EarlyTox Cell Integrity Kit

The EarlyTox™ Cell Integrity Kit from Molecular Devices is an optimized set of reagents that simplifies the measurement of live and dead cells in a single well. The assay uses two nuclear dyes that allow you to detect changes in outer cell membrane permeability resulting from cell damage or cell death via necrosis, apoptosis, or other mechanisms. Cell viability is assessed by counting the number or percent of live and damaged cells. Use the assay for studies of the effects of different treatments on cell viability, evaluation of toxicity effects of pharmaceutical compounds or other chemicals, studies of necrosis and apoptosis, and many other applications. The EarlyTox Cell Integrity Kit is designed to work with many cell types, both adherent and non-adherent.

The simple workflow and reagent performance of the protocol make it amenable to high-throughput screening using the ImageXpress® Micro XLS Widefield High Content Analysis System, ImageXpress® Micro 4 Automated Imaging System, ImageXpress® Nano Automated Imaging System, or the SpectraMax® i3x Multi-Mode Microplate Reader with the SpectraMax® MiniMax™ 300 Imaging Cytometer. See Setup for Imaging Instruments on page 11 and Setup for SpectraMax i3x Reader With the Spectra Max MiniMax 300 Imaging Cytometer on page 9.

Available Kits

Assay Kit	Explorer Kit	Bulk Kit
EarlyTox™ Cell Integrity Kit (200 Tests, two (2) 96-well or 384-well plates)	R8213	
EarlyTox™ Cell Integrity Kit (1000 Tests, ten (10) 96-well or 384-well plates)		R8214

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Chapter 1: About the EarlyTox Cell Integrity Kit

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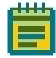
The EarlyTox Cell Integrity Kit provides a fast, simple, and reliable fluorescence-based assay to detect changes in cell viability based on permeability of outer cell membranes (cell integrity).

The kit is based on two nuclear dyes:

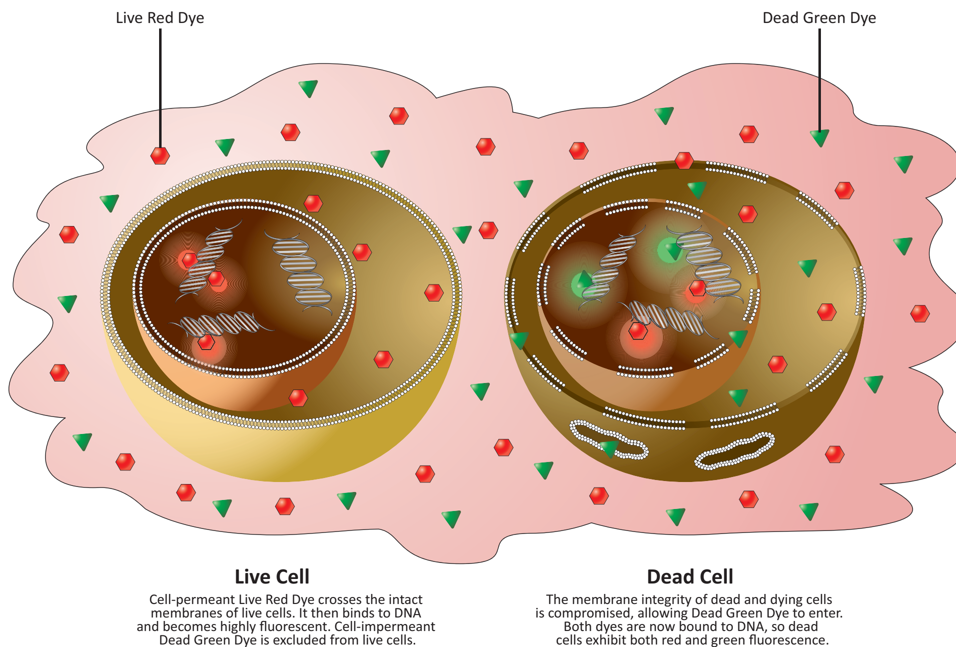
- Live Red Dye is cell permeant and marks both live and dead cells (Excitation: 622 nm/Emission: 645 nm).
- Dead Green Dye is cell impermeant and stains only cells with damaged outer membranes (Excitation: 503 nm/Emission: 526 nm).



Note: Use the appropriate wavelength setting for your instrument. See Setup for SpectraMax i3x Reader With the Spectra Max MiniMax 300 Imaging Cytometer on page 9 or Setup for Imaging Instruments on page 11.

The kit also contains a proprietary EarlyTox Masking Reagent to increase assay performance in a homogeneous format. Each kit enables a simple mix-and-read procedure whereby cells are incubated with reagents for 30 minutes and then transferred directly to an imaging instrument for evaluation. This eliminates time-consuming wash steps.

Assay Principle



EarlyTox Cell Integrity Kit Assay Principle

Chapter 2: Materials and Equipment

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Kit Components

Components of the EarlyTox Cell Integrity Kits

Item	Explorer Kit (R8213)	Bulk Kit (R8214)
EarlyTox Live Red Dye	1 vial	1 vial
EarlyTox Dead Green Dye	1 vial	1 vial
EarlyTox Masking Reagent	1 bottle	3 bottles

- The entire EarlyTox Cell Integrity Explorer Kit (R8213) is sufficient for two (2) 96-well or 384-well plates.
- The entire EarlyTox Cell Integrity Bulk Kit (R8214) is sufficient for ten (10) 96-well or 384-well plates.

Materials Required But Not Provided

Reagents and Supplies

Item	Suggested Vendor
DPBS Buffer (1x Dulbecco's Phosphate Buffered Saline) pH 7.4	Corning Cellgro or equivalent

Storage and Handling

On receipt of the EarlyTox Cell Integrity Kit, store the contents at –20°C. Under these conditions the reagents are stable for six months in the original packaging.

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: support.moleculardevices.com/

Chapter 3: Assay Protocols

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Standard Protocol

To run the standard protocol:

1. Plate the cells at the applicable plating densities (generally overnight) and treat the cells with compounds according to the specific experiment (generally for 24 to 48 hours).

   Note: For imaging applications, use black-well, clear-bottom plates.

2. Prepare 1:2000 dilutions of the dyes (add 10 µL of each reagent to 20 mL of DPBS) immediately before cell staining.
3. Gently remove the cell media from the plates by pipetting or aspirating (do not wash the cells).
4. Add the staining solution directly into the wells.
   - Add 100 µL per well for 96-well plates.
   - Add 25 µL per well for 384-well plates.
5. Return the plates to the incubator and incubate for 15 to 30 minutes at 37°C and 5% CO_2.
6. Proceed with image acquisition using the appropriate wavelengths for the Live Red Dye and Dead Green Dye. See Setup for SpectraMax i3x Reader With the Spectra Max MiniMax 300 Imaging Cytometer on page 9 or Setup for Imaging Instruments on page 11.
   - Live Red Dye can be detected using the 713 channel (Ex: 625 nm/Em: 713 nm).
   - Dead Green Dye can be detected by using the 541 channel (Ex: 456 nm/Em: 541 nm).

   Typical exposure times are 10 to 20 ms.

   Note: Adjust the exposure times for the 713 and 541 channels to achieve the correct color balance between channels. Generally, 541 exposure time is 2x greater than 713 exposure time.

Homogeneous Assay Protocol

Note: In this protocol the culture medium is not removed from the wells. Use the optional Masking Reagent to reduce the background fluorescence typically created by the medium.

To run the Homogeneous Assay protocol:

1. Grow cells in 96-well or 384-well plates with the appropriate treatments. The final volume of media should be approximately 100 µL for 96-well plates and 25 µL for 384-well plates.
2. Prepare 2x (1:1000) solution of staining reagents in DPBS (for example, 10 µL of each reagent in 10 mL of DPBS) immediately before cell staining.
3. Add a volume of 2x staining solution (equal to the volume already in the well) directly to the wells.
   - Add 100 µL for 96-well plates.
   - Add 25 µL for 384-well plates.
4. Return the plates to the incubator and incubate for 30 minutes at 37°C and 5% CO_2.
5. Optional:
   - Prepare a 5x solution of Masking Reagent (add 10 mL of DPBS to the bottle and vortex for ~1 minute).
   - Add the 5x solution of Masking Reagent directly into the media (25 µL per 96-well plate, 6.3 µL per 384-well plate).
   - Image the plate immediately.

   Note: Do not add Masking Reagent simultaneously with staining solution.
6. Proceed with image acquisition using the appropriate wavelengths for the Live Red Dye and Dead Green Dye. See Setup for SpectraMax i3x Reader With the Spectra Max MiniMax 300 Imaging Cytometer on page 9 or Setup for Imaging Instruments on page 11.
   Typical exposure time 10 to 20 ms. If Masking Reagent is used, it is necessary to increase imaging exposure times (100 to 200 ms).

Note: For optimal results it is important to use the recommended reagent volumes and concentrations. However, standard procedures vary across laboratories, and Molecular Devices recognizes that a variety of cell-handling conditions might be adopted at the discretion of the user. Assay development might be required for optimization.

Suggested Plating Volumes and Seeding Densities

Cell Type	96-well plate (100 µL growth medium with compounds)	384-well plate (25 µL growth medium with compounds)
Adherent cells	4000 to 20000 (cells per well)	1000 to 5000 (cells per well)
Non-adherent cells	20000 to 100000 (cells per well)	5000 to 20000 (cells per well)

Setup for SpectraMax i3x Reader With the Spectra Max MiniMax 300 Imaging Cytometer

Acquire images from the prepared plate using the SpectraMax i3x Multi-Mode Microplate Reader with the SpectraMax MiniMax 300 Imaging Cytometer and the SoftMax® Pro Data Acquisition and Analysis Software from Molecular Devices with the following suggested settings.

Wavelength:

- Excitation: 456 nm / Emission: 541 nm for Dead Green Dye
- Excitation: 625 nm / Emission: 713 nm for Live Red Dye

Image Acquisition Settings:

- Select control and treated wells for the **Max Image** and **Min Image** wells
- For Live Red Dye (713 nm), use a typical exposure time of 10 to 20 ms
- For Dead Green Dye (541 nm), use a typical exposure time of 20 ms

Image Analysis Settings:

- Use **Discrete Object Analysis**
- **Wavelength for Finding Objects**: 713 nm
- **Find Objects**
  - **Finding Method**: **Draw on Images**, or **Set Size and Intensity**

    Note: If you are using Masking Reagent, select **Set Size and Intensity**.

  - **Draw on Images** settings: **Nuclei** preset drawing analysis
    Set Size and Intensity settings:
    Min. Width: 5 µm
    Max. Width: 30 µm
    Intensity Above Background: 100–200 (adjust as needed for best results)
  - Click **Apply**
- **Classification**
  - **Settings**: Create New Classification
  - **Type A**: Rename to **Live**
    Select the objects that are red but not green.
  - **Type B**: Rename to **Dead**
    Select the objects that are both green and red.
  - Click **Apply**
- **Measurements**
  - Default (all parameters selected)

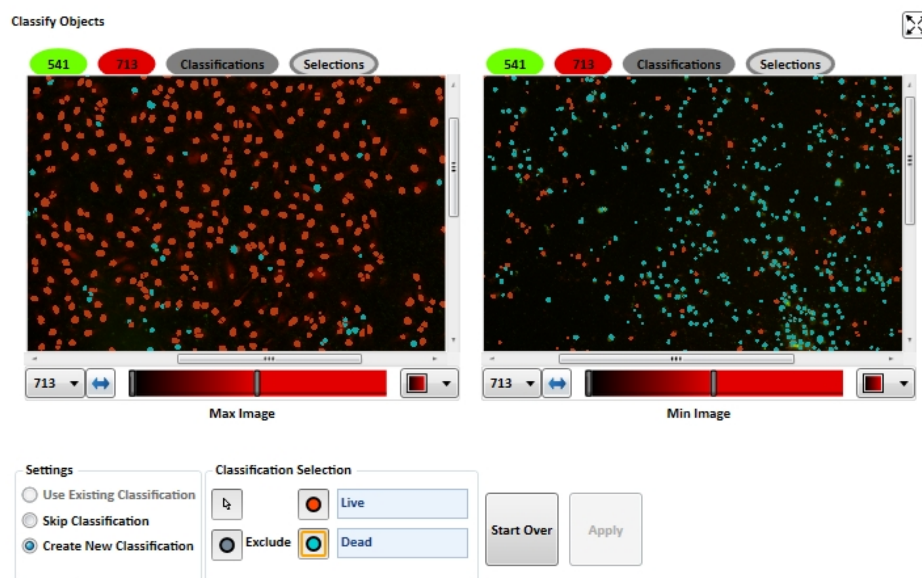
Click **OK** to save the acquisition and analysis settings.

Click **Read** to acquire the images with the defined analysis settings.

In the **Plate** section, define a template for the tested compounds and concentrations.

Create appropriate tables and graphs using the desired output parameters.

Use SoftMax Pro Software or another program for additional analysis.



Example of Results of Classification for EarlyTox Cell Integrity Assay Data Analysis Using the SoftMax Pro Software

Setup for Imaging Instruments

Acquire images from the prepared plate using the ImageXpress Micro XLS Widefield High Content Analysis System, ImageXpress Micro 4 Automated Imaging System, and ImageXpress Nano Automated Imaging System. Use the following suggested configurations and settings in the MetaXpress High-Content Image Acquisition and Analysis Software from Molecular Devices.

- 20x, 10x, or 4x objectives
- Cy5 filter cube (Ex: 628 nm, Em: 692 nm) for Live Red Dye with a typical exposure time of 10 ms
- FITC filter cube (Ex: 485 nm, Em: 530 nm) for Dead Green Dye staining with a typical exposure time of 20 ms
- For 384-well plates with the 10x or 20x objectives, take one (1) or more images per well
- For 96-well plates with the 10x or 20x objectives, take two (2) to four (4) images per well
- For the 4x objective, take one (1) image per well

For image analysis use the MetaXpress Software with the Live-Dead image processing module. The images are first segmented into individual cells using the Live Red nuclear stain and designated Live or Dead based on the presence of the Dead Green stain. Typical settings for image analysis include the following:

Wavelength 1 Parameters (for Live Red Dye):

- **Wavelength 1 Source**: Cy5
- **Approximate min width**: 7 µm
- **Approximate max width**: 30 µm
- **Intensity above local background**: ~500 graylevels

Wavelength 2: Parameters (for Dead Green Dye):

- **Wavelength 1 Source**: FITC
- **Approximate min width**: 5 µm
- **Approximate max width**: 30 µm
- **Intensity above local background**: ~500 graylevels

Note: Nuclear sizes and brightness of staining can vary depending on specific cells and between experiments. The suggested settings should be adjusted for the specific experiment.

Wavelength 1 Parameters

Wavelength 1 Source: Cy5

Display result image: [None]

Adaptive Background Correction™ system

Algorithm: Fast

Stained cell type: All Cells

Stained area: Nucleus

Approximate min width: 7 µm = 11 pixels

Approximate max width: 30 µm = 46 pixels

Intensity above local background: 500 graylevels

☑ Split touching objects

Preview

Wavelength 2 Parameters

Wavelength 2 Source: FITC

Display result image: [None]

Stained cell type: Dead

Stained area: Nucleus

Approximate min width: 5 µm = 8 pixels

Approximate max width: 30 µm = 46 pixels

Intensity above local background: 500 graylevels

☑ Split touching objects

Preview

Configure Summary Log... | Configure Data Log (Cells)...

Save Settings... | Load Settings... | Set to Defaults | Test Run | Close

Suggested Settings for EarlyTox Cell Integrity Assay Data Analysis Using the MetaXpress Software and the Live-Dead Application Module

In the **Configure Summary Log** dialog, select the following parameters for toxicity evaluation:

- Total Cells (number of all cells, nuclear count)
- Live Cells
- Dead Cells
- % Live Cells
- % Dead Cells

Chapter 4: Data Analysis Examples

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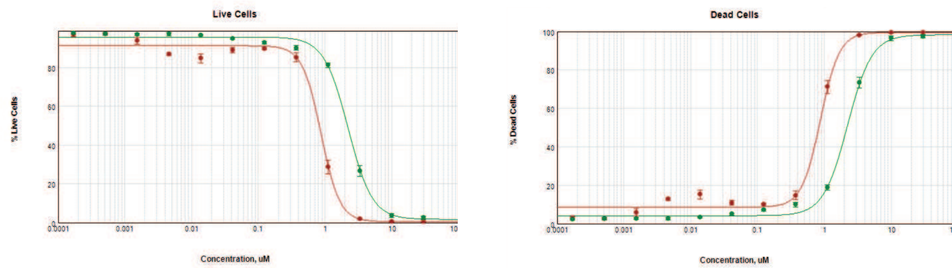

Examples of 4-parametric curve fits for concentration dependence of % of intact (left) or damaged (right) cells. Concentration responses are presented for staurosporine (red), IC_{50} 0.2 µM; and mitomycin C (green), IC_{50} 3.4 µM.

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 level of technical service.

Our Support website—support.moleculardevices.com/—describes the support options offered by Molecular Devices, including service plans and professional services. It also has a link to the Molecular Devices Knowledge Base, which contains documentation, technical notes, software upgrades, safety data sheets, and other resources. If you still need assistance, you can submit a request to Molecular Devices Technical Support.

Please have the instrument serial number (on the rear of the instrument), or reagent kit lot number, and any related sample data files available when you call.

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