TECHNICAL NOTE

Getting the Most Out of Your CAR-T Bioprocessing with the CliniMACS Prodigy® & IsoCode Single-Cell Chip

Automated end-to-end CAR-T cell production and single-cell analysis with the Miltenyi Biotec CliniMACS Prodigy® and Bruker's IsoLight system.

In this Technical Note we outline:

- The use of the CliniMACS Prodigy® (Miltenyi Biotec) to produce CAR-T cell products after T-cell enrichment, activation, viral transduction, expansion, and harvest for downstream use
- Bruker's single-cell adaptive immune application and CAR-T single-cell quality check on the Bruker system
- Protocols that minimize impact on live cells when analyzed on Bruker's IsoLight platform
- Guidelines for most commonly used cell types and applications

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Prep, Run, Analyze

Automated end-to-end CAR-T cell processing and functional analysis

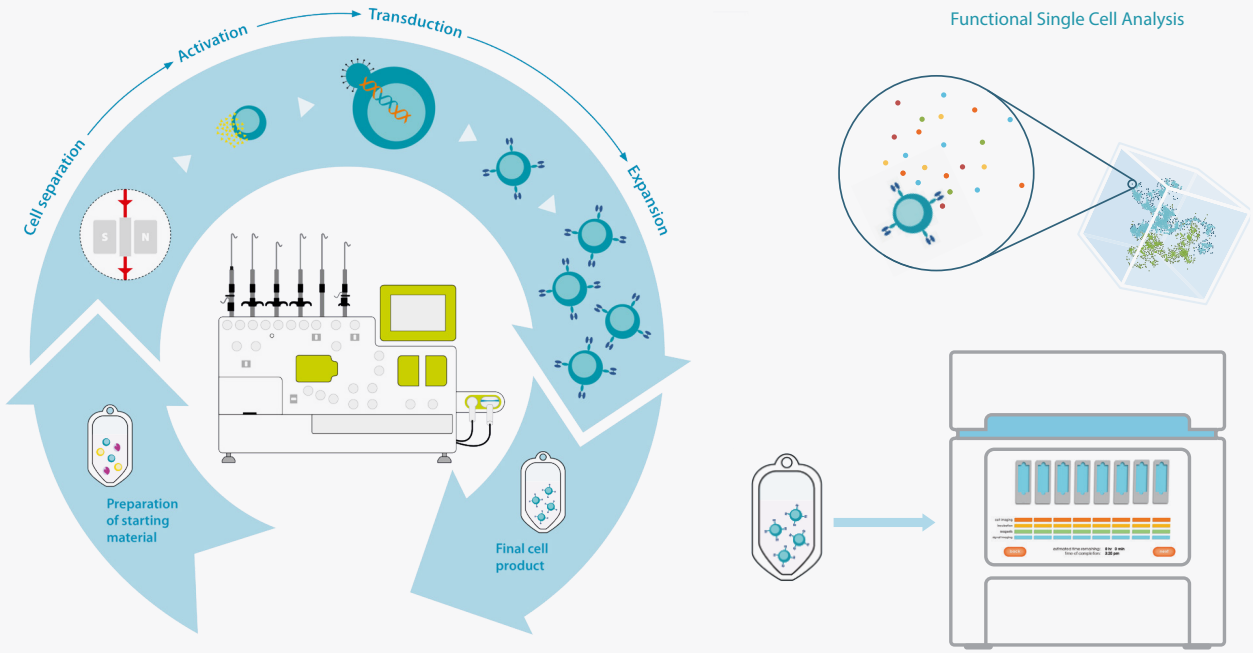

Figure 1 | Overview of the CliniMACS Prodigy® T Cell Transduction (TCT) Process workflow for CAR-T manufacturing (left, image credit: Miltenyi Biotec). Single-cell cytokine profiling of functional quality and potency of the engineered cell product with the IsoLight System (right).

Focus on quality with a comprehensive end-to-end solution from Miltenyi Biotec and Bruker.

The field of cellular immunotherapy has demonstrated significant progresses in the past decade and currently is considered as the most promising treatment for advanced hematologic malignancies[1]. CAR-T cell therapy is a highly personalized cancer treatment where a patient's own T cells are engineered to specifically target and kill the patient's own cancer cells. Unlike most of the off-the-shelf medications, the qualities of cell therapy products vary significantly between batch to batch and patient to patient. In fact, the process of production of this personalized cell product is further complicated by the high requirements numerous Good Manufacture Practice (GMP) instruments and scientific personels with significant and highly specific expertise. Therefore, there is an urgent need for utilizing an automated process which incorporates both cell product processing and functional evaluation. The CliniMACS Prodigy® represents the next generation in automated cell processing[2]. This device offers advanced integrated solutions to streamline cell processing workflows – from cell separation through cell culture to formulation of the final product. In combination with Bruker's IsoCode single-cell polyfunctional platform, it can minimize the need for human involvement in numerous steps, reducing human resource utilization and the risk of cell contamination, and it eventually may be used to ensure successful translation from cell therapy development to clinical approved products [3,4].

Bruker's Single-Cell Adaptive Immune Application:

Bruker's polyfunctional strength index (PSI) consolidates high-dimensional, single-cell protein secretion data into a single metric that represents the overall activity of a sample. It captures two critically relevant factors uniquely: the percentage of polyfunctional cells (single-cells secreting two or more cytokines) in a sample, and the intensity of all profiled secreted cytokines. Polyfunctional cells are recognized as key effector cells contributing to the development of potent and durable cellular immunity against viral infection, cancer, and other disease [2-4].

Bruker's ability to capture the range of relevant cytokines from each immune cell represents a unique secreted protein multiplexing capability. While the percentage of highly polyfunctional cells on its own is a meaningful indicator of potency, the Bruker system quantitates the intensity of the cytokines secreted by these highly polyfunctional cells. Having both of these key factors in tandem has helped capture the potency of important and highly functional T-cell and other immune cell subsets, which has correlated with in vivo response [4,5].

Recently, PSI™ has been employed in the anti-CD19 CAR-T cell pre-infusion products manufactured from apheresis of patients with Non-Hodgkin lymphoma (NHL). It has demonstrated a statistically positive association predictive of objective response (OR) as well as cytokine release syndrome (CRS) in these patients after anti-CD19 CAR-T therapy, outperforming other pre-infusion flow cytometry-based and bulk-level metrics in its ability to differentiate responders and non-responders [5].

Phenotypically identical cells, though functionally highly heterogeneous

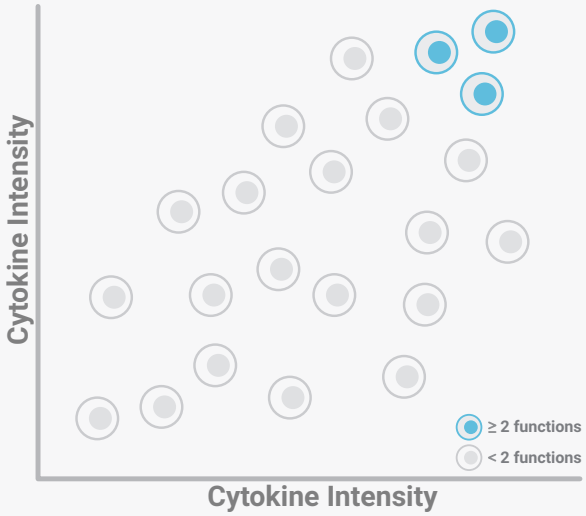

Figure 2 | Highly Polyfunctional cell subsets revealed by Bruker systems enable visualization of immune cells with a high degree of inflammation (cells that secrete two or more cytokines per cell, at a high intensity) that correlate to disease progression.

Prep, Run, Analyze

CAR-T Single-cell Quality Check on the Bruker system

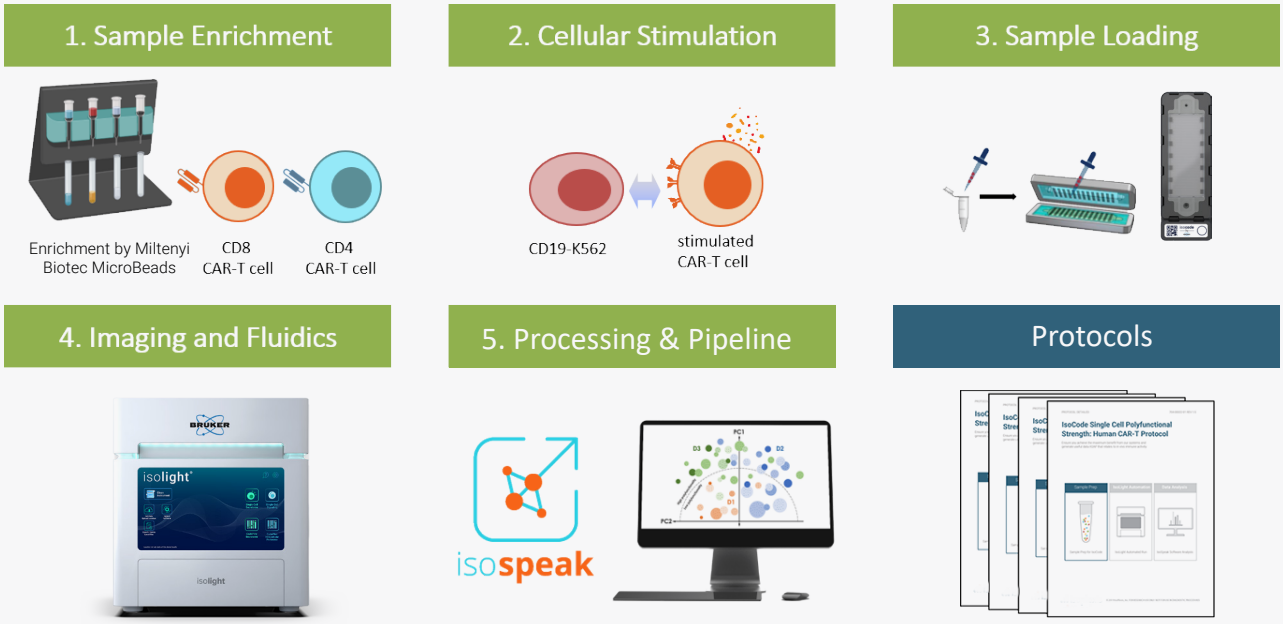

Figure 3 | 1. Sample: Phenotypes of interest are isolated using CD4 and CD8 Miltenyi Biotec MicroBeads. In Bruker CAR-T cell protocol, CD8+ and CD4+ CAR-T cells are enriched for stimulation. 2. Stimulated: CD19 and CD22-specific response at 37°C, 5% CO_2 for 20 hours. 3. Loading: Stimulated samples are collected and loaded onto Bruker's IsoLight system. 4. Automated assay workflow: IsoCode chips are imaged for cell detection, incubated, conducts ELISA protocol and imaged for signal. 5. Automated data processing and interactive analysis: cell detection and cytokine analysis are performed automatically. Easily create advanced bioinformatics visualizations for comparison between samples and generation of data reports. Proven and published sample preparation protocols are available for the end-to-end workflow.

Bruker's Comprehensive Single-Cell Adaptive Immune Workflows:

Our standard CAR-T single-cell quality evaluation has been described previously [3]. In brief, cryopreserved CAR T-cell products are thawed and cultured in complete medium with IL-2 at a density of 1x10^6/mL in a 37°C, 5% CO_2 incubator. After overnight recovery, viable T cells are enriched using Ficoll-Paque Plus medium. CD4+/CD8+ CAR-T cell subsets are separated using anti-CD4 or anti-CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and stimulated with K562 cells transduced with either CD19 or CD22 at a ratio of 1:2 for 20 hours at 37°C, 5% CO_2. The cocultured CD4+ or CD8+ T cells are further enriched using anti-CD19– or anti- CD22–conjugated MicroBeads to deplete target cells. Presence of CD4+ or CD8+ CAR-T cells are then confirmed by staining with fluorochrome conjuagated anti-CD4 or anti-CD8 antibodies. Enriched and stained CAR-T cells are then loaded onto IsoCode chips for single-cell secrtomic analysis. After incubation and signal generation, the generated data is processed automatically by an IsoSpeak software pakage for further bioinformatic analysis.

Polyfunctional activation of actigen specific stimulation compared to NGFR control

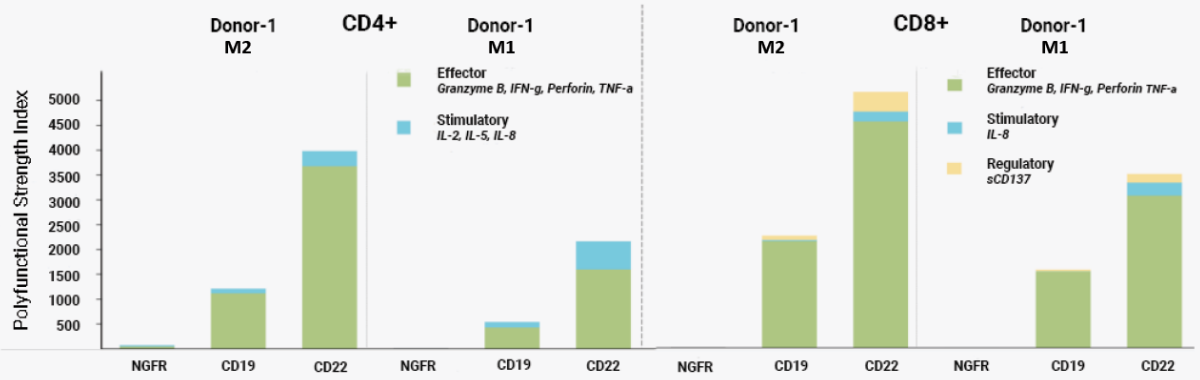

Figure 4 | CD19 and CD22 antigen specific stimulation elicits highly polyfunctional activation of the cell therapy products while NGFR control stimulation does not lead to activation of the cell therapy product.

Optimizing Cell Therapy with CliniMACS Prodigy® and IsoLight System.

The quality of CAR-T products is crucial for this cell-based immunotherapy and optimization of the manufacturing processes will significantly improve the product quality. However, the primary challenge is how to provide an objective functional readout to differentiate different bioprocessing procedures and justify whether a newly developed method is superior than the original method.

In a recent study, researchers used the CliniMACS Prodigy® TCT Process (Miltenyi Biotec) (Figure 1), a closed-system automated device, to produce CAR-T cell products from T-cell enrichment, activation, viral transduction, and expansion to harvest for downstream use [6]. In their study, Method 1 (M1) was terminated with a wash step at day 5, while Method 2 (M2) was terminated with a wash step earlier, at day 3. CD19 and CD22 antigen specific stimulation elicits highly polyfunctional activation of the cell therapy products while NGFR control stimulation does not lead to activation of the cell therapy product (Figure 4).

Improved Engineered Cell Therapy Manufacturing Characterization with the IsoLight

Increased cell quantities and viabilities are not always correlated with enhanced cellular functions. To further understand whether M2 can improve the overall functionalities of CAR-T cell products, we did *in vitro* co-culture with either CD19 or CD22 expressing target cells. After co-culture, the polyfunctional potencies of bispecific CD19/CD22 CAR-T cell products were precisely evaluated by IsoCode Chip technology and presented as IsoPSI (See Figure 3 for the experimental setup).

Consistently more robust polyfunctional response of CD4+ or CD8+ CAR-T cell products to CD19 or CD22 with M2 compared to M1

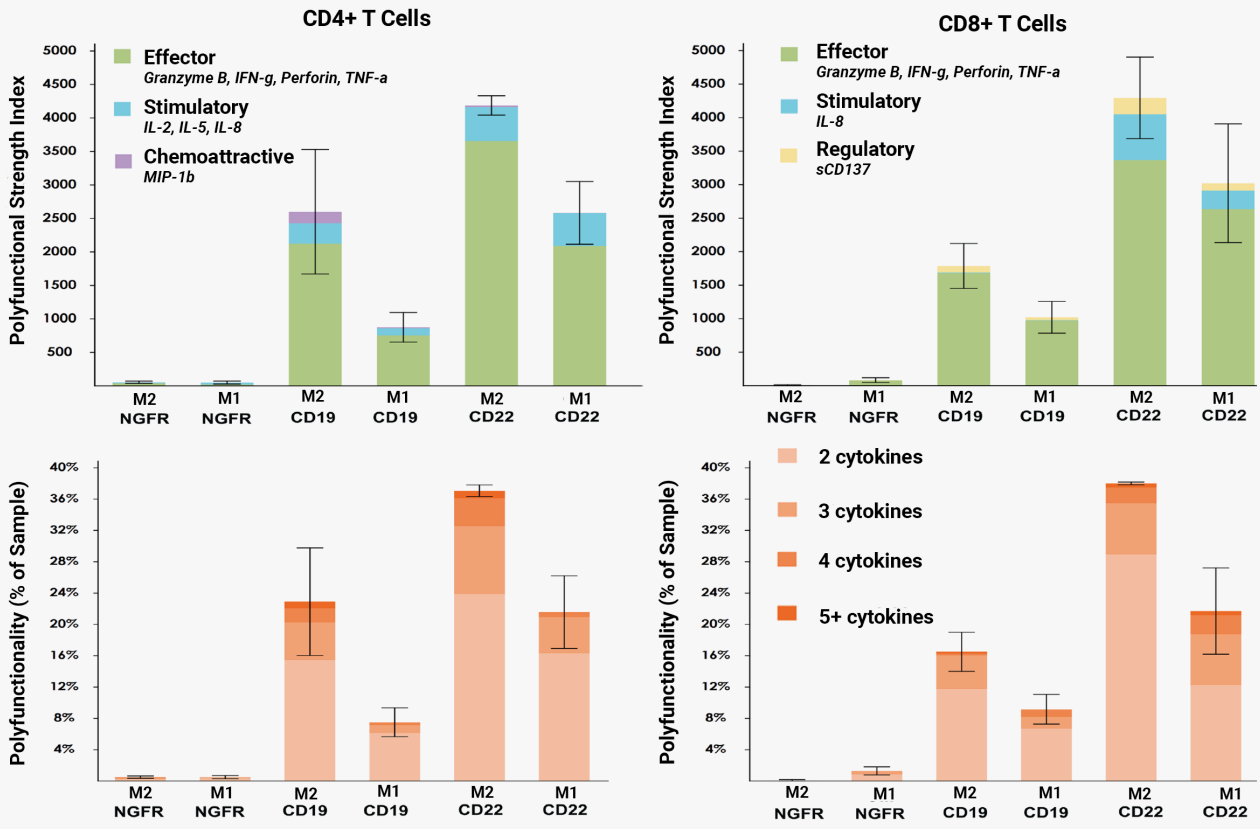

Figure 5 | Recovered CAR-T cell products were separated using anti-CD4 or anti-CD8 microbeads and co-cultured with CD19 or CD22 expressing target cells for 20-24 hours at 37°C, 5% CO_2. After depleting target cells, the CAR-T cell suspension was loaded into an IsoCode Chip. After overnight incubation at 37°C, 5% CO_2, cytokine signals from ~2000 single-cells were captured and analyzed on IsoSpeak software suite. Upper panel: Adaptive Immune Index of CD4+ (left) and CD8+ (right) CAR-T products. Lower panel: Polyfunctionality of CD4+ (left) and CD8+ (right) CAR-T products.

This single-cell polyfunctional data clearly demonstrates that M2 significantly improved the overall polyfunctional responses of bispecific CD19/CD22 CAR-T cell products compare to previously used M1 (Figure 5). Moreover, these data also revealed that CAR-T cell products manufactured by M2 have much higher secretions in multiple secreted proteins, the enhanced IsoPSIs were mainly driven by effector and stimulatory cytokine secretions. Interestingly, chemoattractive (in CD4+) and regulatory (in CD8+) cytokine secretions were also enhanced in M2 group (Figure 5), indicating that in this study, M2 improves the quality of CAR-T cell products in multiple dimensions and is superior in both quantities and qualities.

Protocol frameworks where Bruker systems achieved published correlates: validated enrichment & stimulation workflows

	Immune Cell Type & Immunotherapy Type	Indication	Enrichment	Stimulation Type & Time	PSI Correlate
Cell Engineering & Therapy	Human CD19 CAR-T cells[3]	Cancer: Non-hodgkins Lymphoma	Miltenyi Biotec MicroBeads	CD19 antigen	Responder correlate (p = 0.0117)
	Human TCR-Engineered Mart-1 T-cells[7]	Cancer: Solid Tumor	Tetramer	Mart-1 antigen	Tumor relapse correlate
	Human NK cells[8]	Cancer: Leukemia	Miltenyi Biotec MicroBeads	IL-15 O/N + IL-12 / IL-18	Mouse tumor regression correlate
Checkpoint & Combination Therapies	Human PBMC, pre and post TLR Agonist Therapy[9]	Cancer: Sarcoma	Miltenyi Biotec MicroBeads	CD3 / CD28	Responder correlate
	Human TILs, post checkpoint therapy[10]	Cancer: Solid Tumor	Miltenyi Biotec MicroBeads	CD3	Responder correlate (p = 0.0294)
	Human PBMC, pre and post cancer vaccine[11]	Cancer: Solid Tumor	Miltenyi Biotec MicroBeads	CD3 / CD28	Survival Correlate (p = 0.001)
Inflammation & Autoimmunity	Human Monocyte in healthy and diseased patients[12]	Autoimmune & CNS: Multiple Sclerosis	Miltenyi Biotec MicroBeads	P3C or LPS	MS correlate (p = 0.0348)
	Human NK cells, no therapy[13]	Autoimmune: IBD and Crohn's disease	Flow Cytometry	PMA/Ionomycin	Inflammatory state correlate
Mouse Immunotherapy	Mouse TILS, post combination therapy[14]	Cancer: Solid Tumor	Flow Cytometry	CD3 / CD28	Mouse Response Correlate
	Mouse CD19 CAR-T cells[15]	Cancer, B-ALL	Miltenyi Biotec MicroBeads	CD19 antigen	Product Analysis
	Mouse PBMC, post vaccine[16]	Infectious disease	Miltenyi Biotec MicroBeads	CD3 / CD28	Survival correlate

Figure 6 | The goal for starting with a straightforward protocol that is recommended by Bruker is to ensure success and drive optimization. These protocols have been highly successful in detecting polyfunctional differences in the past (see Technology Note: Validation of Sample Preparation). We recommend not deviating from published protocols, but at the same time simplifying certain aspects that should not impact the success of the study, to ensure success for first time IsoLight users.

Conclusion

- Published enrichment, stimulation, and staining protocols with different immunotherapies and indications can help provide a framework to achieve goals of obtaining single-cell data, proven in multiple publications and presentations
- These enrichment and stimulation protocols minimize impact on live cells that are analyzed with Bruker' systems
- The type of sample preparation protocols and strategies have helped researchers achieve critical correlates to in vivo data in the past as well
- While every user project is specific in terms of requirements, (e.g., stimulation time), sample datasets can act as a rubric for choice of protocol

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