

EZQ® Phosphopeptide Quantitation Kit (E33202)

Quick Facts

Storage upon receipt:

- ≤25°C
- Protect from light

Ex/Em: 300 and 555/580 nm

Number of Assays: 1000

Introduction

The EZQ® Phosphopeptide Quantitation Kit provides a rapid and sensitive assay for phosphopeptide quantitation in solution. No radioactivity is required, and sample analysis can typically be completed within 60 minutes. The phosphopeptide assay requires only 1 µL of sample, and up to 96 samples, including standards, can be assayed simultaneously. The assay is compatible with a variety of commonly used protein kinase buffers and components (Figure 1). The kit is ideal for the analysis of protein kinase or phosphatase activities, as well as for identifying phosphopeptides present in HPLC fractionation samples.

In the assay, the phosphopeptide samples are spotted onto specially prepared assay paper, fixed onto the paper with methanol, and then stained with our proprietary EZQ® phosphopeptide assay reagent. Relative phosphate content is determined from a standard curve of control phosphopeptide, pT1721, or any standard phosphopeptide of interest. Subpicomole amounts of most monophosphorylated peptides can be detected; for peptides that we have tested, the overall dynamic range of detection is over 500-fold, from 0.2–0.8 pmol at the lower end to about 400 pmol, depending on the peptide (Figure 2). The Z-factor for the assay is in the “excellent” range at greater than 0.8 with N = 8.

The EZQ® Phosphopeptide Quantitation Kit is designed for high-throughput analysis. The solid-phase format and special 96-well microplate 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)

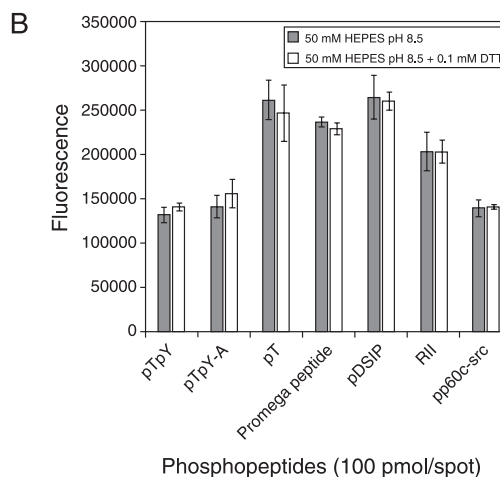
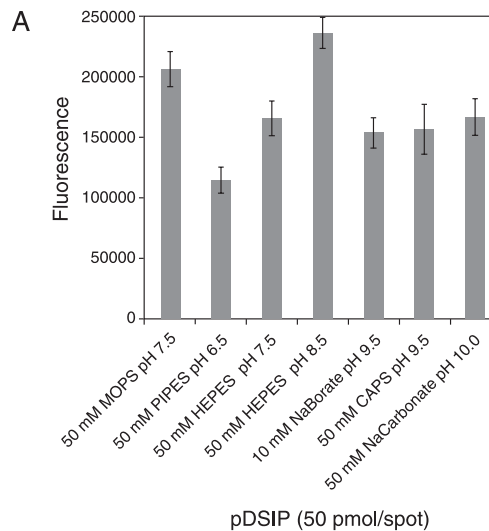


Figure 1. EZQ® phosphopeptide quantitation assay is compatible with many commonly used kinase assay buffers and reagents (panel A) as well as DTT (panel B) (See Figure 3 for sequences of these peptides.)

Materials

Contents:

- Component A: EZQ® phosphopeptide quantitation reagent, 500 mL
- Component B: EZQ® phosphopeptide destain reagent (4X concentrate), 500 mL
- Component C: EZQ® 96-well microplate cassette

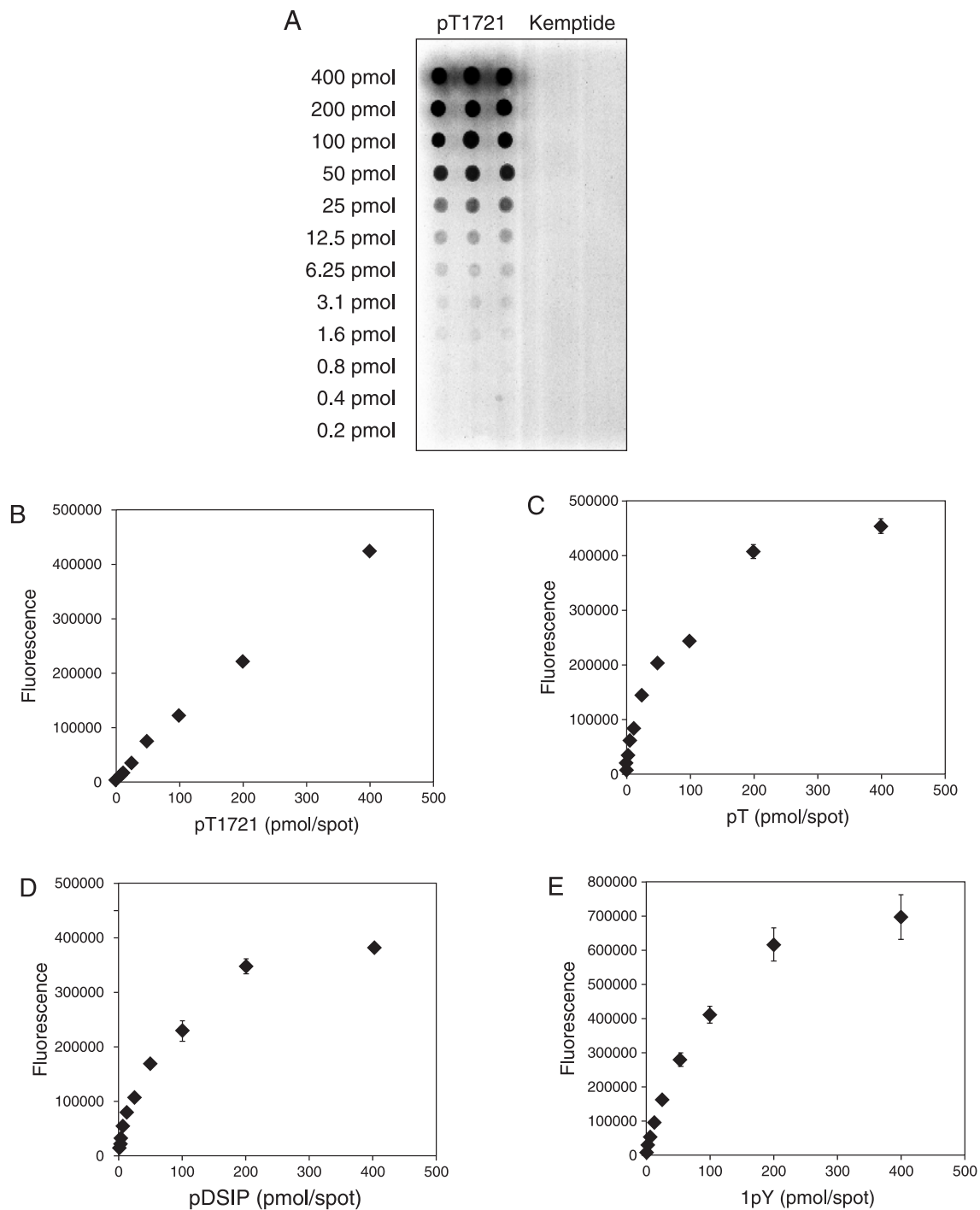


Figure 2. Two-fold dilution series from 400 pmol/ μ L to 0.20 pmol/ μ L were made in 50 mM HEPES, pH 8.5 and spotted in triplicate on assay paper. After staining, the papers were scanned using the Fuji FLA-3000 laser scanner using the filters described in the accompanying protocol. Signal intensities were quantitated using Image Gauge analysis software (Fuji Photo Film, Tokyo, Japan). Panel A shows the spots corresponding to the dilution series for the positive and negative control peptides (pT1721 [VPIPGRFDRRVpTVE] and Kemptide [LRRASLG]), and panel B shows the graph of the data from the pT1721 dilution series. Similar data were gathered for peptides pT, pDSIP, and 1pY (see Figure 3 for sequences of these peptides); the resulting graphs can be seen in panels C, D, and E. The sensitivity of detection for the monophosphorylated peptides ranges from 0.2–0.8 pmol and the dynamic range of detection for all the phosphopeptides is greater than 500-fold.

- Component D: Assay paper, 12 sheets
- Component E: Positive control phosphopeptide, pT1721, (VPIPGRFDRRVpTVE, MW: 1720.9), 1 vial containing 60 nanomoles
- Component F: Negative control peptide, Kemptide, (LRRASLG, MW: 771), 1 vial containing 60 nanomoles

Storage

Upon receipt, store the EZQ® Phosphopeptide Quantitation Kit at $\leq 25^{\circ}\text{C}$, protected from the light. The kit components should be stable for at least 6 months.

Materials Required but Not Provided:

- methanol, 100%
- ethanol, 100%
- sodium borohydride
- 0.1 % SDS in dH₂O
- plastic staining tray

Protocol

Prepare the Control Peptides:

1.1 Make a stock solution of the peptide standards. Prepare the positive control peptide, pT1721 (p-Thr) and the negative control peptide (Kemptide), (Components E and F, respectively) supplied with the kit. Add 30 μ L of dH₂O (or buffer of choice) to each the Component E vial and the Component F vial and mix well. Each vial contains 60 nanomoles of peptide; the final peptide solutions will be 2 mM. The control stock solutions can be prepared in dH₂O, but should always be diluted for use in the same buffer used in experimental samples, as the buffer can have a significant effect on the fluorescence signal (Figure 1). Dispense aliquots of the stock solution into microcentrifuge tubes and store at $\leq -20^{\circ}\text{C}$ for future use.

1.2 Prepare dilutions of the control peptide stock solutions. Prepare dilutions of the 2 mM peptide stocks. The dilution buffer should be the same as that used for the experimental samples (see step 3.1). At least six dilutions should be used to cover the range expected for the experimental samples. We suggest a standard curve ranging from 0.2 pmol/ μ L to 400 pmol/ μ L. This will require an initial 5-fold dilution of the peptide stocks to 400 pmol/ μ L (2 μ L peptide stock in 10 μ L total volume). Then perform six serial 4-fold dilutions (4 μ L peptide + 12 μ L buffer) to a final concentration of 0.2 pmol/ μ L. A buffer blank should be included alongside the dilution series during the assay.

1.3 Prepare 1X EZQ[®] phosphoprotein destain reagent. Dilute the EZQ[®] phosphoprotein destain reagent (4X concentrate, Component B) 1:4 to prepare the 1X working solution. For example, add 30 mL of 4X concentrate to 90 mL of dH₂O 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 96-well Microplate Cassette (Component C)

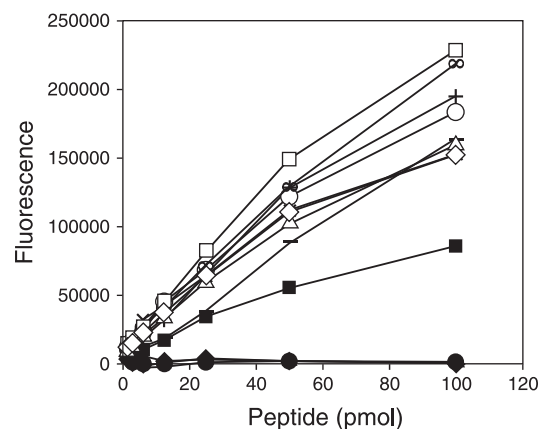
2.1 Insert the assay paper. Place the microplate face down on a clean surface. Wearing gloves, place a sheet of assay paper (Component D) over the microplate, and align the paper with the inner tabs of the top, bottom, and left sides of the plate. Mark one corner of the paper with a pencil to identify 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 Control Peptides and Samples

3.1 Spot the peptide samples onto the assay paper. In general, sample buffers with pH values above 6.5 are recommended for the assay. Several common protein kinase buffers are compatible with the assay (Figure 3). Optimal peptide binding occurs using 50 mM HEPES buffer, pH 8.5. Although not recommended, phosphate buffers can be tested for compatibility with each particular assay. If it is suspected that the concentration of the phosphopeptide will fall outside the limits of the assay (0.2 pmol/ μ L to 400 pmol/ μ L), then several dilutions of the sample can be assayed.



Symbol (in graph above)	Peptide Name	Sequence	# of Phosphates
◇	3pY	TRDIpYETDpYpYRK	3
□	pTpY	DHTGFLpTEpYVATR	2
△	pTpY-A	DHTGFLpTGYVATR	1
×	1pY	TRDIpYETDYRK	1
∞	pT	KRpTIRR	1
○	pDSIP	WAGGNAPSGE	1
+	Promega peptide	RRApTVA	1
-	RII	DLDVPIpGRFDRRVpSVAEE	1
■	pp60c-src	TSTEPQpYQPGENL	1
NA	pT1721	VPiPGRFDRRVpTVE	1
◆	p60c-src	TSTEPQPYQPGENL	0
●	AT	DRVYIHPFHL	0
▲	A8186	RKRARKE	0
NA	Kemptide	LRRASLG	0

NA = Not applicable.

Figure 3. Quantitation of various phosphopeptides using the EZQ[®] Phosphopeptide Quantitation Kit. Fluorescence signals generated by various phosphopeptides in the assay are plotted versus pmol of phosphopeptide. Each point on the graph represents the fluorescence signal at that data point, minus the background signal given by the zero pmol point for that peptide.

Peptide samples can be applied to the assay paper either through the top wells of the microplate or through the backing plate on the bottom of the plate. Apply a 1 μ L volume of the control peptides (prepared in step 1.2) and of each experimental sample to separate wells of the microplate assembly. Include a 1 μ L sample of buffer alone, to serve as a no-peptide 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 Peptide Standards and Samples

4.1 Remove the assay paper from the plate. Wearing gloves, remove the backing plate from the microplate 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 Prepare sodium borohydride reducing reagent. Carefully dissolve 150 mg of solid sodium borohydride in 35 mL of a phosphate buffered saline solution (PBS) by slowly adding the sodium borohydride to the PBS in at least 4 parts. Swirl the solution after each addition of sodium borohydride and allow the solid to dissolve before adding more. After all the sodium borohydride is dissolved in the PBS, add 15 mL of 100% ethanol. Swirl the solution to mix.

Note: Addition of sodium borohydride to an aqueous solution results in the release of hydrogen gas and should be performed in a chemical hood.

4.3 Fixing the samples. Pour ~40 mL of the sodium borohydride solution prepared in step 4.2 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 peptide-spotted assay paper into the sodium borohydride solution and incubate, with gentle agitation, for 5 min at room temperature. This step is important for attaching the peptides permanently to the assay paper.

4.4 Wash the paper. Perform all washes at room temperature. After incubating in the sodium borohydride solution, remove the assay paper from the solution and perform the following washes:

- Rinse the paper in ~40 mL dH₂O two times, 1 minute each with gentle agitation.
- Wash the paper in ~40 mL 0.1% SDS two times, for 1 minute each with gentle agitation.
- Rinse the paper in ~40 mL dH₂O two times, for 1 minute each with gentle agitation.
- Wash the paper in ~40 mL 100% methanol for 1 minute with gentle agitation.

4.5 Dry the assay paper. After washing, ensure the assay paper is completely dry.

4.6 Stain the peptides. Pour 40 mL of the EZQ[®] phosphopeptide 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 min. For half sheets of paper, use ~20 mL and a smaller staining tray.

4.7 Destain the peptides. Wash the assay paper 3 \times 10 min 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 3 quick rinses of the paper in dH₂O.

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

Read and Analyze the Results

5.1 Read the fluorescence. EZQ[®] phosphopeptide 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 4.8) 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 assay plate 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 reads 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. Create a standard curve by plotting the fluorescence values of the control peptides vs. the corresponding pmol of phosphate (control phosphopeptide has 1 phosphate group). Background subtraction can be performed using the fluorescence values of the buffer blank. Estimate the relative phosphate content of the experimental samples by comparison

with the standard curve. Note that while the signal from each individual peptide is linear with concentration, the fluorescence signal can vary from phosphopeptide to phosphopeptide so that direct quantitation of the precise number of moles of phosphate from the standard curve provided is not always possible (see Figure 3). For the most accurate quantitation of the number of phosphate groups on a single peptide, one should use a dilution series of a known amount of the same phosphorylated peptide for the standard curve.

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

Cat #	Product Name	Unit Size
E33202	EZQ [®] Phosphopeptide Quantitation Kit *1000 assays*.....	1 kit

Contact Information

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