Technical Bulletin

PepTag® Assay for Non-Radioactive Detection of Protein Kinase C or cAMP-Dependent Protein Kinase

INSTRUCTIONS FOR USE OF PRODUCTS V5330 AND V5340.

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1. Description

Protein kinases and their roles in cellular regulation are continually growing areas of research. The need for rapid means of detection of kinase activities has grown along with this interest. The most common method for assaying kinases involves measuring the transfer of radioactive phosphate to a substrate protein or peptide. While this is effective, the large quantity of ^{32}P used makes this assay somewhat inconvenient and potentially hazardous. We have developed the PepTag® Non-Radioactive Protein Kinase Assays[a,b] to provide a rapid,

sensitive and non-radioactive method to detect protein kinase C (PKC) and cAMP-dependent protein kinase (PKA).

The PepTag® Assays use brightly colored, fluorescent peptide substrates that are highly specific for the kinases in question. The hot pink color is imparted by the addition of a dye molecule to the PepTag® Peptide substrate. Phosphorylation by PKC or PKA of its specific substrate alters the peptide's net charge from +1 to –1. This change in the net charge of the substrate allows the phosphorylated and non-phosphorylated versions of the substrate to be rapidly separated on an agarose gel. The phosphorylated species migrates toward the positive electrode, while the nonphosphorylated substrate migrates toward the negative electrode (Figure 1). The amino acid sequence of the PKC-specific peptide substrate, PepTag® C1 Peptide, is P-L-S-R-T-L-S-V-A-A-K. The amino acid sequence of the PKA-specific peptide substrate, PepTag® A1 Peptide, is L-R-R-A-S-L-G (Kemptide). Using the PepTag® Assay, less than 10ng of kinase can be detected in under 2 hours. This allows rapid screening of large numbers of samples such as those produced from the assay of multiple column fractions.

The PepTag® Assay has several advantages over other non-radioactive protein kinase assay systems. The success of a phosphorylation reaction can be quickly determined after the electrophoretic separation step. Because the phosphorylation of the colored peptides supplied with the PepTag® Assays is used to measure kinase activity, phosphorylation of other substrates occurring naturally in the sample does not add to the kinase activity measured. This results in minimal or no background when assaying partially purified samples when compared to using [γ-^{32}P]ATP. Quantitation of the phosphorylated peptide can be accomplished using a densitometer, a spectrophotometer, a 96-well plate reader or a fluorometer. Gel photos depicting control reactions using varying amounts of PKC and PKA are shown in Figures 2 and 3, respectively (Sections 3.B and 3.D).

The PepTag® Assay can detect either PKC or PKA in partially purified samples or purified preparations of enzyme, making it a good choice for rapid screening of column fractions or screening of kinase activators or inhibitors. The PepTag® Assays include either purified, active rat PKC or bovine heart PKA as a positive control. PKC has been purified by the method of Walton *et al.* (1), and PKA has been purified by the method of Flockhart and Corbin (2).

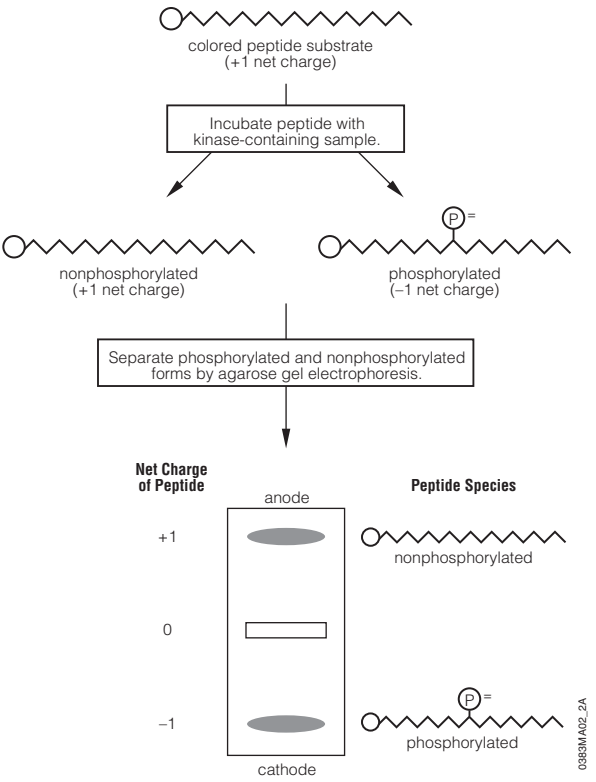

Figure 1. Schematic diagram of the PepTag® Non-Radioactive Protein Kinase Assay procedure. Schematic only—actual data is represented in Figures 2 and 3.

2. Product Components and Storage Conditions

Product	Size	Cat.#
PepTag® Non-Radioactive PKC Assay	120 assays	V5330

Includes:

- 650µl PepTag® PKC Reaction 5X Buffer
- 650µl PKC Activator 5X Solution
- 20µl Protein Kinase C, 0.5µg (control enzyme)
- 650µl PepTag® C1 Peptide (0.4µg/µl)
- 10ml Gel Solubilization Solution
- 250µl Peptide Protection Solution

Product	Size	Cat.#
PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay	120 assays	V5340

Includes:

- 650µl PepTag® PKA Reaction 5X Buffer
- 650µl PKA Activator 5X Solution
- 25µl cAMP-Dependent Protein Kinase, Catalytic Subunit, 2,500u (control enzyme)
- 650µl PepTag® A1 Peptide (0.4µg/µl)
- 10ml Gel Solubilization Solution
- 250µl Peptide Protection Solution

Storage Conditions: Store all components at –70°C. Multiple freeze-thaw cycles of Protein Kinase C and cAMP-Dependent Protein Kinase, Catalytic Subunit, can lead to loss of activity. If all of the control enzyme supplied is not used in one experiment, store in aliquots at –70°C. Do not store diluted control enzyme; it will lose activity.

Do not store diluted control enzyme.

3. Qualitative PepTag® Assay Protocols

The PepTag® Assay can be used with purified samples or partially purified homogenates. Numerous methods exist for the preparation of partially purified homogenates from various sources, including chicken lymphoma (3) and rat brain (1). The following protocols describe methods used at Promega to prepare homogenates for either PKC or PKA.

3.A. Preparation of Tissue or Cell Samples for PKC Assay

Materials to Be Supplied by the User
(Solution compositions are provided in Section 6.A.)

- PKC extraction buffer
- PKC extraction buffer with 200mM NaCl
- phosphate-buffered saline (PBS)
- 1ml column of DEAE cellulose (Whatman® DE52) pre-equilibrated in PKC extraction buffer
- homogenizer (e.g., Polytron® homogenizer or similar homogenizer for tissue samples and a Dounce homogenizer or similar homogenizer for cultured cells)
- microcentrifuge capable of 14,000 × *g*

1. Pre-cool the appropriate homogenizer and PKC extraction buffer to 0–4°C.
2. **For tissue:** Homogenize 1g of tissue in 5ml of cold PKC extraction buffer using a cold homogenizer (e.g., a Polytron® homogenizer).

   For cultured cells: Wash 5×10^6 to 1×10^7 cells with phosphate-buffered saline (PBS; 5ml per 100mm dish), and remove the buffer completely. Suspend the cells in 0.5ml of cold PKC extraction buffer, and homogenize using a cold homogenizer (e.g., a Dounce homogenizer).

   Note: Crude extracts should be assayed the same day they are prepared to retain maximal activity and obtain optimal results.
3. Centrifuge the lysate for 5 minutes at 4°C, 14,000 × *g* in a microcentrifuge, and save the supernatant.
4. Pass the supernatant over a 1ml column of DEAE cellulose that has been pre-equilibrated in PKC extraction buffer. Wash the column with 5ml of PKC extraction buffer, then elute the PKC-containing fraction using 5ml of PKC extraction buffer containing 200mM NaCl. Proceed to Section 3.B.

3.B. PKC Assay Protocol

Materials to Be Supplied by the User
(Solution compositions are provided in Section 6.A.)

- PKC dilution buffer
- horizontal agarose gel apparatus
- glycerol, 80%
- Tris-HCl, 50mM (pH 8.0)
- agarose, 0.8%, in 50mM Tris-HCl (pH 8.0)
- probe sonicator

1. Dilute a portion of Protein Kinase C to 2.5µg/ml in PKC dilution buffer. The Product Information shipped with the system provides the enzyme's starting concentration. Do not store diluted control enzyme; it will lose activity.

3.B. PKC Assay Protocol (continued)

2. Sonicate the PKC Activator 5X Solution using a probe sonicator for 20–30 seconds or until warm.

   Note: The PKC Activator 5X Solution contains phospholipids that aggregate to form micelles. Disruption of the micelles by a probe sonicator is required to achieve maximal PKC activation. The use of a vortex or water bath sonicator will not sufficiently disrupt the phospholipid micelles.

3. For each sample, combine the PepTag® PKC Reaction 5X Buffer, PepTag® C1 Peptide, the sonicated PKC Activator 5X Solution and water in a 0.5ml microcentrifuge tube using the reaction guidelines listed below. Keep on ice until the sample is added. The negative control is required to determine molar absorptivity when quantitating the reaction (Section 4).

   For partially purified enzyme samples, see Note 1.

   Standard PKC assay

PepTag® PKC Reaction 5X Buffer (Note 2)	5µl
PepTag® C1 Peptide (0.4µg/µl)	5µl
PKC Activator 5X Solution, sonicated (Note 2)	5µl
Peptide Protection Solution (optional)	1µl
sample (Step 4)	1–10µl
deionized water to final volume of	25µl

   PKC positive control assay

PepTag® PKC Reaction 5X Buffer (Note 2)	5µl
PepTag® C1 Peptide (0.4µg/µl)	5µl
PKC Activator 5X Solution (Note 2)	5µl
Peptide Protection Solution (optional)	1µl
Protein Kinase C (2.5µg/ml in PKC dilution buffer; Step 4)	4µl
deionized water to final volume of	25µl

   PKC negative control assay (no PKC added)

PepTag® PKC Reaction 5X Buffer (Note 2)	5µl
PepTag® C1 Peptide (0.4µg/µl)	5µl
PKC Activator 5X Solution (Note 2)	5µl
Peptide Protection Solution (optional)	1µl
deionized water to final volume of	25µl

4. At time zero, remove the tube from the ice and incubate in a 30°C water bath for 2 minutes. Then add the sample or Protein Kinase C and incubate at 30°C for 30 minutes (Note 2).

5. Stop the reaction by placing the tube in a boiling water bath or a 95°C heating block for 10 minutes. Store the samples at 4°C or –20°C in the dark until ready to load on the gel.

6. Before loading the samples onto the gel, add 1µl of 80% glycerol to each sample to ensure it remains in the well (Section 3.F).

Notes:

1. When using partially purified homogenates, it may be necessary to add 1µl of Peptide Protection Solution or protease inhibitor to prevent degradation of the peptide. Use protease inhibitors at a final concentration of 0.1–1mM for PMSF, 1–10µM for E-64 or 10–100µM for leupeptin. PMSF irreversibly inhibits serine proteases; E-64 irreversibly inhibits cysteine proteases; leupeptin reversibly inhibits trypsin-like serine proteases and some cysteine proteases. See reference 4 for more information on the specificity and preparation of protease inhibitors.

2. The compositions of the PepTag® PKC Reaction 5X Buffer and PKC Activator 5X Solution are listed in Section 6.A for comparison to other reaction buffers and activators used in radioactive assays. The Reaction Buffer and Activator Solution provided allow phosphorylation of the C1 Peptide by PKC. However, the composition may be altered, or the buffer and solution may be substituted with another buffer or activator solution if previous experience has shown a different concentration or component works better in a specific system. The volume of reaction buffer or activator solution should make up no more than 5µl of the final 25µl reaction mixture to ensure that the concentrations of the other components are not affected.

3. The 30-minute reaction time produces a linear relationship between phosphorylation and PKC activity for 0–20ng of PKC (Cat.# V5261). If the approximate concentration of kinase in the experiment is outside this range, the incubation time can be adjusted to ensure linearity. However, PKC is a labile enzyme at 30°C, and **reaction times should be kept to a minimum to avoid enzyme degradation during the assay.**

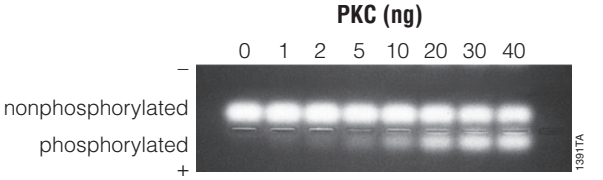

Figure 2. Detection of Protein Kinase C. Two micrograms of PepTag® C1 Peptide were incubated as described in the standard PKC assay (Section 3.B) with varying amounts (0–40ng) of PKC in a final volume of 25µl for 30 minutes at room temperature. The reactions were stopped by heating to 95°C for 10 minutes. The samples were separated on a 0.8% agarose gel at 100V for 15 minutes. Phosphorylated peptide migrated toward the cathode (+), while nonphosphorylated peptide migrated toward the anode (–). The gel was photographed on a transilluminator.

3.C. Preparation of Tissue or Cell Samples for PKA Assay

Materials to Be Supplied by the User
(Solution compositions are provided in Section 6.A.)

- PKA extraction buffer
- phosphate buffered saline (PBS)
- homogenizer: (e.g., a Polytron® homogenizer or similar homogenizer for tissue samples or a Dounce homogenizer or similar homogenizer for cultured cells)
- microcentrifuge capable of 14,000 × *g*

1. Pre-cool the appropriate homogenizer and PKA extraction buffer to 0–4°C.
2. **For tissue:** Homogenize 1g of tissue in 5ml of cold PKA extraction buffer with a cold homogenizer (e.g., a Polytron® homogenizer).

   For cultured cells: Wash 5 × 10^6 to 1 × 10^7 cells with phosphate-buffered saline (PBS; 5ml per 100mm dish) and remove the buffer completely. Suspend the cells in 0.5ml of cold PKA extraction buffer, and homogenize using a cold homogenizer (e.g., a Dounce homogenizer).

   Note: Crude extracts should be assayed the same day they are prepared to retain maximal activity and obtain optimal results.
3. Centrifuge the lysate for 5 minutes at 4°C at 14,000 × *g* in a microcentrifuge, and save the supernatant. Proceed to Section 3.D.

3.D. PKA Assay Protocol

Materials to Be Supplied by the User
(Solution compositions are provided in Section 6.A.)

- PKA dilution buffer
- horizontal agarose gel apparatus
- glycerol, 80%
- Tris-HCl, 50mM (pH 8.0)
- agarose, 0.8%, in 50mM Tris-HCl (pH 8.0)

1. Dilute a portion of the cAMP-Dependent Protein Kinase, Catalytic Subunit, to 2µg/ml in PKA dilution buffer. The Product Information shipped with the system provides the enzyme's starting concentration. Do not store diluted control enzyme; it will lose activity.
2. For each sample, mix the PepTag® PKA Reaction 5X Buffer, PepTag® A1 Peptide, PKA Activator 5X Solution and water in a 0.5ml microcentrifuge tube using the following guidelines. Keep on ice until the sample is added. The negative control is required to determine molar absorptivity when quantitating the reaction (Section 4).

   Note: For partially purified enzyme samples, see Note 1.

Standard cAMP-Dependent Protein Kinase (PKA) assay

PepTag® PKA Reaction 5X Buffer (Note 2)	5µl
PepTag® A1 Peptide (0.4µg/µl)	5µl
PKA Activator 5X Solution (Note 2)	5µl
Peptide Protection Solution (optional)	1µl
sample (Step 3)	1–10µl
deionized water to final volume of	25µl

PKA positive control assay

PepTag® PKA Reaction 5X Buffer (Note 2)	5µl
PepTag® A1 Peptide (0.4µg/µl)	5µl
PKA Activator 5X Solution (Note 2)	5µl
Peptide Protection Solution (optional)	1µl
cAMP-Dependent Protein Kinase, Catalytic Subunit (2µg/ml in PKA dilution buffer; Step 3)	5µl
deionized water to final volume of	25µl

PKA negative control assay (no PKA added)

PepTag® PKA Reaction 5X Buffer (Note 2)	5µl
PepTag® A1 Peptide (0.4µg/µl)	5µl
Peptide Protection Solution (optional)	1µl
PKA Activator 5X Solution (Note 2)	5µl
deionized water to final volume of	25µl

3. At time zero, remove the tube from the ice and incubate in a 30°C water bath for 1 minute. Then add the sample or cAMP-Dependent Protein Kinase, Catalytic Subunit, and incubate at room temperature for 30 minutes (Note 3).

4. Stop the reaction by placing the tube in a boiling water bath or on a 95°C heating block for 10 minutes. Store the samples at either 4°C or –20°C in the dark until ready to load onto the gel.

5. Before loading the samples onto the gel, add 1µl of 80% glycerol to the sample to ensure that it remains in the well (Section 3.F).

Notes:

1. When using partially purified homogenates, it may be necessary to add 1µl of Peptide Protection Solution or protease inhibitor to prevent degradation of the peptide. Use protease inhibitors at a final concentration of 0.1–1mM for PMSF, 1–10µM for E-64 or 10–100µM for leupeptin. PMSF irreversibly inhibits serine proteases; E-64 irreversibly inhibits cysteine proteases; leupeptin reversibly inhibits trypsin-like serine proteases and some cysteine proteases. See reference 4 for more information on the specificity and preparation of protease inhibitors.

3.D. PKA Assay Protocol (continued)

Notes (continued):

2. The compositions of the PepTag® PKA Reaction 5X Buffer and PKA Activator Solution are listed in Section 6.A for comparison to other reaction buffers and activators used in radioactive assays. The Reaction Buffer and Activator Solution provided with the system allow phosphorylation of the PepTag® A1 Peptide by PKA. However, they may be altered or substituted with another buffer or activator solution if previous experience has shown a different concentration or component works better in a specific system. The volume of reaction buffer or the activator should comprise no more than 5µl of the final 25µl reaction mixture to ensure that the concentration of the other components are not affected.

3. The 30-minute reaction time produces a linear relationship between phosphorylation and PKA activity for 0–16ng of PKA (Cat.# V5161). If the approximate concentration of kinase in the experiment is outside this range, the incubation time can be adjusted to ensure linearity. However, PKA is a very labile enzyme at room temperature, and **reaction times should be kept to a minimum to avoid enzyme degradation during the assay.**

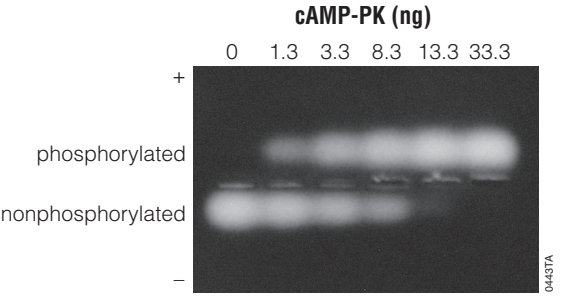

Figure 3. Detection of cAMP-Dependent Protein Kinase. Two micrograms of PepTag® A1 Peptide were incubated as described in the standard PKA assay (Section 3.D) with varying amounts (1.3–33.3ng) of PKA in a final volume of 25µl for 30 minutes at room temperature. The reactions were stopped by heating to 95°C for 10 minutes. The samples were separated on a 0.8% agarose gel at 100V for 15 minutes. Phosphorylated peptide migrated toward the cathode (+), while nonphosphorylated peptide migrated toward the anode (–). The gel was photographed on a transilluminator.

3.E. Gel Preparation

1. Assemble the horizontal agarose gel apparatus following the manufacturer's instructions. If only one comb is used, place it in the center of the gel tray. If more than one comb is used, arrange them with at least 1 inch of gel space between the combs.

   Note: If many samples are to be read simultaneously, place two or more combs in a single gel to double the number of samples per gel, but arrange them with at least 1 inch of gel space between the combs.

2. Prepare a 0.8% agarose solution in 50mM Tris-HCl (pH 8.0). Heat the solution until the agarose is completely dissolved. Allow the mixture to cool to approximately 60°C before pouring the solution into the gel tray. Allow the gel to solidify for about 20 minutes (the amount of time may vary, depending on the type of agarose used; see Notes). For a 7 × 10cm gel, use 30ml of agarose solution. For a 10 × 15cm gel, use 70ml of agarose solution. Thicker gels can be used, but problems may result from the volume of agarose in excised gel slices. For larger or smaller gels, adjust the volume of agarose accordingly.

3. If the gel will not be used for several hours, cover it with a thin layer of 50mM Tris-HCl (pH 8.0), and store at room temperature.

Notes:

1. The type of agarose used could affect the results of the assay. The procedure described assumes the use of standard-melting-point agarose. Low-melting-point agarose is effective, but the gel is more fragile and could dissolve if the gel is placed on a warm surface such as a densitometer.

2. Varying the concentration of agarose in the gel has little or no effect on mobility or resolution of the peptide. The concentration described in the protocol, 0.8%, provides enough rigidity to allow the gel to be handled and still be easily solubilized.

3.F. Separation of Phosphorylated and Nonphosphorylated PepTag® Peptides by Electrophoresis

1. Gently pull the comb from the solidified 0.8% agarose gel. Place the gel tray in the electrophoresis chamber. Pour 50mM Tris-HCl (pH 8.0) over the gel until the wells are filled and the buffer covers the gel completely.
2. Load the samples into the wells. Immediately after the last sample is loaded, run the gel at 100V for 15–18 minutes or until the separation of bands is apparent. Visualization of faint bands can be enhanced by viewing the gel under ultraviolet light.
3. When electrophoresis is complete, remove the gel from the chamber and photograph, if desired. While the colored PepTag® Peptide bands are visible to the naked eye, photographing the gel under UV light will provide greater sensitivity.

   At this point, the qualitative assay is complete. By examining the gel under UV light, you can determine which samples contain protein kinase activity and make a qualitative estimate of the relative amounts of activity in the samples. To quantitate kinase activity, continue with one of the procedures in Section 4.

Notes:

1. To minimize diffusion, load the samples as quickly as possible without overflowing the lanes.
2. The voltage can be varied from 60–140V with no apparent alteration of mobility of the PepTag® Peptide. High salt concentrations in the samples may retard the mobility of the peptide. In such cases, the gels should be run slightly longer to ensure that all products have completely left the wells.

4. Quantitation of PepTag® Assay Results

Spectrophotometric, densitometric or spectrofluorometric methods can detect differences in the amount of phosphorylated and nonphosphorylated peptide species and can be used to quantitate kinase activity. Irrespective of the method used, **begin quantitation within 10 minutes of ending gel electrophoresis to avoid diffusion of the PepTag® Peptide into the surrounding agarose.**

We recommend photographing the gel before cutting out the bands of interest. If a large number of samples makes this difficult, run the samples on more than one gel and stagger their starting times. **When quantitating the activity of a kinase, it is necessary to run a negative control containing 5µl of the PepTag® Peptide but no kinase** (Sections 3.B and 3.D). This control is used to determine the exact molar absorptivity of the dye in your system.

4.A. Quantitation by Spectrophotometry

Material to Be Supplied by the User

- glacial acetic acid

1. Using a razor blade or scalpel, excise the negatively charged phosphorylated bands from the gel, keeping the total volume uniform and at **approximately** 250µl (Note 1). Place each excised band into a 1.5ml graduated microcentrifuge tube and heat at 95°C until the gel slice is melted. If the volume of a gel slice is considerably less than 250µl, dilute it to that amount with water.

2. **For a 96-well plate:** Transfer 125µl of the hot agarose to a tube containing 75µl of Gel Solubilization Solution (that has been warmed to room temperature and mixed well) and 50µl of glacial acetic acid. Quickly vortex and transfer 250µl to a well in a 96-well plate. Once in acidified Gel Solubilization Solution, the agarose should remain liquid for several hours (Note 2). After all the samples have been transferred to the plate, read the absorbance at 570nm. Blank the plate reader with liquefied agarose without PepTag® Peptide.

3. **For a cuvette:** Transfer 175µl of hot agarose to a tube containing 75µl of Gel Solubilization Solution (that has been warmed to room temperature and mixed well), 100µl of glacial acetic acid and 150µl of distilled water. Once in the acidified Gel Solubilization Solution, the agarose should remain liquid for several hours. Vortex and then transfer the 500µl of solution to a 0.5ml cuvette. Read the absorbance at 570nm. Zero the spectrophotometer with liquefied agarose without PepTag® Peptide. Cuvettes can be washed with water after use. None of the components in the Gel Solubilization Solution will damage a glass cuvette.

If the A_{570} readings are too low or too high, increase or decrease the dilution of the sample to be within the linear range of enzyme activity.

4.A. Quantitation by Spectrophotometry (continued)

Notes:

1. Excise the bands as quickly as possible to avoid diffusion of the sample after the power supply has been turned off. We recommend using graduated microcentrifuge tubes to measure the volume of the gel slice. Make sure that the volume of the gel slice is ≤250µl.

2. After heating, the liquefied agarose may begin to resolidify before the acetic acid and Gel Solubilization Solution are added. If this occurs, reheat the gel slice until it liquifies.

4.B. Calculation of Activity

One unit of kinase activity is defined as the number of nanomoles of phosphate transferred to a substrate per minute per milliliter. One phosphorylation site exists on each peptide; therefore, the number of moles of peptide present in the negatively charged, phosphorylated bands is equivalent to the number of moles of phosphate transferred. The number of moles of phosphorylated peptide is computed using Beer's Law. Beer's Law states that:

$A = \varepsilon BC$, where:
A = absorbance of the sample;
ε = the molar absorptivity of the peptide in $L/mol \cdot cm^{-1}$;
B = the width of the light cell;
C = the concentration of the peptide in mol/L of the sample read.

Table 1 presents additional information required to perform these calculations.

Table 1. PepTag® Peptide Characteristics.

Peptide	Molecular Weight	Nanomoles per Described Reaction (2µg)
PepTag® A1 Peptide	1,314g/mol	1.522nmol
PepTag® C1 Peptide	1,684g/mol	1.187nmol

1. Calculate the molar absorptivity (ε) of the PepTag® Peptide based on the absorbance reading system used by first calculating the concentration of the **nonphosphorylated** peptide in the negative control and then using Beer's Law.

   Note: The absorbance reading is based only on the percentage of peptide present in the cuvette or well. If you are following the procedure in Section 4.A using either a 96-well plate reader or spectrophotometer, then adjust the absorbance reading by a factor of 1.43 (use 175µl of 250µl of the melted agarose band).

   Example: The following example is for a situation where the volume of melted agarose is 250µl and where half (125µl) of the gel slice was used for quantitation. Two micrograms of the PepTag® A1 Peptide were used in the

negative control assay, and the complete reaction was loaded onto the gel. Half of the melted agarose gel band was used for the spectrophotometer reading. The absorbance at 570nm (A) was 0.157 when read in a spectrophotometer with a 1cm light cell width in a final volume of 0.5ml.

C = (number of mol) ÷ volume in L

C = $(1.522 \times 10^{-9}) \div (500 \times 10^{-6}L) = 3.044 \times 10^{-6}mol/L$

Using Beer's law, find ε:

A = εBC, ε = A/BC

A = 0.157

B = 1cm

C = $3.044 \times 10^{-6}mol/L$

ε = $(0.157 \times 2) \div (1cm \times 3.044 \times 10^{-6}mol/L)$

ε = $103,150L/mol \cdot cm^{-1}$

2. Next, determine the concentration (C) of phosphorylated PepTag® Peptide in mol/L.

   Example: The absorbance at 570nm for one of the phosphorylated bands was 0.0215. From the previous calculation the molar absorptivity has been determined to be $103,150L/mol \cdot cm^{-1}$.

   A = εBC, C = A/εB

   A_{570} = 0.0215

   ε = $103,150L/mol \cdot cm^{-1}$

   B = 1cm

   C = $(0.0215) \div (103,150L/mol \cdot cm^{-1} \times 1cm) = 2.08 \times 10^{-7}mol/L$

3. Calculate the number of moles of the phosphorylated peptide in the sample read by multiplying the concentration (C; from Step 2) by the volume (V) in the cuvette or well.

   Example: The volume of the sample read was 500µl.

   C × V = number of moles in the sample

   C = $2.08 \times 10^{-7}mol/L$

   V = $500 \times 10^{-6}L$

   $(2.08 \times 10^{-7}mol/L)(500 \times 10^{-6}L) = 104pmol$

4. Adjust the concentration determined in Step 3, by the appropriate dilution factor to determine the total amount of phosphorylated peptide in the agarose band slice. If the assay was followed as described, this number is 2 (250µl of melted agarose band).

   Example: One-half of the band slice was quantitated.

   104pmol × 2 = 208pmol

   Note: One phosphorylation site exists on each peptide; therefore, the moles of peptide present in the negatively charged, phosphorylated band are the number of moles of phosphate transferred.

4.B. Calculation of Activity (continued)

5. Calculate the number of units of kinase activity in the reaction. One unit of kinase is the number of nanomoles of phosphate transferred to a substrate per minute per milliliter.

   To determine the number of units, first divide the number of moles in the reaction by the reaction time. Then divide this number by the volume of the sample added to the reaction.

   Example: A 30-minute incubation was used and the original sample volume was 10µl.

$$\frac{208\text{pmol}}{30\text{ minute}} = 6.93\text{pmol/minute} = 0.00693\text{nmol/minute}$$

$$\frac{0.00693\text{nmol}}{10 \times 10^{-3}\text{ml}} = 0.693\text{units/ml}$$

4.C. Quantitation by Densitometry

The intact agarose gel can be analyzed in a scanning densitometer using a wavelength of approximately 570nm. However, because differences may exist between commercial densitometers, we cannot recommend a specific protocol for scanning. Please contact the manufacturer of your densitometer for recommendations. When planning to use densitometry, it is important to allow the agarose gel to run for a longer period of time to allow the colored bands to migrate further from the central well. The well can cause distortion in the scanning of the colored bands. If interference from the well cannot be eliminated, try scanning the gel across the bands on either side of the well. Care must be taken that the heat from the densitometer does not cause the gel to soften and distort.

Note: Densitometry should be performed as rapidly as possible after finishing electrophoresis to minimize band diffusion.

4.D. Quantitation by Spectrofluorometry

Because the peptides are much more intensely visible under fluorescent light, spectrofluorometry provides the most sensitive method of quantitatively detecting protein kinase activity. Subnanogram amounts of kinase can be detected using this method. Figures 4 and 5 show the excitation and emission spectra for PepTag® Peptide A1. Table 2 provides the excitation and emission maxima of the PepTag® Peptides.

When performing quantitation with a spectrofluorometer, excise, melt and solubilize the gel band according to the procedures for a spectrophotometer (Section 4.A). Then dilute the 500µl of solution with distilled water to the volume required by your instrument. The diversity of spectrofluorometers does not allow us to recommend a general protocol. Please contact your manufacturer for recommendations.

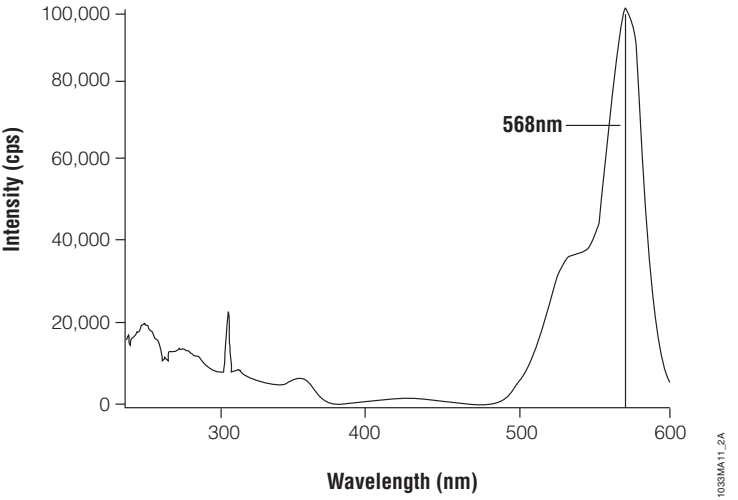

Figure 4. Excitation spectrum of PepTag® Peptide A1. The excitation spectrum of PepTag® Peptide A1 was read on a Spex Fluorograph with model 1680 0.22µm double spectrophotometer using an emission wavelength of 610nm. Five microliters of PepTag® Peptide were diluted to 2ml in water containing 3.75% Gel Solubilization Solution and 5% acetic acid for the reading. Peak excitation occurs at 560nm with a shoulder at 540nm.

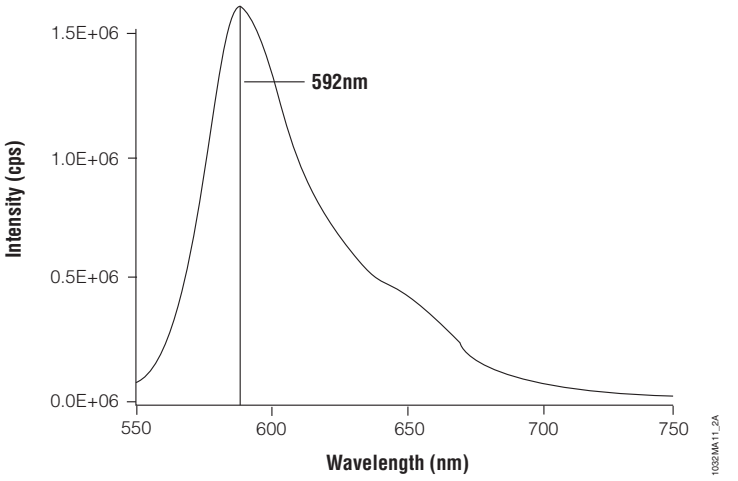

Figure 5. Emission spectrum of PepTag® Peptide A1. The emission spectrum of PepTag® Peptide A1 was read on the Spex Fluorograph as described in Figure 4 except that the excitation wavelength was 540nm.

4.D. Quantitation by Spectrofluorometry (continued)

Table 2. Excitation and Emission Wavelengths of PepTag® Peptides (A1 or C1).

Excitation maximum	568nm
Emission maximum	592nm*

***Note:** Due to the high level of fluorescence of PepTag® Peptides, excitation at 560nm may cause the emission reading to be so high that it is nonlinear. More quantitative values can be obtained using 540nm as the excitation wavelength.

5. References

1. Walton, G.M. *et al.* (1987) A three-step purification procedure for protein kinase C: Characterization of the purified enzyme. *Anal. Biochem.* **161**, 425–37.
2. Flockhart, D.A. and Corbin, J.D. (1984) In: *Brain Receptor Methodologies, Part A,* Marangos, P.J. *et al.*, eds., Academic Press, Orlando, FL, 209.
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4. North, M.J. (1989) In: *Proteolytic Enzymes,* Beynon, R.J. and Bond, J.S., eds., IRL Press, Oxford, UK.
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6. Appendix

6.A. Composition of Buffers and Solutions

PepTag® A1 Peptide

L-R-R-A-S-L-G (Kemptide)

0.4µg/µl in water

PepTag® C1 Peptide

P-L-S-R-T-L-S-V-A-A-K

0.4µg/µl in water

PepTag® PKA Reaction 5X Buffer

100mM	Tris-HCl (pH 7.4)
50mM	$MgCl_2$
5mM	ATP

PepTag® PKC Reaction 5X Buffer

100mM	HEPES (pH 7.4)
6.5mM	$CaCl_2$
5mM	DTT
50mM	$MgCl_2$
5mM	ATP

phosphate-buffered saline (PBS)

0.2g/L	KCl
8.0g/L	NaCl
0.2g/L	KH_2PO_4
1.15g/L	Na_2HPO_4

PKA Activator 5X Solution

5µM	cAMP in water

PKA dilution buffer

350mM	K_3PO_4 (pH 6.8)
0.1mM	DTT

PKA extraction buffer
(see reference 5)

25mM	Tris-HCl (pH 7.4)
0.5mM	EDTA
0.5mM	EGTA
10mM	β-mercaptoethanol
1µg/ml	leupeptin
1µg/ml	aprotinin

Store at 4°C or, for up to six months, at –20°C. Just before use, add 0.5ml of PMSF stock solution (100mM PMSF in 100% ethanol) per 100ml of PKA extraction buffer.

PKC Activator 5X Solution

1mg/ml	phosphatidylserine in water

PKC dilution buffer

100µg/ml	bovine serum albumin (BSA)
0.05%	Triton® X-100

PKC extraction buffer

25mM	Tris-HCl (pH 7.4)
0.5mM	EDTA
0.5mM	EGTA
0.05%	Triton® X-100
10mM	β-mercaptoethanol
1µg/ml	leupeptin
1µg/ml	aprotinin

Store at 4°C or, for up to six months, at –20°C. Just before use, add 0.5ml of PMSF stock solution (100mM PMSF in 100% ethanol) per 100ml of PKC extraction buffer.

6.B. Related Products

Product	Size	Cat.#
SignaTECT® Protein Kinase C (PKC) Assay System	96 reactions	V7470
SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System	96 reactions	V7480
SignaTECT® DNA-Dependent Protein Kinase Assay System	96 reactions	V7870
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System	96 reactions	V8161
SignaTECT® cdc2 Protein Kinase Assay System	96 reactions	V6430
SignaTECT® Protein Tyrosine Kinase Assay System	96 reactions	V6480
cAMP-Dependent Protein Kinase, Catalytic Subunit	2,500u	V5161
InCELLect® AKAP St-Ht31 Inhibitor Peptide	150µl	V8211
InCELLect® St-Ht31P Control Peptide	150µl	V8221
Kemptide (PKA) Peptide Substrate	1mg	V5601
cAMP-Dependent Protein Kinase Peptide Inhibitor	1mg	V5681
PMA	5mg	V1171
4α-PMA	1mg	V1181
LY 294002	5mg	V1201
$Neurogranin_{(28-43)}$ (PKC) Peptide Substrate	1mg	V5611
Myristoylated Protein Kinase C Peptide Inhibitor	1mg	V5691
Protein Kinase C	1µg	V5261

6.B. Related Products (continued)

Product	Size	Cat
Kinase-Glo® Luminescent Kinase Assay	10ml	V6711
	10 × 10ml	V6712
	100ml	V6713
	10 × 100ml	V6714
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	10 × 10ml	V3772
	100ml	V3773
	10 × 100ml	V3774
ProFluor® PKA Assay	4 plate	V1240
	8 plate	V1241
ProFluor® Src-Family Kinase Assay	4 plate	V1270
	8 plate	V1271

[a]U.S. Pat. No. 5,580,747 has been issued to Promega Corporation for a non-radioactive enzyme assay.

[b]Licensed for research and development purposes only under U.S. Pat. No. 5,120,644 and European Pat. No. 0240914.

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