Pro-Q® Diamond LC Phosphopeptide Detection Kit (P33203)

Quick Facts

Storage upon receipt:

- ≤–20ºC
- Protect from light
- Dessicate

Ex/Em: 555/580 nm

Introduction

The Pro-Q® Diamond LC Phosphopeptide Detection Kit provides sensitive and selective fluorescence-based detection of phosphopeptides during high-pressure liquid chromatography (HPLC) separations. Pro-Q Diamond LC reagent interacts selectively with phosphothreonine-, phosphotyrosine-, and phosphoserine-containing peptides (Figure 1) to form unique, highly fluorescent dye–phosphopeptide complexes that elute from the HPLC column with altered retention times, allowing identification and purification of phosphopeptides prior to analysis by mass spectrometry (Figure 2). This kit is ideal for isolating phosphopeptides from chromatographic fractions of semi-complex peptide mixtures or from complex peptide mixtures such as the tryptic digestion of a phosphoprotein (Figure 3). The kit provides sufficient reagents for 20 HPLC phosphopeptide separations; a single separation will selectively detect 20 pmol or less of a monophosphorylated peptide using a standard microbore C_{18} HPLC column. Positive and negative control peptides are included in the kit.

Materials

Kit Contents

- Pro-Q Diamond LC phosphopeptide detection reagent (Component A), 150 µL
- 10X Activation buffer (Component B), 150 µL
- Positive control phosphopeptide (RII, DLDVPIPGRF-DRRVpSVAAE) (Component C), 2 µg
- Negative control peptide (Kemptide, LRRASLG) (Component D), 1 µg

Storage and Handling

Store all ingredients at ≤–20˚C. The Pro-Q Diamond LC phosphopeptide reagent should be desiccated and protected from light. When stored properly, the kit components should remain stable for at least six months from the date of purchase.

Materials Required but Not Provided

- HPLC with fluorescence detector
- C_{18} or C_8 reversed-phase column
- HPLC-grade methanol

Experimental Protocol

A single separation will selectively detect a monophosphorylated peptide, assuming 20 pmol of starting material (or 20 pmol of phosphate). Under these conditions, approximately 30–60% of the monophosphorylated peptides should be in the fluorescent complex, depending on the size and composition of the peptide.

Control and Experimental Sample Preparation

1.1. Prepare the control peptide standards. Dissolve the Kemptide nonphosphorylated peptide control (Component D, MW = 773) in 260 µL HPLC-grade water and mix well by vortexing. Dissolve the RII control phosphopeptide (Component C, MW = 2192) in 180 µl HPLC-grade water and mix well by vortexing. The control peptide concentrations are now at 5 µM. Divide the control peptide solutions into 10 µL aliquots and store at ≤–20˚C.

1.2. Prepare the experimental samples. Dry down the digest or peptide fractions and then resuspend the samples in up to 20 µl of HPLC-grade water. Samples should be free of salts, buffers, and detergents.

Preparation of Reaction Mixtures for HPLC

Prepare reaction mixtures using the amounts listed in Tables 1 and 2 (see page 4). We suggest adding the components in the following order: water, peptides, detection reagent, and activation buffer. Combine all ingredients and vortex briefly. Centrifuge the samples at 12,000 × g for 5 minutes to clear the solution of any insoluble matter. Samples should be analyzed within 1–2 hours of preparation.

If the peptides are suspected to be insoluble in water, up to 20% ethanol can be added to the reaction mix in place of water (5 µL ethanol/25 µL reaction mix). If the amount of experimental sample is not limited, a control reaction without activation buffer can be prepared to establish the profile of the uncomplexed peptides and the dye alone. Note: To analyze lower- or higher-abundance phosphopeptides, the total volume of the sample peptide mix prepared in Table 2 can be less than or greater than 25 µL provided that the volumes of the components are increased or decreased proportionally.

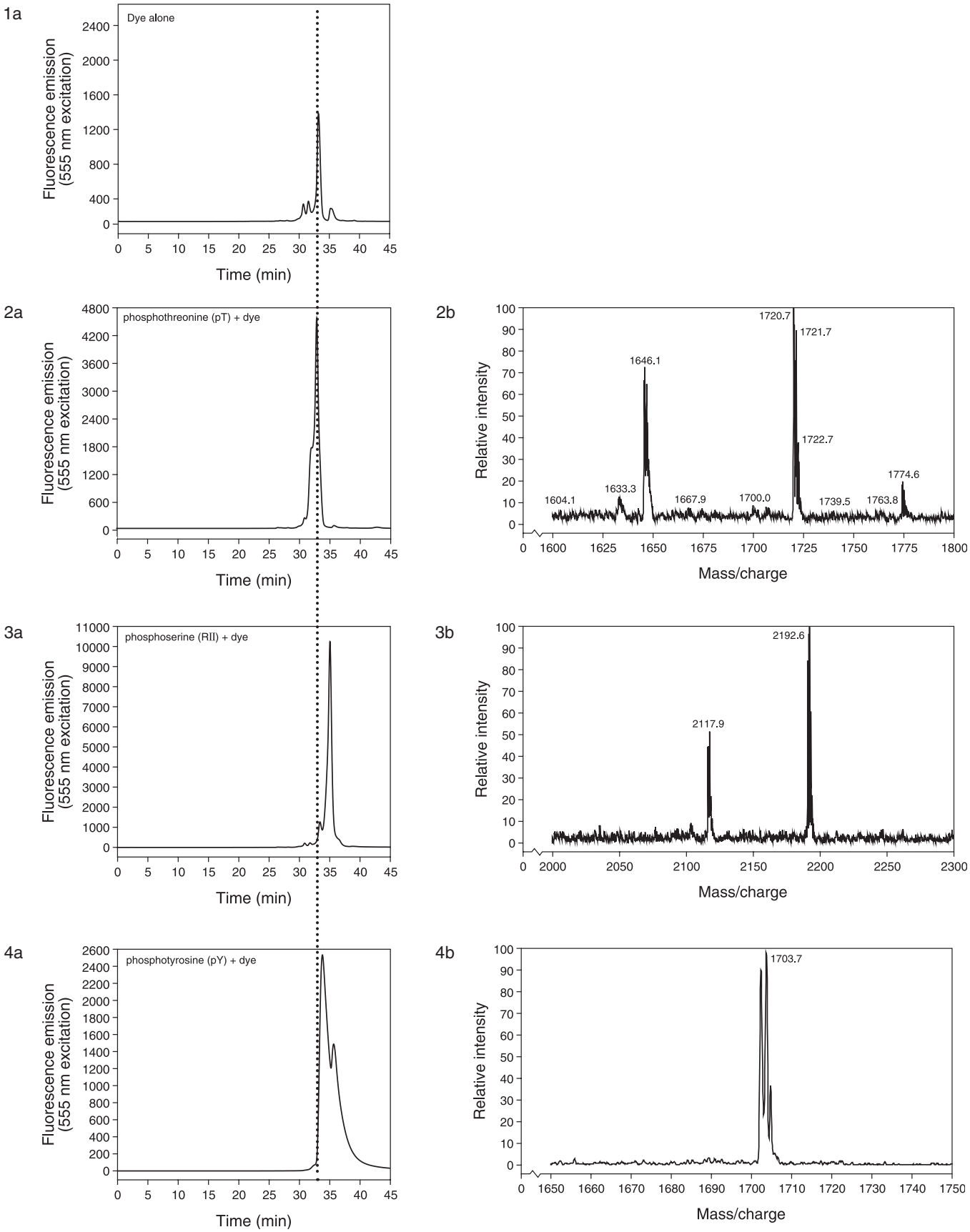

Figure 1. Pro-Q Diamond LC phosphopeptide detection reagent selectively complexes with phosphoserine-, phosphothreonine-, and phosphotyrosine-containing peptides. Complexing reactions were prepared with no phosphopeptide (1a) or with 300 pmol of phosphothreonine peptide (pT, 2a), phosphoserine peptide (RII, 3a), or phosphotyrosine peptide (pY, 4a). Complex peaks show increased or decreased retention times with respect to dye alone, as well as increased fluorescence intensities. Analysis of the complex peaks by MALDI mass spectrometry shows the expected mass weights of pT (2b, MW = 1721), RII (3b, MW = 2192), and pY (4b, MW = 1702). The additional –74 peaks in 2b and 3b represent in-flight loss of phosphate.

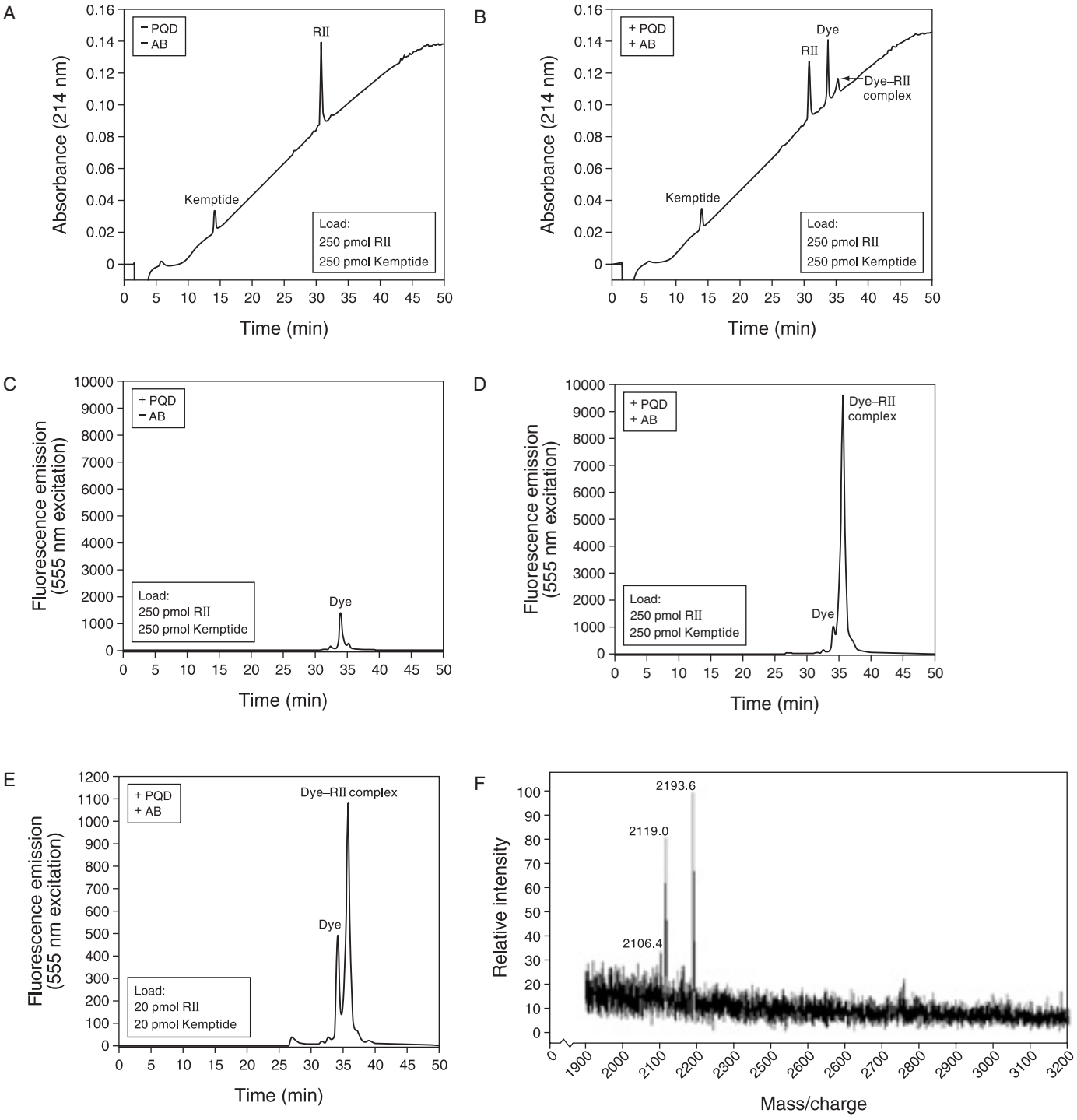

Figure 2. Analysis of control peptides using Pro-Q Diamond LC phosphopeptide detection reagent. Peptides were separated using an HPLC gradient of 5–85% methanol in 15 mM ammonium formate, pH 4, over 40 minutes. A) 214 nm absorbance profile of 250 pmol each of Kemptide (negative control) and RII (positive control). B) 214 nm absorbance profile of 250 pmol each of Kemptide and RII with Pro-Q Diamond LC phosphopeptide detection reagent (PQD) and activation buffer (AB) added. Note the decrease (~35%) in the intensity of the RII peak. C) Fluorescence emission (580 nm) profile of 250 pmol of each peptide with Pro-Q Diamond LC phosphopeptide detection reagent, but without activation buffer. D) Fluorescence emission profile of 250 pmol of each peptide with Pro-Q Diamond LC phosphopeptide detection reagent and activation buffer added. E) Fluorescence emission profile of 20 pmol of each peptide with Pro-Q Diamond LC phosphopeptide detection reagent and activation buffer added. Note the difference in scale between D and E; the peak at 20 pmol of RII is approximately 1/10 the height of the peak at 250 pmol of RII. F) Analysis of the unique fluorescence peak from panel E by MALDI mass spectrometry revealed a mass weight corresponding to the RII phosphopeptide (MW = 2192). The additional –74 peak represents the in-flight loss of phosphate.

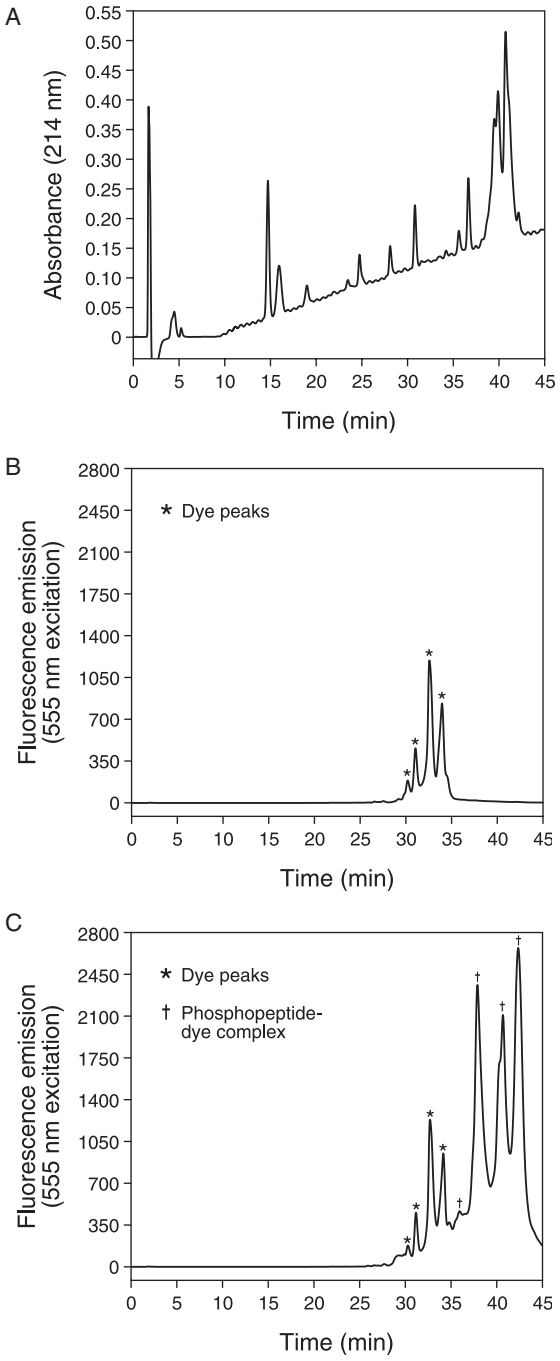

Figure 3. Detection of phosphorylated β-casein phosphopeptides using the Pro-Q Diamond LC Phosphopeptide Detection Kit. 20 µg of β-casein was digested with trypsin and 6 µg was injected on a reversed-phase HPLC gradient of 5–85% methanol in 15 mM ammonium formate, pH 4, over 40 minutes. A) 214 nm absorbance profile of the β-casein digest; B) fluorescence emission (580 nm) profile of the β-casein digest with Pro-Q Diamond LC reagent; C) fluorescence emission (580 nm) profile of the β-casein digest with Pro-Q Diamond LC reagent and activation buffer. Note the appearance of unique peaks in the profile. The peaks were collected, combined, concentrated, and analyzed by MALDI mass spectrometry; two β-casein phosphopeptides (MW = 3123 and MW = 2062) were identified in the combined peak fractions.

Table 1. Control peptide mixes.

	Uncomplexed Control Peptide Mix	Complexed Control Peptide Mix
Water	12.5 µL	10 µL
RII phosphopeptide (5 µM)	5 µL	5 µL
Kemptide peptide (5 µM)	5 µl	5 µL
Pro-Q Diamond detection reagent (Component A)	2.5 µL	2.5 µL
10X activation buffer (Component B)	0 µL	2.5 µL
Total volume	**25 µL**	**25 µL**

Table 2. Sample peptide mixes.

	Uncomplexed Sample Peptide Mix (Optional)	Complexed Sample Peptide Mix
Water	Adjust relative to sample volume	Adjust relative to sample volume
Experimental phosphopeptide sample	Up to 20 µL	Up to 20 µL
Pro-Q Diamond detection reagent (Component A)	2.5 µL	2.5 µL
10X activation buffer (Component B)	0 µL	2.5 µL
Total volume	**25 µL**	**25 µL**

Performing HPLC Analysis

We suggest using high-quality, end-capped columns such as the Waters XTerra® C18 or C8 series columns. For 20 pmol or less of digest, we generally use a 1.0 × 100–150 mm microbore column running at 50–70 µL/min. The following HPLC reagents and conditions are suggested:

- Solvent A: 15 mM ammonium formate, pH 4, 5% methanol
- Solvent B: 15 mM ammonium formate, pH 4, 85% methanol
- Gradient: 0–100% Solvent B over 30–45 minutes

Note: The Kemptide negative control peptide is a relatively small hydrophilic peptide that may not bind to a C_8 HPLC column. When using a C_8 HPLC column, any nonphosphorylated peptide can be used as a replacement for the negative control.

2.1 Inject 20 µL of the Uncomplexed Control Peptide Mix (prepared as in Table 1). The Pro-Q Diamond detection reagent has fluorescence excitation/emission maxima of 555/580 nm; monitor the peaks using those excitation and emission wavelengths. If UV absorbance is also used, monitor the separations at 214, 280, and 555 nm. Depending on the sensitivity of the HPLC detector, the control phosphopeptides and nonphosphopeptides may not be visible by UV absorbance; however, a peak corresponding to the uncomplexed fluorescent dye reagent should be visible at all wavelengths.

2.2 Inject 20 µL of the Complexed Control Peptide Mix (prepared as in Table 1). Under the conditions described, a new peak containing the RII phosphopeptide–dye complex should appear with a retention time greater than that of the uncomplexed dye (Figure 1b). Although this peak will generally show a strong fluorescence response at 580 nm, absorbance at 555 nm will be small or absent compared to that of the free dye. Collect all fluorescence peaks for further analysis by mass spectrometry.

2.3 (Optional) Inject 20 µL of the Uncomplexed Sample Peptide Mix (prepared as in Table 2). This sample will serve as a negative control for mass spectrometry. Collect all fluorescence peaks for further analysis by mass spectrometry.

2.4 Inject 20 µL of the Complexed Sample Peptide Mix (prepared as in Table 2). Collect all fluorescence peaks for further analysis by mass spectrometry.

The retention times of the complexed phosphopeptides may be shorter or longer than those of the uncomplexed dye alone. Occasionally, the peaks representing the complexes will overlap with the peak representing the uncomplexed dye; therefore, all peaks should be collected and analyzed by mass spectrometry, including the uncomplexed dye peak.

Column Evaluation and Cleaning

Evaluate the column after 5–8 runs by running a blank (water-only injection) gradient. If there is a peak in the fluorescence profile of the blank run that is greater than one-tenth the size of the control uncomplexed dye peak, we recommend cleaning the column. The procedure for cleaning the column is as follows:

- 20 column volumes of 1M sodium chloride, 5 mM EGTA, in 5% acetonitrile
- Equilibrate with 0.1% TFA
- Run a gradient of 0–100% acetonitrile in 0.1% TFA over 15 minutes (repeat if fluorescent peak persists)
- Equilibrate the column with Solvent A (15 mM ammonium formate, pH 4, 5% methanol) and repeat the blank injection to assure removal of bound dye

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

Cat #	Product Name	Unit Size
P33203	Pro-Q® Diamond LC Phosphopeptide Detection Kit	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 Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

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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 • order@probes.com

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Technical Assistance: 8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353 • Toll-Free (800) 438-2209
Fax: (541) 335-0238 • tech@probes.com

Molecular Probes Europe BV
Poortgebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 • Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 • Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 • Fax: +31-71-5241883
eurotech@probes.nl