GE Healthcare

Amersham p42/p44 MAP kinase enzyme Biotrak assay system

Product Booklet

Code: RPN84

Page Finder

1. Legal

http//www.gehealthcare.com/lifesciences

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2. Handling

2.1. Safety warnings 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: For use with radioactive material

This product is to be used with radioactive material. Please follow the manufacturer's instructions relating to the handling, use, storage and disposal of such material.

Warning: Contains bioactive peptide.

The substrate mixture contains bioactive peptide related to sequences of the epidermal growth factor receptor. These sequences are believed to be essential for signal transduction by the receptor. The peptide in this kit may inhibit or block this transduction and interfere with normal cell growth and development. It may be active at very low concentration. The chemical and toxicological properties of this compound have not been fully investigated.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

Warning: Contains azide.

Some components contain sodium azide in dilute solution. Dispose of waste by flushing with copious amounts of water

to avoid the build up of explosive metallic azides in copper and lead plumbing. The total azide present in each pack is 1.3 mg.

2.2. Storage

Store at -15°C to -30°C.

2.3. Expiry

The expiry date is stated on the package and will normally be at least 4 weeks from the date of despatch.

3. Contents of the assay system

The assay system contains the following components.

1. Substrate buffer
A ready to use solution of peptide in a buffer containing Hepes, sodium orthovanadate, and 0.05% sodium azide, pH 7.4.

2. Magnesium/ATP buffer reagent
A ready to use optimized solution of ATP in a buffer containing HEPES and magnesium chloride, pH 7.4.

3. Stop reagent
A ready to use solution of orthophosphoric acid containing carmosine red. This component is not considered harmful under the Chemical Hazard Information and Packaging (CHIP) regulations, 1993, but is considered harmful under the OSHA regulations.

4. Peptide binding papers
Twenty sheets of peptide binding paper containing 12 assay discs (3.0 cm diameter) allowing 240 assays to be performed. Each disc is individually numbered.

4. Description

The BIOTRAK™ MAP kinase assay from GE Healthcare provides a simple and reliable means of detecting MAP kinase in a variety of samples. The assay system is based upon the p42/44 MAP kinase catalyzed transfer of the γ-phosphate group of adenosine-5'-triphosphate to a peptide which is highly selective for p42/44 MAP kinase. The assay sensitivity is at least 100 fmoles of purified enzyme and the linear range is between 0.9–27 pmoles Pi transferred per minute.

The reaction is initiated by the addition of [γ-^{32}P]ATP or [γ-^{33}P]ATP (not provided). Incubation proceeds for thirty minutes and the peptide is separated from unincorporated activity using a binding paper separation step. Each pack contains sufficient reagents for 240 assay tubes.

- Rapid and convenient assay employing optimized assay reaction buffers
- Specific peptide substrate
- Good linear range for quantitative measurement
- Minimal sample preparation
- Only 1 µCi phosphorus-32 per assay

To be used in conjunction with adenosine-5'-[γ-^{32}P] or [γ-^{33}P] triphosphate (not provided), available from GE Healthcare as:

PB168	[γ-^{32}P]ATP
AA0068	[γ-^{32}P]ATP RedivueTM (2–8°C storage)
BF1000	[γ-^{33}P] ATP
AH9968	[γ-^{33}P] ATP Redivue (2–8°C storage)

5. Introduction

A key question in eukaryotic intracellular signal transduction is how tyrosine kinase activity of ligand bound receptors (eg. the EGF receptor) is relayed to the final targets as a serine or threonine phosphorylation (1).

In 1984, Cooper (2) noted that a cytoplasmic protein of 42 kDa mass became rapidly phosphorylated at tyrosine residues upon ligand binding to growth factor receptors. This protein was shown to account for the majority of cellular tyrosine phosphorylation. It was predicted that isolation and characterization of this protein would answer some of the key questions regarding intracellular signal transduction, and this has proved to be the case. The 42 kDa protein was designated myelin basic protein kinase (after the substrate used to assay it), mitogen activated protein kinase (MAP kinase, after one of the activating mechanisms), or microtubule associated protein 2 kinase (MAP2 kinase). The variety of nomenclature has led to some confusion which has prompted Boulton and Cobb to propose the name extracellular signal regulated kinase (ERK) to describe these enzymes and to accommodate the known wide variety of cellular stimuli that activate these enzymes (3).

MAP kinases (ERKs) are now known to be a family of serine/threonine kinases which are activated by threonine and tyrosine phosphorylation by MAP kinase kinase in an unexpectedly complex chain of events leading from the activated receptor(4). Proteins of 42 kDa, 44 kDa and 54 kDa have been isolated and characterized. All show very similar substrate specificity and activation mechanisms but their individual roles still await elucidation (3).

Recent research has shown that p42 and p44 MAP kinase (ERK2 and ERK1) are activated very rapidly by a range of cellular stimuli, in particular growth factors. Increased enzyme is detected within 1 to 10 minutes of cellular stimulation and the activity usually decreases to a

basal level within 60 minutes (5). The enzymes are also characterized by their elution profiles on high resolution anion exchange columns, a technique which has greatly facilitated the understanding of MAP kinase activation and identification of MAP kinase activators (eg MAP kinase kinase) (6, 7).

MAP kinases occupy a central position in the relay of signals from tyrosine kinases to serine/threonine kinase activity and are directly involved in the regulation of transcription factor activity by phosphorylation (8). Stimuli known to activate MAP kinases include growth factors (EGF, PDGF, NGF and others) (9), tumour promotors (phorbol 12-myristate 13-acetate) (10), antigen binding to T cell receptors (11), infection of cells by Salmonella sp. (12), and okadaic acid and other phosphatase inhibitors (13). MAP kinase is also involved in cell cycle control and becomes activated during M-phase of the cell cycle; an involvement in oocyte maturation has also been demonstrated (14, 15).

To assay for p42/p44 MAP kinase activity the protein substrates myelin basic protein and microtubule associated protein 2 are commonly used either in a phosphocellulose binding paper assay format (16) or by use of an 'in gel' format (17). These assays can be expensive, time consuming and utilize large amounts of phosphorus-32 radiolabel. GE Healthcare's p42/p44 MAP kinase assay uses a synthetic peptide substrate that is highly selective for p42/p44 MAP kinase and uses only 1 µCi of [^{32}P]ATP per assay tube. The assay is linear over a wide range of enzyme concentration and up to 10% incorporation of radiolabel. The assay is sufficiently sensitive to detect p42/p44 MAP kinase activity using 100 fmol of purified enzyme.

Studies on MAP kinase activation and regulation will lead to an improved understanding of intracellular signal transduction and involvement of this enzyme in key signalling processes. The p42/p44 MAP kinase kit from GE Healthcare provides a convenient and rapid assay format with which to perform many of these studies.

5.1. Peptide specificity

p42/p44 MAP kinase recognizes the phosphorylation sequence PLS/TP as a site for phosphorylation (18). The peptide used in this assay contains this sequence and no other phosphorylation sites; the cell cycle dependent enzyme p34cdc2 prefers the sequence (basic or polar)S/TP(polar)(basic) (19). It is observed, therefore, that *in vitro* p34cdc2 kinase will phosphorylate substrates that contain the p42/p44 MAP kinase phosphorylation site. The peptide used in this assay is based on the Thr^{669} phosphorylation site of the EGFr and has been modified to contain only one phosphorylation site. Our experiments show that the peptide used in this kit, although not totally specific for p42/p44 MAPK, is much more specific for p42/p44 MAPK than the commonly used substrate (myelin basic protein) when comparing p42/p44 MAPK and p34cdc2 kinase activities. In addition, myelin basic protein contains other phosphorylation sites that are phosphorylated by other kinases (eg. protein kinase C and protein kinase A (21)), whilst the peptide used in this kit contains only one phosphorylation site. Thus this assay has significant advantages in substrate specificity compared to the commonly used substrate myelin basic protein.

Many experiments performed to study p42/p44 MAPK use cells that are quiescent; in such cases the level of active p34cdc2 is minimal as p34cdc2 shows a highly regulated cell cycle dependence on activity, with activity only at the G2/M phase transition. In this situation it is not necessary to perform a control for p34cdc2 activity. However if cells or tissue samples are used that contain non-quiescent cells then we recommend that a control assay for p34cdc2 activity be performed. GE Healthcare's p34cdc2 kinase assay (RPN 83) uses a totally specific peptide for p34cdc2 (20) and this may be used as a control system if required.

6. Summary of the assay

The system is designed to detect p42/p44 MAP kinase in lysed tissues, cells and column fractions, etc. Enzyme present in the samples will catalyze the transfer of the γ-phosphate of adenosine-5′-triphosphate to the threonine group on a peptide which is highly selective for p42/p44 MAP kinase.

The assay is performed at pH 7.4 in an optimized HEPES buffer with Mg^{2+} as the essential metal ion. The optimum assay temperature has been determined to be 37°C for HeLa cell p42/p44 MAPK. The assay will give linear incorporation of phosphorus-32 into substrate peptide corresponding to at least 10% ATP incorporated, provided samples are suitably diluted.

Phosphorylated peptide is separated from unincorporated label on binding paper. After washing the paper the extent of phosphorylation may be detected by scintillation counting.

$$[\text{Peptide}] + [\gamma\text{-}^{32}\text{P}]\text{ATP} \xrightarrow[Mg^{2+}]{\text{MAPK}} [^{32}\text{P}]\text{ peptide} + \text{ADP}$$

⇓

Apply to paper

⇓

Wash then count

7. Assay methodology

Users are recommended to read this entire section before starting work

7.1. Materials and equipment required

The following materials and equipment are required but not provided:

- Adjustable pipettes with disposable polypropylene tips capable of pipetting in the range 1–1000 µl.
- Disposable polypropylene or polystyrene tubes (0.5–1.5 ml capacity). Suitable tubes are Eppendorf polypropylene microfuge tubes.
- Water bath
- Benchtop microfuge appropriate for centrifuging Eppendorf tubes.
- Dishes or trays suitable for washing binding papers.
- 5000 ml of 75 mM orthophosphoric acid or 1% (v/v) acetic acid (wash reagent) for washing binding papers (240 assays).
- 20 ml scintillation counting vials.
- Scintillation counter suitable for phosphorus-32 counting.
- Liquid scintillation cocktail (see complete cocktail mix from GE Healthcare) and dispenser.

7.2. Sample preparation

Cell samples from tissue culture should be lysed and homogenized in a buffer containing protease and phosphatase inhibitors and all operations should be performed on ice. Researchers are free to use their own lysis regimes, however a recommended procedure is as follows.

Cells may be lysed in 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin (CB),

10 µg/ml aprotinin, pH 7.4 measured at 4°C. Cellular debris should be precipitated at 25 000 x g for 20 minutes and the supernatant retained to obtain cytoplasmic MAP kinase. The enzyme preparation can be frozen at -80°C or assayed immediately if kept on ice. MAP kinase is not stable if freeze/thawed and has limited stability at -15°C to -30°C.

Samples should be diluted and assayed as described. Calculations of total apparent p42/p44 MAP kinase activity may be obtained from consideration of the dilution factor and the extent of phosphorylation using the formula described on page 17.

Apparent low values obtained for concentrated samples may be due to interfering effects of some reagents used for sample preparation and competing enzyme activities in the sample (ATP hydrolyzing and proteolytic enzymes). Dilution may reduce or obviate these effects.

7.3. Incubation conditions

The assay is designed to measure the incorporation of phosphorus-32 into peptide at 30°C for a period of 30 minutes. The assay is linear to incorporation of up to 10% of total ATP. Further incubation or incorporation may be non-linear and may therefore not be a true indication of the MAP kinase levels in the sample.

7.4. Reagent preparation

All assay components should be thawed at room temperature for around 20 minutes, before beginning the assay. Do not use extended thawing times. Once thawed mix thoroughly by swirling and inversion and place reagents on ice. Refreeze reagents once required quantities have been taken and store at -15°C to -30°C. The reagents supplied are stable for up to four weeks under these conditions.

7.5. Assay procedure

7.5.1. Preparation of magnesium [^{32}P]ATP buffer

The assay is designed to use a minimum of 37 kBq, 1.0 µCi, of [^{32}P]ATP per tube.

Dispense sufficient magnesium ATP buffer to perform the number of assays required. Add [^{32}P]ATP (code PB 168) to a concentration of 200 µCi/ml (PB 168 is supplied at an activity concentration of 2 mCi/ml). Each reaction requires 5 µl of magnesium [^{32}P]ATP buffer. It is advisable to prepare a small excess to allow for discrepancies in pipetting. The magnesium ATP buffer has been overdispensed by 10% to facilitate this and still ensure sufficient materials are present for at least 240 reactions. Keep the solution on ice and use as soon as possible. Dispose of any remaining in an appropriate manner. Do not refreeze for use at a later date.

Use a 5 µl aliquot to determine the radioactive concentration of the solution.

7.5.2. Assay protocol

The assay procedure is summarized in table 1. It is advisable to set up the reactions on ice.

Kinases, depending on their source, may exhibit different optimum reaction temperatures. We have found, for example, that for MAP kinase from HeLa cells the optimum reaction temperature is 37°C. Therefore, the most suitable incubation temperature may need to be determined for each application. In the absence of optimization data we recommend an incubation temperature of 30°C.

1. Prepare the reagents as described previously.

2. Label tubes (in duplicate or as appropriate) for samples and place in a rack.

3. Pipette 15 µl of sample or lysis buffer into each appropriate tube.

4. Pipette 10 µl of substrate buffer into each tube.

5. Start the reaction by adding 5 µl of magnesium [^{32}P]ATP buffer. Mix the contents of the tube. Microfuge for 15 seconds to wash all reagents into base of tube. Place tubes in water bath at 30°C.
6. Incubate for 30 minutes. During the incubation prepare the binding discs. Without removing discs from the sheet place the required number of discs in a dry tray suitable for washing the papers.
7. Terminate the reaction with 10 µl stop reagent.
8. Mix the terminated reaction. Microfuge tubes for 15 seconds to wash all reagents into base of the tube.
9. Separate phosphorylated peptide as described in the next section.

7.5.3. Separation procedure

Pipette 30 µl of terminated reaction mixture on to the centre of each paper disc. Wash the papers as follows:

1. Add 250 ml 75 mM orthophosphoric acid or 1% acetic acid to the wash tray containing the paper discs. Do not wash more than 2 sheets (24 discs) per wash tray. Rock the tray gently for 2 minutes. Decant wash reagent and dispose of as phosphorus-32 liquid waste. Add a similar volume of wash reagent and repeat once.
2. Add 250 ml of distilled water to each wash tray. Rock the tray gently for 2 minutes. Decant water and dispose of as phosphorus-32 liquid waste. Add a similar volume of water and repeat once.
3. Add 200 ml of water to the wash tray. Rock the tray gently to float paper discs. Using forceps remove individual paper discs from the paper sheet and place each disc in a 20 ml scintillation vial. Dispose of water as phosphorus-32 liquid waste and paper sheet as phosphorus-32 solid waste.
4. Add 10 ml liquid scintillation cocktail to each vial and count in an appropriate scintillation counter for phosphorus-32.

Table 1. Recommended assay procedure - all volumes in microlitres.

	sample	blank
Sample	15	-
Lysis buffer	-	15
Substrate buffer	10	10
Magnesium [^{32}P]ATP	5	5
⇓		
Mix centrifuge for 15 seconds at 14 000 rpm Incubate for 30 minutes		
⇓		
Stop reagent	10	10
⇓		
Centrifuge for 15 seconds at 14 000 rpm Aliquot 30 µl on to binding paper Wash papers and count		

7.5.4. Blanks and controls

Suitable blanks should always be carried out.

A no enzyme blank will correct for any non-specific effects of [^{32}P]ATP or its radiolytic decomposition products binding to papers.

A no peptide control may also be performed to assess the degree of endogenous protein phosphorylation.

If non-quiescent cells are used note the controls suggested in peptide specificity, page 6.

7.6. Calculation of results

The phosphorus-32 incorporated into the peptide is quantitatively measured by the binding papers. In the presence of enzyme the [^{32}P] counted on the papers is the sum of non-specific [^{32}P]ATP binding, specific binding of phosphorylated peptide and binding of

phosphorylated proteins in the cellular extract (A).

In the absence of enzyme the [^{32}P] counted on the papers is the non-specific binding of [^{32}P]ATP or its radiolytic decomposition products (B).

MAP kinase activity is therefore obtained from [A–B].

Calculation of specific activity (R) of 1.2 mM magnesium [^{32}P]ATP

$$\frac{\text{5 µl of 1.2 mM Mg[}^{32}\text{P]ATP contains 6 x 10}^{-9}\text{moles ATP}}{6}$$

R= cpm per 5 µl Mg[^{32}P]ATP cpm/nmole.

Calculation of total phosphate (T) transferred to peptide and endogenous proteins

30 µl spotted on to binding paper.
Total terminated volume 40 µl

T= [(A)–(B)] x 1.33

Calculation of pmoles phosphate (P) transferred per minute

$$P = \frac{T \times 1000}{I \times R} \text{ pmoles/minute}$$

where I = incubation time (minutes)

The data obtained by users will depend upon the type of experiment performed. Figure 1 shows a typical dose response using purified p42/p44 MAPK.

The data for this graph were obtained by scintillation counting. We have also found Cerenkov counting of [^{32}P] labelled samples useful in determining phosphorylation activity, provided that the total counts sample is treated in the same manner.

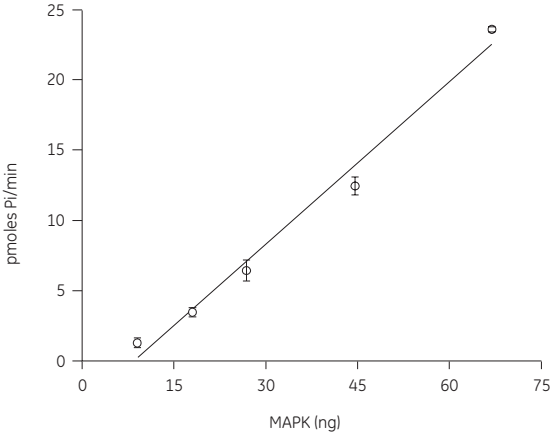

Figure 1. MAPK standard curve

8. Additional information

8.1. Alternative radiolabel for assay.

AH 9968 [γ-^{33}P]ATP, Redivue, was used in place of [γ-^{32}P]ATP (GE Healthcare PB168) as the radiolabel. A standard curve of enzyme titration was established using each label and the results compared. [γ-^{33}P]ATP may be used to directly substitute for [γ-^{32}P]ATP in the p42/p44 MAP kinase assay (figure 2).

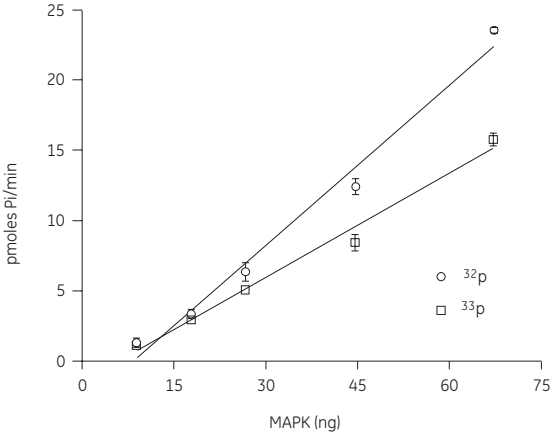

Figure 2. MAPK standard curves

8.2. Reproducibility

The within assay variation was determined by repeatedly measuring the activity of purified p42/p44 MAPK in a single experiment.

The between assay variation was determined from a total of 10 assays carried out by three different operators on purified p42/p44 MAPK. The data are summarized in table 2.

Table 2. Assay reproducibility

	pmoles phosphate/min.	sd	%CV	n
Within assay	8.6	0.99	11.6	10
Between assay	5.7	0.59	10.3	10

8.3. Specificity

The activity of a number of protein kinases assayed using optimum conditions for p42/p44 MAP kinase is shown in table 3. The specificity of the peptide used in this kit and myelin basic protein are compared.

Table 3. Assay specificity

Enzyme	pmoles phosphate/min.	
	Peptide substrate	MBP substrate
15 ng p44 MAPK	18.10	9.50
15 ng p34cdc2	0.62	3.21
15 ng PKC	0.01	0.60

On a mole to mole basis p44 MAPK will transfer one mole of phosphate to the peptide substrate in comparison to approximately 0.1 mol transferred by p34cdc2 using assay conditions optimized for p42/p44 MAPK.

9. References

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10. Related products

Biotrak assay systems in the signal transduction range

Kinase assay systems

p34cdc2 kinase enzyme assay system	EA	RPN83
ds DNA-activated protein kinase enzyme system	EA	RPN85
Casein kinase enzyme assay system	EA	RPN86
Protein kinase C enzyme assay system	EA	RPN77
EGF receptor tyrosine kinase enzyme assay system	EA	RPN78

Other assay systems in the range

Cyclic AMP	EIA	RPN225
Cyclic AMP, [^{3}H]	RR	TRK432
Cyclic AMP + Amprep columns, [^{3}H]	RR	TRK4329
Cyclic AMP, [^{125}I]	RIA/AM	RPA509
Cyclic AMP + Amprep columns, [^{125}I]	RIA/AM	RPA5099
Cyclic AMP, [^{125}I]	SPA	RPA538
Cyclic AMP, [^{125}I] (500 tubes)	SPA	RPA542
Cyclic GMP	EIA	RPN226
Cyclic GMP, [^{3}H]	RIA	TRK500
Cyclic GMP + Amprep columns, [^{3}H]	RIA	TRK5009
Cyclic GMP, [^{125}I]	RIA/AM	RPA525
Cyclic GMP + Amprep columns, [^{125}I]	RIA/AM	RPA5259
Cyclic GMP, [^{125}I]	SPA	RPA540
Cyclic GMP, [^{125}I] (500 tubes)	SPA	RPA541
sn-1,2-Diacylglycerol (DAG)	EA	RPN200
sn-1,2-Diacylglycerol (DAG) +	EA	RPN2009

Amprep columns		
D-myo-Inositol 1,4,5-trisphosphate (IP_3), [^{3}H]	RR	TRK1000
D-myo-Inositol 1,4,5-trisphosphate (IP_3) + Amprep columns, [^{3}H]	RR	TRK10009

Key:

AM	=	Amerlex-M separation	RIA	=	Radioimmunoassay
EA	=	Enzyme assay	RR	=	Radioreceptor assay
EIA	=	Enzymeimmunoassay	SPA	=	Scintillation proximity assay

[^{32}P] and [^{33}P] Nucleotides

(To be ordered separately for use with the kinase assay systems)

[γ-^{32}P]ATP	~3000 Ci/mmol		PB1688
[γ-^{32}P]ATP	~3000 Ci/mmol	Redivue formulation	AA0068
[γ-^{33}P]ATP	~1000–3000 Ci/mmol		BF1000
[γ-^{33}P]ATP	~1000–3000 Ci/mmol	Redivue formulation	AH9968

Pipettes and pipette tips

A comprehensive range of variable, fixed and multi-channel pipettes and disposable pipette tips.

Variable volume pipettes:		
0.5–10 µl	1 pipette	RPN2340
5–50 µl	1 pipette	RPN2341
50–200 µl	1 pipette	RPN2342
200–1000 µl	1 pipette	RPN2343
1–5 ml	1 pipette	RPN2344

Liquid scintillation cocktails

BCS	4 x 4 litres	(easy disposal)	NBCS104
PCS	4 x 4 litres	(maximum efficiency)	NPCS104

For a full range of safety accessories, please refer to the GE Healthcare catalogue.

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