



## Efferocytosis Assay Kit

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Item No. 601770

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## GENERAL INFORMATION

### Materials Supplied

Item Number	Item	Quantity/Size	Storage
600121	CFSE Stock Solution	1 vial/100 µl	-20°C
601771	CytoTell™ Blue Stock Solution	1 vial/25 µl	-20°C
601772	Staurosporine Apoptosis Inducer	1 vial/100 µl	-20°C
10009322	Cell-Based Assay Buffer Tablet	1 ea	RT

NOTE: CytoTell™ Blue is a product of AAT Bioquest, Inc

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.



**WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.**

## Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

## Precautions

**Please read these instructions carefully before beginning this assay.**

## If You Have Problems

### Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

## Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

## Materials Needed But Not Supplied

1. Phagocytic cells and bait cells (we have had success with PMA-differentiated THP-1 cells and human neutrophils), as well as culture medium appropriate for those cells.
2. Flow cytometer or fluorescence microscope equipped with lasers and filters capable of exciting and detecting the wavelengths shown in Table 1, on page 7.
3. A source of pure water; glass distilled water or deionized water acceptable. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*
4. A plate centrifuge.
5. FACS tubes or v-bottom staining plates.

## Background

Efferocytosis is a specialized form of phagocytosis in which macrophages and other phagocytic cells clear dead and dying cells to promote homeostasis, embryonic development, regulation of the immune system and the resolution of inflammation.<sup>1</sup> Efficient clearance of cells is essential for the removal of the nearly 1 million cells per second undergoing apoptosis in a human adult.<sup>2</sup> Efferocytic removal of apoptotic cells before they become necrotic and release their contents can prevent an immune response to these non-pathogenic antigens.

Macrophages recognize apoptotic cells *via* “eat me” signals, such as externalized phosphatidylserine, which signals through a variety of receptors on the macrophage cell surface. Some intracellular pathogens can circumvent an immune response by induction of these “eat me” signals, stimulating the non-inflammatory process of efferocytosis.<sup>2</sup> On the other hand, efferocytosis has also been implicated in antigen cross-presentation. Engulfment and digestion of infected apoptotic cells provides a significant source of external antigens, which are normally processed and presented to MHC class II, to instead be displayed to cytotoxic T-cells by MHC class I. This pathway is vital to the ability of the immune system to clear some pathogens such as *M. tuberculosis*.<sup>3</sup> Annexin A1 and other specialized pro-resolving mediators, such as Resolvin E1 and Protectin D1, have been shown to upregulate efferocytosis, promoting the resolution of inflammation.<sup>3,4</sup>

## About This Assay

Cayman’s Efferocytosis Assay Kit employs CytoTell™ Blue to visualize effector (phagocytic) cells, CFSE to visualize bait (apoptotic) cells and staurosporine, a protein kinase C inhibitor, to induce apoptosis. The Efferocytosis Assay Kit is a convenient tool to study modulators of efferocytosis, apoptosis, pro-resolving mediators and cross-presentation. The reagents provided in this kit are sufficient to stain  $5 \times 10^7$  effector cells and  $2 \times 10^8$  bait cells.

Reagent	Ex/Em Filters	Flow cytometric laser/filter sets	What it stains
CFSE	493/518	488/525	Bait (apoptotic) cells
CytoTell™ Blue	403/454	405/450	Effector (phagocytic) cells

**Table 1. Dye combination included in this kit and optimal detection methods for fluorescence microscopy and flow cytometry.**

## Preparation of Labeling Reagents

### Assay Buffer Preparation

Dissolve each Cell-Based Assay Buffer Tablet (Item No. 10009322) with 100 ml of pure water. Mix well to ensure that the tablet dissolves completely. Sterile filter the buffer through a 0.2  $\mu\text{m}$  filter. The buffer is stable for one year at room temperature.

### CFSE Bait Cell Staining Solution (2X) Preparation

Prepare a 2X CFSE Bait Cell Staining Solution by diluting the CFSE Stock Solution (Item No. 600121) 1:200 with assay buffer. Prepare 1 ml of solution for every  $1 \times 10^7$  cells to be stained. This 2X CFSE Bait Cell Staining Solution is stable for one hour at room temperature. *Protect from light. If multiple uses of the CFSE Stock Solution are anticipated, make smaller aliquots and store at  $-20^\circ\text{C}$  to minimize freeze-thaw cycles.*

### CytoTell™ Blue Effector Cell Staining Solution (2X) Preparation

Prepare a 2X CytoTell™ Blue Effector Cell Staining Solution by diluting the CytoTell™ Blue Stock Solution (Item No. 601771) 1:200 with assay buffer. Prepare 1 ml of solution for every  $1 \times 10^7$  cells to be stained. This 2X CytoTell™ Blue Effector Cell Staining Solution is stable for one hour at room temperature. *Protect from light. If multiple uses of the CytoTell™ Blue Stock Solution are anticipated, make smaller aliquots and store at  $-20^\circ\text{C}$  to minimize freeze-thaw cycles.*

## Labeling of Bait Cells with CFSE

*Perform all steps under sterile conditions.*

1. Obtain cells that will be stimulated to undergo apoptosis.
2. Centrifuge the cells at  $300 \times g$  for five minutes and aspirate the supernatant.
3. Wash cells with assay buffer and centrifuge as above to remove serum.
4. Resuspend to  $1 \times 10^7$  cells/ml in assay buffer in a 50 ml conical tube.
5. Add an equal volume of 2X CFSE Bait Cell Staining Solution to the cell suspension, being sure to add the entire volume quickly, and mix immediately. The final dilution of CFSE is 1:400.
6. Incubate in a  $37^\circ\text{C}$  water bath, protected from light, for 30 minutes. Invert the tube occasionally to make sure there is even labeling of cells with CFSE.
7. Add 25 ml of media containing 10% FBS and centrifuge the cells at  $300 \times g$  for five minutes.
8. Aspirate the supernatant and repeat wash step two more times.
9. Cells are now ready to undergo apoptosis.

## Inducing Apoptosis in Bait Cells

*NOTE: It is recommended to label bait cells with CFSE prior to inducing apoptosis.*

1. Dilute Staurosporine Apoptosis Inducer (Item No. 601772) 1:1000 in complete medium and incubate cells at 37°C for 3-6 hours.
2. After incubation, centrifuge the cells at 300 x g for five minutes and aspirate the supernatant.
3. Wash cells with 10-20 ml of media and centrifuge as above. Repeat wash step a second time.
4. Cells are now ready for the efferocytosis assay.
5. **OPTIONAL:** Primary human neutrophils can be induced to undergo apoptosis by culturing in serum-free RPMI overnight at 37°C. Washing after the overnight incubation is not necessary.

## Labeling of Effector Cells with CytoTell™ Blue

*NOTE: If using differentiated THP-1 cells as effector cells, it is recommended to stain with CytoTell™ Blue prior to differentiation.*

1. Obtain phagocytic cells to be used as effector cells.
2. Centrifuge the cells at 300 x g for five minutes and aspirate the supernatant.
3. Wash cells with assay buffer and centrifuge as above to remove serum.
4. Suspend effector cells to  $1 \times 10^7$  cells/ml in assay buffer.
5. Add an equal volume of 2X CytoTell™ Blue Effector Cell Staining Solution to the cell suspension, being sure to add the entire volume quickly and mixing immediately. *NOTE: If cells are already adherent, alternatively aspirate medium, wash once with PBS and add a 1X CytoTell™ Blue Effector Cell Staining Solution. However, staining with CytoTell™ Blue may cause some cell types to become dislodged. If this occurs it is recommended to repeat with staining prior to differentiation as recommended above.*
6. Incubate in a 37°C water bath, protected from light, for 30 minutes. Invert the tube occasionally to make sure there is even labeling of cells with CytoTell™ Blue.
7. Add 25 ml of media containing 10% FBS and centrifuge the cells at 300 x g for five minutes.
8. Aspirate the supernatant and repeat wash step two more times.
9. Resuspend in appropriate complete medium for differentiation or incubation until starting your efferocytosis assay.
10. Seed cells in plates to be used in your final assay. **Optional:** To differentiate labeled THP-1 cells, resuspend cells in complete medium with 200 nM PMA (Item No. 10008014), seed at desired density into 24 well plate and incubate 2-3 days at 37°C. *NOTE: If analyzing by flow cytometry, it is recommended to leave at least one well without effector cells to be used as a compensation/gating control.*

## Determination of Optimal Bait:Effector Cell Ratio

*NOTE: This protocol is designed for use in a 24-well plate. For different vessel sizes, adjust volumes accordingly.*

1. Replace differentiation media with 0.5 ml of culture media.
2. Seed CytoTell™ Blue labeled (and differentiated if necessary) effector cells in a 24-well plate at a density of  $2.5 \times 10^5$  cells/well in 500  $\mu$ l of culture media.
3. Dilute CFSE labeled, apoptotic bait cells to a density of  $2 \times 10^6$  cells in media.
4. Add bait cells to the effector cells at a various bait:effector ratios. An example is shown in table 2 below.
5. Follow the Assay Procedure on page 13 starting at step 3.

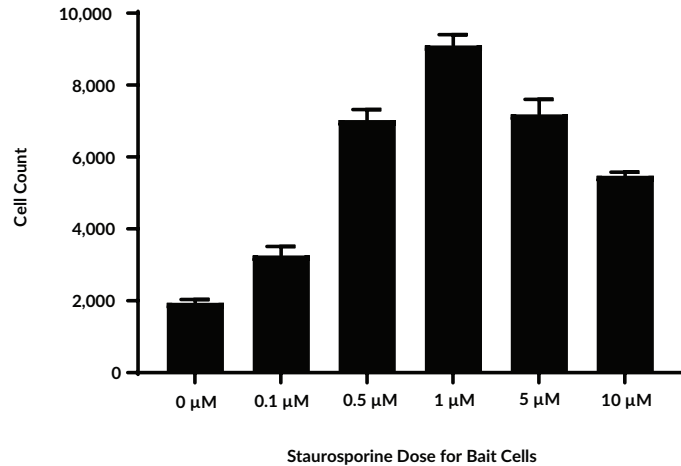
Bait:Effector Ratio	Effector Cell Suspension	Bait Cell Suspension	Bait Cell Medium	Final Volume
No Bait Control	0.5 ml	0 ml	0.5 ml	1 ml
1:1	0.5 ml	0.125 ml	0.375 ml	1 ml
2:1	0.5 ml	0.25 ml	0.25 ml	1 ml
4:1	0.5 ml	0.5 ml	0 ml	1 ml
Bait Only Control	0 ml	0.5 ml	0.5 ml	1 ml

**Table 2.** Example dilution scheme for finding the optimal bait:effector cell ratio based on a 24-well plate.

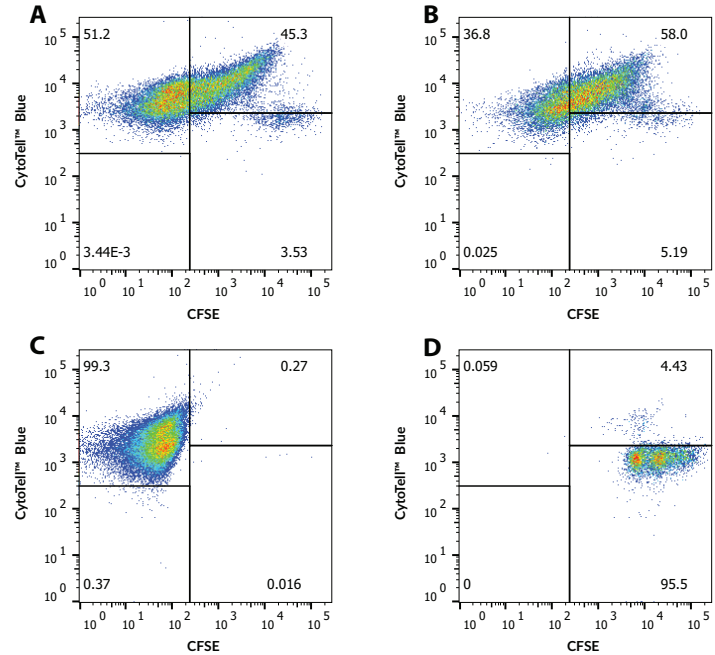
## Assay Procedure

1. Treat cells with or without experimental compounds to be used in your assay.
2. Dilute bait cells according to the ratio determined previously on page 12 under the Determination of Optimal Bait:Effector Cell Ratio section and add the bait cells to the effector cells. *NOTE: If analyzing by flow cytometry, it is recommended to have at least one well of each cell type alone to be used as compensation/gating controls.*
3. Incubate 6-24 hours at 37°C.
4. At the end of the incubation, carefully aspirate media.
5. Gently wash with 500  $\mu$ l of assay buffer and aspirate.
6. For flow cytometry: Add 500  $\mu$ l of assay buffer and triturate or gently scrape cells to remove adherent effector cells from the plate. Collect cells into a v-bottom plate or FACS tubes and collect events using the laser lines shown in Table 1, on page 7.
7. For fluorescence microscopy: Add 500  $\mu$ l of assay buffer and image cells using the filter sets shown in Table 1, on page 7.

## Performance Characteristics

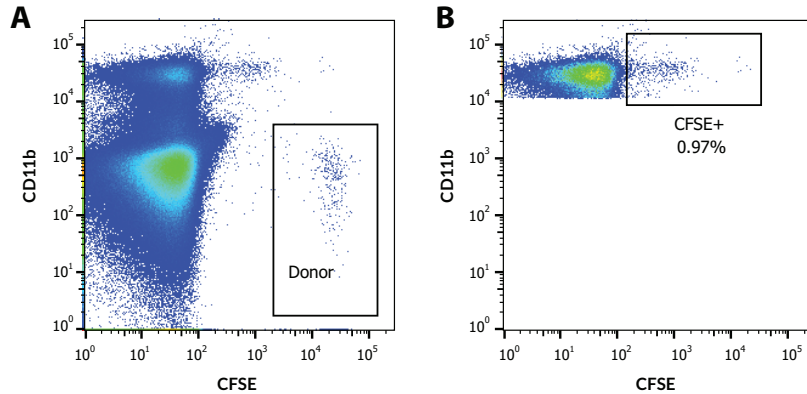


**Figure 1. Staurosporine dose response curve using J774A.1 effector and Jurkat bait cells.** Jurkat cells were stained with CFSE according to the protocol outlined in this kit booklet before being treated with indicated concentrations of staurosporine for three hours at 37°C. Cells were then fed to CytoTell™ Blue labeled J774A.1 cells at a ratio of 2:1 (bait:effector) and incubated overnight at 37°C before processing for flow cytometry following the protocol outlined in this kit booklet. For analysis, cells were gated on CytoTell™ Blue positivity before counting the number of events that were also CFSE positive.



**Figure 2. Neuraminidase upregulates efferocytosis in differentiated THP-1 cells.** THP-1 effector cells were stained with CytoTell™ Blue before differentiating with 200 nM PMA for 72 hours at 37°C. Human neutrophil bait cells were stained with CFSE and incubated overnight at 37°C in serum-free RPMI. The following day, the bait cells were collected and pre-treated with 1 U/ml neuraminidase for 1 hour at 37°C before being fed to effector cells at a ratio of 2:1 (bait:effector). The cells were incubated together overnight at 37°C before processing for flow cytometry. For analysis, cells were first gated on CytoTell™ Blue positivity (control: *Panel C*) before counting the number of events that are also CFSE positive (control: *Panel D*). Neuraminidase-treated cells (*Panel B*) show an increase in CFSE positivity as compared with untreated cells (*Panel A*).





**Figure 3. Apoptotic thymocytes are taken up by myeloid cells *in vivo*.** Thymocytes from naïve mice were labeled with CFSE and cultured overnight in 250 nM staurosporine.  $1 \times 10^7$  cells were then transferred i.v. into naïve syngeneic recipients, which were euthanized 3 hours later. Splenocytes were dissociated, stained for myeloid markers, and subjected to flow cytometry. Donor cells are identified in *Panel A* as non-myeloid (CD11b<sup>-</sup>), CFSE<sup>bright</sup> cells, while cells which have efferocytosed those apoptotic cells (*Panel B*) are CD11b<sup>+</sup>, CFSE<sup>mid</sup>.

## RESOURCES

### Troubleshooting

Problem	Possible Causes	Recommended Solutions
Loss of effector cells	Carryover staurosporine has killed cells	Wash bait cells well before adding to effectors
No uptake of apoptotic cells	Bait cells are not apoptotic	A. Titrate staurosporine B. Test apoptosis using the Annexin V APC Kit (Item No. 601410)
No uptake of apoptotic cells	Effector cells are not phagocytic	Try different effector cells or differentiation procedure

## References

1. Korn, D., Frasn, C., Fernandez-Boyanapalli, R. *et al.* Modulation of macrophage efferocytosis in inflammation. *Front. Immunol.* **2(57)**, 1-10 (2011).
2. Karaji, N. and Sattentau, Q.J. Efferocytosis of pathogen-infected cells. *Front. Immunol.* **8(1863)**, 1-10 (2017).
3. Tzelepis, F., Verway, M., Daould, J., *et al.* Annexin 1 regulates DC efferocytosis and cross-presentation during *Mycobacterium tuberculosis* infection. *J. Clin. Invest.* **125(2)**, 752-768 (2015).
4. Schwab, J.M., Chiang, N., Arita, M., *et al.* Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature* **447(7146)**, 869-874 (2007).

## NOTES

### Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

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