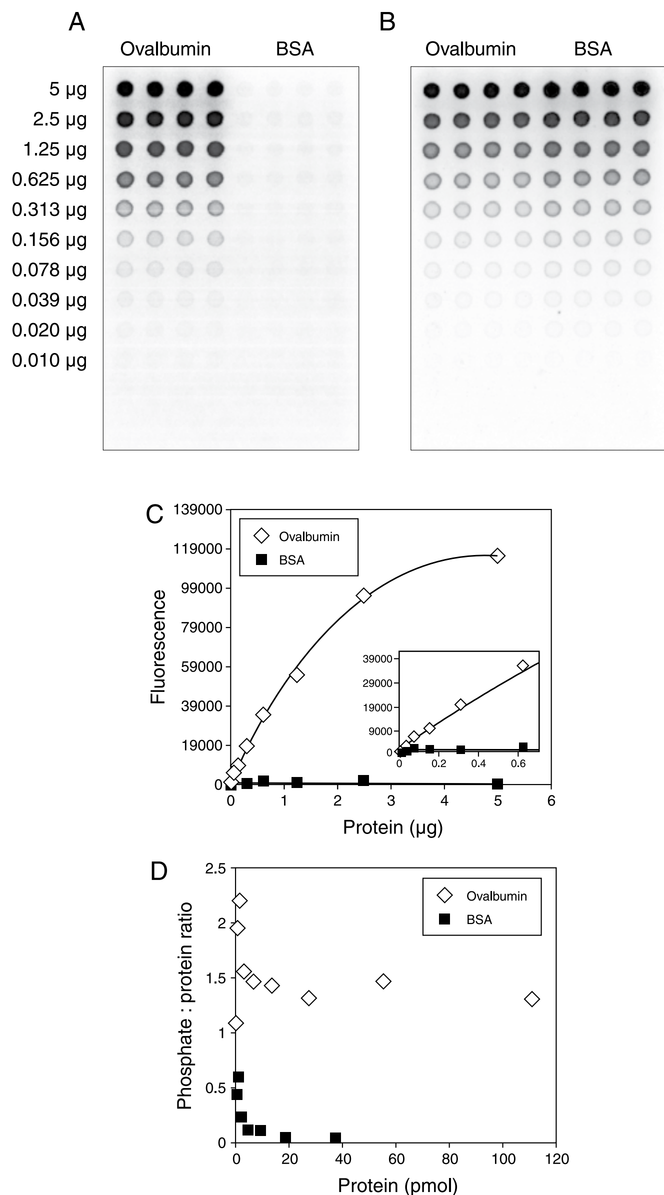

Figure 2. Phosphoprotein detection with EZQ® phosphoprotein detection reagent. Two-fold dilution series of ovalbumin and BSA (5 µg to 10 ng) were spotted on assay paper using 1X NuPAGE® LDS Sample Buffer plus TCEP and stained with EZQ® phosphoprotein quantitation reagent as described. Subsequently, the paper was stained for total protein using the EZQ® Protein Quantitation Kit. Total assay time, including both stains, was approximately 2 hours. The assay paper was scanned on the Fuji FLA-3000 fluorescence laser scanner (Fuji Films, Tokyo, Japan) using the filters described for laser-based scanners in the accompanying protocol. Figure 2A shows the FLA-3000 image acquired after staining with the EZQ® phosphoprotein quantitation reagent, and Figure 2B shows the image acquired after subsequent staining with the EZQ® protein quantitation reagent. Analysis of the data acquired is shown in Figure 2C. The assay was performed over a broad range, and the inset shows the low range in greater detail. Assays were performed in quadruplicate as shown, and the mean values in arbitrary fluorescence units were plotted after subtracting background values. Panel D shows the ratio of EZQ® phosphoprotein quantitation reagent fluorescence to EZQ® protein quantitation reagent fluorescence, taken from panels A and B respectively.

Storage

Upon receipt, store the EZQ® Phosphoprotein Quantitation Kit at ≤25ºC, protected from the light. The kit components should be stable for at least 6 months.

Materials Required but Not Supplied

- methanol, 100%
- plastic staining tray

Protocol

Prepare the Protein Standards

1.1 Make a stock solution of ovalbumin. The ovalbumin (Component E) supplied with the kit can be used to make protein standards for the assay. To make a 10 mg/mL stock solution, add 200 µL of buffer to one vial containing 2.0 mg of ovalbumin, and mix well. The buffer used should be the same as that used for the experimental samples. Dispense aliquots of the stock solution into microcentrifuge tubes and store at ≤–20°C for future use.

1.2 Prepare dilutions of the ovalbumin stock solution. Prepare standards by making dilutions of the 10 mg/mL ovalbumin stock solution. The dilution buffer should be the same as that used for the experimental samples. Buffers containing reducing agents, such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP), are recommended. At least six different concentrations should be used to cover the range expected for the experimental samples. The full effective phosphate detection range for this assay is ~0.9–220 picomoles. This translates to an ovalbumin standard range of ~0.02–5 µg. Volumes of 1 µL are used in the assay.

1.3 Prepare 1X EZQ® phosphoprotein destain reagent. Dilute the EZQ® phosphoprotein destain reagent (4X concentrate, Component B) 4-fold to prepare the 1X working solution. For example, add 30 mL of 4X concentrate to 90 mL of dH_2O to make the 1X destain solution. This volume (120 mL) is sufficient for one assay paper. For destaining half sheets of assay paper, a 60 mL volume of 1X destain reagent is sufficient.

Prepare the EZQ® 96-well Microplate Cassette (Component C)

2.1 Insert the assay paper. Place the microplate face down on a clean surface. Wearing gloves, place a sheet of assay paper (Component D) over the microplate, and align the paper with the inner tabs of the top, bottom, and left sides of the plate. Mark one corner of the paper with a pencil to identify the orientation. If desired, assay papers can be cut in half and used in the device.

2.2 Insert the backing plate. To insert the stainless steel backing plate into the microplate, hold the backing plate so that the flexible bar is at the top. Place the bottom tabs of the backing plate along the bottom, inner edge of the microplate. Apply gentle pressure to the flexible bar and guide the top edge of the plate into position. The top tabs should fit into the top, inner edge of the microplate. Release the pressure on the flexible bar and check that the paper is securely in place.

Note: To ensure a tight fit, the width of the opening beneath the flexible bar should not be narrower than 2 mm before inserting the stainless steel backing plate into the microplate. The opening can be manually adjusted to this width if required.

Spot the Protein Standards and Samples

For the following procedure, the concentration of the protein samples should not exceed 5 mg/mL. If the concentration is

estimated to be higher than this, the samples can be diluted in buffer. Buffers containing reducing agents, such as DTT or TCEP, are recommended as they can enhance dye access to phosphate groups.

3.1 Spot the protein samples onto the assay paper. Protein samples can be applied to the assay paper either through the top wells of the cassette or through the backing plate on the bottom. Apply a 1 µL volume of each protein standard (prepared in step 1.2) and each experimental sample to separate wells of the microplate assembly. Include a 1 µL sample of buffer alone, to serve as a no-protein control. Loading each sample in triplicate is recommended. Be careful not to puncture or scratch the paper with the pipet tip. Gently dispense samples from the pipet tip onto the paper without touching the pipet tip to the paper. Once in contact with the paper, the sample should wick out from the tip. Pipetting accuracy can be improved by gently wiping away any sample on the outside of the pipet tip before spotting the sample onto the paper.

3.2 Dry the samples. After spotting samples, allow the assay paper to dry completely. A hair dryer can be used to reduce the drying time.

Stain the Protein Standards and Samples

4.1 Remove the assay paper from the cassette. Wearing gloves, remove the backing plate from the cassette by depressing the flexible bar on the stainless steel backing plate and lifting it away from the assay paper. Using forceps, remove the protein-spotted assay paper.

4.2 Fix and wash the protein spots. Pour ~40 mL of 100% methanol into a plastic staining tray. Use a plastic tray slightly larger than the assay paper. If half sheets of paper are used, use ~20 mL and a proportionally smaller tray. Place the protein-spotted assay paper into methanol and wash, with gentle agitation, for 5 minutes. This step fixes the proteins and removes contaminating substances including detergents, reducing agents, chaotropes, salts, and dyes that may be present.

4.3 Dry the assay paper. After washing, dry the assay paper. A hair dryer can be used to reduce drying time.

4.4 Stain the proteins. Pour 40 mL of the EZQ® phosphoprotein quantitation reagent (Component A) into a staining tray. Place the assay paper into the stain solution and agitate gently on an orbital shaker for 30 minutes. For half sheets of paper, use ~20 mL and a smaller staining tray.

4.5 Destain the proteins. Wash the assay paper 3 × 15 minutes in ~40 mL of 1X destain reagent (prepared in step 1.3). If half sheets of paper are used, use ~20 mL per wash and a proportionally smaller tray. After the final wash, perform three quick rinses of the paper in dH_2O.

4.6 Prepare the assay paper for analysis. If protein spots will be analyzed using a laser-based scanner or UV illuminator, wet or dry assay papers can be used. The phosphoprotein stain is brighter when the paper is dry, however, the dynamic range and sensitivity are identical whether assayed wet or dry. If the protein spots are to be analyzed using a fluorescence-based microplate reader, the assay paper must be dry. Allow the assay paper to dry on a clean, flat surface. A hair dryer can be used to reduce drying time.

Read and Analyze the Results

5.1 Read the fluorescence. EZQ® phosphoprotein quantitation reagent has two excitation maxima, one at ~300 nm and another at ~555 nm. The emission maximum is at ~580 nm. Various instruments can be used to read the fluorescence, as described below.

Microplate readers: Place the dried paper (from step 4.6) back into the microplate and secure the backing plate. A hand-held UV light can be used to ensure the correct alignment of the paper to the wells. The dry assay paper can be flattened under a heavy object for several minutes to facilitate placement into the plate.

Note: The stainless steel backing plate must be securely in place before placing the cassette into the plate reader. If it is not secure, there is risk of jamming the instrument. Refer to step 2.2 to ensure proper adjustment. If desired, the backing plate may be taped into place using tape that does not autofluoresce.

The stained protein spots can be analyzed in a fluorescence-based microplate reader using excitation/emission settings of ~550/580 nm. Top- or bottom-reading microplate readers may be used, and it makes no difference whether the protein samples were spotted from the top or from the bottom. Best results are obtained with 10 or more readings per well, and the microplate reader should be set in a rastering mode so that data is gathered from the largest possible area in each well.

Laser-based scanners: The stained assay paper can be visualized using an imaging system equipped with a 532–560 nm laser and an appropriate emission filter (e.g. 580 nm longpass filter, or 600 nm bandpass filter).

UV illuminators: To visualize the stained protein spots, the assay paper can be illuminated with a 300 nm transilluminator, a UV top-illuminating system, or a hand-held UV-B light source. A photographic or CCD camera, with an appropriate filter, can be used to generate digital images of the stained paper, which then can be quantitated with the appropriate software.

Note: Analysis using trans- or epi-UV illumination results in a reduction of sensitivity of detection of about 8 times compared with that obtained using visible-light excitation.

5.2 Analyze the results: Calculate the fluorescence values of the ovalbumin control protein spots and experimental samples by subtracting the fluorescence value of the averaged buffer-only spots. Plot the background-subtracted fluorescence values of ovalbumin vs. picomoles of phosphate (ovalbumin has two phosphates per molecule). The standard curve serves as a positive control demonstrating assay sensitivity and dynamic range. The dynamic range should extend from 0.02 to 5 µg of ovalbumin (~0.9 picomoles to 220 picomoles of phosphate).

Protein kinase and phosphatase assays: Plot the background subtracted fluorescence intensity values vs. time to evaluate relative phosphate incorporation. If desired, estimate the stoichiometry of phosphate incorporation from the standard curve.

Note: The staining of pure phosphoproteins using the EZQ® phosphoprotein reagent can vary depending upon a number of factors including the size of the protein, the number of phosphates, and the proximity of phosphates to each other within the protein. The ovalbumin standard curve can be used to estimate the stoichiometry of phosphate incorporation, however the most accurate quantitation requires a standard curve using the phosphorylated protein of interest with a known stoichiometry of phosphorylation.

Protein phosphatase assays: Plot the background-subtracted fluorescence intensity values vs. time to evaluate relative phosphate release. If desired, estimate the stoichiometry of phosphate release from the standard curve.

Normalizing to total protein: The EZQ® phosphoprotein quantitation can be normalized to total protein (see step 5.3). This is useful when evaluating relative phosphate content of different protein extracts such as those obtained during phosphoprotein enrichment chromatography. For example, the extent of enrichment can be determined by evaluating the phosphoprotein to total protein ratios of the starting materials, column flow-throughs, and column eluates. To determine the phosphoprotein to total protein ratios, post-stain the membrane in the EZQ® protein quantitation reagent (see step 5.3).

Divide the fluorescence intensities of the ovalbumin phosphoprotein standard curve by the fluorescence intensity of the corresponding total protein standard curve. The ratio should be relatively constant over the range of 0.08 to 5 µg of ovalbumin (see Figure 2D). When determining relative phosphate amounts of experimental samples (e.g. phosphoprotein column flow-through samples vs. eluates), the total protein amount should be within this range for accurate quantitation.

5.3 Determine total protein (Optional). The total amount of protein in the samples can be determined using the EZQ® Protein Quantitation Kit (E33200). After analyzing phosphoprotein staining, place the assay paper in 40 mL of the EZQ® protein quantitation reagent for 30 minutes and analyze as described in the product information sheet. The amount of total protein can be used to normalize the phosphate signal to the amount of protein loaded. This normalization will correct for any variation in protein loading.

Product List

Current prices may be obtained from our Web site or from our Customer Service Department.

Cat #	Product Name	Unit Size
E33201	EZQ® Phosphoprotein Quantitation Kit *2000 assays*	1 kit

Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Assistance Department in Eugene, Oregon.

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