Technical Bulletin

# Hyaluronidase Inhibitor Screening Assay Kit

**Catalogue number MAK458**

## Product Description

Hyaluronidases are a family of enzymes that catalyze the degradation of the glycosaminoglycan hyaluronic acid. Hyaluronic acid is one of the major constituents of the extracellular matrix in organisms where it contributes to both cell proliferation and migration. The role of hyaluronidases in breaking down this key factor in cell proliferation makes them a possible target for cancer treatment. One hypothesis is increased hyaluronidase may help prevent tumor invasion by breaking down the extracellular matrix needed for tumor expansion. Conversely, decreasing hyaluronidase activity might prevent metastasis by stopping cancer cells from escaping primary tumor masses. The study to determine hyaluronidases' exact role in cancer pathology is ongoing.

The Hyaluronidase Inhibitor Screening Assay Kit uses a two-step turbidimetric reaction to measure hyaluronidase activity by the amount of hyaluronic acid that is hydrolyzed. A stop reagent halts the enzymatic reaction and forms turbidity with any residual hyaluronic acid in the well. The decrease in turbidity at 600 nm ($OD_{600}$) is directly proportional to the hyaluronidase activity in the sample.

The kit is suitable for the high-throughput screening for evaluation of hyaluronidase inhibitors.

## Components

The kit is sufficient for 100 colorimetric assays in 96-well plates.

- Substrate — 1.5 mL
  Catalogue Number MAK458A
- Stop Reagent — 20 mL
  Catalogue Number MAK458B
- Assay Buffer — 5 mL
  Catalogue Number MAK458C
- Enzyme Buffer — 10 mL
  Catalogue Number MAK458D

## Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (example, multichannel pipettor)
- Spectrophotometric multiwell plate reader
- Clear flat-bottom 96-well plates. Cell culture or tissue culture treated plates are not recommended.
- Purified human or bovine hyaluronidase.
- Hyaluronidase inhibitor (optional) (for example, 6-O-Palmitoyl-L-Ascorbic Acid, Catalogue Number 76183)

## Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

## Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C.

## Preparation Instructions

Briefly centrifuge small vials prior to opening. Prior to assay, equilibrate all components to room temperature.

### Enzyme Preparation

Hyaluronidase (not included in the kit) should be prepared in Enzyme Buffer and used fresh. Albumin and other proteins interfere with this assay and should not be included in the Enzyme Buffer.

The protocol is optimized for Human Hyaluronidase (Acro Biosystems Catalogue Number PH0-H5225) or Bovine Hyaluronidase (US Biological Catalogue Number H7981-01). If using a different species or brand of enzyme, experimentally determine the optimal amount of enzyme to use per well (see Frequently Asked Questions section for instructions).

If using human hyaluronidase, dilute to 20 U/mL in Enzyme Buffer.

If using bovine hyaluronidase, dilute to 10 U/mL in Enzyme Buffer.

### Test Compound Preparation

Dissolve the test compounds (i.e., inhibitors) in solvent of choice. It is prudent to first test the tolerance of the hyaluronidase enzyme to the solvent. DMSO at concentrations of 1% (v/v) or less in the 100 μL enzymatic reaction will not interfere. Therefore, the 20 μL of test compound solution may contain up to 5% (v/v) DMSO for this procedure.

## Procedure

All Samples should be run in duplicate.

**Note:** The assay can be run in 384-well plates by using ¼ of the volumes specified for the 96-well plate assay.

### Test Compound and Control Preparations

1. For each inhibitor and inhibitor concentration being tested, transfer 40 μL of hyaluronidase into separate wells of a 96-well plate.
2. Transfer 40 μL of hyaluronidase into separate wells for the No Inhibitor Control (NIC).
3. Transfer 40 μL of Enzyme Buffer into separate wells for the No Enzyme Control (NEC).
4. To the NIC and NEC wells, add 20 μL of the solvent in use with the test compounds.
5. To the Sample wells, add 20 μL of each respective test compound.

   **Note:** The concentration of test compound in the 20 μL should be 5× the desired final concentration in the 100 μL enzymatic reaction (see Frequently Asked Questions for additional information).

6. Incubate wells with test compounds for 15 minutes at room temperature.

### Working Reagent

**Note:** This assay is based on a kinetic reaction. To ensure identical incubation time, addition of Working Reagent should be quick and mixing should be brief but thorough. Use of a multi-channel pipettor is recommended.

**Note:** Prepare the Working Reagent fresh for each assay and use within 2 hours of preparation.

Mix enough reagents for the number of assays to be performed. For each well, prepare 45 μL of Working Reagent according to Table 1.

**Table 1.**
Preparation of Working Reagent

| Reagent | Working Reagent |
|---|---|
| Assay Buffer | 35 μL |
| Substrate | 10 μL |

### Assay Reaction

1. Add 40 μL of Working Reagent to every well and tap plate immediately to mix.
2. Incubate the plate for 20 minutes at room temperature.
3. Add 160 μL of Stop Reagent to each well. Tap plate to mix briefly and thoroughly. Incubate for 10 minutes at room temperature.

### Measurement

Read optical density (OD) at 600 nm.

## Results

Calculate the % inhibition using the equation below:

$$\%\ \text{Inhibition} = \left(1 - \frac{OD_{NEC} - OD_{Test\ Compound}}{OD_{NEC} - OD_{NIC}}\right) \times 100$$

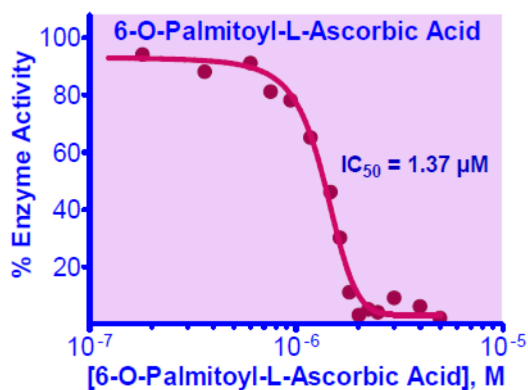
where:

$OD_{NEC}$ = OD value at 600 nm of the No Enzyme Control (NEC)

$OD_{NIC}$ = OD value at 600 nm of the No Inhibitor Control (NIC)

$OD_{Test\ Compound}$ = OD value at 600 nm of the Test Compound

**Figure 1.**
6-O-Palmitoyl-L-Ascorbic Acid Titration: Recombinant human hyaluronidase was incubated with various concentrations of 6-O-Palmitoyl-L-Ascorbic Acid. Each concentration of inhibitor contained 5% (v/v) DMSO (final 1% (v/v) in 100 µL enzymatic reaction).




## Frequently Asked Questions

**How do I calculate the concentration of the test compound in the reaction?**

The final concentration of the test compound in the 100 µL reaction is 5× lower than the 20 µL of test compound. For example, if 20 µL of 100 µM test compound was added to the well, the final concentration of test compound in the 100 µL enzymatic reaction would be 20 µM. The final concentration after adding 160 µL Stop Reagent is irrelevant because the reaction is stopped after its addition.

**My percent inhibition readout is greater than 100%. What does this mean?**

Inhibition readouts slightly over 100% (example., 100%to110%) are not an issue and can be assumed to be 100% inhibition. Inhibition readouts significantly over 100% (>110%) may mean there is a substance interfering with the assay.

**My percent inhibition readout is negative. What does this mean?**

Inhibition readouts that are slightly negative (example., -1% to -10%) are not an issue and can be assumed to be 0% inhibition. Inhibition readouts that are significantly negative may mean there is a substance interfering with the assay or that the test compound is acting as an activator to improve the enzyme's activity, rather than inhibit it.

**I am using a different species or a different brand of hyaluronidase. How do I determine the optimal hyaluronidase concentration for inhibitor screening?**

Determine the optimal hyaluronidase concentration for inhibitor screening by following the below protocol:

1. Prepare a stock Hyaluronidase solution in Enzyme Buffer (start at a high concentration and dilute down if necessary).
2. In Eppendorf tubes, serially dilute 50 µL of stock Hyaluronidase in Enzyme Buffer.
3. Transfer 40 µL of each Hyaluronidase dilution into separate wells of a clear, flat bottom 96-well plate.
4. Transfer 40 µL of purified water into two separate wells for the No Enzyme Control (NEC) and No Substrate Control (NSC).
5. Prepare enough Working Reagent for each well by combining 10 µL of Substrate and 35 µL of Assay Buffer.
6. Add 40 µL of Working Reagent to Hyaluronidase dilution Sample wells and the NEC. Add 40 µL of Assay Buffer to the NSC well.
7. Tap plate briefly to mix and incubate the plate for 20 minutes at room temperature.
8. Add 160 µL of Stop Reagent to each well. Tap plate to mix briefly and thoroughly.
9. Incubate for 10 minutes at room temperature and read the optical density at 600 nm.

Select a concentration of enzyme for use in the Screening Procedure that yields an $OD_{600}$ reading about halfway between the NEC and NSC $OD_{600}$ readings.

**Note:** The low concentrations of enzyme may produce $OD_{600}$ readings slightly higher than the NEC. This is normal.

## References


1. Lokeshwar, V.B., *et al*., HYAL1 Hyaluronidase in prostate cancer: a tumor promoter and suppressor. *Cancer Res*., **65**, 7782-7789 (2005).
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4. Gong, H., *et al*., Hyaluronidase to enhance nanoparticle-based photodynamic tumor therapy. *Nano Lett*., **16**, 2512-2521 (2016).

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