REFERENCES

APPENDIX
The following buffers\(^1\) did not interfere at concentrations ≤ 100 mM: Tris-HCl pH 9.0, HEPES pH 7.5, MOPS pH 7.0, Imidizole pH 7.0, and MES pH 5.0.

The following detergents and common reagents were tested for interference in the phosphate assay. The effects occurred at concentrations above those listed.

<table>
<thead>
<tr>
<th>Detergents(^2)</th>
<th>Level</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton(^2) X-100</td>
<td>0.3%</td>
<td>Increased Blank</td>
</tr>
<tr>
<td>Tween(^2) 20</td>
<td>0.1%</td>
<td>Reduced Sensitivity</td>
</tr>
<tr>
<td>NP-40 Alternative</td>
<td>1%</td>
<td>None</td>
</tr>
<tr>
<td>CHAPS</td>
<td>1%</td>
<td>None</td>
</tr>
<tr>
<td>SDS</td>
<td>≤ 0.01%</td>
<td>Increased Blank</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>≤ 0.01%</td>
<td>Precipitates, Increased Blank</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Common Reagents(^2)</th>
<th>Level</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>5%</td>
<td>Reduced Sensitivity</td>
</tr>
<tr>
<td>DMSO</td>
<td>10%</td>
<td>Reduced Sensitivity</td>
</tr>
<tr>
<td>Ethanol</td>
<td>25%</td>
<td>Reduced Sensitivity</td>
</tr>
<tr>
<td>BSA</td>
<td>0.05 mg/mL</td>
<td>Reduced Sensitivity</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
<td>None</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>3 mM</td>
<td>Reduced Sensitivity</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>10 mM</td>
<td>None</td>
</tr>
<tr>
<td>Na/OD</td>
<td>1 mM</td>
<td>Reduced Sensitivity</td>
</tr>
<tr>
<td>NaCl</td>
<td>100 mM</td>
<td>None</td>
</tr>
<tr>
<td>KCl</td>
<td>100 mM</td>
<td>None</td>
</tr>
<tr>
<td>CaCl(^2)</td>
<td>10 mM</td>
<td>None</td>
</tr>
</tbody>
</table>

\(^1\)Tested using the microplate assay protocol with or without 1 nmol phosphate (KH\(_2\)PO\(_4\)).

\(^2\)Tested using the microplate assay protocol in 25 mM Tris-HCl, pH 7.5, with or without 1 nmol phosphate (KH\(_2\)PO\(_4\)).
MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at room temperature. Do not use past kit expiration date.

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>PART #</th>
<th>DESCRIPTION</th>
<th>STORAGE OF OPENED MATERIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malachite Green Reagent A</td>
<td>89585</td>
<td>5 vials (3 mL/vial) of ammonium molybdate in 3 M sulfuric acid</td>
<td>Store at room temperature. *</td>
</tr>
<tr>
<td>Malachite Green Reagent B</td>
<td>89586</td>
<td>5 vials (3 mL/vial) of malachite green oxalate and polyvinyl alcohol</td>
<td>Store at room temperature for up to 6 months after initial use*</td>
</tr>
<tr>
<td>Phosphate Standard</td>
<td>892869</td>
<td>1 mL of 1 M phosphate (KH2PO4)</td>
<td></td>
</tr>
</tbody>
</table>

* Provided this is within the expiration date of the kit.

SOLUTIONS REQUIRED

Assay Buffer - The composition of the Assay Buffer will vary with the user’s application and must be optimized for each sample type. Refer to the Appendix for a list of tested interfering and compatible compounds. Prepare fresh buffer as needed.

MATERIALS REQUIRED

- Microplate reader or spectrophotometer capable of measuring absorbance at 620 nm.
- Microplate or spectrophotometer cuvettes.
- Eppendorf microcentrifuge tubes.
- Pipettes and pipette tips.

PRECAUTIONS

The Malachite Green Reagent A, Malachite Green Reagent B, and Phosphate Standard supplied with this kit are acidic solutions. Wear eye, hand, face, and clothing protection when using these materials.

MICROPLATE ASSAY PROTOCOL

**Note:** An initial 1:100 dilution of the 1M phosphate standard in Assay Buffer is needed before preparing the standard curve. To avoid precipitation of the standard, dilute it to 10 mM (1:100) with divalent cation-free buffer before making dilutions into buffers containing these ions.

1. Prepare a six point standard curve using 2-fold serial dilutions with a high standard of 100 μM. Dilutions should be made in Assay Buffer.
2. Add 50 μL of sample, phosphate standard or blank (Assay Buffer) per well. If desired, the assay volume may be increased by adding proportionately larger volumes of sample, phosphate standard, blank, Malachite Green Reagent A, and Malachite Green Reagent B.
3. Add 10 μL of Malachite Green Reagent A to each well. Mix thoroughly and incubate for 10 minutes at room temperature.
4. Add 10 μL of Malachite Green Reagent B to each well. Mix thoroughly and incubate for 20 minutes at room temperature.

**Note:** For best inter-assay consistency, read plates at a fixed time after adding Malachite Green Reagent B.

5. Determine the optical density of each well using a microplate reader set to 620 nm.

**Microplate Assay Phosphate Standard Dilution**

<table>
<thead>
<tr>
<th>Phosphate Standard</th>
<th>Phosphate (μM)</th>
<th>nmol/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>1.25</td>
</tr>
<tr>
<td>4</td>
<td>12.5</td>
<td>0.625</td>
</tr>
<tr>
<td>5</td>
<td>6.25</td>
<td>0.312</td>
</tr>
<tr>
<td>6</td>
<td>3.12</td>
<td>0.156</td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

OPTIONAL HIGH SENSITIVITY PROTOCOL

**Note:** When using high sensitivity protocol, the kit contains enough reagents to run 5 plates.

1. Prepare a six point standard curve using 2-fold serial dilutions with a high standard of 100 μM. Dilutions should be made in Assay Buffer.
2. Add 50 μL of sample, phosphate standard or blank (Assay Buffer) per well. If desired, the assay volume may be increased by adding proportionately larger volumes of sample, phosphate standard, blank, Malachite Green Reagent A, and Malachite Green Reagent B.
3. Add 30 μL of Malachite Green Reagent A to each well. Mix thoroughly.
4. Add 100 μL of distilled water to each well.
5. Add 30 μL of Malachite Green Reagent B to each well. Mix thoroughly and incubate for 20 minutes at room temperature.

**Note:** For best inter-assay consistency, read plates at a fixed time after adding Malachite Green Reagent B.

6. Determine the optical density of each well using a microplate reader set to 620 nm.

TECHNICAL HINTS & LIMITATIONS

- For best inter-assay consistency, read plates at a fixed time after adding Malachite Green Reagent B.
- It is recommended that all phosphate standards and samples be assayed in duplicate or triplicate.
- This Malachite Green Phosphate Detection Kit should not be used beyond the expiration date on the label.
- Malachite Green is a highly sensitive phosphate detection method. Many soaps and dish detergents contain phosphate and will leave a residue that will increase the background absorbance of the assay. Containers should be rinsed extensively with distilled or deionized water before use.
- Cell and tissue extracts/lysates also contain phosphates from the breakdown of nucleic acids, lipids, etc. Excessive free phosphate should be removed from samples to be analyzed. Two commonly used methods of removing phosphate from samples are desalting columns and immunoprecipitation capture of the phosphate-generating enzymes being studied.
- Divalent cations such as calcium, magnesium, and manganese form phosphate salts that have low water solubility.
- If a precipitate forms, check water purity, and consider using water from a different source.
- Sample dilution may be required because high concentrations of phosphate in the sample can cause precipitation.
- To reduce high assay background, dilution may be required with samples containing greater than 100 μM ATP.
- If color development is low, there may be insufficient phosphate present in the sample. Prepare several dilutions of the sample to determine optimal sample concentration for the assay.
- Malachite Green measures only inorganic, water-soluble phosphate. To measure protein- or lipid-bound phosphate, the samples must be hydrolyzed. This can be achieved by heating the samples with NaOH for proteins or perchloric acid for lipids. After hydrolysis, the samples must be neutralized before phosphate measurements are performed. A protocol example is listed below.

Protein Hydrolysis and Microplate Assay Protocol

- Add 25 μL of 4 M NaOH to 50 μL of sample.
- Heat at 100 °C for 30 minutes.
- Cool to room temperature and centrifuge briefly.
- Add 25 μL of 4 M HCl to the sample.
- Transfer the sample to a microplate well.
- Add 20 μL of Malachite Green Reagent A and incubate for 10 minutes at room temperature.
- Add 20 μL of Malachite Green Reagent B and incubate for 20 minutes at room temperature.
- Read the absorbance at 620 nm.

Lipid Hydrolysis and Microplate Assay Protocol

- See Reference # 4.