Phosphodiesterase [^{3}H]cAMP SPA enzyme assay

TRKQ 7090

Phosphodiesterase [^{3}H]cGMP SPA enzyme assay

TRKQ 7100

500 assays

STORAGE

Store at -15°C to -30°C

EXPIRATION

The expiry date is stated on the kit and will be at least 4 weeks from the date of dispatch.

Warning: For research use only.

Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

PerkinElmer, Inc.
Boston, MA 02118 USA
Phone: (800) 762-4000 or (+1) 203-925-4602
www.perkinelmer.com
For a complete listing of our global offices, visit www.perkinelmer.com/lasoffices

CONTENTS

COMPONENTS OF THE ASSAY SYSTEM

[^{3}H]cAMP tracer (supplied with TRKQ7090)
A solution of [2,8-^{3}H]adenosine 3',5'-cyclic phosphate in ethanol: water [1:1 (v/v)].
Concentration: 37MBq/ml (1mCi/ml), Specific activity 0.925-1.48TBq/mmol (25-40Ci/mmol).
Total volume supplied 40µl/1.48MBq/40µCi.
Store at -15°C to -30°C.

[^{3}H]cGMP tracer (supplied with TRKQ7100)
A solution of [8-^{3}H]guanosine 3',5' cyclic phosphate in ethanol: water [1:1(v/v)].
Concentration: 37MBq/ml (1mCi/ml), Specific activity: 185-555GBq/mmol (5-15Ci/mmol).
Total volume supplied 40µl/1.48MBq/40µCi.
Store at -15°C to -30°C.

Yttrium SPA PDE beads, lyophilized.
Approximately 500 mg of beads containing 18mM zinc sulfate.
Store at -15°C to -30°C.

10x PDE assay buffer.
5.5ml of assay buffer is supplied as a 10x concentrate, to be stored at -15°C to -30°C. This buffer contains;

- 500mM Tris/HCl pH7.5
- 83mM $MgCl_2$
- 17mM EGTA

SAFETY WARNING AND PRECAUTIONS

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

Caution: Radioactive material
Instructions relating to the handling, use, storage and disposal of radioactive materials.

1. Upon receipt, vials or ampoules containing radioactive material should be checked for contamination. All radioactive materials should be stored in specially designated areas and suitable shielding should be used where appropriate. Access to these areas should be restricted to authorized personnel only.
2. Radioactive material should be used by responsible persons only in authorized areas. Care should be taken to prevent ingestion or contact with skin or clothing. Protective clothing, such as laboratory overalls, safety glasses and gloves should be worn whenever radioactive materials are handled. Where this is appropriate, the operator should wear personal dosimeters to measure radiation dose to the body and fingers.
3. No smoking, drinking or eating should be allowed in areas where radioactive materials are used. Avoid action that could lead to the ingestion of radioactive materials, such as the pipetting of radioactive solutions by mouth.
4. Vials containing radioactive materials should not be touched by hand; wear suitable protective gloves as normal practice. Use forceps when handling vials containing 'hard' beta emitters such as phosphorus-32 or gamma emitting labeled compounds. Ampoules likely to contain volatile radioactive compounds should be opened only in a well ventilated fume cabinet.
5. Work should be carried out on a surface covered with absorbent material or in enamel trays of sufficient capacity to contain any spillage. Working areas should be monitored regularly.
6. Any spills of radioactive material should be cleaned immediately and all contaminated materials should be decontaminated or disposed of as radioactive waste via an authorized route. Contaminated surfaces should be washed with a suitable detergent to remove traces of radioactivity.
7. After use, all unused radioactive materials should be stored in specifically designated areas. Any radioactive product not required or any materials that have come into contact with radioactivity should be disposed of as radioactive waste via an authorized route.
8. Hands should be washed after using radioactive materials. Hands and clothing should be monitored before leaving the designated area, using appropriate instruments to ensure that no contamination has occurred. If radioactive contamination is detected, hands should be washed again and rechecked. Any contamination persisting on hands and clothing should be reported to the responsible person so that suitable remedial actions can be taken.
9. Certain national/international organizations and agencies consider it appropriate to have additional controls during pregnancy. Users should check local regulations. Most countries have legislation governing the handling, use, storage, disposal and transportation of radioactive materials. The instructions set out above complement local regulations or codes of practice. Such regulations may require that a person be nominated to oversee radiological protection. Users of radioactive products must make themselves aware of and observe the local regulations or codes of practice which relate to such matters.

Warning: Contains yttrium silicate, ethanol and zinc sulfate.

DESCRIPTION

- **Scintillation proximity assay (SPA)**
- **Measures either cAMP or cGMP dependent PDE isoenzymes**
- **Homogeneous assay, performed in microplates or tubes**
- **Suitable for high throughput screening**
- **Ready to use buffer**
- **[^{3}H] tracer included**

The assay concept from is based on the observation that linear nucleotides bind preferentially to SPA yttrium silicate beads compared to cyclic nucleotides in the presence of zinc sulfate. Therefore, under optimized conditions, the product of the enzyme reaction binds directly to the SPA beads, and the enzyme substrate will not.
The binding of the radiolabeled product to the bead brings the isotope into close enough proximity to allow radiation from the tritium to excite the scintillant within the bead. Any unbound radiolabel is not close enough to the scintillant to allow this energy transfer, so no signal is generated. In addition, as the cyclic substrate does not bind effectively to the bead, the background signal generated is very low.
A complex ion chelating mechanism enables the linear nucleotide to bind to the bead. Optimal concentrations of zinc sulfate enhance the binding and ensure robust and efficient capture. The addition of zinc sulfate in SPA bead suspension also has the inherent ability to terminate PDE reactions. (see figure 1)

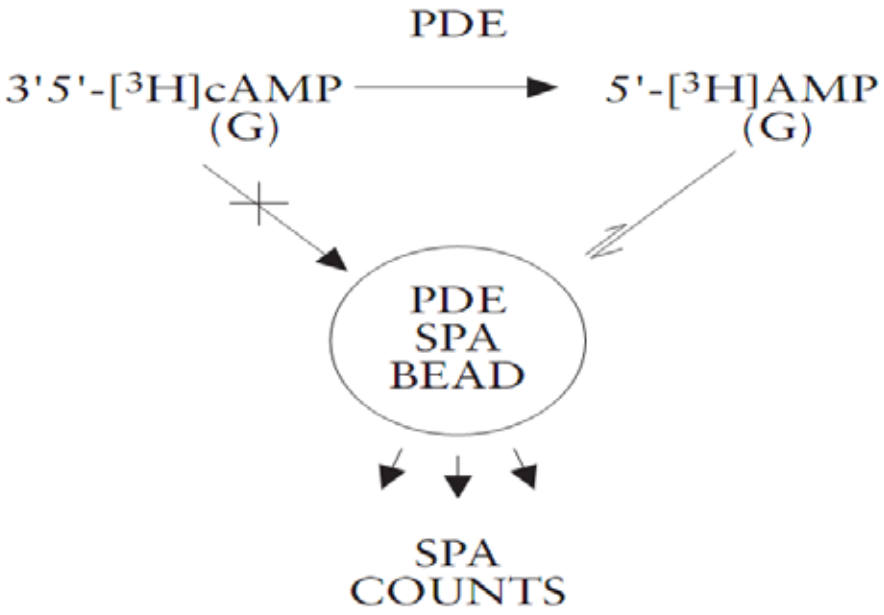

Figure 1. Diagrammatic representation of the PDE [^{3}H] SPA assay

CRITICAL PARAMETERS

The following points are critical.

- Use the recommended volume of water to reconstitute the beads. Using a different volume will alter the concentration of the bead/zinc sulfate suspension and affect assay performance.
- Ensure that the beads are uniformly suspended before use by inverting the vial several times and/or placing on a vortex mixer for a few seconds.
- The beads will settle completely after standing for **15 minutes**. During dispensing steps it is recommended that the beads are stirred continuously using a magnetic stirrer to ensure an even suspension.
- Variation on the recommended amount of bead added per assay well or tube may affect assay characteristics.
- The assay buffer supplied may not be optimal for all PDE isotypes.
- The total assay volume should **not exceed 100µl**. If greater than 10µl of either PDE or inhibitor are required reduce the quantity of water accordingly.
- Ensure suitable blanks and controls are included in all assays.
- After mixing, the samples should be allowed to stand for 20 minutes before being counted to allow for bead settling.
- Antimicrobial agents are not included in assay components. Users should be aware that contamination may occur when reconstituted beads are stored for long periods. If such agents are added, it remains the user's responsibility to assess the effects on the assay.

ADDITIONAL EQUIPMENT AND REAGENTS REQUIRED

The following equipment and reagents are required but not supplied:

- Phosphodiesterase enzyme
- Distilled or deionized water
- Pipettes or pipetting equipment with disposable polypropylene tips (100µl to 1ml)
- Disposable polypropylene tubes, microplates or trays
- A water bath or incubator capable of uniformly heating tubes or trays at 30°C
- Vortex mixer
- Microplate shaker
- Counting equipment

SYSTEM PROTOCOL

Reagent preparation

Note: Use distilled or deionized grade water for reconstitution and dilution of components.

Yttrium silicate beads

1. Reconstitute using 28ml of distilled or deionized grade water to give a final concentration of ~20mg/ml.
2. Ensure that a uniform suspension is obtained by inverting the vial several times and/or placing on a vortex mixer for a few seconds.

DO NOT FREEZE THIS REAGENT. Reconstituted SPA beads **must** be stored at 2-8°C.

10x PDE assay buffer

1. Supplied ready for use. Once thawed the buffer may be stored at 2-8°C for convenience.
2. The 10x buffer may be diluted with analytical grade water to provide a 1x solution, for use as an enzyme diluent.

The beads and buffer are stable for at least 4 weeks when stored at 2-8°C.

[^{3}H]cAMP/ [^{3}H]cGMP tracer

1. The [^{3}H] tracer requires diluting to a concentration of 1:200 using distilled or deionized water before use. This gives a dilution with a final concentration of 0.005µCi/µl.
2. Each assay requires 10µl of this diluted tracer, i.e. 0.05µCi is used per assay.

For example, 5µl of tracer diluted in 995µl of water is sufficient for 100 assays.
Note: Remember to include extra diluted tracer as needed for controls, or the estimation of total SPA counts.

PDE enzyme

1. The assay has been developed using human type IV PDE enzyme. It is recommended that the enzyme being used is diluted using **1x** PDE buffer and held on ice before use.

For example, dilute to a concentration of 0.05µg/µl in **1x** PDE buffer. Each assay uses 10µl of diluted enzyme, so the final concentration is 0.5µg PDE/100µl.

Assay protocol

The following protocol illustrates the assay procedure using microplates counted in a PerkinElmer MicroBeta^2™. Microplates compatible with the PerkinElmer TopCount® NXT can also be used, as can T-Trays™ compatible with the PerkinElmer 1205 Betaplate™.
Polypropylene screw cap tubes can also be used and counted using a LKB-Wallac 1209 Rackbeta™.

A general assay procedure is shown in **table 1.**

	Protocol	Notes
1	Prepare reagents as described in the 'reagent preparation' section.	Diluted enzyme, tracer and reconstituted beads are required before starting the assay. In addition an incubator set at 30°C will be needed.
2	Label as necessary appropriate plates.	
3	Into each well pipette: 60µl of water 10µl of 10x assay buffer 10µl of diluted PDE enzyme* 10µl of inhibitor sample 10µl of diluted tracer* 100µl total	*The assay can be started by adding diluted enzyme or tracer. If many wells are being assayed it will be more convenient to prepare a bulk solution of: Water/ buffer/ enzyme, or Water/ buffer/ tracer. This is then added as a combined single volume to replace pipetting steps and minimize error.
4	Incubate for a defined time at 30°C.	The assay is linear for at least 10 minutes. A time course held over 30 minutes sampling at 5 minute intervals is routinely applied to check assay performance.
5.	Terminate the reaction by adding 50µl of beads.	Ensure that the beads are uniformly in suspension before being used. The zinc sulfate present effectively terminates PDE activity.
6	Seal the plates and, using a plate shaker, shake well to redistribute the settled beads.	The SPA counts will remain stable for at least 56 hours after being counted.
7	Allow all plates to settle for 20 minutes before being counted.	
8	Count on a scintillation counter for an appropriate time.	See the section on 'counting'.

Table 1. Recommended assay protocol
The assay conditions used are outlined below:

	Control	**Sample**	**Blank**
10x assay buffer	10µl	10µl	10µl
PDE enzyme	10µl	10µl	-
Water	60µl	60µl	70µl
Inhibitor sample	-	10µl	-
Inhibitor diluent *	10µl	-	10µl
[^{3}H]cAMP or cGMP	10µl	10µl	10µl
Incubate for a defined time at 30°C			
SPA beads	50µl	50µl	50µl
Mix well and allow to stand at room temperature for 20 minutes			
Count tubes/trays/plates in a scintillation counter			

*for example, if the inhibitor is diluted in organic solvent, add the relevant solvent.

Enzyme parameters

Figure 2. [^{3}H]cAMP time course using human type IV

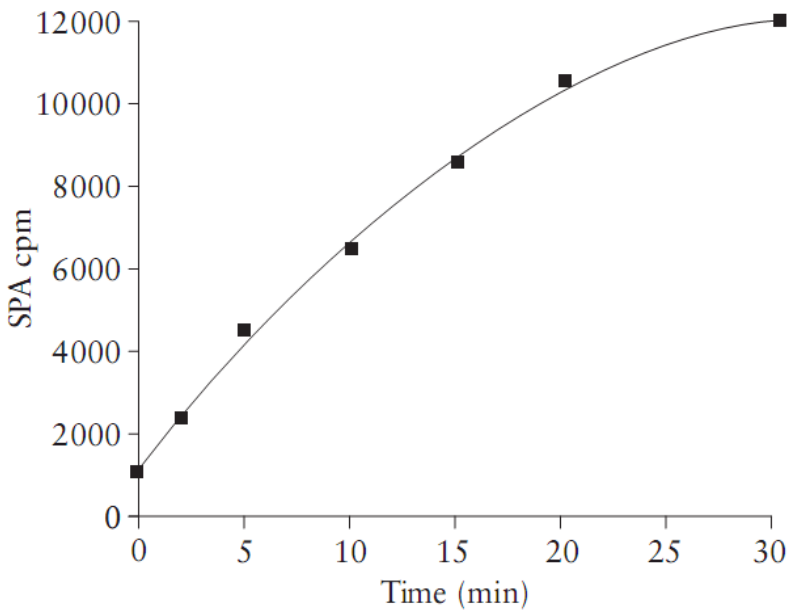

Figure 2 shows typical data obtained using human type IV PDE.

The time course was performed under conditions described in the protocol. The reaction was stopped at time intervals by the addition of 50µl (1mg) of SPA beads in zinc sulfate.

Figure 3. The dose dependent relationship of [^{3}H]AMP binding to SPA

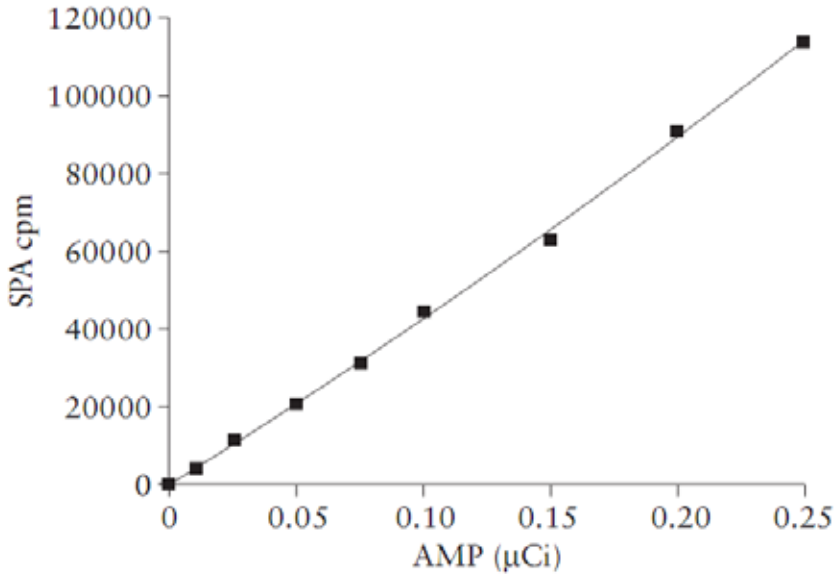

Figure 3 shows the dose dependant relationship of [^{3}H]AMP binding to SPA beads, under conditions described in the protocol. Binding of AMP is linear significantly above the level that could be produced in the assay.

Figure 4. Rolipram inhibition of human type IV PDE

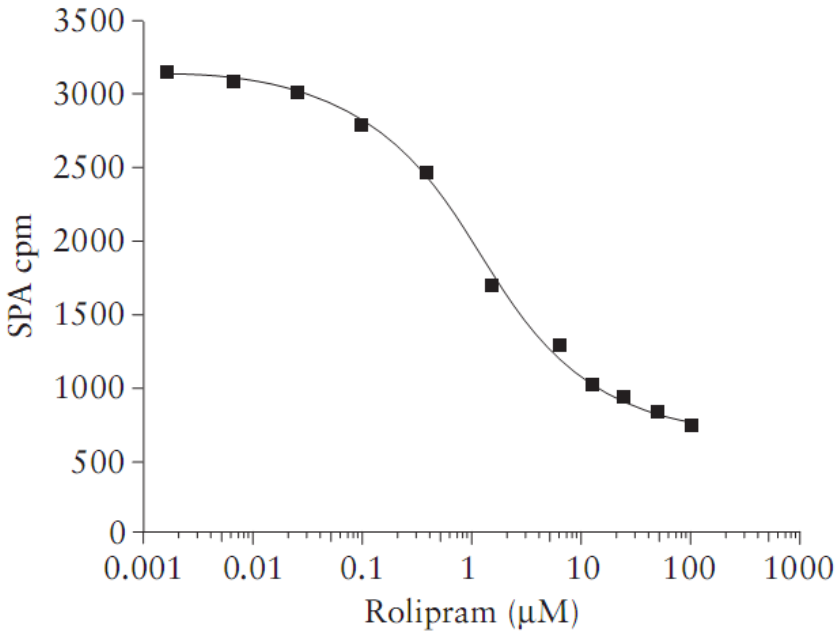

Figure 4 shows the inhibitor profile for the type IV PDE specific inhibitor, rolipram, using conditions described in the protocol. A classical inhibition curve was obtained with an estimated IC_{50} value of 1.8µM.

Figure 5. Dose-dependent hydrolysis of [^{3}H]3',5'-cAMP

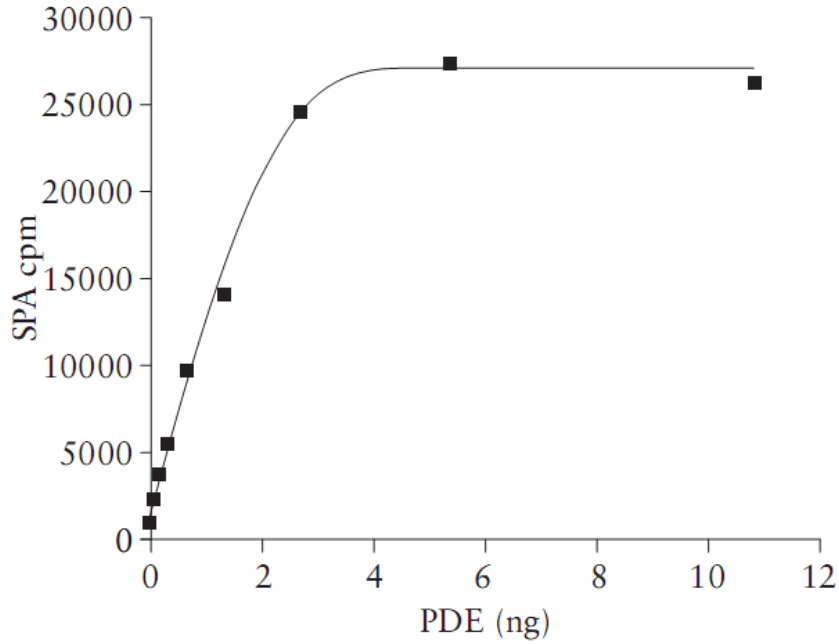

Figure 5 illustrates that the rate of hydrolysis is dependent on the amount of enzyme used. A linear range is observed before saturation is reached.

ADDITIONAL INFORMATION

The effect of various agents on assay performance

The enzyme assay is based on the selective binding of AMP/GMP over cAMP/cGMP by a complex binding mechanism. For this reason it is important that the user is aware of the effects of interfering substances on assay performance.

Table 2 shows the effect of a limited range of reagents.
The effect of using biological buffers such as Tris, HEPES, and MOPS on assay characteristics is pH and enzyme dependent, and should be tested thoroughly as part of any optimization studies.

Table 2. The effects of potentially interfering substances using PDE type IV enzyme.

Agent	Concentration	% of control signal
Potassium dihydrogen phosphate	20µM	94
Potassium chloride	20µM	99
Adenosine triphosphate	20µM	66
Theophylline	20µM	103
Tween™ 20	0.1%(w/v)	92
DMSO	5%(w/v)	102
Ethanol	5%(w/v)	102
Bovine serum albumin	0.33mg/ml	91
Glutathione	0.1mM	81
b-Mercaptoethanol	0.1mM	91
HEPES buffer pH8	50mM	50
MOPS buffer pH8.5	50mM	55
Tris buffer pH6.5	50mM	70
Tris buffer pH8	50mM	70

Counting

1. SPA PDE beads in general will yield approximately 65-70% of the cpm expected from conventional liquid scintillation counting.
2. Scintillant should not be added to the assay.
3. If assay tubes are used and the scintillation counter has removable racks, tubes can be loaded directly into the racks. Alternatively, assay tubes can be placed into standard liquid scintillation vials and loaded into the counter.
4. For counters fitted with spectrum analysis packages the suitable window opening should be determined and the machine set accordingly. Windows for other counters should be set wide open.
5. When researchers are using highly colored samples, color quench correction may be necessary. Please contact your local PerkinElmer representative for further information.

6. The following machines are compatible with SPA technology :
   1205 Betaplate, 96-well T-trays
   MicroBeta^2, VisiPlate or IsoPlate (96-well microplates)
   TopCount NXT, Optiplate or ProxiPlate (96-well microplates)
   Conventional scintillation counters

Please note: The SPA counts obtained will depend on the type of counter used and the absolute efficiency of the instrument.

TROUBLESHOOTING GUIDE

Problem	**Checks**
Low counts	Ÿ Check the amount of enzyme used Ÿ Check the incubation temperature and time Ÿ Check that the [^{3}H] tracer has been diluted correctly. Ÿ Total counts for a 1:200 dilution should range from 60,000 - 80,000 cpm Ÿ Check for poor direct product binding caused by an interfering substance
Poor curve shape/ reproducibility	Ÿ Check for uneven distribution of beads during pipetting steps Ÿ Check for uneven heat distribution in tubes/wells Ÿ Ensure that all reagents have been pipetted into the bottom of the tube/well. Tap if necessary Ÿ Check precision by including more replicates Ÿ Check pipette calibration

BACKGROUND

This novel assay has been developed for use in the detection of phosphodiesterase (PDE) inhibitors. The format of the assay system, which is dependent on the [^{3}H] cyclic nucleotide used, enables the product of the PDE reaction, either adenosine 5'-monophosphate (AMP) or guanosine 5'-monophosphate (GMP), to be detected in a direct and novel signal increase assay.
It is suitable for testing both cAMP and cGMP substrate dependent isoforms in a high throughput screening format. Conventional assay techniques in this field have used column chromatography[(1)] or selective precipitation[(2,3)] to isolate the product of the PDE reaction, and are not ideal for high throughput applications.
Currently at least seven different PDE families have been identified, namely PDE I-VII. These differ with respect to substrate specificity, tissue distribution, sensitivity to inhibitors and their regulatory characteristics[(4,5)]. Inhibition of phosphodiesterases results in elevated cellular levels of either cAMP or cGMP, both of which are involved in a wide variety of biochemical and physiological responses.
Interest in the potential of PDE isozymes as drug targets derives from their involvement in a multitude of cellular processes, and the discovery of isozyme-selective inhibitors. The restricted pattern of PDE isozyme expression in different tissue types and the involvement of individual isotypes with specific processes, make them particularly good candidates for inhibition. In particular, inhibitors of PDE IV[(6)] are receiving attention as novel anti-asthmatic agents due to their ability to attenuate the activation of inflammatory cells[(7)].

Reference

1. THOMPSON, W.J. *et al., Advances in Cyclic Nucleotide Research,* 10, pp.69-92, 1979.
2. CHAN, P.S. and LIN, M.C., *Methods in Enzymology*, 38, pp.38-41, 1974.
3. SCHILLING, R.J. *et al., Analytical Biochem.,* 216, pp.154-158, 1994.
4. BEAVO, J.A., *Advances in Second Messenger and Phosphoprotein Research,* 22, pp.1-38, 1988.
5. BEAVO, J.A., *Cyclic Nucleotide Phosphodiesterases: Stucture, Regulation and Drug Action,* pp.3-15, 1990.
6. TORPHY, T.J. *et al., New drugs in Allergy and Asthma,* pp.51-71, 1993.
7. GIEMBYCZ, M.A., *Biochem. Pharmacol.,* 43, pp.2041-2051, 1992.

RELATED PRODUCTS

PerkinElmer sells a wide range of enzyme and receptor SPA assays for pharmaceutical customers. Product specification documents (PSD) containing further details of each available assay can be obtained from your local PerkinElmer representative.