*Revised: 09–February–2001*

# *Vybrant™ Cell Adhesion Assay Kit (V-13181)*

**Quick Facts**

***Storage upon receipt:***

- –20°C
- Desiccate
- Protect from light

***Note:*** Prepare the working solution of calcein AM just prior to use.

## *Introduction*

The Vybrant™ Cell Adhesion Assay Kit utilizes the fluorogenic dye calcein acetoxymethyl ester (calcein AM) to provide a fast and sensitive method for measuring cell adhesion with a variety of cell types (Figure 1). Calcein AM is nonfluorescent but, once loaded into cells, is cleaved by endogenous esterases to produce highly fluorescent calcein. Calcein provides a brightly fluorescent, pH-independent, cytoplasmic cell marker, which interferes minimally with the cell adhesion process[1] and has proven to be an excellent reagent for measuring the effects of E-selectin–binding peptides[2] and integrins[3] on neutrophil adhesion, integrin-mediated cell adhesion in transfected K562 cells[4,5] and BSC-1 cells,[6] leukocyte[7,8] and neutrophil[9] adherence to endothelial cells and monocyte adhesion in HIV-infected cells.[10] The Vybrant cell adhesion assay is designed for use with fluorescence microplate readers. This simple procedure avoids problems associated with assays that utilize radioisotopes, which generate hazardous waste, and with assays that rely on the use of covalently coupled cell-surface labels, which can potentially alter cell function.[8] Calcein's absorbance maximum of 494 nm and emission maximum of 517 nm are ideally suited for detection by instruments equipped with standard fluorescein filters.

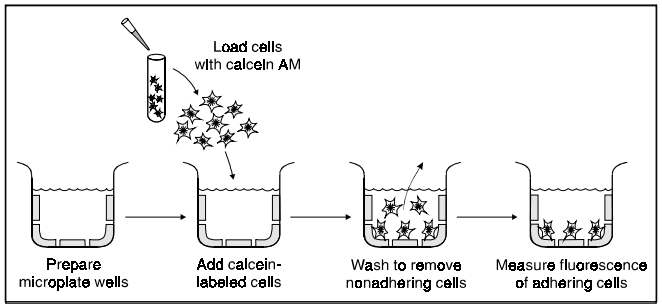


***Figure 1****. Principle of the Vybrant Cell Adhesion Assay. Microplate wells may be left untreated, cultured with monolayers of cells or precoated with extracellular matrix proteins, antibodies or other reagents.*

In addition to calcein AM, the Vybrant Cell Adhesion Assay Kit also includes SYTOX® Green nucleic acid stain, an easy-to-use dead-cell indicator, to assess overall health of cells prior to performing the cell adhesion assay. As a fluorescent substitute for trypan blue, this high-affinity nucleic acid stain easily penetrates cells that have compromised membranes but will not cross the membranes of live cells. Upon binding to nucleic acids, the SYTOX Green dye exhibits a >500-fold fluorescence enhancement (Ex/Em = 504 nm/523 nm).

## *Materials*

### *Kit Contents*

- **Calcein AM** (Component A; MW 995), 10 vials, each containing 50 µL of a 1 mM solution in anhydrous DMSO
- **SYTOX Green nucleic acid stain** (Component B; MW ~600), 1 vial containing 25 µL of a 1 mM solution in anhydrous DMSO

When used at the concentration suggested in the protocol below, this kit provides sufficient calcein AM to perform about 1000 assays using a fluorescence microplate reader.

### *Storage and Handling*

Kit reagents should be stored at -20°C, upright, sealed, desiccated and protected from light. Allow the reagents to warm to room temperature and centrifuge briefly before opening. Before refreezing, seal all stock solutions tightly.

Calcein AM is susceptible to hydrolysis when exposed to moisture. Prepare aqueous working solutions containing calcein AM immediately prior to use, and use within one day.

Stock solutions of SYTOX Green dye may be subjected to many freeze-thaw cycles without reagent degradation. When stored properly, the stock solution of SYTOX Green dye is stable for at least one year.

**Caution:** No data are available addressing the mutagenicity or toxicity of calcein AM and SYTOX Green stain. In particular, because SYTOX Green stain binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care. The DMSO stock solution of SYTOX Green stain should be handled with caution as DMSO is known to facilitate the entry of organic molecules into tissues. We strongly recommend using double gloves when handling the DMSO stock solutions. As with all nucleic acid stains, solutions of this reagent should be poured through activated charcoal before disposal. The charcoal must then be incinerated to destroy the dye.

## *Suggested Protocol*

The general protocol presented below is based on the method of Akeson and Woods[8] and was developed to measure adhesion

of calcein-labeled human monocytic (THP-1) and human T (Jurkat) cell lines to confluent monolayers of human aortic (HAEC) and umbilical vein (HUVEC) endothelial cells.

In our protocol, calcein AM is used at a concentration of 5 µM for cell loading. Researchers are encouraged to use this protocol as a starting point for developing and optimizing the cell adhesion assay for their particular requirements. For some cell types it may be necessary to increase or decrease the loading concentration of calcein AM to maximize assay sensitivity. Please refer to *Additional Information* for additional references on calcein AM–based cell adhesion assays. SYTOX Green stain may be used prior to performing the cell adhesion assay to assess the general health of cells prior to loading with calcein AM (see *Use of SYTOX Green Nucleic Acid Stain to Assess Cell Viability*).

In preparation for the cell adhesion assay, be sure to include appropriate controls for measuring total fluorescence of labeled cells before adhesion (note **A**) and controls for measuring autofluorescence of unlabeled cells or tissue culture medium. Absolute cell numbers may be determined by establishing a standard curve correlating fluorescence with cell number. The lower limit of detection for NIH 3T3 fibroblasts loaded with 5 µM calcein AM is about 5000 cells/well (Figure 2).

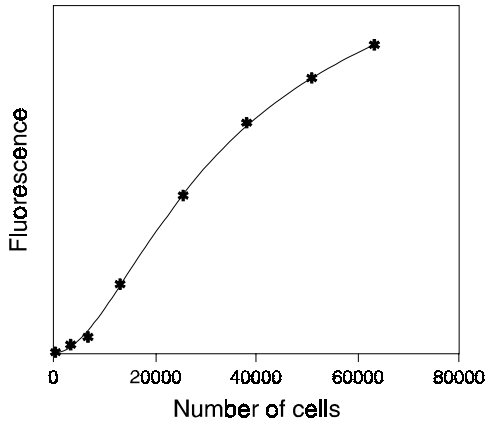


***Figure 2.*** *Fluorescence of NIH 3T3 fibroblasts loaded with 5 µM calcein AM at 37°C for 30 minutes and washed with PBS. Fluorescence was measured in a fluorescence microplate reader using a standard fluorescein filter set and plotted versus cell number.*

**1.** Determine the number of assays to be run and the number of cells required. Generally, $5 \times 10^5$ cells per microplate well provides sufficient cells for each adhesion assay.

**2.** Wash cells twice with PBS. Resuspend cells in RPMI medium[8] (or other suitable tissue culture medium without serum) at $5 \times 10^6$ cells/mL.

**3.** Add 5 µL of the calcein AM stock solution (Component A) per mL of cell suspension to achieve a final concentration of 5 µM. Mix well.

**4.** Incubate at 37°C for 30 minutes.

**5.** Wash cells twice with prewarmed (37°C) RPMI. Resuspend the cells in RPMI at $5 \times 10^6$ cells/mL.

**6.** Add 100 µL of the calcein-labeled cell suspension ($5 \times 10^5$ cells) to prepared microplate wells containing confluent cells (medium removed) or coated with ECM proteins or antibodies.

**7.** Incubate at 37°C for 30 to 120 minutes (the optimal incubation time for adhesion may vary according to cell type and other experimental parameters).

**8.** Remove nonadherent calcein-labeled cells by *careful* washing. Add prewarmed RPMI to each well and gently swirl. Invert the plate and blot excess liquid onto filter paper or paper towels.

**9.** Repeat the washing procedure four times. Add 200 µL of PBS to each well.

**10.** Measure the fluorescence using a fluorescein filter set (calcein has an absorbance maximum of 494 nm and an emission maximum of 517 nm).

**11.** Determine the percentage of adhesion by dividing the corrected (background subtracted) fluorescence of adherent cells by the total corrected fluorescence of cells added to each microplate well and multiplying by 100% (note **A**). In some experimental situations, especially when calcein AM has been loaded at higher concentrations (>5 µM), some calcein may leak out of the cells during the course of the experiment. For accurate quantitative determinations, this loss of calcein to the medium should be measured by performing suitable control experiments.

## *Use of SYTOX Green Nucleic Acid Stain to Assess Cell Viability*

SYTOX Green stain is provided for determining the overall viability of cells exposed to various adhesion modulators, ECM proteins or antibodies prior to performing the adhesion assay. This dye is included as a fluorescent substitute for the commonly used trypan blue. ***Note:*** *Calcein and nucleic acids stained with the SYTOX Green dye have similar fluorescence emissions and must be added to separate samples of cells that have been treated in the same manner*. After a brief incubation with the SYTOX Green dye, the nucleic acids of dead cells fluoresce bright green; live cells with intact membranes will exclude the dye. Best results are obtained in buffers that do not contain phosphate. SYTOX Green stain, at concentrations ranging from 10 nM to 1 µM, should be added to test aliquots of live and dead cells. In initial experiments, it may be best to try several dye concentrations over this suggested range, to determine the concentration that yields optimal staining.

Prepare samples of live cells as well as of dead cells on glass coverslips. Cells may be killed using any preferred method (e.g., treatment with 0.1% saponin for 10 minutes, 0.1–0.5% digitonin for 10 minutes, 70% methanol for 30 minutes or complement and the appropriate IgG for 30 minutes). Prepare samples with various ratios of dead and live cells. Allow 5 minutes or more for staining of cells to reach completion. The fraction of dead cells under various experimental conditions may be observed in a fluorescence microscope or quantitated in a fluorescence microplate reader using a standard fluorescein filter set.

## Additional Information

Researchers are encouraged to consult the primary literature for further information. A list of cell types that have been used in calcein AM–based cell adhesion assays and literature citations is presented below:

- BSC-1 (African green monkey kidney) [6]
- CEM (human T cell) [11]
- CHO (chinese hamster ovary) [12]
- HAEC (human aortic endothelial) [8]
- HIMEC (human intestinal microvascular endothelial) [13]
- HL-60 (human neutrophil) [2,13]
- HSB$^{c}$ (human T cell) [11]
- Human foreskin fibroblasts [1]
- Human foreskin keratinocytes [1]
- Human peripheral lymphocytes [1]
- Human peripheral monocytes [10,14]
- Human peripheral neutrophils [9]
- HUVEC (human umbilical endothelial) [8,9,13]
- Jurkat (human T cell) [8,11,15]
- LTK$^{-}$, LB7$^{+}$ (murine T cell hybridoma) [16]
- N9 (mouse microglial) [14]
- Primary rat microglial [14]
- THP-1 (human monocyte) [8]

## Notes

**[A]** To measure the percentage of adherent cells, assays of total cells must be performed. Total cells can be assayed with this protocol by using a *separate* microplate, omitting the wash steps (steps 8 and 9) and adding 100 µL of PBS to each sample just prior to measuring fluorescence (step 10).

## References

**1.** J Immunol Methods 178, 41 (1995); **2.** J Biol Chem 270, 21129 (1995); **3.** J Immunol Methods 172, 25 (1994); **4.** J Cell Biol 130, 745 (1995); **5.** J Cell Biol 127, 1129 (1994); **6.** Proc Natl Acad Sci USA 90, 5700 (1993); **7.** J Immunol Methods 172, 115 (1994); **8.** J Immunol Methods 163, 181 (1993); **9.** J Biol Chem 269, 10008 (1994); **10.** J Immunol 156, 1638 (1996); **11.** J Immunol Methods 192, 165 (1996); **12.** J Cell Biol 134, 1531 (1996); **13.** J Immunol 156, 2558 (1996); **14.** Nature 382, 716 (1996); **15.** J Immunol Methods 175, 59 (1994); **16.** J Biol Chem 271, 9403 (1996).

## Product List

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

| Cat # | Product Name | Unit Size |
|---|---|---|
| V-13181 | Vybrant™ Cell Adhesion Assay Kit | 1 kit |

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