Instructions for use:

For accurate measurement of creatine kinase activity in a variety of biological samples.

This product is for research use only and is not intended for diagnostic use.
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1. BACKGROUND

Creatine Kinase Activity Assay kit (ab155901), creatine kinase converts creatine into phosphocreatine and ADP. The generated phosphocreatine and ADP reacts with CK Enzyme Mix to form an intermediate, which reduces a colorless Probe to a colored product with strong absorbance at 450 nm. This kit is high-throughput adaptable, simple and sensitive. This assay kit can detect Creatine Kinase activity less than 1 mU.

Creatine Kinase (CK) also known as creatine phosphokinase (CPK) and ATP: creatine N-phosphotransferase is a common cellular enzyme. It catalyzes the reversible conversion of creatine and ATP into ADP and phosphocreatine. CK is widely expressed in various tissues and cell types, with highest activity in striated muscles, heart tissue and brain. CK consists of two subunits: M (muscle) and B (brain), and has three isoenzymes: CK-MM (skeleton muscle), CK-MB (cardiac muscle), and CK-BB (brain). Increased CK level is associated with many diseases such as myocardial infarction, muscular dystrophy, pulmonary infarction and brain tumors. Accurate measurement of CK is crucial for early diagnosis, prediction and therapeutic strategy.
2. ASSAY SUMMARY

Standard curve preparation

Sample preparation

Add reaction mix

Measure optical density (OD450 nm) in a kinetic mode (T1)

Incubate at 37°C 20 - 40 minutes

Measure optical density (OD450 nm) in a kinetic mode (T2)
3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. STORAGE AND STABILITY

Store kit at -20ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**
5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK Assay Buffer</td>
<td>25 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>CK Substrate</td>
<td>1 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>ATP (Lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>CK Enzyme Mix (Lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>CK Developer (Lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>NADH Standard (Lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Positive Control (Lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at OD 450 nm
- MilliQ water or other type of double distilled water (ddH₂O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- Dounce homogenizer (if using tissue)
- (Optional) 10 kD Spin Columns (ab93349) – to remove small molecules from sample
8. TECHNICAL HINTS

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure plates are properly sealed or covered during incubation steps.

- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.

- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.

- Make sure you have the right type of plate for your detection method of choice.

- Make sure all necessary equipment is switched on and set at the appropriate temperature.
9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening

9.1. **CK Assay Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2. **CK Substrate:**

Ready to use as supplied. If necessary, warm in 37°C water bath to dissolve any precipitate, then vortex to mix thoroughly. Aliquot substrate so that you have enough to perform the desired number of assays. Store at -20°C.

9.3. **ATP:**

Reconstitute with 220 µL dH₂O. Pipette up and down to dissolve completely. Aliquot ATP so that you have enough to perform the desired number of assays. Store at -20°C. Use within 2 months.

9.4. **CK Enzyme Mix:**

Reconstitute with 220 µL CK Assay Buffer. Pipette up and down to dissolve completely. Aliquot enzyme mix so that you have enough to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw cycles. Keep on ice whilst in use.

9.5. **CK Developer:**

Reconstitute with 220 µL ddH₂O. Pipette up and down to dissolve completely. Aliquot developer so that you have enough to perform the desired number of assays. Store at -20°C. Use within 2 months.

9.6. **NADH Standard:**

Reconstitute with 50 µL CK Assay Buffer to generate 10 mM (10.0 nmol/µL) NADH Standard solution. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C. Keep on ice while in use.
9.7. **Positive Control:**

Reconstitute with 200 µL CK Assay Buffer to generate 10 mU/µL stock and mix thoroughly. Aliquot positive control so that you have enough to perform the desired number of assays. Store at -20°C.
10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be prepared immediately prior use. Do not store for future use.

10.1. Prepare 1mM of NADH Standard by adding 10 µL of 10 mM NADH Standard to 90 µL Assay Buffer.

10.2. Using 1 mM standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of NADH Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End Conc in well (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>144</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>138</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>132</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>126</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>120</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).
11. SAMPLE PREPARATION

General Sample Information

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1. Cell (adherent or suspension) samples:

11.1.1. Harvest the amount of cells necessary for each assay (initial recommendation = 2 x 10^6 cells).

11.1.2. Wash cells with cold PBS.

11.1.3. Resuspend cells in 100 µL ice cold CK Assay Buffer.

11.1.4. Homogenize cells quickly by pipetting up and down a few times.

11.1.5. Centrifuge sample for 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.1.6. Collect supernatant and transfer to a new tube.

11.1.7. Keep on ice.

11.2. Tissue samples:

11.2.1. Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).

11.2.2. Wash tissue in cold PBS.

11.2.3. Resuspend tissue in 100 µL of ice cold CK Assay Buffer.
11.2.4. Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.2.5. Centrifuge samples for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.2.6. Collect supernatant and transfer to a new tube.

11.2.7. Keep on ice.

Tissue samples (liver, for example) may contain small molecules such as ADP or NADH that generate background. Remove these molecules from sample by using a 10kD Spin Column (ab93349).

11.3. **Plasma and Serum samples:**

Plasma and serum samples can be tested directly by adding sample to the microplate wells.

**NOTE:** We suggest using different volumes of sample to ensure readings are within the Standard Curve range.
12. ASSAY PROCEDURE

- Equilibrate all materials and prepared reagents to correct temperature prior to use.
- We recommended to assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

12.1. Plate Loading:

Standard wells = 50 µL standard dilutions
Sample wells = 1 – 50 µL samples (adjust volume to 50 µL/well with CK Assay Buffer).
Sample Background control wells= 1 - 50 µL samples (adjust volume to 50 µL/well CK Assay Buffer).
Positive control = 2 - 10 µL Positive control (adjust volume to 50 µL/well with CK Assay Buffer).

12.2. Reaction Mix:

12.2.1. Prepare 50 µL of Reaction Mix for each reaction. Mix enough reagents for the number of assays (samples and controls) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:
X µL component x (Number reactions +1).

<table>
<thead>
<tr>
<th>Components</th>
<th>Reaction Mix (µL)</th>
<th>Background Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK Assay Buffer</td>
<td>34</td>
<td>36</td>
</tr>
<tr>
<td>CK Enzyme Mix</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>CK Developer</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ATP</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CK Substrate</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

12.2.2. Add 50 µL of Reaction Mix into each standard, sample and positive control sample wells. Mix well.

12.2.3. Add 50 µL of Background Reaction Mix to Background control sample wells.
12.2.4. Mix well.

12.3. **Plate Measurement:**

12.3.1. Measure output at OD 450 nm on a microplate reader in a kinetic mode, every 2 – 3 minutes, for at least 20 – 40 minutes at 37°C protected from light.

**NOTE:** Incubation time depends on the Creatine Kinase (CK) Activity in the samples. We recommend measuring OD in a kinetic mode, and choosing two time points (T1 and T2) in the linear range (OD values A1 and A2 respectively) to calculate the CK activity of the samples.

For standard curve, do not subtract A2 from A1. Standard curve can also be read in end point mode (i.e. at the end of incubation time).
13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

13.1. Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.2. Standard curve calculation:

13.2.1. Average the duplicate reading for each standard.

13.2.2. Plot standard curve readings and draw the line of the best fit to construct the standard curve. Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.3. Measurement of CK activity in sample:

13.3.1. Calculate $\Delta OD$ for sample as follows:

$$\Delta OD_{450nm} = (A_2 - A_{2BG}) - (A_1 - A_{1BG})$$

Where:

- $A_1$ is the sample reading at time T1.
- $A_{1BG}$ is the background control sample at time T1.
- $A_2$ is the sample reading at time T2.
- $A_{2BG}$ is the background control sample at time T2.

13.3.2. Use the $\Delta OD_{450nm}$ to calculate amount of NADH generated by CK during the reaction time ($\Delta T$).
13.4. CK activity (nmol/min/mL or mU/mL) in the test samples is calculated as:

\[
CK \text{ Activity} = \left( \frac{B}{\Delta T \times V} \right) \times D
\]

Where:
- \( B \) = Amount of NADH in sample well calculated from standard curve (nmol).
- \( \Delta T \) = Reaction time (minutes).
- \( V \) = Original sample volume added into the reaction well (mL).
- \( D \) = Sample dilution factor.

CK activity can also be expressed as U/mg of total protein in the sample.

**Unit Definition:**

1 Unit CK activity = amount of CK that will generate 1.0 µmol of NADH per minute at pH 9.0 at 37°C.
14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Figure 1: Typical NADH standard calibration curve using colorimetric reading.
Figure 2: Creatine kinase activity was tested in human serum (5 µL) and rat heart lysate (192 ng).
15. QUICK ASSAY PROCEDURE

**NOTE:** This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare standard, ATP, developer, positive control and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare NADH standard dilution [2 – 10 nmol/well].
- Prepare samples in optimal dilutions so that they fit standard curve readings.
- Set up plate in duplicate for standard (50 µL), samples (50 µL), positive control (50 µL) and background wells (50 µL).
- Prepare a master mix for CK Reaction Mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Reaction Mix (µL)</th>
<th>Background Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK Assay Buffer</td>
<td>34</td>
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</tr>
<tr>
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<td>2</td>
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</tr>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ATP</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CK Substrate</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

- Add 50 µL of Reaction Mix to the standard and sample wells.
- Add 50 µL of Background Reaction Mix into the background sample control wells. Mix well.
- Measure absorbance at OD 450 nm on a microplate reader in a kinetic mode at 37°C for 20 – 40 minutes protected from light.
## 16. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Samples not deproteinized (if indicated on protocol)</td>
<td>Use provided protocol for deproteinization</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer, increase number of strokes</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/Higher readings in samples and Standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
</tbody>
</table>
## RESOURCES

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard readings do not follow a linear pattern</td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions described in the protocol</td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
</tr>
</tbody>
</table>
17. INTERFERENCES
These chemicals or biological materials will cause interference in this assay causing compromised results or complete failure:
- RIPA buffer – it contains SDS which can destroy/decrease the activity of the enzyme.
- Small molecules (ADP, NADH) – may generate high background levels.

18. FAQs
Which protein assay is compatible with this kit?
We suggest you use a detergent compatible BCA assay kit, such as BCA Protein Quantification Kit (ab102536).

What is the sample volume to be used with this kit for plasma samples from rat?
This depends on the amount of active CK enzyme in the sample. The sample volume per well would need to be optimized to make sure that the values obtained are within the linear range of the standard curve.
19. NOTES
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