Vybrant™ Cytotoxicity Assay Kit (V-23111)

Quick Facts

Storage upon receipt:

- –20°C
- Dessicate
- Protect from light

Abs/Em of reaction product: 563/587 nm

Introduction

Cell death is often assayed using probes that enter the interior of the cell through areas of plasma membrane damage. The Vybrant™ Cytotoxicity Assay Kit (V-23111) provides an alternative method that monitors the release of the cytosolic enzyme glucose 6-phosphate dehydrogenase (G6PD) from damaged cells into the surrounding medium. G6PD is a ubiquitous enzyme that is part of the pentose phosphate pathway. Because G6PD generates NADPH,[1,2] it plays a crucial role in cellular antioxidant defense.

The Vybrant cytotoxicity assay detects G6PD through a two-step enzymatic process that leads to the reduction of resazurin into red-fluorescent resorufin (Figure 1). The resulting fluorescence signal is proportional to the amount of G6PD released into the cell medium, and this release correlates with the number of dead cells in the sample (Figure 2). The fluorescence emission of resorufin (Abs/Em 563/587 nm) is beyond the autofluorescence of most biological samples. In addition, this assay results in lower background signals than are typically observed in lactate dehydrogenase–based assays (Figure 3), because the level of G6PD activity in common cell culture sera is typically lower than the level of lactate dehydrogenase activity. The Vybrant Cytotoxicity Assay Kit contains all of the enzymes and substrates needed to detect the release of G6PD from damaged and dying cells. The assay can be completed in less than an hour and is effective with samples containing as few as 500 cells.

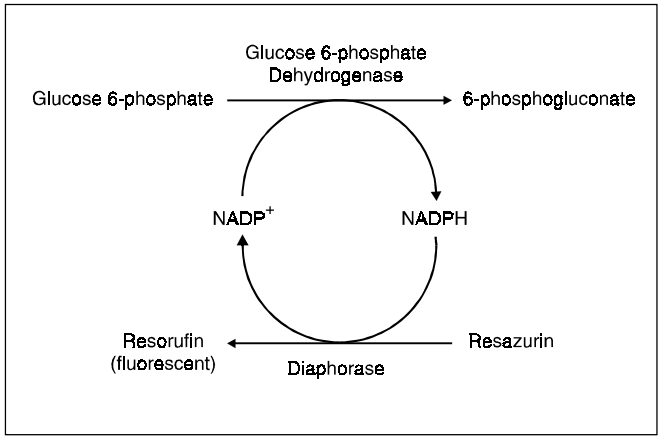

Figure 1. *Principle of the coupled enzymatic assay for detection of glucose 6-phosphate dehydrogenase activity. Oxidation of glucose 6-phosphate by glucose 6-phosphate dehydrogenase results in the generation of NADPH, which in turn leads to the reduction of resazurin by diaphorase to yield fluorescent resorufin.*

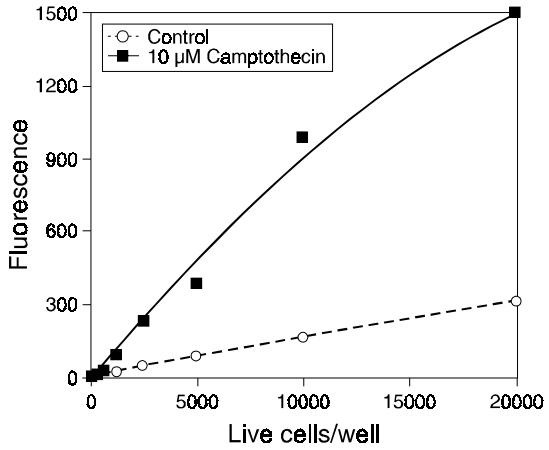

Figure 2. *Detection of dead and dying cells using the Vybrant Cytotoxicity Assay Kit. Jurkat cells were treated with 10 µM camptothecin for six hours, then assayed for glucose 6-phosphate dehydrogenase release. An untreated control sample is shown for comparison. The fluorescence was measured in a microplate reader (excitation/emission ~530/590 nm). A background of 55 fluorescence units was subtracted from each value.*

Materials

Kit Contents

- **Resazurin** (MW = 251.17, Component A), 5 vials, each containing 75 µg of reagent
- **Dimethylsulfoxide (DMSO), anhydrous** (Component B), 500 µL
- **Reaction mixture** (Component C), 5 vials, each containing a lyophilized mixture of diaphorase, glucose 6-phosphate and $NADP^+$
- **5X Reaction buffer** (Component D), 28 mL of 0.50 M Tris, pH 7.5
- **100X Cell-lysis buffer** (Component E), 1.0 mL

Each kit provides sufficient reagents for approximately 1000 assays using a fluorescence microplate reader and reaction volumes of 100 µL.

Storage and Handling

Upon receipt, components should be stored at -20°C, protected from light, until required for use. When stored properly, the kit components should be stable for at least six months. Allow the vials to warm to room temperature before opening.

Assay Protocol

The following procedure is designed for use with a fluorescence multiwell plate scanner. To use this protocol with a standard fluorometer, volumes must be increased accordingly. The absorption and fluorescence of resorufin are pH dependent. Below the pK_a (~6.0), the absorption maximum of resorufin shifts to ~480 nm and the fluorescence quantum yield is markedly lower. For these reasons, the reactions should be performed in the buffer provided.

Stock Solution Preparation

Allow all kit components to warm to room temperature before preparing the stock solutions.

1.1 Prepare a 4 mM stock solution of resazurin by dissolving the contents of the vial (Component A) in 75 µL of DMSO (Component B). This stock solution should be stored at -20°C, protected from light.

1.2 Prepare a 1X reaction buffer by diluting the 5X reaction buffer (Component D) fivefold in deionized water (dH_2O). For example, for approximately 200 assays, mix 2.0 mL of 5X reaction buffer with 8.0 mL of dH_2O.

Cytotoxicity Assay

2.1 Plate the cells to be assayed in a 96-well microplate; between 500 and 25,000 cells/well in a 50 µL volume is sufficient (note **A**). High concentrations of serum will increase the background but will not significantly impair the sensitivity of the assay. In addition to the wells that contain experimental cells, reserve wells for various controls, including a no-cell control (medium alone), untreated cells and fully lysed cells (note **B**). We recommend that all samples (control and experimental) be assayed in triplicate.

2.2 Add the preferred cytotoxic agent(s) to the experimental wells, and incubate for the desired time. However, incubations that are longer than 24 hours will result in significant degradation of G6PD, impairing the assay results.

2.3 Prepare the reaction mixture solution by dissolving the contents of one vial of the lyophilized reaction mixture (Component C) in 400 µL of 1X reaction buffer (prepared in step 1.2). Mix the components gently; do not vortex. Note that each vial of reaction mixture contains enough material for approximately 200 assays. Freeze any unused reaction mixture solution in single-use aliquots.

2.4 Make a 2X resazurin/reaction mixture by combining 75 µL of the 4 mM resazurin stock solution (prepared in step 1.1), 400 µL of the reaction mixture solution (prepared in step 2.3) and 9.52 mL of the 1X reaction buffer (prepared in step 1.2) (note **C**). Mix the components gently, do not vortex. The volumes may be scaled to accommodate a larger or smaller number of assays, but make only enough 2X resazurin/reaction mixture to complete the number of assays prepared in step 2.1. The concentration of resazurin in the final reaction mixture will be 15 µM.

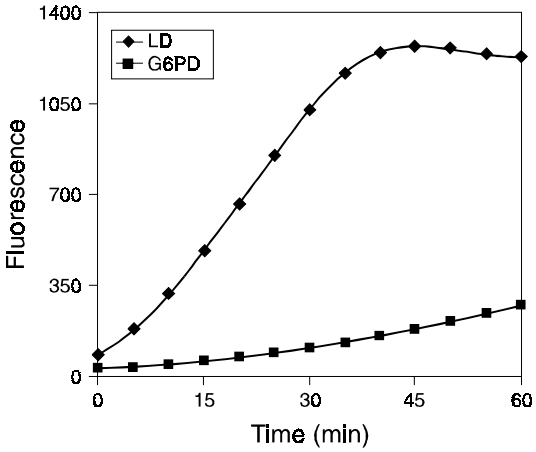

Figure 3: *Comparison of signal levels due to lactate dehydrogenase and glucose 6-phosphate dehydrogenase present in serum. 10% Bovine serum was assayed for the presence of lactate dehydrogenase (LD, diamonds) and glucose 6-phosphate dehydrogenase (G6PD, squares). G6PD was assayed using the reaction scheme shown in Figure 1; LD was detected using a similar scheme, except that in this case LD reduces lactate to generate NADH. The result clearly shows that, over the time course of the experiment, the serum generates a much lower signal in the G6PD assay than in the LD assay.*

2.5 Add 50 µL of the 2X resazurin/reaction mixture to each well.

2.6 To the lysed-cell control wells ONLY, add 1 µL of 100X cell-lysis buffer (Component E).

2.7 Incubate the microplate at 37°C for 10–30 minutes. Take fluorescence readings at 5 minute intervals to determine the optimal time point for the particular experiment. The fluorescence microplate reader should be set up with excitation and emission filters appropriate for resorufin (excitation 530–560 nm, emission 580–600 nm).

Analysis of Results

3.1 Correct the values obtained for the experimental cells and the fully lysed cells by subtracting from each the value obtained from the untreated control cells.

3.2 For each experimental condition, determine the relative cytotoxicity by dividing the corrected fluorescence of the experimental cells by the corrected fluorescence of the fully lysed cells.

Product Notes

[A] Cells may also be grown in a separate plate or under other conditions, then spun down and only the supernatant assayed. The presence of the cells themselves does not affect the assay, but it may be more convenient under certain circumstances to assay the supernatant only.

[B] This control is included to assay the total amount of cellular G6PD; the cells are subsequently lysed to release all G6PD into the assay medium. Inclusion of this control is not necessary if a quantitative determination of cell death is not needed.

[C] The sensitivity of the assay can be increased by reducing the resazurin concentration in the 2X resazurin/reaction mixture. Adjust the recipe given in step 2.4 as follows: add only 25 µL of 4 mM resazurin, and increase the amount of 1X reaction buffer to 9.57 mL. In this case, the concentration of resazurin in the final reaction mixture will be 5 µM. However, this change will also decrease the maximum attainable signal level.

References

1. Nucleic Acids Res 14, 2511 (1986); **2.** J Biol Chem 275, 40042 (2000).

Product List

Current prices may be obtained from our Web site or from our Customer Service Department.

Cat #	Product Name	Unit Size
V-23111	Vybrant™ Cytotoxicity Assay Kit *G6PD release assay* *1000 assays*	1 kit

Contact Information

Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

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