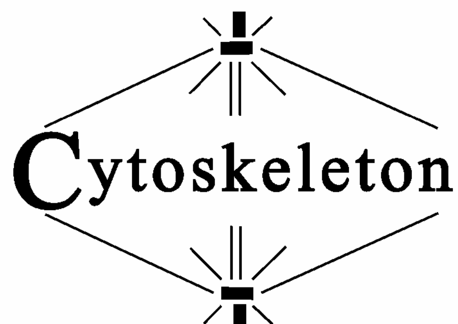

EasyRad Phosphate Assay Biochem Kit

(Cat. # BK055)

ORDERING INFORMATION

To order by phone: (303) - 322 - 2254

To order by Fax: (303) - 322 - 2257
To order by e-mail: cserve@cytoskeleton.com
Technical assistance: (303) - 322 - 2254
tservice@cytoskeleton.com
Visit the web page: www.cytoskeleton.com

EasyRad™ Phosphate Assay Biochem Kit (BK055)

The EasyRad™ Phosphate Assay Biochem Kit is an extremely quick and economical way to measure nucleoside triphosphate hydrolysis. There is minimal interference from other common ions and reagents used in molecular biology and biochemistry. This kit is designed to guide you through the process of measuring end-point and kinetic assays. There is special consideration for low activity ATPase and GTPase assays which can be performed easily with this kit. The kit is separated into six sections:

1) Overview and uses of the kit
2) Equipment and Materials required
3) Contents
4) Methods
5) Trouble-shooting
6) Associated products

1. Overview

The EasyRad™ Phosphate Assay is a simple two step assay based on the separation of $^{32 \text{ or } 33}$Phosphate from $^{32 \text{ or } 33\text{Gamma-}}$Phosphate-nucleoside triphosphate (NTP). This is the highest sensitivity phosphate assay available and it is capable of measuring Kcat as low as 0.00001 (NTPs hydrolysed per sec per molecule). The assay is based on a phosphate pre-loaded activated carbon which differentially binds NTPs rather than phosphate. The unparalleled sensitivity makes this assay a first choice for determining low activity ATPases and GTPases (i.e. Kcat between 0.00001 and 0.05). There are two formats which differ in their quantity of through put, the bench scale format is for up to 24 reactions which can be processed using a microfuge for sedimentation separation. Then there is the high through version which requires vacuum filtration for separation.

Uses:

1) To measure low activity ATPases or GTPases.

2) To measure phosphate release from other phosphatase catalyzed reactions.

Cytoskeleton Inc. Tel: 303-322-2254 Fax: 303-322-2257 E-mail: cserve@cytoskeleton.com
WWW.CYTOSKELETON.COM

2. Equipment and Materials required

1) Scintillation counter (bench scale), or 96-well scintillation counter (HTS).
2) 1.5ml tubes (bench scale) or 96-well filter plates (e.g. Whatman Cat# 7700-3308).
3) Pipettors 20,200 and 1000ul capacity (bench scale), or multichannel pipettor (HTS) wit wide bore tips (2mm) for 400ul pipetting of Separator Solution.
4) Microfuge (bench scale), or 96-well filtration device (HTS).
5) Ice supply.
6) Radioactive nucleotide, one of the following:
   i) ATP with gamma 33P e.g. Amersham Cat# BF1000
   ii) ATP with gamma 32P e.g. Amersham Cat# PB10168
   iii) GTP with gamma 33P e.g. Amersham Cat# not avail.
   iv) GTP with gamma 32P e.g. Amersham Cat# PB10244

3. Contents

1) 1 x 100ml Reaction Buffer (sealed). Store at 4°C.
2) 1 x 250ml Separator Solution (sealed). Store at 24°C. Shake before use.
3) 1 x 1ml ATP stock solution (100mM, cat# BSA04) Store at -70ºC.
4) 1 x 100ul GTP stock solution (100mM, cat# BST06) Store at -70ºC.
5) 1 x manual EasyRad™ Phosphate Assay Manual.

4. Methods

Preliminaries:

In order to set up your EasyRad™ Phosphate Assay you need to determine what format is best for you. There are end-point or kinetic assays, and high or low activity assays to choose from. Usually for bench scale operations where you are looking at a only a few variables, it is useful to start with a kinetic assay because there is more information to be obtained. If you are considering HTS then the endpoint reaction is useful. The assay is not disturbed by different concentrations of protein so it is useful to start at 1mg/ml or 0.1mg/ml protein initially, then depending on your results you can modify that variable later (see Appendix 1 for Assay Optimization Parameters).

The concentration of radionucleotide is a key factor in obtaining a sensitive assay, essentially you will be diluting radionucleotide with cold NTP to produce a solution that is high enough concentration so the enzyme still binds to the NTP but not too high because that will dilute the radionulceotide too much and cause very little difference in the signal. A good ball park to start is 10uM final concentration, so usually that means adding 10uM cold NTP and 100nM radionucleotide. However for very low activities you can use just radionucleotide at 1uM, the activity may be reduced in this scenario because the concentration of NTP is possibly well below the enzymes K_{aff} for NTP binding. For example tubulin polymerization requires >250uM GTP for hydrolysis. Finally it is worthwhile using ^{33}P if possible because you only lose two fold in sensitivity whereas the radioactive exposure is about 10 fold less.

Preparing solutions

1. Reconstitute the ATP (1ml) or GTP (100ul) with nanopure water and freeze in 100ul or 10ul aliquots respectively.
2. Prior to the assay defrost an aliquot and dilute 1000 fold with nanopure water to 100uM . Use 99ml water for the 100ul ATP aliquot, and 9.9ml for the 10ul GTP aliquot.

Endpoint assay

1. Label three tubes T = 0, T = 30, and Ref. Place on ice and pipette 400ul of Separator Solution (mix before pipetting and use a wide bore tip) into T = 0 and T = 30. Pipette 400ul of nanopure water into Ref.

2. Set up your Reaction Tube on ice with the following components to make 50ul final volume. Add any cofactors necessary (see Appendix 1) at this stage and adjust the volume of Reaction Buffer to make a final volume of 50ul.

i) 19-29ul of Reaction Buffer.
ii) 5-15ul of enzyme solution (final concentration 0.1 to 1.0mg/ml).
iii) 5ul of cold 100uM NTP solution.
iv) 1ul of radionucleotide and mix well.

2. Pipette 10ul of the Reaction Tube mixture into tubes T = 0 and Ref, and mix well and leave on ice.

3. Incubate your Reaction Tube at 37ºC for 30min in a covered water bath.

4. Pipette 10ul of the Reaction Tube post-incubation mixture into T = 30.

5. Microfuge T = 0 and T = 30 tubes for 10min at room temperature at 14,000rpm.

6. Carefully pipette off 200ul of supernatant directly into a scintillation vial containing 5ml of scintillant. Also pipette 200ul of Ref solution into 5ml of scintillant.

7. Count the cpm for 1min each, possibly the results will look like this :

i) T = 0	3,500 cpm
ii) T = 30	12,000 cpm
iii) Ref	50,000 cpm

This tells you that 12,000 – 3,500 = 8,500 cpm came from the enzyme's activity.

And that this corresponds to (8,500 / 50,000) x 10uM = 1.7uM NTP hydrolyzed during 30min.

If you had a 1mg/ml solution of protein (50Kdal) = 20uM protein concentration.

Then 1.7uM of NTP was hydrolyzed in 30min by 20uM protein.

Cytoskeleton Inc. Tel: 303-322-2254 Fax: 303-322-2257 E-mail: cserve@cytoskeleton.com

Then in 1min, 0.057uM of NTP is hydrolyzed by 20uM protein.

Then in 1sec, 0.00094uM of NTP is hydrolyzed by 20uM protein.

Then for 1 umole of protein (i.e. divide by 20), there is 0.000047 umoles of NTP hydrolyzed.

This result can be transposed directly to the Kcat,

therefore Kcat = 0.000047 moles NTP hydrolyzed per molecule of protein per sec.

This measurement of Kcat or V (velocity of the reaction, usually measured in nmoles NTP / min / mg protein) assumes a linear reaction from zero to 30min, this is not usually the case because of factors such as denaturation and substrate limitation. However this format is suitable for HTS applications when you have determined that the endpoint does lie with the linear portion of the reaction by using the kinetic assay described next.

Kinetic Assay

This assay has the same set-up as the Endpoint except that the reaction volume is twice as large and the tubes for collecting the time point samples are greater in number.

1. Label eight tubes 0, 1, 2, 5, 10, 20, 30, and Ref. Place on ice and pipette 400ul of Separator Solution (mix before pipetting and use a wide bore tip) into 0,1,2,5,10,20 and 30. Pipette 400ul of nanopure water into Ref.

2. Set up your Reaction Tube on ice with the following components to make 100ul final volume. Add any cofactors necessary (see Appendix 1) at this stage and adjust the volume of Reaction Buffer to make a final volume of 100ul.

i) 58-78ul of Reaction Buffer.
ii) 10-30ul of enzyme solution (final concentration 0.1 to 1.0mg/ml).
iii) 10ul of cold 100uM NTP solution.
iv) 2ul of radionucleotide and mix well.

2. Pipette 10ul of the Reaction Tube mixture into tubes T = 0 and Ref, and mix well and leave on ice.

3. Incubate your Reaction Tube at 37ºC for 30min in a covered water bath, and immediately start a timer counting from zero to 30 min.

4. At Time = 1min, pipette 10ul of the Reaction Tube mixture into tube 1, mix well and place on ice. Remember to keep the Reaction Tube in the waterbath at all times.

5. At Time = 2min, pipette 10ul of the Reaction Tube mixture into tube 2, mix well and place on ice. Remember to keep the Reaction Tube in the waterbath at all times.

6. At Time = 5min, pipette 10ul of the Reaction Tube mixture into tube 5, mix well and place on ice. Remember to keep the Reaction Tube in the waterbath at all times.

7. At Time = 10min, pipette 10ul of the Reaction Tube mixture into tube 10, mix well and place on ice. Remember to keep the Reaction Tube in the waterbath at all times.

8. At Time = 20min, pipette 10ul of the Reaction Tube mixture into tube 20, mix well and place on ice. Remember to keep the Reaction Tube in the waterbath at all times.

9. At Time = 30min, pipette 10ul of the Reaction Tube mixture into tube 30, mix well and place on ice. Remember to keep the Reaction Tube in the waterbath at all times.

10. Microfuge tubes 0,1,2,5,10,20 and 30 for 10min at room temperature at 14,000rpm.

11. Carefully pipette off 200ul of supernatant directly into pre-labeled scintillation vials containing 5ml of scintillant. Also pipette 200ul of Ref solution into 5ml of scintillant.

12. Count the cpm, and use the process in the “End Point Assay” protocol to calculate the Kcat or Vmax. The only difference is that Vmax can be calculated using software such as Sigma Plot, where the initial time points reveal more of the actual initial reaction rate. Using the rate in cpm/min calculate the uM change per sec (using the Ref value) and then use the same process in the “End Point Assay” protocol to calculate the Kcat or Vmax. Vmax is usually quoted as nmoles NTP / min / mg protein, whereas Kcat relates the rate to one molecule of protein.

High-throughput Applications

This application is essentially the same as the Endpoint Assay protocol except that you will be using a larger volume of reaction buffer and you may want to start the reaction with the addition of the NTP (hot and cold mixed together). Using the filter plates from Whatman (Cat# 7700-3308) it is possible to filter the reaction after incubation, rather than centrifuging, then measuring the filtrate for radioactivity. The absolute counts can be compared to the control reaction with or without an inhibitor.

Cytoskeleton Inc. Tel: 303-322-2254 Fax: 303-322-2257 E-mail: cserve@cytoskeleton.com

5. Trouble-shooting

Observation	Possible cause	Remedy
1. No increase in cpm over time of incubation.	1. Denatured protein. 2. Incorrect labeling of tubes. 3. Sample too dilute. 4. Reaction needs cofactor. 5. NTP is not a substrate.	1. Prepare another batch of intact protein. 2. Repeat experiment. 3. Use more concentrated protein. 4. See Appendix 1. 5. Try CTP, TTP or UTP or other substrate.
2. In the kinetic assay, plotted phosphate versus time is not linear	1. Enzyme is denaturing during assay. 2. Enzyme is hydrolyzing all the substrate.	1. Change buffer conditions to stabilize enzyme, try reducing agent DTT at 0.5mM and glycerol 5% v/v, or immobilize the protein to stabilize the enzyme. 2. Use less enzyme.
3. In the kinetic assay, the large number of samples is difficult to process quickly enough to reduce the background hydrolysis.	1. Separator Solution is hydrolyzing the NTP substrate.	1. Use a higher through-put method e.g. Multichannel pipettors and 96-well plates. 2. Perform a “buffer plus substrate” parallel reaction and subtract the background of this control from the master reaction’s measurements.
4 High initial cpm and little increase towards T=30min.	1. Radionucleotide is bad. 2. Interfering substances in reaction, e.g. 100mM guanidine or adenine ? 3. Early time points were not stopped on ice.	1. Purchase a different Lot #. 2. Test each component of your reaction in an endpoint assay and remove or replace the offending chemical, sometimes the preservative in the radionucletide can interfere with the reaction. 3. Use ice.

6. Associated Products

Advanced Protein Assay (0.25-10ug/ml reagent) ADV01
Precision Red Protein Assay (5-100ug/ml reagent) ADV02
PhosFree Phosphate One-Step Assay Kit (medium to high activity enzymes) BK054
PhosFree Realtime Phosphate Assay (medium to high activity enzymes) BK051
PhosFree Phosphate Assay (medium to high activity enzymes with high protein content) BK050

Appendix 1: Optimizating your reaction

Optimizations are best performed in sets of two parameters titrated against one another in a 6 x 6 matrix format. For example a good pair of parameters is ATP and Mg2+ or pH and Salt concentration. The following series of conditions should be tested for optimization:

Protein pair:
1. Protein concentration 0, 10ng/well, 30ng/well, 100ng/well, 300ng/well, 1000ng/well, and 3000ng/well
2. Protein cofactor 0, 10ng/well, 30ng/well, 100ng/well, 300ng/well, 1000ng/well and 3000ng/well.

ATP/covalent cation pair:
1. Magnesium chloride or calcium chloride 0, 10uM, 100uM, 1mM, 3mM 10mM 30mM.
2. ATP concentration 0, 10uM, 30uM, 100uM, 300uM, 1mM, 3mM.

Salt and pH pair:
1. Sodium chloride or potassium chloride 0, 25mM, 50mM, 75mM, 100mM, 200mM, 500mM.
2. pH use MES, PIPES, Tris at 500mM each and pH 50ml each at pH 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5.

Cytoskeleton Inc. Tel: 303-322-2254 Fax: 303-322-2257 E-mail: cserve@cytoskeleton.com
WWW.CYTOSKELETON.COM