EarlyTox Caspase-3/7 R110 Assay Kit

The EarlyTox™ Caspase-3/7 R110 Assay Kit enables analysis of apoptosis based on caspase-3/7 activity and its consensus target sequence, Asp-Glu-Val-Asp (DEVD), using a fluorescence microplate reader.

Table 1-1: Available Kits

Assay Kit	Explorer Kit	Bulk Kit
EarlyTox Caspase-3/7 R110 Assay Kit	R8346	R8347

Contents

Product manufactured by Molecular Devices, LLC.
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Molecular Devices, LLC is ISO 9001 registered.

Chapter 1: About the EarlyTox Caspase-3/7 R110 Assay Kit

The EarlyTox™ Caspase-3/7 R110 Assay Kit provides a convenient tool for apoptosis assays based on caspase-3/7 activity and its consensus target sequence, Asp-Glu-Val-Asp (DEVD). The key features of this kit include the following:

- Optimized for fluorescence microplate readers such as SpectraMax® readers
- Simple workflow – direct measurement in wells without medium removal with a single addition of substrate in lysis buffer
- Increased sample throughput with microplate format
- Preconfigured protocol in SoftMax® Pro Software

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 execution phase of apoptosis. The EarlyTox™ Caspase-3/7 R110 Assay Kit provides a single-step, homogenous assay that is specifically designed for microplate readers. The fluorogenic substrate $(Ac\text{-}DEVD)_2$-R110 contains two DEVD consensus target sequence and is completely hydrolyzed in cell lysate by the enzymes in two successive steps[2]. Hydrolysis of both DEVD peptides releases the green fluorescent dye R110, resulting in a substantial fluorescence increase, with excitation at 490 nm and emission at 520 nm.

The assay kit includes the competitive caspase-3/7 inhibitor Ac-DEVD-CHO for use as a negative control. R110 is also provided in the kit for generating a standard curve, which can be used for quantifying caspase-3 activity.

Chapter 2: Materials and Equipment

Kit Components

Table 2-1: Components of the EarlyTox Caspase-3/7 R110 Assay Kit

Item	Explorer Kit (R8346)	Bulk Kit (R8347)
Cell Lysis / Assay Buffer	2 x 10 mL	1 x 100 mL
Enzyme Substrate: $(Ac\text{-}DEVD)_2$-R110, 2 mM	2 x 500 µL	1 x 5 mL
Enzyme Inhibitor: Ac-DEVD-CHO, 5 mM	2 x 20 µL	2 x 100 µL
R110, 80 µM	1 x 1 mL	1 x 1 mL

- 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 –20°C. When stored as directed, the 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 2-2: 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

- 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

Chapter 3: Assay Protocols

Example of Assay Protocol

The following was used for an endpoint apoptosis assay with HeLa cells in a 96-well format. Optimal assay conditions for different cell types and plate formats can vary. Molecular Devices recommends testing a range of substrate concentrations, generally from 20 µM to 100 µM, to determine the optimal conditions for your assay system.

Substrate Assay Buffer Preparation

Use the following procedure to prepare 100 µL per sample of the substrate assay buffer:

Add enzyme substrate $(Ac\text{-}DEVD)_2$-R110 (2 mM) to cell lysis/assay buffer at a ratio of 50 µL substrate per 1 mL buffer, and then mix well.

Assay Setup

Molecular Devices recommends that you perform the following controls:

- Negative control: Cells not induced to undergo apoptosis.
- Positive control: Cells induced to undergo apoptosis.
- Inhibitor control: Cells induced to undergo apoptosis, and then Caspase-3 inhibitor is added.

Use the following procedure to set up the assay:

1. Plate 20000 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.
   Molecular Devices recommends seeding cells at sufficient density to form a confluent.
   - For suspension cells, the recommended cell density is 40000 to 200000 cells per well.
   - For adherent cells, the recommended cell density is 20000 to 80000 cells per well.
2. Induce apoptosis in cells using the methods of your choosing for cell viability assays. Remember to include untreated wells as controls.
3. Add 100 µL of the substrate assay buffer to each well, resulting in a final volume of 200 µL per well and a final concentration of 50 µM. Mix gently by pipetting up and down slowly.
4. Incubate the samples at room temperature for 30 to 60 minutes (or longer), protected from light.
5. Measure fluorescence in a fluorescence microplate reader with excitation at 490 nm and emission at 520 nm.

Inhibitor Control Preparation

To verify that the signal detected by the kit is the result of Caspase-3 activity, use the following procedure to prepare the inhibitor control:

1. Mix 100 µL of cell lysis/assay buffer and 2 µL of enzyme inhibitor Ac-DEVD-CHO (5 mM) for each inhibitor control well.
2. Add 100 µL per well of cells. Incubate at room temperature for 15 minutes.
3. Add 5 µL enzyme substrate $(Ac\text{-}DEVD)_2$-R110 (2 mM), and then mix well.
4. Incubate at room temperature for 30 to 60 minutes, or longer.
5. Measure fluorescence in a fluorescence microplate reader with excitation = 490 nm and emission = 520 nm.

R110 Reference Standard Preparation (Optional)

Optionally, to use the R110 standard to quantitate the amount of R110 that is generated at the assay endpoint, use the following procedure:

1. Dilute R110 (80 µM) to 20 µM in Cell Lysis Buffer.
2. Do 1:2 serial dilutions to obtain concentrations of 10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.625 µM, 0.313 µM, and 0.156 µM of R110 and Cell Lysis Buffer.
   Include blank (0 µM R110) samples of Cell Lysis Buffer only.
3. Add 100 µL per well of the serially diluted R110 solutions from 20 µM to 0 µM into a 96-well plate.
4. Measure the fluorescence intensity of the standards with excitation at 490 nm and emission at 520 nm.
5. Subtract the fluorescence reading from the blank (0 µM R110) from each fluorescence value to calculate relative fluorescence units (RFU).
6. Plot RFU versus R110 concentration to generate a standard curve. See Figure 3-1.

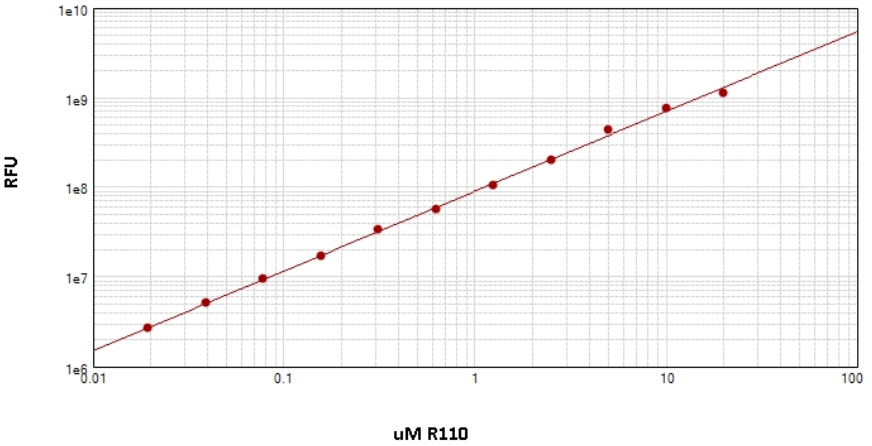


Figure 3-1: R110 Standard Curve

R110 standard concentrations were prepared as described. Fluorescence was measured on a SpectraMax i3 microplate reader, read from the top.

Note: Because the two-step cleavage of the substrate generates an intermediate and an end-product with different fluorescence intensities, the kinetics of fluorescence generation as the result of substrate cleavage are not linear.(See Assay Principles on page 3.) Consequently, the R110 standard can be used to quantitate the amount of R110 that is generated at the assay endpoint, but it cannot be used for kinetic studies.

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 Caspase-3/7 R110 protocol that is available in the protocol library.

Table 3-1: EarlyTox Caspase-3/7 R110 Assay protocol

Parameter	Setting
Read Mode	Fluorescence
Read Type	Endpoint
Wavelengths	Excitation = 490 nm Emission = 520 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.

Chapter 4: Data Analysis Examples

Example Results

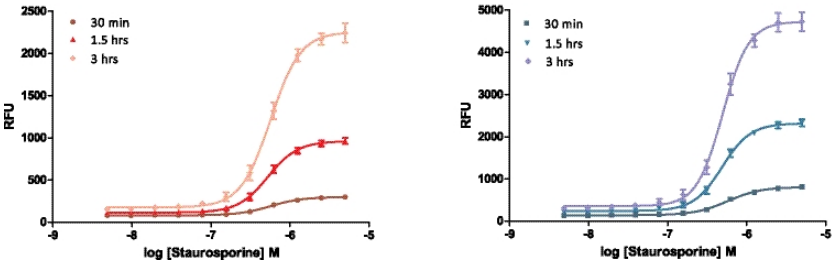
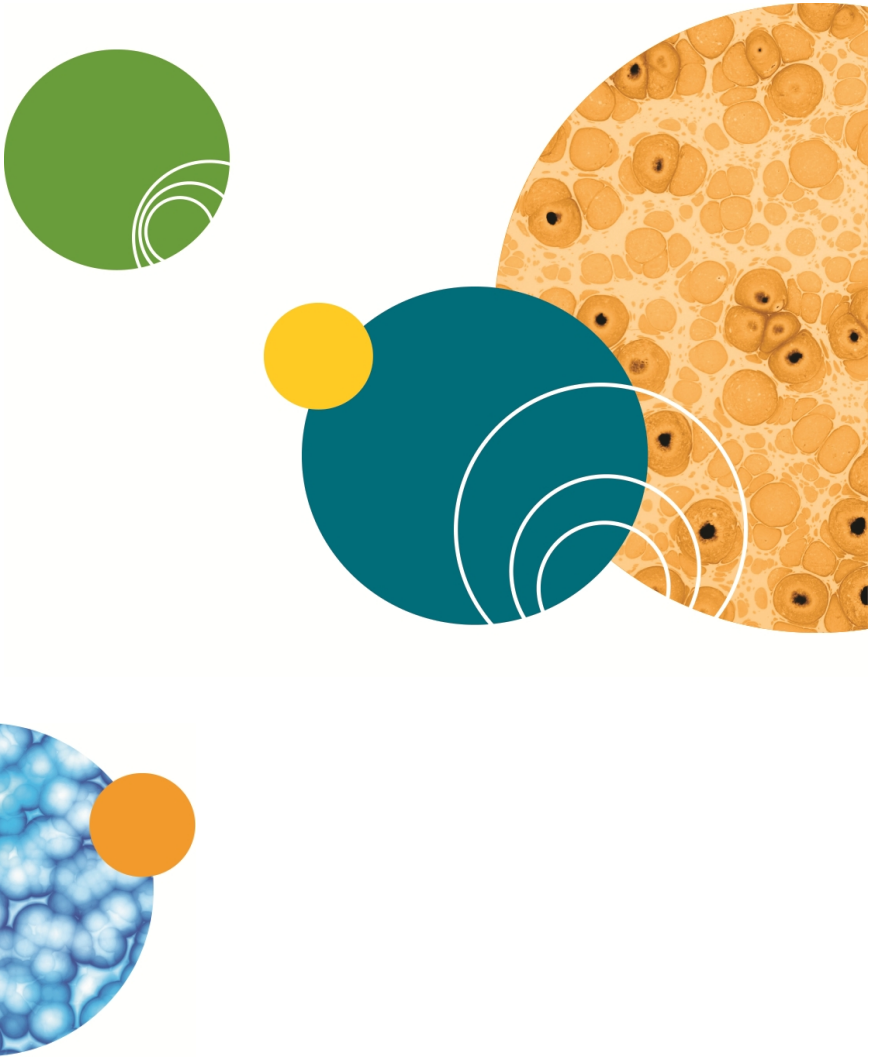


Figure 4-1: Apoptosis assay: HeLa cells treated with staurosporine

HeLa cells plated in a 96-well microplate overnight were treated with staurosporine for 4 hours at 37°C to induce apoptosis. 100 µL of 2x substrate assay buffer was added directly into each well. The samples were incubated at room temperature. Fluorescence was measured at 30 minutes, 1.5 hours, and 3 hours on a SpectraMax M5 microplate reader, read from the top (left) and the bottom (right).

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 concentrations of the two dyes, generally between 20 and 100 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.
- Try measuring the signals with both top and bottom read to determine the best instrument settings.
- The Well Scan read type might improve the data quality and consistency.



Chapter 5: 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 5-1: 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. 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).

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