sartorius

MultiCyt® Apoptosis Kits

intellicyt®
A SARTORIUS BRAND

Protocol Guide

Store at 2 – 8 °C.
For Research Use Only.

Notice to Purchaser
The MultiCyt Apoptosis Kits are members of the MultiCyt® product line that has been extensively tested for live cell analysis applications. MultiCyt screening kits are validated as complete screening assays and are optimal for use in high content flow screening applications. IntelliCyt reagent kits are specifically formatted for optimal performance on IntelliCyt screening platforms.

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Limited Warranty
These products are offered under a limited warranty. The products are guaranteed to meet appropriate performance specifications described in the product insert at the time of shipment. IntelliCyt Corporation will provide product replacement for valid claims. All claims should be made within five (5) days of receipt of order.

Trademarks and Patents
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This product contains licensed NucView™ substrate from Biotium, Inc.

List of Catalog Numbers

Description	Catalog No.			
	1 x 384 wells	5 x 384 wells	20 x 384 wells	50 x 384 wells
MultiCyt 4-Plex Apoptosis Screening Kit	90053	90054	90155	90156
MultiCyt Caspase 3/7 Reagent	N/A	91034	91035	91036
MultiCyt Annexin V Reagent	N/A	91030	91031	91032
MultiCyt FL3 Cell Membrane Integrity Reagent	N/A	90346	90347	90348
MultiCyt Mitochondrial Membrane Potential Reagent	N/A	91038	91039	91040

Kit Contents

MultiCyt Apoptosis Screening Kits (Catalog #90053, 90054, 90155, 90156)	
Caspase Detection Reagent	FL1
Annexin V Binding Reagent	FL2
FL3 Cell Membrane Integrity Reagent	FL3
Mitochondrial Membrane Potential Reagent	FL4
10X Annexin Binding Buffer	

MultiCyt Caspase 3/7 Reagent (Catalog #91034 – 91036)	
Caspase Detection Reagent	FL1

MultiCyt Annexin V Binding Reagent (Catalog #91030 – 91032)	
Annexin V Binding Reagent	FL2
10X Annexin Binding Buffer	

MultiCyt FL3 Cell Membrane Integrity Reagent (Catalog #90346 – 90348)	
FL3 Cell Membrane Integrity Reagent	FL3

MultiCyt Mitochondrial Membrane Potential Reagent (Catalog #91038 – 91040)	
Mitochondrial Membrane Potential Reagent	FL4

Materials Needed but not Provided

- IntelliCyt Screening System
- ForeCyt Screening Software
- 384-well plates (recommend: Greiner 781280)
- Appropriate cells and cell culture media
- Positive and negative control(s) appropriate to the cellular model
- DMSO or other vehicle control
- Buffers for S1, S2, S3, and S4 rinse stations

Table of Contents

Introduction

Apoptosis is the process of programmed cell death where cells undergo specific shutdown and digestion mechanisms. It can be triggered as a defense mechanism against toxic events or executed by cells that are no longer necessary. There are numerous methods by which apoptosis is initiated, and clear markers that can be assayed to determine if a cell has become apoptotic.

Apoptosis plays a fundamental role in cell biology, and characterizing this endpoint is an important target across the drug discovery process, from primary screening to toxicity profiling.

The MultiCyt Apoptosis Kits are a family of reagents that each inform on a different target and potential mechanism of apoptosis. As a product family, the reagents can each be run individually, in multiplex with other apoptosis family reagents, or in multiplex with other MultiCyt reagents. All four reagents in the apoptosis panel can also be combined together, and in addition to cell counts, will provide a multi-parameter assay providing 5 different apoptosis endpoints per well, for a robust determination of apoptosis progression and insight into mechanism of action (Figure 1).

The Apoptosis Kits were designed for ease of use in multiplexing, enabling a straightforward workflow to measure multiple mechanisms and hallmarks of apoptosis. Compared with other apoptosis reagents, MultiCyt reagents offer several unique advantages:

- **Minimal cytotoxicity**
- **Streamlined, no wash protocols**
- **Optimized for multiplexing**
  Each reagent has been specifically titrated for robust signal stability with minimized fluorescence spill-over to adjacent detectors.

Assay Principles

The MultiCyt Apoptosis Kits are comprised of 4 spectrally distinct and mechanistically unique reagents that can either be utilized individually or in multiplex. Each proprietary reagent has been carefully developed and optimized to match the detection capabilities of IntelliCyt's screening platforms.

- Activation of Caspase 3 and 7 is detected by the use of NucView 488 Caspase-3/7 substrate, which upon cleavage by activated enzyme, results in a fluorescent signal.
- Surface expression of phosphatidylserine is detected by the binding of Annexin V to the cell surface.
- Cell viability as measured by membrane integrity is determined by the inability to exclude a DNA binding dye due to compromised (porous) membranes.
- Mitochondrial membrane potential is determined by sequestration of a small fluorescent molecule inside the lumen of intact mitochondria with an active membrane potential. Upon mitochondrial depolarization the dye leaks into the cytoplasm and the cell exhibits a decrease in fluorescence.

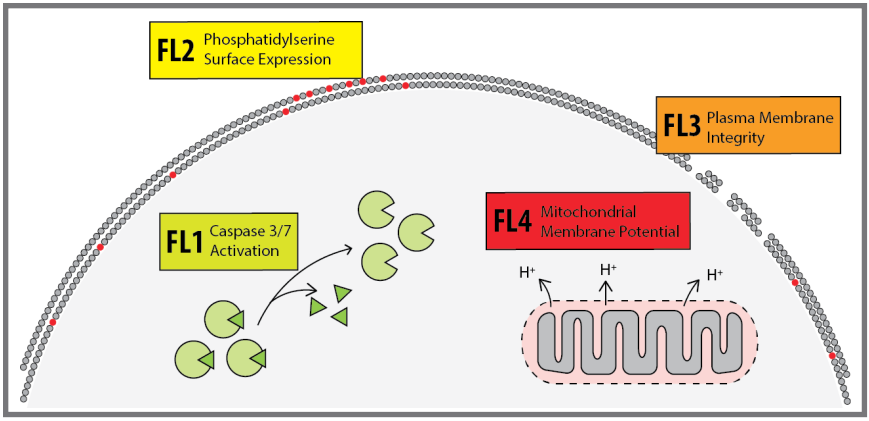
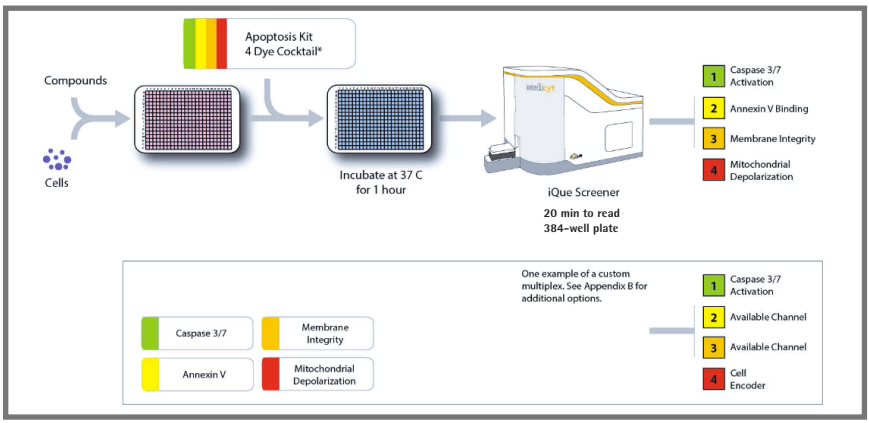


Figure 1. Apoptotic markers measured by the MultiCyt Apoptosis Kits. 1) Caspase 3/7 activation, 2) Phosphatidylserine surface expression, 3) Cell viability, and 4) Mitochondrial membrane potential. The kit components can be utilized either separately or in combination in a no wash format. Apoptosis reagents can also be mulitplexed with other MultiCyt reagents in no wash assays.

To quantify the apoptotic profile, the percentage of cells that are negative or positive for each endpoint is determined. In the case of caspase 3/7 activation, Annexin V binding, and cell membrane integrity, stained cells show an increase in fluorescence and are scored as positive for an apoptotic response. In cells with depolarized mitochondria the loss of fluorescence is indicative of an apoptotic response.

Mix-and-Read Assay Workflow Overview



*Apoptosis reagents can be ordered separately for custom multiplexing.

Before Beginning

1. Ensure that all reagents are completely thawed. If necessary, place vials in a 37°C water bath for 5-10 minutes before use.
2. Briefly centrifuge the vials before use to prevent reagent loss.
3. Gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution.
4. Prepare 2X working stock of staining cocktail as follows.

Dilute each reagent to the appropriate dilution factor noted below in cell culture media. Dilution factors for each component will be the same, regardless of whether the dye will be used in singleplex or multiplex. The specified volumes for culture media are for the complete 4-plex stain.

To create singleplex staining cocktails, increase the volume of culture media to achieve the proper dilution factor for each reagent.

Only include the Annexin 10X buffer when using the Annexin V reagent.

	Culture Media	Caspase (1:500)	Annexin (1:1000)	Viability (1:100)	Mitochondrial (1:1000)	Annexin Binding Buffer (1:10)
1 x 384 wells	7.1 mL	16 µL	8 µL	80 µL	8 µL	800 µL
5 x 384 wells	36 mL	80 µL	40 µL	400 µL	40 µL	4.0 mL
20 x 384 wells	155 mL	350 µL	175 µL	1.75 mL	175 µL	17.5 mL
50 x 384 wells	395 mL	880 µL	440 µL	4.4 mL	440 µL	40 mL

The volumes above are specified to create enough prepared dye for adding 20 µL per well for a full plate with minimal overage. To prepare stain for partial plates or with more overage, dilute the reagents at the indicated dilution factors in cell culture medium to the desired total volume.

Cell Seeding/Treatment Protocol

1. Seed cells and treat with the desired compounds in a 384-well plate. The total assay volume should be 20 µL with a final cell density of ~ 1 x 10^6 cells/mL.

For plates prepared without the use of automation, IntelliCyt recommends seeding 10 µL of cells from a stock cell solution of 2 x 10^6 cells/mL followed by treatment of 10 µL of the desired compound at 2X concentration. This will result in a final assay volume of 20 µL, a cell density of 1 x 10^6 cells/mL, and treatment with your compound of interest at the appropriate concentration. Incubate cells with treatment.

2. Mix the plate using a plate shaker and ensure thorough mixing. The shaker on your IntelliCyt screening system can be utilized for this step. Refer to Appendix A for shaking recommendations.

Staining Protocol

1. Once assay treatment is complete, prepare working solution of the desired staining cocktail.
2. Add 20 µL of prepared 2X working stock of the staining cocktail to each well.
3. Mix the plate using a plate shaker and ensure thorough mixing. The shaker on your IntelliCyt screening system can be utilized for this step. Refer to Appendix A for shaking recommendations.
4. Incubate the plate for 1 hour at 37˚C, 5% CO_2.
5. Acquire data on your IntelliCyt screening system.

Cell Treatment Protocols: Recommendations

While application of this reagent kit will differ between users, we offer general recommendations for assay development that will help ensure success.

- **Include assay controls:** Positive compounds such as staurosporine (not provided) can be used at a final concentration of 5 - 10 µM to induce apoptosis in most cell lines. Appropriate control compounds and concentrations for each assay model will need to be established. Unstained and untreated cells should be included on every plate to verify if the assay has been successful.
- **Know your cells:** The cell density plated in the assay will need to be independently determined for the cell type and experiment. The variables to consider are the compound treatment time and sensitivity of cells. For optimal results, select a density that ensures that cells will remain in log-phase growth for the duration of the experiment. For sensitive cells or other conditions where high amounts of toxicity (low viable cells) are expected, the use of IntelliCyt's Markers (In Well or Between Well) is highly recommended to assure proper well ID.
- **Working with adherent cells:** Adherent cells have been successfully utilized with these reagents. Each cell type will be different in terms of the preferred protocol. For some cells types, detaching cells from the culture plate and staining in suspension is preferred. Other cell types can be simultaneously detached and stained in a staining cocktail prepared in a mild cell detachment solution such as Accutase.

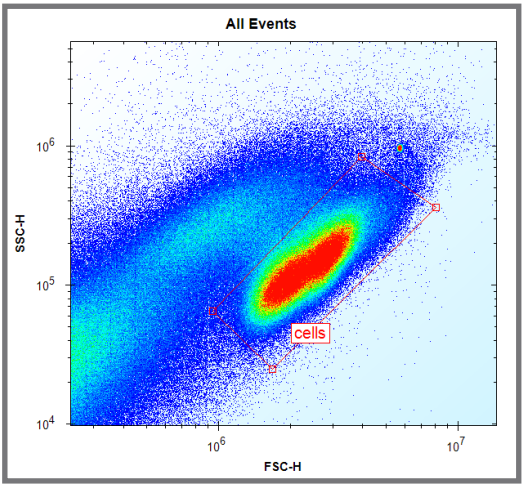
Data Acquisition and Analysis

1. Launch ForeCyt Screening Software.
2. Create a new experiment using the pre-defined assay template provided with your kit.

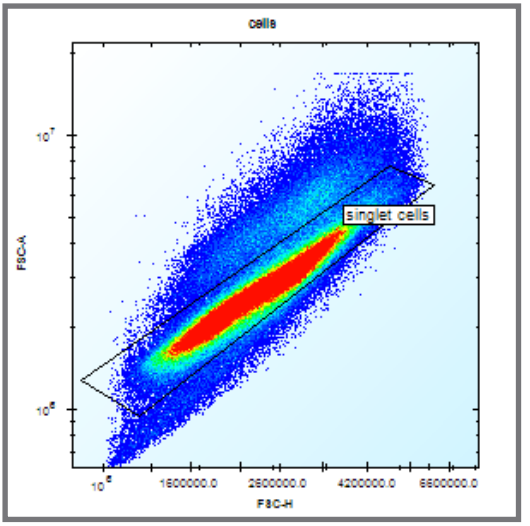
   Template Name: MultiCyt Apoptosis Kits Template

   For detailed instructions on how to load and/or use IntelliCyt's assay templates, refer to the IntelliCyt Video Tutorial Series found at www.intellicyt.com/training.
3. During the plate read, the data will automatically populate into the pre-defined analysis template.
4. A general explanation of each plot and its function is provided below. For detailed information on additional analyses and visualizations that can be performed on this data set, please visit www.intellicyt.com.
5. Verify that the sample data aligns with the pre-defined gating strategy, and if necessary adjust the gates in each plot to encompass the population of interest as shown in the following figures. All gates can be moved by clicking the gate label and dragging to the desired location.

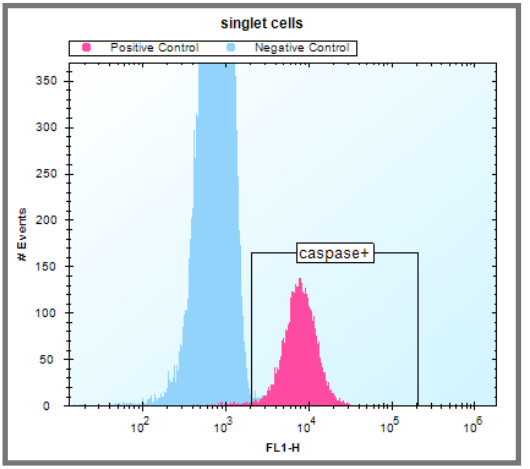
Step 1: Identify Cell Population. If necessary, move the "cells" gate to encompass the main region of interest as shown. If desired, the size of this gate can also be enlarged if additional cell populations are to be included in the analysis.



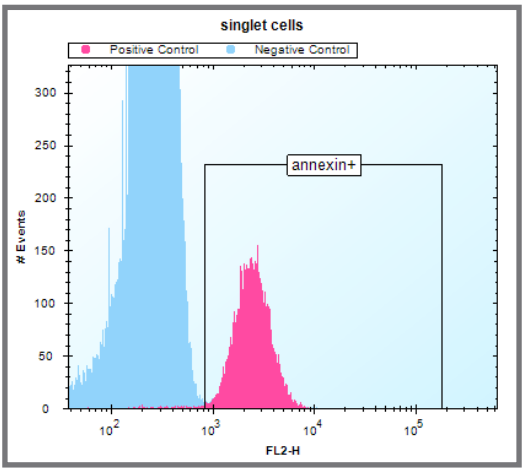
Step 2: Identify Singlet Cells. Analyzing only the single cell population helps avoid analysis artifacts created when aggregates of cells are analyzed. The singlet population will be seen on the ~45° angle on the FSC-H vs FSC-A plot.



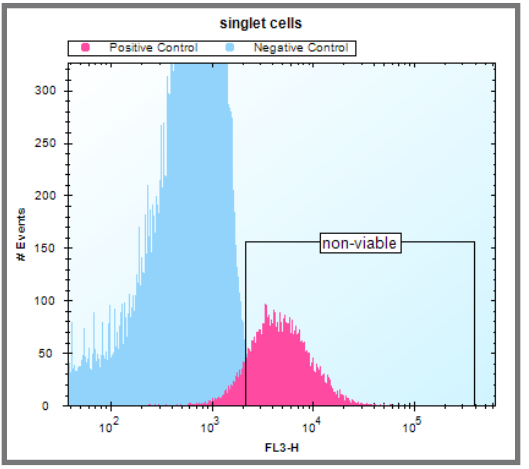
Step 3: Identify Cells Positive for Caspase. Caspase activation is shown in the FL1-H histogram. Adjust the gate as necessary to encompass the entirety of the right (positive) peak. This gate will be used to report the percentage of Caspase positive cells in each well.



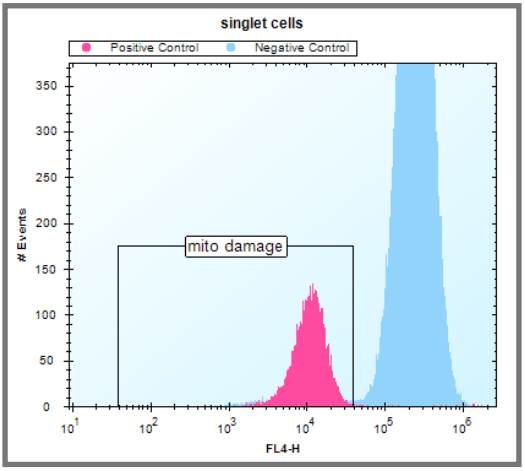
Step 4: Identify Cells Positive for Annexin. Annexin binding is shown in the FL2-H histogram. Adjust the gate as necessary to encompass the entirety of the right (positive) peak. This gate will be used to report the percentage of Annexin positive cells in each well.



Step 5: Identify Non-Viable Cells. Binding of a DNA dye to the cells gives a measurement of cell viability in the FL3-H histogram. Positive cells (right peak) represent the non-viable population. Adjust the gate as necessary to encompass this peak. This gate will be used to report the percentage of non-viable cells in each well.



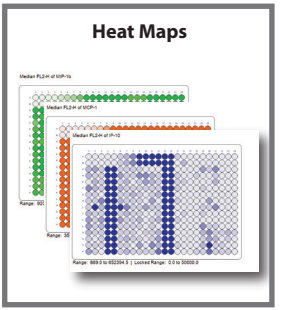
Step 6: Identify Cells with Depolarized Mitochondrial Membranes. Mitochondrial depolarization will cause leakage of the dye out of the mitochondria, and the fluorescence intensity of the cell will decrease. Adjust the gate as necessary to encompass the entirely of the left (depolarized) peaks. Depending on the degree of mitochondrial damage, multiple peaks within the "mito damage" gate are possible.

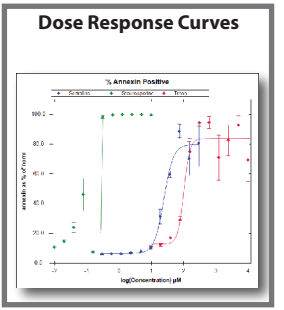


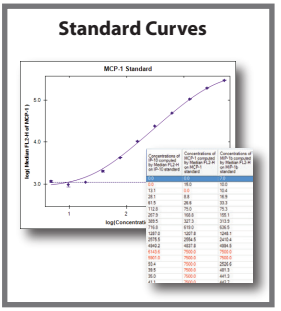
If there are unstained cells on the plate, they may potentially fall inside the "damage" gate due to the low fluorescence intensity of the unstained cells. The size of the gate may need to be decreased to exclude the unstained cells from the "damage" gate.

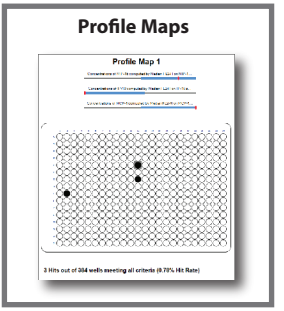
Visualization of Screening Results

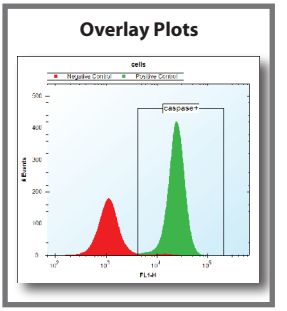
1. After all the gates have been verified and adjusted as necessary for the plate-level data set, all additional analyses including heat maps, etc. will autopopulate for the specified endpoints.
2. As desired, additional data analyses and visualizations can be performed. For detailed information on available ForeCyt software features and instructional tutorials, please visit www.intellicyt.com.











Additional Information: Validation Data

This reagent kit has been validated for screening applications using Jurkat cells, a human T-cell leukemia cell line and staurosporine treatment as an apoptotic inducing compound.

Complete validation data is reported in the application note, which can be downloaded at www.intellicyt.com or obtained by contacting technical support.

	Z'
Caspase Activation Endpoint	0.97
Annexin Binding Endpoint	0.97
Cell Viability Endpoint	0.89
Mitochondrial Integrity Endpoint	0.96

Table 1. Z' results for 4 apoptosis-specfic endpoints, measured by the MultiCyt Apoptosis Screening Kit. Jurkat cells were treated with staurosporine at 5 µM for 24 hours to induce apoptosis.

Additional cell lines successfully tested include:

- U937
- HeLa
- TMD8

For the use of adherent cells, proper detachment protocols will need to be independently established before use. Some modifications and additional optimizations may be required for use with other cell types.

Appendix A: Mixing Samples with the IntelliCyt Shaker

Maximum Fill Volumes & Shake Speed for the iQue Screener and iQue Screener PLUS

Plate Type	Well Volume	Recommended MAX RPM
96-Well	20–40 µL	2600
96-Well	40–60 µL	2200
96-Well	60+ µL	A/O*
384-Well	10–30 µL	3000
384-Well	30–50 µL	2800
384-Well	50+ µL	A/O*

Maximum Fill Volumes & Shake Speed for the HTFC Screening System

Plate Type	Well Volume	Recommended MAX RPM
96-Well	20–40 µL	2800
96-Well	40–60 µL	2400
96-Well	60+ µL	A/O*
384-Well	10–30 µL	3500
384-Well	30–50 µL	3,000
384-Well	50+ µL	A/O*

Maximum Fill Volumes & Shake Speed for the iQue Screener HD

Plate Type	Well Volume	Recommended MAX RPM
96-Well	20–40 µL	3200
96-Well	40–60 µL	2400
96-Well	60+ µL	A/O*
384-Well	10–30 µL	3500
384-Well	30–50 µL	3,100
384-Well	50+ µL	A/O*
1536-Well	up to 5 µL	5,000

***A/O** = Additional Optimization is Necessary. While these volumes are possible to run, they are not routinely tested by the assay development team. To determine ideal shake speeds for high volume assays, IntelliCyt recommends starting at low RPM values and slowly increasing to higher values.

Appendix B: Microplate Recommendations and Wash Protocols

The following plate types have been extensively tested with the QBeads PlexScreen wash protocols:

Plate Type	Well Type	Manufacturer	Manufacturer Product #
384-well	V-bottom	Greiner	781280
96-well	V-bottom	IntelliCyt	10149

When using the above plate types, the following aspiration programs have been tested on a BioTek ELx405 Select. If you have a different plate washer brand or model, it is possible to approximate the aspiration settings on a different system.

It is highly recommended that wash protocols utilize the aid of an automated plate washer. Manual aspiration of plates and/or plate inversion techniques could result in severe sample loss.

Plate Type	Aspiration Height Setting	Aspiration Height Offset	Aspiration Rate Setting	Aspiration Rate
384-well, V-bottom	#31	3.937 mm	#6	15 mm/sec
96-well, V-bottom	#40	5.08 mm	#6	15 mm/sec

NOTES:

Abbreviated List of Consumables for IntelliCyt Screening Systems

iQue®/iQue® Screener PLUS and HTFC® Probes

Part	Description
90659	iQue® Probe & Tubing Assy for Gen 2 iQue® & iQue® HD - 5 Pk
90293	iQue® Probe & Tubing Assy for Gen 1 iQue® (8" Probe) - 5 Pk
90038	HTFC® Probe & Tubing Assy (8" Probe, 40" Tubing) - 5 Pk
91088	iQue® Screener PLUS Probe & Tubing Assy for iQue® Screener PLUS - 5 Pk
91093	iQue® Screener PLUS FluidLink tubing connector - 5 Pk

iQue®/iQue® Screener PLUS and HTFC® Solutions

Part	Description
90077	Decontamination Concentrate Solution for iQue® Screener/HTFC® -5 PK (makes 1 Liter)
90078	Bacteriostatic Concentrate Soln for Sheath Fluid for iQue®/HTFC®/iQue® Screener PLUS
90079	Cleaning Concentrate Solution (makes 1 Liter)
90082	Extended Flow Cell Cleaning Solution for iQue®/HTFC®
90286	iQue®Fluidic Station Buffer Cartridge - 10 Pk
90288	iQue®Fluidic Station Decon/Cleaner Cartridge - 10 Pk
90289	iQue®Fluidic StationWater Cartridge - 10 Pk
91089	PLUS ONE Detector Maintenance Solution only for iQue Screener PLUS (orange label) 5X
91090	PLUS TWO Detector Maintenance Solution only for iQue Screener PLUS (purple label) 5X

iQue®/iQue® Screener PLUS and HTFC® Marker Beads

Part	Description
90040	FL1 In-Well Marker Beads for iQue®/HTFC® - 10 X 384 well plates
90041	FL2 In-Well Marker Beads for iQue®/HTFC® - 10 X 384 well plates
90042	FL3 In-Well Marker Beads for iQue®/HTFC®- 10 X 384 well plates
90043	FL4 In-Well Marker Beads for iQue®/HTFC®- 10 X 384-well plates
90044	In-Well Marker Beads for iQue®/HTFC® Starter Kit (4 colors)
90635	FL1 Between-Well Marker Cartridge for iQue® (ForeCyt 4.0 or later req'd)
90636	FL2 Between-Well Marker Cartridge for iQue® (ForeCyt 4.0 or later req'd)
90637	FL3 Between-Well Marker Cartridge for iQue® (ForeCyt 4.0 or later req'd)
90638	FL4 Between-Well Marker Cartridge for iQue® (ForeCyt 4.0 or later req'd)

iQue®/iQue® Screener PLUS and HTFC® Maintenance

Part	Description
90075	iQue®/HTFC® Fluidics Maintenance Kit
90295	6 peak Validation beads (for Red Laser and FL4 Detector)
90296	8 peak Validation Beads (for Blue Laser and FL1,FL2,FL3 Detectors)
91091	PLUS Validation Beads (all channels) for iQue Screener PLUS
91094	iQue® Screener PLUS Maintenance Kit
91095	iQue® Screener PLUS Maintenance Kit - 10 pack

**Refer to Intellicyt.com for complete list. Contact your local area sales representive for part number and pricing information

Detection Channels for MultiCyt Kits and Reagents on the iQue® Screener and iQue® Screener PLUS

iQue® Screener	iQue® Screener PLUS
FL1	BL1
FL2	BL2
FL3	BL4
FL4	RL1
FL3 (SDS)	RL2

ABOUT THE COVER:

The prickly pear (genus Opuntia) is a common cactus in central New Mexico. There are some 200 species of this genus. The one most commonly used for culinary purposes is O. ficus-indica. They typically grow with flat, round paddle-like structures that contain both large fixed spines and small hair-like prickles. They produce an edible fruit which can be eaten after careful preparation or made into jelly and candies. An unusual feature of Opuntia is the thigmotactic anthers, which when touched curl over and deposit their pollen.

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