In-Cell Western™ Assay (HeLa Cellular Response to Anisomycin Treatment)

LI-COR Biosciences

Abstract

This is a complete apoptosis assay example. It details the seeding, induction, and detection of the HeLa cellular response to Anisomycin treatment.

Developed for:

Aerius,
Odyssey® Classic,
Odyssey CLx, and
Odyssey Sa
Infrared Imaging Systems

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your Odyssey Imager model.

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Guidelines

I. Required Reagents

LI-COR Reagents

- IRDye® 800CW Goat anti-Mouse Secondary Antibody (LI-COR P/N 925-32210 or 926-32210)
- IRDye 680RD Goat anti-Rabbit Secondary Antibody (LI-COR P/N 925-668071 or 926-68071)
- Odyssey® Blocking Buffer (LI-COR P/N 927-40000 or 927-50000)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)(Step 9)
- 20% Tween® 20
- Anisomycin (Sigma®, P/N A9789)
- Trypsin-EDTA Solution (1X) (Sigma, P/N T-3924)
- 37% formaldehyde
- 10% Triton® X-100
- Clear or black 96-well or 384-well microplates (See IX. Experimental Considerations)

NOTE: When using primary antibodies discussed in this protocol, either blocking buffer will provide low background. This may not hold true for other primary antibodies.

- Primary antibodies:
  - Cleaved Caspase-3 (Cell Signaling Technologies, P/N 9661)
  - β-Tubulin (D-10), [Santa Cruz, P/N SC-5274] or Anti-β-Tubulin, clone AA2,

[Upstate Group Inc., P/N 05-661]

IX. Experimental Considerations

Proper selection of microplates can significantly affect the results, as each plate has its own characteristics, including well depth, plate autofluorescence, and well-to-well signal crossover. Use the following general considerations for microplate selection.

- In-Cell Western analyses use detection at the well surface with minimal liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear as well as black-sided plates with clear bottoms. Do not use plates with white walls, since autofluorescence from the white surface will create significant noise.
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR Biosciences:

  96-well format, Eppendorf (P/Ns 0030730119 or 0030741030)
  96-well, clear, Nunc® (P/Ns 167008, 161093)
  96-well, clear, FalconTM (P/Ns 353075, 353948)
  96-well, black with clear bottom, Nunc (P/N 165305)
  96-well format, Corning (P/N 3603)

  384-well, clear, Nunc (P/Ns 164688, 164730)
  384-well, clear, Falcon (P/Ns 353229, 353289)
  384-well, black with clear bottom, Nunc (P/N 142761)
  384-well format, Corning (P/N 3712)

- **Focus Offset Optimization** – If plates other than those recommended are used, the focus offset can be determined empirically by scanning a plate containing experimental and control samples using the following focus offset settings.

Instrument	Focus Offset Determination (mm)
Odyssey Classic & Odyssey CLx	1.0, 1.5, 2.0, 2.5, 3.0, 3.5, & 4.0
Odyssey Sa & Aerius	1.5, 2.0, 2.5, 3.0 & 3.95

Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise ratio for experiments. The actual minimum and maximum focus offset will vary with each instrument. Alternatively, the plate manufacturer can be consulted to obtain the measured distance from the skirt bottom to the bottom of the plate.

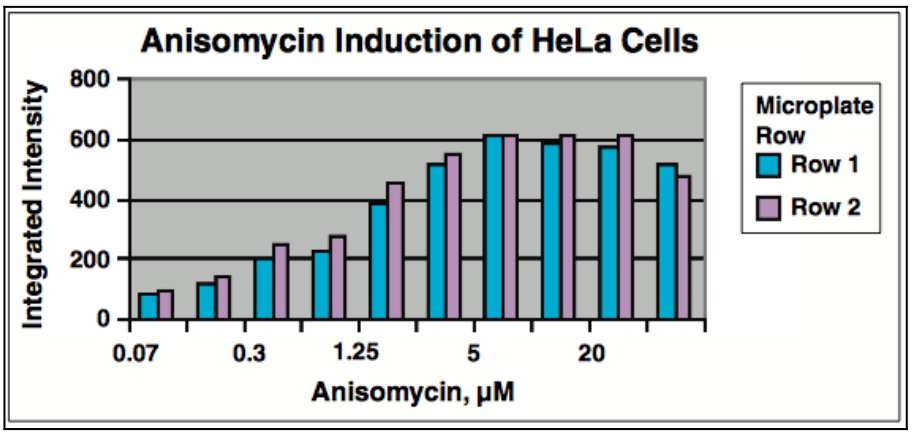
- All Aerius and Odyssey® Imaging systems (excluding Odyssey Fc) require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Aerius and Odyssey Sa instrument and is found by choosing Settings > System Administration, then clicking Scanner Information). When using plates specified for In-Cell Western assays, the recommended focus offset is 3.5 mm or higher.
- **Intensity Setting Optimization -**

Instrument	Initial Intensity Setting (700/800 nm)	Intensity Settings: Weak Signal (700/800 nm)	Intensity Settings: Saturated Signal (700/800 nm)
Odyssey® Classic	5 / 5	7.5 / 7.5	2.5 / 2.5
Odyssey CLx	5 / 5	7.5 / 7.5	2.5 / 2.5
	AutoScan*	-	-
Odyssey Sa	7 / 7	8 / 8	4 / 4
Aerius	7 / 7	8 / 8	4 / 4

**The Odyssey CLx AutoScan function alleviates the need to scan the plate at multiple intensity settings.*

- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at 4 °C.
- Establish the specificity of your primary antibody by screening lysates through Western blotting and detection on the Odyssey instrument. If significant non-specific binding is present, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results.

X. Experimental Results



Induction of apoptosis in HeLa cells was achieved with increasing concentrations of anisomycin. An increase in Cleaved Caspase-3, a cleaved by-product indicative of apoptosis, is illustrated in the graph. The ultimate result of apoptosis induction is cell death. This can clearly be seen at high

concentrations of anisomycin (5 - 40 µM) in this example. The reduction in cell number per well is taken into account when normalizing with another antibody or a DNA stain. In an assay such as this, normalization is very important. See www.licor.com/ICWnormalization for more information on normalization options for In-Cell Western assays
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Materials

Odyssey® Blocking Buffer (PBS) 927-40000 927-40100 by LI-COR

Odyssey Blocking Buffer (TBS) 927-50000 927-50100 by LI-COR

IRDye® 800CW Goat anti-Mouse Secondary Antibody 925-32210 926-32210 by LI-COR

IRDye 680RD Goat anti-Rabbit Secondary Antibody 925-668071 926-68071 by LI-COR

Anisomycin A9789 by Sigma Aldrich

Trypsin-EDTA Solution T-3924 by Sigma Aldrich

Cleaved Caspase-3 9661 by Cell Signaling Technology

β-Tubulin (D-10) SC-5274 by Santa Cruz Biotechnology

Anti-β-Tubulin, clone AA2 05-661 by Emd Millipore

Protocol

Cell Preparation

Step 1.
Allow HeLa (ATCC; CCL-2) cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).

Cell Preparation

Step 2.
Remove growth media, wash cells with sterile 1X PBS, and displace cells with 5 mL Trypsin EDTA solution.

AMOUNT

5 ml Additional info:
Trypsin-EDTA solution

Cell Preparation

Step 3.
Neutralize displaced cells with culture media and clarify by centrifugation.

Cell Preparation

Step 4.
Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube.
To maintain cell integrity, do not pipet or vortex during pellet disruption.

Cell Preparation

Step 5.
Reconstitute cells in complete media so that 50,000 cells/mL is achieved.

Cell Preparation

Step 6.
Manually mix the cell suspension thoroughly.

Cell Preparation

Step 7.
Under sterile conditions, dispense 200 µL of the cell suspension per well in a 96-well plate (10,000 cells plated per well).

AMOUNT

200 µl Additional info:
Cellular suspension per well

Cell Preparation

Step 8.
Incubate cells and monitor cell density until 80% confluency is achieved.

Cell Treatment

Step 9.

Warm serum-free media to 37 °C.

TEMPERATURE

37 °C Additional info:

Cell Treatment

Step 10.

Add either serum-free media for resting cells (mock) or serum-free media containing dilution series (1:2) of Anisomycin ranging in concentration from 0.07 - 40 µM. Add 100 µL of resting or activation media per well.

AMOUNT

100 µl Additional info:
Resting or activation
media per well

Cell Treatment

Step 11.

Transfer media from the dilution plate into the experimental plate.

Cell Treatment

Step 12.

Allow incubation at 37 °C for 4 hours.

TEMPERATURE

37 °C Additional info:
incubation temperature

DURATION

04:00:00

NOTES

James Thornton Jr 10 May 2017

Towards the end of the incubation, prepare the Fixing Solution, as described in Step14.

Fixing/Permeabilizing Cells

Step 13.

When the Cell Treatment incubation period is complete, remove activation media manually or by aspiration.

NOTES

James Thornton Jr 10 May 2017

Immediately fix cells with Fixing Solution (4% formaldehyde in 1X PBS) for 20 minutes at room temperature (RT) as detailed in steps 14-16.

Fixing/Permeabilizing Cells

Step 14.

Prepare fresh Fixing Solution as follows:

1X PBS	45 mL
37% Formaldehyde	5 mL
3.7% Formaldehyde	50 mL

Fixing/Permeabilizing Cells

Step 15.

Using a multi-channel pipettor, add 150 µL of fresh *Fixing Solution* (room temperature). **Add the solution by pipetting down the sides of the wells carefully to avoid detaching the cells from the well bottom.**

AMOUNT

150 µl Additional info:
Fixing Solution

Fixing/Permeabilizing Cells

Step 16.

Allow incubation on bench top for 20 minutes at RT with no shaking.

DURATION

00:20:00

Fixing/Permeabilizing Cells

Step 17.

Wash 5 times with 1X PBS containing 0.1% Triton® X-100 (cell permeabilization) for 5 minutes per wash. Detailed in steps 18 - 34.

NOTES

James Thornton Jr 05 Jan 2017

Do not allow cells/wells to become dry during washing. Immediately add the next wash after manual disposal.

Fixing/Permeabilizing Cells

Step 18.

Prepare Triton Washing Solution as follows:

1X PBS	495 mL
10% Triton X-100	5 mL
1X PBS + 0.1% Triton X-100	500 mL

Fixing/Permeabilizing Cells

Step 19.

Remove Fixing Solution to an appropriate waste container (contains formaldehyde).

Fixing/Permeabilizing Cells

Step 20.

Using a multi-channel pipettor, add 200 µL *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 1/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 21.

Allow plate to shake on a rotator for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 22.

Remove wash manually. (wash 1/5)

Fixing/Permeabilizing Cells

Step 23.

Using a multi-channel pipettor, add 200 µL *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 2/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 24.

Allow plate to shake on a rotator for 5 minutes at RT. (wash 2/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 25.

Remove wash manually. (wash 2/5)

Fixing/Permeabilizing Cells

Step 26.

Using a multi-channel pipettor, add 200 µL *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 3/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 27.

Allow plate to shake on a rotator for 5 minutes at RT. (wash 3/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 28.

Remove wash manually. (wash 3/5)

Fixing/Permeabilizing Cells

Step 29.

Using a multi-channel pipettor, add 200 µL *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 4/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 30.

Allow plate to shake on a rotator for 5 minutes at RT. (wash 4/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 31.

Remove wash manually. (wash 4/5)

Fixing/Permeabilizing Cells

Step 32.

Using a multi-channel pipettor, add 200 µL *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 5/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 33.

Allow plate to shake on a rotator for 5 minutes at RT. (wash 5/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 34.

Remove wash manually. (wash 5/5)

Blocking Cells

Step 35.

Using a multi-channel pipettor, block cells/wells by adding 150 µL of Odyssey® Blocking Buffer to each well. **Add the solution by pipetting down the sides of the wells carefully to avoid detaching the cells.**

NOTES

James Thornton Jr 11 Jan 2017

No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution.

James Thornton Jr 11 Jan 2017

Odyssey Blocking buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4 °C, so diluted antibodies cannot be kept and reused for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammarsten-grade casein is not required).

James Thornton Jr 11 Jan 2017

Blocking solutions containing BSA can be used, but in some cases may cause high membrane background. **BSA-containing blockers are not generally recommended** and should be used only when the primary antibody requires BSA as blocker.

Blocking Cells

Step 36.

Allow blocking for 1.5 hours at RT with moderate shaking on a plate shaker.

DURATION

01:30:00

Primary Antibodies

Step 37.
Dilute the two primary antibodies in Odyssey or StartingBlock™ Blocking Buffer. Combine the following antibodies for Cleaved Caspase-3 target analysis:

- Cleaved Caspase-3; rabbit (1:100 dilution).
- Anti-β-Tubulin, clone AA2; mouse (1:100 dilution), or β-Tubulin (D-10); mouse (1:100 dilution).

Primary Antibodies

Step 38.
Mix the primary antibody solution thoroughly before adding to wells.

Primary Antibodies

Step 39.
Remove blocking buffer and add 50 µL of the desired primary antibody or antibodies in Odyssey or StartingBlock Blocking Buffer to cover the bottom of each well.

AMOUNT

50 µl Additional info:

Primary Antibodies

Step 40.
Make sure to include control wells without primary antibody to serve as a source for background well intensity. To control wells, add 50 µL of Odyssey or StartingBlock Blocking Buffer only.

AMOUNT

50 µl Additional info:
Odyssey or StartBlock
Blocking Buffer

Primary Antibodies

Step 41.
Incubate with primary antibody for 2 hours with gentle shaking at RT.

DURATION

02:00:00

NOTES

James Thornton Jr 11 Jan 2017

NOTE: For greatest sensitivity, continue incubation overnight at 4 °C with no shaking.

Primary Antibodies

Step 42.
Wash the plate 5 times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. Detailed in steps 43 - 57.

Primary Antibodies

Step 43.
Prepare Tween Washing Solution as follows:

1X PBS	495 mL
10% Triton X-100	5 mL
1X PBS + 0.1% Triton X-100	500 mL

Primary Antibodies

Step 44.
Using a multi-channel pipettor, add 200 µL *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 1/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 45.
Allow wash to shake on plate shaker for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Primary Antibodies

Step 46.
Remove wash manually.(wash 1/5)

Primary Antibodies

Step 47.
Using a multi-channel pipettor, add 200 µL *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 2/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 48.
Allow wash to shake on plate shaker for 5 minutes at RT. (wash 2/5)

DURATION

00:05:00

Primary Antibodies

Step 49.

Using a multi-channel pipettor, add 200 µL *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 3/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 50.

Allow wash to shake on plate shaker for 5 minutes at RT. (wash 3/5)

DURATION

00:05:00

Primary Antibodies

Step 51.

Remove wash manually.(wash 3/5)

Primary Antibodies

Step 52.

Using a multi-channel pipettor, add 200 µL *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 4/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 53.

Allow wash to shake on plate shaker for 5 minutes at RT. (wash 4/5)

DURATION

00:05:00

Primary Antibodies

Step 54.

Remove wash manually.(was 4/5)

Primary Antibodies

Step 55.

Using a multi-channel pipettor, add 200 µL *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 5/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 56.

Allow wash to shake on plate shaker for 5 minutes at RT. (wash 5/5)

DURATION

00:05:00

Primary Antibodies

Step 57.

Remove wash manually.(wash 5/5)

Secondary Antibodies

Step 58.

Dilute the fluorescently-labeled secondary antibody in Odyssey® Blocking Buffer and add 0.5% Tween 20 to the diluted antibody to lower background as specified below.

- IRDye® 680RD Goat anti-Rabbit (1:800 dilution)
- IRDye 800CW Goat anti-Mouse (1:800 dilution)

NOTES

James Thornton Jr 11 Jan 2017
Recommended dilution range is 1:200 to 1:1,200.

James Thornton Jr 11 Jan 2017
Avoid prolonged exposure of the antibody vials to light.

Secondary Antibodies

Step 59.

Mix the antibody solutions well and add 50 µL of the secondary antibody solution to each well.

AMOUNT

50 µl Additional info:
secondary antibody

solution

Secondary Antibodies

Step 60.

Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

DURATION

01:00:00

Secondary Antibodies

Step 61.

Wash the plate 5 times with 1X PBS + 0.1% Tween 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer. Detailed in steps 62 - 75.

NOTES

James Thornton Jr 11 Jan 2017

Protect plate from light during washing.

James Thornton Jr 11 Jan 2017

NOTE: If using an Eppendorf glass-bottom plate (P/N 0030741030), rinse briefly with PBS, then wash once with PBS on a rotator for 5 min at room temperature.

Secondary Antibodies

Step 62.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT. **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 1/5)

GOTO

Tween Washing Solution, Step 43 -> go to step #43

Secondary Antibodies

Step 63.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Secondary Antibodies

Step 64.

Remove wash manually.(wash 1/5)

Secondary Antibodies

Step 65.
Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT. **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 2/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 66.
Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 2/5)

DURATION

00:05:00

Secondary Antibodies

Step 67.
Remove wash manually.(wash 2/5)

Secondary Antibodies

Step 68.
Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT. **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 3/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 69.
Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 3/5)

DURATION

00:05:00

Secondary Antibodies

Step 70.
Remove wash manually.(wash 3/5)

Secondary Antibodies

Step 71.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (VI. Primary Antibodies, step 44). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 4/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 72.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 4/5)

DURATION

00:05:00

Secondary Antibodies

Step 73.

Remove wash manually.(wash 4/5)

Secondary Antibodies

Step 74.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (VI. Primary Antibodies, step 44). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom.** (wash 5/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 75.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 5/5)

DURATION

00:05:00

Imaging

Step 76.

After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer

Imaging

Step 77.

For best results, scan plate immediately; plates may also be stored at 4 °C for several weeks (protected from light).

TEMPERATURE

4 °C Additional info:
storage temperature

Imaging

Step 78.

Before plate scanning, clean the bottom plate surface and the Odyssey Imager scanning bed (if applicable) with moist, lint-free paper to avoid any obstructions during scanning.

Imaging

Step 79.

Scan plate with detection in both 700 and 800 nm channels using an Odyssey or Aerius System described following:

NOTE: All settings may require adjustment for optimal data quality.

Instrument	Resolution*	Focus Offset**	Scan Quality*	Intensity Setting (700/800)	Scan Time Medium Quality
Odyssey® Classic	169 µm	3.5	medium-lowest	5 / 5	7 min
Odyssey CLx	169 µm	3.5	medium-lowest	5 / 5	7 min
	169 µm	3.5	medium-lowest	AutoScan	16 min
Odyssey Sa	200 µm	3.5	medium-lowest	7 / 7	3 min
Aerius	200 µm	3.5	medium-lowest	7 / 7	3 min

**Higher resolution or scan quality may be used, but scan time will increase.*

Warnings

See SDS for safety and hazards.