Product Manual

BIOMOL® GREEN

Reagent for phosphate detection

Catalog #: BML-AK111

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Product Manual

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Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

INTRODUCTION

The *BIOMOL® GREEN Reagent* is a convenient 1-step reagent for measuring free-phosphate released during enzymatic phosphatase assays. It is a modification of the classic Malachite green reagent[1, 2] and it offers the following advantages:

- Non-Radioactive
- Convenient 1-Step Reagent -No Mixing!
- Long Shelf-Life > 6 Months at 4°C
- Excellent Sensitivity (See Below)
- Suitable For Microplate Assays (100 µL Volume) or Cuvette Assays (1 mL Volume)

Each *BIOMOL® GREEN Reagent* bottle includes 1 vial of phosphate standard (1 mL) suitable for several phosphate standard curves.

References:

1. B. Martin *et al. J. Biol. Chem.* 1985 **260** 14932
2. K.W. Harder *et al. Biochem. J.* 1994 **298** 395

MATERIALS SUPPLIED

BIOMOL® GREEN Reagent (BML-AK111-1000) or *(*BML-AK111-0250*)*	1000 mL or 250 mL
Phosphate Standard in dH_2O (BML-KI102) (800 µM in distilled water)	1 mL

Storage temp

STORAGE

Room Temperature. Long-term at +4°C. *Please note that BIOMOL® GREEN can be stored frozen without deleterious effects.*

***Note: Due to potential expansion during freezing, it is recommended that the contents be divided into smaller volumes prior to freezing.**

STABILITY

When used and stored as directed, the kit is stable until the expiration date indicated on the box.

ADDITIONAL MATERIALS NEEDED

- Microplate reader or visible wavelength spectrophotometer capable of measuring absorbance at 600 to 680 nm to ≥3-decimal accuracy. Although OD_{620nm} is used in the protocols and examples below, the colored phosphate complex formed in the reaction has a broad absorbance peak with a maximum at ~650 nm. With appropriate calibration with the Phosphate Standard, any wavelength in the 600 to 680 nm range may be used in the assay.
- ½-Volume or standard 96-well microplate (ENZO catalog no. ADI-80-2404) or 1 mL cuvette (plastic/glass/quartz)
- Pipetman capable of pipetting 5 µL -1 mL accurately
- Multi-channel pipettor capable of pipetting 100 µL (optional).

Important/ Warning

PRECAUTIONS

The BIOMOL® GREEN reagent is a highly sensitive phosphate detection solution. Free phosphate present on labware and in reagent solutions will greatly increase the background absorbance of the assay. This is detected visually as a change in color from yellow to green. Detergents used to clean labware may contain high levels of phosphate. Use caution by either rinsing labware with dH_2O or employ unused plasticware.

REAGENT PREPARATION

I. Microplate Assay (Linear Range: 0.03 nmol to 2 nmol):

1. Prepare 1:1 serial dilutions of phosphate standard (BML-KI102) plus a blank (50 µL per well). Dilutions of 40, 20, … 0.625 µM correspond to 2, 1, …0.031 nmol PO_4 (see Table 1):
   a. Dilute 800 µM phosphate standard (BML-KI102) 1/20 into the experimental assay buffer (e.g., 20 µL phosphate + 380 µL assay buffer).
   b. Add 100 µL of the 40 µM phosphate standard to sample #1 of microtiter-plate (1/2 volume or standard).
   c. Add 50 µL assay buffer to samples #2 through #8.
   d. Remove 50 µL from sample #1 and add it to sample #2.
   e. Mix thoroughly by pipetting up and down several times.
   f. Remove 50 µL from sample #2 and add it to sample #3.
   g. Mix thoroughly and repeat for samples #4-7. At sample #7, remove 50 µL and discard. DO NOT PROCEED TO sample #8 (blank).
2. Prepare experimental phosphatase samples in a total volume of 50 µL.

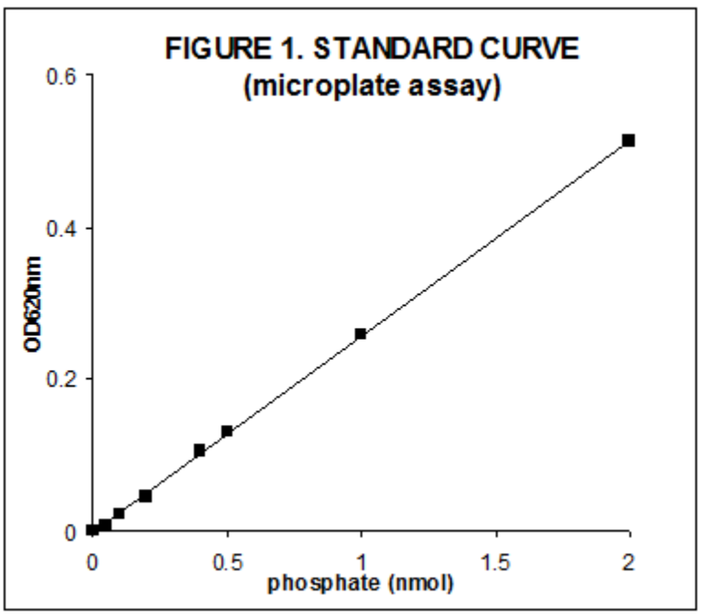
Sample #	†Well #	Phosphate (nmol)
1	A1,2	2
2	B1,2	1
3	C1,2	0.5
4	D1,2	0.25
5	E1,2	0.125
6	F1,2	0.063
7	G1,2	0.031
8	H1,2	0

† For highest accuracy, perform all samples in duplicate.

Table 1. Example of Microtiter Plate Standard Curve Samples

To Terminate Reaction:

1. Add 100 µL *BIOMOL® GREEN* Reagent (BML-AK111) to each well of the microtiter plate (Hint: Use a multichannel pipetman). The experiment should be performed such that all wells will be terminated at approximately the same time.
2. Incubate plate at room temperature for 20-30 minutes to allow development of the green color.
3. Measure OD_{620nm} on a microtiter-plate reader (see Figure 1, 1/2 volume microtiter-plate, below).
4. Perform data analysis (see below).

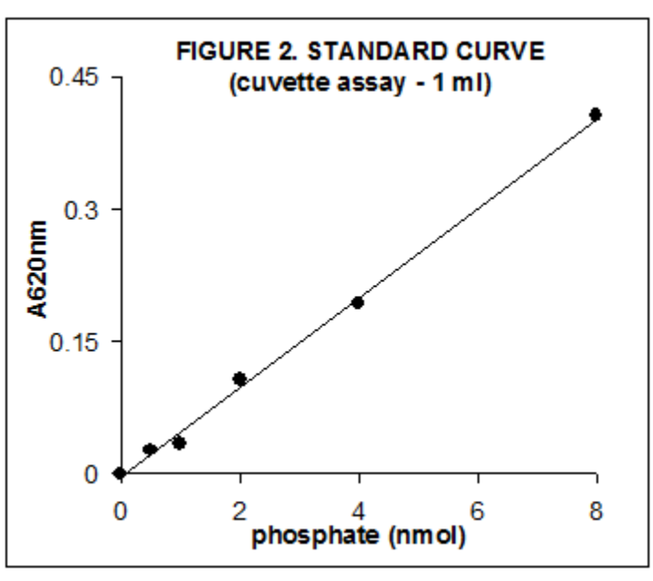


II. Cuvette Assay (Linear Range: 0.05 nmol to 8 nmol:

1. Prepare in tubes 1:1 serial dilutions of phosphate standard (BML-KI102) plus a blank (100 µL per tube). Dilutions of 80, 40, …5 µM correspond to 8, 4, …0.5 nmol PO_4 :
   a. Dilute 800µM phosphate standard (BML-KI102) 1/10 into the experimental assay buffer (e.g., 50 µL phosphate + 450 µL assay buffer).
   b. Add 200 µL of the 80 µM phosphate standard to sample #1.
   c. Add 100 µL assay buffer to samples #2 through #6.
   d. Remove 100 µL from sample #1 and add it to sample #2.
   e. Vortex thoroughly.
   f. Remove 100 µL from sample #2 and add it to sample #3.
   g. Vortex thoroughly and repeat for samples #4-5. At sample #5, remove 100 µL and discard. DO NOT PROCEED TO SAMPLE #6 (blank).
2. Prepare experimental phosphatase samples in a total volume of 100 µL.

To Terminate Reaction:

1. Add 1 mL *BIOMOL® GREEN* Reagent (BML-AK111) to each sample. The experiment should be performed such that all wells will be terminated at approximately the same time.
2. Incubate samples at room temperature for 20-30 minutes to allow development of the green color.
3. Measure OD_{620nm} using 1 mL cuvettes (plastic/glass/quartz) on a visible-wavelength spectrophotometer (see Figure 2, below).
4. Perform data analysis (see below).



DATA ANALYSIS

Phosphate (PO_4) Standard Curve:

NOTE: *For highest accuracy, a standard curve must be performed for each new set of assay data. This will normalize for variations in free phosphate in samples, time of incubation with the BIOMOL® GREEN reagent, and other experimental factors.*

1. Plot standard curve data as OD_{620nm} versus nmol PO_4 (see Figure 1 and 2).
2. Obtain a line-fit to the data using an appropriate routine.
3. Convert OD_{620nm} data into the amount of phosphate released using the standard curve line-fit data, from above:

$$\text{Phosphate released} = (OD_{620nm} - Y_{int})/\text{slope}$$

EXAMPLE:

Std curve slope=0.3 OD_{620nm}/nmol phosphate

Std curve Y_{int}=0.003 OD_{620nm}

Sample OD_{620nm}=0.4

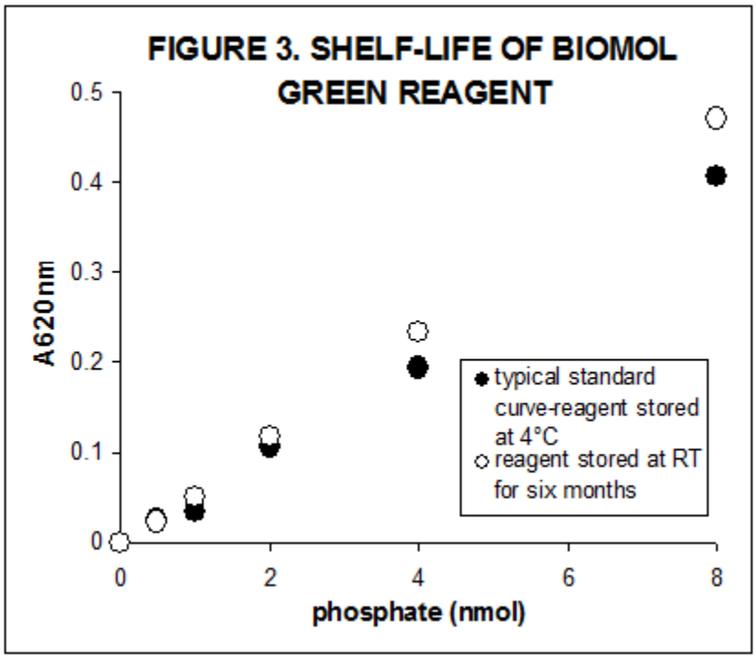
Phosphate released= (0.4-0.003)/0.3 = 1.32 nmol

Other Considerations:

1. The range and background of phosphate standard curves using the *BIOMOL® GREEN* Reagent may be affected by the composition of assay buffers employed. Therefore, phosphate standard curves should always be performed in a buffer identical to that of the experimental samples. The presence of bovine serum albumin (BSA) in an assay buffer can diminish the signal and/or delay color development. If BSA must be used, a concentration ≤0.1 mg/mL and a 60 min. incubation following addition of *BIOMOL® GREEN* are recommended. The detergents NP-40 (0.05%) and Brij 35 (0.01%) are compatible with the assay. Tween detergents can suppress color development, especially at lower phosphate concentrations, and are therefore not recommended.
2. Cell extracts or other samples containing extraneous free phosphate and/or interfering substances may be used if desalted prior to analysis. This can be accomplished by dialysis or by gel-filtration (Hint: Use spin-columns).
3. Suitable substrates for phosphatase assays include phosphopeptides, phosphorylated proteins and phosphoamino acids. The substrate must be matched to the target phosphatase of interest. The minimal amount of substrate used in the assay must consider the usable range defined by the phosphate standard curve (e.g., Figure 1 and 2). Typically, <10% of total substrate should be hydrolyzed during the assay to allow linear

kinetics. Thus, a general minimal concentration of substrate is 200µM for the microplate (50 µL assay volume) or cuvette assay (100 µL assay volume).

REAGENT STABILITY



NOTES

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