# A High-Throughput, Radioactivity-Free Assay For Cell-Mediated Cytotoxicity

Immunotherapy promises to be a powerful approach for treating a variety of diseases—most notably cancer.[1–5] However, development of novel immunotherapeutics has been limited by the lack of high-throughput methods to screen for effective molecular entities or, in the case of adoptive cell therapy, genetically engineered cells. Most current assays that measure cell-mediated cytotoxicity (CMC), such as the chromium release assay, are difficult to perform on large numbers of samples, can only report on a single biological readout like cell membrane integrity, and cannot differentiate between effector and target cells. Methods based on flow cytometry, such as the CFSE assay,[6] can assess CMC at the level of an individual cell and enable discrimination between effector cells and target cells. Traditional flow cytometry is slow, however, making it unsuitable for use as a high throughput screening (HTS) assay.

Here we demonstrate a fast, efficient, radioactivity-free CMC assay with low sample input requirements, enabling miniaturization to 384-well plates. Using the iQue® Screener and differential labeling of effector and target cells, this method can capture multiple facets of biology—apoptosis markers, signal transduction markers, cell permeability, proliferative capability, and more—in a single well, providing a rich and highly quantitative data set specific to each cell type present. With this approach, researchers and drug discovery teams can quickly screen through compounds and conditions, building a detailed understanding of the molecular events occurring in each well and speeding insight into development of immunotherapeutic approaches.

## Materials and methods

Cells and Reagents: Jurkat cells, clone E6.1 (TIB-152 from ATCC), were used as target cells and NK-92 cells (CRL-2407 from ATCC) were used as effector cells. Jurkat cells were maintained in log growth phase at 37°C with 5% $CO_2$ in 1640 RPMI medium supplemented with 10% fetal bovine serum (Seradigm). NK-92 cells were cultured at 37°C with 5% $CO_2$ in MyeloCult medium (Stem Cell Technologies) supplemented with 20 ng/ml rhIL2 (Sigma) and 1 µM hydrocortisone (Sigma). All assays were conducted in Jurkat cell medium. NK-92 cells were used in CMC assays 48 hours after passage into new medium. Signal transduction inhibitors PP2, U73122, Sunitinib, and SP600125 were obtained from Sigma. Each inhibitor was solubilized in 100% DMSO (various concentrations) and stored at -20°C until use.

**CMC titration assays:** The iQue Screener CMC workflow is illustrated in Figure 1. Prior to the start of the assay, Jurkat cells (target) were encoded using the MultiCyt® FL4 Cell Proliferation and Encoder Kit (IntelliCyt) according to the protocol. Briefly, the cells were prepared in batch and excess dye removed by washing before use in the assay. The encoding protocol takes approximately 20 minutes. The following steps were performed in a single 384-well plate with thirty replicates of each condition and a total assay volume of 20 µL/well. Unlabeled NK-92 cells were serially diluted (1:2) over 12 steps from 60,000 cells/well down to 30 cells/well. At the start of the assay, each dilution was mixed with labeled Jurkat cells (6,000 cells/well) and incubated for four hours at 37°C. The resulting effector:target ratios ranged from 10:1 down to 1:200. To assess CMC, we monitored two different readouts—apoptosis using the Caspase 3/7 reagent from the MultiCyt Apoptosis Kit (IntelliCyt), and cell viability using the MultiCyt FL3 Cell Membrane Integrity reagent (IntelliCyt). Both reagents were used as stated in the product protocols, and were added to the reaction at the same time as the NK-92 cells. After four hours, the 384-well plate was placed directly into the iQue Screener and analyzed.

**CMC inhibition assays:** The CMC inhibition assay was performed as described for the CMC titration assay with the following modifications. A 16-step, 1:2 dilution series of each inhibitor was prepared with the following as the highest concentrations: Sunitinib at 25 ng/ml; PP2 at 100 mM; U73122 at 37.5 mM; SP600125 at 150 mM. NK-92 cells (12,000 cells/well) were preincubated at 37°C for 20 minutes with each dilution of inhibitor, and then mixed

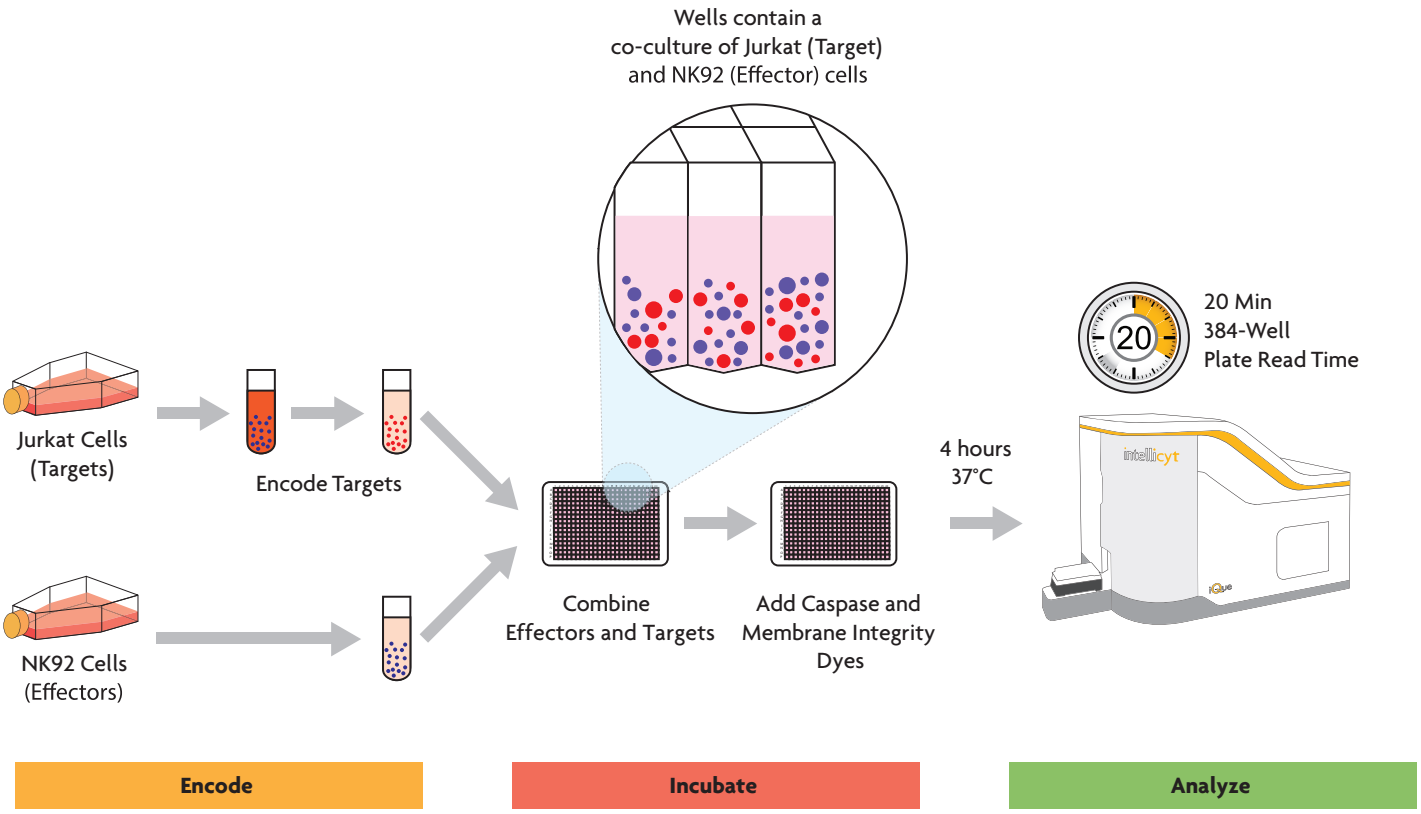


**Figure 1. Three-step iQue Screener cell mediated cytoxicity (CMC) assay workflow.**

with Jurkat cells (6,000 cells/well). An effector:target ratio of 2:1 was used for this study, with a total reaction volume of 20 µL. Each condition was performed in triplicate.

**iQue Screener and ForeCyt® analysis:** The iQue Screener provides high-throughput measurements using a flow cytometry detection engine and patented air-gap delimited sampling technology, which can sample wells with zero dead volume. Samples are delivered in a continuous stream to the detector engine without needing to pause between each sample. Individual wells are separated by discrete air gaps ensuring accurate discrimination of each sample (Figure 2). ForeCyt Software was used to acquire data, set appropriate gates, quantify, and plot CMC titration and inhibition data as described in the Results and Discussion section.

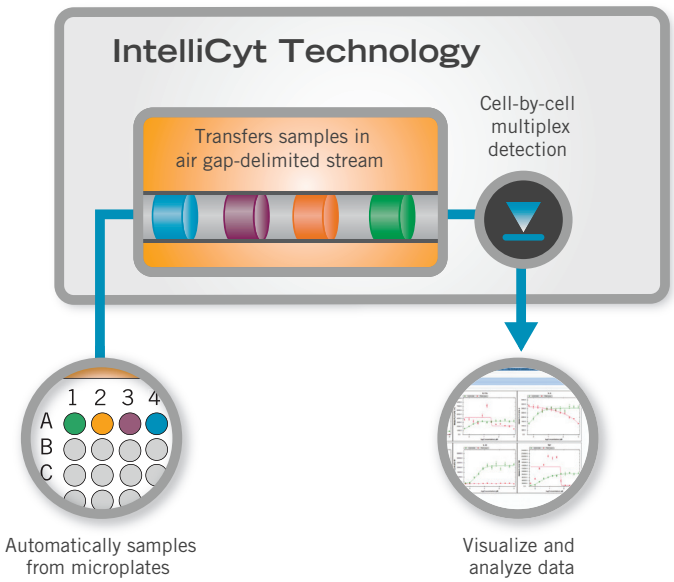


**Figure 2. iQue Screener Technology provides multiparameter, high-content analysis with as little as 1 µL of sample and zero dead volume.**

## Results and discussion

CMC assay overview: The iQue Screener-based CMC assay is a simple, three-step process that enables rapid screening of multiple conditions (Figure 1). First, target cells are labeled with an encoding dye, and then incubated with effector cells and reporter dyes, all in the same well of a multi-well plate. At the end of the incubation, the plate is placed into the iQue Screener for analysis. The resulting data is analyzed using ForeCyt Software to set appropriate gates, allowing discrimination of target cells from effector cells and quantification of cell membrane integrity and caspase 3/7 activation in individual cell types.

The advantages of this assay stem not only from its easy implementation as a high-throughput screen but also from the streamlined protocol, which reduces hands-on time. By adding staining reagents at the beginning of the assay, the labor involved in downstream steps is minimized while still providing robust results.

Data from an example assay using Jurkat target cells and NK-92 effector cells is shown in Figure 3. Figure 3A shows two distinct cell populations, the background fluorescence of unlabeled NK-92 cells and a higher intensity signal generated by fluorescence-encoded Jurkat cells.

Gating the Jurkat cells and analyzing just this population in the FL1 channel for caspase 3/7 activation enables quantification of Jurkat apoptosis. Because only apoptotic cells with activated caspase 3/7 will display fluorescence in the FL1 detection channel, we can determine the percent of apoptotic target cells in the well by comparing the number of FL1-positive cells (Figure 3B, right peak) to FL1-negative cells (Figure 3B, left peak).

Similarly, by analyzing the data for the Jurkat target cells in the FL3 channel, we can measure the relative amount of cell death mediated by the effector cells (Figure 3C). The higher intensity peak consists of FL3-positive cells with damaged membranes (Figure 3C, right peak). A gate can be drawn to quantify the number of target cells that are detected as FL3-positive, and therefore reports on the number of non-viable cells, whereas the lower-intensity "negative" peak is comprised of the viable cells that have excluded the dye and are not fluorescent (Figure 3C, left peak).

Finally, the same analysis can be done on the NK-92 cells to verify that they remain unaffected by incubation with the target cells (Figure 3D, E). In this case, the same gates used to assess activation of caspase 3/7 (FL1) and cell viability (FL3) for Jurkat cells were applied to the NK-92 cells for an unbiased analysis.

Note that for this example we chose to detect caspase 3/7 activation and cell membrane integrity, but a number of other reporters can be simultaneously detected and quantified for each cell type. Examples of other MultiCyt reagents that have been validated in multiplex with either the caspase or the cell viability reagents include Annexin V binding, mitochondrial membrane depolarization (MultiCyt Apoptosis Kits), cell proliferation (MultiCyt Cell Proliferation and Encoding Kit), and detection of secreted cytokines (QBeads® PlexScreen and DevScreen Kits).

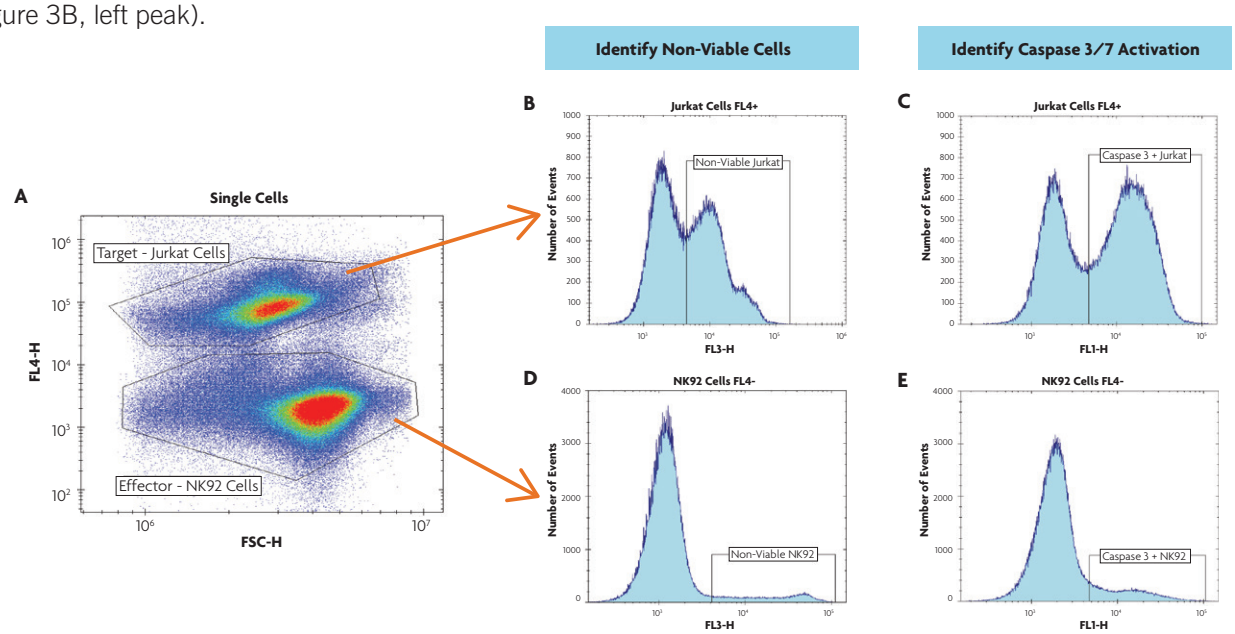


**Figure 3. Analysis of iQue Screener CMC assay. Target cells are encoded with a dye that fluoresces in the FL4 channel, enabling differentiation from unstained effector cells (A). Both target and effector cells can then be queried for viability (B, D) or caspase activation (C, E) separately, even though both are present in the same well.**

Reproducible quantification demonstrated by effector cell titration: To demonstrate the ability of the iQue Screener CMC assay to provide highly quantitative information, we assessed the dose-response relationship of NK-92 effector cells on CMC of Jurkat target cells. Keeping the number of Jurkat cells fixed at 6,000 cells/well, we added increasing amounts of NK-92 cells, changing the effector:target ratio from 1:200 to 10:1 (Figure 4). Each data point is the average of measurements from 30 different wells, with error bars as indicated.

Examination of the caspase 3/7 activation and cell viability plots for the NK-92 cells show background levels of caspase activation and cell viability, with higher variability at the lower NK-92 concentrations likely a reflection of measurement uncertainty at extremely low cell densities.

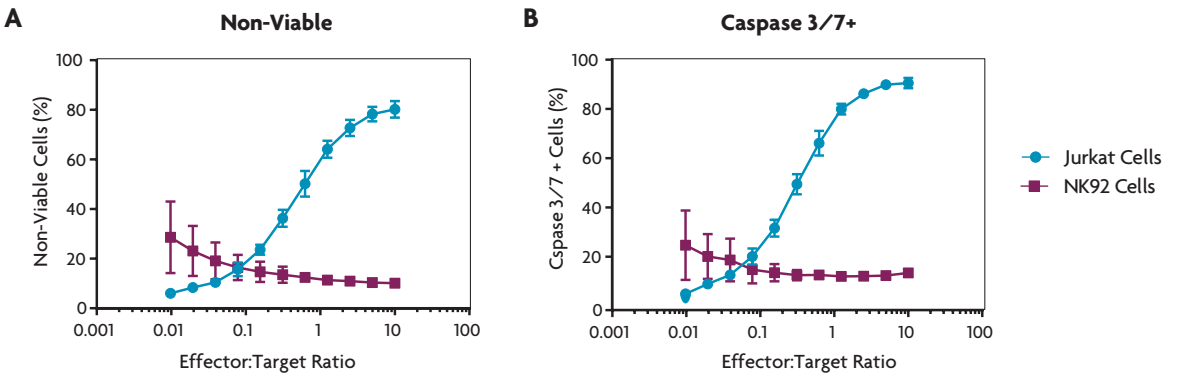


**Figure 4. CMC Titration Assay. Cell viability (A) and Caspase 3/7 activation (B) assessment demonstrate that Jurkat target cells are killed by NK92 effector cells in a dose-dependent manner. For each data point, n = 30 with the curves simply connecting each data point.**

Assay sensitivity, cell type discrimination demonstrated by CMC inhibitor dose-response assay: To demonstrate the sensitivity and specificity of the iQue Screener CMC assay, we performed the assay in the presence of increasing amounts of known CMC inhibitors that target different signaling pathways (Figure 5)—sunitinib (tyronsine kinase inhibitor), PP2 (Src inihibitor), U73122 (PLC inhibitor), or SP600125 (JNK inhibitor). Note that the ability to observe the effects of pathway-specific inhibitors on both effector cells and target cells helped us to quickly uncover an experimental artifact—general cytotoxicity rather than specific CMC inhibition—that could have led to misinterpretation of the results in the absence of the effector cell data.

For this assay we selected a fixed 2:1 ratio of NK-92 cells to Jurkat cells, which results in baseline values of ~75% of Jurkat cells staining positive for caspase 3/7 activation, and ~60% of Jurkat cells staining positive for membrane permeability (non-viability).

The effect of pathway inhibitor compounds on CMC can be seen in Figure 5, with increasing inhibitor concentration generally resulting in less cell membrane permeability (Figure 5A, C, E, and G) and caspase 3/7 activation (Figure 5B, D, F, and H) in Jurkat cells. There are two intriguing exceptions.

Figure 5A and B show the effects of sunitinib on both Jurkat target cells and NK-92 effector cells. While this tyrosine kinase inhibitor appears to have no effect on CMC based on the stable levels of membrane permeability and caspase 3/7 activation in the Jurkat target cells (Figures 5A, B), it increases caspase 3/7 activation in NK-92 effector cells (Figure 5B), while leaving cell membrane permeability in NK-92 cells unaffected. This effect on NK-92 cells would not have been visible using a traditional Cr51 release assay, and raises questions on the interpretation of the Jurkat caspase 3/7 data.

A second intriguing result comes from U73122. At the highest concentration of U73122, Jurkat target cells show a reverse in the trend of decreased cell membrane permeability and caspase 3/7 activation. Examination of these two readouts in NK-92 cells shows that at higher concentrations of U73122, NK-92 cells also display increased cell membrane permeability and caspase 3/7 activation (Figure 5E, F), suggesting that U73122 might possess general cytotoxic activity at these concentrations instead of specific CMC inhibition. This is in contrast to other inhibitors, such as PP2 and SP600125, which show no increased cell membrane permeability or caspase 3/7 activation at any concentration in NK-92 cells.

Thus, the ability to measure endpoints in both effector and target cells enabled a possible explanation of the anomalous U73122 data at high concentrations. Importantly, if only the highest concentration of U73122 had been examined, its effectiveness as an inhibitor of CMC would have been underestimated. In the context of a HTS where primary screening is typically performed using a single concentration of the test compound, this type of underestimation could lead to early elimination of a potentially potent compound.

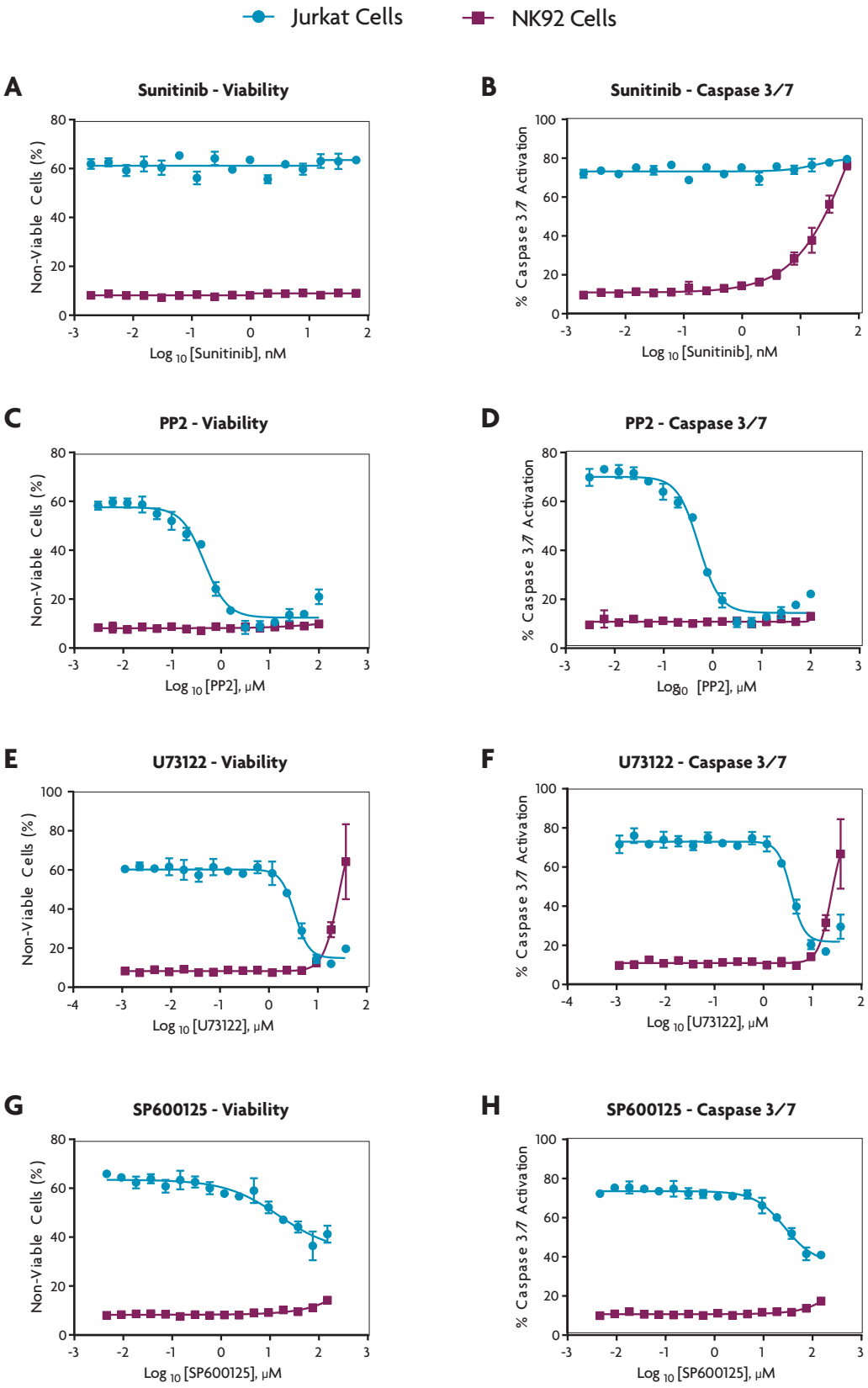


**Figure 5. Pathway-specific inhibition of CMC. Four different compounds show varying degrees of CMC inhibition on Jurkat target cells (blue curves) as assessed by cell membrane permeability (A, C, E, G) and caspase 3/7 activation (B, D, F, H). For each data point n = 3, with the data fit using a four parameter logistic nonlinear regression model. Notably, sunitinib and U73122 also have an effect on the NK-92 effector cells (red curves), which would not have been detected using a traditional Cr51 release assay.**

## Conclusion

The iQue Screener CMC assay is a sensitive, specific, and high-throughput assay that can provide a rich data set of quantitative information in a cell type-specific manner. It overcomes the problems of the traditional Cr51 release assay by removing the need to work with a radiolabeled isotope, reducing hands-on time, and increasing reproducibility and measurement precision. Like flow-cytometry approaches, the iQue Screener CMC assay enables discrimination of signal generated by effector cells versus target cells. However, the iQue Screener CMC assay goes one step further and improves on flow-cytometry-based approaches through its HTS format. Further, the potential for additional multiplexing with readouts such as quantification of secreted cytokines provides even more biological context. By simplifying and miniaturizing the CMC assay, the iQue Screener enables researchers to get results faster while conserving precious sample, thus providing an opportunity to accelerate discovery and development of new immunotherapeutics.

## References


1. Anguille S, Smits EL, Bryant C, et al. Dendritic Cells as Pharmacological Tools for Cancer Immunotherapy. *Pharmacol Rev.* 2015;67(4):731-753. doi:10.1124/pr.114.009456.
2. Chester C, Marabelle A, Houot R, Kohrt HE. Dual antibody therapy to harness the innate anti-tumor immune response to enhance antibody targeting of tumors. *Curr Opin Immunol.* 2015;33:1-8. doi:10.1016/j.coi.2014.12.010.
3. Zamarin D, Postow MA. Immune checkpoint modulation: rational design of combination strategies. *Pharmacol Ther.* 2015;150:23-32. doi:10.1016/j.pharmthera.2015.01.003.
4. Fousek K, Ahmed N. The Evolution of T-cell Therapies for Solid Malignancies. *Clin Cancer Res Off J Am Assoc Cancer Res.* 2015;21(15):3384-3392. doi:10.1158/1078-0432.CCR-14-2675.
5. Pennock GK, Chow LQM. The Evolving Role of Immune Checkpoint Inhibitors in Cancer Treatment. *The Oncologist.* 2015;20(7):812-822. doi:10.1634/theoncologist.2014-0422.
6. Jedema I, Werff NM van der, Barge RMY, Willemze R, Falkenburg JHF. New CFSE-based assay to determine susceptibility to lysis by cytotoxic T cells of leukemic precursor cells within a heterogeneous target cell population. *Blood.* 2004;103(7):2677-2682. doi:10.1182/blood-2003-06-2070.

## Notes

## Notes

IntelliCyt Corporation
Corporate Headquarters
9620 San Mateo Blvd. NE
Albuquerque, NM 87113
+1 505-345-9075 • www.intellicyt.com