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CAR-T Generation for Identity, Purity, Potency and Sterility Assay Testing

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ABSTRACT

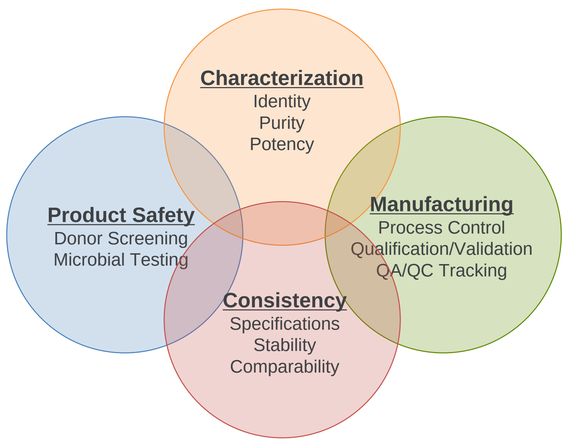
Chimeric antigen receptor T-Cell (CAR-T) has been approved by FDA to treat B-cell leukemias and has seen great clinical success. CAR-T cell manufacturing involves isolating T-cells from a patient, cell activation, engineering in the CAR construct, and expanding the cells to a therapeutic dose. To ensure 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 product. Often times, these assay are procured by multiple sources and may vary in performance resulting in inconsistent outcomes and overall product quality.

To address these issues, CAR-T cells were generated in house and used for the development and optimization of CAR-T specific characterization tools. T-cells were isolated from healthy donors, expanded in vitro, and transduced with a second generation (CD3ζ and 4-1BB) anti-CD19 CAR lentivirus. Using a clinically relevant workflow, expression of the CAR construct was obtained in a high, consistent manner. These CAR-T cells were then extensively characterized for pertinent analytical assays. Our presented workflow details optimized methods of CAR-T Cell characterization that may be used in pre-clinical and clinical CAR-T manufacturing, following proper validation and regulatory approval.

INTRODUCTION

Traditional cancer treatments include surgical resection, chemotherapy, and/or radiotherapy but are non-specific and inadequate to treat relapse/refractory tumors. Immunotherapy is a promising category of cancer therapies and that enhances the immune system ability to mount an anti-tumor response. CAR-T cell therapy is a potent cellular immunotherapy that has seen great success in the treatment of hematological malignancies. CAR-T cell manufacturing involves the isolation, activation, engineering, and expansion of patient derived T-cells. Every step of the manufacturing process can affect the efficacy and consistency of a CAR-T cell product and requires thorough characterization to ensure that the product meets pre-designed specifications.

Characterization of a biological product (includes determination of physiochemical properties, biological activity, immunochemical properties, purity and impurities) is necessary to allow relevant specifications to be established.



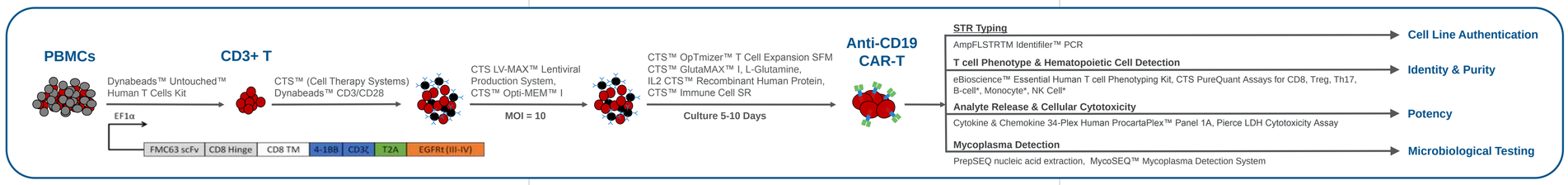
Cell Therapy testing commonly comprises of

- **Microbial Testing**
  - Sterility (Bacterial & Fungal)
  - Mycoplasma
  - Adventitious Agent Testing
- **Identity**
- **Purity**
  - Residual contaminants
  - Pyrogenicity/Endotoxin
- **Potency**
- **Other**
  - General Safety
  - Viability
  - Cell Number/Dose

Validation of analytical procedures are performed as per **ICH Q2 R1** guidelines with the sole objective of demonstrating that the assay is fit for its intended purpose. **Assay qualification** establishes that an assay will provide meaningful data under the specific conditions used. **Assay Validation** further establishes the condition to assure that the assay is working appropriately every time it is run.

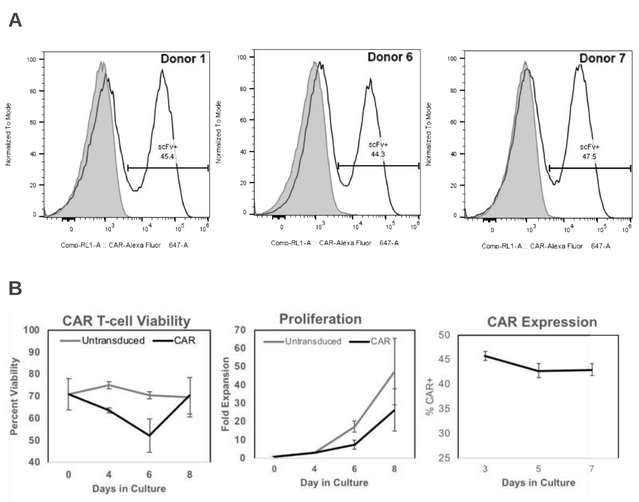
Precision	**8 Steps of Assay / Method Validation**	Specificity
Accuracy		Linear and Range
Limit of Detection		Ruggedness
Limit of Quantitation		Robustness

MATERIALS AND METHODS



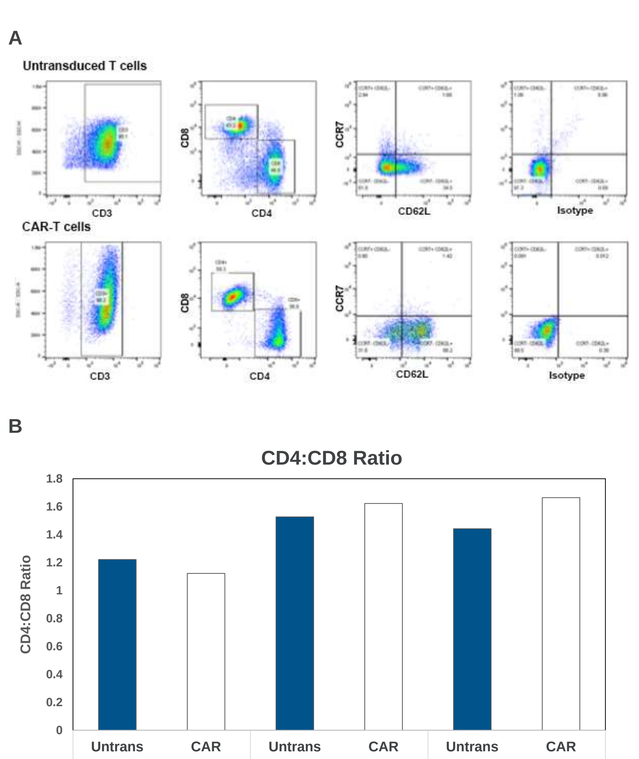
RESULTS

Figure 1: Consistent CAR-T Generation with three different donor T cells.



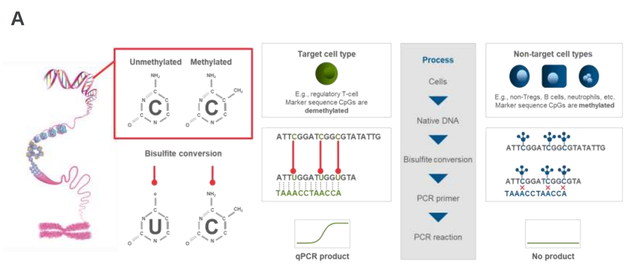
A. Expression of CAR construct detected via goat anti-mouse IgG antibody. Expression is non-variable between donors at the time of co-culture.
B. CAR-T cells expanded similarly to untransduced controls in prolonged culture. Expression of the CAR construct was stable and remained non-variable throughout culture.

Figure 2: Phenotype Assessment with Flow Cytometry



A. The eBioscience Essential Human Phenotyping kit provides ready-to-use, high-quality, and reliable phenotyping panels containing antibodies commonly used to phenotype T cells including CD3, CD4, CD8, CCR7, and CD62L. Show are representative Flow cytometry plots of Donor 1 untransduced and corresponding CAR-T cells
B. CAR-T cells have similar CD4:CD8 ratios compared to untransduced controls for three independent donor T cells.
C. CAR-T cells displayed an expansion of the naïve CD62L+ cells relative to their parental T cells. Shown here is representative data from Donor 1 T cells. The ratio of naïve cells before and after CAR-T generation can be highly variable between different donor T cells.

Figure 3: Identity & purity determination using Methylation PCR Assay



B

	Sample	Reference	Donor 1 CAR-T	Donor 6 CAR-T	Donor 7 CAR-T
T-cell Subsets	CD3	23.81	90.6	82.7	88.1
	CD4	16.16	54.6	51.9	49.9
	CD8	7.08	37.3	32.9	41.3
	Treg	0.97	0.1	0.4	0.3
	Th17	2.15	0.6	0.8	1.3
Contaminating Cells	B cell	4.73	0	0.1	0.1
	Monocyte	8.15	0.6	0.3	0.4
	NK	3.93	0.5	0.6	0.7

A. Methylation patterns specific to the target cell type are detected through bisulfite conversion, a process that converts unmethylated cytosine into uracil. Primers are then designed against the converted targets to yield amplification during qPCR. Using a standardized analysis template, this amplification can be used to determine the starting population of cells with unmethylated target loci.
B. CAR T purity was confirmed with CTS™ PureQuant™ Methylation Assays, which offer a more standardized way to measure immune cell types in a heterogeneous sample. The CAR T product is comprised of T-cells with minimal contaminants.

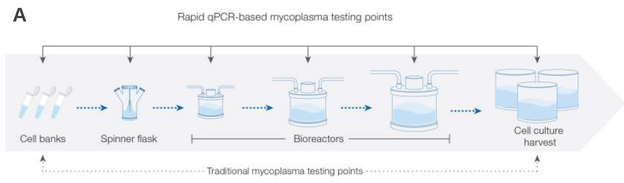
Figure 4: Authentication of CAR-T cells

B

	Donor 1		Donor 6		Donor 7	
	T cells	CAR-T	T cells	CAR-T	T cells	CAR-T
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

A. It is critical to determine cell identity & distinguish it from other products being manufactured in the same facility, thus ensuring no cell-line mix ups & contamination.
B. The AmpFLSTRTM Identifiler™ PCR Amplification Kit matches CAR-T cells to their corresponding parental donors T cells through STR typing for all three donors

Figure 5: Mycoplasma testing

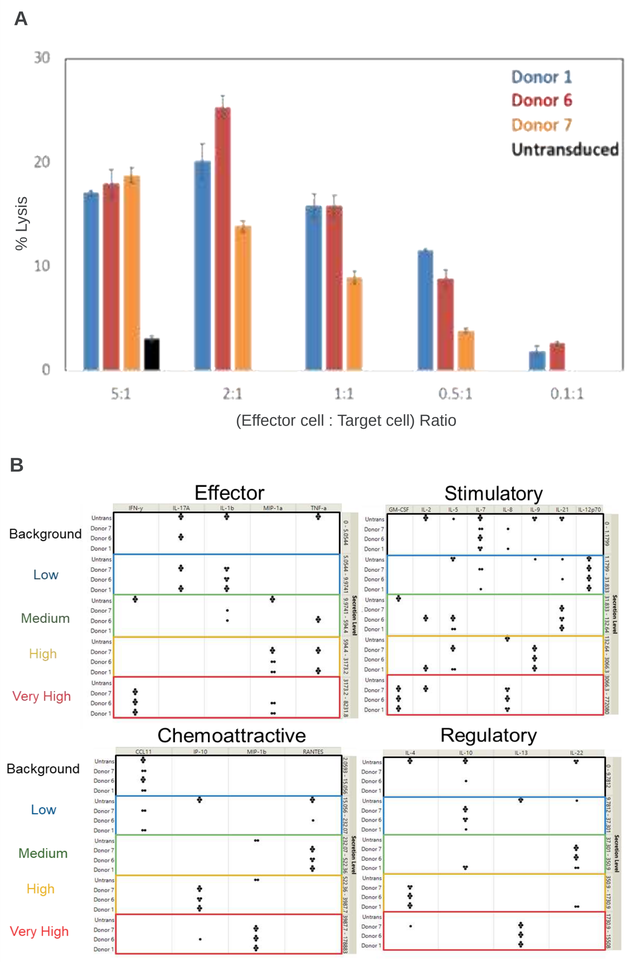


B

Donor	Ct	Tm	Result
1	36.26461	65.55288	Negative
6	36.34592	65.61047	Negative
7	35.84776	64.91937	Negative

A. Rapid PCR-based testing for Mycoplasma infection can be conducted throughout the cell culture manufacturing process, from inoculation through harvest, enabling immediate recognition and response to a mycoplasma contamination.
B. Absence of mycoplasma was confirmed by real-time PCR using the MycoSEQ Mycoplasma detection system, which includes sample prep and proprietary real-time PCR reagents. Samples were analyzed using the 7500 Fast Real-Time PCR instrument and AccuSEQ data analysis software. Acceptance criteria for a negative sample are Cycle Threshold (CT) > 36 and Melt Temperature (Tm) <75.

Figure 6: Potency assessment with direct cytotoxicity measurement and indirect Cytokine quantification



A. In vitro cytotoxicity was detected via lactate dehydrogenase (LDH) release CAR T cells exhibited significantly higher lysis than transduced controls. Percent lysis for all donors after a 16hr co-culture with CD19 K562 Cells.
B. Cytokines produced after overnight stimulation with CD19 expressing K562s detected with 34-Plex Human ProcartaPlex™. CAR T cells produced significantly more cytokines than untransduced controls. Interestingly, IFN-γ production was not different between donors despite differences seen in other characterization assays.

CONCLUSIONS

- CAR T cells were generated and expanded using chemically defined, serum-free reagents and growth kinetics did not differ significantly from un-transduced controls
- Identity and purity of the CAR T cells was confirmed through flow cytometry and epigenetic assays demonstrating minimal contaminating cell types were present
- STR typing confirmed that the of the CAR T cells matched the starting T cell population, ensuring the identity of the final CAR T product.
- The absence of mycoplasma supports the sterility of the CAR T product and manufacturing workspace.
- CAR T cells, but not untransduced controls, displayed cytotoxicity against CD19 expressing target cell line and secreted many effector cytokines correlated with CAR T cell potency

TRADEMARKS/LICENSING

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* Products used are currently in development

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