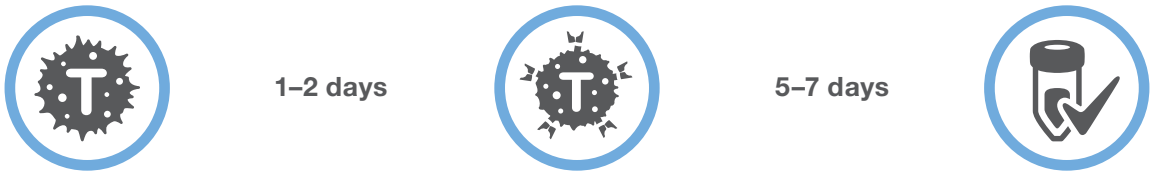
Integrated generation and characterization of CAR T cells

Introduction

Chimeric antigen receptor (CAR) T cell therapies have been approved by the FDA and other global public health agencies to treat B cell leukemias and have seen great clinical success. Autologous CAR T cell manufacturing involves isolating T cells from a patient, activating these cells, introducing an engineered CAR construct, and expanding the cells to a scale appropriate for therapeutic dosing. Patient samples from multiple sources result in inconsistent clinical outcomes and overall product quality. To ensure patient safety and product quality, a number of quality control (QC) tests must be performed throughout the manufacturing process and for product release. These include confirming the identity, purity, potency, and safety of the final CAR T cell product.

Therefore, it is critical to develop robust processes that can reduce variability through the seamless integration of systems and improve the repeatability of testing with high-quality reagents.

Here, we present workflow details and optimized methods for the generation and characterization of CAR T cell products (Figure 1). T cells were isolated from healthy donors, expanded *ex vivo*, and transduced with a second-generation (4-1BB and CD3ζ domains) anti-CD19 CAR lentivirus. Robust expression of the CAR construct was obtained consistently using a clinically relevant workflow. These CAR T cells were then extensively characterized using pertinent analytical assays.



Isolate, activate, and expand	Engineer and expand	Analyze
• CTS (Cell Therapy Systems) Dynabeads CD3/CD28	• CTS LV-MAX Lentiviral Production System	• eBioscience Essential Human T Cell Phenotyping Kit
• CTS OpTmizer T Cell Expansion SFM	– CTS Viral Production Cells	• PureQuant identity and purity assays
• CTS GlutaMAX-I Supplement	– CTS LV-MAX Production Medium	• Cytokine & Chemokine 34-Plex Human ProcartaPlex Panel 1A
• L-Glutamine	– LV-MAX Lentiviral Packaging Mix	• CyQUANT LDH Cytotoxicity Assay
• IL-2 Recombinant Human Protein	– CTS LV-MAX Transfection Kit	• MycoSEQ Mycoplasma Detection Kit
• IL-15 Recombinant Human Protein	– CTS Opti-MEM I Medium	• AmpFLSTR Identifiler Direct PCR Amplification Kit
• CTS Immune Cell Serum Replacement (SR)		

Figure 1. Integrated and optimized workflow for consistent CAR T cell generation.

Materials and methods

Donor cells

Peripheral blood mononuclear cells (PBMCs) from three donors (labeled A, B, and C) were isolated from fresh Leukopak™ apheresis product (AllCells, Alameda, CA) from healthy donors using Ficoll™ gradient centrifugation. Donors used in this study were: donor A, 39-year-old male (Cat. No. LP101-1/8, Lot No. A5708, ID No. 9745); donor B, 39-year-old male (Cat. No. LP101-1/4, Lot No. 3018208, ID No. 12990); donor C, 43-year-old female (Cat. No. LP101-1/4, Lot No. 3018209, ID No. 12117).

T cell isolation, activation, and expansion

T cells were isolated from fresh PBMCs and activated using Gibco™ CTS™ (Cell Therapy Systems) Dynabeads™ CD3/CD28 (**Cat. No. 40203D**). PBMCs were analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer using the Invitrogen™ eBioscience™ Essential Human T Cell Phenotyping Kit (**Cat. No. A42923**) to obtain the percentage of $CD3^+$ cells. This value was used to determine the total number of T cells required for addition of Dynabeads CD3/CD28 at a 3:1 ratio of beads to T cells. T and CAR T cells were expanded in T cell expansion medium made with Gibco™ CTS™ OpTmizer™ T Cell Expansion SFM (**Cat. No. A1048501**), 2 mM Gibco™ CTS™ GlutaMAX™-I Supplement (**Cat. No. A1286001**), 2 mM Gibco™ L-Glutamine (**Cat. No. 25030081**), 100 U/mL Gibco™ IL-2 Recombinant Human Protein (**Cat. No. CTP0023**), 0.5 ng/mL Gibco™ IL-15 Recombinant Human Protein (**Cat. No. PHC9151**), and 2% Gibco™ CTS™ Immune Cell SR (**Cat. No. A2596101**).

Lentivirus generation and T cell transduction

Lentivirus was generated from an anti-CD19 (clone FMC63), second-generation (CD3ζ and 4-1BB domains), truncated EGFR (EGFRt) CAR vector from Creative Biolabs using the Gibco™ CTS™ LV-MAX™ Lentiviral Production System. The CTS LV-MAX system is composed of Gibco™ CTS™ Viral Production Cells (**Cat. No. A3152801**), Gibco™ CTS™ LV-MAX™ Production Medium (**Cat. No. A4124001**), Gibco™ LV-MAX™ Lentiviral Packaging Mix (**Cat. No. A43237**), and the Gibco™ CTS™ LV-MAX™ Transfection Kit (**Cat. No. A4132601**). T cells activated for 24 hours were transduced with CAR lentivirus at a multiplicity of infection (MOI) of 10 at 10^6 cells/mL with 8 µg/mL Polybrene™ reagent. After 3 days posttransduction, cells were analyzed for CAR expression. Detailed functional characterization was performed on day 5 posttransduction.

Characterization of CAR T cells

Beads were removed from cells prior to further analysis. CAR expression was measured using Invitrogen™ Goat Anti–Mouse IgG (H+L) Secondary Antibody (**Cat. No. 31800**) and Invitrogen™ Streptavidin APC (**Cat. No. SA1005**) on the Attune NxT Flow Cytometer. T cell and CAR T cell phenotyping was carried out using the Invitrogen™ eBioscience™ Essential Human T Cell Phenotyping Kit (**Cat. No. A42923**). Determination of various T cell subtypes, contaminating B cells, and monocytes was carried out using Applied Biosystems™ PureQuant™ assays for CD8 (**Cat. No. A43674**), Treg (**Cat. No. A43675**), Th17 (**Cat. No. A43676**), CD3 (**Cat. No. A47197**), CD4 (**Cat. No. A47194**), B cells (**Cat. No. A47196**), and monocytes (**Cat. No. A47195**) using the Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System (**Cat. No. 4471134**). Potency of CAR T cells was measured via direct killing of target cells using the Invitrogen™ CyQUANT™ LDH Cytotoxicity Assay (**Cat. No. C20300**) and indirect cytokine measurement using the Invitrogen™ Cytokine & Chemokine 34-Plex Human ProcartaPlex™ Panel (**Cat. No. EPX340-12167-901**) and the Luminex® 200™ Instrument System (**Cat. No. APX10031**). The absence of mycoplasmas was determined using the Applied Biosystems™ MycoSEQ™ Mycoplasma Detection Kit (**Cat. No. 4460626**), and cell identity was authenticated using the Applied Biosystems™ AmpFLSTR™ Identifiler™ Direct PCR Amplification Kit (**Cat. No. 4467831**).

Results

CAR lentivirus production

The second-generation CAR used in this study contains an anti-CD19 (clone FMC63) single-chain variable fragment (scFv), 4-1BB costimulatory domain, CD3ζ signaling domain, and truncated EGFRt domain (Figure 2A). Viruses were generated under serum-free conditions, and the infectious titer was determined with Jurkat cells (ATCC TIB-152), pooled primary T cells, and the HT-1080 fibrosarcoma cell line (ATCC CCL-121). HT-1080 cells were chosen for further titer determination since the transduction efficiency and infectious titer were comparable to those of primary T cells (Figure 2B). To compare production efficiencies at different scales, lentivirus was produced at less than 100 mL or more than 1 L, and high titers were achieved in both cases (Figure 2C). Transduction conditions were optimized using T cells from donor C that were activated for 24 hours. Cells were transduced at various MOIs in T cell medium with or without Polybrene reagent. An MOI of 10 with Polybrene reagent was identified as the optimal condition and used for further studies (Figure 2D).

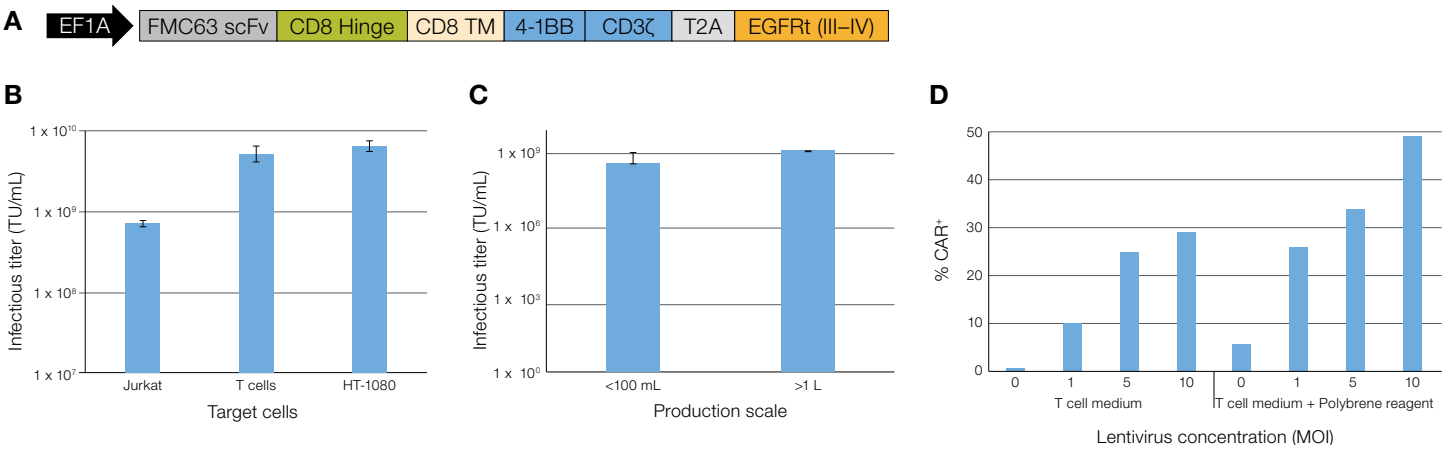


Figure 2. CAR lentivirus generation and transduction optimization. (A) Composition of CAR encoded by the vector. **(B)** Evaluation of infectious titer using different cell types. **(C)** Lentiviral production efficiencies at two different scales. **(D)** Optimization of transduction conditions using Polybrene reagent and various MOIs.

CAR T cell generation

T cells isolated from three independent donors were transduced with lentivirus at an MOI of 10. After 5 days in culture, CAR expression was detected on T cells immunostained with goat anti–mouse IgG antibody and analyzed by flow cytometry. All donors showed >40% CAR expression, with 45.4%, 44.3%, and 47.5% CAR+ cells from donors A, B, and C, respectively (Figure 3A). Expression of the CAR construct over one week was stable, as indicated by the average CAR expression of the three donors' CAR T cells at 3, 5, and 7 days posttransduction (Figure 3B).

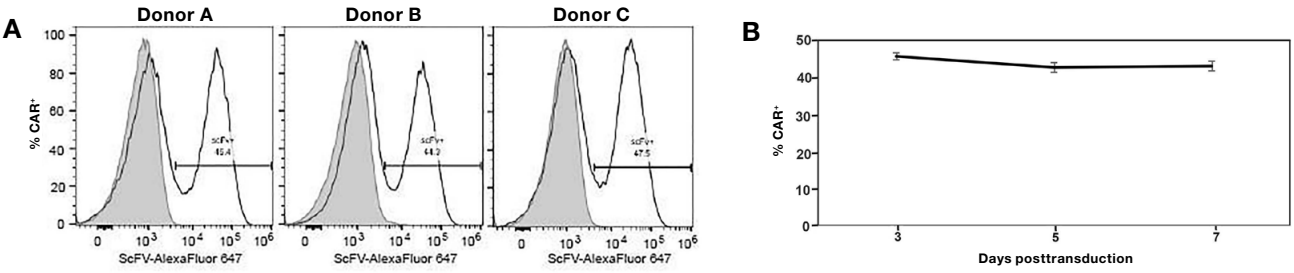


Figure 3. Consistent CAR T cell generation from three different donors' T cells. (A) CAR expression on T cells from three donors, as determined by flow cytometry. **(B)** Average CAR expression on T cells from the three donors over one week.

Cell identity and contamination testing

To determine cell identity and to ensure that cell-line misidentification or cross-contamination did not occur, the AmpFLSTR Identifiler Direct PCR Amplification Kit was used. This kit uses a short tandem repeat (STR) multiplex PCR assay to amplify unique STR loci and the gender-determining marker amelogenin. The combination of genotypes creates a unique and highly discriminatory pattern that can be used for identification and identity confirmation. STR genotyping was carried out, and CAR T cells were confirmed to match with the corresponding parental donor PBMCs (Table 1).

The absence of mycoplasmas was confirmed by real-time PCR using the MycoSEQ Mycoplasma Detection Kit. The acceptance criteria for a negative sample were a threshold cycle (C_t) of >36 and a melting temperature (T_m) of <75°C (Table 2).

Table 1. Confirmation of the identity of CAR T cells.

	Donor A		Donor B		Donor C	
	PBMCs	CAR T cells	PBMCs	CAR T cells	PBMCs	CAR T cells
AMEL	X, Y	X, Y	X, Y	X, Y	X, X	X, X
CSF1PO	10, 11	10, 11	11, 12	11, 12	10, 12	10, 12
D13S317	8, 14	8, 14	10, 12	10, 12	12, 13	12, 13
D16S539	11, 12	11, 12	10, 12	10, 12	13, 13	13, 13
D18S51	14, 17	14, 17	12, 14	12, 14	12, 16	12, 16
D19S433	13, 16.2	13, 16.2	13, 13	13, 13	13, 13	13, 13
D21S11	26, 30	26, 30	29, 32.2	29, 32.2	29, 31	29, 31
D2S1338	18, 20	18, 20	17, 19	17, 19	20, 24	20, 24
D3S1358	15, 16	15, 16	15, 17	15, 17	16, 19	16, 19
D5S818	11, 11	11, 11	12, 12	12, 12	11, 14	11, 14
D7S820	10, 11	10, 11	10, 11	10, 11	10, 10	10, 10
D8S1179	10, 11	10, 11	13, 15	13, 15	12, 13	12, 13
FGA	21, 22	21, 22	25, 25	25, 25	20, 22	20, 22
TH01	8, 9.3	8, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3
TPOX	8, 11	8, 11	9, 9	9, 9	9, 11	9, 11
vWA	15, 17	15, 17	18, 18	18, 18	17, 18	17, 18

Table 2. Verification of the absence of mycoplasmas.

CAR T cells derived from:	C_t	T_m	Result
Donor A	36.26	66	Negative
Donor B	36.35	66	Negative
Donor C	35.85	65	Negative

Purity assessment

The purity of the CAR T cells was assessed using the eBioscience Essential Human T Cell Phenotyping Kit. The CAR T cells derived from each donor's T cells had CD4:CD8 ratios comparable to those of their respective untransduced controls (Figure 4).

CAR T cell purity was confirmed with Applied Biosystems™ PureQuant™ assays that offer more standardized and reproducible data than flow cytometry, to determine immune cell types in a heterogeneous sample. This assay relies on methylation patterns, specific to target cell types, that are detected through bisulfite conversion, a process that converts unmethylated cytosine residues to uracil. Primers for qPCR are then designed to amplify the converted targets. Using a standardized analysis template, this amplification can be used to determine the starting population of cells with unmethylated target loci. Results indicate that the CAR T cell population is composed of the expected T cell subsets with minimal contaminating cells (Table 3).

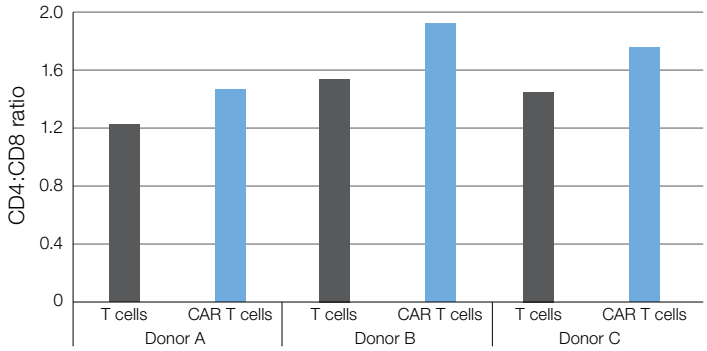


Figure 4. Determination of CD4:CD8 ratio of CAR T cells by phenotyping using flow cytometry.

Table 3. Purity assessment of CAR T cells with PureQuant methylation assays.

Category	Cell type	Positive cells (%)		
		Donor A CAR T cells	Donor B CAR T cells	Donor C CAR T cells
T cell subsets	CD3	90.6	82.7	88.1
	CD4	54.6	51.9	49.9
	CD8	37.3	32.9	41.3
	Treg	0.1	0.4	0.3
	Th17	0.6	0.8	1.3
Contaminating cells	B cell	0	0.1	0.1
	Monocyte	0.6	0.3	0.4

Potency assessment

The potency of the three donors' CAR T cells was determined by measuring their *in vitro* cytotoxicity. Lactate dehydrogenase (LDH) release from CD19-expressing K562 cells was measured after a 16 hr coculture with CAR T cells. Untransduced T cells were used as controls. The cocultures with CAR T cells exhibited higher LDH signals, indicating more lysis than the untransduced control (Figure 5A).

Cytokines produced after overnight stimulation with CD19-expressing K562 cells were detected using the Cytokine & Chemokine 34-Plex Human ProcartaPlex Panel. CAR T cells produced higher cytokine signals than untransduced controls, albeit with differences among the three donors' CAR T cells in expression levels of different markers (Figure 5B).

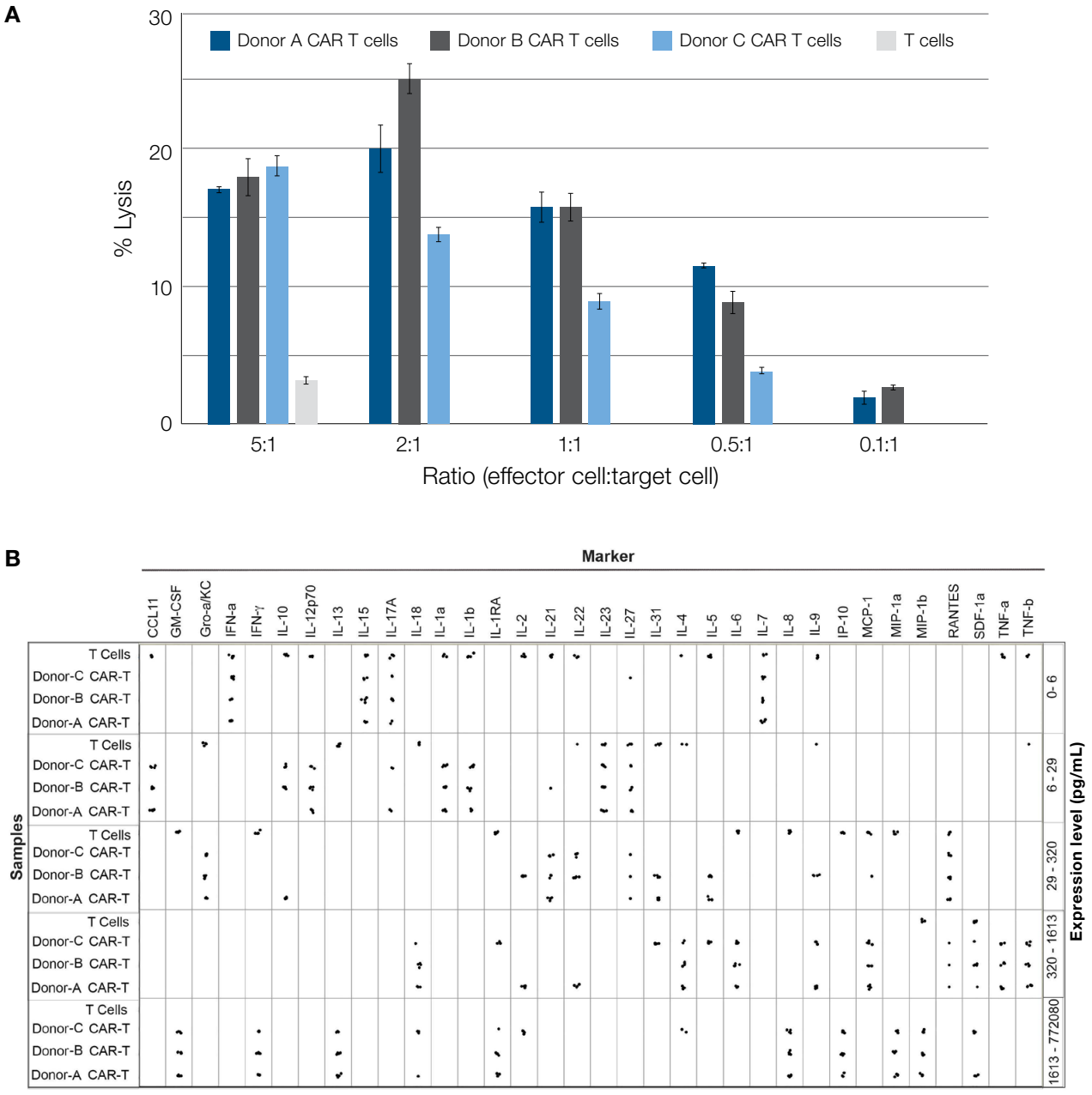


Figure 5. Potency assessment of CAR T cells after overnight coculture with CD19-expressing K562 cells. (A) Direct cytotoxicity measurement using the CyQUANT LDH Cytotoxicity Assay. **(B)** Indirect cytotoxicity measurement using the Cytokine & Chemokine 34-Plex Human ProcartaPlex Panel.

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Conclusions

- CAR T cells were generated and expanded using chemically defined, serum-free reagents designed for adoptive cell therapy and GMP applications.
- High-titer lentivirus was generated at <100 mL and >1 L scales.
- Lentiviral transduction at an MOI of 10 in the presence of Polybrene reagent resulted in >40% CAR+ T cells from three different donor cells.
- Identity and purity of the CAR T cells were confirmed through flow cytometry and an orthogonal PCR-based methylation assay, which demonstrated that minimal amounts of contaminating cell types were present.
- STR typing confirmed that the donor identity of the CAR T cells matched that of each starting T cell population, ensuring the identity of the final CAR T cell product.
- CAR T cells were confirmed to be mycoplasma-negative.
- CAR T cells, but not untransduced controls, displayed cytotoxicity against a CD19-expressing target cell line and secreted high levels of cytokines associated with CAR T cell potency.

Ordering information

Product	Quantity	Cat. No.
Isolate, activate, and expand		
CTS (Cell Therapy Systems) Dynabeads CD3/CD28	10 mL	**40203D**
CTS OpTmizer T Cell Expansion SFM, bottle format	1,000 mL	**A1048501**
CTS OpTmizer T Cell Expansion SFM, no phenol red, bottle format	1,000 mL	**A3705001**
CTS GlutaMAX-I Supplement	100 mL	**A1286001**
L-Glutamine	100 mL	**25030081**
IL-2 Recombinant Human Protein	1 mg	**CTP0023**
IL-15 Recombinant Human Protein	1 mg	**PHC9153**
CTS Immune Cell SR	50 mL	**A2596101**
Engineer		
CTS Viral Production Cells	1 mL	**A3152801**
CTS LV-MAX Production Medium	1 L	**A4124001**
LV-MAX Lentiviral Packaging Mix	1.5 mL	**A43237**
CTS LV-MAX Transfection Kit	1 L	**A4132601**
CTS Opti-MEM I Medium	100 mL	**A4124801**

Product	Quantity	Cat. No.
Analyze		
eBioscience Essential Human T Cell Phenotyping Kit	100 reactions	**A42923**
PureQuant CD8+ T Cell Assay	28 reactions	**A43674**
PureQuant Treg Assay	28 reactions	**A43675**
PureQuant Th17 Assay	28 reactions	**A43676**
PureQuant CD4+ T Cell Assay	28 reactions	**A47194**
PureQuant Monocyte Assay	28 reactions	**A47195**
PureQuant B Cell Assay	28 reactions	**A47196**
PureQuant CD3+ T Cell Assay	28 reactions	**A47197**
CyQUANT LDH Cytotoxicity Assay	200 assays	**C20300**
Cytokine & Chemokine 34-Plex Human ProcartaPlex Panel	96 tests	**EPX340-12167-901**
MycoSEQ Mycoplasma Detection Kit	100 reactions	**4460626**
AmpFLSTR Identifiler Direct PCR Amplification Kit	200 reactions	**4467831**

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