BIOLOGICS VS ATMPS

For Biologics:

- Well-characterized cell lines for production
- Scalable manufacturing processes
- Defined and controlled regulatory processes

For Biologics: Cell is NOT the product

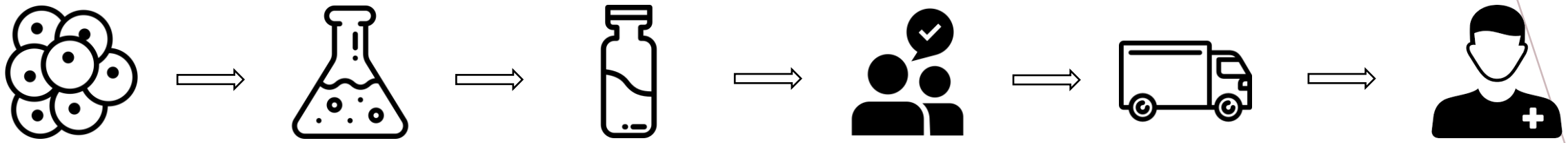
For ATMPs:

- Highly heterogeneous: Primary cells with high donor-to-donor and patient-to-patient variation
- Limited commercial therapies, primarily research-based and often point-of-care
- New and evolving regulatory landscape

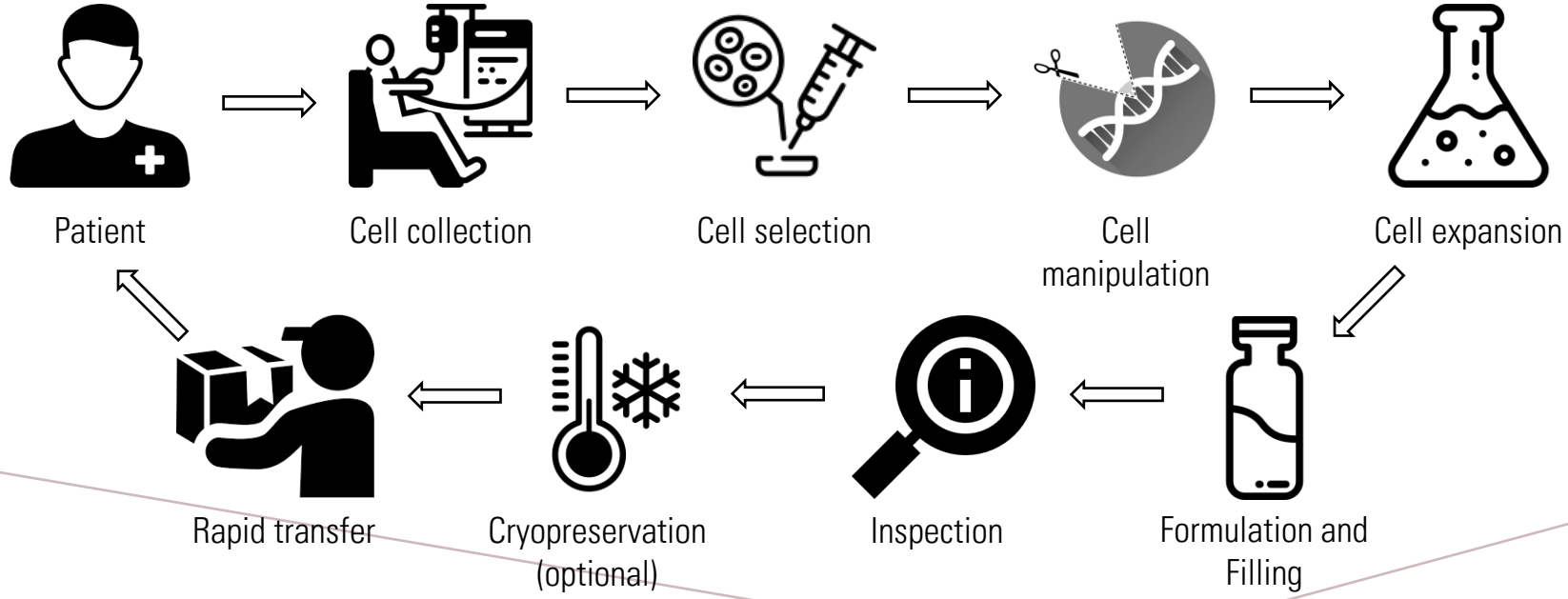
For ATMPs: Cell is injected therapy

Manufacturing process

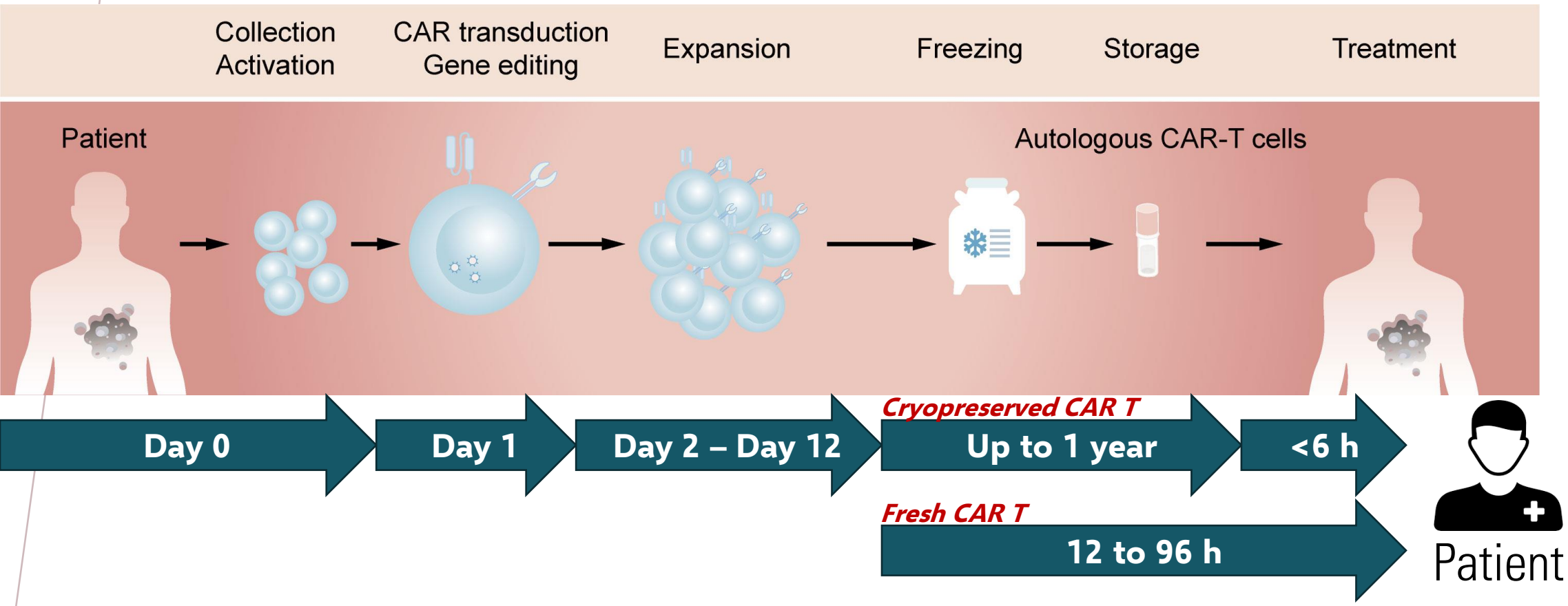
For Biologics: Linear process



For ATMPs: Circular process



Workflow for CAR T production

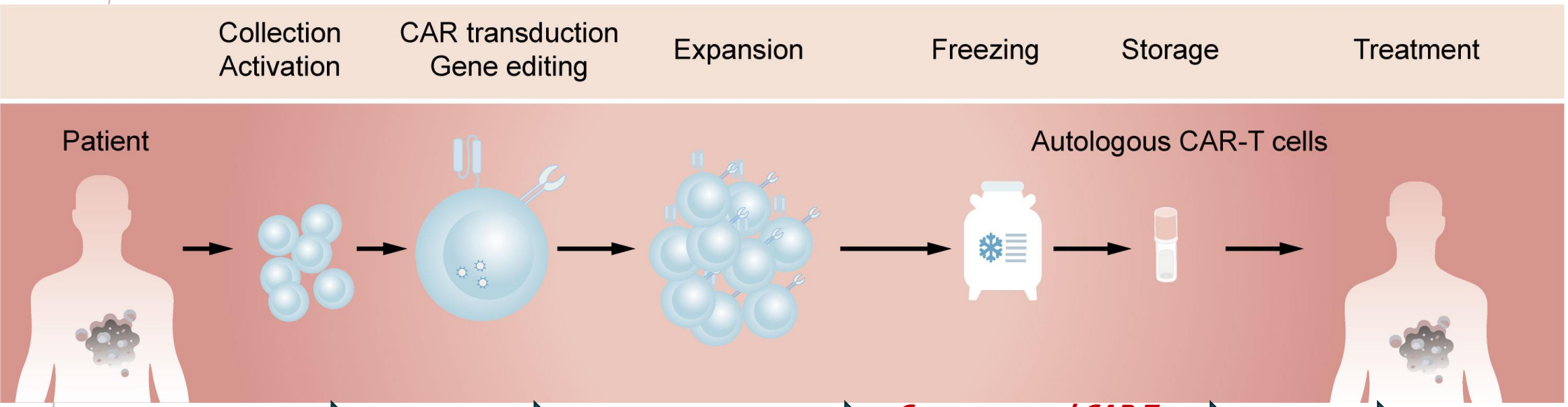


Ref: Lv Z, Luo F, Chu Y. Strategies for overcoming bottlenecks in allogeneic CAR-T cell therapy. Front Immunol. 2023 Jul 24;14:1199145.

EXAMPLE TARGET PRODUCT PROFILES OF CAR T

TPP parameters	Target
Product dosage form	Suspension for IV infusion, Fresh CAR T product
Total cells per dose	30 to 1,000 million T cells with at least 10% CD19 CAR T cells
Net volume	30 mL
Process lot size	4 bags of cells (2 for retention, 1 for release testing, 1 for quarantine)
Administration time	IV infusion 30 minutes within 96 h after product release
Transfer conditions	2-8 °C
Container closure system	Identical primary packaging to RLD
Package integrity	No failure
Potency	• Phenotypic profile confirmation of CD19 CAR T cells • Transgene expression • Vector copy number • Cytotoxic assay of CD19+ tumor cells • Cytokine release assay • Cell viability
Safety	• Insertional mutagenesis risk • Replication competent lentivirus • Sterility testing • Endotoxin testing • Mycoplasma contamination testing

So, what happens if they need to do every single test for each batch of CAR T cells before they can release it?



Day 0 → **Day 1** → **Day 2 – Day 12** → ***Cryopreserved CAR T*** **Up to 1 year** → **<6 h** → Patient

Fresh CAR T **12 to 96 h** → Patient

Potency: Tumor cytotoxic assay (~1-2 days)

Safety: NGS for possible genome insertion(~1-2 days)

Safety: Sterility tests (~6-14 days)

Safety: Mycoplasma contamination tests (~28 days)

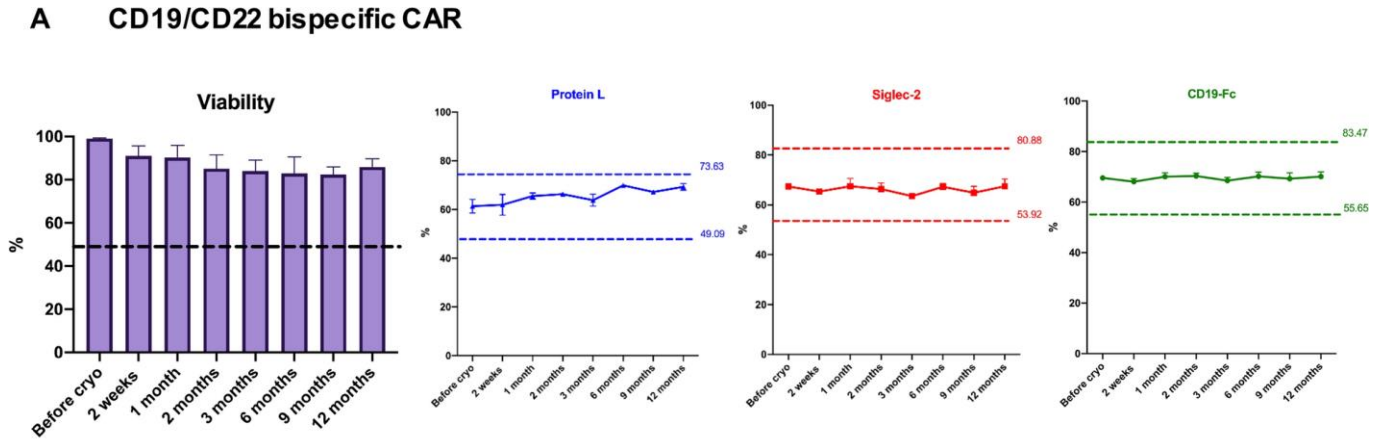
Why are fresh CAR-T cell products still used?

If frozen CAR-T cells offer greater advantages, particularly the ability to perform full lot release testing.

Fresh CAR T products
- Limited shelf life
- The timeframe for full release tests is limited

Frozen CAR T products
- Sufficient time for full release testing
- High flexibility in scheduling patients
- Allow manufacturing at a central location

- Reduced viability after a cycle of freeze-and-thaw



Ref: Cai Y, Prochazkova M, Jiang C, Song HW, Jin J, Moses L, Gkitsas N, Somerville RP, Highfill SL, Panch S, Stroncek DF, Jin P. Establishment and validation of in-house cryopreserved CAR/TCR-T cell flow cytometry quality control. J Transl Med. 2021 Dec 24;19(1):523.

- The potential harm from cryoprotectants
- The validated thawing of the product at the clinical site

That is the reason why…

“Cell therapy products are really only defined by their process.”

David Courtman
Director, Cell Manufacturing, Biotherapeutics Core Facilities,
Ottawa Hospital Research Institute

“In other words, everything you do in your manufacturing, you need to have evidence that it is safe and that it’s producing what you expect. If you have that, you are in a much better space in terms of moving ahead in translation.”

A short guide to cell therapy manufacturing, part 1
https://medium.com/the-expression/a-short-guide-to-cell-therapy-manufacturing-part-1-ad61e29f94b2

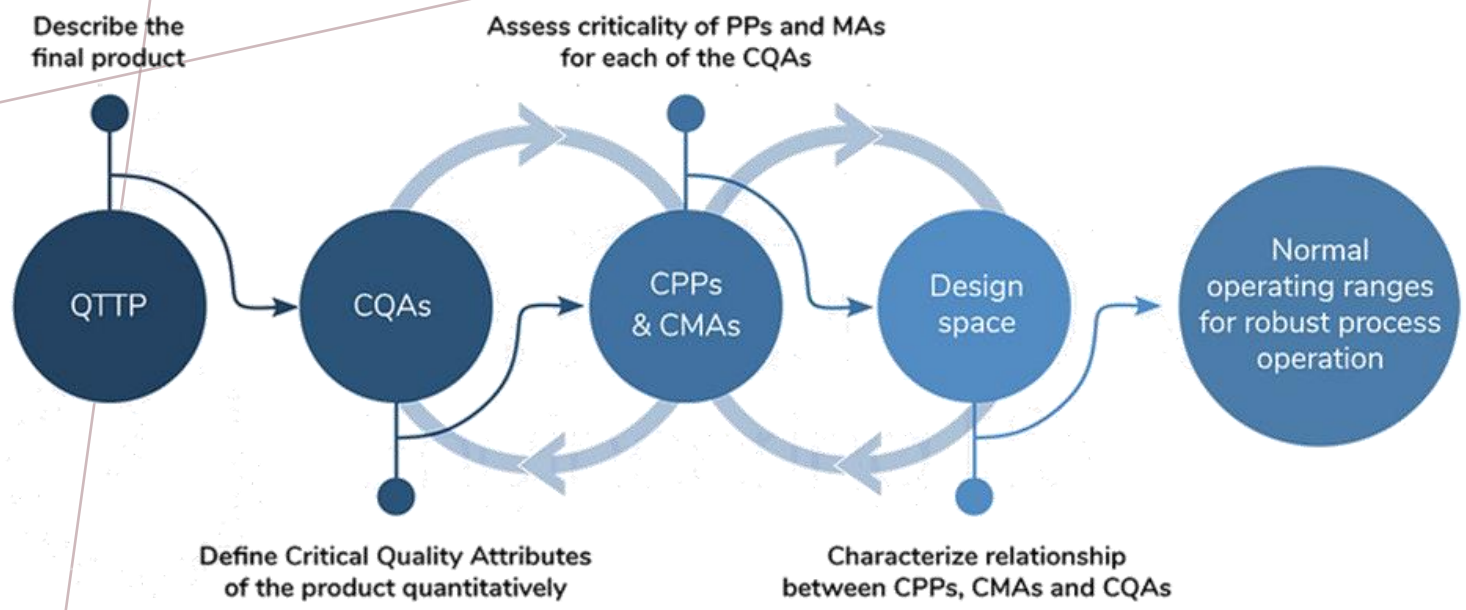
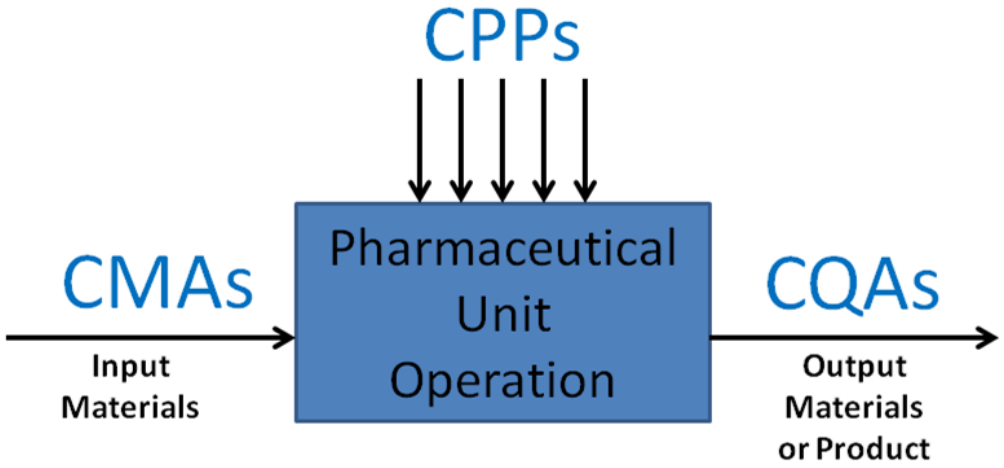


Image courtesy of Antleron

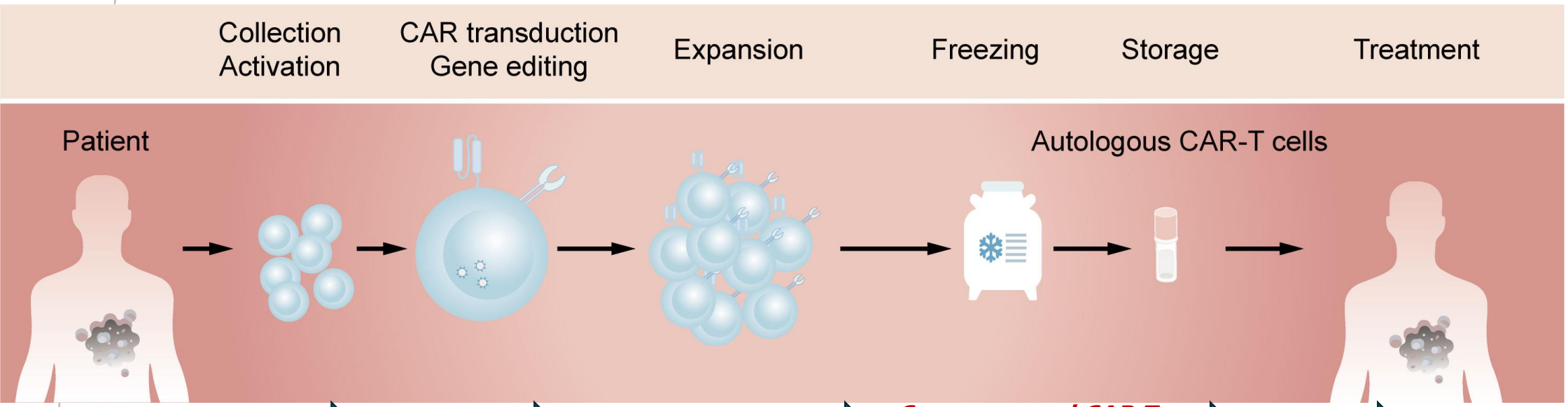


$$CQAs = f\,(CPP_1, CPP_2, CPP_3 \ldots CMA_1, CMA_2, CMA_3 \ldots)$$

Image courtesy of ISPM

Quality-by-Design approach for ATMP development and manufacturing

Overview of quality controls of CAR T products



Day 0 → **Day 1** → **Day 2 – Day 12** → ***Cryopreserved CAR T*** **Up to 1 year** → **<6 h**

Fresh CAR T **12 to 96 h**

Patient

Starting materials
- Cell identity
- Impurities
- Cell count
- Cell fitness and differentiation status
- T cell enrichment

Antigen stimulation
- Immunophenotype (CD69, CD25)
- Cytokine production (IFN-γ, IL-2, Granzyme B)

Transduction efficiency
- CAR expression
- Vector copy number

During expansion
- Cell count and viability
- Sterility testing of the supernatant

Quarantine
- Cell count and viability
- CAR expression and VCN
- Functional tests
- Sterility and endotoxin testing

For in process testing and release testing, the criteria set should be those that indicate quality, safety, and potency relevant to the biological mechanism of action.

Overview of quality controls of CAR T products

Table 6. Sampling strategy of in-process control samples

Parameter	In process	Product ID	Product Potency
Cell count	X		X
Viability	X		X
Transgene presence	X (After transduction – depends on process length)	X	
VCN	(Sample collection)	X	
Transduction efficiency (PCR)		X	
Transduction efficiency (Prot)		X	
Appearance		X	
Purity (immunophenotype specific to mechanism of action)	X	X	
Characterisation • Activation • Therapeutic cell subpopulation (effector/memory) • Secondary elements • Metabolites	X	X	
Impurities	X	X	
Potency/potency panel	(Sample collection)		X
Safety	X	X	

British Pharmacopoeia: Advanced Therapy Medicinal Products Guidance, T Cell and NK Cell Characterisation Assays

A GOOD BEGINNING IS HALF SUCCESS

The quality of starting materials directly impacts the quality of the final product

Table 7. Leukapheresis product characteristics variability amongst donations, between healthy donors and patients, and across clinical indications[31]

Characteristics	Healthy donors (n=30)	Lymphoma (n=32)	ALL (n=6
Total nucleated cells ($x10^8$), median (range)	149.0 (66.4–392.7)	100.4 (9.3–340.5)	62.5 (19.7–156.0)
Total CD3+ cell count ($x10^8$), median (range)	72.0 (4.1–185.9)	41.0 (4.2–231.8)	26.0 (4.0–68.0)
Haematocrit (%), median (range)	3.9 (1.8–7.4)	2.6 (1.3–7.4)	2.5 (1.1–3.3)
Monocytes (%), median (range)	N/A	24.7 (8.1–53.8)	14.7 (6.2–33.0)
Platelets ($x10^8$), median (range)	987.0 (418.0–7551.0)	1088.0 (147.0–3120.0)	615.5 (170.0–1310.0)
Viability (%), median (range)	99.8 (99.6–100)	99.9 (99.6–100)	99.8 (99.6–99.9)

Recommended acceptance criteria:

- Minimum cell number

(e.g., total nucleated cells $\geq 20x10^8$ cells)

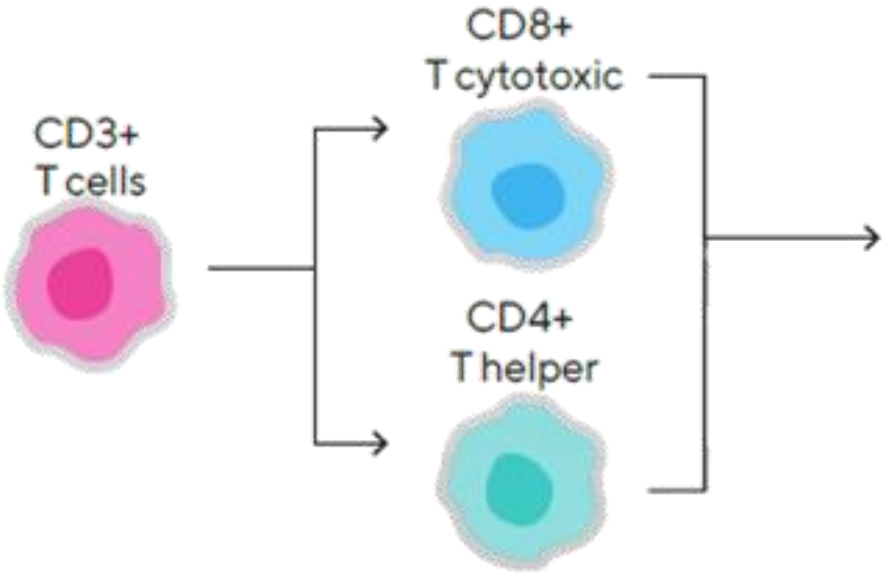
- Viability
- Percentage or absolute number of CD3+ cells

(e.g., CD3+ cells $\geq 10x10^8$ cells)

- Absolute number of CD4+, CD8+ T cells
- Impurities (NK cells, monocytes, B cells)
- (optional) Percentage of naïve and memory T cells

Quick recap: T cell subtype and surface markers

Image courtesy of AZOnetwork



Self-Renewal Potency

	T_N	T_{SCM}	T_{CM}	T_{TM}	T_{EM}	T_{EMRA}	T_{TE}
CD45RA:	+	+	-	-	-	+	+
CD45RO:	-	-	+	+	+	+	-
CD27:	+	+	+	+	-	-	-
CD62L:	+	+	+	-	-	-	-
CD95:	-	+	+	+	+	+	+

- Two subtypes of T cells, CD4+ T cells and CD8+ T cells, both are CD3+
- Majority of CD4+ T cells are T helper and regulatory T cells, and T helper cells include various subtypes, e.g., Th1, Th2, Th17, T follicular helper, and Th22
- CD4+ and CD8+ T cells are categorized based on their surface markers into memory T cells, e.g., naïve T cells, T stem cell memory (Tscm), T central memory (Tcm), T effector memory (Tem), and T effector (Teff)
- CAR T cells with an early Tscm and Tcm memory phenotype have been associated with better clinical outcomes, because there was a correlation between the proportions of naïve/stem cell memory (Tn/Tscm) and the ability of CD4 and CD8 T cells to proliferate.

Here comes our protagonist!!!

Flow cytometry

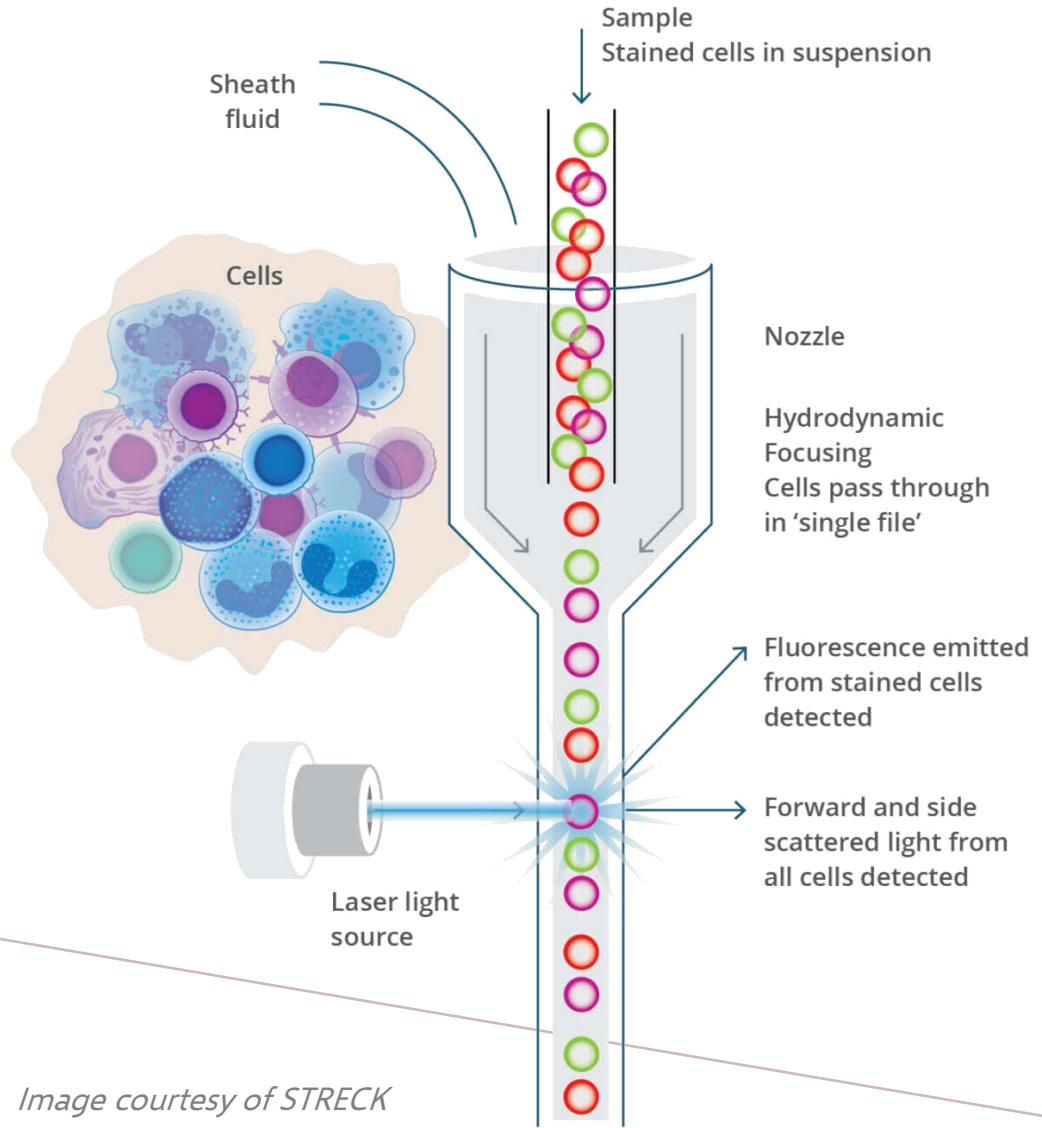


Image courtesy of STRECK

Flow cytometry is a widely used but technically complex tool; the practical application of which is recognised to be diverse and often challenging to standardise. Standardisation is critical in supporting robust data generation, enabling data comparability between users and instruments, and when applied to development of ATMPs, ensuring reproducible product quality, safety, and efficacy throughout the entire product lifecycle.

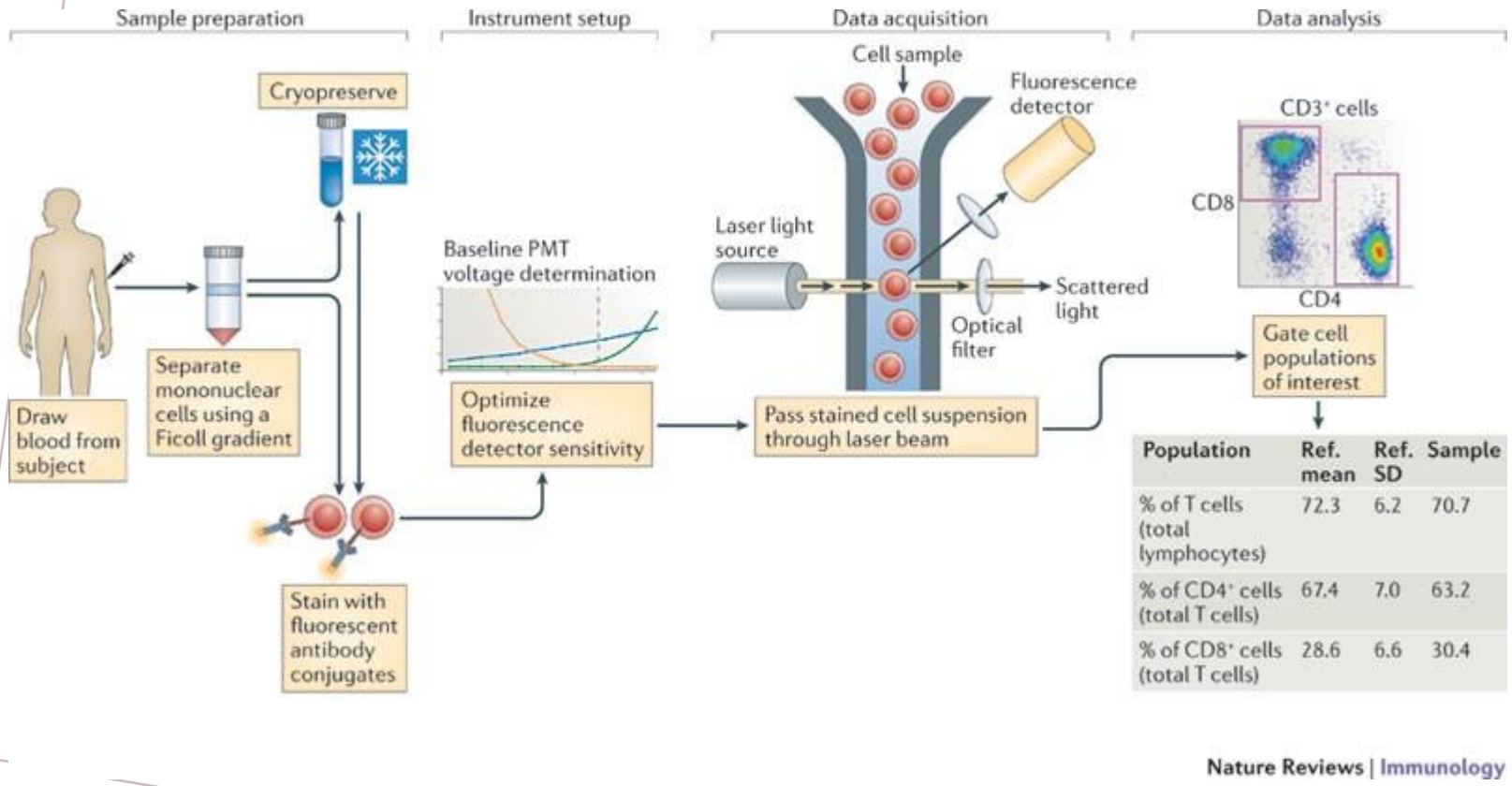
British Pharmacopoeia: Advanced Therapy Medicinal Products Guidance, Application of Flow Cytometry

Applications of Flow cytometry in CAR T production: Examples

- Cell identity and impurities of leukapheresis products
- T cell enrichment
- T cell activation markers
- Transduction efficiency (CAR expression)
- Viability

Flow cytometry applications:

T cell identification and impurity screening



Recommended surface markers:

T cells: CD3 (to define T cells), CD4, CD8, CD45RA and CCR7

B cells: D19 and CD20 (to define B cells), CD38 (plasmablasts and transitional B cells), CD24 (transitional B cells), and IgD and CD27 (naive and memory B cell)

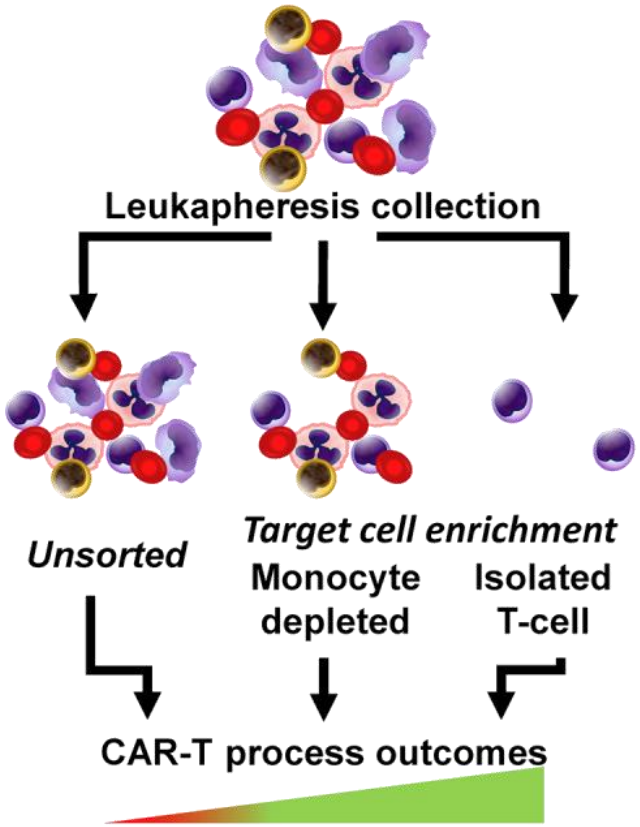
NK cells, Dendritic cells, and Monocytes: HLA-DR, CD11c, CD14, CD16, CD56 and CD123

Recommended articles for T cell characterization by Flow cytometry

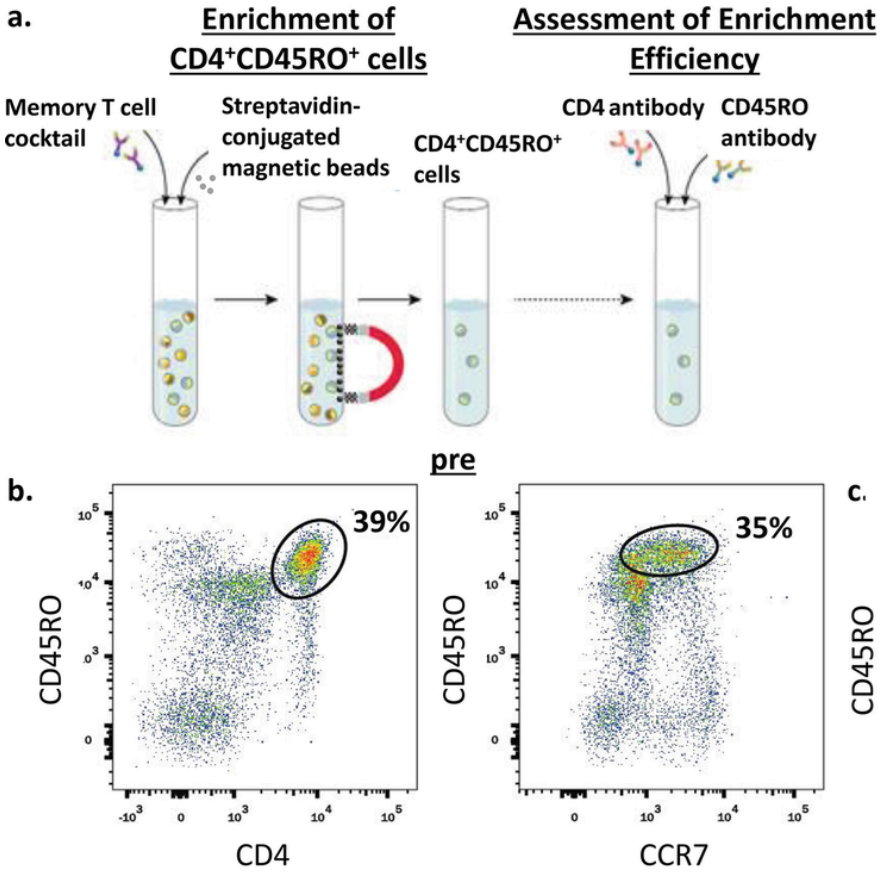
- Maecker, H., McCoy, J. & Nussenblatt, R. Standardizing immunophenotyping for the Human Immunology Project. *Nat Rev Immunol* **12**, 191–200 (2012). https://doi.org/10.1038/nri3158

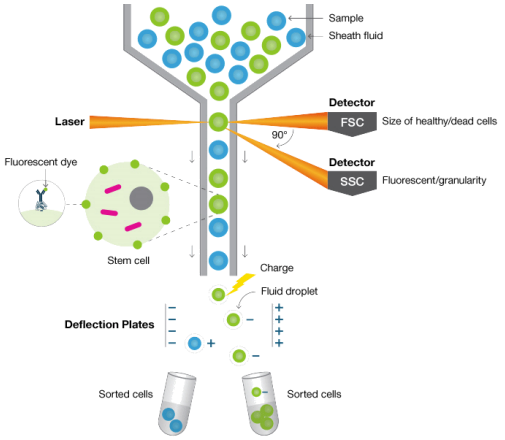
Flow cytometry applications:

T cell enrichment



Noaks E, Peticone C, Kotsopoulou E, Bracewell DG. Enriching leukapheresis improves T cell activation and transduction efficiency during CAR T processing. Molecular therapy. Methods & Clinical Development. 2021





Goetz, C., Hammerbeck, C., Wyman, A., Huh, JB. (2018). Cell Enrichment. In: Flow Cytometry Basics for the Non-Expert. Techniques in Life Science and Biomedicine for the Non-Expert. Springer, Cham.

Flow cytometry applications:

T cell activation markers

- **T-cell activation** is the process by which resting T cells become functional.
- T cell activation refers to a process in which mature T cells can express antigen-specific T cell receptors on their surface to recognize their cognate antigens and respond by entering the cell cycle, secreting cytokines or lytic enzymes, and initiating the cell-based functions of the immune system.

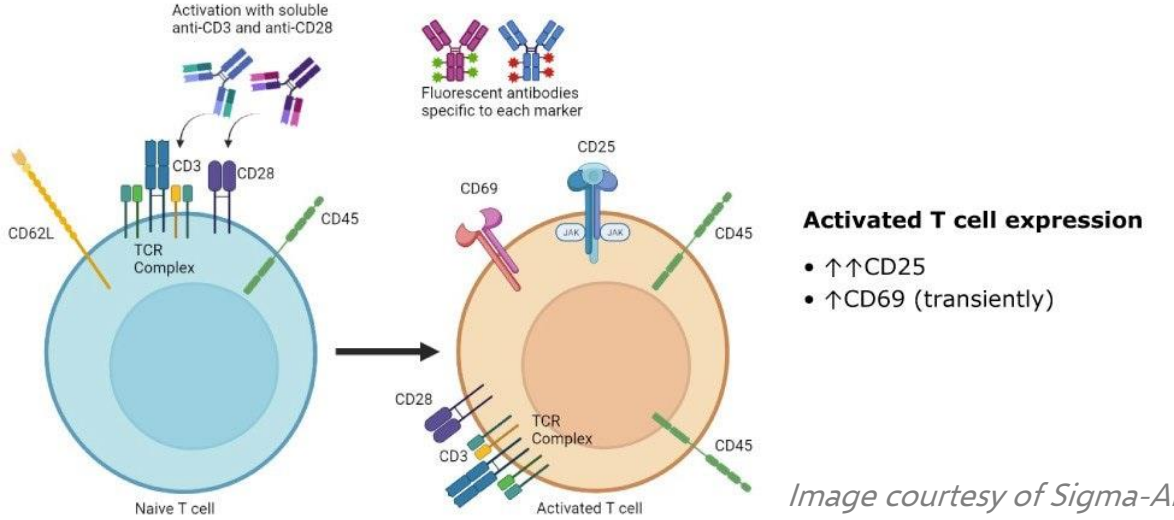


Image courtesy of Sigma-Aldrich

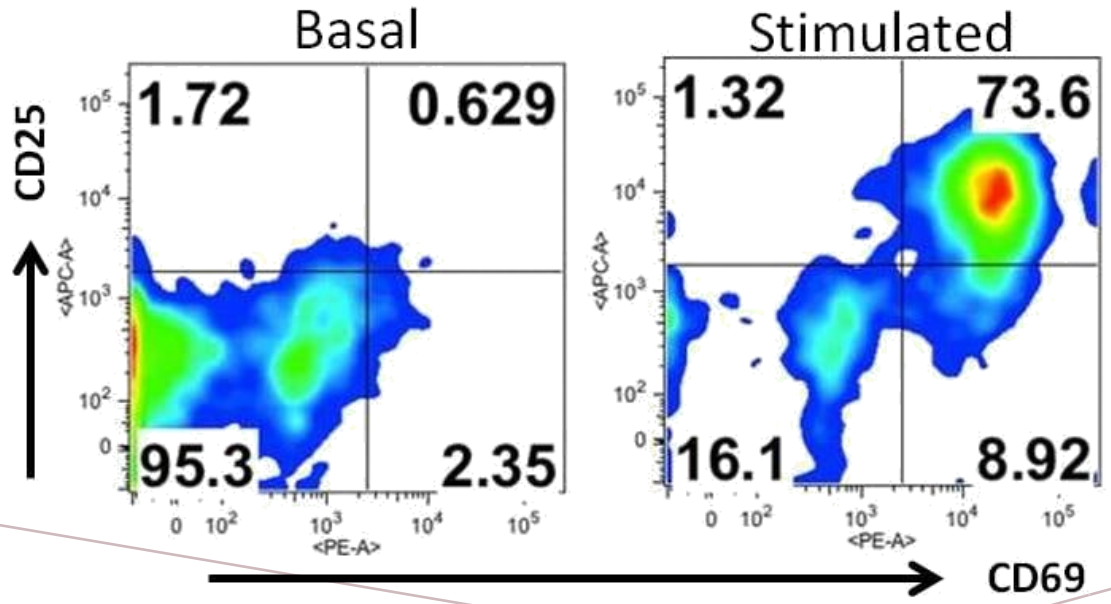
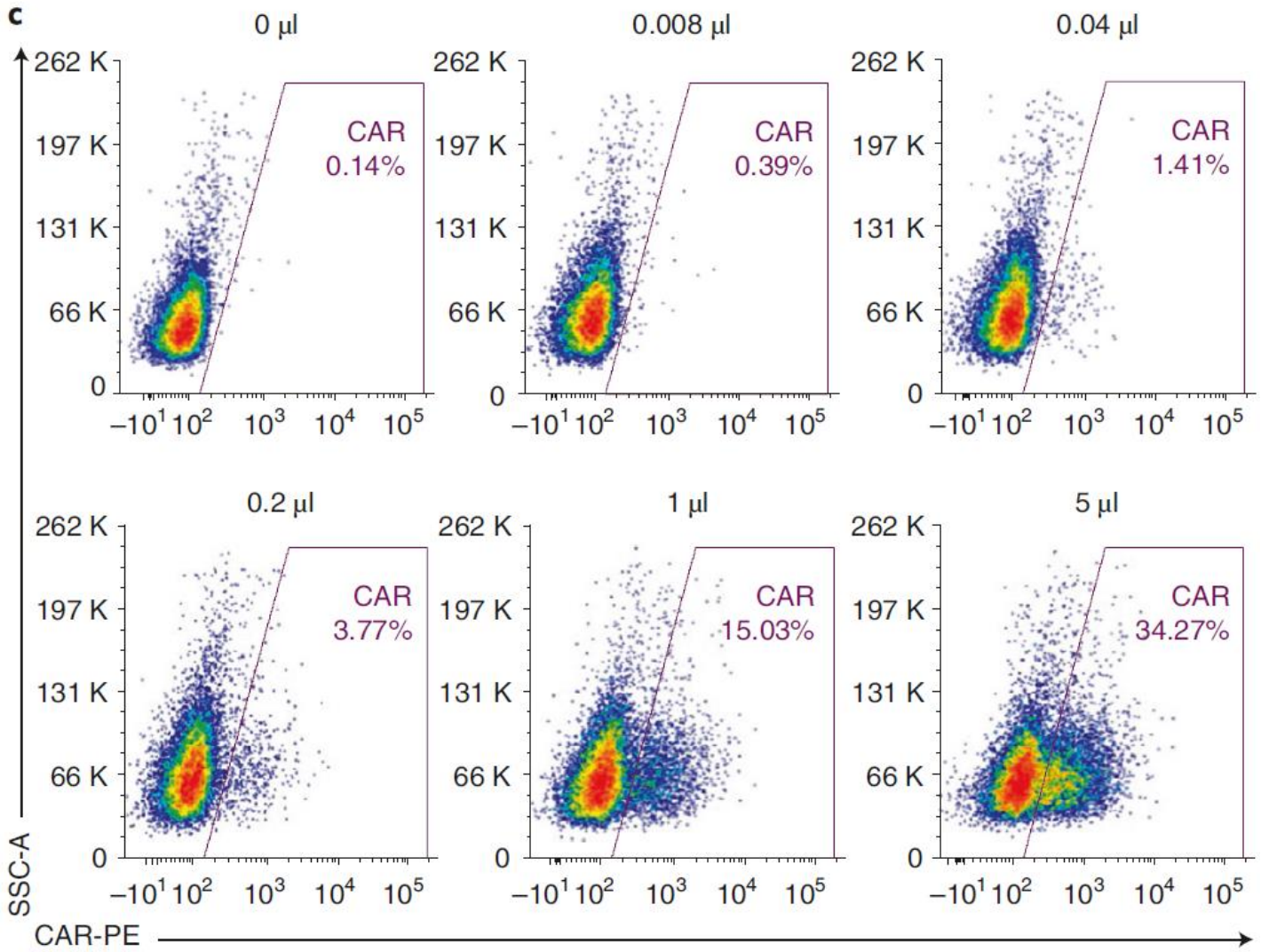


Image courtesy of Anirban Banerjee, Surgery, George Washington University, Washington DC, Post-doctoral Fellow

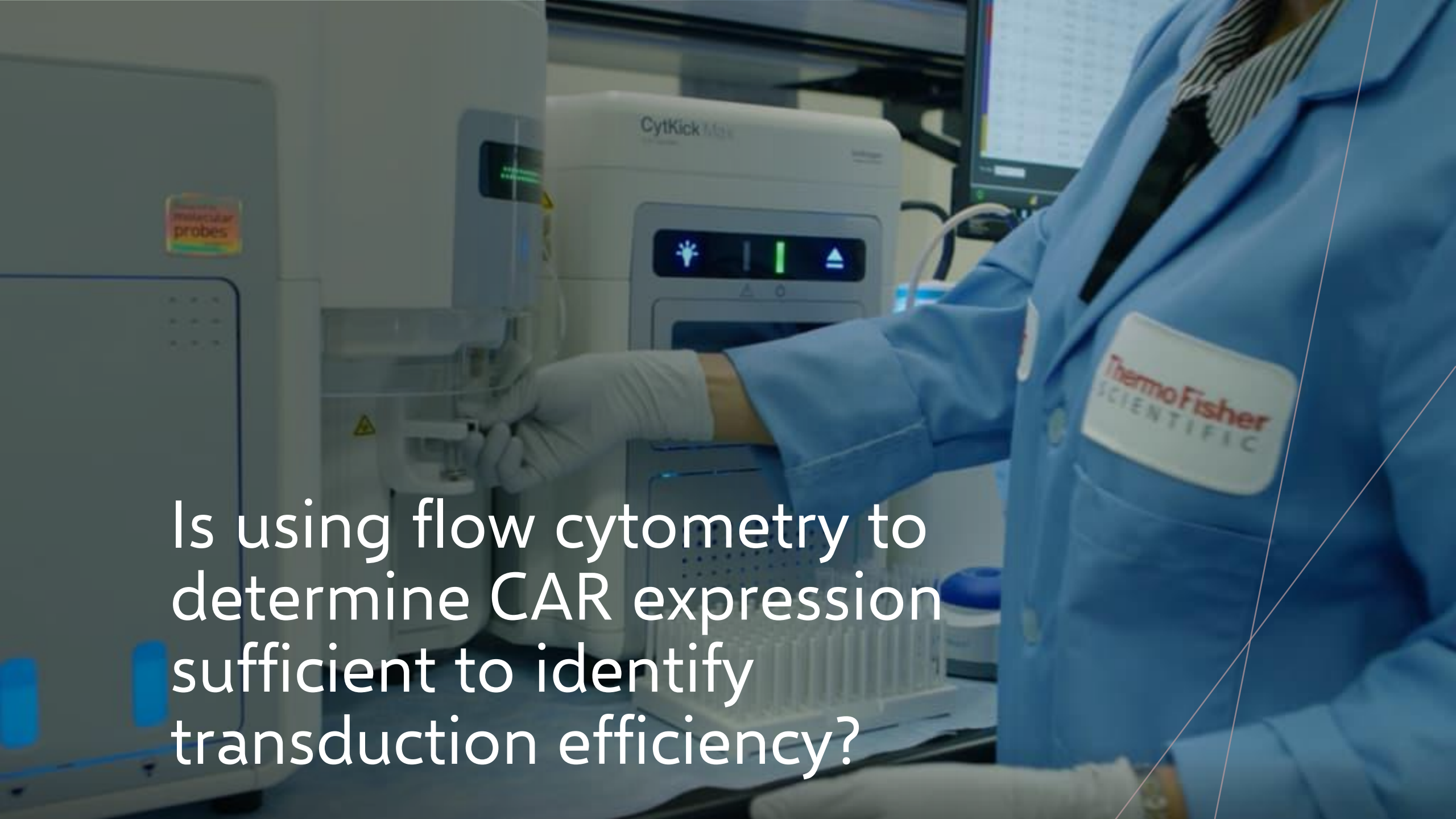
Flow cytometry applications:

Transduction efficiency (CAR expression)



Gene transfer activity of a CD8-LV stock encoding the CD19-CAR. Serial dilutions of the vector stock were incubated with Molt4.8 cells. CAR expression was measured by the expression of the myc-tag after 4 d via flow cytometry. Side scatter area (SSC-A) is displayed on a linear scale, whereas the axis scale for the CAR (PE) is logarithmic.

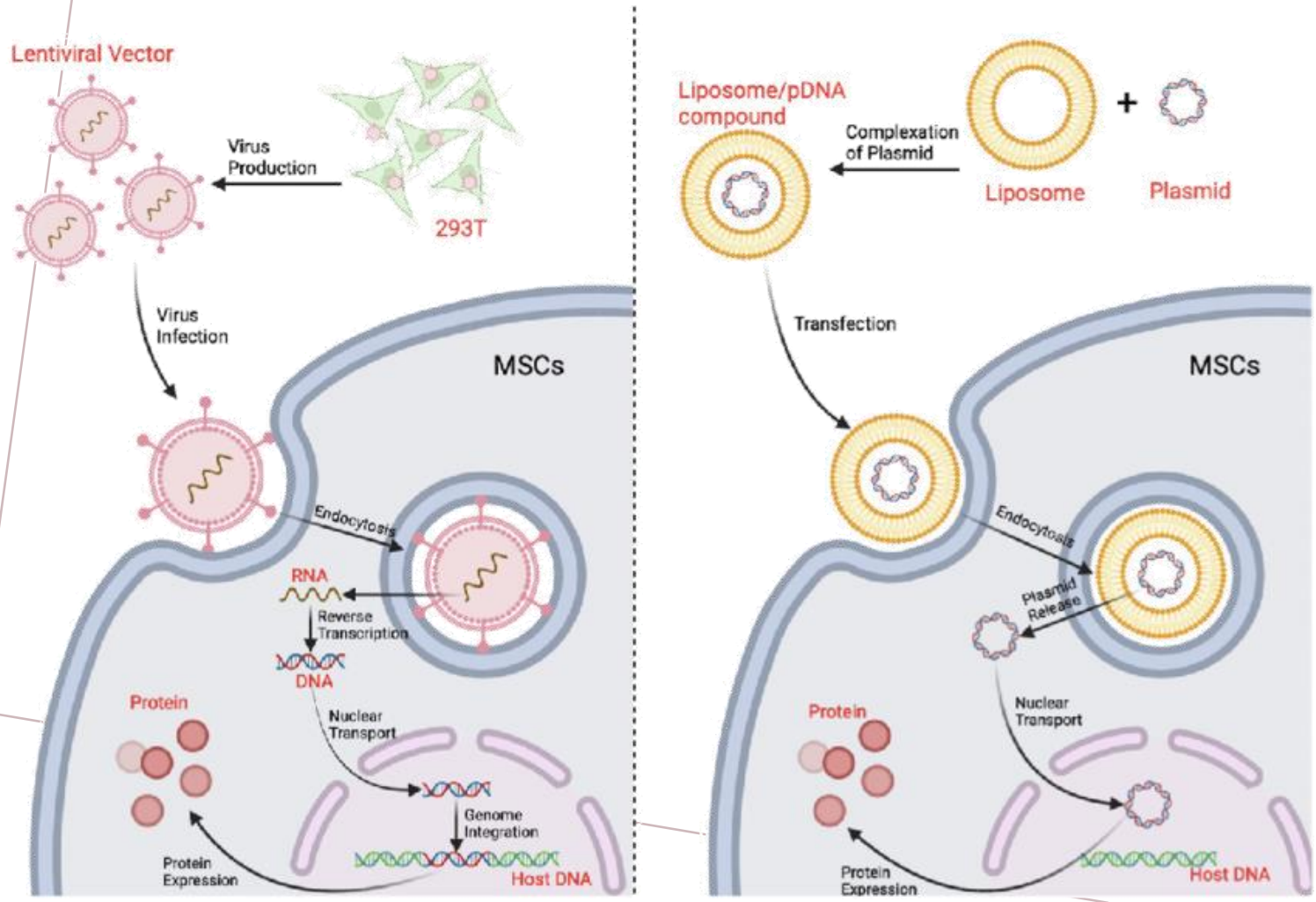
*Weidner, T., Agarwal, S., Perian, S. et al. Genetic in vivo engineering of human T lymphocytes in mouse models. Nat Protoc **16**, 3210–3240 (2021).*



Is using flow cytometry to determine CAR expression sufficient to identify transduction efficiency?

- Flow cytometry quantitates the amount of CAR/TCR that is expressed on the cell surface using specific fluorochrome-conjugated antibodies.
- A single-chain fragment variable can also be used but tends to have higher non-specific staining / binding and may require additional staining optimisation.

Therefore, the **Vector Copy Number (VCN)** for T cells is needed

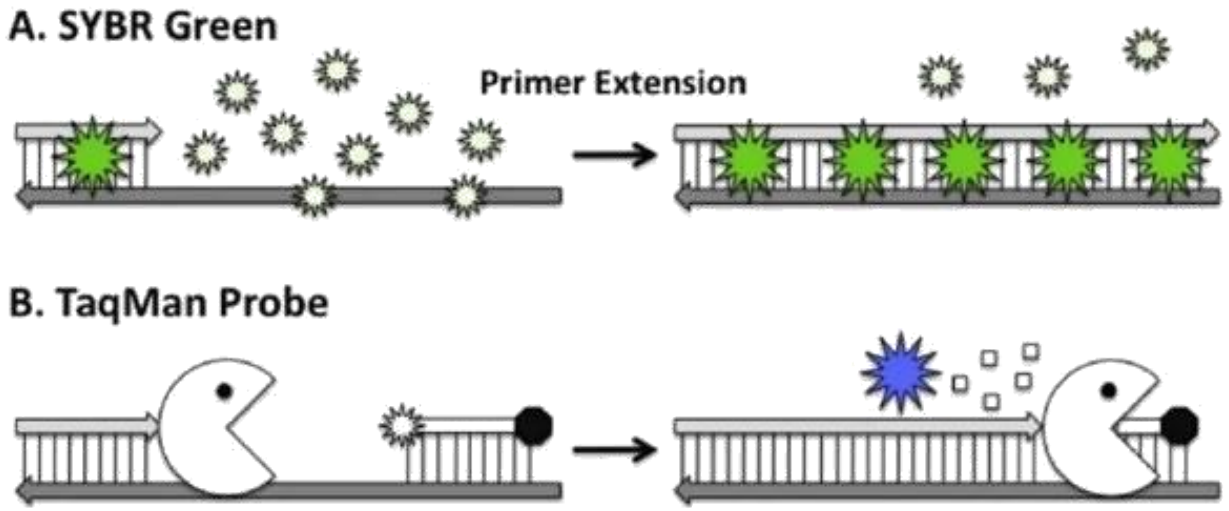


- **Vector copy number (VCN)** refers to the number of vector genomes integrated into the genome of the target cells.
- **Lentiviral vectors** are the preferred choice for ex vivo CAR transduction of T cells due to their integrase enzyme, which enables stable integration of the CAR gene into the dividing T cells.

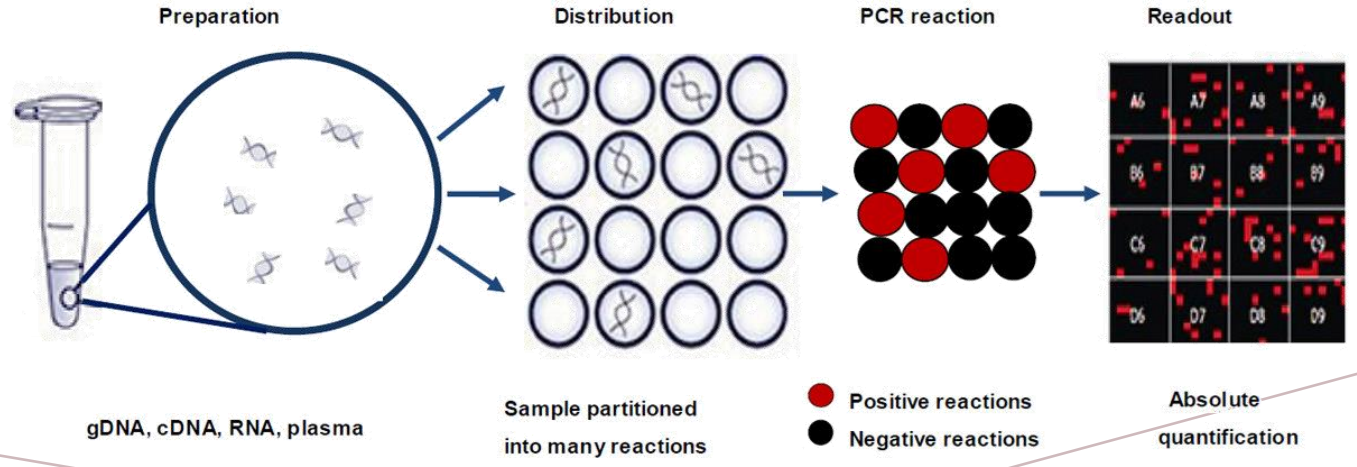
Ref: Zhou H, He Y, Xiong W, Jing S, Duan X, Huang Z, Nahal GS, Peng Y, Li M, Zhu Y, Ye Q. MSC based gene delivery methods and strategies improve the therapeutic efficacy of neurological diseases. Bioact Mater. 2022 Nov 30;23:409-437.

Most common method for VCN determination:
Quantitative polymerase chain reaction (PCR)

Real-time quantitative PCR (RT-qPCR)



Droplet Digital PCR (ddPCR)



RT-qPCR vs ddPCR

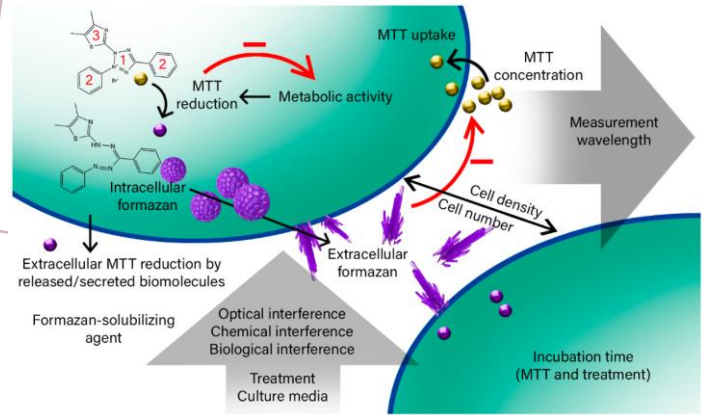
Comparative Strengths of qPCR and dPCR	
Strengths of qPCR	Strengths of dPCR
Established technology	Emerging technology
Relative measurement, ideally suited for gene expression analysis	Absolute measurement eliminates need for standard curve
Wide choice in detection chemistry and reaction volume equates to flexible running costs	High precision for better reproducibility for low input target concentrations
Large dynamic range	Greater sensitivity for rare mutation detection
Higher throughput, automation compatibility	Improved precision for higher copy number variation analysis

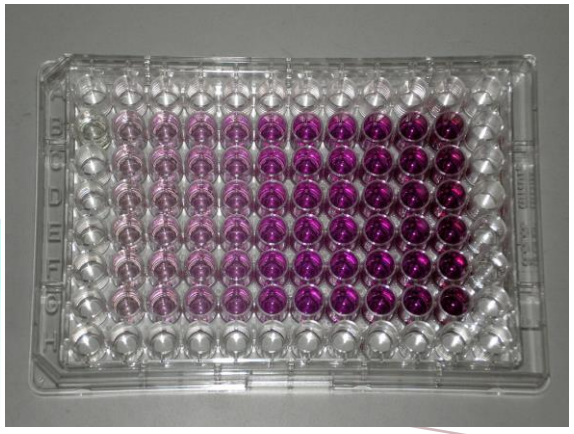
Image courtesy of Drug Discovery World

Cell viability

- **Viability** = The absolute number of living cells
- Can be based on
  - cellular membrane integrity (membrane integrity dyes),
  - cellular function such as enzymatic activity (enzyme activity substrates),
  - or metabolic activity (metabolic activity reagents).
- Can be measured by
  - fluorescence microscopy,
  - flow cytometry,
  - and microplate readers.

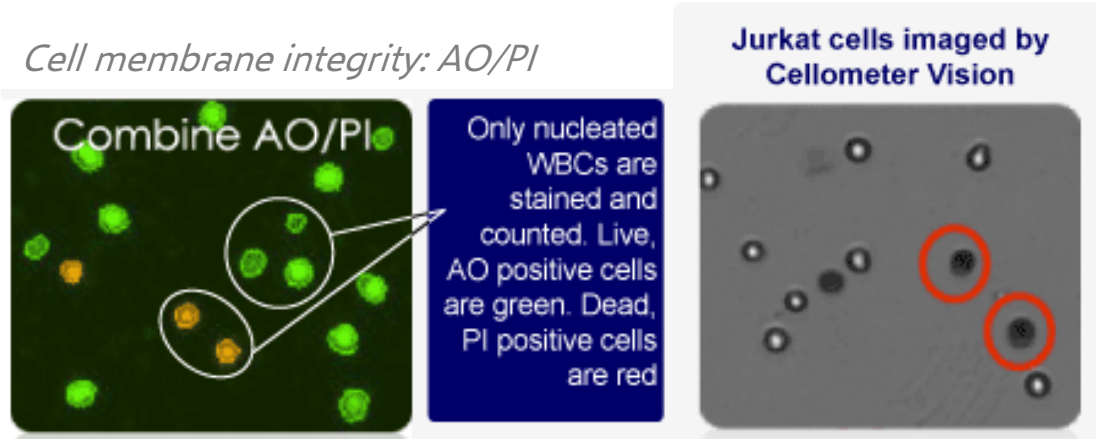
Cell membrane integrity: AO/PI



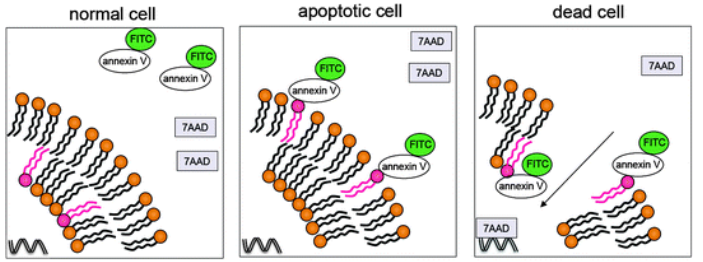


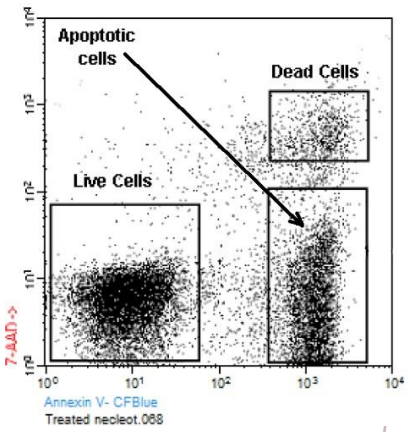
Cell membrane integrity: Trypan Blue

Cell membrane integrity: AO/PI

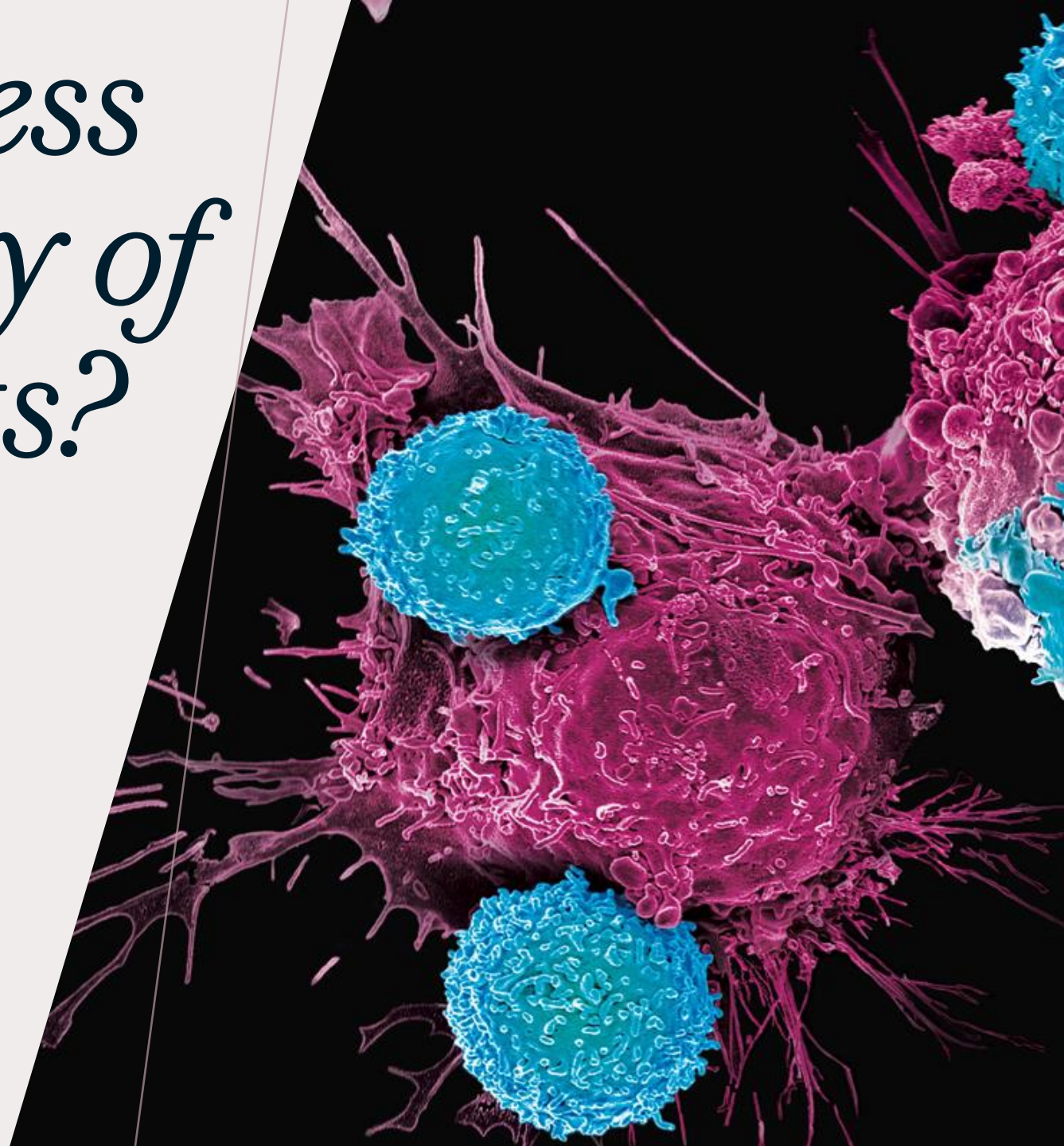


Cell membrane integrity: Annexin V/7-AAD

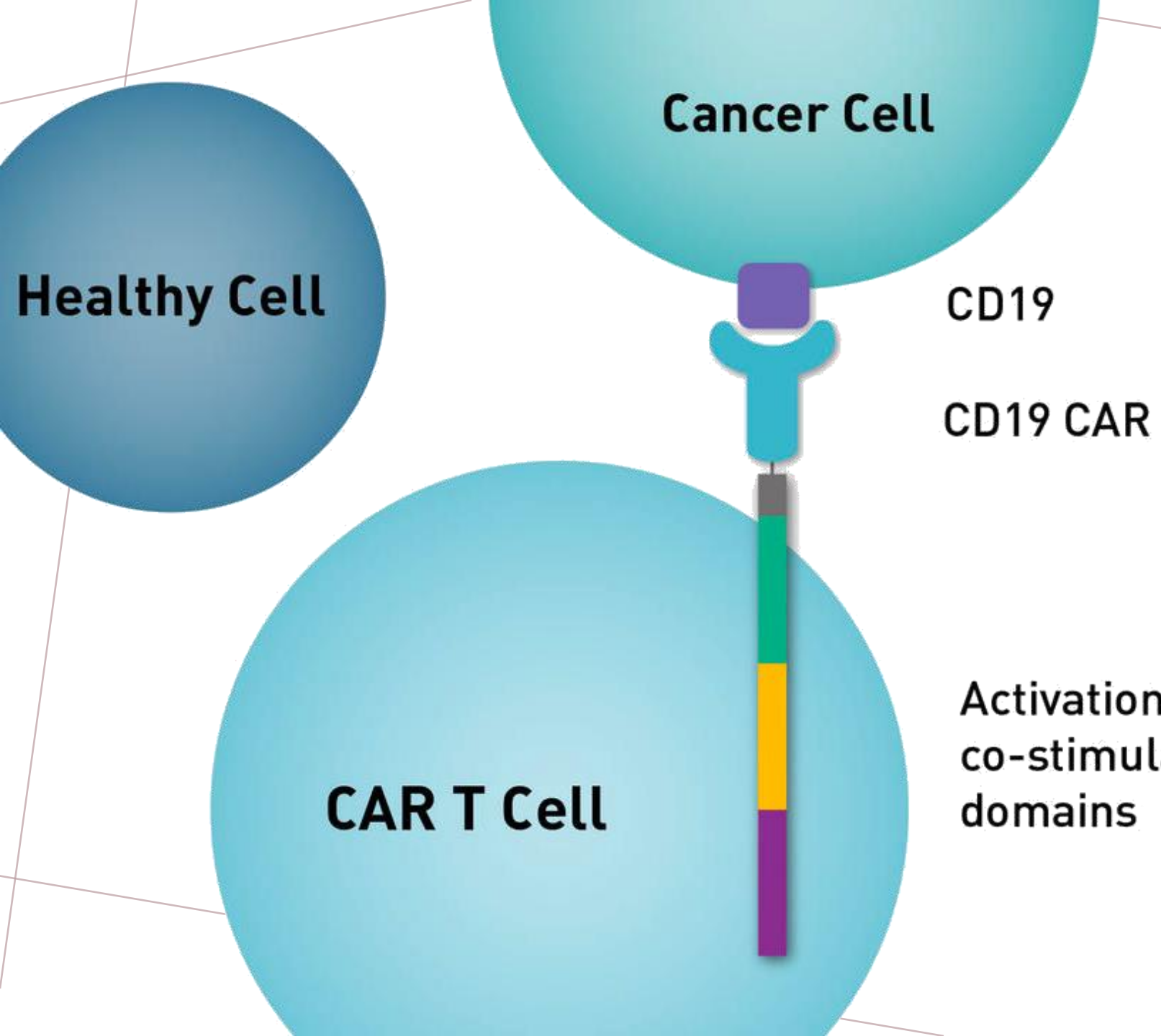




How do we assess the true efficacy of CAR T products?



Functional assay: Direct Tumor Killing/Cytotoxic assay



The gold standard of assessing CAR T function is *In vitro* cytotoxic assays which involve co-culturing the T cells with the target cells that express the specific antigen.

Example: Cytotoxicity determination

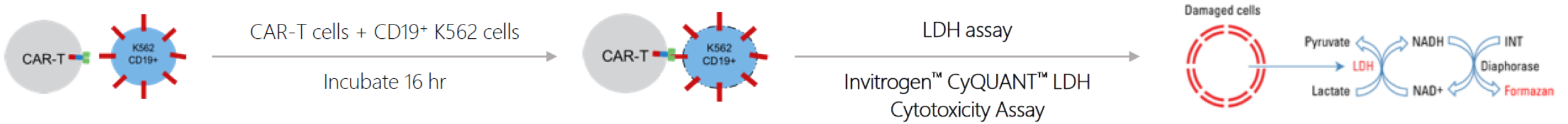


Image courtesy of Invitrogen, Thermo Fisher Scientific

Viability measurement could be conducted using:

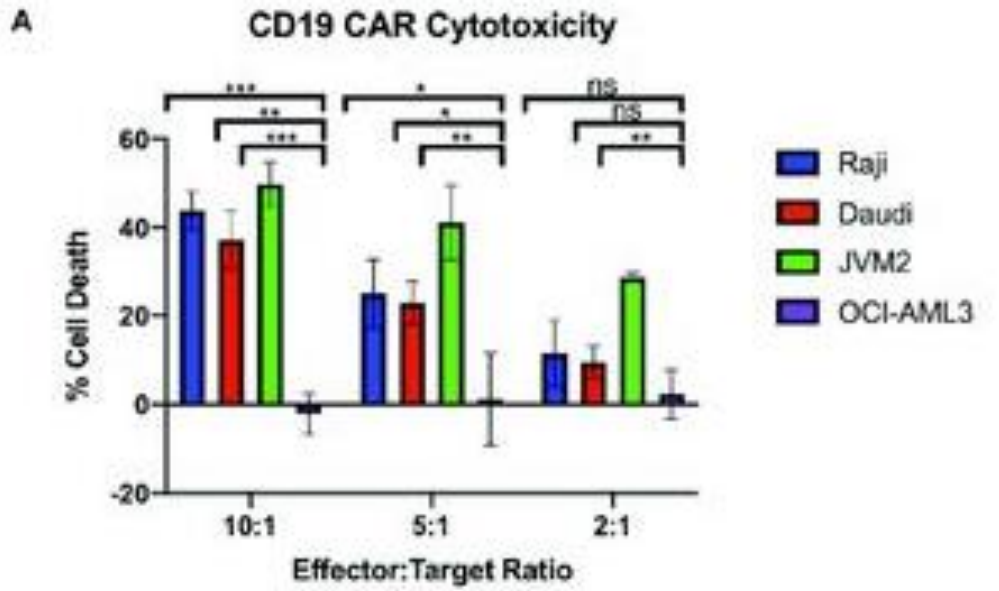
- MTT uptake,
- Chromium-51 release (although there is an industry-wide move away from radioactivity-based assays),
- Lactate dehydrogenase activity,
- and luciferase activity (which requires a luciferase expressing tumour target cell line).

General concerns

- Tumour target cells and level of antigen expression within the target cells.
- Other cell subtypes included in the assays (such as CD4 T cells which affect both CD8 T cell function and exhibit individual cytotoxic function).
- Effector:Target ratios and effectors based on total T cells or CAR+ T cells
- Controls used and the choice and read out of the assay itself.

Control selection

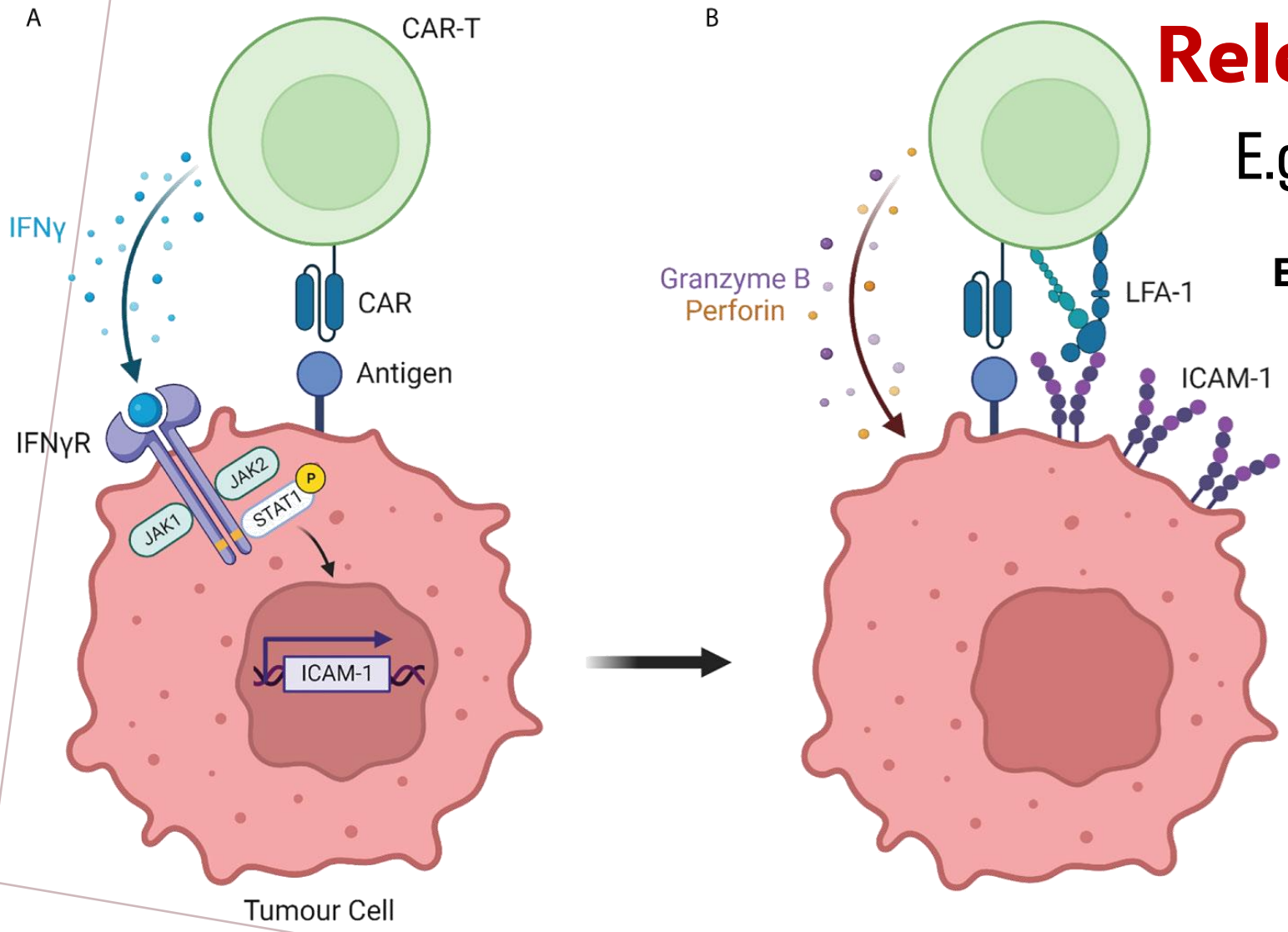
- Untransduced T cells to show background non-specific killing
- Target cell line with low or no expression of TAA to confirm the specifics of the T cell killing



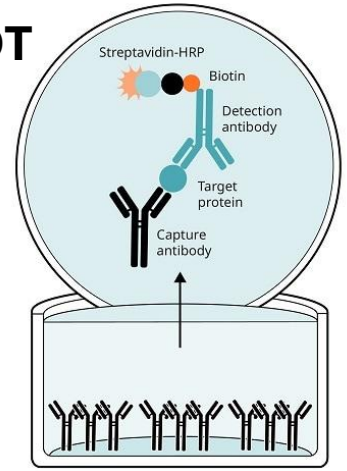
Fenlu Zhu, Nirav N. Shah, Huiqing Xu, Dina Schneider, Rimas Orentas, Boro Dropulic, Parameswaran Hari, Carolyn A. Keever-Taylor; CAR-T Cell Production Using the Clinimacs® Prodigy System. Blood 2016; 128 (22): 5724.

Functional assay:
Release of key cytokines

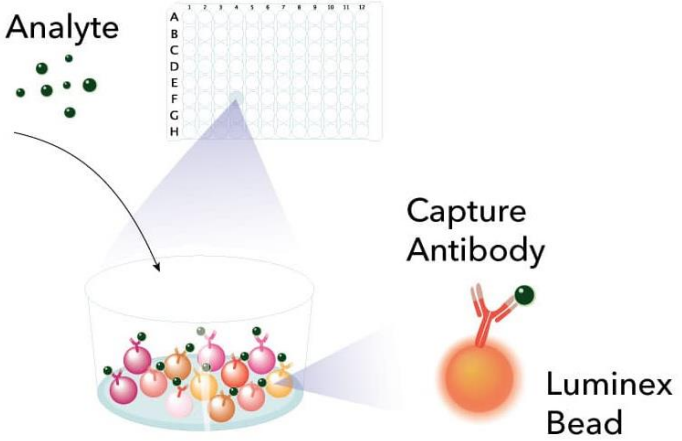
E.g., IFN-γ, IL-2, and Granzyme B



ELISA or ELISPOT

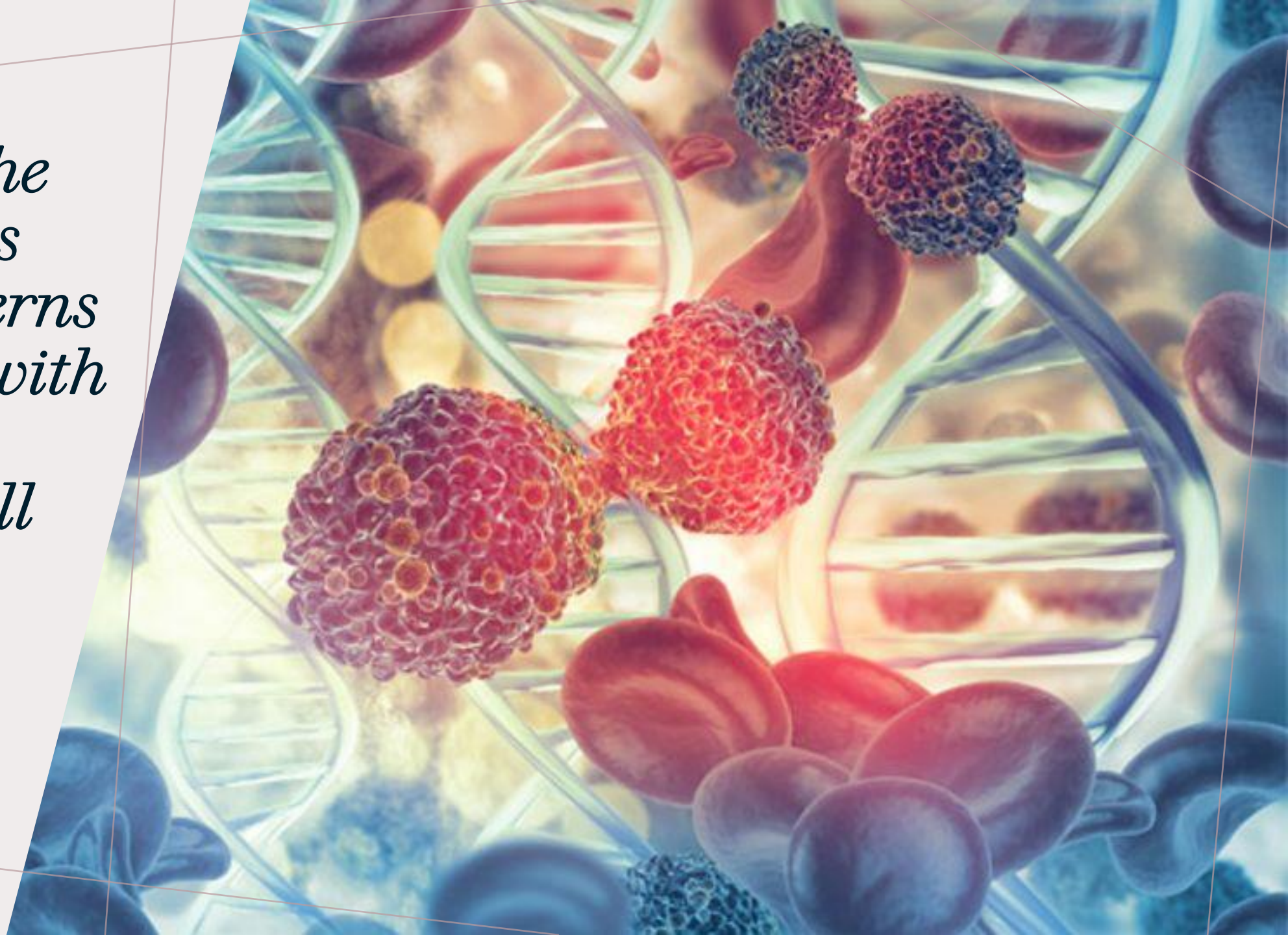


Multiplex assay

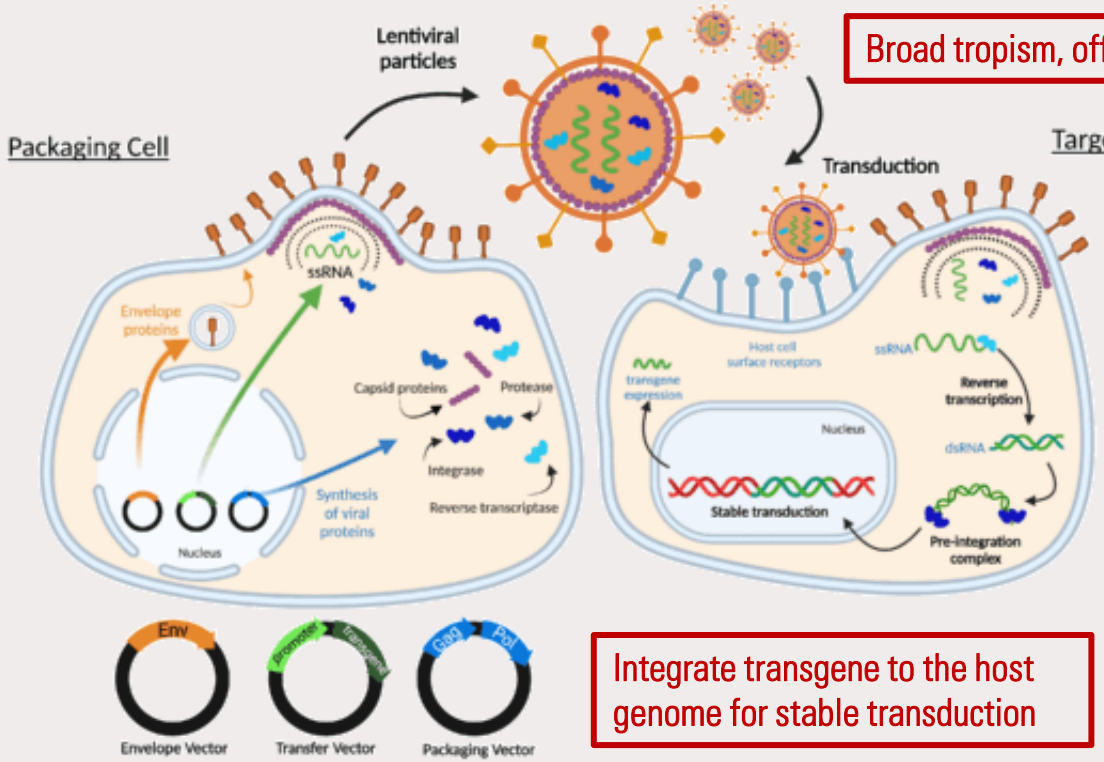


Ref: Espie D and Donnadieu E (2022) New insights into CAR T cell-mediated killing of tumor cells. Front. Immunol. 13:1016208.

What are the most serious safety concerns associated with genetically modified cell therapies?



Vectors we used are safe?



Broad tropism, off-target transduction

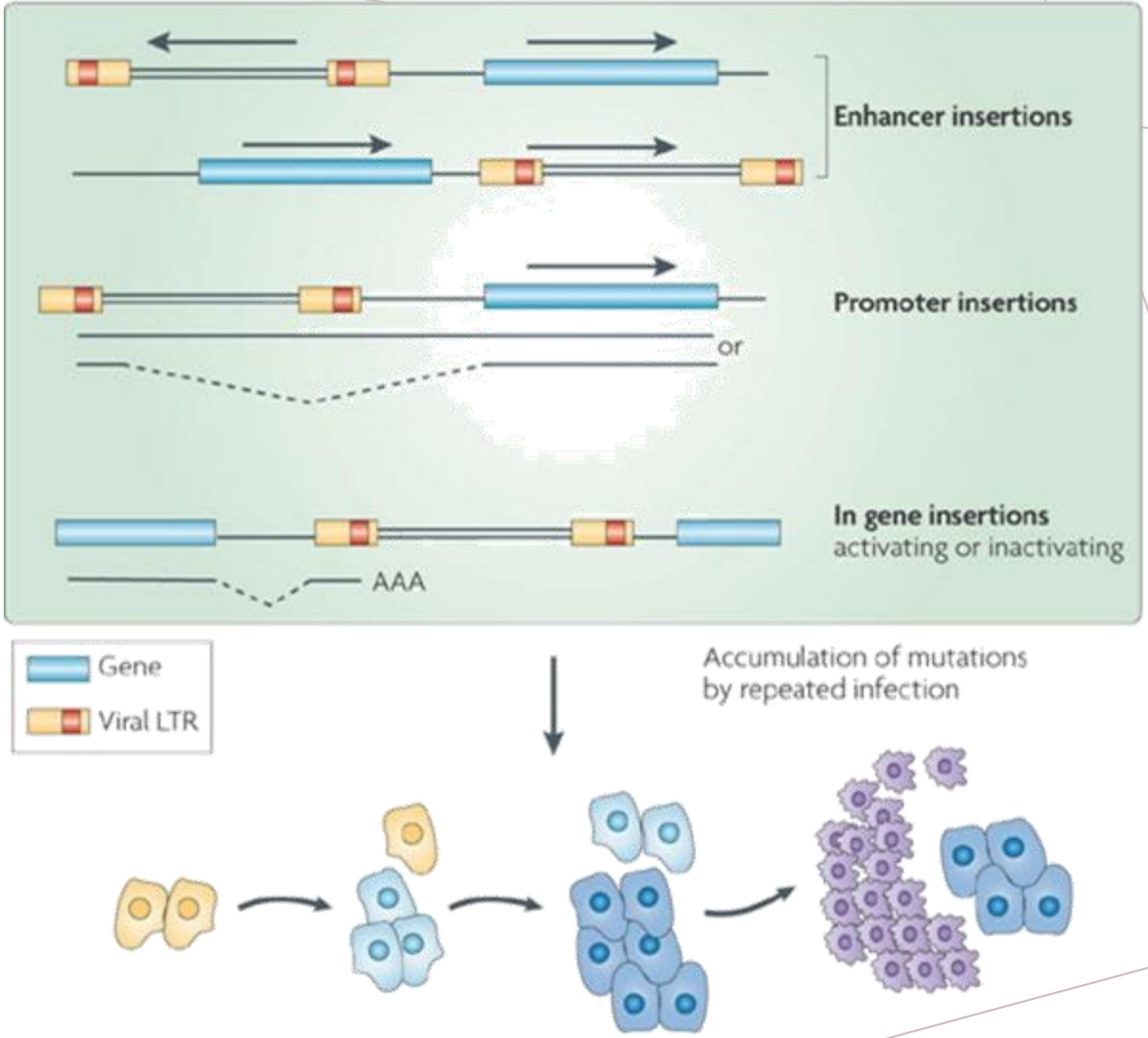
Enter cell nucleus and carry the integration machinery

Integrate transgene to the host genome for stable transduction

Insertional mutagenesis

ICH Considerations, General Principles to Address the Risk of Inadvertent Germline Integration of Gene Therapy Vectors

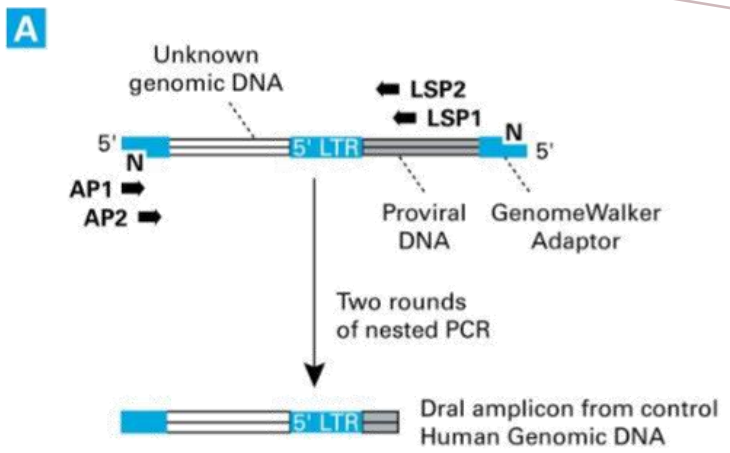
What happens when a lentiviral vectors integrate the transgene at the wrong location?

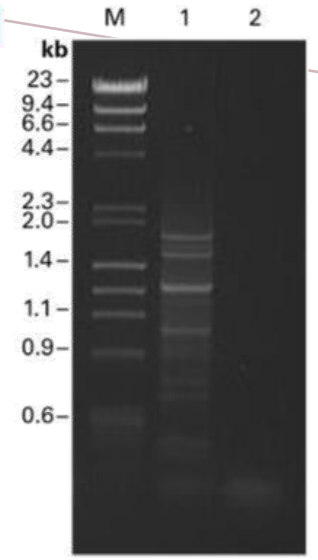


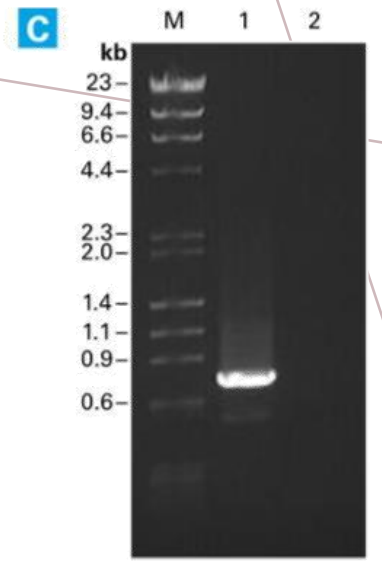
*Ref: Kool, J., Berns, A. High-throughput insertional mutagenesis screens in mice to identify oncogenic networks. Nat Rev Cancer **9**, 389–399 (2009).*

How to...

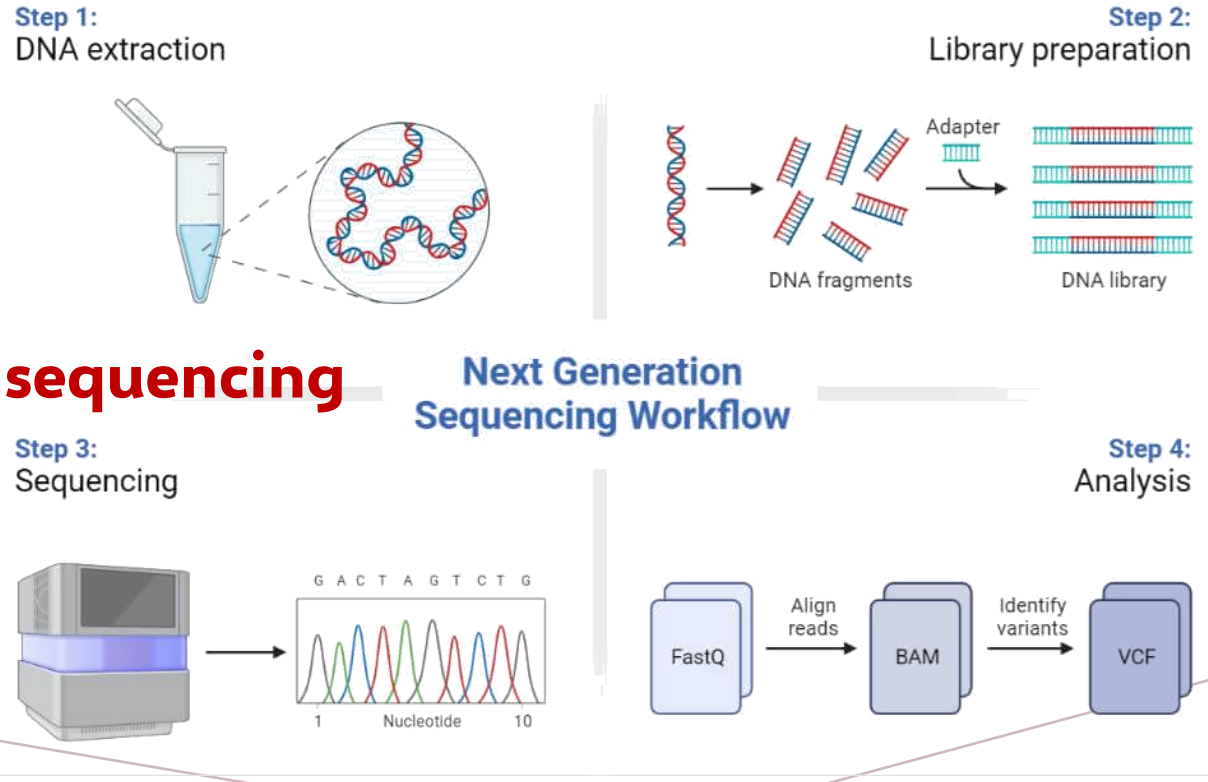
PCR





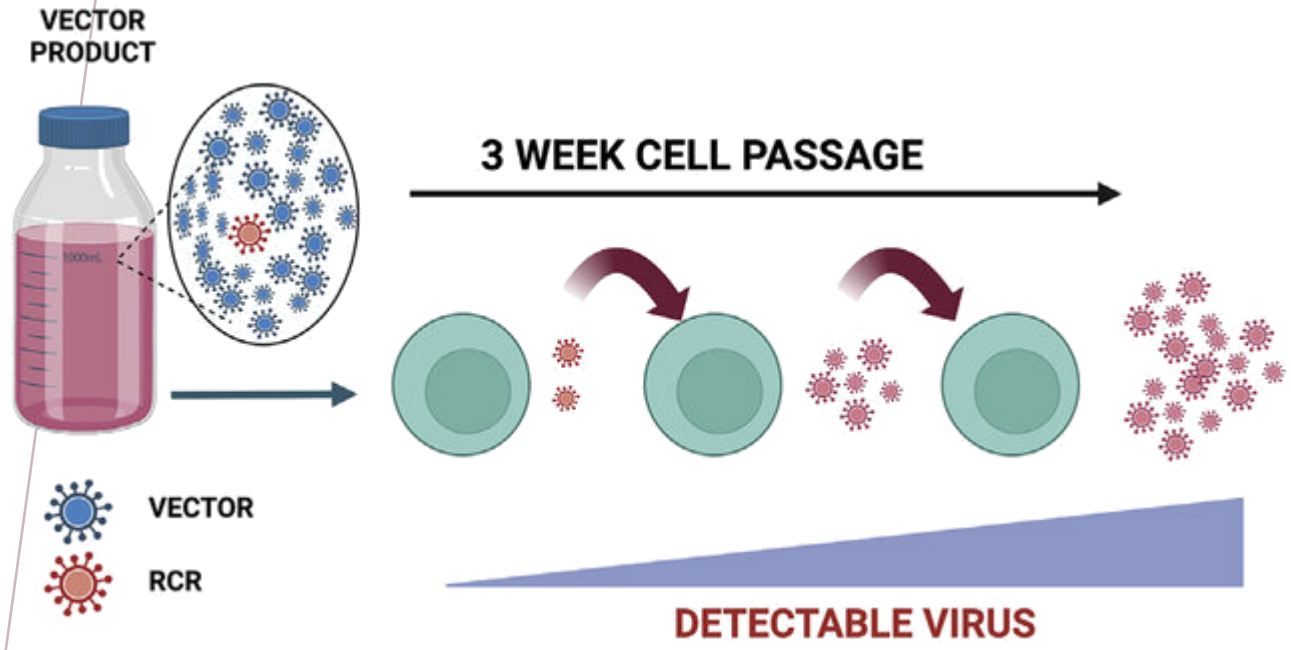


Whole genome sequencing



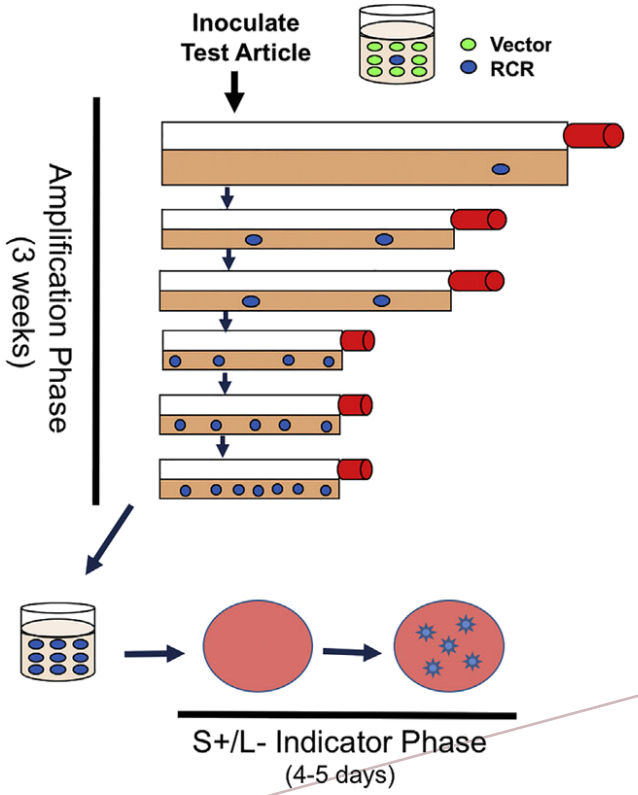
While uncommon, another risk to consider is…

Replication competent lentivirus (RCL)



Cornetta K, Lin TY, Pellin D, Kohn DB. Meeting FDA Guidance recommendations for replication-competent virus and insertional oncogenesis testing. Mol Ther Methods Clin Dev. 2022 Dec 2;28:28-39.

USFDA, Testing of Retroviral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up: Guidance for Industry



Ref: Cornetta K, Duffy L, Feldman SA, Mackall CL, Davila ML, Curran KJ, Junghans RP, Tang JY, Kochenderfer JN, O'Cearbhaill R, Archer G, Kiem HP, Shah NN, Delbrook C, Kaplan R, Brentjens RJ, Rivière I, Sadelain M, Rosenberg SA. Screening Clinical Cell Products for Replication Competent Retrovirus: The National Gene Vector Biorepository Experience. Mol Ther Methods Clin Dev. 2018 Aug 17;10:371-378.

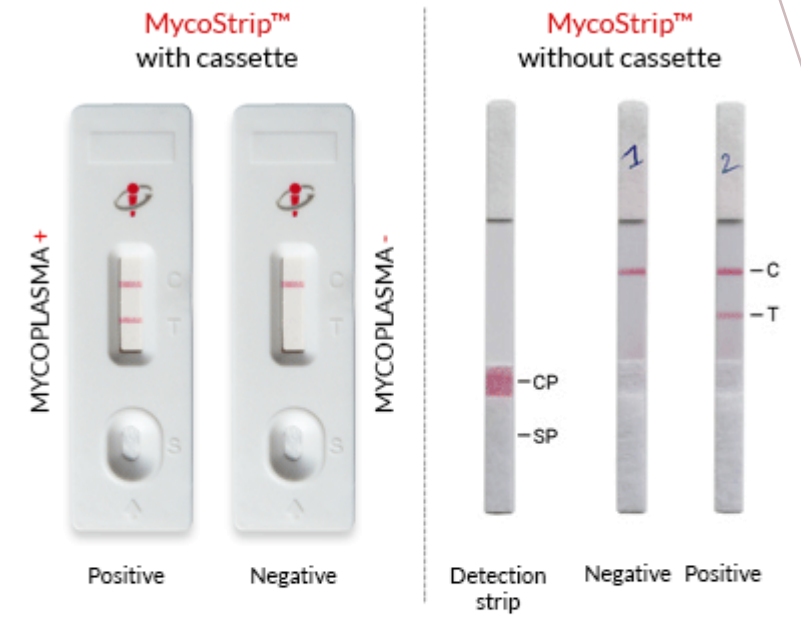
Traditional approach with a modern twist:

Sterility and Mycoplasma testing

Example: Rapid Sterile Testing

Image courtesy of Thermo Fisher Scientific

Example: Mycoplasma detection kit



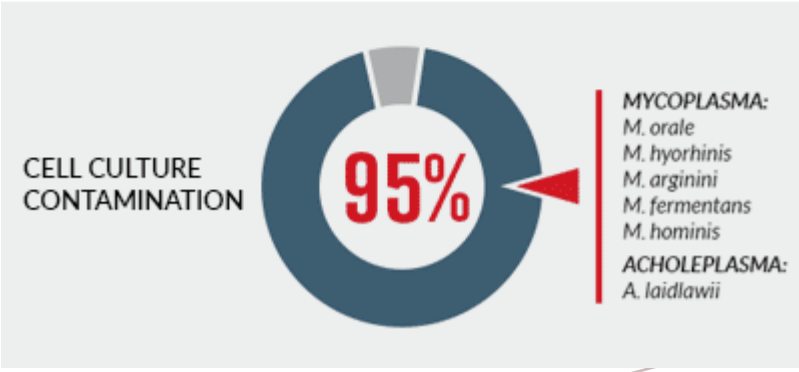


Image courtesy of Invivogen

The information presented here is for informational purposes only and is compiled from various established sources. This information may not be applicable to all situations and is not a substitute for legal advice. For specific guidance regarding your product or situation, please consult with the appropriate regulatory authorities.

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Email: Chavee.L@chula.ac.th

RELEVANT DOCUMENTS

FDA:

Considerations for the Development of Chimeric Antigen Receptor (CAR) T Cell Products: Guidance for Industry
https://www.fda.gov/media/156896/download

Potency Assurance for Cellular and Gene Therapy Products: Draft Guidance for Industry
https://www.fda.gov/media/175132/download

British Pharmacopoeia: Advanced Therapy Medicinal Products Guidance

- Application of Flow Cytometry
- Vector Copy Number
- Characterisation of the Capsid Particle Population for rAAV products
- T Cell and NK Cell Characterisation Assays

https://www.pharmacopoeia.com/guidance/atmp

EMA:

Quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells
https://www.ema.europa.eu/en/quality-non-clinical-clinical-aspects-medicinal-products-containing-genetically-modified-cells-scientific-guideline

Quality, preclinical and clinical aspects of gene therapy medicinal products
https://www.ema.europa.eu/en/quality-preclinical-clinical-aspects-gene-therapy-medicinal-products-scientific-guideline

Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials
https://www.ema.europa.eu/en/guideline-quality-non-clinical-clinical-requirements-investigational-advanced-therapy-medicinal-products-clinical-trials-scientific-guideline