

# Building a Better Cell Therapy Potency Assay

High-Throughput, Multiplex Approach to Characterize CAR-T and CAR-NK Cell Protein Secretion Profiles

### Introduction

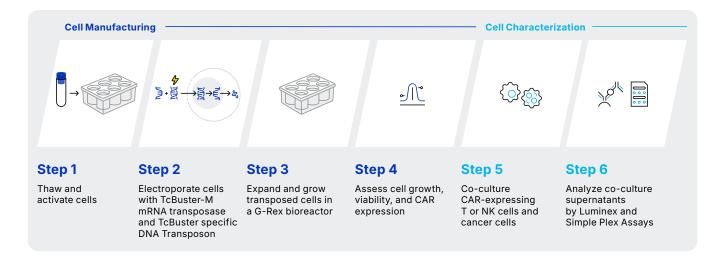
Chimeric antigen receptor (CAR) T and natural killer (NK) cell therapies harness genetically engineered immune cells to specifically recognize and destroy cancer cells. To date, seven CAR-T products have received U.S. Food and Drug Administration (FDA) approval for blood cancers, while CAR-NK therapies remain under investigation. Despite differences in cell type, manufacturing for both therapies typically involves apheresis, enrichment, activation, genetic modification, expansion, and rigorous quality testing. A critical step before patient infusion is demonstrating potency, ensuring that the engineered cells achieve a defined functional threshold.

Currently, most potency assays measure interferongamma (IFN-γ) secretion following overnight co-culture of CAR-T or CAR-NK cells with antigen-expressing target cells¹. All CAR-T therapies that have disclosed their protocols rely on this single-analyte ELISA readout. Regulatory agencies, including the FDA, are increasingly calling for deeper characterization of immune cell function to better define potency and therapeutic relevance². This application note highlights a high-throughput immunoassay workflow designed to broaden the evaluation of CAR-T and CAR-NK cell function. The

approach combines the multiplexing power of R&D Systems™ Assays for Luminex® Instruments to screen a wide range of secreted analytes with the sensitivity of R&D Systems Simple Plex™ Assays on the automated Ella™ System to validate key targets. Together, these complementary platforms enable reliable profiling of cytokine and effector molecule secretion during antigen-specific killing.

In this study, summarized in Figure 1, primary human CD4+ and CD8+ T cells or NK cells from three healthy donors were engineered with R&D Systems TcBuster™-M transposon system to express a CD19-CAR. The TcBuster transposon system is a non-viral gene editing technology that is used in CAR-T and CAR-NK cell therapy manufacturing³. To characterize protein secretion profiles and investigate cell potency, the manufactured CAR-T and CAR-NK cells were co-cultured with CD19-positive Nalm-6 cells and the resulting supernatants were analyzed by both Luminex and Simple Plex Assays.

R&D Systems pipeline for editing and characterizing cells to be used in immune cell therapies



## Methods

### Manufacturing CAR-T cells

CD4<sup>+</sup> and CD8<sup>+</sup> T cells were enriched from PBMCs by magnetic selection and activated for two days in R&D Systems GMP Human T Cell Media supplemented with 5% human AB serum and 10 ng/mL each of IL-7 and IL-15 at 37 °C. Cells were engineered to express CD19-CAR using the non-viral TcBuster transposon system delivered via the MaxCyte® GTx electroporation platform.

Briefly, 1 × 10<sup>7</sup> activated T cells were resuspended in HyClone™ buffer containing 1 µg TcBuster mRNA and 3 µg of a TcBuster-compatible transposon encoding CD19-CAR and eGFP. Cells were electroporated in an OC-100x2 cuvette (program Expanded T cell1) and transferred to G-Rex® 6M-well plates with 1 mL GMP Human T Cell Media lacking serum and cytokines but supplemented with 10 µg/mL DNase. After 15 minutes at 37 °C, wells were volume-filled with GMP Human T Cell Media containing 5% human AB serum and IL-7/IL-15 (10 ng/mL each). Cells were expanded for seven days. Growth and viability were monitored by cell counts, and transposition delivery efficiency was assessed by flow cytometry.

### **CAR-NK Cell Manufacturing**

CD56<sup>+</sup>, CD3<sup>-</sup> NK cells were enriched from PBMCs by magnetic selection and activated with irradiated feeder cells for four days in R&D Systems ExCellerate™ Human NK Cell Expansion Media containing 50 IU/mL IL-2. Activated NK cells were then engineered to express

CD19-CAR and eGFP using the TcBuster system with MaxCyte electroporation.

Briefly,  $5 \times 10^6$  activated NK cells were resuspended in HyClone buffer containing 1  $\mu g$  TcBuster-M mRNA and 7.5  $\mu g$  of a TcBuster-compatible transposon encoding CD19-CAR and eGFP. Cells were electroporated in an OC-100x2 cuvette (program NK6) and transferred to G-Rex 6-well plates with 1 mL Human NK Cell Expansion Medium supplemented with 10  $\mu g/mL$  DNase and incubated for 15 minutes at 37 °C. Wells were volume-filled with Human NK Cell Expansion Medium with 50 IU/mL IL-2 and 10 ng/mL IL-15 for two days at 37 °C. After 2 days, NK cells were re-stimulated with irradiated feeder cells and expanded for seven days. A second feeder-cell stimulation was performed at day 9, with expansion continued until day 16. Cell counts tracked growth and viability, and flow cytometry measured editing efficiency.

### **Luciferase-Based Killing Assay**

Cytotoxicity assays were performed by co-culturing CD19-CAR-T or CAR-NK effector cells with CD19<sup>+</sup> Nalm-6 target cells in 96-well plates at varying effector-to-target (E:T) ratios. For CAR-T cells, E:T ratios were calculated based on CAR<sup>+</sup> cells and co-cultures were maintained for 24 hours. For CAR-NK cells, ratios were calculated on total NK cells with a 12-hour co-culture.

Following incubation, plates were centrifuged, supernatants collected, and cells lysed according to manufacturer's instructions.

Luminescence was measured after addition of assay buffer and substrate. Specific killing was calculated as:

Wells containing only target cells served as controls.

### **Secretome and Cell Potency Evaluation**

Supernatants from the killing assays were analyzed using R&D Systems Luminex and Simple Plex Immunoassays. Initial, broad analyte profiling was performed with the Human Luminex Discovery Assay, Human XL Cytokine Luminex Performance Assay, and Human Immuno-Oncology Luminex Performance Assay following catalog protocols.

In total, 123 unique analytes—including cytokines, chemokines, growth factors, extracellular matrix proteins, and cytotoxic granule proteins—were quantified to define CAR-T and CAR-NK secretion profiles. A subset of 25 analytes, representing both core and peripheral secretome markers identified by Luminex, were subsequently validated with Simple Plex Assays (see Materials section) to confirm reproducibility and assess cross-platform translatability.

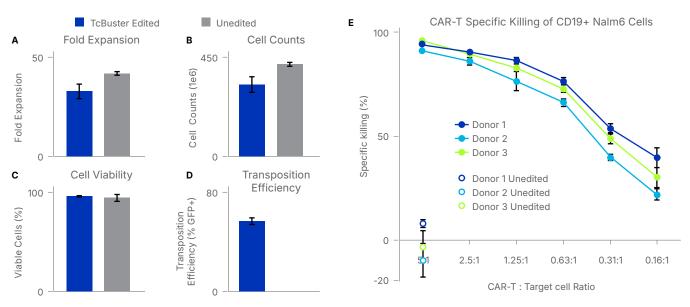
### Results

### **TcBuster Generates Functional CAR-T Cells**

CD4\* and CD8\* T cells from three healthy donors were activated for two days before engineering with the TcBuster transposon system on the MaxCyte platform. Edited T cells were expanded for seven days and evaluated for growth, viability, and transposition efficiency.

TcBuster-edited T cells expanded robustly, similar to unedited controls (Figure 2A), yielding an average of 3.3 × 10<sup>8</sup> cells per well (Figure 2B) while maintaining high viability (Figure 2C). Flow cytometry confirmed stable cargo delivery, with an average of 57% eGFP+ cells compared to no expression in unedited controls (Figure 2D). Functional testing with a luciferase-based killing assay demonstrated that CD19-CAR-T cells achieved robust, antigen-specific cytotoxicity at high effector-totarget (E:T) ratios, whereas unedited T cells showed no activity (Figure 2E). These data confirm that TcBuster efficiently inserts a functional CD19-CAR into T cells without compromising growth or viability.

T cells undergo robust expansion and maintain viability after editing with TcBuster and specifically kill CD19-expressing target cells.



T cells from 3 donors were activated and expanded for 9 days in GMP Human T Cell Media, supplemented with 5% hAB serum and 10 ng/mL each of IL-7 and IL-15, in a 6M well G-Rex. On day 2 of culture, activated T cells were unedited, or edited to express CD19-CAR using the TcBuster transposon system. At day 7 post-electroporation, cells were assessed for (A) fold expansion, (B) cell counts, (C) cell viability, (D) and genome modification (GFP+) by flow cytometry. (E) TcBuster-edited and unmodified T cells from 3 donors were incubated with luciferase-expressing CD19+ Nalm6 target. Luciferase activity of remaining target cells was assessed after 24 hours of co-culture. (A-D) Data points represent the average of 3 donors ±SD. (E) Data is normalized to target only samples. Each donor was run in triplicate and data points represent the average of 3 replicates per donor ±SD.

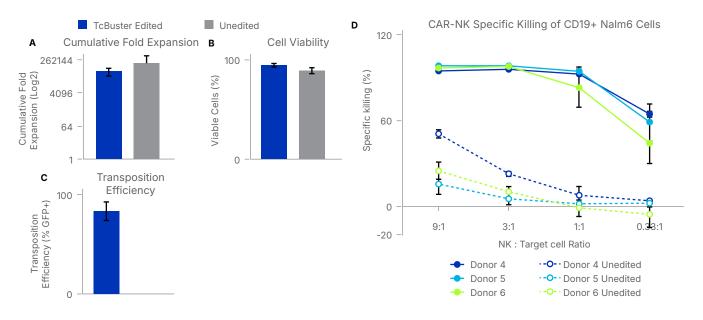
### **TcBuster Generates Functional CAR-NK Cells**

CD56\*, CD3<sup>-</sup> NK cells from three donors were activated for four days before TcBuster-mediated engineering on the MaxCyte platform, followed by 16 days of culture with two rounds of feeder stimulation.

TcBuster-edited NK cells exhibited strong cumulative expansion (Figure 3A) and high viability comparable to unedited controls (Figure 3B). Flow cytometry confirmed successful transposition, with an average of 84% eGFP<sup>+</sup> NK cells compared to none in unedited cells (Figure 3C).

Luciferase-based killing assays demonstrated that CD19-CAR-NK cells mediated potent, antigen-specific cytotoxicity at high E:T ratios, while unedited NK cells showed minimal activity (Figure 3D). Together, these findings establish TcBuster as an effective system for generating functional CAR-NK cells without impairing expansion or viability.

NK cells undergo robust expansion and maintain excellent viability after editing with TcBuster and specifically kill CD19-expressing target cells.



NK cells were isolated from 3 donors and edited with TcBuster in triplicate. Unedited and TcBuster-transposed NK cells were expanded with irradiated target cells and supplemented with 50 IU/mL IL-2 for 16 days post editing in R&D Systems ExCellerate™ Human NK Cell Expansion Media. At day 16 post-electroporation, cells were assessed for (A) cumulative fold expansion, (B) cell viability, (C) and genome modification (GFP+) by flow cytometry. (D) TcBuster-edited and unmodified NK cells from 3 donors were incubated with luciferase-expressing CD19+ Nalm6 target cells. Luciferase activity of remaining target cells was assessed after 12 hours of co-culture. (A-C) Data points represent the average of 3 donors ±SD. (D) Data is normalized to target only samples and not adjusted for CD19+ CAR percentage. Each sample was run in triplicate and data points represent the average of 3 samples per donor for TcBuster-modified ±SD and 1 sample for unmodified.

## Multiplexing Defines CAR-T and CAR-NK Protein Secretion Profiles

Supernatant samples from the CD19-CAR-T and -NK killing assays were analyzed using R&D Systems Luminex Assays to establish secretion profiles for both types of effector cell. A total of 123 analytes from a wide range of protein families were selected for initial screening using the Human Luminex Discovery Assay, Human XL Cytokine Luminex Performance Assay, and the Human Immuno-Oncology Luminex Performance Assay. Luminex protein quantitation revealed distinct secretion signatures between CD19-CAR-T and CD19-CAR-NK cells when cocultured with Nalm-6 target cells (Figure 4) and provided insight into the unique mechanisms of action behind these two cell therapies. Compared to the Nalm-6-only controls, 29 analytes were identified to be differentially secreted from CD19-CAR-T cells and 13 from CD19-CAR-NK cells that ranged in concentrations from thousands of pg/mL to <10 pg/mL on average across three donors.

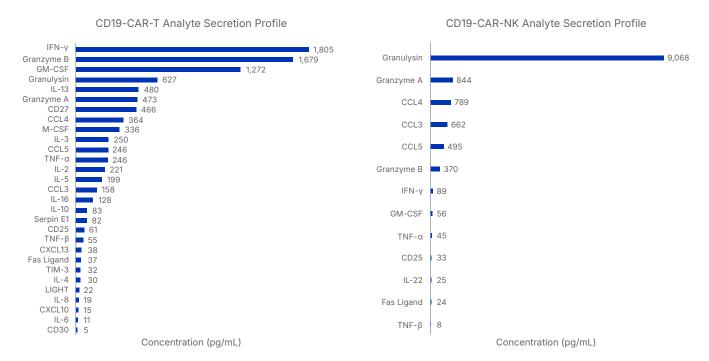
Secreted proteins from the two types of CD19-CAR effector cells spanned diverse functional classes, including:

- Pro-inflammatory cytokines:
   IFN-y, IL-2, TNF-α, TNF-β
- Anti-inflammatory cytokines: IL-4, IL-10, IL-13
- Cytotoxic granule proteins:
   Granzyme A, Granzyme B, Granulysin
- Stimulatory cytokines:
   GM-CSF, M-CSF, IL-3, IL-5
- Chemokines:
   MIP-1α, MIP-1β, RANTES

αβγ

FIGURE 4.

Secretion profiles of CD19-CAR-T and -NK cells when co-cultured with Nalm-6 cells were established using R&D Systems Assays for Luminex Instruments.



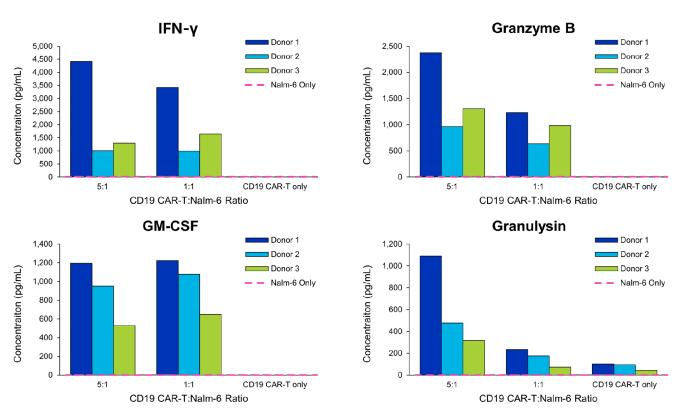
123 proteins were screened using R&D Systems Luminex Assays for presence in supernatant samples collected from CD19-CAR-T and -NK killing assays. Analyte secretion profiles were curated and ranked based on average concentrations measured across three T cell and three NK cell donor samples. Sample concentrations from the largest effector: target cell ratio tested (5:1 for the CD19-CAR-T cells, 3:1 for the CD19-CAR-NK cells) were averaged among the three donor sets to include low-abundance proteins and generate the most comprehensive analyte secretion profile.

Among activated T cells, IFN- $\gamma$ , Granzyme B, GM-CSF, and granulysin were the most abundant secreted analytes (Figure 5), while activated NK cells predominantly secreted granulysin, followed by Granzyme A and MIP-1 $\beta$  (Figure 6). While IFN- $\gamma$  was selectively secreted across all donors for both activated CD19-CAR-T and CD19-CAR-NK cells in the presence of Nalm-6 cells compared to controls, it is evident from the diverse secretion profiles that additional protein biomarkers could be monitored for a more representative measure of effector cell potency. Luminex Multiplex results also revealed donor-specific

heterogeneity in analyte concentrations (Figures 5 and 6), an important characteristic when vetting viability of immune cell therapies. With Luminex Multiplex Assays being able to measure entire panels of analytes in a single well, cell therapy potency could initially be investigated on the scale of entire secretion profiles as demonstrated here, prior to narrowing in on a subset of analytes representative of the effector cell's mechanism of action.

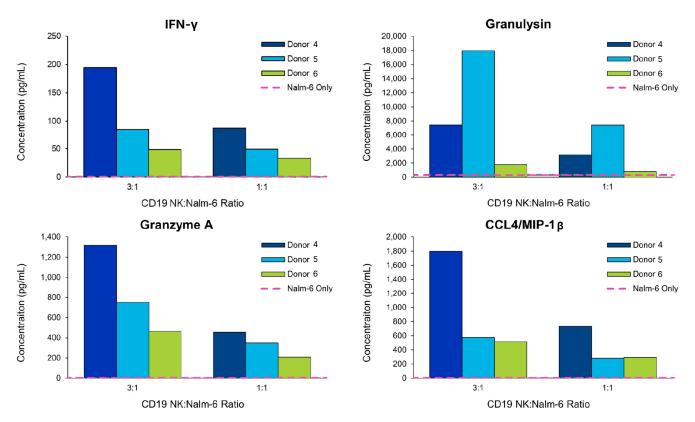
FIGURE 5.

IFN-γ, Granzyme B, GM-CSF, and Granulysin were the highest abundant proteins secreted from CD19-CAR-T cells when co-cultured with Nalm-6 cells



Concentrations presented here were determined using the R&D Systems Luminex Human Discovery Assay. These analytes are representative of trends observed for the remaining analytes of the observed CD19-CAR-T secretome. The different levels of analyte concentration highlight the variability of CAR-T cell potency from donor to donor.

FIGURE 6. Granulysin, Granzyme A, and CCL4/MIP-1  $\beta$  were the most abundant proteins secreted from CD19-CAR-NK cells when co-cultured with Nalm-6 cells.



Concentrations presented here were determined using the R&D Systems Luminex Human Discovery Assay. While IFN- $\gamma$  was secreted from activated CD19-CAR-NK cells in the presence of Nalm-6 cells, six additional analytes from the secretion profile were detected at higher concentrations. These results demonstrate the potential significance of multi-analyte cell potency measurements to achieve a more holistic representation of immune cell therapy mechanism of action in a patient.

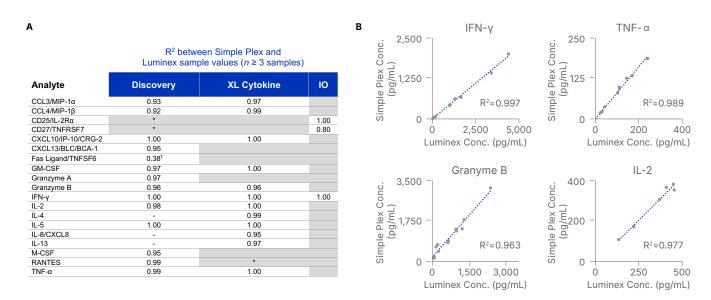
## Simple Plex Validates and Extends Secretome Findings

While the Luminex Platform excels at multiplexing capabilities and establishing analyte profiles, Simple Plex Assays stand out in speed, sensitivity, and reproducibility via its automated ELISA workflow on the Ella instrument. To explore the transition between a Luminex Discovery approach to more tailored, automated Simple Plex Assays, we analyzed the same supernatant samples collected from the CAR-T and CAR-NK killing assays on the Simple Plex Platform and compared analyte concentrations across the two immunoassay types. We investigated 25 analytes with retail Simple Plex Assays (e.g., Cell Activation Panel 1 and 2) or custom multianalyte assay cartridges that included proteins from the

CAR-T and CAR-NK secretomes in addition to multiple negative control proteins (Table 5). Luminex results were substantiated by Simple Plex with 17 analytes showing strong cross-platform correlation ( $R^2 \ge 0.92$ ). Simple Plex Assays further enabled sensitive quantification of low-abundance proteins, such as Perforin, selectively secreted by both CD19-CAR-T and CD19-CAR-NK cells compared to controls. Simple Plex Cell Activation Panel 1, which includes IFN- $\gamma$ , TNF- $\alpha$ , Granzyme B, and IL-2, all had  $R^2 \ge 0.96$  when comparing concentrations to either the Human Luminex Discovery Panel (Figure 7B) or the Human XL Cytokine Luminex Performance Assay.

FIGURE 7.

Luminex Assay screening correlated with automated Simple Plex Assays



(A) Sample values from CD19-CAR-T and -NK supernatants measured using Simple Plex Assays revealed an excellent degree of correlation with results from the Human Discovery, XL Cytokine, and Immuno-Oncology (IO) Assays. Six analytes (CCL17, IL-6, IL-10, IL-15, IL-18/IL-1F4, and Perforin; not shown) were also confirmed to be low abundance, with sample concentrations falling near or below the limits of quantitation in both immunoassay platforms. Gray: Analytes were not tested in the designated Luminex Assay; \*: Analytes were available in the designated Luminex assay, but not tested due to already being included in another panel; -: Analytes were not detectable in a sufficient quantity of samples for the designated Luminex Assay to reliably calculate R<sup>2</sup>; †: Fas Ligand was a low abundance analyte with narrow distribution in sample concentrations. (B) This subset of correlation data showcases the exceptional translatability between the four analytes in the Simple Plex Activation Panel 1 and the R&D Systems Human Discovery Assay.

## **Conclusions**

This study establishes an effective immunoassay workflow for characterizing the complex secretomes of activated CAR-T and CAR-NK cells that were engineered using the TcBuster transposon system. TcBuster editing yielded high percentages of CAR-expressing cells that are viable and underwent robust expansion after electroporation. These CAR-T and -NK cells are highly functional, as demonstrated by cell killing assays and effector protein secretion profile. Through Luminex multiplex screening, we defined distinct cytokine and effector protein profiles for CD19-CAR-T versus CAR-NK cells and revealed donor-specific heterogeneity in cell potency across an initial set of 123 analytes.

Key findings were validated on the Simple Plex Platform, demonstrating strong cross-platform correlation and enabling rapid, highly sensitive quantitation of select proteins. Together, these complementary technologies provide a streamlined approach to move from broad discovery to focused, multi-analyte potency assays.

Beyond B cell malignancies, this workflow is readily adaptable for investigating CAR-T and CAR-NK mechanisms of action, assessing patient-to-patient variability, or expanding to other target antigens for immune cell therapies.

## **Materials**

### TABLE 1. T CELL MANUFACTURING

Supplier	Catalog Number
R&D Systems	CCM038-GMP-1L
R&D Systems	BT-007-GMP
R&D Systems	BT-015-GMP
R&D Systems	HABS001-GMP-100ML
Scale Ready	80660M
Miltenyi	130-111-160
	R&D Systems R&D Systems R&D Systems R&D Systems Scale Ready

### TABLE 2. NK CELL MANUFACTURING

Material	Supplier	Catalog Number
ExCellerate Human NK Cell Expansion Media, Animal Component-Free	R&D Systems	CCM037
Recombinant Human IL-2 GMP Protein, CF	R&D Systems	BT-002-GMP
Recombinant Human IL-15 GMP Protein, CF	R&D Systems	BT-015-GMP
G-Rex 6-Well Plate	Scale Ready	80240M
Feeder cell line	In House	-

### TABLE 3. NON-VIRAL GENOME ENGINEERING AND DETECTION OF CD19-CAR AND LUCIFERASE-BASED KILL ASSAY

Material	Supplier	<b>Catalog Number</b>
TcBuster-M Transposase mRNA	R&D Systems	TCB001.1
TcBuster Transposon CD19CAR-DHFR-eGFP	R&D Systems	TCBP001
Hyclone Electroporation Buffer	MaxCyte	EPB-1
OC-100×2™ Processing Assembly	MaxCyte	SOC-1×2
MaxCyte® Electroporation System	MaxCyte	GTx
DNase I	Various	Various
Recombinant Human CD19 Protein, Atto 647N Conjugate	R&D Systems	ATM9269
Luciferase Assay System	Promega	E1501
5x Lysis Buffer	Promega	E153A

### TABLE 4. LUMINEX DETECTION OF SECRETED PROTEINS

Material	Supplier	Catalog Number	Analyte Plex Size
Human Luminex Discovery Assay	R&D Systems	LXSAHM	Four plexes, 104 analytes total: 44-plex, 26-plex, 17-plex, 17-plex*
Human XL Cytokine Luminex Performance Assay 46-plex Fixed Panel	R&D Systems	LKTM014B	44-plex (RANTES and IL-17E included in Discovery Assay testing)
Human Immuno-Oncology Panel 1 Luminex Performance Premix Kit	R&D Systems	FCSTM24	14-plex (Entire analyte panel)
Luminex 200 RUO System w/xPONENT 4.3	R&D Systems	LX200-XPON-RUO	-
xMAP Sheath Fluid PLUS	R&D Systems	40-50021	-

<sup>\*</sup>This plex was comprised of 17 analytes new to the Human Discovery Assay, including IL-22, Granulysin, and Perforin, that will be available in 2026.

TABLE 5. SIMPLE PLEX DETECTION OF SECRETED PROTEINS

Material	Supplier	Catalog Number	Analyte(s)
Simple Plex Cell Activation Panel 1	R&D Systems	ST01C-CS-003222	Granzyme B, IFN-γ , IL-2 , TNF-α
Simple Plex Cell Activation Panel 2	R&D Systems	ST01C-CS-007366	Granzyme B, IFN- $\gamma$ , Perforin, TNF- $\alpha$
Simple Plex Human CD27/TNFRSF7 Cartridge	R&D Systems	SPCKB-PS-000639	CD27/TNFRSF7
Simple Plex Custom Multiplex Cartridge	R&D Systems	SPCK-PANEL (Custom-Select)	GM-CSF, IL-6, IL-8/CXCL8, M-CSF
Simple Plex Custom Multiplex Cartridge	R&D Systems	SPCK-PANEL (Custom-Select)	CCL17, IL-4, IL-5, IL-13
Simple Plex Custom Multiplex Cartridge	R&D Systems	SPCK-PANEL (Custom-Select)	CCL3/MIP-1α, CD25/IL-2R alpha, Granzyme A, IL-18/IL-1F4
Simple Plex Custom Multiplex Cartridge	R&D Systems	SPCK-PANEL (Custom-Select)	CD25/IL-2R alpha, Fas Ligand/TNFSF6, IL-10, IL-18/IL-1F4
Simple Plex Custom Multiplex Cartridge	R&D Systems	SPCK-PANEL (Custom-Select)	CCL4/MIP-1β, CXCL10/IP-10/CRG-2, IL-15
Simple Plex Custom Multiplex Cartridge	R&D Systems	SPCK-PANEL (Custom-Select)	CCL5/RANTES
Simple Plex Custom Multiplex Cartridge	R&D Systems	SPCK-PANEL (Custom-Select)	CXCL13/BLC/BCA-1
Ella Automated Immunoassay System	R&D Systems	600-100	-

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