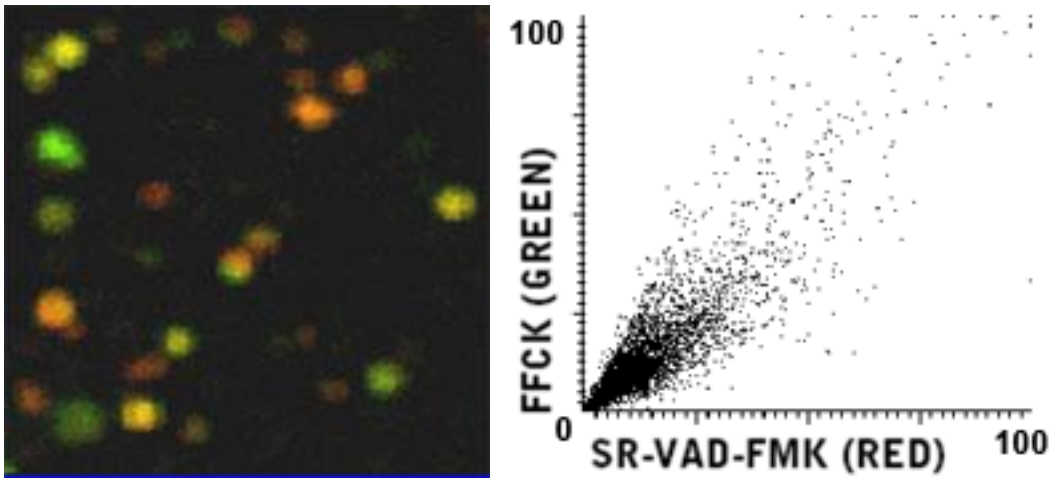
NEUROMICS

All FLISP™ (Fluorescent Labeled Inhibitors of Serine Proteases) Kits.

FLISP™ Manual



Images: HL-60 cells were stained with the FLISP Kit containing the FAM-labeled peptide FFCK and FLICA Kit containing SRVAD-FMK after incubation with camptothecin for 3 hours. Cells with active serine proteases stain green (FFCK-FAM) and cells with active caspases stain red (SR-VAD-FMK). Cell displaying either or both activities are easily detected using fluorescence microscopy (left) and FACS (right).

Introduction

Serine proteases are defined by the presence of a serine residue at the active center of the enzyme, which participates in the formation of an intermediate ester to transiently form an acyl-enzyme complex. Activated serine proteases play major roles in several different functions, including: Apoptosis (1); Markers of tumor malignancy (2-5); Diagnostic and prognostic indicators of breast carcinomas (6, 7) and neck and head carcinomas (8); and Activities are also altered in a variety of other cell-mediated diseases related to transplant rejection and infections (9-14).

The most characterized enzymes of this type are trypsin and chymotrypsin. Involvement of serine proteases in apoptosis has been mostly studied by observing whether particular apoptotic events can be prevented by the specific inhibitors of these enzymes. Fragmentation of DNA in HL-60 cells, treated with DNA topoisomerase inhibitors to induce apoptosis, was prevented by the use of an irreversible serine protease inhibitor such as N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) which inhibits chymotrypsin (15). The same inhibitor also inhibited nuclear fragmentation as well as fragmentation of DNA in other cell types, including thymocytes treated with the corticosteroid prednisolone (16, 17).

It is now possible to measure serine protease activity in whole living cells using the FLISP™ (**F**luorescent **L**abeled **I**nhibitors of **S**erine **P**roteases) line of products (18, 19). These patent pending products are fluorochrome-labeled analogs of serine protease inhibitors, labeled with either Carboxyfluorescein (FAM) or Sulforhodamine 101 (also known as Texas Red™). Current products include FAM-Phenylalanine chloromethyl ketone (FFCK), SR 101-phenylalanine chloromethyl ketone (SFCK), FAM-Leucine chloromethyl ketone (FLCK), and SR101-Leucine chloromethyl ketone (SLCK). The FAM labeled products are also available with a spacer, FSFCK and FSLCK. [Note: the FFCK, FSFCK and SFCK terminology represents the current nomenclature of "F" for phenylalanine as opposed to "P" which was used when the TPCK inhibitor was originally described.]

The FLISP™ reagents are cell permeable and can be used to detect serine proteases in suspension cells and adherent cells. Once they enter the cell, they irreversibly bind to the active catalytic site of the respective active serine protease. The unbound reagent is washed from the cells and the results are read using flow cytometry, fluorescence microscopy or fluorescence plate readers. The reagents are used at low concentrations and normally do not have any adverse effects on the cells. The FLISP™ products can also be used with Neuromics' caspase detection FLICA™ kits, allowing the researcher to monitor serine protease and caspase activites simultaneously (18, 19). The FLICA™ kits employ the use of fluorochrome-labeled analogs of fluoromethyl ketone inhibitors of caspase activity (20-24).

Preparation of FLISP Serine Protease reagent. The FLISP reagent is supplied as a concentrated lyophilized powder. Unopened vials may be stored at -20º C for up to 18 months. The reagent should be reconstituted just prior to use or freezing.

1. **Preparation of 250X FLISP reagent stock.** Dissolve lyophilized FLISP reagent in 0.050 mL (50 µL) of DMSO. Mix thoroughly, washing the base and lower sides of the vial to ensure complete solubilization of the FLISP reagent. DMSO reconstituted vials of FLISP reagent may be stored for up to 1 year at -80º C.
2. **Preparation of 50X FLISP reagent.** Add 0.2 mL (200 µL) of PBS, pH 7.4, to the 250X stock solution of FLISP reagent. Once diluted in PBS, the FLISP reagent must be used as soon as possible, the 50X FLISP reagent is ready to add to the cells (i.e. 0.010 mL (10 µL) of 50X FLISP reagent added to 0.490 mL (490 µL) of cells).
3. FAM labeled FLISP reagents require a 488 ± 10 nm excitation and > 520 nm emission optics pairing.
4. Sulforhodamine 101 labeled FLISP reagents require a 590 ± 10 nm excitation and > 610 nm emission optics pairing.

Preparation of 1X Wash Buffer. The wash buffer comes as a 10X concentrate which must be diluted to 1X with DI H_2O 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 FLISP kit:** Add the entire contents of the 10X wash buffer (15 mL) to 135 mL of DI H_2O, making a total of 150 mL of 1X wash buffer.
3. **For the 100 test FLISP kit:** Add the entire contents of the 10X wash buffer (60 mL) to 540 mL of DI H_2O, making a total of 600 mL of 1X wash buffer.
4. 1X wash buffer may be stored up to 30 days at 2º to 8º C. If any salt crystals form, warm to room temperature and stir 5 minutes until all crystals have dissolved.

10X Fixative. The fixative comes as a 10X concentration of a formaldehyde solution designed to cross-link cell components and will not interfere with the carboxyfluorescein or sulforhodamine 101 labeling once the FLISP reaction has taken place.

1. After FLISP labeling, add the 10X Fixative to the cell solution at a volume that will give a 1:10 dilution. To 0.450 mL (450 µL) of cells add 0.050 mL (50 µL) of 10X Fixative.
2. Fixed cells may be stored on ice or at 2º to 8º C for up to 24 hours.
3. Do not attempt to fix cells that will be stained with Propidium Iodide or Hoechst Stain.

Propidium Iodide. Propidium Iodide (PI) is provided ready-to-use at a concentration of 250 µg/mL. PI stains necrotic, dead and membrane compromised cells and can be used to distinguish between live cells and dead cells.

1. Propidium Iodide can be used with FAM labeled FLISP reagent for bi-color analysis.
2. The PI solution is added to the suspended cells at a 0.5% v/v amount. In a typical reaction mixture, 2.5 µL of PI is added to 0.500 mL (500 µL) of suspended cells or overlay medium for adherent monolayer cells.

3. 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).

Hoechst Stain. Hoechst stain is provided ready-to-use at a concentration of 200 µg/mL. It can be used to label the nuclei of dying cells after labeling with the FLISP reagent.

1. Hoechst stain can be used with FAM or sulforhodamine 101 labeled FLISP reagent for bi-color analysis.
2. The Hoechst solution is added to the suspended cells or adherent monolayer at a 0.5% v/v amount. In a typical reaction mixture, 2.5 µL of Hoechst solution is added to 0.500 mL (500 µL) of suspended cells or overlay medium for adherent monolayer cells.
3. Hoechst stain is detected using a UV-filter with excitation at 365 nm and an emission at 480 nm.

Suspension Cell Procedure

1. Cultivate cells under optimal growth conditions to a concentration where cells remain healthy and do not exhibit signs of over growth or natural induction of apoptosis (for most cell lines, the cell concentrations will be less than 1 x 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 x 10^6 to 1 x 10^7 cells per mL are optimal for incubation with the FLISP reagent. For lower initial cell counts, cells may be concentrated by centrifugation at <400 x g for 5 minutes, then resuspend cells in an appropriate amount of cell media to achieve the desired concentration.
5. Remove 0.490 mL (490 µL) of each cell suspension and place into sterile tubes. To each tube add 0.010 mL (10 µL) of 50X FLISP reagent (the 50X FLISP reagent is always diluted 1:50 in the cell suspension, this gives a final concentration of 20 µM FLISP reagent). This is a suggested starting concentration for the FLISP reagent, optimal FLISP reagent concentration may be as low as 5 µM. The incubation time and FLISP concentration should be optimized for cell type and experimental conditions.
6. Incubate the cells with the FLISP reagent between 30 minutes and 2 hours 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 FLISP reagent enters the cell and binds irreversibly to the catalytic site of the active serine proteases.
    a. If cells are to be stained with Hoechst stain, add 2.5 µL (0.5% v/v) of the Hoechst solution at the end of the FLISP incubation step and incubate for an additional 5 minutes.
7. After incubation with the FLISP reagent, gently centrifuge the cells at less than 400 x g for 5 minutes to pellet the cells.
8. 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 FLISP 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 µL) of 1X Wash Buffer or PBS. If the cells are to be analyzed by a fluorescence plate reader, the negative control and test samples should all be adjusted to approximately the same concentration of cells per mL. The cells are now ready for analysis.
    a. If cells are to be stained with Propidium Iodide, add 2.5 µL (0.5% v/v) of the PI solution to the resuspended cells.
11. 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 2º to 8º C protected from light for up to 24 hours. Do not fix cells if they are to be counterstained with PI or Hoechst stain.

Adherent Cell Procedure

1. Cultivate cells under optimal growth conditions to a concentration where cells are not becoming overgrown and beginning to slough off of the flask surface. When cells are overcrowded, the level of apoptotic cells tends to increase.
2. Trypsinize healthy growing cells 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 monlayer of cells add an amount of the 50X FLISP Reagent to make a 1:50 dilution in the cell media (i.e. if 0.490 mL (490 µL) of cell media is used to culture the cells, add 0.010 mL (10 µL) of 50X FLISP reagent). The final concentration of FLISP reagent will be 20 µM. This is a suggested starting concentration for the FLISP reagent, optimal FLISP reagent concentration may be as low as 5 µM. The incubation time and FLISP concentration should be optimized for cell type and experimental conditions.
5. Incubate the cells with the FLISP reagent between 30 minutes and 2 hours at 37º C. The overlay medium containing the FLISP reagent should be agitated periodically every 20 to 30 minutes to ensure an even labeling process.
    a. If cells are to be stained with Hoechst stain, add 2.5 µL (0.5% v/v) of the Hoechst solution at the end of the FLISP incubation step and incubate for an additional 5 minutes.
6. After FLISP labeling, the adherent cells can be processed by two different methods for analysis. The adherent monolayer can be analyzed directly (go to step 13) or the cells can be removed prior to analysis (beginning with step 7).
7. Carefully aspirate the cell supernatant from the monolayer and place into a sterile centrifuge tube. Trypsinize 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 FLISP reagent.
10. Wash the cells two more times by repeating steps 8 and 9.
11. Resuspend the final washed cell pellet in 0.5 mL (500 µL) of 1X Wash Buffer or PBS. If the cells are to be analyzed by a fluorescence plate reader, the negative control and test samples should all be adjusted to approximately the same concentration of cells per mL. The cells are now ready for analysis.

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 mix. The fixed cells may be stored at 2º to 8º C protected from light for up to 24 hours.
13. For direct analysis of the FLISP 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 FLISP reagent.
15. Wash the adherent monolayer two more times by repeating steps 13 and 14. Care must be taken to minimize the number of adherent cells displaced during the washing process.
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 2º to 8º C protected from light for up to 24 hours. Do not fix cells if they are to be counterstained with PI or Hoechst stain.

Analysis

1. **Flow Cytometry**
   - **a.** Set up acquisition template dot plots SS vs. FW (Fig 1), FL-1 vs. FL-2 (Fig 2) and FL-1 histogram (Figs 4 and 5).
   - **b.** Open compensation and voltage/gain controls. Although the FLISP products are analyzed on a histogram, FL-1 vs. FL-2 dot plot should be used to properly setup interment.
     - **i.** Run the negative control, non-stimulated FLISP reagent stained cells and set up FW vs. SS by drawing a gate around target cell population (Fig 1).
     - **ii.** Gating on the target cell population, setup the Fl-1 vs. Fl-2 dot plot. Readjust voltage/gains to place the cells in the lower left quadrant (Fig 2).
     - **iii.** Run the positive control, stimulated FLISP reagent stained cells and check to confirm that the population of FLISP labeled cells have moved to the right (Fig 3). If the cells move up or down the Y axis, adjust the compensation.
     - **iv.** If compensation was adjusted in the previous step, run the negative control again to ensure the proper setup.
     - **v.** Running the negative control, setup a histogram on the FL-1 channel. Make sure that the peak is to the right of the Y axis and within the first decade, set markers as demonstrated in figure 4.
     - **vi.** By setting the marker as in step **1.b.v.**, the flow cytometer can calculate percentages of FLISP positive cells (Fig 5).

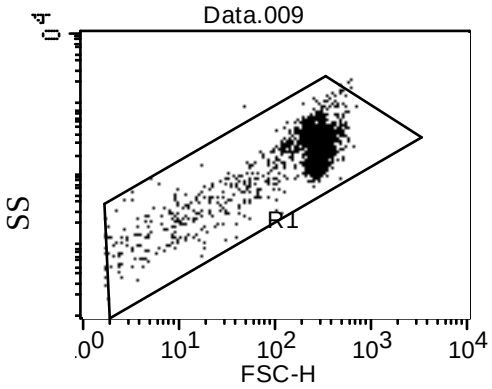
2. **Fluorescence Plate Reader**
   - **a.** Use black welled plates only.
   - **b.** For a 96 well plate, transfer 0.050 mL (50 µL) to 0.300 mL (300 µL) of suspended cells per well. Once the volume has been optimized, deliver equal volumes to all wells for each experiment.
   - **c.** Set fluorescence plate reader to endpoint read.

d. To read cells treated with FAM labeled FLISP reagent, set excitation to 488 nm and emission to 530 nm with a 515 nm cut-off filter.
e. To read cells treated with Sulforhodamine 101 labeled FLISP reagent, set excitation to 590 nm and emission to 620 nm with a 610 nm cut-off filter.

3. Fluorescence Microscopy

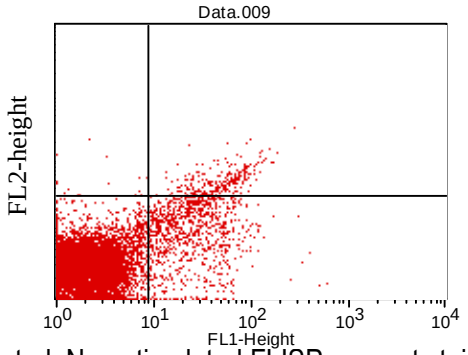
a. To view suspension cells, place one drop of the cell suspension onto a microscope slide and cover with a cover slip.
b. To view adherent cells, mount a cover slip with cells facing down onto a microscope slide containing a drop of 1X Wash Buffer or remove the plastic frame of the chamber slide and add a drop of 1X Wash Buffer and cover with a cover slip.
c. Observe cells treated with FAM labeled FLISP reagent using a broad bandpass filter with an excitation at 490 nm and an emission greater than 520 nm. Cells with active serine protease activity will appear green when using the FAM labeled FLISP reagent.
d. Observe cells treated with Sulforhodamine 101 labeled FLISP reagent using a broad bandpass filter with an excitation at 590 nm and an emission greater than 610 nm. Cells with active serine protease activity will appear red when using the Sulforhodamine 101 labeled FLISP reagent.
e. Hoechst stain can be observed by using a UV-filter with excitation at 365 nm and an emission at 480 nm.
f. Propidium Iodide (PI) can be observed using a broad bandpass filter with an excitation at 490 nm and an emission greater than 610 nm (optimal settings would be an excitation at 535 nm and emission at 617 nm).

Figure 1.



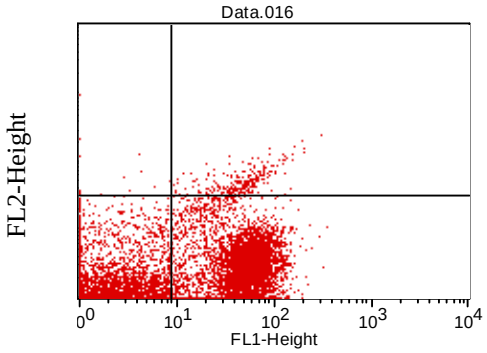
Negative control, Non-stimulated FLISP reagent stained cells. FW vs SS dot plot.

Figure 2.



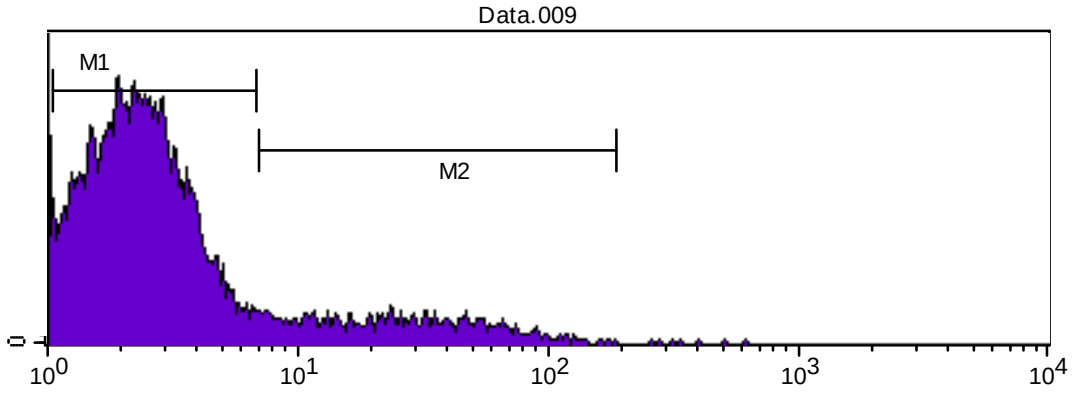
Negative control, Non-stimulated FLISP reagent stained cells. Fl-1 vs. Fl-2 dot plot. Readjust voltage/gains to place the cells in the lower left quadrant.

Figure 3.



Positive control, stimulated FLISP reagent stained cells. Fl-1 vs. Fl-2 dot plot.

Figure 4.

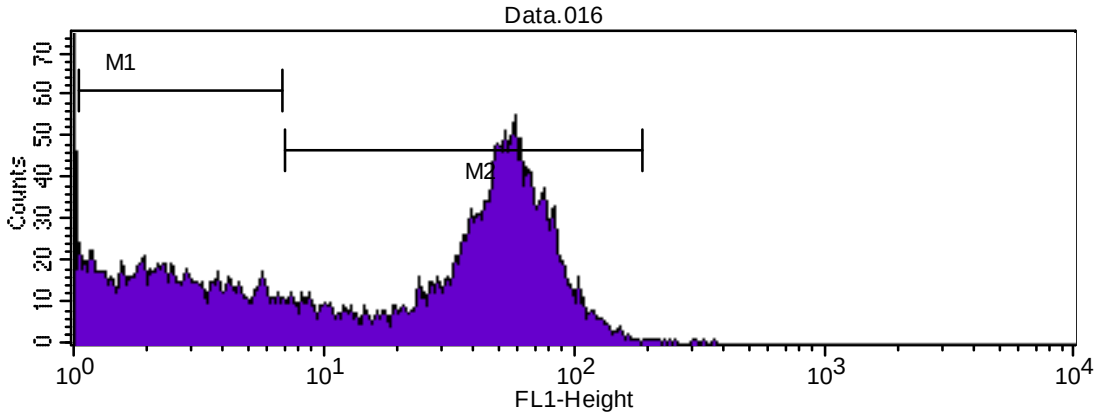


Sample ID: TL-3 NEG
Acquisition Date: 18-May-04
Gate: G1
Gated Events: 10026
Total Events: 10000
X Parameter: FL1-Height (Log

Marker	% Gatec	% Tota	Mean
All	100.00	99.48	6.52
M1	75.30	74.91	2.51
M2	13.50	13.43	31.46

Negative control, Non-stimulated FLISP reagent stained cells. FL-1 histogram.

Figure 5.



Sample ID: TL-3 POS
Acquisition Date: 18-May-04
Gate: G1
Gated Events: 9952
Total Events: 10000
X Parameter: FL1-Height (Log

Marker	% Gatec	% Tota	Mean
All	100.00	98.70	29.74
M1	30.10	29.71	2.84
M2	56.28	55.55	50.44

Positive control, stimulated FLISP reagent stained cells. FL-1 histogram.

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