

# Sample Preparation Guide for the Assay of Protein Kinase A, Protein Kinase C and Protein Tyrosine Kinase

3747 N. Meridian Road  
P.O. Box 117  
Rockford, IL 61105

0617

## I. Introduction

**Important Note to the Kinase Analyst:** Due to the diversity of sample sources which could be assayed for Protein Kinase A (PKA), Protein Kinase C (PKC) or Protein Tyrosine Kinase (PTK) activity, it would be impractical to suggest definitive preparation methods which would apply to each potential species, tissue or cell source, and guarantee success. Among the experienced analysts of kinase activity, it has long been known that the preparation of the sample is a critical step in any protocol to assay kinase activity. The assay of crude extracts are difficult and should be avoided. Typically, several attempts are required to develop the proper conditions which result in preserving the activity of these enzymes prior to assay.

It is the intent of this guide to offer the kinase analyst, especially the first time kinase analyst, general guidelines and suggestions which can be applied to a given sample prior to assay with the Pierce Colorimetric Assay Kit for PKA or PKC, or the Pierce Immunoassay Kit for PTK. The information provided here is intended to allow the analyst to customize his/her approach to sample preparation based on the requirements of a specific tissue or cell source. Careful experimentation is often required to arrive at a protocol which optimizes the integrity and activity of the kinase to undergo analysis.

### Important Considerations for Kinase Sample Preparation Success

Successful preparation of tissue or cells for the subsequent assay of kinase activity depends largely on the following three factors.

1. Maintaining the sample at low temperature during the sample preparation process, i.e.  $\leq 5^{\circ}\text{C}$ .
2. Minimizing actual elapsed sample preparation time, i.e. complete the sample preparation protocol as rapidly as possible. Time from sacrificing an animal or from cell harvesting to homogenization of the sample can be critical for obtaining an active preparation in many cases. All procedure steps should be kept constant and as short as possible.
3. Inclusion of Protease Inhibitors and Phosphatase Inhibitors in the preparation scheme to retain the structural and functional integrity of the kinase present in the sample.

## II. MiniReviews

### PKA MiniReview

Protein Kinase A, adenosine cyclic 3',5'-monophosphate (cAMP) dependent protein kinase transfers a  $\gamma$ -phosphoryl group from ATP in the presence of  $\text{Mg}^{+2}$  to specific seryl or threonyl side chains of numerous protein substrates *in-vivo*.<sup>1</sup> This kinase is recognized as the principal receptor for cAMP, an intracellular second messenger in eukaryotic cells.<sup>2</sup> As such, PKA plays an important role in the regulation of hormone and neurotransmitter action and other signaling pathways. Activation of PKA is initiated via the binding of cAMP to an inactive tetrameric form of the enzyme *in-vivo*.<sup>3</sup> The regulatory subunit dimer (RR) of PKA acts to inhibit the activity of the two identical catalytic monomers (2C) in the absence of cAMP. However, upon binding of cAMP to RR, the catalytic subunits dissociate from the complex, becoming fully active. Two classes of PKA arise from isozymes of the regulatory dimer. Designated type I and II, these PKA isozymes, which have been identified in many tissues, are distinguished by their elution order from a DEAE-cellulose column.<sup>4</sup>

**PKA Distribution in Tissue/Cells:** This cyclic-AMP regulated protein kinase is found in most, if not all, mammalian tissue.<sup>5</sup> Within the cell, this enzyme is found compartmentalized within the cytoplasm in proximity of its substrates. The most common tissue sources from which this enzyme is studied have been rabbit skeletal<sup>6</sup> and bovine cardiac muscle tissue<sup>7</sup>. Other prime tissues often targeted for the study of PKA are liver, brain, adrenal gland, lung and testes.<sup>8-10</sup> Cultured cell line sources for PKA study include HeLa and lymphoma cells.

**PKC MiniReview**

Protein Kinase C represents a family of at least 11 isozymes that exhibit phospholipid-dependent protein-serine/threonine kinase activity.<sup>1-4</sup> In their unactivated state, most of these isoforms are soluble (cytoplasmic) or loosely membrane-associated, but, upon activation, they can be selectively and very tightly localized to cellular membranes.<sup>1,2</sup> In general, these PKC isozymes can be divided into three classes.

1. The conventional PKCs (cPKCs) that require both Ca<sup>+2</sup> and diacylglycerol (or phorbol ester) for maximal kinase activity and are represented by the  $\alpha$ ,  $\beta$ 1,  $\beta$ 2 and  $\gamma$  isoforms.
2. The novel PKCs (nPKCs) that are Ca<sup>+2</sup> independent but phorbol ester-responsive and include the  $\delta$ ,  $\epsilon$ , ( $\epsilon'$ ),  $\eta$  (formerly known as PKC-L),  $\theta$  and possibly  $\mu$  isoforms.
3. The atypical PKCs (aPKCs) which are Ca<sup>+2</sup> independent and phorbol ester-unresponsive isoforms identified as  $\zeta$  and  $\iota$  (known as  $\lambda$  in the murine system).

These various isoforms appear to be important in mediating a large array of biological signals, and accordingly one or more of these enzymes has been identified in nearly every mammalian tissue and cell line studied to date.<sup>1,2</sup>

**PKC Distribution in Tissue/Cells:** Although PKC, the diacylglycerol regulated kinase, enjoys a wide tissue distribution, the brain has been the major tissue source for purifying and analyzing many of the known PKC isoforms.<sup>1,2</sup> Nonetheless, PKC has been studied in other such diverse tissues as heart, spleen, lymphocytes, skin and lung. In addition, many cell lines have been well characterized to contain PKC,<sup>1,2</sup> but the principally studied cell lines include fibroblasts (various 3T3 lines), promyelocytic cells (HL-60 and U937) and neuron-like cell lines (PC12).

**Protein Tyrosine Kinase MiniReview**

Since 1979, a diverse array of extracellular agents have been found to influence intracellular protein-tyrosine phosphorylation, including growth factors, cytokines, hormones, extracellular matrix proteins, immunoglobulins, toxins and neurotransmitters.<sup>1,5</sup> The protein-tyrosine kinases that are involved in these signaling events can be loosely separated into two classes, the receptor tyrosine kinases and the non-receptor tyrosine kinases. The receptor tyrosine kinases are generally regulated by ligand binding and receptor clustering, whereas the controlling mechanism for the non-receptor tyrosine kinases are not as well defined. However, several non-receptor tyrosine kinases have been reported to be regulated upon binding to cellular components, such as certain hormone receptors (that do not possess intrinsic tyrosine kinase activity), cytoskeletal elements or other membrane/receptor associated proteins.

**PTK Distribution in Tissue/Cells:** Multiple tyrosine kinases have been identified in essentially all mammalian cell types.<sup>1-5</sup> Accordingly, almost any cell or tissue type can be used for analysis of this class of enzyme activity, although easily grown and transfected cell types, such as 3T3 fibroblasts or cell types like A431 epidermoid carcinoma cells (which overexpress the epidermal growth factor receptor tyrosine kinase) have been among the most widely characterized systems.<sup>1-5</sup>

**III. Kinase Sample Preparation General Guidelines for PKA, PKC and PTK**

**Cell Lysis/Mechanical Tissue Homogenization Options:** Lysis of the sample, independent of the source, usually occurs in hypotonic media, pH 7.5. The chart below outlines the mechanical sample disruption options normally applied to samples independent of the kinase activity to be assayed.

**Buffer Options for Sample Preparation:** The composition of the homogenization/lysis buffers used in kinase preparations, is in most cases independent of the sample source. The recipes published in the literature are all very similar. Concentrations of components may vary somewhat, but generally the components or classes of components in these complex formulations remain remarkably similar.

Some commonly used buffer components have been found to interfere with the Pierce colorimetric PKA and PKC assays. Interference effects can be avoided by keeping the respective concentration of these components at the levels specified below:

Phosphate < 20 mM; DTT < 50 mM; EDTA < 4 mM; EGTA < 4 mM

Any non-ionic detergent: < 0.02% final concentration

If cellular organelles or plasma membrane fractions are to be subjected to kinase assay, the integrity of these subcellular structures is preserved by the osmotic strength provided by the inclusion of 250-500 mM Sucrose in the resulting formulation.

## Typical Sample Preparation/Homogenization Buffer Composition

### Components and Concentrations

|  |   | PKA     | PKC    | PTK     |
|--|---|---------|--------|---------|
| <b>BUFFER</b>  | 20-100mM Tris, or   | X       | X      | X       |
|  | Hepes, or   | X       | X      | X       |
|  | MES, or   | X       |        |         |
|  | $\beta$ -Glycerolphosphate<br>(Glycerol 2-phosphate) or<br>Potassium Phosphate (<20 mM) |         | X      |         |
| <b>pH RANGE</b>  |   | 6.5-8.0 | 7.5    | 7.2-7.5 |
| <b>CHELATING AGENTS</b>                                | 1-4 mM EDTA and   | X or    | X      | X       |
|  | EGTA  | X       |        |         |
| <b>REDUCING AGENT</b>                                  | 1% (v/v) $\beta$ -Mercaptoethanol or  | X       | X      |         |
|  | 1-5 mM DTT  | X       | X      | X       |
| <b>PROTEASE INHIBITOR(S)</b>                           | <b>Common Protease Inhibitors</b>   | X       | X      | X       |
|  | Leupeptin (2-10 $\mu$ g/ml)   |         |        |         |
|  | 0.1-1 mM PMSF   |         |        |         |
|  | <b>Less Common Inhibitors</b>   | X       | X      | X       |
|  | 25 mg/ml Aprotinin  |         |        |         |
|  | 10 mM Benzamidine   |         |        |         |
| Chymostatin  |   |         |        |         |
| 10 $\mu$ g/ml Pepstatin A                              |   |         |        |         |
| 10 $\mu$ g/ml STI                                      |   |         |        |         |
| 0.1 mM TLCK  |   |         |        |         |
| (N $\alpha$ -p-tosyl-L-<br>lysine chloromethyl ketone) |   |         |        |         |
| <b>PHOSPHATASE INHIBITORS</b>                          | 5-50 mM Fluoride or   | X       |        | X       |
|  | 0.05-0.5 mM Na Vanadate   | X       | X      |         |
|  | Na Pervanadate  |         | X      |         |
|  | Na Orthovanadate  |         |        | X       |
|  | 0.01-1 $\mu$ M Okadaic acid or<br>20-200 nM Microcystin                                 |         | X<br>X |         |
| <b>DETERGENTS</b>                                      | Triton® X-100 or  |         | X      | X       |
|  | NP-40   |         | X      | X       |
|  | Tween®  |         | X      |         |
| <b>DIVALENT METAL IONS</b>                             | Mg <sup>+2</sup> or   | X       |        | X       |
|  | Zn <sup>+2</sup>  |         | X      |         |
|  | Mn <sup>+2</sup>  |         |        | X       |
| <b>SALTS</b>   | 100-200 mM NaCl or  |         | X      | X       |
|  | KCl   |         |        |         |
| <b>GLYCEROL*</b>                                       | 10% Glycerol  | X       | X      | X       |

\* found in kinase storage buffer formulations

**Notes on Fractionation of the Lysed Tissue and Cell Samples:** Following tissue or cell membrane disruption, the sample is centrifuged. The result is a soluble fraction or cytosolic fraction, as it is often described, and an insoluble particulate/membrane fraction. In the case of **PKA**, the soluble fraction is retained for further treatment and assay, the particulate fraction is discarded. The cytosolic fraction may be directly assayed for PKA activity. This fraction may also be purified further prior to submission to the assay.

Telephone 800-8-PIERCE or 815-968-0747

Fax 815-968-7316 or 800-842-5007

Both the cytosolic and insoluble particulate fractions may be retained for assay of PKC activity. The insoluble fraction pellet can be dissolved by addition of Triton® X-100 or Nonidet® P-40 to a concentration of 0.5-1% (v/v) in the homogenization buffer formulation. The supernatant of this step can be purified further by way of anion exchange chromatography prior to submission to the assay. A typical final concentration of these nonionic detergents when used in the assay or in storage buffers is 0.02% (v/v).

One important consideration in evaluating the level of PKC activity in a crude cell or tissue extract involves assessing the differential localization of PKC activity to the particulate fraction. This generally reflects the activated PKC isoforms, versus its presence in the soluble fraction, which reflects unactivated PKC isoforms.<sup>1,2,5,6</sup> Although optimal conditions for cell lysis and fractionation need to be determined for each tissue and/or cell type, initial approaches can take advantage of the observation that the activated, membrane associated PKC isoforms are usually chelator-resistant in their membrane binding and require high detergent concentrations for extraction, whereas the unactivated PKC forms are loosely associated (if at all) with cellular membranes and can be readily extracted upon treatment with low detergent levels (0.1% Triton®) or by Ca<sup>+2</sup> chelation with EDTA or EGTA.<sup>5,6</sup>

Given that PTKs are known to associate with most cellular structures, an analysis of their kinase activity can be performed using a broad range of preparations including cell extracts and plasma membrane, soluble (cytosolic) and cytoskeletal fractions. Because of the large number of tyrosine kinases that may be found in a typical cell, it is often best to fractionate the cell into membrane, cytosolic and cytoskeletal preparations prior to analysis of enzyme activity. This will attenuate the contribution of other competing kinases and simplify data evaluation.

Cells that have been harvested for tyrosine kinase assay are typically pelleted at 200 x g for 5 minutes and lysed either via freeze-thawing, Dounce homogenization or sonication in an ice-cold isotonic buffer containing 5-10 mM HEPES (pH 7.4), detergent and other agents. Refer to the example sample protocols for more details on PTK fractionation.

#### IV. Example Sample Preparation Protocols

The following protocols are given only as examples of approaches to sample preparation prior to the assay of kinases from a few representative sources. Application of these protocols to your specific sample will serve as an excellent starting point. However, further protocol development may be necessary to achieve a preparation of optimal activity for your specific needs.

##### Protein Kinase A

Preparation from heart muscle: Finely chopped heart muscle is washed with ice-cold homogenization buffer [10 mM Potassium Phosphate, 1 mM EDTA, 0.1% Triton®-X100, 0.1 mM DTT, pH 6.8] and homogenized in a Waring Blender. Protease and phosphatase inhibitors are usually added by preference to the homogenization buffer. The ratio of buffer to tissue is typically 3:1 v/v. The homogenate is centrifuged at 20,000 x g for 30 minutes. The supernatant can be retained for assay of PKA activity.<sup>11</sup>

Further purification of the sample prior to assay can be achieved by use of a DEAE-cellulose column equilibrated with 55 mM Potassium Phosphate containing 1 mM EDTA and 0.1 mM DTT. The type II holoenzyme found in heart binds to the anion exchange material under these conditions. Recover the bound catalytic C subunit for assay by eluting with 45 mM Potassium Phosphate containing 0.1 mM DTT and 0.1 mM cAMP.

**Preparation from rabbit skeletal muscle:** For this tissue a typical Tris-HCl homogenization buffer is preferred e.g. [0.05 M Tris-HCl, pH 8.2 containing 4 mM EDTA and 6 mM 2-mercaptoethanol] in addition to suitable protease and phosphatase inhibitors. Centrifuge the homogenate at 20,000 x g for 30 minutes. Saturate the supernatant (soluble fraction) to 50% ammonium sulfate and centrifuge again at 20,000 x g. Dissolve the precipitate pellet in homogenization buffer and clarify by spinning at 65,000 x g for 3 hours. The clarified supernatant can be retained for assay or applied to a DEAE-cellulose column for partial purification. The column is equilibrated with 0.02 M Tris-HCl, pH 7.5, 6 mM 2-merc-apt ethanol. Elute the PKA for assay with a linear gradient from 0-0.4 M NaCl.<sup>12</sup>

**Preparation of recombinant catalytic subunit from *E. coli*:** 30 mM MES, pH 7.2 containing 50mM KCl, 1 mM EDTA and 5 mM mercaptoethanol is a buffer formulation compatible with the use of a French pressure cell to achieve lysis of bacterial cells. The lysate is centrifuged at 12,000 x g for 15 minutes. Phosphocellulose has been used as a chromatographic step in the preparation. Equilibrate with 30 mM MES, pH 6.45, containing 1 mM EDTA and elute with the same buffer adjusted to pH 7.0 and containing 0.5 M potassium phosphate.<sup>13</sup> Prior to assay, the concentration of phosphate must be reduced to 20 mM or less.

## Protein Kinase C

A typical starting procedure with freshly isolated tissues involves washing the tissue free of blood with ice-cold phosphate-buffered saline, Tris-buffered saline or homogenization buffer, followed by homogenization using a tissue blender and/or Dounce homogenizer.<sup>6-8</sup> A typical homogenization buffer would contain Tris-HCl (20-25 mM, pH 7.5), EGTA (0.5-4 mM), EDTA (2mM) and dithiothreitol (DTT) (1-5 mM). DTT appears to facilitate the activator dependency of PKC by preventing the oxidation of critical thiols. In addition, protease inhibitors such as PMSF (1mM) and leupeptin (10-20 µg/ml) are often also included.

Homogenized tissue is then separated into cytosolic (soluble) and particulate (insoluble) fractions by centrifugation. Typical conditions for centrifugation are 15,000 - 100,000 x g for 20-60 minutes. The supernatant can be directly assayed for soluble PKC activity. The membrane pellets obtained by centrifugation can be resuspended and the PKC solubilized in this fraction via sonication in ice-cold homogenization buffer containing 1% Triton® X-100.<sup>5,6</sup> The membrane extract is then usually centrifuged again (100,000 x g) to isolate and separate the supernatant containing the solubilized PKC from other insoluble cellular components such as cytoskeletal elements. This extract can then be assayed for PKC activity that was originally membrane-associated after further purification over a DEAE-cellulose anion exchange column.

When working with a cell line, which has the advantage of allowing for easy hormonal manipulation, the cells can be scraped into Ca<sup>+2</sup> free phosphate-buffered saline (145 mM NaCl/10mM sodium phosphate, pH 7.4) containing 2.5 mM EDTA and EGTA, followed by cell pelleting via centrifugation at 200 x g for 10 minutes. The pelleted cells can then be subjected to a similar sample preparation procedure as described above, except only Dounce homogenization is required, owing to the smaller cell numbers and ease of cell disruption versus tissue disruption.<sup>5,6</sup>

**Partial Purification of the Sample Prior to Assay:** The solubilized fractions are often subjected to chromatographic steps to further purify the crude extracts before an assay is conducted. The most common approach is the passage of the extract through a DEAE cellulose anion exchange column.<sup>6,7,9</sup> Other anion exchange materials have also been used successfully such as DEAE-Sephadex® and MonoQ™.<sup>6,7,9,10</sup> PKC activity is eluted using an increasing NaCl or KCl salt gradient. The composition of a typical equilibration buffer for this purification step is 20 mM Tris-HCl, pH 7.5 containing 1-2mM EDTA, 0.1-2mM DTT with/without 10% glycerol.

The column is washed with several volumes of the equilibration buffer to remove the unbound material. The PKC activity is eluted using a linear salt gradient in the range of 0.0-0.5M. More shallow gradients such as 0-0.3M have also been used.

The sample recovered from an anion exchange step can also be subjected to hydrophobic chromatography over phenyl-agarose.<sup>6,7,10</sup> The base buffer is similar to the equilibration buffer for anion exchange chromatography. During hydrophobic chromatography, the PKC activity is eluted via a linear gradient beginning with high-salt (non-phosphate) and diminishing to no salt. Starting salt concentrations are in the range of 1.5-0.6M.

**Special Notes and Precautions for PKC Sample Preparation:** In general, deep-freezing (dry-ice, liquid nitrogen) cell or tissue extracts leads to a diminution of total PKC activity. Accordingly, it is best to work with freshly isolated cells or tissues if possible.<sup>6,7</sup> In addition, when evaluating hormone effects on PKC activity (and membrane association) using cultured cells, a stronger response is generally seen if the cells are made quiescent (serum-starved) prior to hormone or phorbol ester stimulation. This allows for a greater proportion of the cellular PKC to be in the unactivated state before treatment.

## Concerns with general PKC assay conditions

### 1. The use of detergents to solubilize PKC from membrane fractions

In this case, it is important to sufficiently dilute the detergent present in the final assay in order to minimize any interference of the detergent with the various lipids, PKC and other assay factors. Nonionic detergent concentration prior to assay should be no higher than 0.02%.

### 2. Activator dependency of the cPKCs in a given sample

Here, it is best to assay the activity ± phospholipid (phosphatidyl serine) and have Ca<sup>+2</sup> present in all samples so as to eliminate any contribution from other Ca<sup>+2</sup>- regulated protein kinases (such as the calmodulin-dependent kinases) that are not members of the PKC family.

### 3. PKC substrate specificity

The choice of the protein/peptide substrate will influence which PKC isoforms can be readily determined. Although the isoforms have somewhat different substrate specificities, most of them will phosphorylate general substrates such as

peptides derived from glycogen synthase, myelin basic protein, the epidermal growth factor receptor and serine-substituted derivatives of the PKC pseudosubstrate region. However, it should be noted that certain substrates may bypass various activator dependencies. For example, arginine-rich proteins like protamine can be phosphorylated by cPKCs in the absence of  $\text{Ca}^{+2}$ , diacylglycerol and the phospholipid,<sup>6,8</sup> whereas other substrates, such as myelin basic protein, can be phosphorylated by the cPKCs in the absence of  $\text{Ca}^{+2}$  but still requires phospholipid.<sup>6</sup>

### Protein Tyrosine Kinase

To most clearly assess the activity of a particular kinase, it may be best to first partially isolate the protein (e.g. via immunoprecipitation). This is often not feasible, however. Thus, if possible, it is advantageous to treat quiescent (serum-starved) cells with a factor or condition known (or suspected) to activate a specific tyrosine kinase followed by cell fractionation. By using quiescent cells, the background signal will be minimized, the activation by a specific factor or condition will enhance the tyrosine kinase signal above the background activity, and the use of a specific cell fraction will further restrict which kinases are being assayed and will also lower the background signal.

It should also be noted that due to the use of detergents to prepare some of these cell extracts and cellular fractions for tyrosine kinase assays, it is important to sufficiently dilute the sample so that the detergent (Triton® X-100) level present in the final assay is less than 0.1% in order to minimize any interference of the detergent with the enzyme or other assay factors.<sup>6,10</sup>

Examples of protocols are given below for preparing (EGF receptor) for assay of tyrosine kinase activity in cell extracts, plasma membranes or cytoskeletal preparations isolated from the commonly used human A431 epidermoid carcinoma cells. These procedures are applicable to both the assay of receptor tyrosine kinases<sup>2-4,11,12</sup> and non-receptor tyrosine kinases. For general review, see the references on pages 11 and 12.<sup>1,5,11,12</sup>

**Cell Culture:** A431 cells are plated on 10 cm plastic tissue culture dishes at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup> in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and are grown for 3 days to approximately 80% confluence.<sup>7,8</sup> If the cells are to be treated with EGF before harvest, they can first be rinsed twice with DMEM containing 0.1% bovine serum albumin (DMEM/BSA), and then treated at 37° C for various times with or without (0.1-100 nM) EGF in DMEM/BSA. The medium is then removed and the cells are quickly washed with ice-cold DMEM. The cells can then be removed from the dishes with gentle agitation in phosphate-buffered saline (10mM  $\text{NaPO}_4$ ; pH 7.4, 145 mM NaCl) containing 2.5  $\mu\text{M}$  of both EDTA and EGTA (PBS-EDTA/EGTA).

**NOTE:** Alternatively, the EGF receptor can be activated by incubating the cell extracts or preparations described below with 1  $\mu\text{M}$  EGF for 20 minutes at room temperature or 45 minutes on ice. The EGF is prepared in PBS or PBS with leupeptin.

**Cell Extracts:** A431 (or other) cells that have been harvested as described above, can be pelleted at 200 x g for 5 minutes and lysed either via freeze-thawing, Dounce homogenization or sonication (3-4 short bursts, 10-second duration each, high power) in an ice cold isotonic buffer containing 5-10 mM HEPES (pH 7.4), 1% Triton® X-100 and various stabilizing agents, such as DTT (1mM), EDTA (1-5 mM), and protease inhibitors (10-20  $\mu\text{g}/\text{ml}$  leupeptin). After gentle mixing (10-30 min) of this solution on ice, the sample can be clarified by centrifugation at 10,000 x g for 10 min. and the supernatant used as the source of enzyme activity.<sup>7,8</sup>

**Preparation of Cell Membranes:** Membranes from A431 cells can be prepared in reasonable quantity using ten large (15 cm diameter) plates of near confluent cells.<sup>8,9</sup> The cells are washed three times with ice-cold  $\text{Ca}^{+2}$ -free phosphate-buffered saline containing 2.5 mM EDTA and EGTA and then lysed in a 20 mM HEPES, pH 7.4 buffer containing 1.5 mM  $\text{MgCl}_2$ , 2.5 mM EDTA and EGTA, 1 mM DTT, 1 mM PMSF, 0.1 unit/ml of aprotinin and 20  $\mu\text{g}/\text{ml}$  leupeptin. All procedures should be performed at 0-4°C to minimize degradation. The cells are then subjected to Dounce homogenization followed by sonication. Next, the homogenates are centrifuged at 1500 x g for 10 minutes and the supernatant is then centrifuged at 18,000 x g for 30 minutes. The final membrane pellet can be resuspended in a 20 mM HEPES, pH 7.4 buffer containing 1 mM DTT, 10  $\mu\text{g}/\text{ml}$  leupeptin and 1 mM EDTA. Aliquots can be stored at -70°C.

**Preparation of the Detergent-Insoluble Cytoskeleton:** A431 cells are pelleted at 200 x g for 5 min, resuspended to  $5 \times 10^5$  cells/ml in ice-cold lysis-buffer [25 mM HEPES, pH 7.4, 2 mM  $\text{MnCl}_2$ , 1mM PMSF, 10 $\mu\text{M}$   $\text{Na}_3\text{VO}_4$  and 0.15-1% Triton® X-100] and then incubated on ice for 10 minutes. The cell solution is mixed by inverting the tubes at approximately 30-second intervals during the incubation. The mixture is then centrifuged for 5 minutes at 15,000 x g. The supernatant is transferred to a separate tube and this fraction is defined as the detergent-soluble (cytosolic) and membrane-associated enzyme fraction.<sup>9,10</sup> The pellets can be resuspended in a volume of lysis-buffer equal to that removed as the detergent soluble fraction and this solution is defined as the detergent-insoluble or cytoskeletal fraction. To ensure a homogeneous suspension, all samples should be sonicated at low power, on ice, for five 1-second intervals.

Telephone 800-8-PIERCE or 815-968-0747

Fax 815-968-7316 or 800-842-5007

## V. Helpful Literature References

The references provided below contain many recently published papers describing protocols for kinase sample preparation. Each kinase has its own reference section. References cited in the preceding text under a specific kinase can be found in the respective literature section below. The information contained in these references will be helpful in guiding the analyst in developing a suitable protocol for his/her specific sample source.

### Protein Kinase A

- Taylor, S.S. (1989). cAMP-dependent Protein Kinase. Minireview. *J. Biol. Chem.* **264**, 8443-8446.
- Flockhart, D.A. and Corbin, J.D. (1982). Regulatory mechanisms in the control of protein kinases. *CRC Crit. Rev. Biochem.* **12**, 133-186.
- Corbin, J.D., Sugden, P.H., West, L., Flockhart, D.A., Lincoln, T.M. and McCarthy, D. (1978). Studies on the properties and mode of action of the purified regulatory subunit of bovine heart adenosine 3',5'-monophosphate-dependent protein kinase. *J. Biol. Chem.* **253**, 3997.
- Hofmann, F., Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1975). Comparison of adenosine 3',5'-monophosphate dependent protein kinases from rabbit skeletal and bovine heart muscle. *J. Biol. Chem.* **250**, 7795-7801.
- Kuo, J. F. and Greengard, P. (1969). Cyclic nucleotide-dependent protein kinase. Widespread occurrence of adenosine 3',5'-monophosphate-dependent protein kinase in various tissues and phyla of the animal kingdom. *Proc. Natl. Acad. Sci. USA* **64**, 1349.
- Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968). An adenosine 3',5'-monophosphate-dependent protein kinase from rabbit skeletal muscle. *J. Biol. Chem.* **243**, 3763.
- Shoji, S., Ericsson, L.H., Walsh, K.A., Fischer, E. H. and Titani, K. (1983). Amino acid sequence of the catalytic subunit of bovine type II adenosine cyclic 3,5'-phosphate dependent protein kinase. *Biochemistry* **22**, 3702-3709.
- Kumon, A., Nishiyama, K., Yamamura, H. and Nishizuka, Y. (1972). Multiplicity of adenosine 3',5'-monophosphate-dependent protein kinases from rat liver and mode of action of nucleoside 3',5'-monophosphate. *J. Biol. Chem.* **247**, 3726-3735.
- Yamamura, H., Kumon, A., Nishiyama, K., Takeda, M., and Nishizuka, Y. (1971). *Biochem Biophys. Res. Commun.* **45**, 1560.
- Hunter, T. (1987) A thousand and one protein kinases. *Cell* **50**, 823-829.
- Flockhart, D.A. and Corbin, J. D. (1984) Preparation of the catalytic subunit of c-AMP-dependent protein kinase. *Brain Receptor Methodologies (A)*, Academic Press, **209-215**.
- Yamamura, H., Nishiyama, K., Shimomura, R., and Nishizuka, Y. (1973). Comparison of catalytic units of muscle and liver adenosine 3',5'-monophosphate-dependent protein kinase. *Biochemistry* **12**, 856-862.
- Slice, L.W. and Taylor, S.S. (1989). Expression of the catalytic subunit of cAMP-dependent protein kinase in *Escherichia coli*. *J. Biol. Chem.* **264**, 20940-20946.

### Protein Kinase C

- Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607-614.
- Hug, H. and Sarre, T.F. (1993). Protein kinase C isoenzymes: divergence in signal transduction? *Biochemical J.* **291**, 329-343.
- Johannes, F-J, Prestle, J., Eis, S., Oberhagemann, P. and Pfizenmaier, K. (1994). PKC is a novel, atypical member of the protein kinase C family. *J. Biol. Chem.* **269**, 6140-6148.
- Selbie, L.A., Schmitz-Peiffer C, Sheng, Y. and Biden, T.J. (1993). Molecular cloning and characterization of PKC, an atypical isoform of protein kinase C derived from insulin-secreting cells. *J. Biol. Chem.* **268**, 24296-24302.
- Ha, K.-S. and Exton J.H. (1993). Differential translocation of protein kinase C isozymes by thrombin and platelet-derived growth factor. *J. Biol. Chem.* **268**, 10534-10539.
- Eband, R.M.(1994). In vitro assays of protein kinase C activity. *Anal. Biochem.* **218**, 241-247.
- Walton, G.M., Bertics, P.J., Hudson, L.G. Vedvick, T.S., and Gill, G.N. (1987). A three-step purification of protein kinase C: Characterization of the purified enzyme. *Anal. Biochem.* **161**, 425-437.
- Leventhal, P.S. and Bertics, P.J. (1993). Activation of protein kinase C by selective binding of arginine-rich polypeptides. *J. Biol. Chem.* **268**, 13906-13913.
- Mahoney, C.W., Azzi, A., and Huang, K-P. (1990). Effects of Suramin, an anti-human immunodeficiency virus reverse transcriptase agent, on protein kinase C. *J. Biol. Chem.* **265**, 5424-5428.
- Masmoudi, A, Labourdette, G., Mersel, M., Huang, F.L., Hujang, K-P., Vincendon and Malviya, A.N. (1989). Protein kinase C located in rat liver nuclei. Partial purification and biochemical and immunochemical characterization. *J. Biol. Chem.* **264**, 1172-1179.

### Protein Tyrosine Kinase

- Brickell, P.M. (1992). The p60src family of protein-tyrosine kinases: Structure, regulation and function. *Crit. Rev. Oncogen* **3**, 401-446.
- Carpenter G. and Cohen, S. (1990). Epidermal growth factor. *J. Biol. Chem.* **265**, 7709-7712.
- Carpenter, G. and Wahl, M.I. (1990). The EGF family. *Handbook of Exp. Pharmacol.* **95I**, 69-171.
- Ullrich, A., Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212.
- Hunter, T. and Cooper, J.A., (1985). Protein-tyrosine kinases. *Ann. Rev. Biochem.* **54**, 897-930.
- Weber, W., Bertics, P.J. and Gill, G.N. (1984). Immunoaffinity purification of the epidermal growth factor receptor. *J. Biol. Chem.* **259**, 14631-14636.
- Lin, P.H. Selinfreund, R., Wakshull, E. and Wharton, W. (1987). Rapid and efficient purification of plasma membrane from cultured cells: Characterization of epidermal growth factor binding. *Biochemistry* **26**, 731-736.
- Hubler, L. Leventhal, P.S. and Bertics, P.J. (1992). Alteration in the kinetic properties of the epidermal growth factor receptor protein tyrosine kinase by basic proteins. *Biochem J.* **281**, 107-114.
- Roy, L.M., Gittinger, C.K. and Landreth, G.E. (1989). Characterization of the epidermal growth factor receptor associated with cytoskeletons of A431 cells. *J. Cell Physiol.* **140**, 295-304.
- Gronowski, A.M. and Bertics, P.J. (1993). Evidence for the potentiation of EGF receptor tyrosine kinase activity by association with the detergent-insoluble cellular cytoskeleton: Analysis of intact and carboxy-terminally truncated receptors. *Endocrinology* **133**, 2838-2846.
- Hardie, D.G. (Ed.) (1993). Protein phosphorylation a practical approach. IRL Press.
- Hunter, T. and Sefton, B.M. (Eds.) (1991). *Methods in Enzymology.* **200**.

©Copyright Pierce Chemical Company 4/1995. Printed in U.S.A. Product #1600065

Telephone 800-8-PIERCE or 815-968-0747

Fax 815-968-7316 or 800-842-5007

