EarlyTox Caspase-3/7 NucView 488 Assay Kits

The EarlyTox™ Caspase-3/7 NucView 488 Assay Kit and the EarlyTox Caspase-3/7-D NucView 488 Assay Kit enable detection of apoptosis in intact cell populations based on the measurement of caspase-3/7 activity using either a fluorescence microplate reader or a fluorescence imaging system.

Table 1-1: Available Kits

Assay Kit	Formulation	Explorer Kit	Bulk Kit
EarlyTox Caspase-3/7-D NucView 488 Assay Kit	DMSO	R8348	R8349
EarlyTox Caspase-3/7 NucView 488 Assay Kit	PBS	R8350	R8351

Contents

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Product manufactured for Molecular Devices, LLC
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Chapter 1: About the EarlyTox Caspase-3/7 NucView 488 Assay Kits

The EarlyTox™ Caspase-3/7 NucView 488 Assay Kits are used to detect apoptosis in intact cell populations, based on the measurement of caspase-3/7 activity with either a fluorescence plate reader or a fluorescence imaging system. The key features of this kit include the following:

- Optimized for fluorescence microplate readers and fluorescence imaging
- Simple workflow with single reagent addition
- Optional Masking Reagent to reduce background fluorescence
- Preconfigured protocol in SoftMax® Pro Software

The NucView 488 Caspase-3 substrate is offered in phosphate-buffered saline (PBS) and DMSO formulations. Use the PBS formulation with cells that are sensitive to DMSO.

Assay Principles

Apoptosis is a highly regulated and controlled process that leads to characteristic cellular changes, such as blebbing, cell shrinkage, chromatin condensation, chromosomal DNA fragmentation, apoptotic body formation[1], and eventually cell death. Caspase-3 and caspase-7 are proteases that are activated during the exection phase of apoptosis. NucView 488 Caspase-3 substrate is used to detect caspase-3/7 activity within intact cells without interfering with caspase activity.[2,3,4] The substrate consists of a fluorogenic DNA dye that is coupled to the caspase-3/7 DEVD recognition sequence. Initially non-fluorescent, the substrate permeates the plasma membrane and enters the cytoplasm. In apoptotic cells, caspase-3/7 cleaves the substrate, releasing a high-affinity DNA dye that migrates to the cell nucleus and stains DNA with bright green fluorescence, with excitation at 500 nm and emission at 530 nm. See Figure 1-1. NucView 488 staining is formaldehyde-fixable and compatible with subsequent immunostaining.

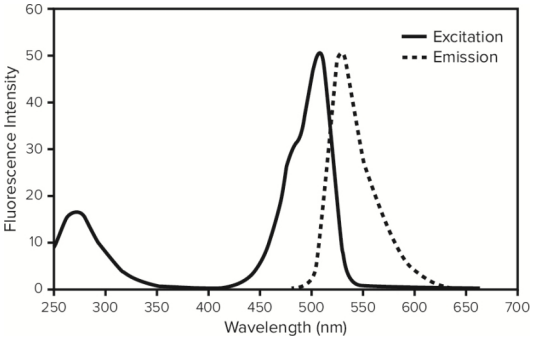

Figure 1-1: Excitation/emission maxima of NucView 488 bound to DNA: excitation = 500 nm and emission = 530 nm

Chapter 2: Materials and Equipment

Kit Components

Table 2-1: Components of the EarlyTox Caspase-3/7-D NucView 488 Assay Kit

Item	Explorer Kit (R8348)	Bulk Kit (R8349)
NucView 488 Caspase-3 Substrate, 1 mM in DMSO	1 x 200 µL	1 x 1 mL
Masking Reagent	2 vials	1 vial

Table 2-2: Components of the EarlyTox Caspase-3/7 NucView 488 Assay Kit

Item	Explorer Kit (R8350)	Bulk Kit (R8351)
NucView 488 Caspase-3 Substrate, 1 mM in PBS	1 x 200 µL	1 x 1 mL
Masking Reagent	2 vials	1 vial

For both kits:

- The Explorer kit is sufficient for two 96-well microplates.
- The Bulk kit is sufficient for ten 96-well microplates.

The number of microplates is based on the example protocol that is detailed in this document.

Storage and Handling

Reagents in this kit should be stored as follows:

- Store reagents in the EarlyTox Caspase-3/7 NucView 488 Assay Kit (PBS formulation) at –20°C.
- Store reagents in the EarlyTox Caspase-3/7-D NucView 488 Assay Kit (DMSO formulation) at 4°C.

When stored as directed, each kit is stable for at least 6 months from the date it is received.

WARNING! Reagents can contain chemicals that are harmful. Exercise care when handling reagents as described in the related safety data sheet (SDS or MSDS). The safety data sheet is available in the Knowledge Base on the Molecular Devices support web site: www.moleculardevices.com/support.

Materials Required but Not Provided

Table 2-3: Reagents and Supplies

Item	Suggested Vendor
Black-walled, clear bottomed microplates (96-well)	Corning 3904 or equivalent
Phosphate-Buffered Saline (PBS)	Corning 21-030 or equivalent

Compatible Molecular Devices Microplate Readers and Imaging Systems

- SpectraMax® i3x Multi-Mode Detection Platform
- SpectraMax® M2 and M2e Multi-Mode Microplate Readers
- SpectraMax® M3 Multi-Mode Microplate Reader
- SpectraMax® M4 Multi-Mode Microplate Reader
- SpectraMax® M5 and M5e Multi-Mode Microplate Readers
- SpectraMax® Paradigm® Multi-Mode Detection Platform
- FlexStation® 3 Multi-Mode Microplate Reader
- Gemini™ EM and XPS Fluorescence Microplate Readers
- FilterMax™ F5 Multi-Mode Microplate Reader
- SpectraMax® MiniMax™ Imaging Cytometer

Chapter 3: Assay Protocols

Example of Assay Protocol

The following were used for an endpoint apoptosis assay with HeLa cells in a 96-well format, with detection done with either a microplate reader or an imaging system. NucView 488 substrate can also be incubated with cells for extended periods of time for time-course studies. See also NucView 488 Frequently Asked Questions on page 11.

Optimal assay conditions for different cell types and plate formats can vary. Molecular Devices recommends testing a range of NucView 488 substrate concentrations, generally between 2 µM and 8 µM, to determine the optimal conditions for your assay model.

NucView substrate can be diluted to form a working solution, for example, 2X or 4X, and then added to assay wells at a volume that gives the appropriate final substrate concentration. Cells can be incubated with substrate in culture medium, PBS, or another buffer of your choice. If high background is observed in cells that contain non-apoptotic cell populations, then medium can be removed and replaced with fresh PBS containing substrate. Washing after substrate incubation is also optional. Care must be taken not to dislodge cells during medium removal or washing. An optional Masking Reagent is included that can be used to reduce fluorescence background.

Assay Setup For Fluorescence Microplate Readers

Molecular Devices recommends that you perform the following controls:

- Negative control: Cells not induced to undergo apoptosis.
- Positive control: Cells induced to undergo apoptosis.

Use the following procedure to set up the assay:

1. Plate 15000 HeLa cells in 100 µL culture medium per well in a black-walled, clear-bottomed, 96-well microplate. Incubate in a 37°C, 5% CO_2 incubator overnight. If needed, include wells without cells as a background control.
2. Induce apoptosis in cells using the methods of your choosing. Remember to include untreated wells as controls.
3. Prepare a working solution of substrate in either culture medium (for example, if cells are to be incubated in substrate for a long-term experiment) or PBS and add it to the cells to make a final concentration of 5 µM. For example, prepare 10 mL of a 2X concentration at 10 µM.

   Note: A final concentration of 2 µM to 8 µM of Caspase 3/7 NucView substrate is usually sufficient.

4. Add 100 µL of substrate directly to wells containing 100 µL of cells and medium and mix well, being careful not to dislodge adherent cells.
5. Incubate the samples at room temperature for 15 to 30 minutes, protected from light.
6. Optionally, after incubation is complete, use a masking agent to reduce background fluorescence before reading the plate on a fluorescent microplate reader. See Using a Masking Agent in the Assay on page 7.
7. Measure the fluorescence in a fluorescence microplate reader with excitation at 490 nm and emission at 535 nm.

Using a Masking Agent in the Assay

Using a masking agent to reduce background fluorescence is always optional.

CAUTION: Add masking reagent after substrate incubation is complete. Do NOT add masking reagent at the same as the substrate.

1. Reconstitute Masking Reagent. For either the Explorer kit or Bulk kit, add 10 mL buffer (PBS, HBSS, or a buffer of your choosing) to the vial to make a working solution.
2. Vortex for at least 30 seconds to dissolve.
3. After substrate incubation is complete, add a dilution of the Masking Reagent working solution to each assay well.
   - For the Explorer kit, the recommended dilution is 1:4.
   - For the Bulk Kit, the recommended dilution is 1:40 (You can make an intermediate dilution for easier liquid handling).

Note: The amount of Masking Reagent added should be optimized for each cell type and assay condition used.

Assay Setup For Fluorescence Imaging

The assay setup for fluorescence imaging is identical to the assay setup for fluorescence microplate readers with the exception of the Masking Reagent, which is not required for imaging cells.

Molecular Devices recommends that you perform the following controls:

- Negative control: Cells not induced to undergo apoptosis.
- Positive control: Cells induced to undergo apoptosis.

Use the following procedure to set up the assay:

1. Plate 15000 HeLa cells in 100 µL culture medium per well in a black-walled, clear-bottomed, 96-well microplate. Incubate in a 37°C, 5% CO_2 incubator overnight. If needed, include wells without cells as a background control
2. Induce apoptosis in cells using the methods of your choosing. Remember to include untreated wells as controls.
3. Prepare a working solution of substrate in either culture medium (for example, if cells are to be incubated in substrate for a long-term experiment) or PBS and add it to the cells to make a final concentration of 5 µM. For example, prepare 10 mL of a 2X concentration at 10 µM.

   Note: A final concentration of 2 µM to 8 µM of Caspase 3/7 NucView substrate is usually sufficient.

4. Add 100 µL of substrate directly to wells containing 100 µL of cells and medium and mix well, being careful not to dislodge adherent cells.
5. Incubate the samples at room temperature for 15 to 30 minutes, protected from light.
6. Observe cells by fluorescence microscopy or another fluorescence imaging system using a FITC or GFP filter set.

Fluorescence Microplate Reader Setup with SoftMax Pro Software

Table 3-1 displays typical fluorescence microplate reader settings. In SoftMax® Pro Software, use the preconfigured EarlyTox NucView 488 protocol that is available in the protocol library.

Table 3-1: EarlyTox NucView 488 Assay protocol

Parameter	Setting
Read Mode	Fluorescence
Read Type	Endpoint
Wavelengths	Excitation = 490 nm, Bandwidth = 15 nm Emission = 535 nm, Bandwidth = 25 nm
PMT and Optics	PMT Gain: Automatic Flashes per read: 10 Read From Bottom*

*Bottom read is preferred for cell-based assays, but top read can be used with microplate readers that do not have the bottom read option.

Fluorescence Microscopy or Fluorescence Imaging System Setup

Observe cells by fluorescence microscopy or another fluorescence imaging system using a standard filter set for FITC/GFP, with excitation at 485 nm and emission at 515 nm.

Chapter 4: Data Analysis Examples

Example Results: Fluorescent Microplate Reader

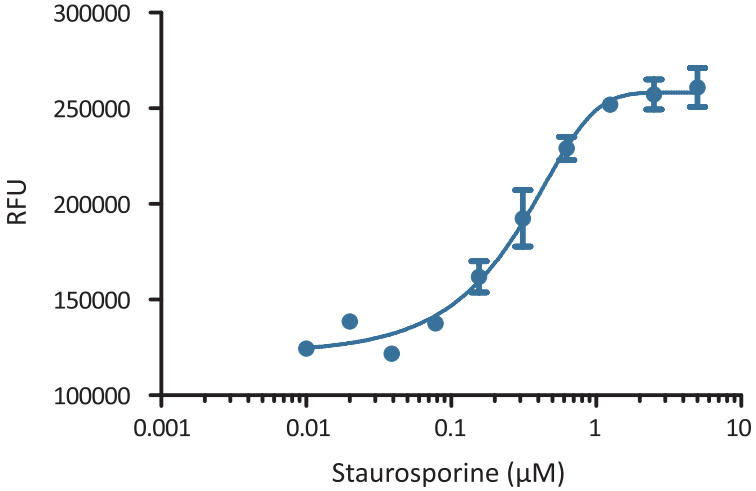

Figure 4-1: Apoptosis assay: HeLa cells treated with staurosporine (fluorescent microplate reader)

HeLa cells plated at 15000 cells per well in a 96-well microplate were treated with staurosporine for 20 hours at 37°C to induce apoptosis. 100 µL of 2X NucView 488 substrate at 10 µM in medium was added to each well for a final concentration of 5 µM. After a 30 minute incubation at room temperature, Masking Reagent was added to wells. Fluorescence was immediately measured using theSpectraMax i3x Multi-Mode microplate reader.

Example Results: Fluorescence Imaging System

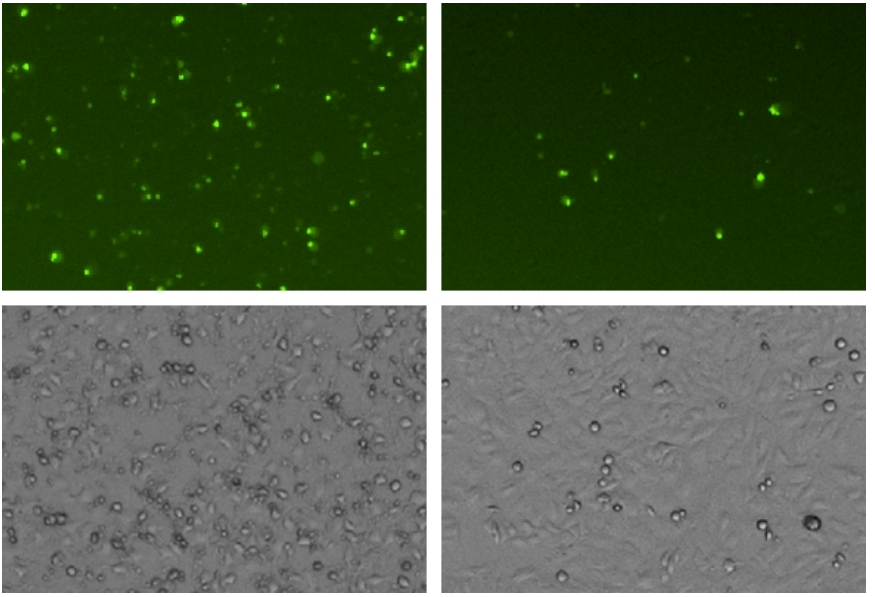

Figure 4-2: Apoptosis assay: HeLa cells treated with staurosporine (fluorescence imaging system)

HeLa cells plated at 15000 cells per well in a 96-well microplate were treated with 0.16 µM staurosporine for 20 hours at 37°C to induce apoptosis (left column) or untreated (right column). 100 µL of 2x NucView 488 substrate at 10 µM in medium was added to each well for a final concentration of 5 µM. Cells were imaged on the SpectraMax SpectraMax MiniMax Imaging Cytometer using the green fluorescence channel (top row) and transmitted light channel (bottom row).

Assay Optimization Tips

- The example protocol was developed using HeLa cells, and can be adapted for use with other cell types. Assay signals can be optimized by adjusting the cell number and concentration of the substrate, generally between 2 µM and 8 µM.
- The example protocol described is a homogeneous protocol that does not replace the cell medium or use a wash step to simplify workflow and avoid disturbing cells. If needed, to reduce background fluorescence, cell medium can be replaced with PBS prior to the addition of reagents. These manipulations, however, might result in inconsistent well-to-well signals because of disturbance or loss of cells.
- Alternatively, to reduce background fluorescence, Masking Reagent can be used. The dilution of Masking Agent might need to be optimized for the particular cell type and assay conditions.
- If a low amount of fluorescent signal is detected when using the PBS formulation of the substrate, then the addition of a small amount of DMSO at the time of substrate addition might help to increase the signal. The addition of DMSO is not recommended, however, if the cells are DMSO sensitive.

Chapter 5: NucView 488 Frequently Asked Questions

Table 5-1 lists the most frequently asked questions about the NucView 488 Caspase-3/7 substrate and the answers to these questions.

Table 5-1: NucView 488 Frequently Asked Questions

Question	Answer
How stable is NucView 488 Caspase-3/7 substrate?	Very stable. Users have reported performing time course assays with NucView 488 Caspase-3/7 Substrate for 5 days at 37°C.
When should I add NucView 488 Caspase-3/7 substrate to my cells?	You can add it at the start of the experiment or at the end. NucView 488 Caspase-3/7 substrate does not affect the time course of apoptosis progression, so it can be used to monitor caspase-3/7 activity in real time
Can I fix NucView 488 Caspase-3/7 substrate for subsequent immunostaining?	Yes. Molecular Devices recommends fixation with 2 to 4% paraformaldehyde for 10 to 15 minutes at room temperature. Over-fixing can cause the signal to decrease. NucView 488 staining can withstand permeabilization with 0.1% Triton X-100, although signal intensity might be diminished after permeabilization and washing. Methanol fixation is not recommended.
How specific are NucView Caspase-3/7 substrates for caspase-3 and caspase-7?	NucView caspase-3/7 substrates are based on a DEVD caspase-3/7 consensus sequence. Because of overlapping substrate specificity among caspases, other caspases might also cleave DEVD substrates.
Which cell types can be used with NucView 488 Caspase-3/7 substrate?	NucView 488 Caspase-3/7 substrate has been reported to work in a wide variety of primary cells and immortalized cell lines in the published scientific literature.

Chapter 6: Obtaining Support

Molecular Devices is a leading worldwide manufacturer and distributor of analytical instrumentation, software, and reagents. We are committed to the quality of our products and to fully supporting our customers with the highest possible level of technical service.

Our support web site, www.moleculardevices.com/support, has a link to the Knowledge Base with technical notes, software upgrades, safety data sheets, and other resources. If you do not find the answers you are seeking, follow the links to the Technical Support Service Request Form to send an email message to a pool of technical support representatives.

You can contact your local representative or contact Molecular Devices Technical Support by telephone at 800-635-5577 (U.S. only) or +1 408-747-1700. In Europe call +44 (0) 118 944 8000.

Please have the product name, part number, and lot number available when you call.

EarlyTox Product Family

Table 6-1: EarlyTox Product Family: Available Kits

Assay Kit	Explorer Kit	Bulk Kit
EarlyTox Live/Dead Assay Kit	R8340	R8341
EarlyTox Live Cell Assay Kit	R8342	R8343
EarlyTox Glutathione Assay Kit	R8344	R8345
EarlyTox Caspase-3/7 R110 Assay Kit	R8346	R8347
EarlyTox Caspase-3/7-D NucView 488 Assay Kit (DMSO Formulation)	R8348	R8349
EarlyTox Caspase-3/7 NucView 488 Assay Kit (PBS Formulation)	R8350	R8351

References

1. Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6, 99 (1999).
2. Cen H, Mao F, Aronchik I, Fuentes RJ, Firestone GL. "DEVD-NucView488: a novel class of enzyme substrates for real-time detection of caspase-3 activity in live cells." *FASEB J* **22**, 2243 (2008).
3. Monaco G, Decrock E, Akl H, Ponsaerts R, Vervliet T, Luyten T, De Maeyer M, Missiaen L, Distelhorst CW, De Smedt H. "Selective regulation of IP3-receptor-mediated Ca2+ signaling and apoptosis by the BH4 domain of Bcl-2 versus Bcl-Xl". *Cell Death Differ* **19**, 295 (2012).
4. Schmalz H, Lenzen S, Baltrusch S. "Glucokinase mediates coupling of glycolysis to mitochondrial metabolism but not to beta cell damage at high glucose exposure levels." *Diabetologia* **54**, 1744 (2011).

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