

## Amplex<sup>®</sup> Red Neuraminidase (Sialidase) Assay Kit (A22178)

### Quick Facts

#### Storage upon receipt:

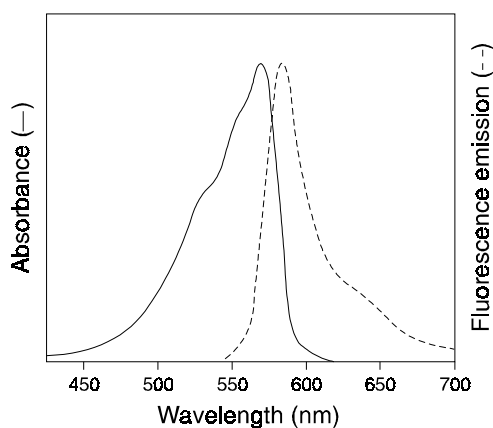
- $\leq -20^{\circ}\text{C}$
- Desiccate
- Protect from light

**Abs/Em of reaction product:** 571/585 nm

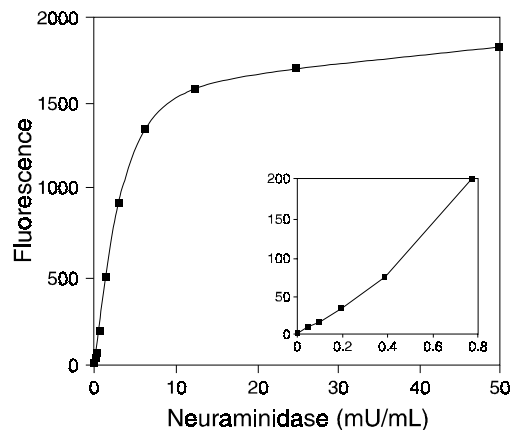
### Introduction

Neuraminidase (also known as sialidase) is a very common enzyme that hydrolyzes terminal sialic acid residues on polysaccharide chains, most often exposing a galactose residue. Although neuraminidase is found in mammals, it is predominantly expressed in microorganisms such as bacteria and viruses.<sup>1</sup>

The negative-stranded RNA influenza virus contains two surface glycoproteins, hemagglutinin (HA) and neuraminidase. Neuraminidase is thought to play a key role in the invasion of target cells and the subsequent replication of the influenza virus through its cleavage of target cell receptor sialic acid moieties. This action prevents further interaction of the virus with the target cell and facilitates elution of progeny virions from the infected cell.<sup>2,3</sup> Additionally, newly synthesized neuraminidase and HA on virions may also contain sialic acid residues that can be cleaved by neuraminidase in order to prevent self-aggregation. It is also thought that the penetration of mucosal linings by the virus is enhanced by neuraminidase hydrolytic action on fetuin, a major component of these membranes. These essential activities



**Figure 1.** Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex Red reagent.



**Figure 2.** Detection of neuraminidase activity using the Amplex Red reagent-based assay. Each reaction contained 50  $\mu\text{M}$  Amplex Red reagent, 0.1 U/mL HRP, 2 U/mL galactose oxidase, 250  $\mu\text{g/mL}$  fetuin and the indicated amount of neuraminidase in 1X Reaction Buffer. Reactions were incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at  $530 \pm 12.5$  nm and fluorescence detection at  $590 \pm 17.5$  nm. A background fluorescence of 70 fluorescence units was subtracted from each data point.

make neuraminidase an important target for influenza drug development.

Various methods using chemiluminescence, absorption and fluorescence have been developed to quantitate neuraminidase in biological fluids for detection of influenza virus<sup>2</sup> and for screening inhibitors of neuraminidase activity in drug development.<sup>2,4</sup> The ultimate goal has been to develop a rapid, single-step assay that is sensitive and adaptable for a high throughput screening format. The development of an assay system utilizing the Amplex<sup>®</sup> Red reagent with superior spectral and chemical characteristics meets these needs.

The Amplex Red Neuraminidase (Sialidase) Assay Kit provides an ultrasensitive method for detecting neuraminidase activity. This assay utilizes Amplex Red to detect  $\text{H}_2\text{O}_2$  generated by galactose oxidase oxidation of desialated galactose, the end result of neuraminidase action. The  $\text{H}_2\text{O}_2$  then, in the presence of horseradish peroxidase (HRP), reacts with a 1:1 stoichiometry with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin.<sup>5</sup> Resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively (Figure 1), and because the extinction coefficient is high ( $54,000 \text{ cm}^{-1}\text{M}^{-1}$ ), the assay can be performed either fluorometrically or spectrophotometrically. In a purified system with fetuin as the substrate, neuraminidase levels as low as 0.2 mU/mL have been detected with the Amplex Red Neuraminidase (Sialidase) Assay Kit (Figure 2). Neuraminidase activity can also be detected in biological samples such as serum (Figure 3).

## Materials

### Kit Contents

- **Amplex Red reagent** (MW = 257, Component A), two vials, each containing 0.26 mg
- **Dimethylsulfoxide (DMSO)**, anhydrous (Component B), 500  $\mu$ L
- **Horseshoe peroxidase (Component C)**, 20 U, where 1 unit is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C
- **Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)** (MW = 34, Component D), 500  $\mu$ L of a stabilized ~3% solution; the actual concentration is indicated on the component label
- **5X Reaction Buffer** (Component E), 10 mL of 0.25 M Tris-HCl, pH 7.2, 5 mM CaCl<sub>2</sub>
- **Galactose oxidase**, from *Dactylium dendroides* (Component F), 100 U, where one unit will produce a change in absorbance of 1.0 at 425 nm per minute at pH 6.0 at 25°C using a peroxidase/*o*-tolidine coupled assay with galactose as the substrate
- **Fetuin, from fetal calf serum** (MW ~67 kDa, Component G), 12 mg
- **Neuraminidase, from *Clostridium perfringens*** (Component H), one vial containing approximately 0.1–0.5 U, where one unit will liberate 1.0  $\mu$ mole of *N*-acetylneuraminic acid per minute at pH 5.0 at 37°C using bovine submaxillary mucin

Each Kit provides sufficient reagents for approximately 400 assays using either a fluorescence or absorbance microplate reader and reaction volumes of 100  $\mu$ L per assay.

### Storage and Handling

Upon receipt, the kit should be stored frozen at  $\leq -20^{\circ}\text{C}$ , protected from light. Stored properly, the kit components should remain stable for at least six months. Allow reagents to warm to room temperature before opening vials. The Amplex Red reagent is somewhat air sensitive. Once a vial of Amplex Red reagent is opened, the reagent should be used promptly. **PROTECT THE AMPLEX RED REAGENT FROM LIGHT.**

## Experimental Protocol

The following procedure is designed for use with a fluorescence or absorbance multiwell plate scanner. For use with a standard fluorometer or spectrophotometer, volumes must be increased accordingly.

Please note that resorufin, the product of the Amplex Red reaction, is unstable in the presence of thiols such as dithiothreitol (DTT) and 2-mercaptoethanol. For this reason, the final DTT or 2-mercaptoethanol concentration in the reaction should be no higher than 10  $\mu$ M.

The absorption and fluorescence of resorufin are pH-dependent. Below the  $\text{pK}_a$  (~6.0), the absorption maximum shifts to ~480 nm and the fluorescence quantum yield is markedly lower. In addition, the Amplex Red reagent is unstable at high pH (>8.5). For these reasons, the reactions should be performed at pH 7–8. We recommend using the included Reaction Buffer (pH 7.2) for optimal performance of the Amplex Red reagent.

### Stock Solution Preparation

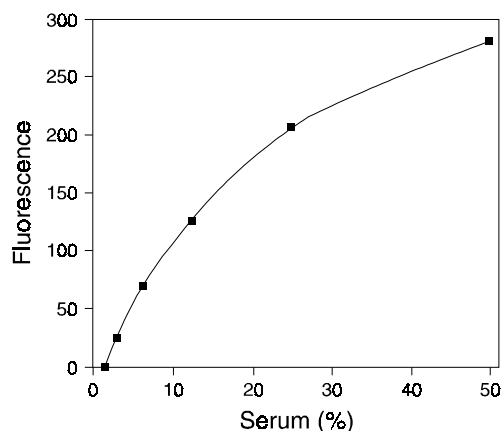
**1.1** Prepare a 10 mM stock solution of Amplex Red reagent: Allow one vial of Amplex Red reagent (Component A) and DMSO (Component B) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex Red reagent (0.26 mg) in 100  $\mu$ L of DMSO. Each vial of Amplex Red reagent is sufficient for approximately 200 assays, with a final reaction volume of 100  $\mu$ L per assay. If not used immediately, the stock solution should be stored frozen at  $\leq -20^{\circ}\text{C}$ , protected from light.

**1.2** Prepare a 1X working solution of Reaction Buffer by adding 4 mL of 5X Reaction Buffer stock solution (Component E) to 16 mL of deionized water (dH<sub>2</sub>O). This 20 mL volume of 1X Reaction Buffer is sufficient for approximately 200 assays of 100  $\mu$ L each with a 10 mL excess for making stock solutions and dilutions. The 1X Reaction Buffer should be stable for several weeks if stored at 4°C.

**1.3** Prepare a 100 U/mL stock solution of horseradish peroxidase (HRP) by dissolving the contents of the vial of HRP (Component C) in 200  $\mu$ L of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at  $\leq -20^{\circ}\text{C}$ .

**1.4** Prepare a 20 mM H<sub>2</sub>O<sub>2</sub> working solution by diluting the ~3% H<sub>2</sub>O<sub>2</sub> stock solution (Component D) into the appropriate volume of dH<sub>2</sub>O. The actual H<sub>2</sub>O<sub>2</sub> concentration is indicated on the component label. For instance, a 20 mM H<sub>2</sub>O<sub>2</sub> working solution can be prepared from a 3.0% H<sub>2</sub>O<sub>2</sub> stock solution by diluting 23  $\mu$ L of 3.0% H<sub>2</sub>O<sub>2</sub> into 977  $\mu$ L of dH<sub>2</sub>O. Please note that although the ~3% H<sub>2</sub>O<sub>2</sub> stock solution has been stabilized to slow degradation, the 20 mM H<sub>2</sub>O<sub>2</sub> working solution will be less stable and should be used promptly.

**1.5** Prepare a 200 U/mL galactose oxidase stock solution by dissolving the contents of the vial of galactose oxidase



**Figure 3.** Detection of neuraminidase activity in serum using the Amplex Red reagent-based assay. Each reaction contained 50  $\mu$ M Amplex Red reagent, 0.1 U/mL HRP, 2 U/mL galactose oxidase, 250  $\mu$ g/mL fetuin and the indicated amount of serum in 1X Reaction Buffer. Reactions were incubated at 37°C. After 60 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at  $530 \pm 12.5$  nm and fluorescence detection at  $590 \pm 17.5$  nm. A background fluorescence of 112 units was subtracted from each data point.

(Component F) in 500  $\mu\text{L}$  of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored at  $\leq -20^\circ\text{C}$ .

**1.6** Prepare a 10 mg/mL fetuin stock solution by dissolving the contents of the vial of fetuin (Component G) in 1.2 mL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored at  $\leq -20^\circ\text{C}$ .

**1.7** Prepare a neuraminidase stock solution by dissolving the contents of the vial of neuraminidase (Component H) in 100  $\mu\text{L}$  of 1X Reaction Buffer. The concentration of this stock solution is approximately 1–5 U/mL. After use, the remaining solution should be divided into small aliquots and stored at  $\leq -20^\circ\text{C}$ .

### Neuraminidase Assay

The following protocol describes the assay of neuraminidase activity in a total volume of 100  $\mu\text{L}$  per microplate well. The volumes recommended here are sufficient for ~100 assays. The kit provides sufficient material for ~400 assays.

**2.1** Dilute the neuraminidase-containing samples in 1X Reaction Buffer. A variable dilution will be required depending on the neuraminidase activity present in the sample. In the first trial the samples should be serially diluted to determine the optimal amount of sample for assay. Pipet 50  $\mu\text{L}$  of each sample into separate wells of a 96-well microplate. Note that the sample concentrations will be twofold lower in the final reaction volume.

**2.2** Prepare a no-neuraminidase negative control by pipeting 50  $\mu\text{L}$  of 1X Reaction Buffer alone into one well of the microplate.

**2.3** If desired, prepare a neuraminidase positive control by diluting the neuraminidase stock solution (prepared in step 1.7) 50-fold in 1X Reaction Buffer. Pipet 50  $\mu\text{L}$  of the dilute neuraminidase solution into a separate well of the microplate. Please note that the provided neuraminidase (Component H) serves only as a positive control for the Amplex Red Neuraminidase Assay and should not be relied upon as a quantitation standard for enzyme activity.

**2.4** If desired, prepare an  $\text{H}_2\text{O}_2$  positive control by diluting the 20 mM  $\text{H}_2\text{O}_2$  working solution (prepared in step 1.4) to 10  $\mu\text{M}$  in 1X Reaction Buffer. Pipet 50  $\mu\text{L}$  of the 10  $\mu\text{M}$  stock solution into a separate well of the microplate.

**2.5** Prepare a 2X working solution of 100  $\mu\text{M}$  Amplex Red reagent containing 0.2 U/mL HRP, 4 U/mL galactose oxidase and 500  $\mu\text{g}/\text{mL}$  fetuin by mixing:

- 50  $\mu\text{L}$  of the Amplex Red reagent stock solution (prepared in step 1.1)
- 10  $\mu\text{L}$  of the HRP stock solution (prepared in step 1.3)
- 100  $\mu\text{L}$  of the galactose oxidase stock solution (prepared in step 1.5)
- 250  $\mu\text{L}$  of the fetuin stock solution (prepared in step 1.6)
- 4.59 mL of 1X Reaction Buffer

This 5 mL volume is sufficient for ~100 assays. Note that the final concentration of each component will be twofold lower in the final reaction volume.

**2.6** Begin the reaction by adding 50  $\mu\text{L}$  of the Amplex Red reagent/HRP/galactose oxidase/fetuin working solution to each microplate well containing the samples and controls.

**2.7** Incubate the reaction for 30 minutes or longer at  $37^\circ\text{C}$ , protected from light. Because the assay is continuous (not terminated), fluorescence or absorbance may be measured at multiple time points to follow the kinetics of the reactions.

**2.8** Measure the fluorescence or absorbance in a microplate reader using excitation in the range of 530–560 nm and emission detection at ~590 nm or absorbance at ~560 nm (see Figure 1).

**2.9** For each point, correct for background fluorescence or absorbance by subtracting the value derived from the no-neuraminidase control.

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## References

1. J Biochem Biophys Methods 22, 23 (1991); 2. J Med Chem 13, 697 (1970); 3. Antiviral Res 47, 1 (2000); 4. Anal Biochem 280, 291 (2000); 5. J Immunol Methods 202, 133 (1997).

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## Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
A22178	Amplex <sup>®</sup> Red Neuraminidase (Sialidase) Assay Kit *400 assays*	1 kit
A12222	Amplex <sup>®</sup> Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A22177	Amplex <sup>®</sup> Red reagent *packaged for high-throughput screening*	10 x 10 mg
A36006	Amplex <sup>®</sup> UltraRed reagent	5 x 1 mg

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