# FLISP Serine Protease Detection Kit

BIO-RAD

## FAM FLISP Kits

| Inhibitor | Cat # | Moiety |
|---|---|---|
| FAM-Phe-CMK | APO006 | Chymotrypsin |
| FAM-Leu-CMK | APO007 | Chymotrypsin C |
| FAM-Leu-DAP | APO008 | Chymotrypsin C |
| FAM-Phe-DAP | APO009 | Chymotrypsin |

## Kit Components

| Component | Storage Conditions | Quantity |
|---|---|---|
| 1 vial Green FAM FLISP Inhibitor Reagent | -20°C | |
| 1 vial fixative | 2-8°C | 6 ml |
| 1 vial 10x cellular wash buffer | 2-8°C | 15 ml |
| 1 vial Hoechst 33342, 200 µg/ml | 2-8°C | 1 ml |
| 1 vial propidium iodide, 250 µg/ml | 2-8°C | 1 ml |

Each kit is provided in two boxes which should be stored at different temperatures:

- Box one contains the FLISP Reagent which should be stored at -20°C. Once reconstituted with DMSO, use immediately or store at -20°C for up to 6 months. Avoid repeat freezing and thawing
- Box two contains all the other kit components which should be stored at 2-8°C. Do not freeze

## Background

FLISP (Fluorescent Labeled Inhibitors of Serine Protease) Serine Protease Detection Kits enable researchers to quantitate intracellular chymotrypsin-like serine protease activity in vitro without lysing cells. FAM FLISP reagents are non-cytotoxic, cell-permeant green fluorescent inhibitors that covalently bind to active serine protease enzymes.

Simply add FLISP directly to cells in culture, incubate and wash to detect active chymotrypsin-like enzyme inside the cell. FLISP will covalently bind an active chymotrypsin–like enzyme and be retained within the cell, whereas non-bound reagent will be able to diffuse out. Cells containing lower levels of active enzyme will bind less FLISP and therefore be less fluorescent after the wash steps.

Serine proteases, such as trypsin and chymotrypsin, 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. Activation of serine proteases plays major roles in several different cellular functions including apoptosis, tumor malignancy, and as diagnostic and prognostic indicators of breast, head and neck carcinomas. Serine protease activation is also thought to play a role in infections and transplant rejection.

Proteolysis is tightly controlled during apoptosis and often mediated by caspases. However there is increased serine protease activity in apoptotic cells, downstream of caspase activation, and this may complement caspase mediated cell death. When FLISP Kits are used in combination with FLICA Caspase Kits, it is possible to discriminate between caspase activity and serine protease activity in apoptosis. Counterstaining with a viability dye such as propidium iodide (contained in the kit) allows identification of necrotic cells by flow cytometry. Nuclear morphology may be observed using Hoechst (contained in the kit) using fluorescence microscopy.

FLISP inhibitors consist of fluorochrome-labeled analogs of the first serine protease inhibitor, tosyl-phenlalanylchloromethyl ketone (TPCK) labeled with carboxyfluorescein (FAM) which fluoresces green (Max Ex. 488 nm, Max Em. 520 nm). They are available with either a chloromethyl ketone (CMK) or diphenyl 1-(N-peptidylamino) alkanephosphonate (DAP) reactive group containing compound with different inhibitor sequences (Phe or Leu) to allow identification of chymotrypsin or chymotrypsin C.

## Experimental Set-Up

Staining with FLISP may require cell culture for a period of time to obtain the correct concentration of cells for cell lines and allow experimental treatment to take effect. The recommended staining is 10 µl of 50x FLISP per 490 µl of cells at a cell concentration of 5 x $10^5$ cells/ml, however this may vary depending upon the cells and treatment used.

Include appropriate controls for your experiment, such as:

- Unlabeled treated and untreated cells
- Labeled treated, untreated and vehicle (e.g. DMSO) treated cells
- As a positive control, cells treated with staurosporine or camptothecin may also be required
- A single stained sample with propidium iodide is useful to determine underlying levels of necrosis within your sample
- A single stained sample of FAM FLISP, propidium iodide and any additional fluorophores may be required to determine compensation levels

Adherent cells may be stained before or after trypsinization but ensure sufficient washes are performed to remove excess trypsin.

## Reagent Preparation

50 µl of DMSO to reconstitute FAM FLISP Reagent (250x). Once reconstituted it can be aliquoted and stored at -20°C for 6 months.

135 ml of $ddH_2O$ to dilute cellular wash buffer to 1x. If a precipitate is visible, gently warm. Do not boil. 1x wash buffer may be stored at 2-8°C for 1 week or frozen and used within 6 months.

## General Protocol for Suspension Cells

1. Prepare samples (1 x $10^6$ cells /ml) and appropriate controls.
2. Dilute 10x cellular wash buffer 1:10 with $ddH_2O$.
3. Reconstitute FLISP with 50 µl DMSO (250x stock).
4. Immediately before use dilute FLISP 1:5 by adding 200 µl PBS (50x stock).
5. Add 10 µL FLISP to 500 µl of cells.
6. Incubate at 37°C avoiding direct light. This can be 30 min to several hours depending on experiment. Gently re-suspend every 20 min to ensure even distribution of FLISP.
7. Add 2 ml wash buffer, gently mix, incubate for 5 min at room temperature (RT) or 37°C, then centrifuge cells at 300 g and discard supernatant. Repeat this wash step.

8. **For fluorescence microscopy**:
   - Re-suspend cells in 300 µl of wash buffer.
   - At this point, cells may be dual stained with 3 µl of PI per 300 µl sample or 1.5 µl of Hoechst 33342 per 300 µl of sample. Incubate for 5 min at RT or 37°C and wash cells as in step 7. Re-suspend in 300 µl wash buffer.
   - If not viewing immediately cells may be fixed, although do not fix cells stained with Hoechst 33342. To fix add 30 µl fixative and incubate for 5 min at RT. Cells can then be mounted onto slides and stored at 2-8°C in the dark for 24 hr.
9. **For flow cytometry:**
   - Re-suspend cells in 300 µl of wash buffer.
   - Add 3 µl of PI.
   - Incubate for five min and read immediately on the flow cytometer.
10. **For fluorescence plate reader analysis:**
   - Re-suspend in 500 µl PBS. Do not add Hoechst 33342 or PI. Adjust the volume so the cell density is the same for each population.
   - Pipette 100 µl into microtiter plate, analyze at least 2 aliquots per sample and perform an endpoint read.
11. FAM FLISP excites at 488 nm and emits at 520 nm.

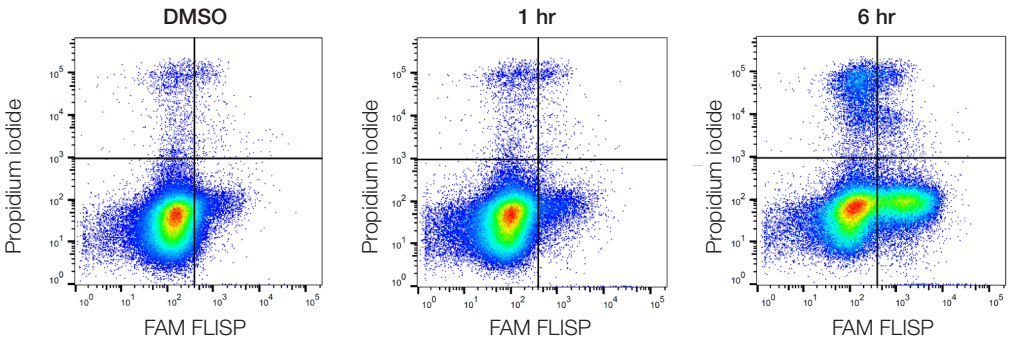


**Figure 1. Active chymotrypsin-like serine protease activity staining by flow cytometry.** Jurkat cells were treated as shown with 1 µM staurosporine to induce apoptosis. The cells were then stained using the FLISP FAM-Phe-DAP Serine Protease Assay Kit, including propidium iodide (#APO009). Apoptotic cells positive for serine protease activity can be seen in the bottom right quadrant and dead cells positive for PI in the top left and right quadrants. Healthy cells are negative for both stains. Data acquired on the ZE5™ Cell Analyzer.

## General Protocol for Adherent Cells

Adherent cells may be labeled with FLISP and analyzed, or labeled before or after trypsinization and treated as suspension cells (see suspension cell staining protocol). Cells may round up and lift off the adherent surface during treatment or washing steps. Therefore careful cell collection and washing is required to minimize loss of cells. Loose cells may be harvested and treated as adherent cells and can be combined with the trypsinized cells. Trypsinization may adversely affect the cells so appropriate controls should be included such as positive and negatively treated cells.

1. Dilute 10x cellular wash buffer 1:10 with dd$H_2O$.
2. Reconstitute FLISP with 50 µl DMSO (250x stock).
3. Immediately before use dilute FLISP 1:5 by adding 200 µl PBS (50x stock).
4. Dilute the 50x FLISP 1:50 in cell culture media (10 µl FLISP in 500 µl media) and add to cover the monolayer.
5. Incubate at 37°C avoiding direct light. This can be 30 min to several hours depending on experiment. Gently swirl every 20 min to ensure even distribution of FLISP.
6. If cells are to be labeled with Hoechst 33342, add 2.5 µl per 500 µl and incubate for 5 min.
7. Remove the FLISP and add 1-2 ml wash buffer, gently mix, incubate for 5-10 min. Discard supernatant.
8. Add 1-2 ml wash buffer, incubate for 15-30 min. Discard supernatant.
9. If cells are to be labeled with PI, add 5 µl per 500 µl and incubate for 1 min. Rinse cells with wash buffer.
10. Add a coverslip and view under a fluorescence microscope and analyze immediately.
11. If cells cannot be analyzed immediately they may be fixed, although do not fix cells stained with Hoechst 33342 or PI. To fix, dilute fixative 1:10 with 1x wash buffer, place one drop on the cell surface and add a coverslip. Store at 2-8°C for up to 24 hr avoiding direct light.

If adherent cells are to be trypsinized for analysis by flow cytometry, after step 7 trypsinize cells and neutralize the trypsin using standard protocols. Add 2 ml wash buffer, gently mix, incubate for 5 min at RT or 37°C, then centrifuge cells at 300 g and discard supernatant. Repeat this wash step twice. Continue from step 9 of Suspension Cell Protocol.



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