Urokinase Activity Assay Kit, Fluorogenic Cat. No. QIA125

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Storage
Store unopened kit at –20°C, urokinase standard at -70°C. Once opened, kit components can be stored as follows: microplate and cell lysis buffer at room temperature, dilution buffer, assay buffer, and urokinase substrate at 4°C, and urokinase standard at -70°C. Upon reconstitution of urokinase standard, aliquot and freeze at -70°C to avoid freeze/thaw cycles. Kit should not be used beyond the expiration date stated on the label.

Intended Use
This Calbiochem® Urokinase Activity Assay Kit is a sensitive fluorogenic assay for the measurement of urokinase activity (excitation: 360 - 380 nm; emission: 430 - 460 nm) in cell lysates, plasma, and serum, as well as for the screening of urokinase inhibitors.

Background
Urokinase or urokinase-type plasminogen activator (uPA) has been implicated in many physiological and pathological processes including cancer invasion and metastasis. The involvement of uPA in the degradation of extracellular matrix components by the generation of pericellular proteolytic activity is important in tissue remodeling and repair, and in invasive cell migration (1, 2, 3). uPA is a 54 kDa serine protease with an extremely limited substrate specificity, cleaving the Cys-Pro-Gly-Arg560⊥Val561-Val-Gly-Gly-Cys that constitutes a small disulfide-bridged loop in plasminogen. Synthetic peptide substrates based on this sequence like Glutaryl-Gly-Arg AMC are widely used to determine urokinase activity.
Principle of the Assay

This assay is designed for the quantitative in vitro determination of urokinase activity in microplates. It utilizes the unique ability of urokinase to digest the synthetic substrate Glutaryl-Gly-Arg AMC. Released free AMC is determined fluorometrically at excitation 360-380 nm and emission 440-460 nm. The activity of the urokinase can be quantified with a urokinase standard, or can be displayed as RFUnits. For the urokinase standard, 1 unit is defined as the amount of enzyme equal to an international standard as tested by the fibrinolytic method. The kit provides all of the components required to perform the assay.

The assay range is between 0.78 and 5.0 units/ml. The sensitivity is 0.01 units/ml.

Materials Provided

- **Urokinase Standard**: 1 x 20 units, human urokinase (Cat. No. 672112), liquid
  
  Note: 1 unit=1CTA=0.7 Ploug units

- **Substrate**: 1 x 100 µl, 100X concentrated Glutaryl-Gly-Arg AMC in DMSO

- **Assay Buffer**: 1 x 20 ml, HEPES buffer with surfactant

- **Dilution Buffer**: 1 x 20 ml, low ionic strength HEPES buffer with surfactant

- **Cell Lysis Buffer**: 1 x 10 ml, CytoBuster ™ Protein Extraction Buffer

- **Microplate**: 1 each

- **Sealer**: 1 each

Materials Required but not Provided

- Pipetman or multi-channel pipetman; use only pipettes that are carefully calibrated to their target volume.

- Microplate reader capable of measuring fluorescence at wavelengths of 360 - 380 nm (excitation) and 430 - 460 nm (emission).

- 37°C incubator

- Ice bucket to keep reagents cold until use.

Preparation of Reagents

All of the reagents necessary to perform the assay are supplied with this kit. Bring all reagents to 15-25°C before use.

1. **Standard**: the urokinase standard is supplied as a 20 units/vial stock solution. Add 200 µl of chilled dilution buffer to the vial to obtain a 100 units/ml solution. Prepare a calibration curve by making serial dilutions of the standard in the range of 5 to 0.078 units/ml. Example: label 8 tubes. Pipet 570 µl of the assay buffer into the first tube and 300 µl into the remaining tubes. Add 30 µl of the 100 units/ml urokinase standard to the first tube. Vortex and transfer 300 µl from this tube to the next tube. Prepare serial dilutions by vortexing and transferring 300 µl up to tube 7. The tube labeled 8 will contain assay buffer only (blank).

2. **Samples**: if necessary dilute samples with assay buffer. Cell lysates with a concentration above 3 mg/ml should be diluted 5 times. If the measured RFU exceeds 12000 the sample should be diluted further.

3. **Cell lysate**: wash cell pellet with ice-cold PBS. Add 500-1000 µl of cell lysis buffer (approximately 1 ml per 1 x 10^7 cells) and incubate on ice for 30 minutes. Vortex and centrifuge the lysate at 14,000 x g in a pre-cooled tabletop microcentrifuge. Immediately transfer the supernatant to a fresh microcentrifuge tube and discard the pellet. Dilute the lysate 1:5 or 1:10 before determining the protein concentration via BCA protein assay.

4. **Substrate**: dilute 100 times with dilution buffer.
Protocol

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standard, and controls be assayed in duplicate.

1. Prepare dilutions of standard, samples, and substrate. Remove microplate from the foil pouch.
2. Pipet 50 µl of assay buffer into each well.
3. Add 100 µl of standard or sample to the desired wells.
4. Add 50 µl of substrate solution to each well.
5. Incubate the microplate at 37°C for 1 hour.
6. Read the fluorescence of the free AMC on a fluorescence plate reader at excitation 360-380 nm and emission 440-460 nm.

Calculation of Results

The data obtained can be displayed in two ways.

1. As RFU: correct the fluorescence value of the samples by subtracting the value of the blank, and then calculate the mean fluorescence value of each sample in duplicate.
2. As units/ml: calculate the mean fluorescence value as stated above. Plot a graph correlating the mean fluorescence values of the standards (y-axis) to the concentration of the urokinase standard in units/ml (x-axis). The urokinase activity of the unknown samples can be read from the standard calibration curve.

Assay Characteristics

Sensitivity: the minimum detectable activity of urokinase by this assay is estimated to be 0.01 units/ml.

Standard curve: generated with human urokinase (Cat. No. 672112). Enzyme activity was measured with Glutaryl-Gly-Arg AMC, pH 8.0, at 37°C.
Time course profile: 5 units/ml of human urokinase measured with Glutaryl-Gly-Arg AMC substrate, pH 8.0, at 37°C for 1 hour.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Units/mg (ml for fluids) at 1 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>None detected</td>
</tr>
<tr>
<td>A431</td>
<td>9.75</td>
</tr>
<tr>
<td>HL-60</td>
<td>None detected</td>
</tr>
<tr>
<td>HeLa</td>
<td>12.60</td>
</tr>
<tr>
<td>U251</td>
<td>31.33</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.619</td>
</tr>
</tbody>
</table>

Urokinase activity in five human cell lines and human plasma measured with the Urokinase Activity Assay, Cat. No. QIA125. Urokinase activity was not detected in Jurkat and HL-60 10X diluted lysates, with protein concentrations of 0.365 and 0.295 mg/ml, respectively. CytoBuster™ (Cat. No. 71009) was used to prepare lysates.

References