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Application Note

November 2, 2020

Keywords or phrases:
Natural Killer cells, NK cells, NK cell killing, CAR-NK, ADCC, Antibody-dependent cellular cytotoxicity

Evaluating NK Cell Killing With Advanced Flow Cytometry

Introduction

Natural Killer (NK) cells are an essential part of the innate immune system and play a crucial role in immune surveillance and anti-tumor responses. Currently, several types of NK cell-related immunotherapeutics are being developed for the treatment of cancers. In particular, tumor-specific monoclonal antibodies (mAb) that are able to induce NK cell-mediated antibody dependent cellular cytotoxicity (ADCC) have proven to be successful against several types of cancer. Multiple bispecific and trispecific NK cell engaging antibodies (BiKES, and TRiKES) are also currently in preclinical and clinical development (1-4). Another promising strategy is the production of CAR-NK cells with a modified or high affinity IgG Fc receptor III (FcγRIIIa; CD16a), which is essential for NK cell binding of antibodies (Abs) attached to tumor cells and triggering ADCC (5-7). These types of CAR-NK cells may provide even more specific, enhanced killing of tumor cells when combined with tumor specific mAbs, BiKES, or TRiKES.

A decisive factor in evaluating the potency of any new tumor-specific Ab is whether it can effectively induce NK cell-mediated ADCC against the targeted tumor cells. Complexity arises from donor cell variability and this modulates the degree of NK cell-mediated ADCC. Thus, in addition to assessing cytolytic potential, it is also important to characterize the number and activation state of the donor NK cells utilized in order to provide insight into the potential treatment efficacy. Traditional cytotoxicity assays, such as Chromium-51 (Cr-51) release or use of fluorescent DNA binding vital dyes with standard flow cytometry, are time intensive and require additional downstream assays in order to characterize donor effector cells. The iQue® Human NK Cell Killing Kit is a novel, multiplexed, high throughput assay that simultaneously measures target cell killing, expression of NK cell phenotypic and activation markers, and quantification of secreted effector proteins and cytokines in a single well. The assay can be done in a 96 or 384-well microtiter plate, and is performed using pre-mixed reagents in a fast, simple workflow. In addition, the low volume sample requirement (10 µL) of this assay allows for measuring more endpoints with smaller amounts of precious or rare samples. Assay data acquisition is performed on the iQue® 3 platform for advanced flow cytometry using the integrated iQue Forecyt® software package with pre-set compensation matrices that enable data acquisition of the multiplexed, phenotyping assay without the need for single stain color compensation, and allows real time data analysis and visualization. Thus, the multiplexed iQue® Human NK Cell Killing Kit collapses traditional workflows into a single assay platform which allows for streamlined and rapid data acquisition.

Assay Principle

NK cells mediate ADCC through engagement of their FcγRIIIa (CD16a) with the constant (Fc) region of Abs bound to a target cell. This process triggers strong NK cell activation including the release of cytotolytic granules containing perforin and proteases known as granzymes, up-regulation of Fas ligand expression, and production of cytokines, such as Interferon gamma (IFNγ). The iQue® Human NK Cell Killing Kit provides a multiplexed approach to simultaneously measure ADCC, assess the NK cell activation state, and quantitate effector proteins secreted in a single well of a 96 or 384-well plate using either PBMCs or enriched NK cell populations as the source of effector cells (Figure 1). In each assay well, target cells are distinguished from effector cells by staining with a fluorescent encoder dye. Live and dead populations are separated by staining with a fluorescent membrane integrity dye that enters only dead cells or those with a compromised membrane, and stains nucleic DNA by intercalation. ADCC activity is then determined by quantifying the number of dead target cells/well. Quantification of the pro-apoptotic protease, Granzyme B, is also included in the assay as another, indirect measure of NK cell cytolytic activity. At the same time, the number and phenotype of the NK cells are also ascertained. Live NK cells are first immunophenotyped by staining with a fluorescent antibody panel to identify CD3- CD56+ NK cells, and then expression of CD16 can be assessed. This is important since differences in CD16 expression levels have been associated with differences in ADCC activity.[8] The phenotyping panel also includes two NK cell functional activation markers; CD69 and CD25.[9] CD69 is a cell surface glycoprotein known to be quickly upregulated upon activation of NK cells. CD25 is required for expression of the high affinity IL-2 receptor which is also often upregulated on activated NK cells. Finally, production of IFNγ is quantified using 2-plex iQue Qbeads® in a sandwich immunoassay format in the same well. Combining this assay with other iQue® Human NK Cell Companion Kits allows the flexibility to quantitate additional effector proteins, including Granzyme A, CD178 (Fas Ligand), TNF, GM-CSF, MIP-1α (CCL3), and RANTES (CCL5), all in the same well.

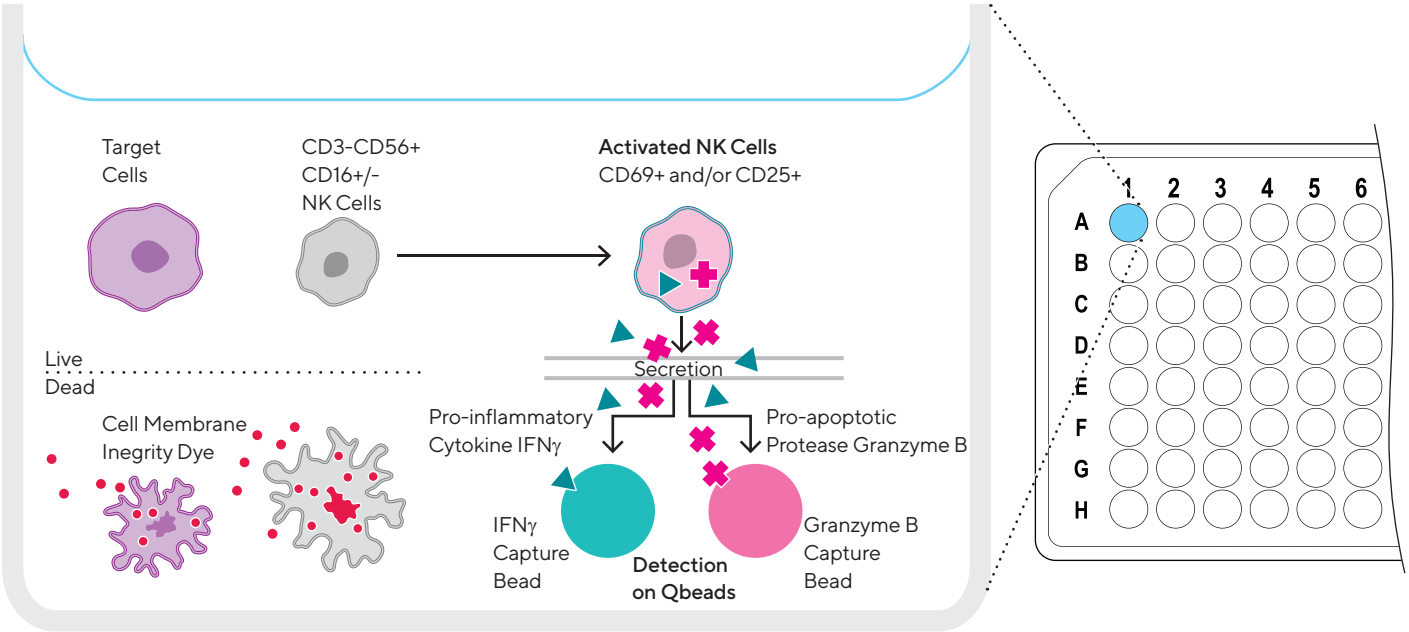


Figure 1. Illustration of multiplexed approach to measure NK cell activation state and target cell killing in a single well. Target cells are distinguished from effector cells by a fluorescent encoder dye, and tumor cell killing is determined with a fluorescent cell membrane integrity dye. NK cells are identified using CD3, CD56, and CD16. Their activation state is assessed with CD69 and CD25 expression. Production of the cytokine, Interferon gamma (IFNγ), and the pro-apoptotic serine protease, Granzyme B, are quantified using 2-plex iQue Qbeads® in the same well. Analysis of additional cytokines are available with iQue® Human NK Cell Companion Kits.

Materials and Methods

Co-culture of NK Cells and Tumor Target Cells With Anti-CD20 mAbs

One day prior to the assay, effector cells (Cellero; human PBMCs or enriched, negatively selected human NK cells) were thawed and allowed to recover in media (Corning; RPMI 1640 medium with 10% fetal bovine serum for enriched NK cells, or RPMI 1640 containing 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, and 1% Pen/Strep for PBMCs) for 16-18 h overnight. On the day of the assay, a frozen aliquot of CD20+ Raji tumor target cells (ATCC, Burkitt's lymphoma cell line) previously stained with a fluorescent encoder dye were thawed, counted, and plated in triplicate in a 96 well plate at 20K/well in the presence of individual anti-CD20 mAbs at concentrations ranging between 0- 10 µg/mL. The anti-hCD20 mAbs used in these studies were all obtained from InvivoGen and included, hcd20-mab1 (Rtx-G1), hcd20ga-mab1 (Ob-G1), hcd20ga-mab13 (Ob-G1nF) and hcd20ga-mab7 (Ob-A2). The encoded target cells and mAbs were allowed to interact for 30 min at room temperature before adding effector cells at Effector to Target (E:T) ratios ranging between 1:1 and 10:1, and then the co-culture was incubated at 37°C, 5% CO_2 . In parallel, effector cells were cultured in triplicate in media alone, or co-cultured with targets alone to evaluate direct tumor cell killing without antibody. Direct antibody-mediated killing of tumor cells was also assessed by culturing Raji cells with the anti-CD20 mAbs in the absence of effector cells.

ADCC Assay Workflow

After a 4 h co-culture, an aliquot sample (10 µL) of the cells/supernatant mixture from each well was transferred into 96 well plates and assessed using the iQue® Human NK Cell Killing Kit combined with the iQue® Human NK Cell Companion Kits to simultaneously measure the following:

- Target cell identification
- Cell count and cell membrane integrity (effectors and targets)
- Cell phenotype markers: CD3, CD56, and CD16
- NK cell functional markers: CD69 and CD25
- Secreted effector proteins (IFNγ, Granzyme B, Granzyme A, CD178 (Fas Ligand), TNF, GM-CSF, MIP-1α (CCL3), and RANTES (CCL5)

The assay was performed using pre-mixed reagents in a fast, simple workflow and requiring minimal hands-on time (Figure 2). First, the Cytokine Capture Beads Cocktail was added and the plate incubated at room temperature for 60 minutes. Next, the Cytokine Detection Cocktail was added, and the assay plates again incubated for 60 minutes at room temperature. After the second incubation, a fluorescent Antibody Detection Cocktail containing iQue® Cell Membrane Integrity Dye was added, and the assay plates incubated for another 60 minutes. Following the final incubation, the assay plates were washed once before acquisition on the iQue® platform (VBR).

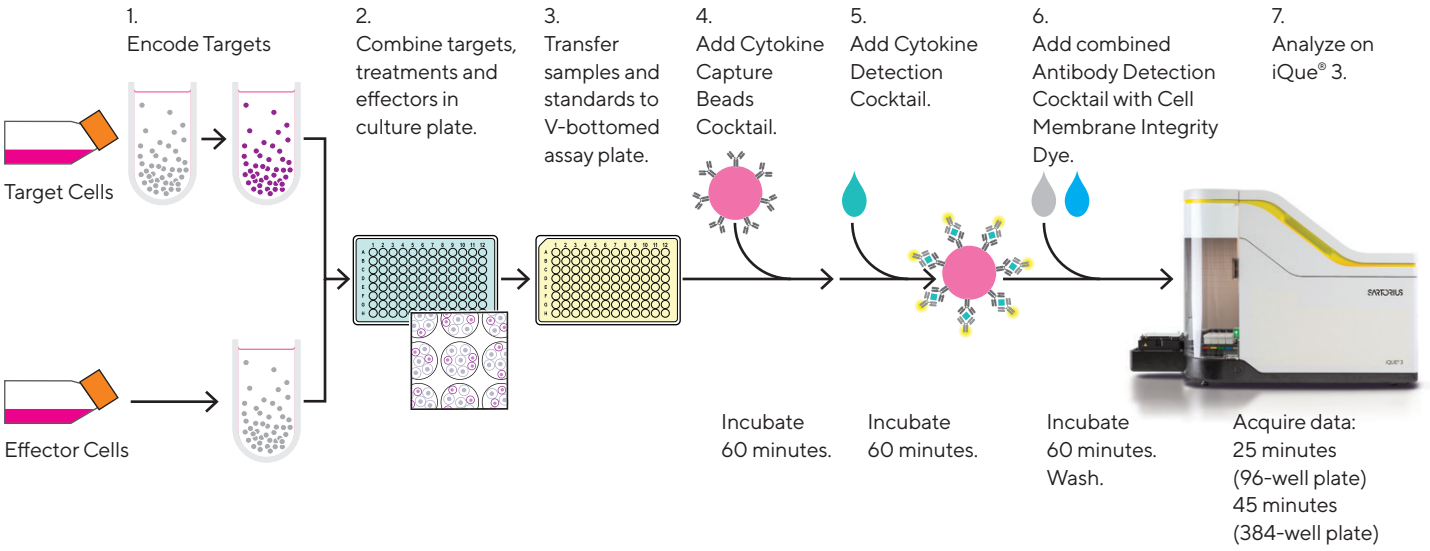


Figure 2. Assay Workflow:

- Following the appropriate co-culture time period, an aliquot sample of the cells/supernatant mixture (10 µL) from each well is transferred into 96 or 384-well assay plates.
- Cytokine/effector protein Capture Beads Cocktail is added, and the assay plates are incubated at room temperature for 60 minutes.
- Next, the Cytokine Detection Cocktail is added, and the assay plates are again incubated for 60 minutes at room temperature.
- After the second incubation, a fluorescent Antibody Detection Cocktail (anti-CD3, CD56, CD16, CD69 and CD25 mAbs) containing iQue® Cell Membrane Integrity Dye is added, and the assay plates are incubated for another 60 minutes at room temperature.
- Following the final incubation, the assay plates are washed once before acquisition on the iQue® platform (VBR).

Data Acquisition and Analysis

Data acquisition was performed using the iQue® platform (VBR) which combines high throughput sampling, flow cytometry detection and plate-level data analytics. Sample acquisition can be attained in 25 minutes for a 96 well plate and 45 minutes for a 384-well plate. The integrated iQue Forecyt® software enables rapid analysis and dynamic data visualization tools that are specifically designed to process and compare plate-level data. The platform and data analysis package streamlines data acquisition, analysis workflow and solves data synchronization problems. The acquisition protocol and data analysis, along with the event gates and gating strategy, activation metrics, standard curves, IC/EC_{50} curves and color compensation matrices are auto-generated using the iQue® Human NK Cell Killing Kit template and iQue Forecyt® software. Templated analysis workflows reduce time to actionable results due to the inclusion of pre-set color compensation matrices, circumventing the need for single color compensation controls and subsequent color compensation analysis as illustrated in Figures 3A and 3B. Standard curves to quantitate effector proteins were generated using 4-parameter curve fit with $1/Y^2$ weighting factor using the iQue Forecyt® software, which also automatically determines the linear range for each standard curve.

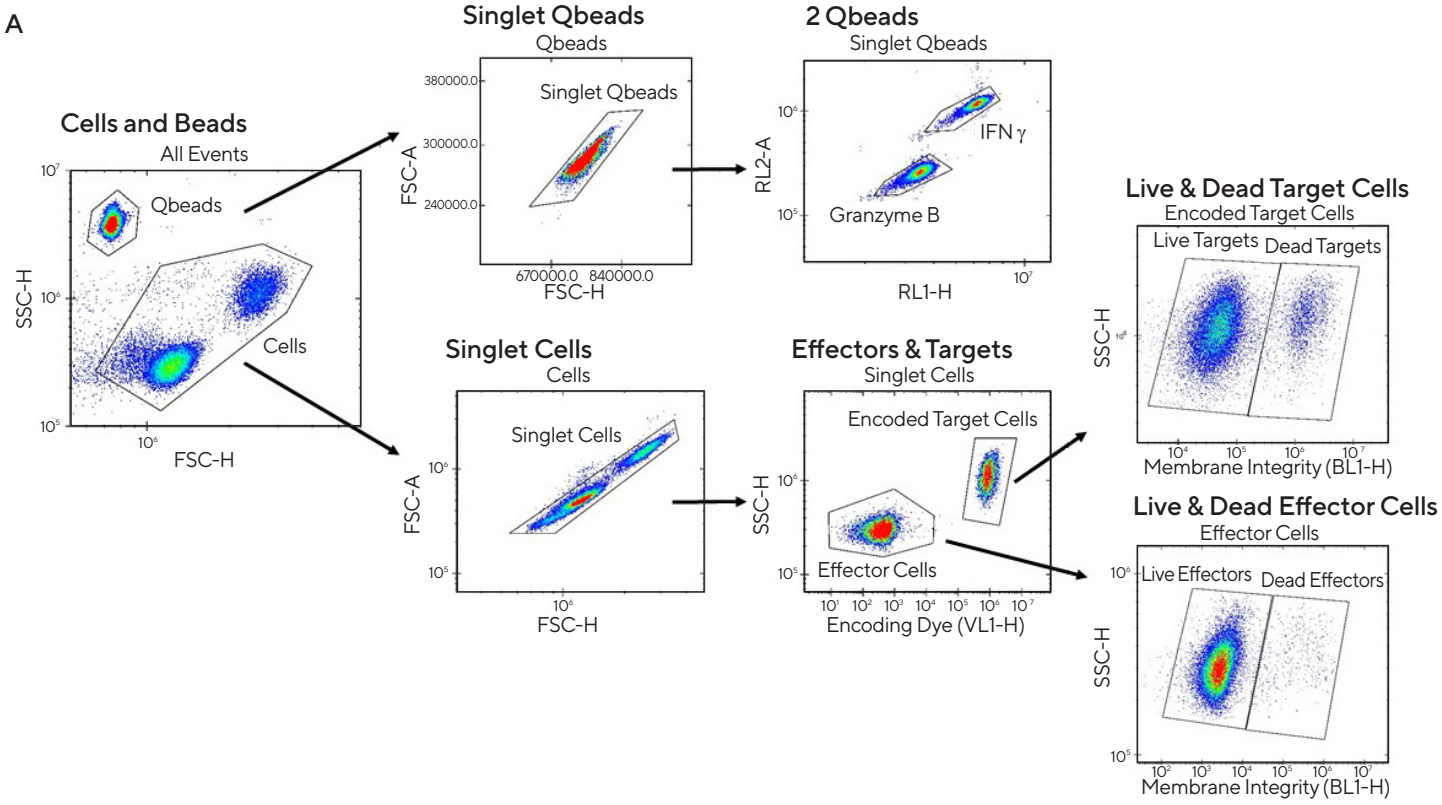


Figure 3A. Simultaneous assessment of NK cell cytolytic activity along with NK cell phenotypic and activation markers. Templated gating allows individual cytokine populations to be identified from the singlet iQue Qbeads® population. Separate effector and target cell populations are identified from the singlet cell population based on an encoding dye. Live/dead cell populations can then be further identified for both the effector and target cell populations using a cell membrane integrity dye.

B

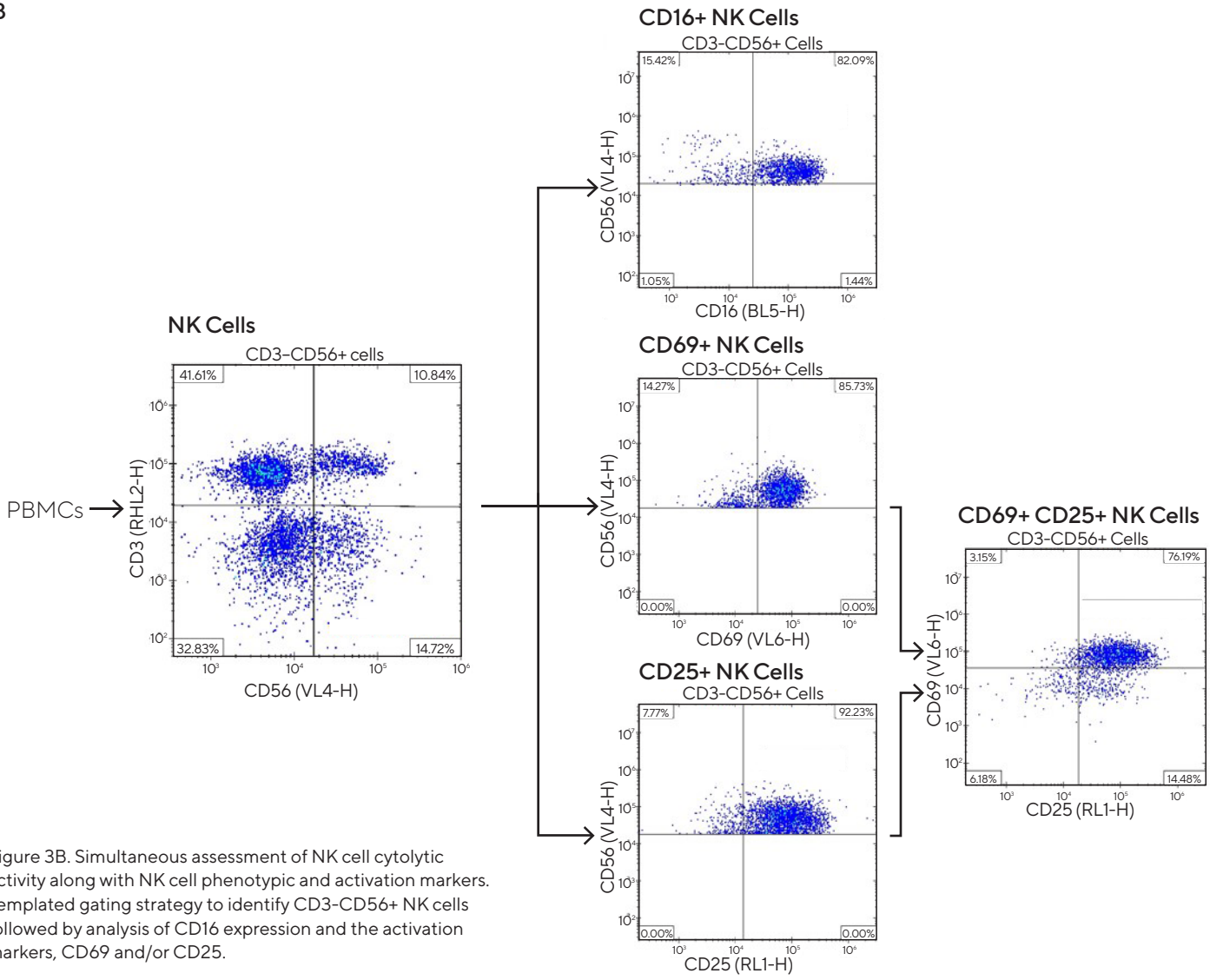


Figure 3B. Simultaneous assessment of NK cell cytolytic activity along with NK cell phenotypic and activation markers. Templated gating strategy to identify CD3-CD56+ NK cells followed by analysis of CD16 expression and the activation markers, CD69 and/or CD25.

Results and Discussion

Multiplexed Measurements of Target Cell Number, Effector Cell Phenotype and Cytokine Expression

Assessing NK cell-mediated ADCC is an important tool in determining the potential clinical efficacy of newly developed tumor-specific Abs. To fully characterize induction of NK cell-mediated ADCC against targeted tumor cells requires concurrent analysis of multiple factors, including evaluation of target cell death, effector cell phenotype and expression of cytokines. The iQue® Human NK Cell Killing Kit (Table 1) and iQue® Human NK Companion Kits (Table 2) allows for simultaneous measurements of these factors for a comprehensive assessment of the potency of tumor-specific mAbs.

For this study, a B cell non-Hodgkin's lymphoma model was chosen to demonstrate how the iQue® Human NK Cell Killing Kit plus iQue® Human NK Cell Companion Kits can be utilized to easily evaluate and compare the

Cell Type	NK Cell ID			Cell Surface Activation Markers		Secreted Effector Proteins		Cell Count	Cell Viability
	CD3	CD56	CD16	CD69	CD25	IFNγ	Granzyme B		
NK cells	-	+	+/-	+/-	+/-	+/-	+/-	#	0-100%
Target cells	-	-	-	-	-	-	-	#	0-100%

Table 1. iQue® Human NK Cell Killing Kit assay result readouts.

iQue® Human NK Cell Companion Kits	
Granzyme A	RANTES (CCL5)
CD178 (Fas Ligand)	GM-CSF
TNF	MIP-1α (CCL3)

Table 2. Additional effector protein analysis available.

ADCC potential of various mAbs, along with examining the level of ADCC induced by a mAb across donors.

Monoclonal Abs targeting CD20 have long been used in the treatment of B cell lymphomas. Rituximab is a type I, chimeric mAb (mouse variable region + human IgG1 Fc region) that targets the human (h) CD20 antigen. It has been used clinically for over 20 years and is approved by the FDA for the treatment of various B cell malignancies, including a B-cell non-Hodgkin's lymphoma (follicular lymphoma) and B-cell chronic lymphocytic leukemia (CLL) (10, 11). More recently, a type II, fully humanized IgG1 monoclonal antibody, obinutuzumab, was approved by the FDA for use in treatment regimens for patients with CLL and certain patients with follicular lymphoma (12, 13). Obinutuzumab binds a partially overlapping, but unique epitope on CD20 compared to rituximab, and was glycoengineered to reduce fucosylation in the Fc region which improved the binding affinity for FcγRIIIa (CD16a), resulting in enhanced ADCC (12, 14).

Anti-hCD20 mAbs similar to rituximab and obinutuzumab were utilized in the current study. These include an anti-hCD20 mAb featuring a human IgG1 Fc region and the variable region of rituximab (Rtx-G1), a fully humanized mAb with a native human IgG1 Fc region and the variable region of obinutuzumab (Ob-G1), and a mAb with the variable region of obinutuzumab and a non-fucosylated human IgG1 Fc region (Ob-G1nF). Another anti-hCD20 mAb, Ob-A2, was used as a negative control. It also possesses the variable region of obinutuzumab, but has a human IgA2 constant region and does not bind FcγRIIIa (CD16a).

Assessment of mAb Potency

The ADCC potential of the Rtx-G1 and Ob-G1 anti-hCD20 mAbs was assessed using PBMCs from 2 separate donors. Encoded CD20+ Raji tumor cells were first incubated with the anti-hCD20 mAbs for 30 minutes before adding donor PBMCs at a 10:1 E:T ratio and then the co-culture was incubated at 37°C, 5% CO_2 . In parallel, donor PBMCs were cultured in media alone or co-cultured with targets without antibody to assess the baseline activation state of the NK

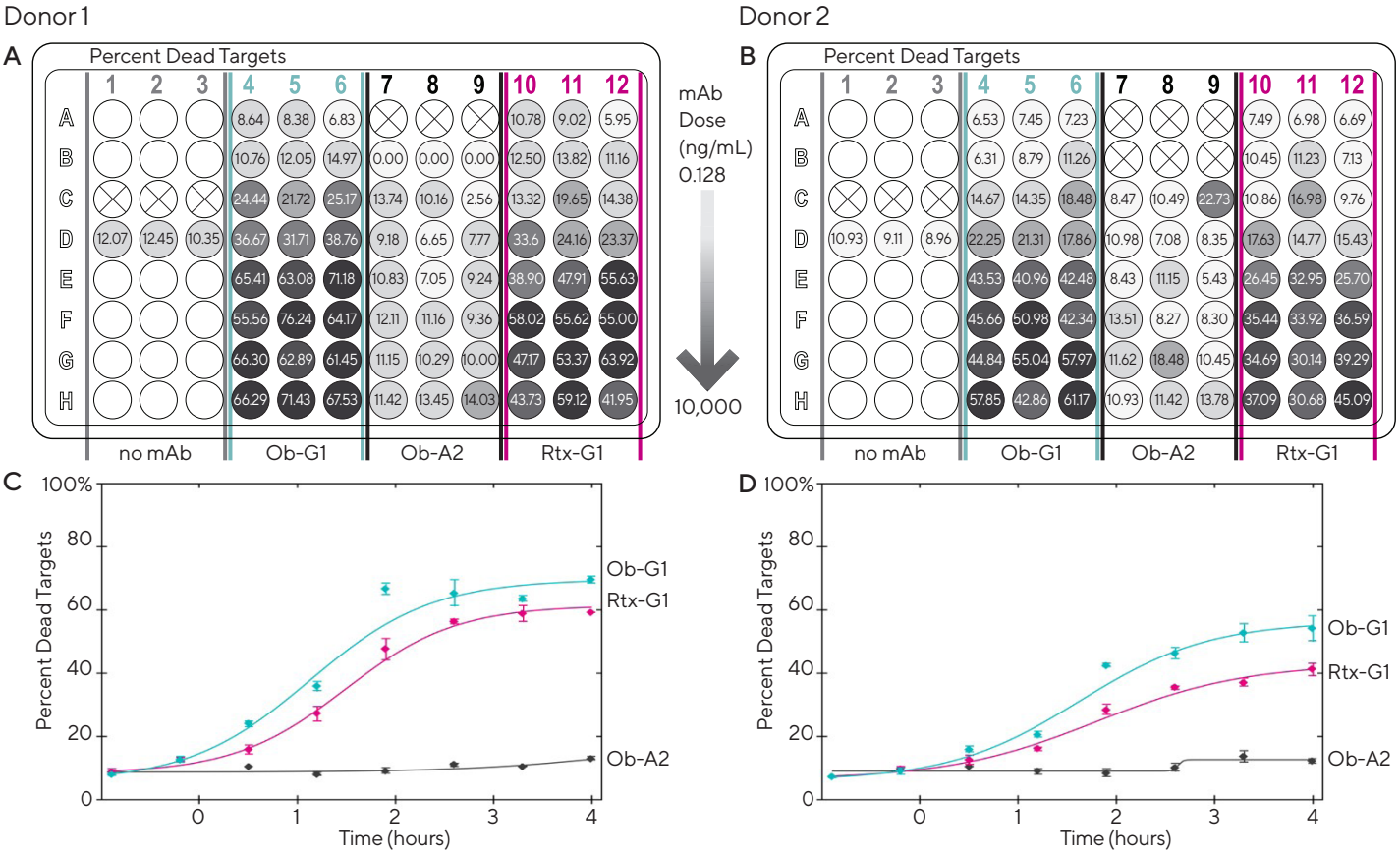


Figure 4. NK cell-mediated ADCC is mAb-dependent. PBMCs (200K/well) from two separate donors were co-cultured with encoded Raji tumor cells (20K/well) in the presence of different anti-hCD20 mAbs: Ob-G1, Rtx-G1, or Ob-A2, as a negative control. ADCC was assessed over five-fold serial dilutions of mAb starting at 10 µg/mL. After 4h, 10 µL samples were analyzed to assess tumor cell killing using the iQue® Human NK Cell Killing Kit plus iQue® Human NK Cell Companion Kits. Greater ADCC was seen with the Ob-G1 anti-hCD20 mAb as compared to the Rtx-G1 anti-hCD20 mAb for both donors: Plate heat maps for (A) Donor 1 and (B) Donor 2 and EC_{50} curves for (C) Donor 1 and (D) Donor 2.

Donor ID	Source of NK cells	% NK cells (CD3-CD56+)	% CD16+ NK Cells	Direct NK cell Killing (%)	mAb EC_{50} (ng/mL)	
					Rtx-G1	Ob-G1
Donor 1	PBMCs	12	91	12	31	13
Donor 2	PBMCs	14	89	10	64	42

Table 3. Comparison of anti-hCD20 mAb EC_{50} between donors

cells, as well as their ability to directly kill the tumor cells. Raji cells were also incubated with the anti-hCD20 mAbs alone to evaluate direct Ab-mediated cytotoxicity.

After co-culture for 4 h, sample aliquots (10 µL) were transferred into 96 well assay plates and analyzed using the iQue® Human NK Cell Killing Kit combined with additional iQue® Human NK Cell Companion Kits and the iQue® 3. Data acquisition was performed using the iQue® platform (VBR) which combines high throughput sampling, flow cytometry detection and plate-level data analytics. Use of the integrated iQue Forecyt® software automatically generates plate heat maps of the data to allow fast, actionable data visualization as well as automated concentration-response curves and EC_{50} values.

The results showed that the fully human Ob-G1 anti-hCD20 mAb demonstrated greater ADCC potency as compared to the mouse/human chimeric anti-hCD20 mAb (Rtx-G1) for both donors tested (Figure 4 and Table 3). No increase in ADCC above baseline levels was seen when the Ob-A2 anti-hCD20 mAb was included in the co-cultures, and only very low levels of direct killing of Raji cells was observed using the anti-hCD20 mAbs alone (< 12%; Ob-G1, < 6% Rtx-G1).

Donor to Donor Comparison

Using separate donors to assess the ADCC potency of the Rtx-G1 and Ob-G1 anti-CD20 mAbs in this study also provided insight into donor to donor variability. Similar numbers of NK cells were present in the PBMCs obtained from the two donors, and both donor NK cell populations showed high CD16 expression (Table 3). However, differences were detected in the level of tumor cell killing observed between the two donors, with PBMCs from Donor 1 exhibiting greater ADCC than Donor 2 using either Ob-G1 (Figure 5A) or Rtx-G1 (Figure 5C).

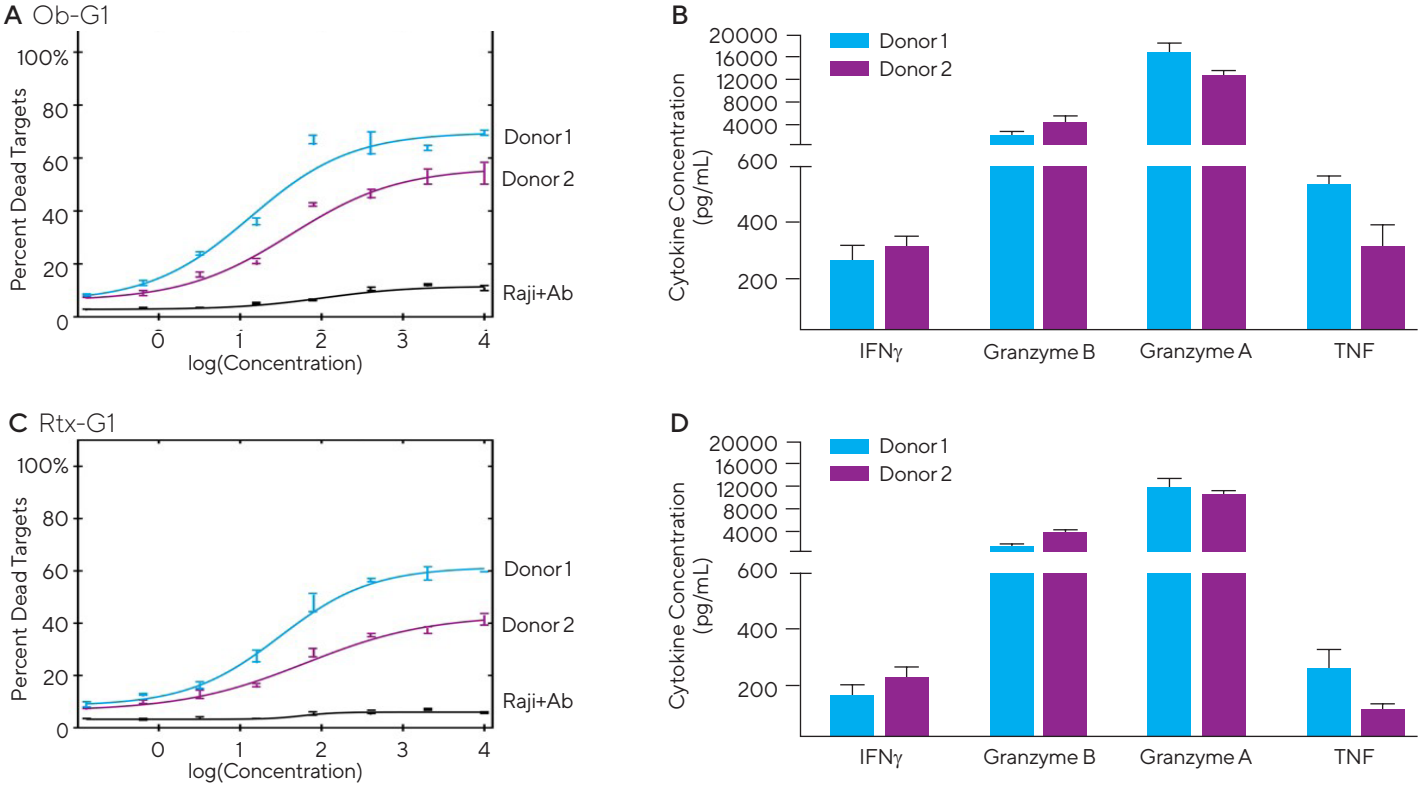
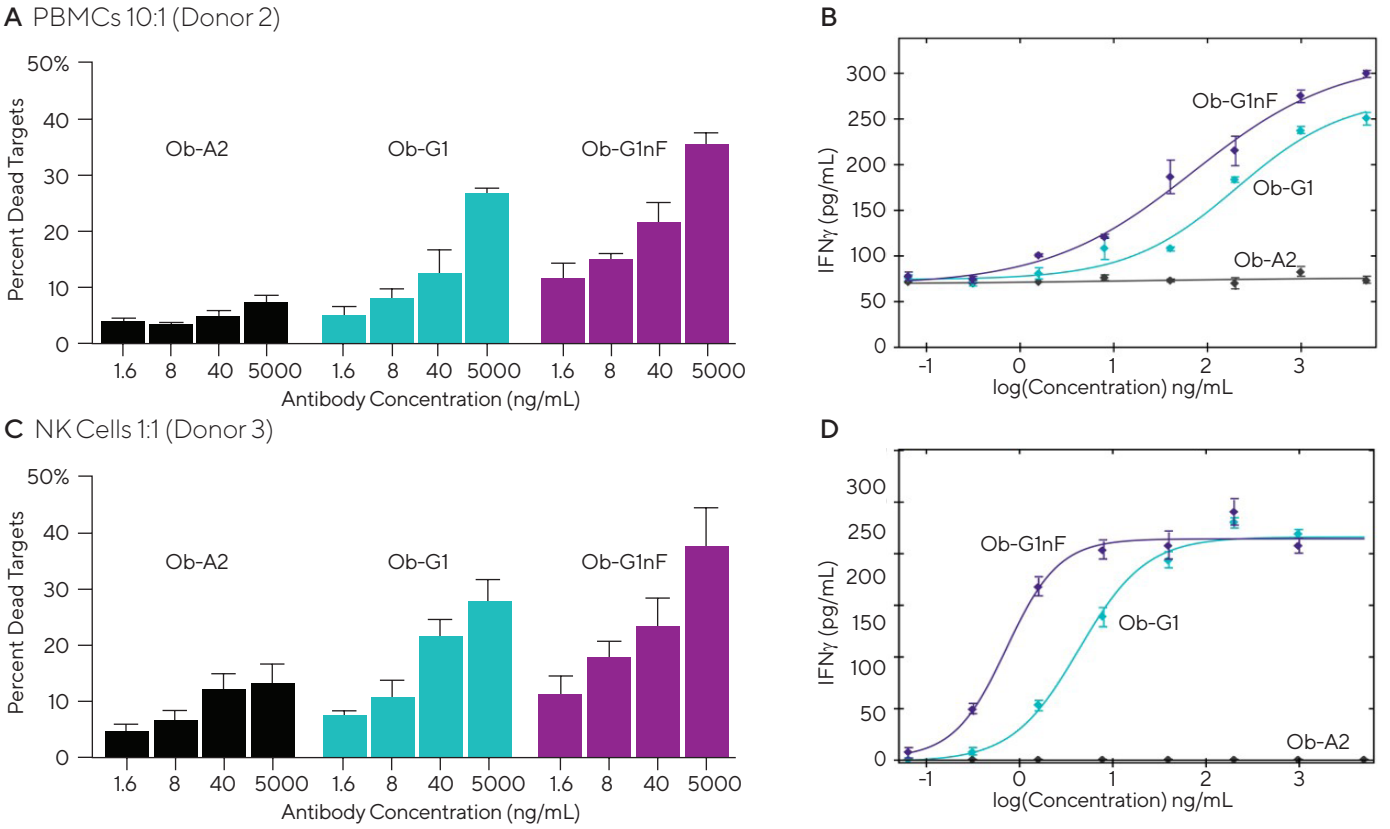


Figure 5. The degree of NK cell-mediated ADCC and secretion levels of effector proteins are donor-dependent. PBMCs from two separate donors were co-cultured with encoded Raji tumor cells (20K/well) in the presence Ob-G1 or Rtx-G1 anti-hCD20 mAbs at concentrations ranging between 0 - 10µg/mL, as described in Fig. 1 . Raji cells were also incubated with the mAbs alone to evaluate direct Ab-mediated cytotoxicity (Raji + Ab). Comparison of ADCC activity was analyzed after a 4 h co-culture for the 2 donors using (A) Ob-G1 or (C) Rtx-G1 anti-hCD20 mAbs. Levels of Granzyme B, Granzyme A, IFNγ and TNF secreted during tumor cell killing induced at a 10 µg/mL dose for the 2 donors using (B) Ob-G1 or (D) Rtx-G1 anti-hCD20 mAbs. Data presented as Mean +/- SD, 3 replicates/mAb dose.

Interestingly, differences were also observed in the levels of effector proteins produced by the two donors for both anti-CD20 mAbs in relation to ADCC. Donor 2 showed higher secretion levels of the cytolytic serine protease, Granzyme B, while higher levels of Granzyme A, along with increased TNF production was seen for Donor 1 (Figure 5B and D). One known difference between Donor 1 and Donor 2 was their FcγRIIIa genotype. It has been shown that having a valine (V) as opposed to phenylalanine (F) at amino acid position 158 of FcγRIIIa results in a higher binding affinity to IgG1 and enhanced ADCC (8, 15-17). The FcγRIIIa-158 genotype of Donor 1 was V/V, while Donor 2 was V/F. In addition, Donor 1 was seropositive for cytomegalovirus (CMV) which can also impact NK cell function (18, 19), while Donor 2 was CMV negative. Both of these factors may have contributed to the differences in the NK cell-mediated ADCC and cytokine secretion observed in this experiment.

Assessment of Fucosylated vs. Non-fucosylated mAb Potency

Next, a study was conducted to determine whether tumor cell killing by PBMCs from Donor 2 would be improved with the use of a mAb featuring the variable region of obinutuzumab and a non-fucosylated human Fc region (Ob-G1nF) as compared to Ob-G1 with a native IgG1 Fc region. As seen in Figure 6A, the non-fucosylated mAb, Ob-G1nF showed greater ADDC potency than Ob-G1 (Table 4). The ADCC potential of Ob-G1 and Ob-G1nF was next compared using enriched NK cells from another donor with a V/F FcγRIIIa-158 genotype. Again, greater tumor cell killing (Fig. 6B) and a lower EC_{50} (Table 4) was seen with the use of Ob-G1nF compared to Ob-G1. In addition, a mAb dose-dependent increase in IFNγ production was also observed using either PBMCs (Fig. 6C) or purified NK cells (Fig. 6D), with relatively higher expression levels induced by the non-fucosylated mAb, Ob-G1nF, as compared to Ob-G1.



Data Figure 6. ADCC was enhanced with the use of a non-fucosylated anti-CD20 mAb. Encoded Raji tumor cells (20K/well) were co-cultured with (A, B) PBMCs at an E:T of 10:1, or (C,D) enriched NK cells at an E:T of 1:1 in the presence of the Ob-G1, Ob-G1nF, or Ob-A2 anti-hCD20 mAbs at concentrations ranging between 0 - 5 µg/mL. After co-culture for 4h, 10 µL samples were analyzed using the iQue® Human NK Cell Killing Kit to assess tumor cell killing (A and C) and IFNγ production was also assessed (B and D). Data presented as Mean +/- SD, 3 replicates/mAb dose.

Donor ID	Source of NK cells	% NK cells (CD3-CD56+)	% CD16+ NK Cells	Direct NK cell Killing (%)	mAb EC_{50} (ng/mL)	
					Ob-G1	Ob-G1nF
Donor 2	PBMCs	14	89	5	45	28
Donor 3	Enriched NK cells	96	79	4	20	11

Table 4. EC_{50} for fucosylated and non-fucosylated anti-hCD20 mAbs using PBMCs or enriched NK cells.

Conclusions

In summary, the iQue® Human NK Cell Killing Kit is a novel multiplexed assay that simultaneously measures target cell killing, expression of NK cell activation markers, and quantification of IFNγ and Granzyme B with the flexibility to quantitate up to six additional effector proteins and cytokines using the iQue® Human NK Cell Companion Kits. The results of these studies demonstrate that use of the iQue® Human NK Cell Killing Kit plus iQue® Human NK Cell Companion Kits enables rapid assessment of the potency of different mAbs in ADCC mediated by NK cells, and can effectively distinguish differences in mAb efficacy between donors. In addition, the assay also allows for rapid characterization of NK-cell surface marker expression and cytokine secretion which may reveal potential mechanistic differences related to the variation in tumor cell killing observed between donors. Overall, these data show that the iQue® Human NK Cell Killing Kit combined with the advanced flow cytometry iQue® platform, is a powerful tool to rapidly screen NK cell effector functions and new, potential NK cell-based therapeutics.

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