Cellometer® Spectrum

ViaStain™ Cell Fitness Panel for Cellometer Spectrum

Product Number: CSK-V0023-1

Nexcelom Bioscience

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Description

Cell Fitness Panel Kit is designed for researchers interested in acquiring data on cell sample quality/health during of product development or manufacturing. Many samples, in cellular therapy, immunotherapy, and cell line development, are by their nature are unique and precious. Patient derived primary samples are often comprised of a heterogeneous cell population and display a wide range of physiological behaviors. Whether these samples underwent a freeze/thaw cycle, or cell isolation procedure, or have been cultured within a bioreactor for an extended period of time, the Cell Fitness Panel is used to assess not only the wellbeing of cell samples but also offer potential insights into samples with low or crashing viabilities. It consists of five individual tests to assess viability, vitality, mid and late stage apoptosis, and reactive oxidative stress.

Materials

Materials Supplied

The kit contains enough reagents to perform 25 individual test for each assay catagory

Assay	Reagent	Component	Amount	Storage
Viability	AO/PI	A	500 µL	4°C
Enzymatic Activity	Calcein AM	B	50 µL	-20°C
	Propidium iodide	C	125 µL	-20°C
Mid-Stage Apoptosis	AnnexinV	D	125 µL	4°C
	PI	E	125 µL	4°C
	AnnexinV Buffer	F	2.5 mL	4°C
Late-State Apoptosis	Caspase3	G	50 µL	4°C
Oxidative Stress	Reactive Oxygen Species (ROS)	H	1 Vial	-20°C
	ROS Buffer	I	20 mL	-20°C
	DMSO	J	200 µL	-20°C

Materials Required

1. Micro centrifuge tubes
2. Pipettes
3. Cellometer SD100 or PD100 slides
4. Cellometer Spectrum
5. FCS Express Software

AO/PI Viability Assay

Assay	Reagent	Component	Amount	Storage
Viability	AO/PI	A	500 µL	4°C

1. Description of Assay

The ViaStain™ AOPI Staining Solution in PBS enables the user to quantitatively distinguish live and dead nucleated cells from a variety of primary mammalian cell samples, even in the presence of a high background of non-lysed red blood cells, platelets and/or debris using the Cellometer system. This formulation has been optimized to work with whole blood, peripheral blood mononuclear cells (PBMC), bone marrow, splenocytes, thymocytes, lymph node and hepatocytes but also works in various other digested tissues and cultured cell lines. The solution contains a combination of the green fluorescent nucleic acid stain, acridine orange, and the red-fluorescent nucleic acid stain, propidium iodide. Propidium iodide is a membrane exclusion dye that only enters cells with compromised membranes while acridine orange penetrates all cells in a population. When both dyes are present in the nucleus, propidium iodide causes a reduction in acridine orange fluorescence by fluorescence resonance energy transfer (FRET). As a result, nucleated cells with intact membranes stain fluorescent green and are counted as live, whereas nucleated cells with compromised membranes only stain fluorescent red and are counted as dead when using the Cellometer system. Non-nucleated material such as red blood cells, platelets and debris do not fluoresce and are ignored by the Cellometer software.

2. Experimental Protocol
    1. Pipette 20 µL of cell sample into a microcentrifuge tube.
    2. Add 20 µL of AOPI Staining Solution to microcentrifuge tube and mix well by pipetting up and down 3-5 times.
    3. Load 20 µL of stained sample into a counting chamber
        a. If using SD100 slides, peel plastic film off both sides before loading).
    4. Select the appropriate assay type for AOPI viability measurement.
    5. Preview bright field and fluorescent images
    6. Press Count

Calcein AM/PI Enzymatic Activity Assay

Assay	Reagent	Component	Amount	Storage
Enzymatic Activity	Calcein AM	B	50 µL	-20°C
	Propidium iodide	C	125 µL	-20°C

1. Description of Assay

Calcein AM (Calcein acetoxymethyl ester) is a cell permeable, non-fluorescent compound. Upon crossing the cell membrane, calcein AM is rapidly hydrolyzed by cellular esterases inside live cells. The hydrolysis cleaves the AM group, converting the non-fluorescent calcein AM to a strongly green fluorescing calcein. The more hydrophilic calcein is trapped inside the cell (1). Cells that do not possess active cytoplasmic esterases are unable to convert calcein AM to calcein, and therefore do not fluoresce green. This allows for a quick and easy detection of metabolically-active cells in a sample. Stains such as propidium iodide (PI), 7-AAD, and ethidium bromide (EB), are membrane exclusion dyes that are frequently used to stain non-viable nucleated cells with compromised membranes. Acridine orange freely diffuses across the cell membrane and stains DNA in all nucleated cells. When AO and PI are combined it is possible to determine % viability for nucleated cells. When calcein AM is used in conjunction with PI, it is possible to determine % vitality / viability based on the number of metabolically-active (green fluorescent) and non-viable (red fluorescent) cells in a sample. Since calcein AM does not require DNA binding, it stains all metabolically-active cells and can be used to measure metabolic activity in non-nucleated cells, such as platelets (4). Because calcein AM is photostable, shows low cytotoxicity, does not affect cellular functions, and requires cellular esterases for conversion to green fluorescing calcein, it is a popular stain for the examination of cell vitality and viability. (1,2,3).

1. Braut-Boucher, F. et al. Journal of Immunological Methods. Vol. 178, Issue 41 (1995).

2. Luc S. De Clerck. et al. Journal of Immunological Methods. Vol. 172, Issue 1, (1994).

3. Parish, CR. Immunology and Cell Biology. Vol. 77 (1999)

4. Verheul , HW. et al. Blood. Vol. 96 No. 13 (2000)

2. Experimental Protocol
3. Prepare calcein AM Working Solution

Note: Calcein AM working solution is stable up to 3 hrs

1. Pipette 2 µl Calcein-AM (Component B) into 18 µl of dH2O. This is now calcein-AM working solution.
    a. Mix by pipetting up and down at least 15 times or vortex.

4. Staining Procedure
    1. Add 5 µl of Calcein-AM working solution and 5 µl of PI Staining Solution (Component C) to 40 µl of cell sample.
    2. Gently pipette the sample up and down ten times, then incubate for 20 min at 37°C in the dark.
    3. After the 20 minute incubation, the sample is ready for analysis.
    4. Gently mix the cell sample by pipetting up and down at least ten times
    5. Then load 20 µL into the Cellometer counting chamber
    6. Insert into the Cellometer instrument.
    7. Select the appropriate assay type for calcein AM vitality measurement.
        a. Verify that the dilution factor is set to 1.25
    8. Preview bright field and fluorescent images
    9. Press Count

AnnexinV/PI Mid-Stage Apoptosis Assay

Assay	Reagent	Component	Amount	Storage
Mid-Stage Apoptosis	AnnexinV	D	125 µL	4°C
	PI	E	125 µL	4°C
	AnnexinV Buffer	F	2.5 mL	4°C

1. Description of Assay

Apoptosis, or *programmed cell death*, is a natural process of cellular self-destruction. Apoptosis is a part of routine cell turnover and tissue homeostasis, prevalent in epithelial cells, erythrocytes, and other cell types genetically programmed to have a limited life span. It is also important in embryogenesis, maintenance of immune tolerance, and development of the nervous system. Apoptosis can be induced either by a stimulus, such as irradiation or toxic drugs, or by removal of a repressor agent. The cells disintegrate into membrane-bound particles that are then eliminated by phagocytosis. Problems with the regulation of apoptosis are thought to be linked to many cancers, degenerative diseases, and autoimmune diseases, making apoptosis a key target in many fields of clinical research.

Necrosis is the death of cells or tissues from severe injury or disease, especially in a localized area of the body. Causes of necrosis include inadequate blood supply (as in infarcted tissue), bacterial infection, traumatic injury, and hyperthermia.

Annexin V and propidium iodide are used to measure apoptosis and necrosis. Annexin V is a member of the annexin family of intracellular proteins that binds to phosphatidylserine (PS) in a calcium-dependent manner. PS is normally only found on the intracellular leaflet of the plasma membrane in healthy cells, but during early apoptosis, PS translocates to the external leaflet. Fluorochrome-labeled Annexin V can then be used to specifically target and identify the PS on the surface of apoptotic cells. Annexin V binding alone cannot differentiate between apoptotic and necrotic cells. Propidium Iodide (PI) solution is a membrane-exclusion dye that permeates cells with compromised cell membranes and binds to DNA. Early apoptotic and healthy cells with intact membranes will exclude PI, while late stage apoptotic and necrotic cells with compromised membranes are stained. Use of both Annexin V-FITC and PI allows researchers to characterize a cell population based on % normal, % apoptotic, and % necrotic /very late-stage apoptotic cells.

2. Experimental Protocol
3. Generation of a Positive Control
    1. A positive control should be used to check exposure time and optimize the y-axis gate in FCS Express for apoptotic and non-apoptotic cells. A positive control may be generated by exposing cells to an apoptosis inducing pharmacological agents such as α-TOS and etoposide. For Cellometer examples, Jurkat cells were incubated overnight with 10 µM α-TOS.
    2. A negative control (untreated cells) should be tested to determine baseline cell concentration, % viability, and % apoptotic cells. The negative control is used to optimize the x-axis gate for live and dead cells in FCS Express.

Note: Positive and negative controls should be processed at the same time using the staining and data acquisition procedures outlined below.

4. Staining Procedure
    1. Into a new tube, pipette 50 µl of cells that are at a concentration of 2-3 x 10^6 cells/mL. Spin down cell sample at 200 - 400 x g (~1,000 to 2,000 rpm) for 5 minutes
    2. Carefully aspirate medium from the tube

Note: Take care not to aspirate off cells. It's ok if several microliters remain in the bottom

3. Resuspend cells in 40 µl of Annexin V Binding Buffer (Component F). Mix by pipetting up and down at least 10 times.
4. Add 5 µl of Annexin V-FITC (Component D) to the same tube
5. Add 5 µl of PI solution (Component E) to the same tube
6. Gently pipette the cells up and down ten times, then incubate for 15 min at RT (25°C) in the dark.
7. Add 250 µl of 1x PBS to the sample and spin down the cell sample at 200 - 400 x g (~1,000 to 2,000 rpm) for 5 minutes.
8. Carefully aspirate off the medium. Once again take care not to aspirate off any cells
9. Resuspend cell pellet in 50 µl of Annexin V Binding Buffer (Component F)
10. Mix by pipetting up and down ten times
11. Then load 20 µL into the Cellometer counting chamber
12. Select the appropriate assay type for AnnexinV/PI apoptosis measurement.
13. Preview bright field and fluorescent images
14. Press Count
15. Once cells are correctly counted, click on the report icon at the top left of the screen
16. Then click the Export button at the bottom right of the Counting Results screen.
17. Confirm that "Export data to Nexcelom Data Package" is selected, and click Continue
18. Open FCS Express AnnexinV template and import the positive and negative controls

Caspase3/7 Late-Stage Apoptosis Assay

Assay	Reagent	Component	Amount	Storage
Late-State Apoptosis	Caspase3	G	50 µL	4°C

1. Description of Assay

The reagent (NucView™) consists of a nucleic acid-binding dye with a fluorescent probe that is attached to a four-amino acid peptide sequence DEVD (Asp-Glu-Val-Asp) forming a cell membrane-permeable DEVD-DNA complex. While the nucleic-acid dye is linked to the DEVD peptide sequence, the dye is unable to bind to DNA and remains non-fluorescent. During apoptosis, caspase 3/7 proteins cleave the DEVD-DNA dye complex and thereby release the high-affinity DNA dye, which translocates to the nucleus and binds to the DNA, producing a bright green fluorescent signal.

2. Experimental Protocol
3. Generation of a Positive Control
    1. A positive control may be generated by exposing cells to an apoptosis inducing pharmacological agents such as α-TOS and etoposide. For Cellometer examples, Jurkat cells were incubated overnight with 20 µM α-TOS.
4. Staining procedure
    1. Into a new tube, pipette 398 µl of cells that are at a concentration of 2-3 x 10^6 cells/ml
    2. Add 2 µl of Caspase 3 (Component G) to the cells.
    3. Incubate for 30 mins at 37°C
    4. Mix by pipetting up and down ten times
    5. Then load 20 µL into the Cellometer counting chamber
    6. Select the appropriate assay type for Caspase3 apoptosis measurement.
    7. Preview bright field and fluorescent images
    8. Press Count

Reactive Oxygen Species (ROS) Oxidative Stress Assay

Assay	Reagent	Component	Amount	Storage
Oxidative Stress	Reactive Oxygen Species (ROS)	H	1 Vial	-20°C
	ROS Buffer	I	20 mL	-20°C
	DMSO	J	200 µL	-20°C

1. Description of Assay:

Reactive oxygen species (ROS) are natural byproducts of the normal metabolism of oxygen and play important roles in cell signaling. However, during oxidative stress-related states, ROS levels can increase dramatically. The accumulation of ROS results in significant damage to cell structures. The role of oxidative stress in cardiovascular disease, diabetes, osteoporosis, stroke, inflammatory diseases, a number of neurodegenerative diseases and cancer has been well established. The ROS measurement will help to determine how oxidative stress modulates varied intracellular pathways. The ROS Assay Kit uses our unique ROS sensor to quantify ROS in live cells. ROS Green is cell-permeable. It generates the green fluorescence when it reacts with ROS. The kit is an optimized "mix and read" assay format, without a washing step.

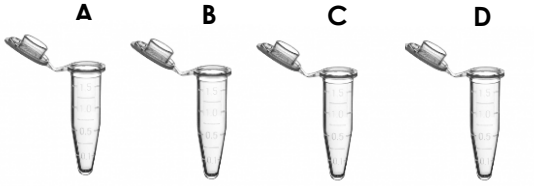
2. Prepare ROS Green Stock Solution:

Note: *All unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles.*

1. To ViaStain™ Total ROS Green for Cellometer Spectrum (Component H) add 65 µL of DMSO (Component J) to produce **stock solution**
2. Mix well, protect from light and store at -20 °C

3. Multi-Sample Experimental Protocol:

1. Add 2 µL of Total ROS Green **stock solution** (from step 2.1) into 600 µL of ROS buffer (component I). This is your ROS **working solution**
2. Obtain 4 small empty centrifuge tubes
3. Obtain 600 µL of cells at $3x10^6$ cells/ml
4. Aliquot 150 µL of cells per each empty tube
5. Centrifuge all four tubes for 5 minutes at 1200 rpm.
6. Decant media from each tube
7. Resuspend cells in 150 µL in ROS **working solution** from step 1.
8. Incubate for 1 hr at 37°C



(+) Control | (-) Control | (1) Sample | (2) Sample

9. After 1 hr incubation treat cells with desired compound(s) or move to step 11
    a. For negative control, add equal volume of 1x PBS
    b. For positive control, add 5.3 µL of 2.9 mM TBHP. See Section 4 for detail
10. Incubate/treat cells for an appropriate amount of time
    a. Treat for 30 minutes at 37°C if using TBHP
11. After incubation, add 20 µL of cell sample into a Cellometer counting chamber
12. Insert into Cellometer Spectrum and image

4. Single-Sample Experimental Protocol:

1. Obtain 600 µL of cells at $3x10^6$ cells/ml
2. Centrifuge cells for 5 minutes at 1200 rpm.
3. Decant media and resuspend cells in 610 µL of ROS buffer (component I)
4. Add 2 µL of Total ROS Green stock solution (from step 2.1)
5. Incubate for 1 hr at 37°C
6. After 1 hr incubation either treat cells with desired compound or load 20 µL of cell sample into a Cellometer counting chamber
7. Insert into Cellometer Spectrum and image

5. Generation of Single-Sample Positive Control:

1. Prepare *tert*-Butyl hydroperoxide solution (TBHP 70X) working solution by adding 2 µL TBHP stock solution to 5 mL of Water (this makes a 2.9 mM working solution)
2. Obtain 600 µL of cells at $3x10^6$ cells/ml
3. Centrifuge cells for 5 minutes at 1200 rpm.
4. Decant media and resuspend cells in 362 µL of ROS buffer (component I)
5. Add 2 µL of Total ROS Green stock solution (from set 2.1)
6. Incubate for 1 hr at 37°C
7. After incubation add 21 µL of 2.9 mM TBHP to induce ROS.
    a. Final TBHP concentration is 100 µM
    b. **Note**: Need to add 5.3 µL of 2.9 mM TBHP to 150 µL cells (tube A, step 9 for multi-sample protocol)
8. Incubate for 30 minutes at 37°C
9. After 30 minutes load the 20 µL of treated sample into a Cellometer counting chamber
10. Insert into Cellometer Spectrum and image

Storage and Handling

Store the Cell Fitness Panel components at the correct designated temperatures. Refrigerated samples store between 2°-8°C. Freezer samples store between -16° to -24°C protected from light. AVOID REPEATED FREEZE THAW CYCLES. Please consult the Safety Data Sheet for more safety information, found on www.nexcelom.com/Products.

Warranty

This product is for RESEARCH USE ONLY and is not approved for diagnostic or therapeutic use. Product is warranted to meet the specifications outlined in the Certificate of Analysis when stored and used according to the manufacturer's instructions. No other warranty, expressed or implied (such as merchantability, fitness for a particular purpose, or non-infringement) is granted. Warranty is valid until the expiration date stated on the product label. If no expiration is listed, the warranty is valid for 6 months from the date of product receipt.

Warranty will be void if product is stored incorrectly, the recommended protocol is not followed, or the product is used for a different application.

Ordering Information

When ordering with a Purchase Order:

Fax a copy of the order to 978-327-5341

Email a copy of the order to sales@nexcelom.com

When ordering with a Credit Card:

Visit www.shop.nexcelom.com and place your order