SARTORIUS

Product Guide

iQue® Apoptosis Kits

Product Information

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

Product Name	**Cat. No.**			
	1 × 384-well	5 × 384-well	20 × 384-well	50 × 384-well
iQue® Human 4-Plex Apoptosis Kit	90053	90054	90155	90156
iQue® Caspase 3/7 Detection Reagent	N/A	91034	91035	91036
iQue® Annexin V Binding Reagent	N/A	91030	91031	91032
iQue® Cell Membrane Integrity (B/Red) Dye	N/A	90346	90347	90348
iQue® Mitochondrial Depolarization Detection Reagent	N/A	91038	91039	91040

Safety data sheet (SDS) information can be found on our website at **www.sartorius.com**

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.

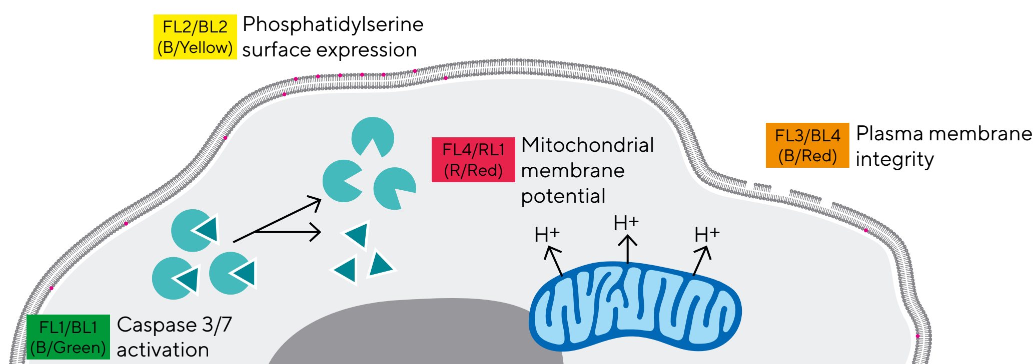
Apoptosis plays a fundamental role in cell biology, and characterizing this endpoint is an important target across the drug discovery process, from primary screening to toxicity profiling.

The iQue® 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 iQue® reagents. All four reagents in the apoptosis panel can also be combined together. In addition to cell counts, the kits provide a multi-parameter assay with 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, iQue® reagents offer these 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.

Figure 1



Note. Apoptotic markers measured by the iQue® Apoptosis Kits. Caspase 3/7 activation (red), Phosphatidylserine surface expression (yellow), Cell viability (orange), and Mitochondrial membrane potential (red). The kit components can be used either separately or in combination in a no-wash format. Apoptosis reagents can also be mulitplexed with other iQue® reagents in no-wash assays.

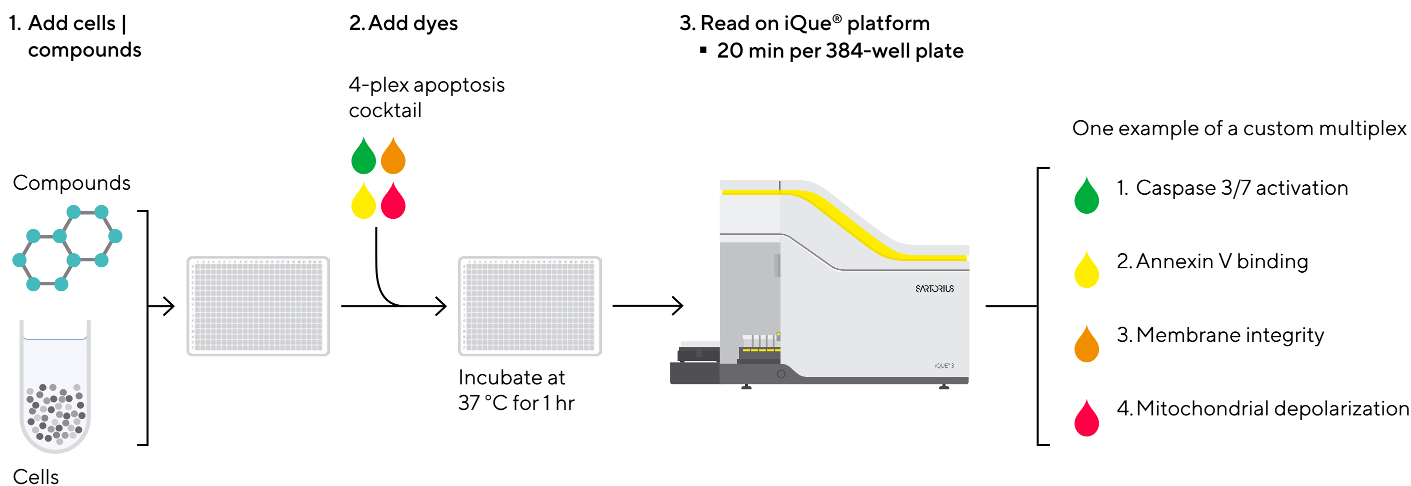
Assay Principles

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

- Activation of Caspase 3 and 7 is detected by the 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.

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.

Figure 2
Mix-and-Read Assay Workflow Overview



*Apoptosis reagents can be ordered separately for custom multiplexing.

Kit Contents and Detection Channel

Kit	Channel (iQue® Screener Plus and iQue® 3)*
iQue® Apoptosis Screening Kits (Cat. No. 90053, 90054, 90155, 90156)	
iQue® Caspase 3/7 Detection Reagent	B/Green
iQue® Annexin V Binding Reagent	B/Yellow
iQue® Cell Membrane Integrity (B/Red) Dye	B/Red
iQue® Mitochondrial Depolarization Detection Reagent	R/Red
10X Annexin Binding Buffer	
iQue® Caspase 3/7 Detection Reagent (Cat. No. 91034–91036)	
iQue® Caspase 3/7 Detection Reagent	B/Green
iQue® Annexin V Binding Reagent (Cat. No. 91030–91032)	
iQue® Annexin V Binding Reagent	B/Yellow
10X Annexin Binding Buffer	
iQue® Cell Membrane Integrity (B/Red) Dye (Cat. No. 90346–90348)	
iQue® Cell Membrane Integrity (B/Red) Dye	B/Red
iQue® Mitochondrial Depolarization Detection Reagent (Cat. No. 91038–91040)	
iQue® Mitochondrial Depolarization Detection Reagent	R/Red

*Refer to Appendix C : Detection channel for Kits and Reagents on iQue® Screener, iQue® Screener Plus and iQue® 3.

Protocols and Procedures

Storage and Stability

Store at 2–8 °C.

Materials Needed but Not Provided

- iQue® platform
- iQue Forecyt®
- 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

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.

# of Wells	Culture Media	Caspase (1:500)	Annexin (1:1000)	Viability (1:100)	Mito-chondrial (1:1000)	Annexin Binding Buffer (1:10)
1 × 384	7.1 mL	16 µL	8 µL	80 µL	8 µL	800 µL
5 × 384	36 mL	80 µL	40 µL	400 µL	40 µL	4.0 mL
20 × 384	155 mL	350 µL	175 µL	1.75 mL	175 µL	17.5 mL
50 × 384	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 × 10^6 cells/mL.
   Note. For plates prepared without the use of automation, iQue® 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 iQue® platform can be utilized for this step. Refer to Appendix A for speed recommendations.

Staining Protocol

1. Once the 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 iQue® platform 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 iQue® platform.

Cell Treatment Protocols: Recommendations

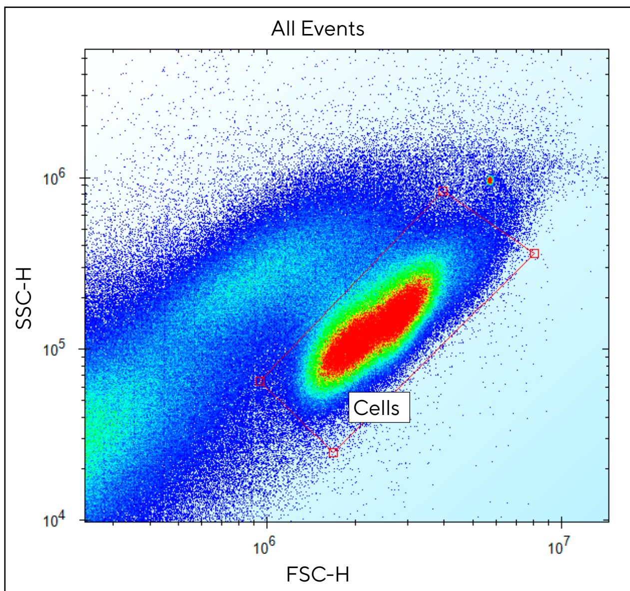
While application of this reagent kit will differ between users, we offer general recommendations for assay development that will help ensure success.

- Include assay controls: Positive compounds such as staurosporine (not provided) can be used at a final concentration of 5–0 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 iQue® 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.

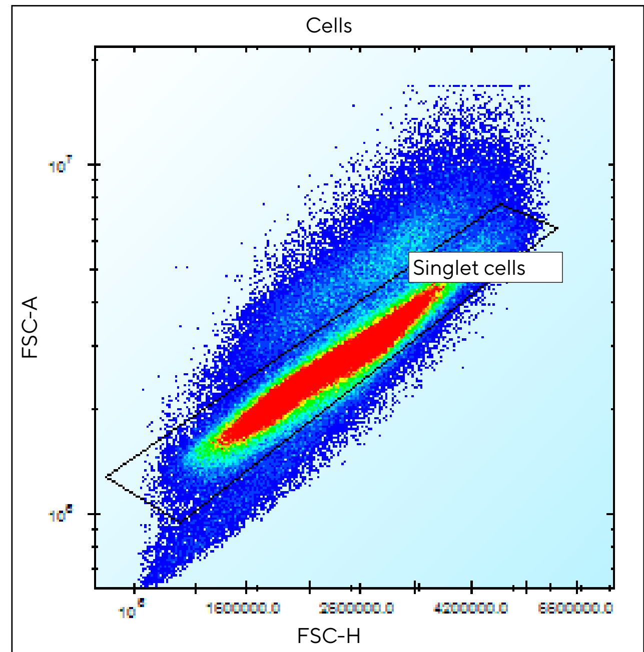
Data Acquisition and Analysis

1. Launch iQue Forecyt® Software.
2. Create a new experiment using the predefined assay template provided with your kit.
   Template Name: iQue® Apoptosis Kits Template.
3. During the plate read, the data will automatically populate into the predefined 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.sartorius.com.
5. Verify that the sample data aligns with the predefined 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.

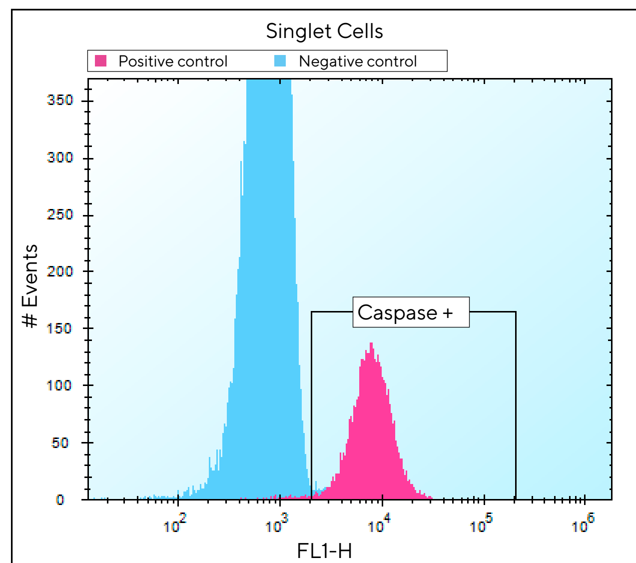
Step 1: Identify Cell Population.
If necessary, move the "cells" gate to encompass the main region of interest as shown. If desired, the size of this gate can also be enlarged if additional cell populations are to be included in the analysis.



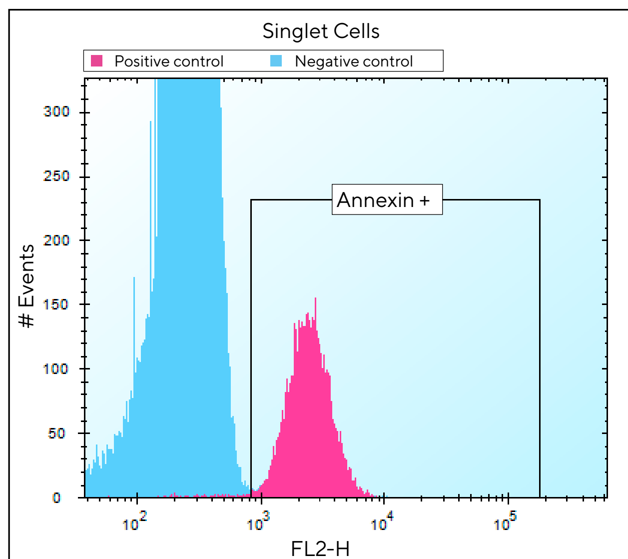
Step 2: Identify Singlet Cells.
Analyzing only the single cell population helps avoid analysis artifacts created when aggregates of cells are analyzed. The singlet population will be seen on the ~45° angle on the FSC-H vs FSC-A plot.



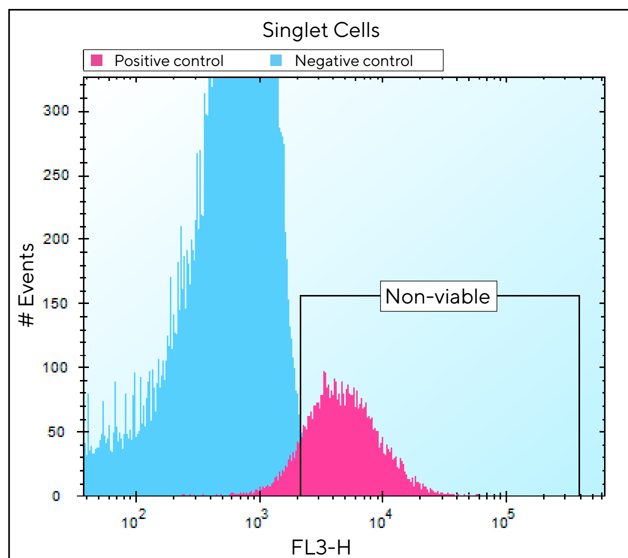
Step 3: Identify Cells Positive for Caspase.
Caspase activation is shown in the FL1-H histogram. Adjust the gate as necessary to encompass the entirety of the right (positive) peak. This gate will be used to report the percentage of Caspase positive cells in each well.



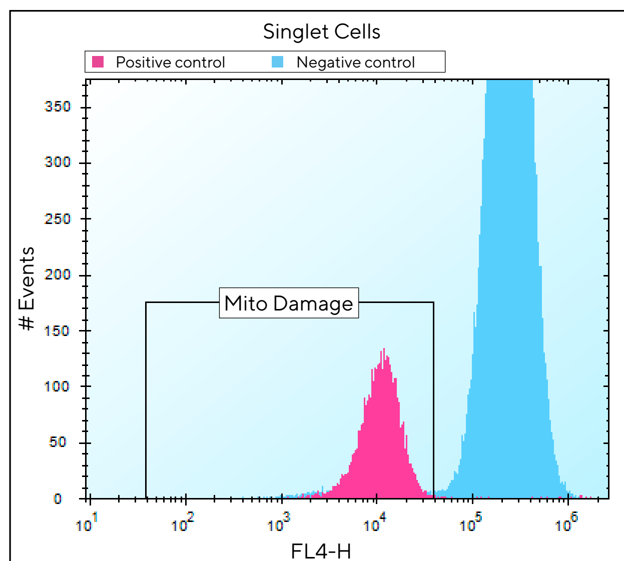
Step 4: Identify Cells Positive for Annexin.
Annexin binding is shown in the FL2-H histogram. Adjust the gate as necessary to encompass the entirety of the right (positive) peak. This gate will be used to report the percentage of Annexin positive cells in each well.



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.

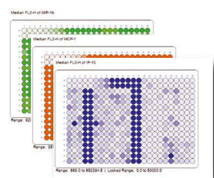


If there are unstained cells on the plate, they may potentially fall inside the "damage" gate due to the low fluorescence intensity of the unstained cells. The size of the gate may need to be decreased to exclude the unstained cells from the "damage" gate.

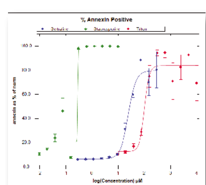
Visualization of Screening Results

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 auto-populate for the specified endpoints.
2. As desired, additional data analyses and visualizations can be performed. For detailed information on available iQue Forecyt® software features and instructional tutorials, please visit www.sartorius.com.

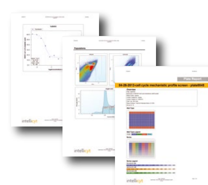
Heat Maps



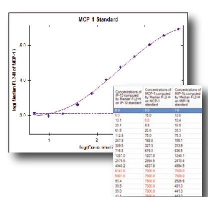
Dose Response Curves



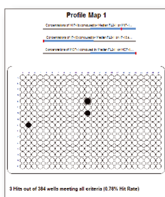
PDF Data Reports



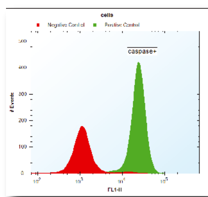
Standard Curves



Profile Maps



Overlay Plots



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.

Z′ Results for 4 Apoptosis-Specific Endpoints, Measured by the iQue® Apoptosis Screening Kit

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

Note. 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 iQue® Shaker

Maximum Fill Volumes and 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 and 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	3000
384-Well	50+ µL	A/O*

Maximum Fill Volumes and 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	3100
384-Well	50+ µL	A/O*
1536-Well	up to 5 µL	5000

*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, iQue® 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 iQue Qbeads® PlexScreen wash protocols:

Plate Type	Well Type	Manufacturer	Manufacturer Cat. No.
384-well	V-bottom	Greiner	781280
96-well	V-bottom	iQue®	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

Appendix C: Detection Channels for Kits and Reagents on the iQue® Screener, iQue® Screener Plus and iQue® 3

iQue® Screener	iQue® Screener Plus and iQue® 3
FL1	B/Green
FL2	B/Yellow
FL3	B/Red
FL4	R/Red
FL3 (SDS)	R/Crimson

Notes

For Research Use Only

This product is manufactured and sold by iQue® for research use only. The kit and components are not intended for diagnostic or therapeutic use. Purchase of the product does not include any right or license to use, develop, or otherwise exploit this product commercially. Any commercial use, development or exploitation of this product without the express written authorization of iQue® is strictly prohibited.

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. iQue® will provide product replacement for valid claims. All claims should be made within five (5) days of receipt of order.

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

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