

**SerPase<sup>™</sup> Kits:**  
*Detection of Active Serine  
Proteases*

**Cat. No.**

**IMI-2301-25, IMI-2301-100: FAM-Phe-CMK (FFCK)**  
**IMI-2303-25, IMI-2303-100: TR-Phe-CMK (TRFCK)**



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**Note: Please read through the entire manual before beginning any experiments.**

# 1. Introduction

Serine proteases, defined by the presence of a serine residue in their active domain, belong to a multigene family of proteins that are involved in the post-translational processing of many polypeptides (reviewed in 1). These proteases have central roles in regulating a wide range of physiological processes, including digestion, coagulation, malignancy, inflammation, fertilization, and development.

## 1a. Serine Protease Activation

Serine proteases, like other proteases, are synthesized as larger pro-enzyme or zymogen inactive precursor forms. During activation the pro-enzyme is cleaved resulting in the active enzyme. The active site region of serine protease enzymes typically includes a serine, a histidine, and an aspartate residue, although some members may utilize glutamic or lysine groups. Serine proteases differ in their substrate specificity through variations in the amino acid composition of the S1 binding region of the enzyme.

Activated serine proteases play key roles in regulating and amplifying signal transduction cascades by proteolytically activating inactive zymogen precursors. Often, the protease substrates in the signaling cascades are the inactive form, or zymogen, of a downstream serine protease. Examples of this type of serine protease-mediated regulation include blood coagulation, kinin formation, and the complement system.

The chymotrypsin-like serine protease subfamily, which selectively hydrolyzes peptide bonds often C-terminal to a basic amino-acid residue, have well-documented roles in the digestive process. However, a growing body of evidence indicates that they also have a pivotal role in a number of diverse physiological processes including the maintenance of homeostasis, inflammation and immune reactions, apoptosis, and cell survival. For example, analogous to caspases, serine proteases are involved in protein degradation during apoptosis (2-4). Caspase inhibitors have been instrumental in defining apoptosis pathways, and analogously inhibitor technology is also emerging as a powerful research tool for elucidating the role of serine proteases in apoptosis. Data using the chymotrypsin serine protease inhibitor 1-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) suggests that a chymotrypsin-like protease mediates caspase independent apoptosis-like cell death or cell death that can not be fully blocked by the broad spectrum caspase inhibitor Z-VAD-FMK (4). Additionally, TPCK has been shown to induce apoptosis by blocking  $\text{I}\kappa\text{B}\alpha$  degradation; blocking  $\text{I}\kappa\text{B}\alpha$  degradation prevents NF- $\kappa\text{B}$  activation and can result in cell death through loss of cell survival signals (5).

## 1b. Principles of the SerPase™ Assays

SerPase™ Kits are research tools for measuring chymotrypsin-like serine protease activation. The serpase methodology is based on fluorochrome-labeled, cell permeable inhibitors of chymotrypsin-like serine proteases (2 and 3). The inhibitors in the SerPase™ Kits are fluorochrome-labeled analogs of N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). TPCK is an inhibitor that covalently binds to the active centers of chymotrypsin-like enzymes. The kits contain either carboxyfluorescein (FFCK) or Sulforhodamine 101 [also know at Texas-Red™ (TRFCK)] labeled inhibitor to measure green or red fluorescence, respectively. [Note: the FFECK and TRECK terminology represents the current nomenclature of “F” for phenylalanine as opposed to “P” which was used when the TPPCK inhibitor was originally described (2)].

The use of labeled inhibitors as activation markers, first described for measuring caspase activity (6), is an emerging, cutting-edge technology (2, 3, 6). A key advantage of this technology for intracellular flow cytometry and fluorescence microscopy is the cell membrane permeability of these inhibitors. FFCK and TRFCK inhibitors are designed to be used with living cells and do not require any cellular fixation or permeabilization steps (2,3). The nomenclature “serpase” was first defined in analogy to caspases to describe serine proteases that are activated during apoptosis (2). However, when compared to caspases much less information is known about apoptosis-associated serpases (2-4). The SerPase™ Kits should be useful research tools for helping to elucidate the roles of serine proteases in apoptosis, cell survival, and other signal transduction pathways.

The SerPase™ Kits can be used with both suspension and adherent cells. When added to a population of cells, the fluorescently labeled inhibitor [FFCK or TRFCK] diffuses into the cells and covalently binds to active sites of proteases (2). Bound inhibitor is retained within the cell, and unbound inhibitor that diffuses out of the cell is washed away. The remaining fluorescent signal is a measure of the active proteases that were present in the cell at the time the FFCK or TRFCK inhibitor was added.

FFCK and TRFCK have been used to detect serine protease activation in live cells by flow cytometry (2,3), fluorescence microscopy (2,3) and 96-well microtiter plate fluorometry. Cell lysates prepared from FFCK and TRFCK labeled cells have also been used to detect FFCK-reactive proteins by western blot using anti-FITC second step antibodies (3). For optimal results, cells labeled with the FFCK and TRFCK reagents should be read immediately; they may also be preserved until the following day using the fixative provided with the kits.

## 2. FFCK and TRFCK SerPase™ Kits

Each Kit contains either an FFCK [IMI-2301-25/-100] or a TRFCK [IMI-2303-25/-100] labeled inhibitor to measure green or red fluorescence, respectively and essential Wash Buffer. The Kits also contain Fixative and Hoechst Stain for optional use. Additionally, the FFCK Kits contain Propidium Iodide, also for optional use. The Kits are available in both 25 Test [IMI-2301-25 and IMI-2303-25] and 100 Test [IMI-2301-100 and IMI-2303-100] sizes. For suspension cells, a test size is defined as a 500 µl cell suspension volume. For adherent cells, a test size is defined as a 500 µl overlay media volume. The actual amount of tests obtained from the kits may vary depending on the experimental model system used.

This Manual is designed for use with both the FFCK and TRFCK SerPase™ Kits and contains suggested protocols for using the SerPase™ Kits to measure activated serine proteases in several assays, including flow cytometry, fluorescence microscopy, and microtiter plate fluorometry. Every lot of the SerPase™ Kits is quality control tested using apoptosis as a model system for serine protease activation. Researchers may want to use an apoptosis model system as a positive control when evaluating their systems for serine protease activation. A protocol to induce apoptosis and sample data from HeLa [human cervical carcinoma (ATCC: CCL-2)], Jurkat (T cell leukemia, ATCC: TIB 152), and RAW 264.7 (ATCC: TIB-71) is provided. These three cell types were selected because many labs have access to one or more of them, or they can readily be ordered from the ATCC ([www.atcc.org](http://www.atcc.org)).

### 2a. Kit Contents and Storage

Serpase™ Kits are shipped on ice packs or on dry ice for overseas destinations. Store the unopened vials of the TRFCK and FFCK Reagents at  $\leq -20^{\circ}\text{C}$  for up to 18 months; protect from light at all times. Store the other Kit reagents at  $2^{\circ}\text{C}$  to  $8^{\circ}\text{C}$ . Refer to the Reagent Preparation section for additional information on reagent storage conditions following reconstitution and dilution.

#### Catalog Numbers:

##### Green Fluorescence

IMI-2301-25: FAM-Phe-CMK (FFCK), 25 Test Size

IMI-2301-100: FAM-Phe-CMK (FFCK), 100 Test Size

##### Red Fluorescence

IMI-2303-25: \*Sulforhodamine 101-Phe-CMK (TRFCK), 25 Test Size

IMI-2303-100: \*Sulforhodamine 101-Phe-CMK (TRFCK), 100 Test Size

\*Sulforhodamine 101 is also known as Texas Red™

## Kit Contents:

### **IMI-2301-25, FAM-Phe-CMK (FFCK), 25 Test Size**

IMI-0107	FFCK Reagent, lyophilized (1 vial)
IMI-0103	10X Wash Buffer, 15 ml
IMI-0104	Fixative, 6 ml
IMI-0106	Hoechst Stain, 1 ml
IMI-0105	Propidium Iodide, 1 ml

### **IMI-2301-100, FAM-Phe-CMK (FFCK), 100 Test Size**

IMI-0107	FFCK Reagent, lyophilized (4 vials)
IMI-0102	10X Wash Buffer, 60 ml
IMI-0104	Fixative, 6 ml
IMI-0106	Hoechst Stain, 1 ml
IMI-0105	Propidium Iodide, 1 ml

### **IMI-2303-25, Sulforhodamine 101-Phe-CMK (TRFCK), 25 Test Size**

IMI-0108	TRFCK Reagent, lyophilized (1 vial)
IMI-0103	10X Wash Buffer, 15 ml
IMI-0104	Fixative, 6 ml
IMI-0106	Hoechst Stain, 1 ml

### **IMI-2303-100, Sulforhodamine 101-Phe-CMK (TRFCK), 100 Test Size**

IMI-0108	TRFCK Reagent, lyophilized (4 vials)
IMI-0102	10X Wash Buffer, 60 ml
IMI-0104	Fixative, 6 ml
IMI-0106	Hoechst Stain, 1 ml

## 2b. Safety Information

Use gloves while handling the FFCK and TRFCK reagents, Propidium Iodide, Hoeschst Stain and Fixative.

Dispose of all liquid components down the sink and flush with copious amounts of water. Solid components may be disposed of as regular trash.

## 2c. Materials, Equipment, and Instrumentation

In addition to the reagents provided in the Serpase™ Kits, the following materials and equipment are also required or recommended depending on the model system or method of detection utilized.

- Cultured cells with media
- Reagents to induce serine protease activation
- Amber vials or polypropylene tubes for storage of 250X concentrate at  $\leq -20^{\circ}\text{C}$ , if aliquoted

- 150 ml or 600 ml graduated cyclinder
- Slides
- Hemocytometer
- Clinical centrifuge at <400 X g
- 37°C CO<sub>2</sub> incubator
- Pipette(s) capable of dispensing at 10µl, 50µl, 200µl, 300µl, 1ml
- Phosphate Buffered Saline (PBS) pH 7.4, up to 100 ml needed
- Dimethyl Sulfoxide (DMSO), 50µl or 200µl needed
- Ice or 4°C refrigerator to store cells

The instrumentation needs and settings vary according to the technique, fluorochromes, and dyes used. Please refer to the **Reagent Preparation** and section for details regarding excitation and emission ranges for the fluorochromes and dyes used in the Serpase™ Kits, and to the **Cell Analysis** section for information on settings.

1. Flow cytometer equipped with appropriate excitation laser and filters.
2. Fluorescence microscope with appropriate filters.
3. 96-well fluorescence plate reader and black round or flat bottom well microtiter plates

### 3. Reagent Preparation

The FFCK and TRFCK reagents are prepared first as Stock Solutions. A 50X Working Solution is prepared immediately prior to use. The Wash Buffer is diluted from a 10X concentrate to 1X prior to use. The Fixative, Hoechst Stain, and Propidium Iodide are diluted by adding directly to cell suspensions or cell overlay solution.

#### 3a. FFCK (FCK) and TRFCK (FCK) Reagents.

The FCK reagents [FFCK (IMI-2301-25/-100) and TRFCK (IMI-2303-25/-100)] reagents are supplied as concentrated lyophilized powders. Unopened vials may be stored at -20° C for up to 18 months. FCK reagents are first prepared as a Stock Solution and then used as a Working Solution. The lyophilized FFCK or TRFCK reagents should be reconstituted to a Stock Solution just prior to use or freezing. The Working Solution, prepared from the Stock Solution, must be used as quickly as possible after preparation (ideally within 30 min) and should not be stored.

##### 3a.1. 250X FCK Stock Solution

- A. Dissolve one vial of the lyophilized FCK reagent in 50 µl (0.050 ml) of DMSO to yield an FFCK or TRFCK 250X Stock Solution. Mix thoroughly, around the base and lower sides of the vial to

ensure complete solubilization of the FCK reagent.

- B. The Stock Solution must be used immediately by diluting into a Working Solution (see below) or DMSO reconstituted vials of FCK reagents may be stored for up to 1 year at -80° C.
- C. Avoid repeated freeze/thaws of individual vials of FCK Stock Solutions as this may compromise its integrity. Hence we recommend that when making vials of FCK Stock Solution for freezing, each aliquot contains only the amount that will likely be needed at the time of thawing.

### **3a.2. 50X FCK Working Solution for Immediate Use.**

Add 200 µl (0.2 ml) of PBS, pH 7.4, to the 250X FCK Stock Solution to yield a 50X FCK Working Solution (e.g. 4 µl PBS/ 1µl FCK Stock solution). Use immediately, e.g., add 10 µl (0.010 ml) of 50X FCK reagent to every 490 µl (0.490 ml) of cell suspension or cell culture media in the case of adherent cells. Discard left over FCK Working Solution.

- a. FFCK (FAM-Phe-CMK): optimal excitation at 488 +/- 10 nm and emission  $\geq$ 520 nm
- b. TRFCK (TR-Phe-CMK): optimal excitation at 590 +/- 10 nm and emission  $\geq$ 610 nm

**Note:** The Working Solution dilution is a general guideline, researchers may need to optimize the amount of FCK reagents for their particular cell line and model system.

### **3b. Wash Buffer.**

The Wash Buffer is supplied as a 10X concentrate which is diluted to 1X with DI H<sub>2</sub>O prior to use.

1. If necessary, gently warm the 10X concentrate to completely dissolve any salt crystals that may have formed during storage.
2. For the 25 Test Size FCK Kits (IMI-2301-25 and IMI-2303-25): Add the entire contents of the 10X wash buffer (15 ml) to 135 ml of DI H<sub>2</sub>O, making a total of 150 ml of 1X Wash Buffer.
3. For the 100 Test Size FCK Kits (IMI-2301-100 and IMI-2303-100) Add the entire contents of the 10X Wash Buffer (60 ml) to 540 ml of DI H<sub>2</sub>O, making a total of 600 ml of 1X Wash Buffer.
4. The 1X Wash Buffer may be stored for up to 30 days at 2°-8°C. If any salt crystals form, warm to room temperature and stir 5 min until all crystals have dissolved.

### **3c. Fixative**

The Fixative is supplied as a 10X concentrate of a formaldehyde solution. Fixing cells is an optional step. The Fixative is added directly to cells at a volume that will give a 1:10 dilution. For example, 50 µl

(0.050 ml) of Fixative to 450  $\mu$ l (0.450 ml) of cell suspension or overlay medium for suspension cells. Fixative should not be added to cells that will subsequently be counterstained with Propidium Iodide or Hoechst dyes.

### **3d. Hoechst Stain**

Hoechst Stain is provided ready-to-use at a concentration of 200  $\mu$ g/ml. It can be used to label the nuclei of cells at the end of the FCK incubation step. Do not fix cells prior to staining with Hoechst.

1. Hoechst Stain can be used with FFCK or TRFCK reagents for two-color analysis in fluorescence microscopy.
2. The Hoechst solution is added to the suspended cells or adherent monolayer at a 0.5% v/v amount. For example, 2.5  $\mu$ l of Hoechst solution is added to 500  $\mu$ l (0.5 ml) of suspended cells or overlay medium for adherent monolayer cells.
3. After a 5 min incubation with Hoechst Stain, cells are washed in Wash Buffer to remove excess dye.
4. Hoechst Stain is detected using a UV-filter with excitation at 365 nm and an emission at 480 nm.

### **3e. Propidium Iodide**

Propidium Iodide (PI) (IMI-2301-25/-100) is supplied ready-to-use at a concentration of 250  $\mu$ g/ml. PI is a vital dye that binds to double-stranded DNA. It crosses the membrane of non-viable cells, including late apoptotic, necrotic, and other membrane compromised cells and complexes with DNA in the nucleus. PI is used to distinguish between live cells and dead cells. In general, cells in the early and mid stages of apoptosis exclude PI, whereas cells later in the process have compromised membranes and hence stain with PI. PI is added at the end of the FCK labeling step. Do not fix cells prior to staining with PI.

1. PI can be used with the FFCK reagent for two color analysis in flow cytometry.
2. The PI solution is added to suspended cells at a 0.5% v/v amount. For example, 2.5  $\mu$ l of PI is added to 500  $\mu$ l (0.5 ml) of suspended cells or overlay medium for adherent monolayer cells.
3. After a 5 min incubation with PI, cells can be washed in Wash Buffer.
4. PI has a broad excitation range, 488 to 492 nm will provide enough excitation (the optimal excitation is 535 nm) and has an emission maximum at greater than 610 nm (peak emission is 617 nm).

## 4. Methods for Culturing and Staining Cells

The Serpase™ Kits are compatible with various cell model systems and treatment protocols. The Kits can be used with both adherent and suspension cells.

### a. Staurosporine Treatment Protocol

The positive control sample data shown in this manual was generated from staurosporine treated cultures. Staurosporine (*Streptomyces staurospores*) is a relatively non-selective kinases inhibitor, which blocks many kinases to different degrees. Staurosporine has been used extensively in the literature for inducing apoptosis.

1. Prepare a 1 mM Stock Solution of Staurosporine (e.g. Sigma 85658) in DMSO.
2. Add 1-4  $\mu\text{M}$  (final concentration) Staurosporine to cell suspension or to overlay medium.
3. Perform a time course to monitor results; a 1-16 h incubation at 37° C is suggested.
4. Proceed with assays designed to evaluate induction of serine protease activation or apoptosis.

### 4b. Suspension Cells

1. Cultivate cells under optimal growth conditions to a concentration where cells remain healthy and do not exhibit signs of overgrowth (for most cell lines, the cell concentrations will be  $\leq 1 \times 10^6$  cells per ml).
2. Split cells into two or more cultures with one culture serving as a negative control (no treatment or apoptosis induction) and the other cultures as the test samples (cells receiving treatment or apoptosis induction).
3. Treat test samples with your reagent or induce apoptosis. Continue to culture negative control and test samples for the same length of time under the same conditions as determined by your treatment.
4. Following treatment, count cells. Cell concentrations between  $0.5 \times 10^6$  to  $1 \times 10^7$  cells per ml are generally optimal for incubation with the FCK reagents. For lower initial cell counts, cells may be concentrated by centrifugation at  $< 400 \times g$  for 5 min, then resuspended in an appropriate amount of cell media to achieve the desired concentration.
5. Remove 0.490  $\mu\text{l}$  (490 ml) of each cell suspension and place into

sterile tubes. To each tube add 10  $\mu$ l (0.010 ml) of 50X FCK Working Solution. Diluting the 50X FCK reagents 1:50 in the cell suspension yields a final concentration of 20  $\mu$ M FCK reagent. This is a suggested starting concentration. The optimal FCK concentration may vary and should be determined for each individual experimental system.

6. Incubate the cells with the FCK reagent between 30 min and 2 h at 37° C, periodically resuspending the cells every 20 to 30 minutes. Incubation time will vary, depending on cell count and cell type. During the incubation period the FCK reagent enters the cell and binds irreversibly to the catalytic site of the active serine proteases.
  - a. **Note: Hoechst Stain or PI.** If cells are to be stained with Hoechst or PI, add 2.5  $\mu$ l (0.5% v/v) of the Hoechst or PI solution at the end of the FCK incubation step and incubate for an additional 5 min.
7. After incubation with the FCK reagent, gently centrifuge the cells at less than 400 x g for 5 min to pellet the cells.
8. Carefully pull off the supernatant and discard it. Resuspend the cell pellet in 1.0 ml of 1X Wash Buffer to remove any unbound FCK reagent.
9. Wash the cells two more times by repeating Steps 7 and 8.
10. Resuspend the final washed cell pellet in 0.5 ml (500  $\mu$ l) of 1X Wash Buffer or PBS.
  - a. **Note: Microtiter Plate Fluorometry:** The negative control and test samples should be adjusted to approximately the same concentration of cells per ml prior to analysis.
11. Optimally, cells should be analyzed immediately. If the cells cannot be analyzed immediately, they may be fixed by making a 1:10 v/v dilution of the 10X Fixative into the cell suspensions from Step 10 and mix. The fixed cells may be stored at 2° to 8° C protected from light until the next day at which time they should be analyzed. It is not recommended to fix cells that have been stained with PI or Hoechst dyes.

#### 4c. Adherent Cells

1. Cultivate cells under optimal growth conditions to a concentration where cells remain healthy.
2. Trypsinize or otherwise prepare healthy growing cells for splitting, and transfer to cell culture flasks, slides or chambers. Cells should be seeded at a sufficient number to provide an initial coverage of 30% to 50% confluency. Include at least one flask, slide or chamber as a negative control that will receive no treatment.
3. When the cells have reached a level between 60% and 80%

confluency, they can be treated with your reagent or induced into apoptosis. Continue to culture negative control and test samples for the same length of time under the same conditions as determined by your treatment.

4. To each monolayer of cells add an amount of the 50X FCK Working Solution to make a 1:50 dilution in the cell media (i.e. if 0.490 mL (490  $\mu$ L) of cell media is used to culture the cells, add 0.010 ml (10  $\mu$ L) of 50X FCK reagent). The final concentration of the FCK reagent will be 20  $\mu$ M. This is a suggested starting concentration. The optimal FCK concentration may vary and should be determined for each individual experimental system.
5. Incubate the cells with the FCK reagent between 30 min and 2 h at 37<sup>o</sup> C. The overlay medium containing the FCK reagent should be agitated periodically every 20 to 30 min to ensure an even labeling process.
  - a. **Note: Hoechst Stain or PI.** If cells are to be stained with Hoechst or PI, add 2.5  $\mu$ l (0.5% v/v) of the Hoechst or PI solution at the end of the FCK incubation step and incubate for an additional 5 min.
6. After FCK labeling, the adherent cells can be processed by two different methods for analysis.
  - a. Processing cells
    - A. Direct analysis of adherent monolayers. Adherent monolayers can be analyzed directly by fluorescence microscopy (proceed to Step 13)
    - B. Trypsinizing monolayers prior to analysis. Single cell suspensions are prepared for analysis by flow cytometry or microtiter plate fluorometry (proceed to Step 7).
7. Carefully aspirate the cell supernatant from the monolayer and place into a sterile centrifuge tube. Trypsinize or otherwise remove the remaining cells off of the monolayer surface and combine with the cells from the original supernatant in cell culture media containing fetal bovine serum to inactivate the trypsin enzymatic activity.
8. Gently centrifuge the cells at less than 400 x g for 5 minutes to pellet the cells.
9. Carefully pull off the supernatant and discard it. Resuspend the cell pellet in 1.0 to 2.0 mL of 1X Wash Buffer to remove any unbound FCK reagent.
10. Wash the cells two more times by repeating Steps 8 and 9.
11. Resuspend the final washed cell pellet in 500  $\mu$ l (0.5 ml) of 1X Wash Buffer or PBS.
  - a. **Note: Microtiter Plate Fluorometry.** Adjust the negative control and test samples to approximately the same concentration of cells/ml prior to transferring to the

microtiter plates.

12. If the cells cannot be analyzed immediately, they may be fixed by making a 1:10 v/v dilution of the 10X Fixative into the cell suspensions from Step 11 and mixing. The fixed cells may be stored at 2° to 8° C protected from light for up to 24 hours.
13. For direct analysis of the FCK reagent probed adherent monolayer, carefully remove the overlay medium and discard.
14. Add 1.0 to 2.0 ml of 1X Wash Buffer to the monolayer and allow the 1X Wash Buffer to sit over the monolayer for 3 to 5 minutes to remove any unbound FCK reagent.
15. Wash the adherent monolayer two more times by repeating Steps 13 and 14.
16. Following the final wash, mount a cover slip with cells facing down onto a microscope slide containing a drop of 1X Wash Buffer or remove the plastic frame of the chamber slide and add a drop of 1X Wash Buffer and cover with a cover slip. The adherent monolayer cells are now ready for analysis.
17. If the adherent monolayer cells cannot be analyzed immediately, they may be fixed by making a 1:10 v/v dilution of the 10X Fixative into 1X Wash Buffer and add one drop of this to the cell surface followed by a cover slip. The fixed slides may be stored at 2° to 8° C protected from light for up to 24 hours. It is not recommended to fix cells that have been stained with PI or Hoechst dyes.

## 5. Cell Analysis

Cells may be analyzed by flow cytometry, fluorescence microscopy or microtiter plate fluorometry.

### 5a. Flow Cytometry

Flow cytometry analysis is useful for quantitative evaluation of serpase activation in individual cells within a population.

#### 5a.1. One Color Flow Cytometry Analysis

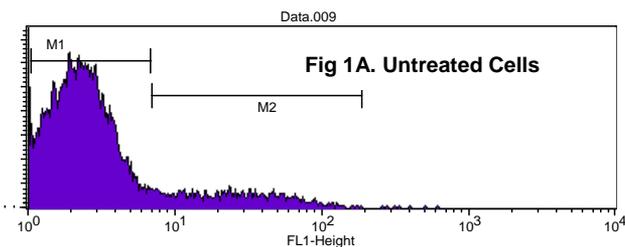
FFCK and TRFCK labeled cells may be read unfixed or fixed first and then analyzed. However, in some model systems, fixation may result in some loss of signal. The following samples are recommended:

5. Unstained cells (untreated and treated)
6. Cells stained with FFCK or TRFCK (untreated and treated)

Measure FFCK (green) fluorescence on the FL1 channel. Generate a log FL1 (X-axis) versus number of cells (Y-axis) histogram. Measure TRFCK (red) fluorescence on the FL2 channel. Generate a log FL2 (X-axis) versus number of cells (Y-axis) histogram. An example of single color flow cytometry analysis is shown in Fig. 1.

Untreated (negative control) samples. The majority of the cells in the negative control population should be FCK negative and fall within the first decade of the X axis (Fig 1A). A small population may show increased fluorescence intensity and appear as a separate peak or a shoulder of the first peak. These cells may be undergoing serine protease activation due to apoptosis or other factors. Position the vertical cursor in the gap between the two peaks. Events falling to the left of the vertical cursor (M1) should be counted as negative, whereas events falling to the right of the vertical cursor (M2) should be counted as activated serine protease positive. A large population of FCK positive cells in the negative control population may be an indication that the cell culture is not healthy. However, the actual number of FCK positive cells in untreated samples may naturally vary according to cell type, cell model system, or other factors.

Serine protease activated (positive control) samples. On the histograms, generally two cell populations represented by two peaks will be present (Fig 1B). The majority of the FCK negative cells will fall within the first log decade of the X axis (first peak, M1), whereas the FCK positive cell population will appear as a separate peak or as a shoulder of the first peak showing increased fluorescence intensity (M2). Cells with activated serine proteases will fluoresce green (FFCK, FL1) or red (TRFCK, FL2).



Sample ID: TL-3 NEG

Acquisition Date: 18-May-04

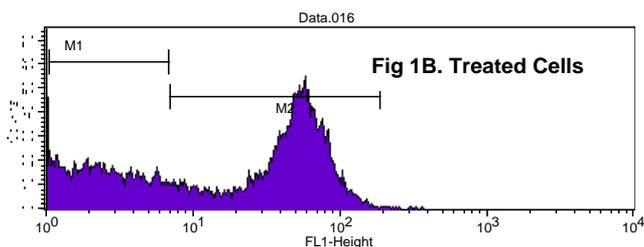
Gate: G1

Gated Events: 10026

Total Events: 10000

X Parameter: FL1-Height (Log)

Marker	% Gated	% Total	Mean
All	100.00	99.48	6.52
M1	75.30	74.91	2.51
M2	13.50	13.43	31.46



Sample ID: TL-3 POS

Acquisition Date: 18-May-04

Gate: G1

Gated Events: 9952

Total Events: 10000

X Parameter: FL1-Height (Log)

Marker	% Gated	% Total	Mean
All	100.00	98.70	29.74
M1	30.10	29.71	2.84
M2	56.28	55.55	50.44

**Figure 1. Flow cytometry analysis of serine protease activity.** (A) Untreated (negative control) and (B) staurosporine treated ( $1\mu\text{M}$ , 4 h) positive control Jurkat cells were labeled with the FFCK reagent. The cells were not fixed prior to analysis.

A comparison of flow cytometry in three different cell types treated with staurosporine and labeled with FFCK is shown in Figure 2. A comparison of flow cytometry and fluorescence microscopy with the TRFCK reagent is shown in Figure 3.

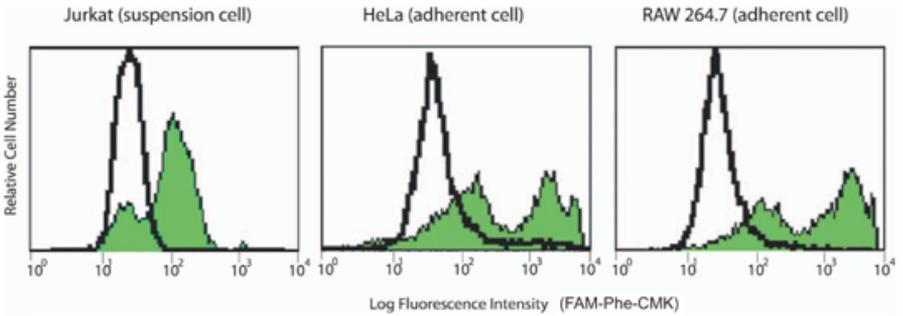


Figure 2. Flow cytometry analysis of serine protease activity in different cell types. Control (unshaded histograms) and staurosporine-treated (shaded histograms) human Jurkat and HeLa cells and mouse RAW cells were stained with the FFCK reagent. Staurosporine treatment (1  $\mu$ M): Jurkat, 3.5 h; HeLa and RAW, 16 h. The cells were not fixed prior to analysis.

### 5a.2. Two Color Flow Cytometry Analysis

For two color analysis, cells may be stained with both FFCK and PI. (PI is not compatible with TRFCK label). PI stains necrotic, dead, and membrane-compromised (late apoptotic) cells. The following samples are recommended:

1. Unstained cells (untreated and treated)
2. Cells stained with FFCK only (untreated and treated)
3. Cells stained with PI only (untreated and treated)
4. Cells stained with FFCK and PI (untreated and treated)

Measure FFCK (green) fluorescence on the FL1 channel and PI (red) fluorescence on the FL2 channel. Generate a log FL1 (X-axis) versus log FL2 (Y-axis) dot plot. Four cell populations may be present:

- i. Lower left quadrant: PI negative, FFCK negative
- ii. Lower right quadrant: PI negative, FFCK positive
- iii. Upper left quadrant: PI positive, FFCK negative
- iv. Upper right quadrant: PI positive, FFCK positive

### 5b. Fluorescence Microscopy

Fluorescence microscopy is useful for qualitative analysis of serpase activation in individual cells within a population. The inhibitor probes can be used in conjunction with Hoechst Stain for correlation of chromatin status with serpase activation. When observed by fluorescence

microscopy, FCK positive cells will appear more brightly fluorescent than negative cells.

Slide preparation. For suspension cells, place one drop of the cell suspension onto a microscope slide and cover with a cover slip. For adherent cells, mount a cover slip with cells facing down onto a microscope slide containing a drop of 1X Wash Buffer or remove the plastic frame of the chamber slide and add a drop of 1X Wash Buffer and cover with a cover slip.

### **5b.1 One and Two Color Fluorescence Microscopy Analysis**

- a. Observe cells treated with the FFCK reagent using a broad bandpass filter with an excitation at 490 nm and an emission  $\geq 520$  nm. Cells with active serine protease activity will appear green when using the FFCK reagent.
  - a. Observe cells treated with the TRFCK reagent using a broad bandpass filter with an excitation at 590 nm and an  $\geq$  than 610 nm. Cells with active serine protease activity will appear red when using the TRFCK reagent.
  - b. Hoechst stain can be observed by using a UV-filter with excitation at 365 nm and an emission at 480 nm.
  - c. Propidium Iodide (PI) can be observed using a broad bandpass filter with an excitation at 490 nm and an emission  $\geq$  than 610 nm (optimal settings would be an excitation at 535 nm and emission at 617 nm).

Examples of two color fluorescence microscopy are shown in Figs 3 and 4. Figure 3 illustrates analysis of a cell population by both flow cytometry and fluorescence microscopy. Figure 4 illustrates analysis of a cell population by both low and high magnification.

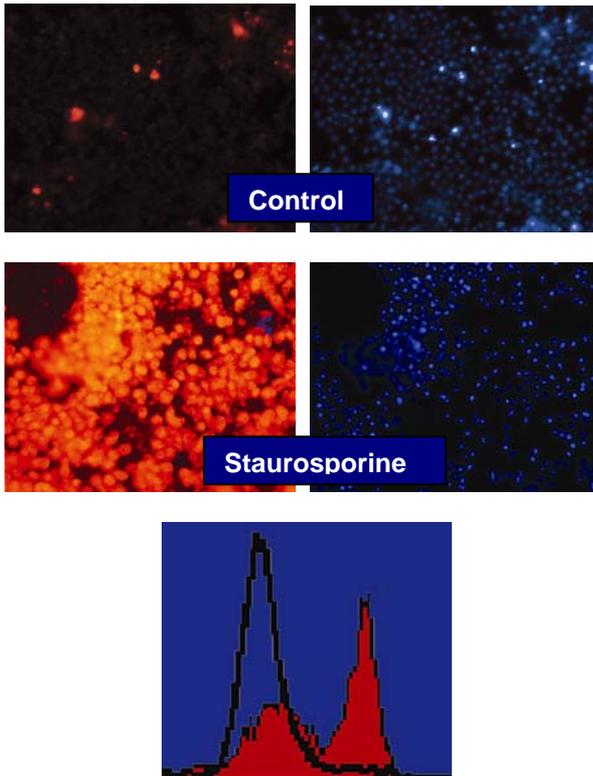
Untreated (negative control) samples. The majority of the cells in the negative control population should appear unstained or dimly fluorescent (Fig. 3 a, a', c, c'). Scattered fluorescent cells are often seen in untreated cells, and may represent serine protease activation due to apoptosis or other factors. Additionally, the basal level of fluorescence, due to serine protease activation or background staining, may vary between different cell types. However, a large population of stained cells with an apoptotic phenotype in the negative control may be an indication of unhealthy cultures.

Serine protease activated (positive control) samples. Generally variable staining intensity will be observed (Fig. 3 b, b', d, d'). Cells with activated serine proteases will fluoresce green (FFCK) or red (TRFCK). Some cells may appear brighter than others depending on the amount of

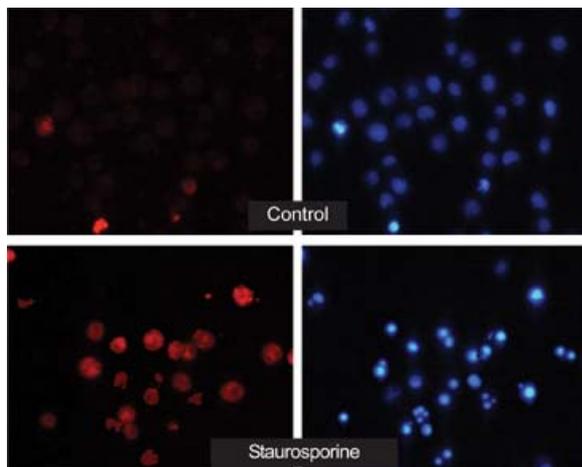
staining or status of the DNA. For example, cells with condensed chromatin generally stain brighter than cells without condensed chromatin.

In general, the amount of active serine protease enzymes capable of binding the FFCK or TRFCK inhibitor probes increases as apoptosis progresses and eventually reaches a maximal level. Therefore, cells in more advanced stages of apoptosis will appear brighter green or red than cells in earlier stages. Cells later in apoptosis will also have more highly condensed chromatin than early apoptotic cells.

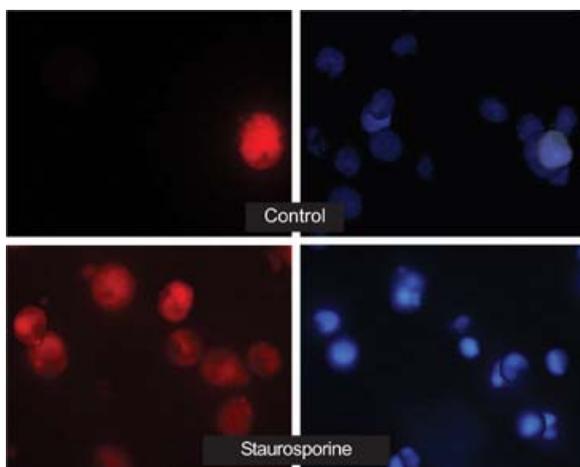
Cells lacking activated serine proteases will appear unstained or dimly fluorescent, depending on the model system or microscope settings.



**Figure 3. Cell analysis by flow cytometry and fluorescence microscopy.** Control and staurosporine treated ( $1\mu\text{M}/16\text{ h}$ ). HeLa cells were labeled with TRFCK for analysis by flow cytometry or with both TRFCK (red) and Hoechst DNA counterstain (blue) for analysis by fluorescence microscopy. Control cells: top micrographs and unshaded histogram. Staurosporine treated cells: bottom micrographs and shaded histogram in red.



**Figure 4 a, a', b, b'. Fluorescence microscopy analysis of serine protease activity.** TRFCK staining in the control population corresponded to cells with condensed chromatin (bright blue). In contrast, TRFCK stained the majority of staurosporine treated cells. (100X magnification). Condensed chromatin is a hallmark of cells undergoing apoptosis, and cells stained in the control population may represent spontaneous apoptosis. The cells were not fixed prior to analysis.



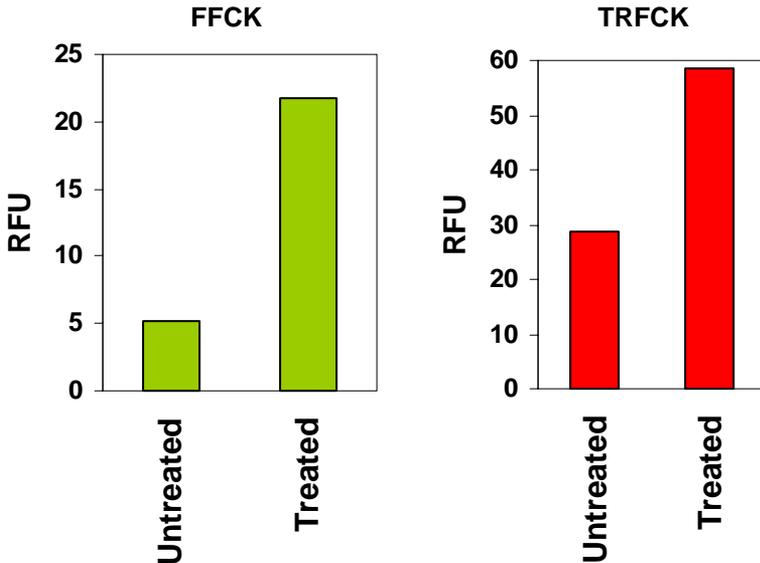
**Figure 4 c, c', d, d'. Variable FCK fluorescence in cell cultures.** Higher magnification (400X) showed that the brightness and subcellular localization of TRFCK varied between cells. The cells were not fixed prior to analysis.

### 5c. Microtiter Plate Fluorometry

Microtiter plate fluorometry is useful for quantitating serpinase activation of a cell population. Using a fluorescence plate reader (with a black microtiter plate), apoptosis can be quantitated as the amount of green or red fluorescence emitted from FFCK or TRFCK probes, respectively. Cell populations in more advanced stages of apoptosis will have a higher RFU intensity than cell populations in earlier stages.

1. Use black well plates only.
2. For a 96 well plate, transfer 0.050 ml (50  $\mu$ l) to 0.300 ml (300  $\mu$ l) of suspended cells per well. Once the volume has been optimized, deliver equal volumes to all wells for each experiment.
3. Set fluorescence plate reader to endpoint read.
4. To read cells treated with the FFCK reagent, set excitation to 488 nm and emission to  $\geq$ 520 nm.
5. To read cells treated with the TRFCK reagent, set excitation to 590 nm and emission to  $\geq$ 610 nm.

An example of analysis by microtiter plate fluorometry is shown in Figure 5.



**Figure 5. Fluorometric analysis of serine protease activation.** (A) Untreated (negative control) and (B) treated with 1  $\mu$ M staurosporine for 4h (positive control). Jurkat cells were labeled with the FFCK or TRFCK reagent. The cells were not fixed prior to analysis.

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