Q-Guide:

Best Practices for Assay Development and Optimization

This Q-Guide is a companion piece to the video tutorial series for ForeCyt 3.0. ForeCyt is the software package that operates IntelliCyt's screening systems including the HTFC® and iQue™ Screener.

In This Guide:

Overview

Cell- and bead-based assays can be utilized to study a staggering variety of biological targets. Increasingly, these assays are being developed in miniaturized and/or high throughput format to increase efficiency and productivity, and to reduce reagent costs. IntelliCyt® High Content Flow (HCF) Suspension Screening Systems are perfectly situated in this process and workflow, and have been successfully utilized in studies spanning the entire drug discovery process, from primary screening to testing drug potency, and for drug purification and production support. Additionally, secondary screening and mechanism of action studies, such as receptor binding, receptor activation, cell signaling, drug internalization, and cytotoxicity are routinely run by end users of these systems.

Depending on what actual endpoint is being assessed, the assay development process can vary widely. Specific to the endpoint, the assay biology needs to be optimized to ensure that accurate and relevant results can be obtained. The process of designing, optimizing, validating and implementing a bioassay can be streamlined by keeping some important factors in mind:

- Select appropriate positive and negative assay controls
- Include treatment and probe controls on EVERY plate
- Determine specificity/background of all reagents, and make sure they are properly titrated
- Check for auto-fluorescence of both the cells and reagents – look at unstained cells with and without treatment for setting baselines and gates

This short assay primer will lay out general parameters that should be evaluated and optimized within an assay, and provide preliminary guidance on the use of the IntelliCyt systems and reagents to facilitate reproducible and streamlined assays. For a detailed development protocol or assay-specific questions, please contact IntelliCyt technical support.

Multiplexing Assay Endpoints

One of the most powerful aspects of flow-based detection is the ability to quickly and easily acquire multiple endpoints from a single sample, however, multiplexing is a double-edged sword. Every biological target has its own, natural variability. Adding targets increases the probability for cross reactivity, non-specific binding, and non-intuitive creation of biological artifacts. Always weigh the value of the data generated, including the time required to interpret the data, against benefits of multiplexing for the specific assay.

Fortunately, IntelliCyt's fixed-voltage detection systems facilitate the detection of multiple endpoints not only across the fluorescence spectrum, but potentially, across an intensity spectrum. The horizontal and vertical multiplexing capability of our systems is shown schematically in Figure 1.

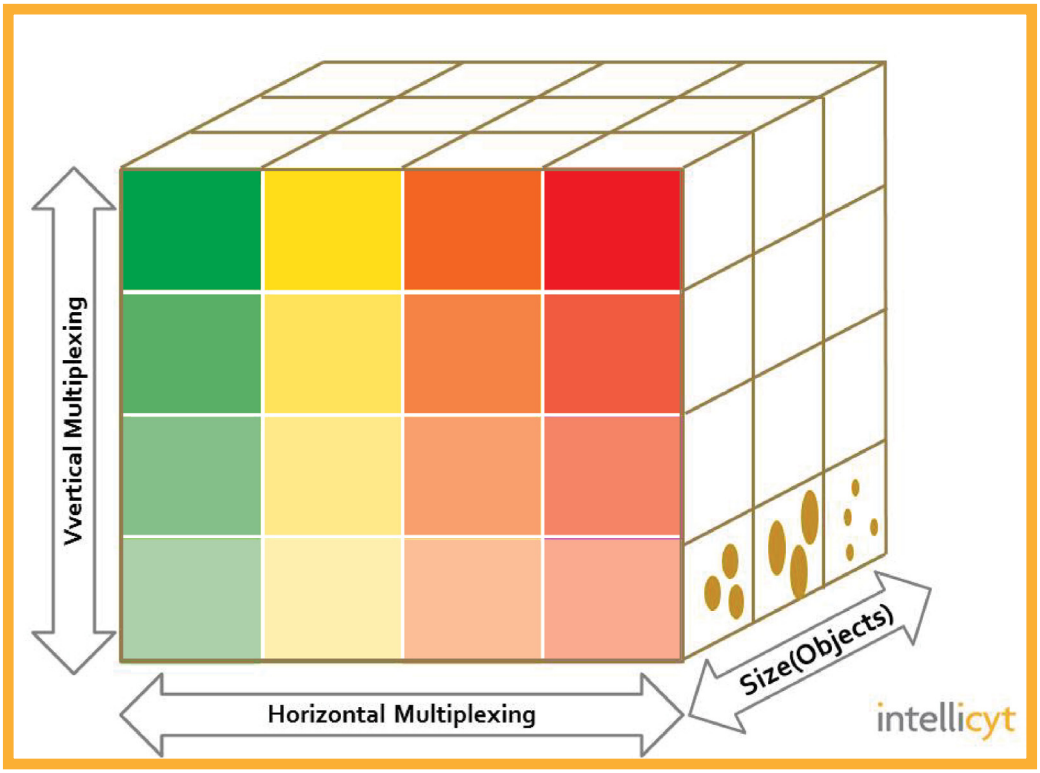


Figure 1. The horizontal and vertical multiplexing capability of Intellicyt systems.

Both the iQue Screener and HTFC Screening System are equipped with four standard detection channels. Within each detection channel, the 7-decade detection range means that it is possible to encode samples with different intensities of a fluorophore and separate them for downstream analysis. This methodology is amenable to both cells and beads. Example applications of vertical multiplexing can be seen in bead-based assays, where different beads representing different targets are labeled with different intensities of a "classifier" signal and physically mixed into a single sample. For cell-based assays, non-specific dyes can be utilized to encode either cellular proteins or membranes at different intensities, to be detected on a single channel. Thus, the combination of vertical and horizontal multiplexing within an assay easily expands the capabilities of the standard detection configuration of your screening system.

Cells: Growth and Maintenance

To achieve optimal and reliable results some recommendations that can ensure the consistency of cells being used in assay are as follows:

1. Cell Biology

- Select a cell line or cell type that is appropriate for the biological target of interest. Know the background and history of the cells that will be used. Do not, as a rule, trust that any sample that is taken from the lab freezer has the correct pedigree and is not contaminated in such a way that its biology may be suspect.
- Getting clones from ATCC or other reputable commercial sources and having the cells tested for mycoplasma as part of the assay development process and before scale-up to an actual screen are good practices to increase success of the screening campaign.

2. Cell Density

- Determine the optimal density desired for assay treatment (growth, compound treatment, time, etc.)
- If the cells need to be grown or treated at lower density, it is possible to perform the assay at lower cell concentration, and then concentrate cells in a final step before sampling.
- For rapid sampling using common suspension cell lines, IntelliCyt recommends a final cell concentration of between 1-3 x10^6 cells/mL. Cell densities that are higher or lower are possible, but should be tested and optimized.

3. Growth Phase of Cells/Cell Cycle

- While cell cycle may not be of primary concern, cells at different phases in their cycle may yield different results.
- Ensure that cells are prepared/passaged similarly for each day/assay.
- Do not use cells fresh out of thaw, they may not exhibit the correct biological response until they overcome thermal shock and/or regenerate surface receptor loads.
- Define a maximum passage number for the cells in the assay and stay well under that number during the screen (using a cell bank). As a general rule do not use cells at very high passage numbers because the risk of phenotypic drift leading to assay variability becomes unacceptably high.

4. Health of Cells

- It is important to use healthy, viable cells in each assay.
- If cells have over-grown in culture or demonstrate low viability, it is best to postpone the assay until viable cells are available.
- Establish an SOP (Standard Operating Procedure) for taking cells from the freezer bank to assay plate and maintain absolute rigor for this process during the course of the screen. Most cell-based assay failures can be attributed to these important protocol “pre steps” so it is best practice to include them as part of the assay.

5. Cell Banking

- It is very important to expand and bank cells for any cell-based screening campaign. Bank enough cell stock to cover the primary screen, potential rescreening, and dose response confirmations of the hits. Take the time to accurately calculate actual cell usage, taking into account any dead volumes in the assay protocols.
- If cells are banked in lots then each lot must be tested during assay development.

Application of Adherent Cells for Suspension Screening

HCF is based on the principles of flow cytometry for detection and, therefore, suspension cells, beads, and microbes are perfectly suited for IntelliCyt System sampling. The system is readily amenable to analysis of suspended adherent cells as well.

It is possible to use adherent cells for assay with appropriate modifications to the assay protocol. Adherent cells will need to be removed from their plate, and brought into suspension before sampling. Depending on the assay, it is possible to either detach the cells and perform all the stain steps with the cells in suspension – or – to perform all the staining steps in the culture plates, and detach cells immediately before sampling. The determination of the ideal method will be highly assay and cell-type specific. For most of the screening kits and protocols developed by IntelliCyt, we recommend completely detaching cells from the well and performing the staining steps on fully suspended samples.

Some factors to consider if adherent cells will be utilized in assay include:

- **When to detach cells** – before or after staining steps. Depending on the assay readout and the cellular target, the timing of the detachment step may affect the end results. If the cellular target is located on the cellular surface, detachment before staining might alter the cellular response.
- **How to detach cells.** There are many reagents and methods to detach cells from their culture plates. Select a reagent that is gentle and effective for the specific cell line. Examples of reagents that have been utilized successfully include Trypsin-EDTA (0.25%, multiple vendors), Versene (Invitrogen, Cat No# 15040-066), Cell Dissociation Reagent (Invitrogen, Cat No #13150-016) , and TrypLE Express reagent (Invitrogen, Cat No # 12605-010).
- **How to keep cells in suspension.** Certain cell types are prone to clustering or “clumping” after detachment. It may be necessary to include small amounts of cell detachment reagent in the sample buffer to minimize this effect, or to take additional precautions to prevent and monitor clumping.

For adherent cell assays, do not use specialty coated plates. Specifically, collagen or poly-lysine treated plates could potentially affect sampling on the system. If the use of a coated plate is required for cell growth, care should be exercised in ensuring complete cell detachment prior to sampling.

Bead-Based Assays

Bead-based assays are typically very simple and robust to perform on the IntelliCyt systems, and are very similar to the use of cells. Often, very few adjustments to the stated suggestions in this chapter for cell-based assays are needed, with the biggest consideration being the different flow rates (see below section Fluidics for more information). Like cell-based assays, careful assay development will help to ensure reliable results. Successful application of many commercially available bead kits, including CBA and CBA-type assays have been shown on the IntelliCyt systems. Bead-based technologies often rely on vertical multiplexing, where a single "classifier" signal is utilized to label beads at different intensities.

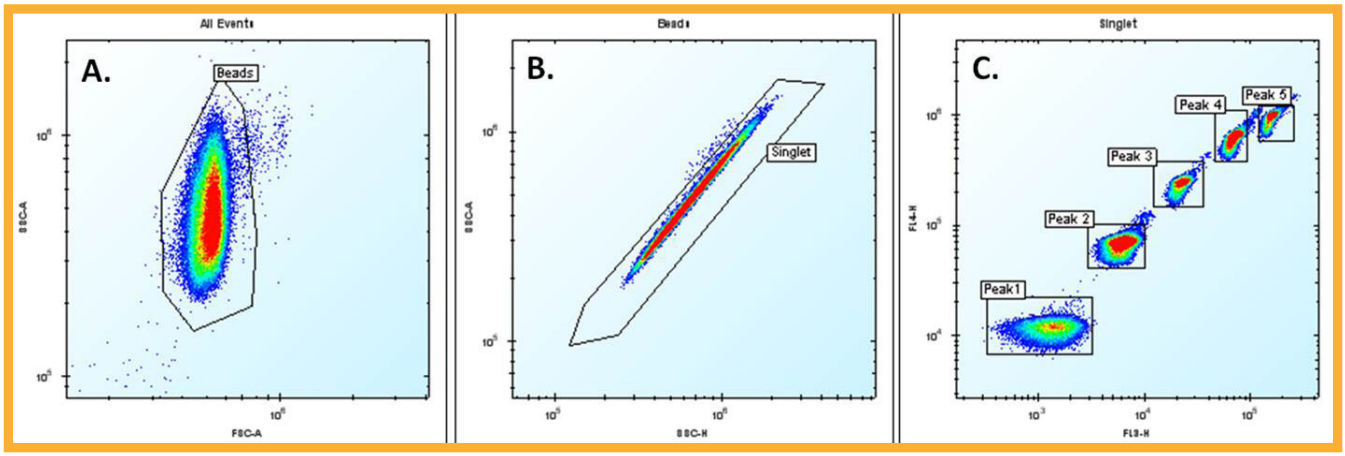


Figure 2. Bead separation for a representative bead set.

Object Sampling for Statistical Relevance

It is optimal to analyze ≥1000 objects (cells, beads, microbes, etc.) per sample/well regardless of the assay type. For the detection of rare events and/or the identification and quantitation of multiple populations, greater object numbers per well will need to be acquired. Certainly, for most assays it is possible to achieve robust and reliable data with fewer objects (as few as ~200-400 events per sample), but a goal of 1000+ objects per well ensures the ability to quickly and efficiently process samples without much downstream adjustment of the well identification (ID) steps. Significant well-to-well fluctuations in the number of objects sampled (i.e., some wells with lots of cells and some wells with very few or no cells in the same plate) could require some manual adjustment to the well ID, or necessitate the use of marker beads for accurate well ID.

To achieve this optimal object count, the factors to consider include:

1. **Increase final cell density.** If the assay treatment is performed at lower cell density, cells can be spun down and resuspended in a lower sample volume to achieve a concentration of ~$1x10^6$ cells/mL. For a typical cell line, IntelliCyt has determined that cell densities of up to ~$5x10^6$ cells/mL do not significantly affect the well-to-well carryover, and that carryover averages ~2% for typical samples. Assays using "sticky" cell lines or samples with higher cell densities may have to institute more extensive cleaning procedures to reduce carryover.
2. **Increase the sip/acquisition time.** If it is not possible or desirable to concentrate the cells, increasing the sip time can achieve the desired number of cells acquired per well. Longer sip times also tend to stabilize cell counts and decrease variability. If cell counts are an important parameter in the assay, longer sip times are an effective mechanism for reducing variability of this parameter.
3. **Thorough mixing on IntelliCyt shaker prior to sampling.** If cells have been previously concentrated or centrifuged, thorough mixing of the assay plate is necessary to ensure that cells have been properly and homogenously resuspended, and will aid in the consistency of cell counts. Periodic mixing of the plate as samples are being read will ensure that cells stay in homogenous suspension. To mix plates using the IntelliCyt shaker, launch ForeCyt and navigate to **Device>Manual Control Mode**.

Rare Event Detection

A growing number of flow cytometry-based assays depend on rare-event detection. These include assays developed for basic science and for clinical applications. The definition of a "rare" event is variable, but often is used to refer to events with anywhere between 5% frequency in the total population down to under 0.00001% frequency. While the very low percentage frequency events are not feasible screening targets for our system, assays with frequencies as low as ~1:1000 events are possible on the IntelliCyt systems.

Established guidelines for minimum numbers of rare events necessary for statistical relevance are shown in the table below (adapted from Terry Hoy, "Rare Event Detection"). For illustration, to achieve significance in an assay with 5% variability, 400 rare events will need to be acquired. If the rare events are at 1:20 frequency, ~8000 total events will need to be acquired. If the frequency of the rare event is 1:1,000, up to ~400,000 events per sample will need to be acquired. This amount of data is possible with the IntelliCyt systems, although definitely at the upper limits of what is recommended.

Coeffiecient of Variation (%)	# of Rare Events Needed to Achieve Statistical Relevance
30	11
10	100
5	400
3	1,111

Table 1

Frequency of Rare Events	Total Events Required to Achieve 10% CV	Total Events Required to Achieve 5% CV
1:20 (5% of total)	2,000	8,000
1:50 (2% of total)	5,000	20,000
1:100 (1% of total)	10,000	40,000
1:1000 (0.1% of total)	100,000	400,000

Table 2

To achieve this statistical relevance, IntelliCyt recommends adjusting the data acquisition threshold parameters to facilitate sampling. The default threshold is set on FSC-H, which means that only objects above a certain size will be registered as events. The default setting for the system is 80,000 which will detect sub-micron sized events. As most cellular objects of interest are micron-sized and above, raising the threshold to 150,000 on FSC-H represents one mechanism to facilitate achieving rare-event detection. Additional threshold settings can also be set on fluoresence parameters. For instance, if all cells in the sample are stained with a certain dye - for example, a nuclear dye - it is possible to set the threshold on fluoresence to analyze only events that are stained, thus reducing the noise/background from cellular debris, etc., in the sample.

CAUTION

Improper setting of acquisition thresholds could result in irrecoverable data loss. If necessary, please consult IntelliCyt Technical Support for assistance and consultation with proper threshold settings.

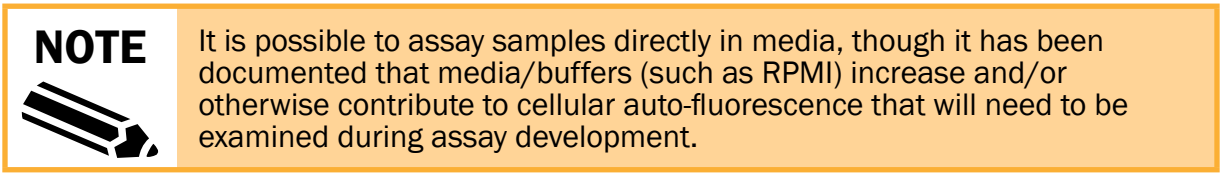
Assay Reagents

General Buffer Considerations

Use standardized (consistent) and pH buffered solutions – changes in buffer/fluid pH as samples travel through the system and tubing may affect fluorescence intensity and calculated ratios. Consistency of solutions will help ensure consistent day-to-day results with the Intellicyt systems. Additional buffer considerations include careful control of buffer viscosity (too high of a viscosity could interfere with sampling), and protein content. While some protein in the solution is suggested, too much protein could cause excessive foaming with shaking. Determine the optimal amount for assay and sampling parameters.

Sample Buffers

Samples should be prepared in PBS with 0.1% BSA (or some other amount/type of protein) in the final step before sampling. Care should be taken if the assay requires addition of greater than 0.1% BSA, as high quantities of protein may lead to excess bubble formation in IntelliCyt sampler tubing.



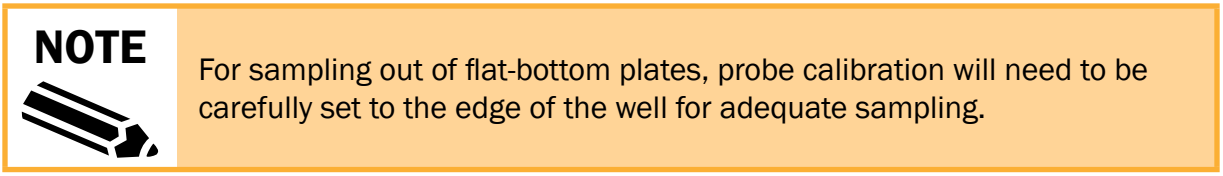
Assay Setup: Plate Types, Assay Volumes and Protocols

The following recommendations for assay setup represent a starting point for new assay development. These recommended parameters will need to be modified and optimized on an assay-by-assay basis.

Assay Plates

Use V-bottom (IntelliCyt Catalog # ICOV181) or U-bottom plates for sampling on the IntelliCyt systems. The probe calibration should be set to sample from the center of the well. This plate geometry allows the generation of a vortex as the sample mixes, and facilitates thorough mixing and homogenous cell/particle distribution in solution.

If necessary, different plate geometries including flat-bottom plates can be used for cell growth. It is possible to directly sample from flat-bottom plates on the IntelliCyt systems, provided cells are brought into homogenous suspension.



Some recommended plate types/vendors that have been successfully utilized on IntelliCyt systems include:

Plate Type	Well Type	Manufacturer	Manufacturer Product #
96-Well	V-Bottom	IntelliCyt	10149
96-Well	Flat-Bottom	Falcon	353075
96-Well	Round-Bottom	Greiner	650101
384-Well	V-Bottom	Greiner	781280
384-Well	Flat-Bottom	Corning	3701
384-Well	Round-Bottom	Corning	3656

Table 3

Assay Volumes

From the robust sampling of the IntelliCyt systems, the per-well assay volumes can be highly flexible to accommodate different assay types, plate geometries, and cell concentrations. The IntelliCyt systems are designed for low volume assays, and can realistically sample as little as several microliters per well, however, for general assay development, we recommend:

20–40 µL final volume per well for 96-well plate assays

10–30 µL final volume per well for 384-well plate assays

Assays Requiring Wash Steps

When possible, it is ideal to limit and/or reduce the number of wash steps required in the assay. Minimized washing not only streamlines the assay workflow and saves time, but will also mitigate loss of cells due to well supernatant aspiration at each wash step.

When washing is necessary, users have found that standard platewashers such as the BioTek ELx405 Select provide robust and consistent performance.

Recommended settings for the ELx405 are provided for specific plate geometries below. These recommendations have been tested extensively by IntelliCyt's Assay Development Group for both cell- and bead-based assays. However, these settings should be taken as guidelines, and additional optimization for specific biologies are potentially necessary.

Recommended Aspiration Settings for BioTek ELx405 Select:

Plate Type	Aspiration Height Setting	Aspiration Height Offset	Aspiration Rate Setting	Aspiration Rate
384-Well, V-Bottom	#31	3.937mm	#6	15mm/sec
384-Well, Flat Bottom	#26	3.302mm	#2	3.4mm/sec
96-Well, V-Bottom	#40	5.08mm	#6	15mm/sec
96-Well, Flat-Bottom	#26	3.302mm	#3	4.0mm/sec

Table 4

Evaluation and Titration of Reagents

For assays where multiple dyes/fluorochromes will be tested, evaluate the dyes separately, and in combination, to ensure:

- No significant fluorescence overlap is encountered between dyes. If fluorescence overlap is unavoidable, characterize the amount of compensation needed and ensure this is a reproducible value
- There is sufficient resolution between positive and negative signals so that gating for positive and negative cells can be done reliably and reproducibly.
- In multiplexed experiments, there is the potential for interacting effects between the dyes and/or antibodies themselves. Making measurements in single-plex then again in cocktail (with all the components included) helps to validate the multiplex experiment.

Total Assay Time and/or Individual Incubation Times

Determine the optimal incubation windows for each reagent/step to attain desired results. Steps including compound incubation, cell treatment, primary and secondary antibody incubations and dye staining windows should be evaluated in terms of both biology and assay workflow. Oftentimes, treatment/compound concentration and incubation time are inversely related and can therefore be manipulated in assay design, i.e., use higher concentrations with shorter incubation windows, or lower concentrations.

Fluidics Speed

The IntelliCyt sampler pump speed is optimally set at 15 RPM. This setting is the fastest sample flow rate that provides adequate data resolution for most screening assays. The chart below provides information on the running samples on the HTFC and iQue Screening Systems. At the recommended cell or object density (1-3 x10^6 cells/mL) this should yield the desired cell count for a one second sip. However, in assays that require greater data resolution (such as cell cycle), a slower pump speed and acquisition speed may need to be used. Slowing the pump speed may require a higher cell density or sip time to maintain appropriate cell counts per well.

Cytometer Speed	Recommended Sampler Pump Speed	Approximate Sample Volume/Second	Recommended Applications	Acquisition Notes
Slow	5 ± 2 RPM		Bead-based assays; cell cycle analysis	Highest resolution of data will be achieved on the slow settings. Object throughput will be decreased due to slower sample introduction.
Medium	15 ± 3 RPM	2 µL/sec	Cell-based assays	This setting is recommended as the starting point for cell-based assay development and optimization
Fast	25 ± 5 RPM		Assays that have cells/beads at low density or high input volume and that do not require tight resolution of populations	Faster acquisition time, and more sample introduced into the system. Data at faster acquisition speeds will exhibit lower resolution. Not suitable for assays that need high resolution in the fluorescence signal. Can do positive/negative discrimination, but potentially not bright/dim

Table 5

Acquisition Parameters

As a starting point for new assay development, recommended settings for initial assay setup are provided below. Details on the importance of each parameter and the effect on data output is presented after the example.

1. Pre-plate prime = 60 seconds. Check box to cycle probe while priming
2. Pre-plate shake = 30 seconds at maximum recommended speed for plate type/volume
3. Sip time = 1 second
4. Additional Up Time = .5 second. For experiments where absolute minimization of cell carryover is desired, set the up time to 0 seconds and perform a 1 second rinse after every well.
5. Inter-well shake = after every row or column for 96-well plates – or every ½ row or column (8 or 12 wells) for 384-well plates. Shake time should be 2-3 x longer than sip time. For instance, with a 1 second sip time, a 3 second shake at maximum speed is recommended.
6. Ensure that rinse station S1 has an appropriate amount of the recommended S1 buffer solution (PBS + 0.1% BSA), or desired assay buffer

Example settings for fully characterized assay in High Throughput Screening Mode (3 minutes for 96-well plate; 15 minutes for 384-well plate) are shown below. The use of robotics can facilitate the automatic loading of plates, and iteration of the plate sampling/acquisition protocol can be coordinated with API software.

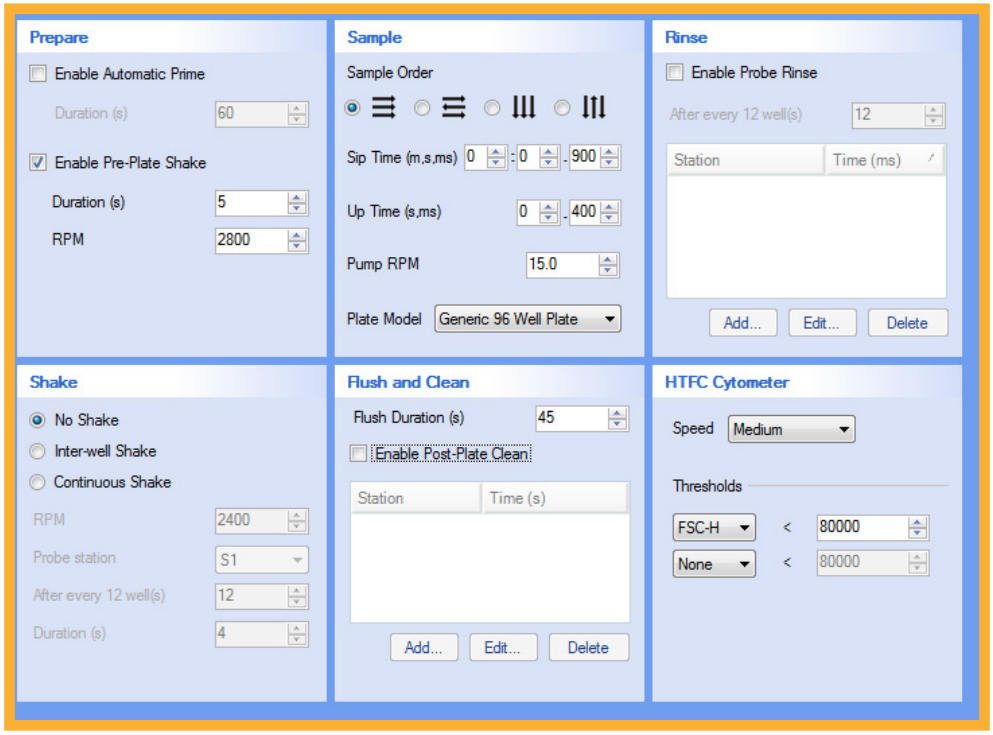


Figure 3. Sample acquisition settings for a fully optimized screening assay. These settings will achieve a throughput of 3 minutes per 96-well plate.

Mixing Samples with the IntelliCyt Shaker

Unless cells and assays are adverse to shaking and/or agitation, shake at maximum speeds for the plate geometry and well volume to ensure best cell resuspension. To mix plates using the IntelliCyt shaker, launch ForeCyt and navigate to **Device>Manual Control Mode**.

The maximum shake speed on standard HTFC systems is 3750 RPM, and 3000 RPM on the iQue Screener. The appropriate shake speed for each individual assay will depend on plate geometry and assay volume.

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

Table 6. iQue Screener – Black Shaker

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

Table 7. Standard HTFC Screening System – Silver Shaker

**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.*

It is recommended to perform a pre-plate shake for all experiments, to ensure maximum homogeneity of sample dispersion immediately prior to sampling. Additionally, one of the optional protocol features within the software is to add inter-well shakes after a specified number of wells. While this is not often necessary to keep cells and objects in suspension for sampling, it is recommended primarily to provide a quick means of doing visual quality control on the well identification.

Adding a longer shake after the acquisition of every row or column breaks the sample set into sections of 8, 12, 16, or 24 wells that are much easier to quickly check and visually verify for integrity, as opposed to 96- or 384-well continuous samples.