New high throughput parallel screening technique for automatic creation of dose-response analysis with multiple E:T ratios, using minimal sample volumes

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Introduction

In vitro analysis of potency using conventional immune cell killing assays requires a large number of cells and considerable manual work to generate Dose/Response (D/R) analyses from these bulk measurements. Each effector cell type is assessed against 3-5 target cell lines with varying sensitivity to the cell-mediated cytotoxicity. Each E:T pair is commonly tested at up to 8 E:T ratios in triplicate, requiring more than a million effector cells, limiting the number conditions that can be tested. In addition, traditional methods typically involve hundreds of error-prone pipetting steps, reducing assay robustness and reproducibility.

Here we present a new automated miniaturized immune-cell killing assay, designed to directly address the industry challenges seen with a semi-automated plate–based method commonly used across cell therapy discovery and development. Based on the VivaCyte platform, this new assay uses the ability of its novel microfluidics to carry out miniaturized immune cell killing assays in which just one or a few immune cells interact with multiple target cells.

Below we explain how the dedicated Workflow 2 (WF2) performs high throughput D/R analysis in parallel enabling direct comparison of multiple samples, edits, or processes, and compare these data against the commonly used plate-based method.

Materials and methods

The fully automated Workflow 2 on VivaCyte® (Cellply) was used to miniaturize the preparation of the multiple E:T ratios required for a D/R analysis, **Figure 1.** The unique microfluidic technology enables automation of:

- co-culture creation
- automated staining to track cell types
- perfusion of culture media with live/dead cell markers
- time-lapse image acquisition and incubation.

Two batches of allogeneic NK cells from a single healthy donor PBMC were used as the effectors; (i) unstimulated NK cells used as sourced without pretreatment, and (ii) stimulated with IL-2 100U/mL, for a period of 72 hours using the standard technique. 315,000 effector cells are required for each D/R curve.

The target cells used were cell line K562 (ATCC), known to be sensitive to NK immune cell mediated cytotoxicity. These cells were tracked using CellTracker™ Blue CMAC (Thermo Fisher). To monitor cell death complete medium containing cell death marker (Propidium Iodide, PI, Thermo Fisher) was used.

Workflow 2 was used to automate creation of the co-cultures with the desired E:T range of 0.25-4, Two channels of the 16 in the CC-Array® plate were required for each assay. The target cells were loaded into both of the channels ensuring that a minimum of 720 microwells had the designated concentration to maximize 4T/well frequency. For each effector cell sample studied a low concentration of E cells was added to the first channel followed by a higher concentration to the second channel to ensure the presence of the required number of cells to create the predefined ratios, **Figure 1.**

The two NK samples were assessed against K562 using E:T ratios ranging from 0.25 to 4 (0.25, 0.5, 1, 2 and 4). Within WF2, AI-based image analysis of the microwells recognizes cells types, classifies each by the number of E and T cells present, then cluster the wells by E:T ratio. WF2 auto-generated 16 E:T ratios per channel pair.

The intra- and inter-assay reproducibility were assessed by performing 8 replicates of the standard D/R assay using the stimulated NK cells, in two runs carried out on two separate days.

An Incucyte™ (Sartorius) was used to represent the standard plate-based technique. The K562 target cells were tracked using Calcein AM (Thermo Fisher) and 10.000 cells were loaded in triplicate for each E:T ratio to generate an E:T D/R curve ranging from 0.25 to 4 (0.25, 0.5, 1, 2 and 4). The manufacturer's recommended assay protocol was used.

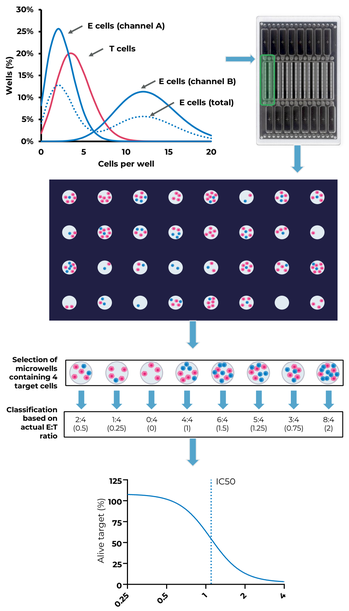

Figure 1: Cartoon of the 5-step assay process used by Workflow 2 to create multiple E:T ratio co-cultures for a dose/response curve

AI-powered analysis allows identification of microwells containing 4 targets, clustering of microwells with specific E:T ratio and building a D/R.

Results

The initial validation of Workflow 2 for automatic creation of the range of E:T ratios needed to generate the desired D/R curve was investigated by assessing the loading profile of the NK cells. NK cells from 3 different donors were used and the data compared. Workflow 2 applied a standard equation to determine the two effector cell concentrations required to cover the entire E:T range (0.25-4) with a set 4 target cells per well. The resulting experimental data were compared to the calculated theoretical Poisson distribution and showed a strong correlation, **Figure 2.**

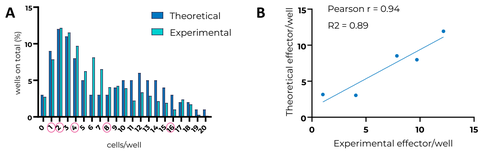

Figure 2: Experimental vs. theoretical E distribution;
(A) NK distribution in triplicate (n=3) for wells with 4T/well.
(B) Correlation between theoretical and experimental distribution in wells with E:T equal to 0.25, 0.5, 1, 2 and 4.

The potency of both stimulated and unstimulated NK against K562 cell line was measured. At the end of the assay, Workflow 2 analysis software automatically clustered the 1,500+ microwells as a function of the number of E and T cells present, **Figure 3A**. It also determined the number of dead cells per well. These data were used to create the corresponding D/R curves and determine the IC50 for each E and T pair. The fit of the 24h D/R curve for stimulated NK was R=0.94 **(3C)** and unstimulated NK was R= 0.82 **(3B).** For both samples, the maximum killing activity was seen in the wells with an E:T ratio of 4 – the highest tested. The IC50 of the post stimulation NK cells equaled 1.10 and the value for unstimulated NK cells equaled 2.24 **(3D)**.

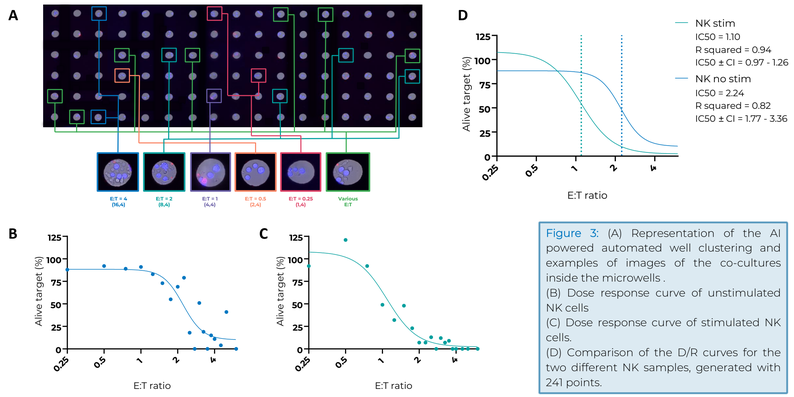

Figure 3: (A) Representation of the AI powered automated well clustering and examples of images of the co-cultures inside the microwells .
(B) Dose response curve of unstimulated NK cells
(C) Dose response curve of stimulated NK cells.
(D) Comparison of the D/R curves for the two different NK samples, generated with 241 points.

Table 1 below shows the number of pipetting steps and the time taken to perform a single high-quality D/R assay using Workflow 2 on the VivaCyte and for using a traditional plate-based method (Incucyte from Sartorius). Workflow 2 needed only two concentrations of E cells compared to the multiple serial dilutions needed for traditional method. With the automation of the WF2 assay protocol, a 16-point D/R curve could be generated in 20 mins, a fraction of the 65+ mins needed for the traditional plate-based method, and the number of pipetting steps significantly reduced from 227 to just 16.

Table 1:

Assay steps	VivaCyte Pipette steps	VivaCyte Time (min)	Traditional technologies Pipette steps	Traditional technologies Time (min)
Count targets and effectors	10	15	10	15
Tubes preparation	6	5	-	-
Target seeding	-	-	55	10
Medium preparation	-	-	5	5
Effectors dilutions	-	-	49	15
Effectors loading	-	-	54	10
Mix	-	-	54	10
Total	**16**	**20**	**227**	**65**

Table 2 shows the number of effector and target cells required to measure the 16-point D/R curve for each of these two techniques. Workflow 2 required just 315,000 effector cells, 30% of the total required using the traditional approach, and approximately a quarter of the number of target cells to generate the high-quality reproducible potency data.

Table 2:

Cell type	VivaCyte Cells/mL (x 10^6)	Traditional technologies Cells/mL (x 10^6)
Effectors	**0.315**	1.020
Target	**0.125**	0.540

Inter-experiment validation was assessed by testing a single NK donor batch against K562 target cells using 8 replicates in the same run. The assay was repeated on two separate days and the data shown in **Figure 4A**.

The bulk immune cell killing of the same donor batch was measured with traditional image-based Incucyte analysis using a calcein release plate-based assay. The reproducibility of these gold standard experiments was extremely low (average SD 28.9%, n=72) if compared to VivaCyte (average SD 9.74%, n=96). Despite this, comparison of these data with the corresponding data from analysis using Workflow 2 gave a 90+% correlation, **Figure 4B**.

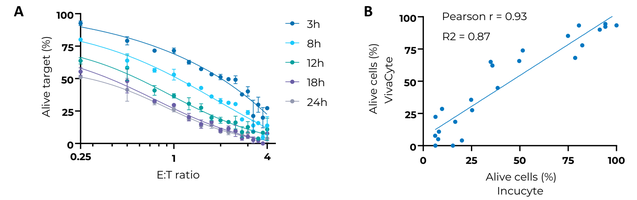

Figure 4: (A) Inter-experiment data for NK killing activity against K562 (data normalized at 0h, n=16).
(B) Comparison of target viability for assays performed using Workflow 2 and a traditional images-based instrument, (n=24).

Conclusion

In this study we have shown that Workflow 2 on VivaCyte, a combination of unique microfluidic technology - enabling protocol automation at the nanolitre scale – coupled with AI based image analysis and robotics, is able to produce consistent potency data for multiple cell therapy samples.

This novel automated immune-cell killing assay protocol has been used to generate high quality, D/R curves with 16 E:T ratios (exceeding the commonly used 5-8 E:T ratios) using a fraction of the usual hands-on time, the smallest number of precious cells, with a high degree of reproducibility.

Experimental data from this technique has been directly compared to data from the current laboratory standard plate-based imaging system (Incucyte) and has been shown to produce more consistent results with notably lower SD values.

This new approach can also provide a kinetic analysis of killing activity at multiple time points and, therefore, can significantly enhance characterization efficiency for these complex heterogeneous therapies.

Reduce amount of sample, time, and errors

3x less number of sample cells needed

3x less hands-on time for each D/R analysis

15x less pipetting steps reducing errors

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