

Advancing CAR-T Manufacturing Through Comprehensive Cellular Analysis and the Development of a Lentiviral Vector Platform

Dissertation

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Table of Contents

List of Abbreviations.....	VIII
List of Figures	XIII
List of Tables	XV
1 Introduction.....	1
1.1 Tumor Immunology.....	1
1.2 T cell Biology.....	3
1.2.1 T cell Maturation from Thymic Development to a Functional T cell.....	3
1.2.2 Epitopes Interaction in T-Cell Activation	4
1.2.3 Intracellular Signaling Cascades and Downstream Activation Pathways ..	5
1.2.4 Differentiation Stages and Subtype Diversity of CD4 ⁺ and CD8 ⁺ T cells....	7
1.2.5 Metabolic Pathways Shaping T-Cell Activation and Differentiation.....	12
1.2.6 T Cell–Based Therapeutic Approaches.....	15
1.3 Lentiviral Vectors.....	16
1.3.1 History of Retroviral Vector Technologies and Early Clinical Trials	16
1.3.2 Understanding Lentiviral Vector Biology.....	16
1.3.3 Advancements in Lentiviral Vectors and Plasmid System Designs.....	18
1.3.4 Scalable Production of Lentiviral Particles	20
1.4 Adoptive T cell therapy and the Application of Lentiviral Vectors.....	28
1.4.1 Chimeric Antigen Receptor T cells	28
1.4.1 Manufacturing of CAR-T Cells and Automation	30
1.4.2 Challenges of CAR-T cells	31
2 Aim and Perspective.....	32
3 Materials and Methods.....	33
3.1 Equipment and devices	33
3.2 Materials	33
3.2.1 Chemicals and Reagents.....	33
3.2.2 Filters and Plastic Consumables	34
3.2.3 Plasmids	35
3.2.4 Cell Culture Supplies	35
3.2.5 Cell Lines.....	36

3.2.6	Flow Cytometry Reagents	37
3.2.7	Kits	37
3.3	Lentivirus Manufacturing	37
3.3.1	Master Cell Bank Generation	37
3.3.2	Suspension HEK293 Cell Culture	38
3.3.3	Cell Cycle Analysis	38
3.3.4	Transfection	38
3.3.5	Bioreactor Setup	39
3.3.6	Bioreactor Inoculation and Cultivation	40
3.3.7	Lentivirus Concentration by Precipitation	40
3.3.8	Clarification	41
3.3.9	Transmembrane Pressure Scouting	41
3.3.10	Normalized Water Permeability Test	41
3.3.11	Ultrafiltration / Diafiltration	41
3.4	Lentivirus Quality Control	42
3.4.1	Cell Titration	42
3.4.2	Host Cell DNA	43
3.4.3	Host Cell Proteins	43
3.4.4	HEK Cell Cycle	43
3.5	CAR-T Cell Manufacturing	44
3.5.1	Manual T cell Isolation and Transduction	44
3.5.2	CliniMACS Prodigy® Manufacturing	45
3.6	Phenotyping, Functional and Intracellular Analysis	46
3.6.1	Cytotox Assay	46
3.6.2	Re-Challenge	46
3.6.3	Flow Cytometry Analysis	46
3.6.4	DigiWest Sample Preparation	48
3.6.5	Statistical Methods	48
4	Results	49
4.1	Lentiviral Vector Manufacturing Platform	49
4.1.1	Master Cell Bank Generation and Evaluation	49
4.1.2	Analysis of Cellular Biology for Optimum Transfection Time	50

4.1.3	Influence of Supplementation on Cell Cultivation	52
4.1.4	Transfection and Initial Functional Test of Lentivirus Production	54
4.1.5	Establishment of a Flow Cytometry-based Lentivirus Titration Method for the Determination of Transducing Units	55
4.1.6	Cell Cultivation-dependent Vector Stability in Varying Buffers.....	59
4.1.7	Adapting Seed Density to Accommodate Vector Stability	64
4.1.8	Scale-Up to Bioreactor Manufacturing	68
4.1.9	Ultrafiltration/Diafiltration Procedure for Lentiviral Vectors	72
4.1.10	Comparative Analysis of Clarification and TFF Setups	74
4.1.11	Evaluation of Cell Culture-related Impurities.....	76
4.2	Method Development and Panel Design for the Prodigy Process Analysis	81
4.2.1	Re-Challenge Model	81
4.2.2	Cellular Marker Panel Setup.....	82
4.2.3	Combining DigiWest with Re-Challenge.....	84
5	Manuscript – CD19-CAR-T phenotyping during Prodigy manufacturing uncovers critical T cell transition	86
6	Discussion.....	138
6.1	Establishment of a Comprehensively Characterized Master Cell Bank	139
6.2	Development of a Robust LV Titration Assay	139
6.3	Pre-Transfection Seeding Density Drives LV Titer and Stability	141
6.4	Bioreactor Cultivation Enables LV Production Scale-Up.....	144
6.5	Combinatorial Testing Enables the Identification of an Optimized Purification Strategy	146
6.6	Host Cell–Derived Impurities: Analysis and Risk Considerations.....	149
6.7	Development of a Tailored Intracellular Marker Panel to Monitor CAR-T Cell Expansion and Functional Dynamics.....	151
6.8	Conclusion and Outlook.....	154
7	Summary	155
8	Zusammenfassung.....	157
9	Statement.....	159
10	Declaration of Contribution	160
11	List of Publications	161

11.1	Scientific journals	161
11.2	Contribution for scientific Meetings	161
11.3	Presentation, Poster	161
12	References	162
13	Appendix	179

List of Abbreviations

ADP	Adenosine diphosphate
AEX	Anion-exchange chromatography
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
AP-1	Activator protein 1
APCs	Antigen presenting cells
ATF	Activating transcription factor
ATMPs	Advanced therapy medicinal products
ATP	Adenosine triphosphate
BCL10	B-cell lymphoma/leukemia 10
Bcl-2	B-cell lymphoma 2
bp	Base pair
CA IX	Carbonic Anhydrase IX
Ca ²⁺	Calcium ions
CAR	Chimeric antigen receptor
CAR	Chimeric antigen receptor
CARMA1	Caspase recruitment domain-containing membrane-associated guanylate kinase protein 1
CBM	CARMA1-BCL10-MALT1 complex
cGMP	Current Good Manufacturing Practice
CM	Co-stimulatory motif
CoA	Acetyl-Coenzyme A
CRAC	Ca ²⁺ release-activated Ca ²⁺ channels
CRS	Cytokine release syndrome
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DAG	Diacylglycerol
DC	Dendritic cell
DN	Double negative
DNA	Desoxyribonucleic acid
DO	Dissolved oxygen
DOPE	1,2-Dioleoyl-sn-glycerophosphoethanolamine
DP	Double positive
dsDNA	Double-stranded DNA
DSP	Downstream processing
DV	Diafiltration volume
E:T	Effector to target ratio

EMA	European Medicines Agency
ER	Endoplasmic reticulum
Erk	Extracellular signal-related kinase
FACS	Fluorescence-Activated Cell Sorting
FAO	Fatty acid oxidation
FasL	Fas-ligand
FBS	Fetal bovine serum
FFA	Free fatty acids
FoxO3a	Forkhead-Box-Protein O3
Foxp3	Forkhead Box P3
GFP	Green fluorescence protein
GVHD	Graft-versus-host disease
HCP	Host cell protein
HIF1	Hypoxia inducible factor 1
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
ICIs	Immune check point inhibitors
IEG	Immediate-early gene
IFN- γ	Interferon- γ
IG	Infectious genomes
IKK	I κ B kinase
IL	Interleukin
IP3	Inositol-1,4,5-trisphosphate
ITAMs	Immunoreceptor tyrosine-based activation motifs
I κ B	Inhibitor of NF- κ B
Kb	Kilo base
LAT	Linker for Activation of T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LDHA	Lactate dehydrogenase A
LOD	Limit of detection
LTR	Long terminal repeats
LV	Lentiviral vectors
MALT1	Mucosa-associated lymphoid tissue translocation protein 1
MAPK	Mitogen-activated protein kinase
MCB	Master cell bank
Mef2	Myocyte enhancer factor 2
MHC	Major histocompatibility complex
MOI	Multiplicity of infection

mTOR	Mammalian target of rapamycin
NF	Nuclear factor
NFAT	Nuclear factor of activated T-cells
NK	Natural killer (cells)
NMI	Naturwissenschaftliches und Medizinisches Institut Reutlingen
NSCLC	non-small-cell lung cancer
NWP	Normalized water permeability
P/S	Penicillin (100 units/mL) / Streptomycin (100 µg/mL)
P/V	Power-to-volume
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed cell death-1
PDK1	Phosphoinositide-dependent kinase 1
PEI	Polyethyleneimine
PES	Polyether sulfone
P _F	Feed pressure
PI3K	Phosphoinositid-3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC γ	Phospholipase C- γ
PM	LV MAX™ Production Medium
PPP	Pentose phosphate pathway
P _R	Retentate pressure
PRR	Pattern recognition receptor
PS	Polysulfone
PTKs	Protein tyrosine kinases
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RC	Regenerated cellulose
RCL	Replication competent lentiviruses
Rib-5P	Ribose-5-phosphat
RNA	Ribonucleic acid
RP-HPLC	Reversed-phase high performance liquid chromatography
rpm	Revolutions per minute
RT	Room temperature
RTC	Reverse transcription complex
scFv	Single-chain variable fragment
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC	Size-exclusion chromatography
SIN	Self-inactivating (lentiviral vector)
SMAC	Supramolecular activation cluster
SP	Single positive
sscDNA	strong stop copy DNA
ssRNA	Single-stranded RNA
STAT	Signal transducers and activators of transcription
TAE	Tris-Acetate-EDTA(-buffer)
TAR	Trans activation response
TC	Tumor contact
TCA	Tricarboxylic acid (cycle)
TCF1	T cell factor 1
T _{CM}	Central memory T cells
TCR	T cell receptor
T _{EFF}	Terminal effector T cells
T _{EM}	Effector memory T cell
T _{EMRA}	Terminally differentiated effector memory cells re-expressing CD45RA
Tet	Tetracycline
TFF	Tangential flow filtration
Tfh	T Follicular cells
TGF- β	Transforming Growth Factor- β
Th	T Helper (cell)
Th17	T helper 17 cells
TIL	Tumor infiltrating lymphocyte
TLR	Toll like receptor
TMP	Transmembrane pressure
T _N	Naïve T cells
TNFR	Tumor necrosis factor receptor
TRAF6	TNF receptor-associated factor 6
TRE	Tetracycline-Response-Element
Treg	Regulatory T cells
tRNA	Transfer RNA
TRUCK	T-cell redirected for universal cytokine-mediated killing
T _{SCM}	Stem-like memory T cells
TU	Transducing unit
UF/DF	Ultrafiltration/Diafiltration
USP	Upstream processing
VCC	Viable cell concentration
VCN	Vector copy number

VSVg	Vesicular stomatitis virus G glycoprotein
WAS	Wiskott-Aldrich syndrome
ZAP70	Zeta chain of TCR-associated protein 70

List of Figures

Figure 1 Schematic representation of the T-cell activation cascade as described in Schinle et al.....	7
Figure 2 Schematic of an HIV-1 particle.	17
Figure 3 Lentiviral generations and plasmid separation.	20
Figure 4 Bag configuration of Sartorius Flexsafe® RM 2 L optical.....	39
Figure 5 Official CliniMACS Prodigy® TS 520 setup	45
Figure 6 Cultivation data for master cell bank generation.....	49
Figure 7 Cultivation data plot of three cultivations compared.	50
Figure 8 Cultivation data plots for 6-day-cultivation.....	51
Figure 9 Cell cycle data collected after split of LV MAX™ HEK293 cells and over the course of 6 days.	52
Figure 10 Cell cultivation of LV MAX™ HEK293 data one day after treatment with either sodium butyrate (NaBu), LV MAX™ Supplement, LV MAX™ Enhancer or untreated....	53
Figure 11 Dot plot of cell cycle data of LV MAX™ HEK293 cells treated with either sodium butyrate (NaBu), LV MAX™ Supplement, LV MAX™ Enhancer or untreated.....	54
Figure 12 Flow cytometry plot of Jurkat cell line 9 days after transduction with construct #119 (CD276-CAR with co-expression of mCherry).	55
Figure 13 Jurkat cultivation optimization for reproducibility.	55
Figure 14 HT1080 cultivation optimization for reproducibility.	56
Figure 15 Lentivirus titration on Jurkat cells via GFP-expression.....	57
Figure 16 Lentivirus titration on HT-1080 cells via GFP-expression.	57
Figure 17 Flow cytometry analysis for detection of CAR and co-expressed CD19 tag. ..	58
Figure 18 Lentivirus titration on HT-1080 cells via antibody-fluorochrome detection. ..	58
Figure 19 Lentivirus stability of clarified supernatant over time.	59
Figure 20 Lentivirus stability in varying buffer conditions over time.....	60
Figure 21 Lentivirus recovery in varying buffer conditions after 4 hours.	61
Figure 22 Lentivirus recovery in varying buffer conditions after one freeze-thaw cycle.	62
Figure 23 Cultivation data of prior to stability assay (Harvest 1 to 4).....	64
Figure 24 Cultivation data of prior to different seed strategies.....	65
Figure 25 Cultivation data of after different seed strategies.	66
Figure 26 Cell cycle data collected between day 10 (day before transfection) and 11 (day of transfection).....	67
Figure 27 Varying seed density and the effect on lentivirus stability in different buffer conditions over time with.	68
Figure 28 Cultivation data prior and during bioreactor cultivation.	69
Figure 29 Bioreactor data on dissolved oxygen (DO) and pH.	71

Figure 30 Agarose gel electrophoresis before and after DNase treatment in the bioreactor.	72
Figure 31 Pressure process data of Harvest 6.	73
Figure 32 Volumetric process data of Harvest 6.	73
Figure 33 Lentivirus yield during purification steps Benzonase (DNase) treatment, clarification, ultrafiltration and diafiltration for Harvest 5 to 7.	75
Figure 34 Lentivirus yield during purification steps Benzonase (DNase) treatment, clarification, ultrafiltration and diafiltration for Harvest 8 to 10.	76
Figure 35 Standard curve of UV signal after SYBR Gold staining of a dilution series of the plasmid for plasmid DNA.	77
Figure 36 x-y plot of SYBR Gold staining linearity by comparison of the expected concentration (according to UV absorption spectrophotometry) to the calculated concentration based on the dilution factor.	77
Figure 37 x-y plot of control measurement with samples from Harvest 10 measured against spiked samples (added 4 ng/ μ L of gag/pol packaging plasmid per sample).	78
Figure 38 DNA clearance during purification steps Benzonase (DNase) treatment, clarification, ultrafiltration and diafiltration for Harvest 8 to 10.	78
Figure 39 Agarose Gel of different purification steps.	79
Figure 40 Standard curve of dilution series of HEK293 host cell protein standard.	79
Figure 41 Host cell protein (HCP) clearance during purification steps Benzonase (DNase) treatment, clarification, ultrafiltration and diafiltration for Harvest 8 to 10.	80
Figure 42 Re-challenge combined with cytotoxicity assay on tumor cell lysis.	82
Figure 43 Matrix plot of DigiWest data on variance during CAR-T and T cell expansion.	84
Figure 44 Matrix plot of DigiWest analytes signals of expansion no. 3.	85
Figure 45 Linear titration range for calculation of Transducing Units (TUs).	141
Figure 46 Schematic overview for a cell cycle.	143
Figure 47 Insights gained from investigating the key factors influencing factors for lentiviral vector stability.	144
Figure 48 Upstream processing (USP) seed train.	146
Figure 49 Selected strategy for Downstream processing of lentiviral vectors.	147
Figure 50 Panel design for DigiWest analysis.	153

List of Tables

Table 1 Differentiation into CD4+ Subsets.....	8
Table 2 T cell development stages.	9
Table 3 Markers to detect differentiation of CD4+ T naïve cells	11
Table 4 Markers to detect differentiation of CD4+ T regulatory (Treg) cells.....	11
Table 5 Biotech terminology and its description.....	23
Table 6 Current regulatory specifications for LV batches	26
Table 7 Summary of CAR construct generations reviewed in	29
Table 8 Cultivation volumes for LV MAX™ HEK293 cells.	38
Table 9 Bag configuration of optical probes of pH and dissolved oxygen (DO).	40
Table 10 Two different dilution series used on effector to target ratios (CAR-T to tumor cell).	46
Table 11 Flow cytometry panel combination for activation and exhaustion profiling and phenotyping of CAR-T cells.	47
Table 12 Gating Strategy for T cell activation.....	47
Table 13 Gating strategy for T cell subtypes.	48
Table 14 Analysis with Radiometer of samples taken from bioreactor, 6 and 24 hours after initiation of medium conditioning.	69
Table 15 Experimental design for the initial purification step clarification.	74
Table 16 Experimental design for the second purification steps ultra-/diafiltration.....	74
Table 17 Sampling pattern for three CAR-T or T cell expansion.....	83
Table 18 Purification setup in direct comparison.....	149

1 Introduction

1.1 Tumor Immunology

Major causes of deaths globally in 2019 in industrialized nations were cardiovascular diseases, ischemic heart disease and stroke together, accounting for roughly 30 – 35 % of all deaths. This is followed by cancers which typically are the second leading cause, responsible for about 20 – 25 % of deaths. Other diseases include chronic respiratory diseases, diabetes, and other metabolic disorders which also add significant mortality [1].

Cancer is caused by progressive growth of a single transformed cell. This cell gained new capabilities which are described as hallmarks of cancer and include recruitment of normal cells, sustained proliferations signaling, evading growth suppressors, invasion and metastasis, replicative immortality, inducing angiogenesis and resisting cell death [2].

The recruitment of normal tissue forms tumor-associated stroma, as active participants in tumorigenesis rather than passive bystanders. These structures have been recognized as organs with complexity approaching or even increasing normal healthy tissue. The biology of a tumor can be studied by observing cell types as well as the tumor microenvironment which is constructed during tumorigenesis. Several cell types contribute to the biology of tumors and regulate signaling that controls their collective functions. Most observations stem from the study of carcinomas in which neoplastic epithelial cells constitute a compartment that is distinct from the mesenchymal cells forming the tumor-associated stroma [2].

The human immune system is a development in response to different diseases, roughly divided into the three pillars of immunology. The first line of defense is the barrier. It consists of the skin, mucosal layer and is a shield for the initial entry. The second line of defense is the innate immunity, acting via pattern recognition receptors (PRRs). The third pillar is represented by the adaptive immunity. This type of immunity is developed after birth and can be described as a system that learns with each encounter of pathogens [3].

In tumor development hallmark is the evasion of immune response. It has been shown that mice develop carcinogen-induced tumors more rapidly if deficient for various components of the immune system. In particular, deficiencies in the development or function of CD8+ cytotoxic T lymphocytes (CTLs) [4], CD4+ Th1 helper T cells and natural killer (NK) cells [5, 6]. It has been shown that tumor clonal evolution patterns during metastatic progression depends on the immune contexture at the metastatic site. Tumor cells and their microenvironment communicate, indicating that the immune system is shaping cancer evolution [7].

Infiltrating cells of the immune system are increasingly accepted to be generic constituents of a tumor. There are both tumor-promoting and tumor-antagonizing leukocytes, in various proportion [2]. Tumor-promoting inflammatory cells include macrophage subtypes, mast cells, neutrophils, as well as T and B lymphocytes [8-12]. Despite this, the best line of defense against tumors remains the adaptive immune system or more precise T cells.

1.2 T cell Biology

1.2.1 T cell Maturation from Thymic Development to a Functional T cell

T cells are one of the most abundant lymphocyte populations in the bloodstream. T cells develop from thymus-migrant hematopoietic lineage cells, particularly common lymphoid progenitors or lymphoid-primed multipotent progenitors derived from the bone marrow or the fetal liver. Developing T cells in the thymus pass through four double-negative stages (DN1-4). This is followed by a double-positive (DP) stage and finally maturation into single-positive (SP) naïve T cells (T_N). DN1-4 cells can be differentiated by their expression of CD44 and CD25 as follows: DN1: $CD44^+CD25^-$, DN2: $CD44^+CD25^+$, DN3: $CD44^-CD25^+$ and DN4: $CD44^-CD25^-$ [13]. An important family of transcription factors contain zinc finger motifs that is involved is the GATA family. The members of the GATA recognize the DNA consensus sequence A/T-G-A-T-A-A/G (Merika and Orkin 1993). GATA-3 was initially identified as a regulator of the *Tcra* gene, which binds to a $T\alpha 3$ element in the *Tcra* enhancer [14]. It turned out that GATA-3 is crucial for T cell development, among other things for the transition from DN2 to DN3 populations (Ting, Olson et al. 1996, Pai, Truitt et al. 2003). In addition, it plays a crucial role in the differentiation of T helper (Th) 2 cells [15]. In the DN3 stage, a pre-T cell receptor (TCR) complex consisting of a pre-TCR α chain and a mature β -chain appears for the first time. The mature β -chain in this complex is a product of somatic DNA rearrangement by recombination. T cells with a functional pre-TCR are positively selected by β -selection and will proliferate [3].

At the DP stage, both the CD4 and CD8 coreceptors are expressed ($CD4^+CD8^+$). The $\alpha\beta$ -TCR is formed in this stage by replacing the pre-TCR α chain with the TCR α chain. DP-T cells encounter further checkpoints: DP-T cells that express $\alpha\beta$ -TCRs that recognize their MHC molecules by TCR rearrangement are positively selected, whereas strongly self-reactive, T cells are partially deleted by negative selection. However, there are exceptions to this, which are discussed later in this chapter. In addition, DP-T cells with dysfunctional TCRs that are unable to receive or transmit TCR-mediated signals undergo apoptosis, while the selected. During development in the thymus, precursors that are $CD4^+CD8^+$ commit to one lineage. Those recognizing major histocompatibility complex (MHC) class II commit to CD4, while those recognizing MHC class I will differentiate into the CD8 lineage [3].

$CD4^+$ T cells are a type of T helper lymphocyte. They are able to recognize antigens presented on MHC class II molecules [16]. The function of MHC class II molecules is to transmit antigens of pathogenic origin through special cells called antigen-presenting cells (APCs), such as macrophages and dendritic cells. In their helper function, $CD4^+$ T cells coordinate in the immune system and interact directly with other immune cells via cytokines and direct cell interaction. TCR affinity is a crucial factor for the development of T helper cells [16].

CD8⁺ T cells are referred to as cytotoxic T lymphocytes that are restricted to MHC class I antigen detection. MHC class I is found on most nucleated cells and is also regarded as a window into the cell. Any malignant developments within the cell or viral infections may be presented to the outside of the immune system. CD8⁺ cells are able to directly kill infected or malignant cells via various cytotoxic functions [17].

1.2.2 Epitopes Interaction in T-Cell Activation

Activation and clonal expansion of T_N cells is often referred to as priming. It is controlled by a variety of signals. The initial signal is the TCR-mediated signal as soon as the TCR-MHC complex is formed with an APC. Cytokines and the interaction with co-stimulators then lead to the full activation and differentiation into various effector cell types.

The encounter between the T cell and the APC is followed by the reorganization of receptors and intracellular proteins into clusters, forming what this group has termed the “immunological synapse” [18]. This structure is organized to form a distinct central region, which contains high concentrations of the (TCR)-CD3 and CD28 clusters as well as the signaling intermediate PKC θ , and also a peripheral adhesion region called the peripheral supramolecular activation cluster (SMAC). The TCR is localized in small structures at the edge of the synapse and then works its way increasingly into the center in an active process [19, 20].

The TCR complex itself consists of the TCR $\alpha\beta$ chains and the CD3 $\gamma/\delta/\epsilon/\zeta$ subunits. The CD3 complex transduces the TCR-triggered signal through immunoreceptor tyrosine-based activation motifs (ITAMs) which present a tail located in the cytoplasm. The CD3 signal is indirect, it is not involved in antigen recognition. The CD3 $\gamma/\delta/\epsilon$ chains each contain one ITAM, while CD3 ζ chains contain 3 motifs [21]. CD4 or CD8 co-receptors bind to MHC on the APC and recruit the lymphocyte-specific protein tyrosine kinase (Lck) to the TCR-CD3 complex. Once the TCR complex is activated, the intracellular signaling cascades are largely similar between CD4⁺ and CD8⁺ T cells [22].

A central principle of T cell activation is that signaling through the TCR alone results in anergy, a state in which T cells no longer respond to re-stimulation. Co-stimulation of other cell surface receptors provides additional signals that are necessary to avoid anergy and to achieve full T cell activation. Although many cell surface receptors can enhance TCR signaling, CD28 does so, more potently than other co-stimulatory molecules [23]. CD28 is one of several members of a subfamily of costimulatory molecules characterized by an extracellular variable immunoglobulin-like domain, other members of the subfamily include ICOS, Cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death-1 (PD-1), PD1H and BTLA [24]. The CD28 ligands B7-1 (CD80) and B7-2 (CD86) differ in their expression patterns, multimeric states and functionality. CD80 is predominantly dimeric on the cell surface, while CD86 is monomeric. CD86 is constitutively expressed on antigen-presenting cells (APCs) and rapidly upregulated by innate stimuli of the APCs. CD80 can bind several receptors, including CTLA4 [25].

It has been discussed whether the TCR and co-receptors induce separate signaling pathways or whether the signaling pathways of both receptor systems completely overlap [26]. An overlapping model of co-stimulatory signaling is strongly supported by a microarray study of Diehn et al., it shows that TCR-induced expression of thousands of genes in primary T cells is enhanced or suppressed to varying degrees by CD28 co-stimulation, but that no new genes are induced [27]. For the remainder of this discussion, this model is taken as the underlying framework, where CD28 acts as a quantitative modifier required to overcome signaling thresholds that cannot be reached by TCR ligation alone.

CTLA-4 and PD-1 are two receptors that limit the expansion and activation of TCR-induced T cells. These molecules are found on activated T cells, with highest expressions 24–48 hours after stimulation. Genetic studies have demonstrated the importance of both receptors for the maintenance of self-tolerance [28, 29]. Like co-stimulatory receptors, inhibitory receptors utilize similar motifs and molecular signaling pathways to transmit a negative signal [30, 31]. Another way in which inhibitory receptors function, is to use the same ligands and signaling molecules as co-stimulatory receptors, thus resulting in ligand competition. This principle is particularly well illustrated by the example of CTLA-4 and CD28. Both utilize the ligands CD80 and CD86, and thus inhibit each other in their regulatory role [32].

1.2.3 Intracellular Signaling Cascades and Downstream Activation Pathways

TCR engagement triggers the activation and recruitment of protein tyrosine kinases (PTKs). One PTK of the Src family is Lck (Figure 1). Another relevant PTK of the Syk family is zeta chain of TCR-associated protein 70 (ZAP-70) [33]. Lck is regulated by C-terminal Src kinase-mediated phosphorylation at its Y505 residue [34]. Phosphorylation of this residue renders Lck inactive [35]. The strict regulation of Lck protects against hyperactivation of T cells and autoimmune reactions. The activated Lck phosphorylates the tyrosine residue of the ITAMs on the CD3 ζ subunit. The phosphorylated CD3 ζ thereby provides a binding site for ZAP-70. Lck then activates ZAP-70 by phosphorylation [36]. ZAP-70 activates and phosphorylates downstream adaptor proteins such as Linker for Activation of T cells (LAT). LAT serve as a nucleate for the larger signaling complex. PLC γ is phosphorylated by Itk, which is essential for T cell activation. Phospho-LAT serves as a scaffold for the assembly of signaling complexes, including PLC γ . Activated PLC γ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) [23].

TCR-induced DAG production leads to the activation of two important signaling pathways involving Ras and PKC θ . Ras proteins form a family of small GTPases. When activated, Ras transmits signals to other proteins and activates genes involved in cell growth, differentiation and survival. All Ras isoforms are expressed in lymphocytes and are

involved in TCR signaling and T cell development and function [37-39]. Ras is required for activation of the serine/threonine kinase Raf-1, which initiates a mitogen-associated protein kinase (MAPK) phosphorylation and activation cascade. Raf-1 is a MAPK kinase that phosphorylates and activates MAPK kinases, which in turn phosphorylate and activate the extracellular signal-regulated kinases 1 (Erk1) and Erk2. The ERK1/2 signaling is regulated by a feedback mechanism that targets ERK1/2 itself or its upstream activators [40]. The Erk kinase activity leads to the activation of the transcription factor Elk1, which contributes to the activation of the transcription complex activator protein-1 (AP-1) by regulating Fos expression. AP-1 is a transcription factor that consists of homodimers or heterodimers of Jun, Fos, and activating transcription factor (ATF) [41].

The second major signaling pathway regulated by DAG is mediated by PKC θ . After T cell activation, the serine/threonine-specific PKC θ is recruited to the immunological synapse. Activated PKC θ phosphorylates a serine residue in the caspase recruitment domain-containing membrane-associated guanylate kinase protein 1 (CARMA1). This then leads to the recruitment of B-cell lymphoma/leukemia 10 (BCL10) and mucosa-associated lymphoid tissue translocation protein 1 (MALT1), which in turn leads to the formation of the active CARMA1-BCL10-MALT1 (CBM) signaling complex [42-44].

Another central kinase regulating this pathway, is the phosphoinositide-dependent kinase 1 (PDK1). PDK1 is required for TCR-mediated activation of NF- κ B and PKC θ . PDK1 activates PKC θ via the phosphoinositide-3-kinase (PI3K) signaling pathway by phosphorylation, which leads downstream to T cell activation and TCR-induced NF- κ B activation. In the resting state of T cells, NF- κ B is present in the cytoplasm bound to I κ B [45, 46]. The CBM complex contributes to the degradation of Inhibitor of NF- κ B (I κ B). I κ B family members keep NF- κ B from moving to the nucleus. Upon T cell activation, I κ B is phosphorylated by the I κ B kinase (IKK) complex, after which it is ubiquitinated and degraded. This allows NF- κ B to move into the nucleus [40, 47].

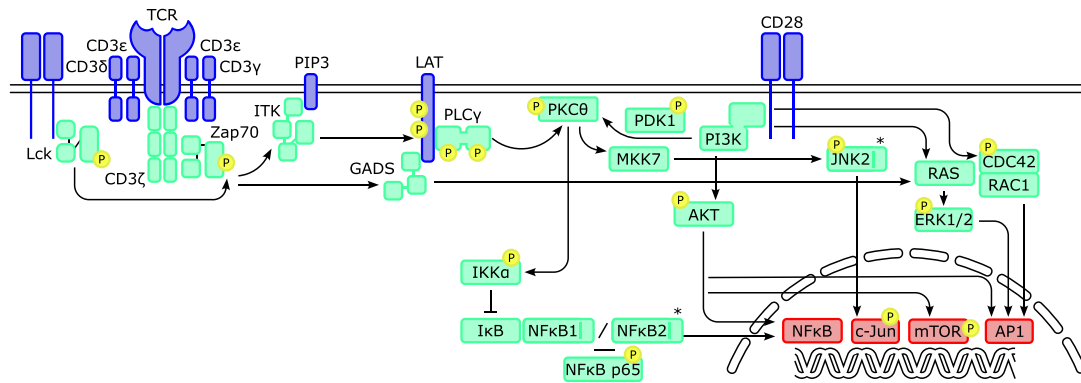


Figure 1 Schematic representation of the T-cell activation cascade as described in Schinle et al. [48].

There is an increasing interest in the understanding of the proteolytic mechanisms involved in the regulation of TCR signal transduction. Proteolysis is mainly mediated by proteasomal and lysosomal pathways. Some short-lived proteins undergo ubiquitination prior to their degradation. TCR signaling through ubiquitination and subsequent degradation of TCR signaling molecules involves CD3ζ, PKCθ, ZAP-70 and PLCγ, PI3K, IκB and Jun [49-53].

1.2.4 Differentiation Stages and Subtype Diversity of CD4⁺ and CD8⁺ T cells

In the previous chapters, the activation cascades resulting in the activated T cell gene expression profiles were described in detail. This chapter will introduce T cell subtypes and how they are activated differently as well as what phenotypes arise from the signaling cascades. This chapter will be the basis for the application of T cell-based cellular therapies.

After development in the thymus, CD4⁺ or CD8⁺ T cells enter the bloodstream. They can leave the bloodstream on reaching a peripheral lymphoid organ. From there they migrate through the lymphoid tissue and can return to the bloodstream via the lymph vessels. Mature, recirculating T cells that have not yet come into contact with their specific antigen are referred to as T_N cells. To proliferate and differentiate, a T cell must encounter its cognate antigen and get stimulated as described in chapter 1.2.2 by forming an immunological synapse with an antigen-presenting cell. Such a cell with the newly acquired activities is referred to as a T effector function (T_{EFF}) cell [3].

CD4⁺ T cell Subtypes

CD4⁺ T cells can be categorized into subtypes: Th1, Th2, Th9, Th17, Th22, regulatory T cells (Treg) and follicular helper T cells (Tfh). All CD4⁺ Th subsets differentiate from naïve CD4⁺ T cells by specific cytokines (Table 1). The development of Th1 cells is driven by interleukin (IL) -12 and interferon γ (IFNγ), a pro-inflammatory cytokine with multiple functions such as increasing Toll-like receptor (TLR) and inducing cytokine secretion of

IFN- γ and TNF [54]. Th2 cells differentiate through IL-4, an important survival factor for B-type lymphocytes, they also release IL-4 as well, as well as IL-5 and IL-13 [55]. Th9 cells are differentiated upon IL-4 and TGF β and secrete the cytokine IL-9 [56]. Th17 produce IL-17, a cytokine that plays an important role in the defense against bacteria and fungi [55]. Th22 cells secrete IL-22, IL-13, IL-26 and TNF α , they are differentiated via IL-6 and TNF α [57]. Treg differentiate through IL-2 and TGF- β . Tregs secrete IL-10, a cytokine with an immunosuppressive function that maintains the expression of the transcription factor Foxp3, which is necessary for the suppressive function of Treg on other cells. In addition, TGF- β is also secreted [58]. Developing Tregs also acquire enhanced survival through upregulation of IL-2R. IL-2 signaling through STAT5 is essential for the survival of Foxp3-expressing cells during Treg development [59]. Tfh cells are differentiated upon IL-6 and IL-21 and secrete IL-21 [57].

Table 1 Differentiation into CD4+ Subsets. Cytokines leading to subsets from naïve CD4 cells and their cytokine profile.

Source	Cytokines for Differentiation	CD4+ Subtype	Cytokine Production
Naïve CD4+ cell	IL-12, INF γ	Th1	TNF, INF γ
	IL-4	Th2	IL-4, IL-5, IL-13
	IL-4, TGF β	Th9	IL-9
	IL-1, IL-6, IL-23, TGF β	Th17	IL-17, IL-21, IL-22, IL-25, IL-26
	IL-6, TNF α	Th22	IL-22, IL-13, IL-26, TNF α
	TGF β , IL-2	Treg	IL-10, TGF β
	IL-6, IL-21	Tfh	IL-21

Models of the development stages

The conventional model proposes that T_N cells differentiate into T_{EFF} cells, which are specialized killer cells that produce cytotoxic molecules and generate effector cytokines. Most effector cells would die during the immune response, the survivors then give rise to memory T cells. This population is able to respond to future pathogenic encounters in a TCR-specific manner [60]. However, there is ongoing debate in the literature as to whether T_{EFF} are fully differentiated cells that cannot be the starting point for memory T cells. There are three models of T cell development stages (Table 2) [61]. In the first model, stem-like memory (T_{SCM}), central memory (T_{CM}) and effector memory (T_{EM}) T cells differentiate stepwise from T_N cells and develop a terminally differentiated phenotype. In the second model, T memory cells are predetermined by intraclonal diversification. In the third model, T memory cells arise during the contraction phase of the immune response and develop directly from effector cells. Two articles published in Nature provided compelling evidence that memory T cells arise from effector T cells through epigenetic modifications. They showed that Dnmt3a, a key DNA methyltransferase, promotes the formation of memory T cells [62, 63].

Table 2 T cell development stages. A: Memory T cells differentiate stepwise from T_N cells, progressing from T_{SCM} to T_{CM} to T_{EM} , a then a terminally differentiated phenotype. B: Memory T cells arise from preset memory precursor genes in the T_N cell population. C Memory T cells arise from surviving T_{EFF} cells.

A	T_N	T_{SCM}	T_{CM}	T_{EM}	T_{EFF}	Cell Death
B	T_N	Memory T cell precursor	Memory T cell			
		T_{EFF}	Cell Death			
C	T_N	T_{EFF}	Memory T cell			
			Cell Death			

Characteristics of each differentiation stage

T_N Cells

T_N cells are actively maintained in a state of hyporesponsiveness, which is characterized by small cell size, low proliferation rate and low basal metabolism [61]. T_N cells are defined by the expression of CD45RA and the lymph node homing markers CD62L and CCR7. These markers enable T_N cells to extravasate from high endothelial venules and migrate into the T-cell zones of peripheral lymph nodes, where they encounter antigens presented by dendritic cells (DCs). Antigen-experienced T cells downregulate CD45RA expression and express CD45RO [61].

T_{EFF} Cells

After T_N cells have differentiated into T_{EFF} cells, they readily release cytotoxic granules and effector cytokines upon full activation (1.2.2 and 1.2.3). T_{EFF} cells are negative for CD27, CD28 and lymph node homing markers such as CD62L, but express markers of terminal T cell activation such as the killer cell lectin-like receptor subfamily G, member 1 (KLGR-1) and the NK marker CD57 [60, 64, 65].

T_{CM} and T_{EM} Cells

Memory T cells can be divided into two groups, T_{CM} and T_{EM} cells are often distinguished on the basis of origin and function. T_{CM} cells express the lymph node homing receptors CD62L and CCR7. They are generally found in lymphoid organs and have no direct lytic function. They are characterized by an increased replicative capacity but limited effector function [60]. The other type is T_{EM} , these do not express CD62L or CCR7 and are characterized by higher cytolytic function and the expression of chemokine receptors and adhesion molecules [66, 67]. This allows them to traffic to peripheral tissues instead of lymph nodes. T_{EM} cells differentiate terminally after a limited number of divisions. Interestingly, however, a part of the population can convert to T_{CM} [68]. It was shown that T cells that were CD62L⁺ and CCR7⁺ (T_{CM}) phenotypically had a lower expression of genes associated with effector function. This is predictive of a less differentiated cell state [69].

A key kinase mentioned in chapter 1.2.3, ZAP-70, is less phosphorylated in memory T cells than in T_N cells [70]. This suggests that memory T cells actually receive weaker signals through their TCRs. However, one study reported that ZAP-70 phosphorylation did not differ between naïve and $CD8^+$ memory T cells. However, increased LAT concentration and phosphorylation in memory T cells led to increased ERK and Jun phosphorylation after activation [71]. Another study showed that SLP-76 was less phosphorylated in memory T cells than in naïve cells, which also indicated weaker TCR signaling in memory T cells [72]. It is an important side note that $CD4^+$ T cells enhance the initial $CD8^+$ T cell response and program the differentiation of responding $CD8^+$ T cells to generate a long-lived, protective memory [73].

Certain transcription factors also promote the development and function of T_{EM} and T_{CM} cells. The expression of T-bet, Blimp1, ID2 and STAT4 is associated with T_{EM} cells, while the expression of Eomes, TCF1, BCL-6, ID3 and STAT3 is associated with T_{CM} cells [74]. In addition, IL-7 is required for the survival of naïve cells and promotes the survival of memory $CD8$ T cells [75].

T_{EMRA} Cells

In humans, a terminally differentiated subpopulation can be identified in addition to T_{CM} and T_{EM} subpopulations. A subset of $CD8^+$ T cells that are terminally differentiated effector memory cells re-expressing CD45RA T_{EMRA} [76].

The initial characterization of these subsets was based on the expression of CD45RA and CD27 [77]. Expression of CD27, a member of the tumor necrosis factor receptor family (TNFR), is restricted to the earliest hematopoietic cell progenitors and cells of the lymphoid cell line [78]. CD27 is highly expressed by T_N , maintained in T_{SCM} and T_{CM} , and expressed at lower levels in only a subset of T_{EM} and T_{EFF} [77, 79]. During the antigen priming phase of T_N cells, the interaction of CD27 with its homotrimeric ligand CD70, which is transiently induced on antigen-presenting cells (APCs), is necessary for determining the T cell memory fate [80-82]. Staining for CD45RA and either CD27 or CCR7 allows the identification of T_N^- ($CD45RA^+/CD27^+/CCR7^+$), T_{EM}^- ($CD45RA^-/CD27^-/CCR7^-$), T_{CM}^- ($CD45RA^-/CD27^+/CCR7^+$) und T_{EMRA}^- ($CD45RA^+/CD27^-/CCR7^-$) $CD8^+$ T cells [60].

With age, memory $CD8^+$ T cells of different phenotypes accumulate. The accumulation of T_{EMRA} cells in humans is influenced by chronic infections such as CMV [83]. It should be noted that researchers do not entirely agree on which markers distinguish T_{CM} , T_{EM} and T_{EMRA} . Other literature suggests the division between T_{CM} and T_{EM} is based on CD95, with CD27 being present on both populations [61].

T_{SCM} Cells

T_{SCM} are a more recently established type. They are characterized by self-renewal capacity and also possess multipotent differentiation capacity [84, 85]. They can be the

origin of T_{CM} and T_{EM} , as well as a pool of more short-lived T_{EFF} . They are phenotypically similar to T_N , i.e. they express CD45RA, CD62L, CCR7, CD27 and CD28, but differ in CD122 and CD95 [84]. Their presence in the blood is very low, with only 2-3% of the circulating T cells corresponding to this phenotype. They express the marker B-cell lymphoma 2 (Bcl-2) in addition to the markers mentioned above [86]. It was shown that the T_{SCM} naïve-like phenotype, with its self-renewal capabilities, can be achieved by inhibiting the Wnt pathway activator TWS119. This suggests a connection with the expression of T cell factor 1 (TCF1) [87].

T_{RM} Cells

Very recently, $CD69^+CD103^+$ tissue-resident memory T cells (T_{RM}) have been identified as non-recirculating immune cells localized in peripheral tissues. The function of tissue surveillance was initially attributed to circulating T_{EM} cells. However, experiments have shown that some cells in the tissue do not circulate but are permanently resident [88]. They have been reported to mediate tumor protection by promoting tumor-immune equilibrium through cytokine secretion and CD103-enhanced tumor cell killing [74].

CD4⁺ Cell Subtype Differentiation

The differentiation of Th type cells into memory and effector T cells plays an important role in immunity. Regulatory T cells (Treg) also differentiate into effector and memory cells. Naïve conventional T cells and regulatory T cells (effector and memory subtypes) differ in their extracellular, intracellular, epigenetic and genetic markers, transcription factors and metabolic pathways (Table 3 and Table 4) [89, 90].

Table 3 Markers to detect differentiation of CD4⁺ T naïve cells [89, 90].

Resting T	Effector T	Memory Th
CCR7+	CCR7-	CCR7-/+
CD25-	CD25+	CD25-
CD45RA+	CD45RA+/-	CD45RA-
CD45RO-	CD45RO+/-	CD45RO+
CD69-	CD69+	CD69+/-
CD127+	CD127-	CD127+

Table 4 Markers to detect differentiation of CD4⁺ T regulatory (Treg) cells [89, 90].

Resting Treg	Effector Treg	Memory Treg
CCR7+	CCR7-	CCR7-
CD25+	CD25+/-	CD25+
CD45RA+	CD45RA-	CD45RA-
CD45RO-	CD45RO+	CD45RO+
CD69-	CD69+	unknown
CD127-	CD127-	CD127+

Circulation T cells

Recent studies in organ donors of different ages have provided some insights into the compartmentalization of human memory subtypes. They describe large populations of T_{RM} cells and regional monitoring by T_{EM} , T_{CM} , and T_{EMRA} cells, which varied by tissue and did not reflect the representation of subtypes in blood [91, 92]. T_{EFF} were originally considered the perfect starting material for adoptive cell therapy. However, it was shown that this phenotype, although possessing potent killing abilities, expands only extremely poorly. As more recent studies show, the better starting condition appears to be the memory phenotype [93, 94]. The cytokines IL-7 and IL-15 are now widely used. It has been shown that their use can be exploited to create the T_{SCM} phenotype. It has been shown that IL7 is critical for polarization to this phenotype. IL15 plays a greater role in expansion [85].

1.2.5 Metabolic Pathways Shaping T-Cell Activation and Differentiation

The metabolism of a cell refers to a complex network of biochemical reactions involved in energy production and macromolecular biosynthesis. The following chapter will discuss the effects on the fate, plasticity, and effector function of T cells.

AMPK Supervisory Control of Cellular Energy Homeostasis

In mammalian cells, fluctuations in cellular energy are monitored by the heterotrimeric AMP-activated protein kinase (AMPK) complex. ATP, ADP, and AMP compete for nucleotide binding sites on the γ -regulatory subunit of AMPK, with AMP (low energy) promoting AMPK activation and ATP (high energy) inhibiting it [95].

Similar to tumor cells, AMPK can regulate a metabolic checkpoint in T cells, thereby slowing down lymphocyte proliferation under poor energy conditions [96]. T_{EFF} cells with defective AMPK signaling would be freed from these metabolic checkpoints and could continue to proliferate and produce cytokines as if cellular bioenergetics could support T cell function. Furthermore, AMPK can regulate the metabolic plasticity of lymphocytes by coordinating metabolic changes in response to nutrient fluctuations, enabling T cells to survive changes in their environment [97]. Furthermore, it has been shown that AMPK-deficient T cells are unable to generate $CD8^+$ memory T cells during infection [98].

AMPK can also be activated by Ca^{2+} (via $CamKK\beta$) and the cytokine TGF- β (via TAK1). Together, LKB1 and AMPK function as part of an evolutionarily conserved energy sensing pathway that maintains cellular energy balance by promoting catabolic pathways of ATP production and limiting processes that consume ATP. When ATP levels drop, AMP binds to AMPK. AMPK is activated to promote ATP production and block ATP consumption. Once ATP homeostasis is restored, the kinase is shut down by increased ATP binding to AMPK [97].

Lymphocytes exclusively express the catalytic $\alpha 1$ subunit of AMPK, and TCR stimulation promotes LKB1-dependent AMPK activation in lymphocytes [99, 100]. Glycolysis is enhanced in resting AMPK α -deficient T cells, consistent with observations that AMPK inactivation promotes the Warburg effect in tumor cells. [100, 101]. LKB1 and AMPK work together to limit the anabolic growth of T cells by suppressing glycolysis and mTOR-dependent biosynthesis. Loss of LKB1-AMPK signaling promotes increased mTORC1 activity in both T_N and T_{EFF} cells, which in turn facilitates the production of the TH1 cytokine IFN γ by T_{EFF} cells [101, 102].

T cell Activation-Dependent Changes in Metabolism

Naïve and effector T cells differ in their metabolic pathways. Resting naïve T or memory cells have a catabolic metabolism, in which nutrients are broken down for energy, while activated T cells have an anabolic metabolism, in which nutrients are used to build molecular complexes and building blocks to support cell proliferation. During differentiation and activation, metabolism switches from oxidative phosphorylation (OXPHOS) to glycolysis [103]. In addition, catabolic processes of ATP formation, such as β -oxidation of fatty acids, are actively suppressed. The predominant metabolic phenotype of activated T cells is a switch to aerobic glycolysis [104].

The main player in anabolic metabolic pathways in activated cells is mTOR. IL-2 and costimulatory CD28 signaling in activated T cells induces a switch to glycolysis with activation of PI3 kinase and downstream AKT. Activated AKT induces the mTOR metabolic pathway and increases the utilization of glucose and amino acids to support the proliferation of activated T cells [97, 105].

Metabolic Programming Governs T-Cell Subtype Differentiation

Metabolic signaling varies depending on the state of T cell differentiation. It is important to note that OXPHOS is still active in T_{EFF} cells, but the production of lactate from pyruvate through aerobic glycolysis is the dominant pathway of glucose metabolism in T_{EFF} cells [106]. The predominant metabolic phenotype of activated T cells is a shift toward aerobic glycolysis [104].

Th1, Th2, and Th17 CD4⁺ cells are primarily glycolytic and show high concentrations of the glucose transporter Glut1 and express active mTOR. Treg cells, on the other hand, are dependent on lipid metabolism and have low concentrations of Glut1 and high concentrations of AMPK [97].

TH17 cells in particular are increasingly dependent on glycolysis during their differentiation, proliferation and maintenance. The development of TH17 cells is promoted by hypoxia-inducible factor-1 α (HIF-1 α), an oxygen-sensitive transcription factor that regulates glycolytic gene expression in TH17 cells. Blocking glycolysis during TH17 cell differentiation reduced the development of TH17 cells and promoted the formation of Tregs [107, 108].

Warburg-Driven Fast Glycolysis Fuels T_{EFF} Cell Activation

Both CD4⁺ and CD8⁺ T_{EFF} cells engage in aerobic glycolysis, which is characterized by the conversion of glucose pyruvate to lactate, even though oxygen is available for complete glucose oxidation. This process, also known as the Warburg effect. It is heavily related to work in cancer biology and is a common feature of actively proliferating cells [109]. The rapid glucose processing promoted by the Warburg effect enables proliferating T cells to produce ATP quickly. Glycolysis also produces metabolic intermediates that are important for cell growth and proliferation.

An important intermediate product of glucose metabolism is ribose-5-phosphate (Rib-5P). It is produced via the oxidative or non-oxidative part of the pentose phosphate pathway (PPP) and is the starting material for nucleotide biosynthesis. The oxidative part of the PPP also produces NADPH. This is the most important metabolic starting product for nucleotide and fatty acid biosynthesis [106].

Glutamine Metabolism and Hypoxia induced Remodeling

Glutamine metabolism in T cells is regulated just as dynamically as glucose metabolism. Glutamine transporters and important glutamine degradation enzymes are upregulated shortly after T cell activation, similar to glycolysis genes. [106, 110]. TCR activation leads to the expression of Slc7a5, an amino acid transporter that mediates the import of large neutral amino acids such as leucine [111]. The influx of amino acids via this transporter is necessary for the activation of mTOR and the expression of c-Myc, thereby coordinating the activation-induced metabolic reprogramming and differentiation of T cells. Amino acids such as glutamine and leucine therefore appear to play additional roles in T cell function beyond protein biosynthesis and may directly influence T cell activation by regulating metabolic reprogramming [97, 106, 112].

T cells often suffer hypoxia in inflammatory tissues [113]. It has been demonstrated that T cells can utilize reductive glutamine metabolism under hypoxic conditions for fatty acid biosynthesis [114]. The stabilization of HIF-1 α and metabolic reprogramming in favor of reductive carboxylation may help T cells maintain their proliferation and effector function at hypoxic inflammatory sites. Interestingly, HIF-1 α protein expression is also observed shortly after T cell activation [115]. HIF-1 α is involved in the differentiation of both proinflammatory TH17 cells and CD4⁺FoxP3⁺ Treg cells [107, 116].

Metabolic Reprogramming in Memory T Cells via Free Fatty Acid Oxidation

Unlike actively proliferating effector CD4⁺ and CD8⁺ T cells, which rely on aerobic glycolysis with production of lactate from glucose-derived pyruvate, memory T cells have different metabolic pathways based on the oxidation of fatty acids [97]. It has been shown that mitochondrial fatty acid oxidation (FAO), which is stimulated downstream of TNF receptor-associated factor 6 (TRAF6), is relevant for the development of CD8⁺ memory T cells [117].

The oxidation of free fatty acids (FFAs) produces acetyl coenzyme A (CoA), which can be further metabolized in the TCA cycle, as well as FADH₂ and NADH, which can be used directly by the electron transport chain to form ATP. FFAs are energy-rich molecules, and FAOs could be a preferred energy source for transmembrane cells, as these rely on an OXPHOS-dependent metabolic program. The administration of metformin, a metabolic stressor that activates the energy sensor AMPK, promotes the formation of CD8⁺ T cell memory cell [117]. One consequence of AMPK activation is the suppression of mTORC1 activity in response to energetic stress [118]. The activation of AMPK and the inhibition of mTOR are both effective activators of autophagy, a catabolic process induced during starvation and, in this case, particularly important for the viability of T cells after activation [119, 120].

1.2.6 T Cell–Based Therapeutic Approaches

With the complex diversity of T cells established, therapeutic applications and exploration of diverse strategies will be described in the following chapter.

TCR $\alpha\beta$ Depletion in Stem Cell Transplantation

TCR $\alpha\beta$ depletion is a technique used in hematopoietic stem cell transplantation. It removes immune cells derived from the donor before the donor cells are transferred to the recipient's body. This approach reduces the risk of severe graft-versus-host disease (GVHD) and post-transplant infections. It also eliminates the need for post-transplant immunosuppressive drugs, which can have side effects. In addition, it accelerates the recovery of blood counts after transplantation. TCR $\alpha\beta$ depletion is particularly helpful when it is not possible to find a fully matched donor. This is often the case for patients from certain ethnic groups [121, 122].

Tumor-Infiltrating Lymphocyte Therapy

In Tumor-Infiltrating Lymphocyte (TIL) TIL therapy, T cells that have naturally infiltrated a patient's tumor are extracted, multiplied ex vivo, and reinfused to attack cancer cells. This method has proven particularly successful in the treatment of metastatic melanoma. In 2024, the FDA approved Amtagvi, a TIL-based therapy for melanoma, representing a major advance in cell-based treatment of solid tumors [123-125].

Cytokine-Induced Killer Cells

Cytokine-Induced Killer (CIK) cells are produced by stimulating peripheral mononuclear blood cells with cytokines such as IFN- γ and IL-2. These cells exhibit both T cell and NK cell properties and can therefore effectively attack tumor cells. CIK therapy has been investigated for various types of cancer, including hematological malignancies and solid tumors [126, 127].

1.3 Lentiviral Vectors

Gene therapy can be broadly defined as the treatment of a disease or disorder by introducing therapeutic genes into the appropriate target cells. These therapeutic genes can correct the harmful effects of specific gene mutations or reprogram cell functions to overcome a disease. For gene therapy to be successful, the exogenous therapeutic gene must be incorporated into the target cell in a specific, efficient and stable manner.

1.3.1 History of Retroviral Vector Technologies and Early Clinical Trials

The concept of gene therapy emerged in the mid-20th century and was considered a revolutionary technology with the promise of being able to cure almost any disease whose molecular basis was understood. Since then, various gene vectors have been developed, and the feasibility of gene therapy has been demonstrated in numerous animal models of human diseases [128]. However, clinical efficacy was not demonstrated until the end of the 20th century and the beginning of the new century [129, 130].

The choice of a suitable tool fell on retroviral vectors due to various advantages, such as their ability to integrate stably. Two vector systems were mainly developed in parallel: γ -retroviral vectors, from murine leukemia viruses, and lentiviral vectors (LV), mainly derived from human immunodeficiency virus (HIV)-1 [131].

The majority of early clinical trials were conducted using γ -retroviruses [132]. One of the first technically successful clinical trials was the treatment of severe combined immunodeficiency (SCID) by Anderson et al. in the 1990s [133]. Another milestone followed with the correction of X-SCID in 11 children in the year 2000 [134]. This initial success following retroviral therapy was later overshadowed by the occurrence of vector-related leukemia in a significant number of the children treated [135-137].

This insight led to the development of next-generation vectors with improved efficiency, specificity, and safety. This was accompanied by a trend toward LVs over γ -retroviral vectors. The integration pattern of LVs differs from that of murine leukemia viruses and is less risky in terms of insertion mutagenesis [138]. LVs are also able to infect non-dividing cells due to their ability to cross the nuclear membrane [139]. Finally, LVs can be processed with higher vector titers making them clinically more relevant [131]. LVs were first considered for rare diseases, in particular primary immunodeficiencies such as Wiskott-Aldrich syndrome (WAS) [140]. Since then, more frequent genetic and acquired diseases have become targets of treatment. These include β -thalassemia [141], Parkinson's disease [142] and chimeric antigen receptor-based immunotherapy [143]. The future success of gene therapy will depend heavily on understanding vector biology.

1.3.2 Understanding Lentiviral Vector Biology

Most LVs are based on HIV-1, which is one of the most thoroughly studied vectors. Alternatives are available but have not yet reached the clinical trial stage: HIV-2 [144],

simian immunodeficiency virus [145], feline immunodeficiency virus [146], bovine immunodeficiency virus [147]. Retroviridae such as HIV-1 are a family of single-stranded RNA (ssRNA) spherical viruses with a diameter between 80 and 120 nm [148].

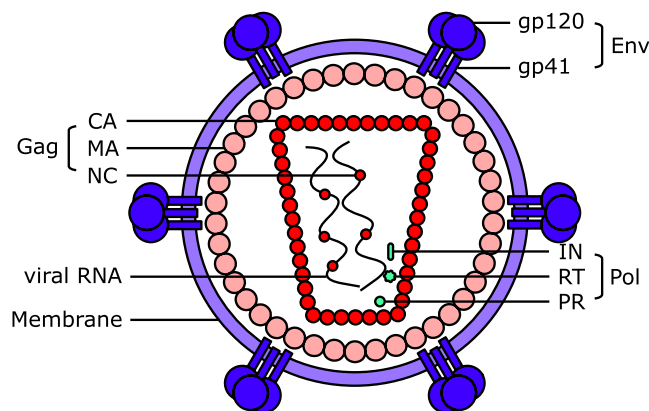


Figure 2 Schematic of an HIV-1 particle. The virion is surrounded by a lipid bilayer containing Env proteins composed of trimeric gp120 and gp41 subunits. On the inner side of the envelope, Gag-encoded MA proteins are present, while CA proteins assemble into the cone-shaped virion capsid. NC proteins associate with the viral RNA genome, and the pol-encoded proteins integrase (IN), reverse transcriptase (RT), and polymerase (PR) are packaged within the virion capsid. Adapted from Liu et al. [149].

The genome consists of two copies of positive-strand RNA and includes the gag, pol, and env genes. Together with important proteins it is complexed in a second protein shell, formed by capsid protein [150]. The gag gene encodes structural proteins such as the viral matrix, the capsid, the nucleocapsid, and the p6 protein [151]. The pol gene encodes proteins that accompany the ssRNA: reverse transcriptase, integrase and protease. Reverse transcriptase transcribes viral ssRNA into double-stranded DNA (dsDNA). The integrase catalyzes the integration of proviral DNA into the host genome. Protease is involved in gag-pol cleavage and virion maturation [152]. The env gene encodes for matrix proteins that form a layer outside the core and interact with a cell-derived lipid envelope containing viral envelope glycoproteins [149].

The genome is flanked by two long terminal repeat (LTR) sequences with elements necessary for gene expression, reverse transcription, and integration into the host chromosomes. The LTRs comprise three domains: U3 and U5 (at the 3' and 5' ends of the RNA, respectively) and R (repeat). Viral transcription begins at the first residue of R in the 5' LTR, and the transcript is polyadenylated at the last residue of R in the 3' LTR [149]. Viral transcription from the LTR begins with the binding of the transactivation response (TAR) and the Rev protein through recognition of the Rev response element (RRE) [153]. The polypurine tract (PPT) is the starting point for positive strand DNA synthesis during reverse transcription [154]. The primer binding site (PBS) is a site to which the transfer RNA (tRNA) primer anneals for reverse transcription [149].

Other important components include the packaging signal (ψ or Ψ), which is required for specific RNA packaging into newly formed virions [155]. HIV-1 also encodes four accessory proteins: Vif, Vpr, Vpu, and Nef. Although these are relevant for infectivity and pathogenicity, they play no role in virus replication [156].

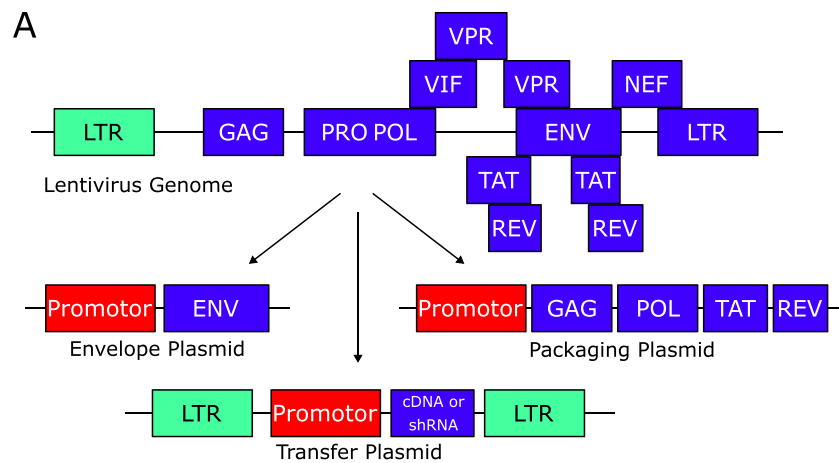
The life cycle begins with the viral envelope docking onto the cell membrane and fusing with it. The viral particle is uncoated and the viral core is released into the cytoplasm. After decapsulation, the remaining viral nucleic acid-protein complex is often referred to as the reverse transcription complex (RTC). This RTC is actively transported to chromosomal DNA, where integration can take place. The ssRNA is reverse transcribed to dsDNA. This process begins with the binding of a tRNA to the primer binding site at the 5' end of the viral RNA genome. While the negative strand of the viral DNA is synthesized by the polymerase activity of the enzyme reverse transcriptase, the viral RNA is degraded by the RNase H activity of the same enzyme. This produces a short fragment of negative-strand, single-stranded DNA, often referred to as strong stop copy DNA (sscDNA). This sscDNA fragment is then transferred to the 3' end of the viral RNA and serves as a primer for the synthesis of the negative strand of the viral DNA. This restores the U3RU5 sequence of the LTR, which is essential for the viral life cycle. The dsDNA is then transported to the nucleus. Transport to the nucleus is active in lentiviral vectors, which means that cell division is not required. The DNA is then integrated into the host DNA (provirus). This then leads to the long-term expression of viral genes. The full-length RNA and the spliced variants of RNA encoding viral proteins enter the cytoplasm and are translated. The full-length RNA is packaged and the viral particles are assembled. Virion maturation occurs together with budding of the particle from the cell. During the budding process, endogenous membrane proteins of the host cell can be incorporated such as MHC molecules. [3, 128, 157]

1.3.3 Advancements in Lentiviral Vectors and Plasmid System Designs

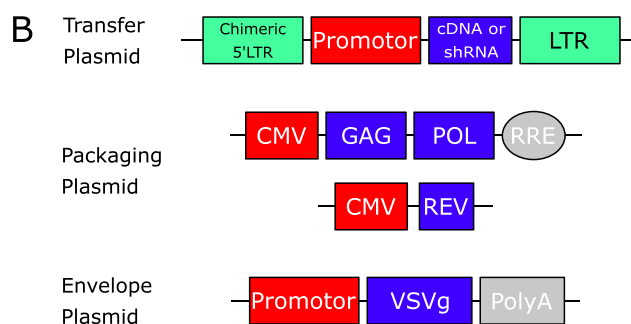
Due to safety concerns mentioned in chapter 1.3.1 and HIV-1's pathogenicity in humans, safety was optimized over several vector generations. The first LVs still contained all intact gag and pol sequences, as well as the regulatory genes tat and rev and the accessory genes vif, vpr, vpr, and nef. Wild-type envelope has a restricted tropism, it was therefore replaced with a vesicular stomatitis virus G glycoprotein (VSVg) in a process termed "pseudotyping". This allows the vector to transduce a wide range of cells [158]. VSVg recognizes the ubiquitously expressed receptor low-density lipoprotein (LDL) [159]. The VSVg gene itself was encoded on a separate plasmid from the other genes. The transfer plasmid consists of an expression cassette and the HIV cis-active factors necessary for packaging, reverse transcription, and integration [128]. It contains the integrating genetic segment and it is as well introduced on a separate plasmid. The principle of separate plasmids is based on a split genome described by Dull et al [160]. The system is therefore based on three plasmids.

In the next step, research was conducted to determine which genes were redundant for the transfer of genetic material into a cell. The findings led to the development of the multiple attenuated second-generation vectors. Here, the accessory genes *vif*, *vpr*, *vpu*, and *nef* were removed. As a result, a potentially replication-competent virus would now be devoid of all virulence factors [161-163]. This was achieved without any loss in vector yield or infectivity [164]. The development of self-inactivating (SIN) transfer vectors by deletion of the enhancer/promoter unit in the U3 region of the 3' LTR would minimize the risk of replication-competent lentiviruses [165, 166].

The third-generation is widely used in research and development and clinical purposes today. It is a four-plasmid system, three helper plasmids and one transfer plasmid. In this subsequent development, *rev* is now located on a separate plasmid [128, 157]. The regulatory *tat* gene was also removed, its transactivation function is dispensable because the U3 promoter of the 5' LTRs in the transfer vector was replaced by a constitutively active promoter sequence, such as the Rous sarcoma virus promoter [160] or the cytomegalovirus promoter [162, 165]. The nuclear import of the transfer construct was improved by incorporating PPT and its central termination sequence. This leads to higher vector titers and improved safety [167]. The addition of further elements, such as the post-transcriptional regulatory element of woodchuck hepatitis B, also improved the design of the lentiviral vector by increasing gene expression through modification of polyadenylation, RNA export and translation [168].



Second Generation



Third Generation

Figure 3 Lentiviral generations and plasmid separation. A: 2nd Generation: The 2nd generation lentiviral system consists of three plasmids: a single packaging plasmid encoding Gag, Pol, Tat and Rev; a transfer plasmid containing the viral LTRs and the psi packaging signal (not pictured); and a separate envelope plasmid encoding Env (usually VSVg). B: 3rd Generation: The 3rd generation lentiviral system improves safety by distributing viral components across four plasmids instead of three and eliminating Tat. The packaging system is split into two plasmids: one encoding Gag and Pol and another encoding Rev. The transfer plasmid contains a hybrid 5'LTR that is partially deleted and fused to a heterologous enhancer/promoter such as CMV or RSV, creating a chimeric 5' LTR that drives Tat-independent transgene expression. Adapted from Addgene [169].

1.3.4 Scalable Production of Lentiviral Particles

The following section examines key factors influencing the establishment and optimization of manufacturing of lentiviral vectors. The section will be broken down and explained in relation to research and the state of the art in industry. This will also include relevant basics, process steps, and requirements with the aim of establishing a flexible platform that allows various lentiviral constructs to be manufactured in accordance with current Good Manufacturing Practice (cGMP) standards.

Producer Cell Lines

In line with established practices for complex biologics, lentiviral vectors are produced in a dedicated manufacturing cell line. Co-transfection of the individual plasmids takes place in human embryonic kidney cells (HEK) 293T or 293 [170]. A packaging plasmid, a transfer plasmid and a shell plasmid, or in the third-generation rev in a separate fourth

plasmid, are required. The presence of the SV40-T antigen (in HEK293T cells) enables more efficient vector production, but raises significant technical and safety concerns due to its oncogenicity [171]. Most small-scale productions are carried out using surface-adherent cells grown in Petri dishes, T-flasks, multi-well systems, or hyperflasks [131]. Process scale-up is hindered by the limited adhesion capacity of HEK293T cells. Larger production scales that employ roller bottles are technically challenging [172]. Production units are often simply expanded via parallel productions in place. However, suspension cultures represent an attractive alternative for scalable industrial manufacturing. In addition to far better scalability, such cell lines benefit from the development and progress of antibody-based production research.

A stable cell line saves significant costs compared to transient transfection. This is partly due to high costs for GMP-compliant DNA as a starting material. Significant cost savings also arise from enhanced reproducibility, compared to transient transfection. Various construction strategies for such cell lines had been developed. Pseudotyping was one of the most important elements in the development of stable producer cell lines. Since the heterologous VSVg envelope, the most commonly used envelope for LV is highly cytotoxic. Its transcription must only be induced during LV production to prevent the death of the packaging cells [158, 173]. Furthermore, the expression of the gag and pol genes of HIV is associated with cytotoxic and cytostatic effects, which is why the high expression of these genes must also be regulated only during the production phase [131]. The first and most commonly used inducible systems are the tetracycline (Tet)-on and Tet-off systems.

Despite the long run of development since the 1990s, stable LV producer cell lines are currently scarcely used as to current knowledge. This and other disadvantages to stable cell lines still argues in favor of transient transfection. Main concerns are flexibility in development and early clinical studies. Since transient expression is considered to be regulatory feasible [174, 175], it opens up the possibility of preparing the existing platform for a new product after approval, which would simplify the characterization of the starting cell line and the starting material (DNA). Validation runs could follow on from the previous approval, whereby the particularly high costs of cell line establishment could be offset by the lower costs of DNA preparation, for example via a supplier. The following sections will therefore focus on the translation of transient systems.

Current Good Manufacturing Production Compliance

Adaptation of media free of bovine serum and animal components is one of the most important prerequisites for current Good Manufacturing Production (cGMP) compliance. All major regulatory and standardization authorities now recommend avoiding animal-derived raw materials, especially fetal bovine serum (FBS), as far as possible or at least subjecting them to strict qualification. The reasons for this are proven

risks such as variability in composition, introduction of adventitious viruses, prions (TSE/BSE) and ethical aspects [176, 177].

Then, suspension-cultivation represents the solution to the scaling problem of adherent systems, minimizing manual handling, making it possible to operate perfusion cultures, apply automation, introduce inline monitoring and control and simplify the use of transfection reagents (reviewed in [178]). A suspension culture can be easily expanded and scaled up in various vessels: shake flasks, glass and stainless-steel bioreactors, or disposable wave bags. For use in these vessels, cells must be first adapted to suspension growth. There are several HEK cell lines that can be used to adapt to suspension culture in chemically defined media, such as 293T, 293FT, and 293SF-3F6. During early development of these cell lines, productivity was significantly lower with bulk titer values of 10^6 TU/mL and below [179]. More recent development has led to titers reaching 10^8 TU/mL through gradual adaptation in suspension media [180]. The GMP-compliant LV-MAX™ (Gibco™) is one example of such an optimized system. It is based on a suspension HEK293 cells adapted to serum-free growth. The cell line does not contain the SV40-T antigen. All media and reagents are serum-free. Transfection is based on a lipid complex consisting of DHDMS, DOPE and cholesterol to increase transfection efficiency. A production enhancer to increase lentiviral production is based on the synergistic three-component enhancer mixture of sodium propionate, sodium butyrate and caffeine [181].

Purification in Downstream Processing

In industry, all steps involved in purifying a biotechnologically produced product are referred to as Downstream Processing (DSP). Owing to their relatively large size, these particles can be purified by simple pelleting. In early research, LVs were often prepared crudely, in most cases by ultracentrifugation as means for concentration, for example at $70\,000 \times g$ [182]. Alternatively, the vector may be concentrated by precipitation. Precipitating agents include poly-L-lysine, polyethylene glycol (PEG), calcium phosphate or a mixture of chondroitin sulfate C and protamine sulfate [178]. There are several commercially available substances [183]. However, these methods leave behind a variety of production-related contaminants, such as host cell proteins, host cell DNA, plasmid DNA from transfection and cell debris. These residues must be removed following DSP steps.

For suspension-based and transient transfection culture, the removal of intact cells and cell debris is the first step in DSP. It reduces complications during later steps. In addition, DNase treatment is introduced as very critical step to degrade viral plasmid and host cell DNA. [178, 184]. Following these initial steps, established industry-standard technologies are employed, which are broadly classified into four major categories: capture, intermediate purification, polishing and sterilization (reviewed in [178, 184], see Table 5).

Table 5 Biotech terminology and its description.

Capture	Initial purification of the target from crude or clarified cell culture and elimination of major contaminants
Intermediate purification	Removal of specific impurities, such as proteins, DNA or endotoxins
Polishing	Removal of trace contaminants leaving an active and safe product
Sterilization	Removal of potential microbial contamination introduced during steps of DSP, in a mostly open system

It is worth noting that the recovery of LV purifications is rather poor compared to DSP of antibody production or recombinant proteins. Yield targets for antibody production are around > 80 % [185, 186]. This stands in stark contrast to what is expected according to literature for the recovery in LV purifications, with < 35 % [187-189].

Clarification with Initial Filtration

Filtration is a highly scalable and efficient downstream processing strategy that predominates in clarification due to its disposable and easy-to-deploy systems. In membrane filters, the feed passes through flat or pleated sheets of an inert polymer with specific pore sizes corresponding to the retention ratings [178]. The filter choice greatly influences efficiency of the filtration step. Cascading filtration systems with progressively decreasing pore sizes are commonly used to prevent premature fouling of the terminal membrane and minimize LV loss [190, 191]. Initial filters in the cascade are depth filters which are a class of filters with a sponge-like structure. They are composed of a polymer, a binder and a filtration aid such as diatomaceous earth [192]. It is important to note that higher concentrations of diatomaceous earth can reduce the LV yield [193].

Tangential Flow Filtration

Tangential flow filtration (TFF) is a well-established method in biotechnological purification. The method uses ultrafiltration/diafiltration (UF/DF) to remove low-molecular-weight impurities, reduce the process volume and exchange the buffer where LVs are suspended in, according to a specific retention rate (typically specified in kDa) [194]. TFF is used both in the initial purification steps and in the subsequent polishing steps [195].

In ultrafiltration, the viral supernatant is pumped tangentially along the surface of a membrane. Unlike conventional direct flow filtration systems, the retained particles do not deposit on the membrane surface. This prevents fouling and enables the processing of large volumes [196]. In LV purification, ultrafiltration enables volume reduction to concentrate the vector to suitable titers and simplifies subsequent processing by reducing the volume feed [196].

Buffer exchange (diafiltration) often follows immediately after the concentration step. Buffer exchange is important for maintaining vector stability over time, during freeze-

thaw cycles, for exchange into optimal chromatography binding buffers as well as for efficient transduction of recipient cells. TFF offers several advantages including automation, little manual intervention and high recoveries.

Two different TFF systems are commonly applied: hollow fiber units and flat sheet cassettes. Both formats are scalable and applicable solutions for vector concentration and buffer exchange. Both offer a high surface area to volume ratio, with flat sheet cassettes being capable of higher flows than hollow fibers, but at the expense of higher shear rates of the vector particles due to their turbulent flow paths. Various materials of these formats are available: polysulfone (PS), polyethersulfone (PES), polyvinylidene fluoride (PVDF) or regenerated cellulose (RC) [197, 198]. Pore sizes vary between 0.001 – 0.1 μm [195]. Larger pore sizes favor flow at the expense of vector adsorption, while lower cut-offs slow down flow to retain the vector. Therefore, membranes in the range of 100 – 750 kDa tend to be used [184]. Membrane fouling can be a significant limitation and lead to a reduction in permeate flow rate and an increase in transmembrane pressure (TMP) during the concentration process. This may lead to longer processing times and require larger membranes with higher dead volume. Vectors can also adsorb or become trapped on the membrane surface [189, 199]. Hence, TFF parameters must be optimized depending on the feed, as flow rate and membrane type can lead to shear damage and vector loss.

Chromatographic Purification

Chromatography is a physical-chemical separation process in which a mixture of substances is distributed between a stationary phase and a continuously flowing mobile phase. The individual analytes adsorb or dissolve to varying degrees on the stationary phase depending on their affinity, causing them to elute with characteristic retention times. By varying the eluent composition, gradient profile or column material, the selectivity and speed of separation can be specifically optimized [200]. The method itself is highly scalable and has been used for years in the preparation of antibodies, recombinant proteins, industrial enzymes and nucleic acids [178, 195]. For LV purification, literature mostly refers to purification using anion-exchange chromatography (AEX), size exclusion (SEC) or affinity-based interaction.

In general, AEX is considered one of the most widely used and promising methods for the scalable purification of viral vectors [179, 195]. Here, the negatively charged LV particles are bound to the positively charged chromatographic matrix. Up to 90 % recovery of vector particles had been reported [201], however, data varies greatly with others reporting recoveries below 30 % [178]. Elution of the bound LV is achieved via highly concentrated salt buffers [202]. These conditions can drastically reduce infectivity of the final product [203]. A major cost advantage is that the column can be regenerated.

Contrary, SEC is based on porous, non-adsorbing material to separate virus particles from contaminants based on size and mass [196]. Recoveries of up to 70 % have been

reported here [204]. One advantage is that the purification conditions in this method are relatively mild compared to AEX. This makes it particularly suitable for the unstable LV particles. A major disadvantage is upscaling. Scaling is accompanied by a reduction in throughput, since low linear flow rates are generally required, this will increase the processing time. This again means lower recovery due to LV instability. Furthermore, a dilution effect is observed during passage, therefore this method requires a downstream concentration step [178]. It is generally used more often as a polishing step to remove remaining small impurities [196].

The third commonly used chromatography method is based on affinity adsorption via heparin affinity. There are promising results, with proven recovery of up to 53 % of infectious LV particles and removal of 94 % of contaminants [179]. A major and critical disadvantage is the heparin. Heparin is an animal-derived raw material, so it is not only heterogeneous, but also poses a high risk if leaching happens. Contaminants of animal origin are critical for clinical products, so further purification steps and additional quality control are required, which increases processing time, reduces yields and significantly increases costs [195].

Formulation and Sterile Filtration

The buffer formulation for LV is necessary to maintain high transducibility of the vector throughout processing and application. The final formulation ultimately varies depending on the application. In vivo therapy requires stricter formulations, such as the use of water for injection [184]. For many ex-vivo therapies, a vector is typically maintained in cell culture medium for cultivation of the recipient cells [188]. However, other buffers may be more suitable for high recovery of LVs. These buffers should maintain a stable pH (ideally physiological) over a range of temperatures. PBS, HBSS, HEPES or histidine are frequently used due to their effective buffer ranges and resistance to pH drift with temperature, in contrast to common buffers such as TRIS, which undergo pH drift [189, 205, 206]. Additional buffer additives serve as preservatives for vector formulations. These may contain recombinant proteins such as human serum albumin (HSA) to stabilize the vector. In addition, sugars such as sucrose or trehalose are often used as cryopreservation agents and osmolarity regulators [207].

Identity, Purity, Potency and Safety

An LV product may be used in clinical studies and human applications only after quality control has confirmed its efficacy and safety. The LV charge must meet all regulatory-mandated specifications and limits. This is controlled by precise and repeatable test protocols that have been verified in several validation runs. The typical LV batch requires specifications regarding identity, purity, potency and safety (Table 6). The stringency increases from development to manufacturing authorization and commercial use. However, LV manufacturing still lacks globally harmonized guidance, with regulatory requirements varying across jurisdictions [178, 184, 208].

Table 6 Current regulatory specifications for LV batches [175, 178, 184, 188, 208, 209].

	Harvest / Final Bulk / Final Lot	Analytical Tests	Acceptance Criterion
Identity	Confirm identity	Western Blot, vector sequencing, restriction enzyme mapping, reverse HPLC, SDS-Page	Qualitative match to reference lot
Potency	Functional viral particles	Flow cytometry, qPCR	Target range 1×10^7 TU/mL ± 1 log)
	Total viral particles	p24 ELISA	Particle to TU ratio target $\leq 10^3$
Purity	Total protein	Bradford, BCA protein assay	
	Host cell protein	ELISA	<100 ng/mL (early clinical studies in vivo)
	Residual endonuclease	ELISA	Not detected
	Residual BSA	ELISA	Not detected
	Total DNA	qPCR, fluorimetry	≤ 10 ng/dose (commercialization)
	DNA size distribution	Agarose gel electrophoresis, capillary electrophoresis	<200 bp
	E1A and SV40 LTA sequence in the vector	qPCR	Not detected
	Transfer of plasmid or host-cell (E1A and SV40 LTA) sequences	qPCR	Not detected
Safety	Replication competent lentiviruses (RCL)	RCL amplification in suitable cell line by serial passages followed by quantification assay (qPCR, p24 ELISA)	Not detected (LOD ≤ 1 RCL/ 10^7 TU)
	Adventitious agents	In vitro testing, PCR-based methods	Not detected
	Mycoplasma	Culture-based methods, PCR-based methods	Negative
	Endotoxins/pyrogens	Lal test, rabbit pyrogen test	≤ 0.5 EU/mL (parenteral)
	Sterility	Bacterial and fungal sterility	Complies
	Appearance / pH / osmolality	Visual / pH probe / osmometer	Clear / pH 7.0 ± 0.5 / 260 – 320 mOsm/kg

Identity can be confirmed by Western blot analysis for gag proteins, vector sequencing, restriction enzyme mapping, reverse phase high-performance liquid chromatography (HPLC) or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [184].

To determine **functionality**, transduction of a cell line is performed then transgene expression is examined. This alone does not take into account multiple integrations that can occur with a high MOI. The method therefore requires titration of the vector over different dilutions. In the next step, the dilutions of the vector solution are mixed with a known number of cells [175]. Polybrene can be added here as an adjuvant to enhance

transduction by minimizing electrostatic repulsion between the envelope protein and the receptor [210]. An incubation period of 2 – 4 days is followed. During this time, cells can expand while the proportion of expression due to episomal transgene expression is being diluted. This minimizes the risk of overestimating the titer due to transgene expression in episomes. The cells are then examined for expression by flow cytometry, typically by staining with antibodies or affinity-based dyes or by direct analysis, e.g. using green fluorescence protein (GFP) [178]. The TUs can be determined based on the percentage of transduced cells, the volume of vector solution added and the cell count. A non-staining protocol for the functional titer can be performed using an integration assay. Here, the transduced cell genomic DNA is extracted, the provirus is quantified by qPCR and compared with a housekeeping gene. For clinical purposes, quantification should be based on integrated reverse transcribed viral genomes [184]. Work is underway to develop a method standard and reference samples for cross-group comparisons [211, 212]. Another important indicator of vector quality, is the ratio of total particles to active particles (TUs), which correlates with the efficacy of the LV batch. If this parameter is too high, the batch must be rejected [195]. Current guidelines do not define a reference value, but ratio values in the order of 10^3 are given [175, 188]. The total particle count is usually determined by p24 titration with ELISA kits [197].

With regard to **purity**, both process- and product-related impurities in the final product should be well characterized. Total proteins can be quantified using colorimetric tests such as Bradford or BCA [184, 188]. The limit value for host cell proteins (HCPs) in early in vivo studies is around <100 ng/mL [178, 213]. For late clinical phases and commercial approval, DNA must be present at a concentration of less than 10 ng per dose and a DNA size of less than 200 base pairs (bp) [209]. Residual DNA can be analyzed using Picogreen or quantitative polymerase chain reaction (qPCR). BP length can include spectrofluorimetry and agarose gel electrophoresis [175, 184, 188]. Since HEK293T exhibits a tumorigenic phenotype, LVs produced with these cell lines must be analyzed to quantify the E1A and SV40 LTA DNA sequences. In transient transfection, the copy number of the plasmid sequences (VSVg and gag-pol) must be determined by qPCR or Southern blot [184, 188]. Other contaminants such as nucleases or HSA can be quantified using ELISA [184, 188].

Finally, each vector batch must be evaluated with regard to various **safety** parameters, with particular attention paid to replication competent lentiviruses (RCL), endotoxins, mycoplasmas, adventitious agents and sterility [208, 209]. Physical and chemical parameters such as pH, osmolarity and appearance supplement the product characterization [208].

1.4 Adoptive T cell therapy and the Application of Lentiviral Vectors

Adoptive cellular immunotherapy describes the therapeutic application of defined immune cell subsets [214]. One of its earliest applications was the use of tumor-infiltrating lymphocytes (TILs) in patients with melanoma [215]. The main obstacles to this approach were the difficulty in culturing and producing TILs, immune tolerance to self-antigens and the need for presentation of antigens by the MHC [216]. In recent years, advances in culture process and in molecular and virological techniques have enabled the generation and expansion of genetically modified anti-tumor T cells, overcoming practical hurdles. This simplified the production of gene-modified T cells from peripheral blood, making them more accessible and thus clinically relevant. Various methods can be used to introduce genes into T cells. Non-viral DNA transfection was originally used due to the cost and low risk of insertional mutagenesis [217, 218]. This method requires long-term culture and antibiotic selection due to the relative inefficiency of gene transfer. Long-term culture can have a detrimental effect on the activity and persistence of the infused cells. In addition, the antibiotic resistance gene products can have an immunogenic effect [216]. Transposon-based systems can integrate transgenes more efficiently than plasmids without an integrating element [219]. Sleeping Beauty has been shown to enable efficient, stable gene transfer and sustained transgene expression in various cell types, including T cells [220]. Again, however, the culture time remains relatively long. LVs, as described in chapter 1.3, came with several advantages in terms of genome integration and scalable manufacturing, and have been applied broadly in this field [217, 221].

1.4.1 Chimeric Antigen Receptor T cells

While TILs belong to the category of non-engineered T cells, which also includes artificially expanded Tregs, viral-specific T cells, and cytotoxic T lymphocytes (CTLs), in so-called engineered T cells, a genetic manipulation takes place prior to expansion. Examples include TCR-engineered T cells [222], chimeric antigen receptor (CAR-)T cells [214], chimeric auto-antibody receptor T (CAART) cells [223] or CAR natural killer (NK) cells [224], to name a few.

The CAR is a chimeric molecule of a T cell receptor (TCR) fused with an antigen recognition domain, such as a single-chain fragment (scFv) of a monoclonal antibody [214]. Thus, despite the native TCR, CAR-T cells can recognize a specific antigen on other cell surfaces via the CAR receptor. Unlike TCR-mediated recognition, antigen recognition by CAR is not dependent on MHC. The antigen can be a protein, a carbohydrate, or a lipid, as antibodies/scFvs can bind to these types of molecules. This approach enables long-term disease control and offers the additional advantage of rapid onset of action. As with all targeted cancer therapies, the target must be specific to the cancer cell to prevent damage to healthy cells and tissues.

Over time, multiple CAR “generations” have emerged, typically referring to changes to the intracellular signaling domains. The first generation consisted only of the scFv and an intracellular CD3 ζ domain (Table 7). Second-generation CARs have a unique co-stimulatory domain derived from CD28 or 4-1BB molecules [214, 225]. This improves the clinical efficacy of the treatment by increasing the survival of modified T cells [226]. Third-generation CARs consist of molecules with three or more cytosolic co-stimulatory domains. In addition to CD28 and 4-1BB, CD27, ICOS or Ox40 can also be coupled, which theoretically increases the activation, survival and efficacy [216]. After years of iterative refinement, chimeric antigen receptors (CARs) have advanced to the fifth generation [225].

Table 7 Summary of CAR construct generations reviewed in [225, 227]. Abbreviations: Co-stimulatory motif (CM); single-chain variable fragment (scFv), T-cell redirected for universal cytokine-mediated killing (TRUCK).

Generation	Design	Signaling / payload	Objectives	Strengths
1G (1993)	scFv CD3 ζ	no CM	- provide HLA-independent activation via ζ chain	- proof of concept - IL-2 secretion
2G	scFv CM CD3 ζ	single CM (e.g. CD28 or 4-1BB)	- reinforce activation & survival	- robust expansion - durable responses (4-1BB) or rapid cytolysis (CD28)
3G	scFv CM1 CM2 CD3 ζ	two costimulatory domains (e.g., CD28 + 4-1BB, CD28 + OX-40)	- combine fast killing with long persistence	- increased cytokine production - in-vivo expansion and tumor clearance improved
4G TRUCK or armored	2G backbone + inducible payload cassette	NFAT-driven Cytokine genes (e.g. IL-12, IL-18, IL-2, IFN- γ) ligands or chemokines released on antigen encounter	- remodel hostile tumor micro-environment - attract innate cells	- local payload delivery - better function in solid-tumor models
5G “next-gen”	2G or 3G scaffold + extra signaling or logic circuitry	Truncated IL-2R β -STAT3/5 tail inside CAR Logic-gated / switchable / multi-target CARs	- tackle antigen loss - low-density targets - suppressive niches without systemic cytokines	- enhanced proliferation without exogenous IL-2 - higher safety by AND/OR/NOT gating or switch control

1.4.1 Manufacturing of CAR-T Cells and Automation

Manufacturing begins with donor cell collection and includes sequential isolation, activation, ex vivo genetic modification to confer receptor expression, expansion, and reinfusion into the patient. Recent technological advances have enabled the automation of this previously manual process. There are several leading cell systems on the market for the automated production of CAR-T cells, with the following being particularly noteworthy due to its versatility and seamless integration. CliniMACS Prodigy® (Miltenyi Biotec), the Lonza Cocoon® platform, the Terumo Quantum® system and the Cytiva Xuri™ platform [228, 229]. CliniMACS Prodigy® functions as a closed “all-in-one” system, automating the entire process from cell isolation (MACS technology) to activation, genetic modification, and expansion, which improves standardization and drastically reduces contamination risks [228, 229]. The Lonza Cocoon® platform offers automated pH control and integrated magnets for cell enrichment. Like the Prodigy, it is based on serial expansion, which limits scalability to one patient per device at a time [228]. The Xuri™ Cell Expansion System (Cytiva) uses gentle rocking technology (WAVE) for optimized nutrient transport, but focuses primarily on the pure expansion step [229]. The Quantum® System (Terumo BCT) scores with continuous media exchange in hollow fiber bioreactors, but is most commonly used for adherent cells [229].

1.4.2 Challenges of CAR-T cells

B cell neoplasms are considered ideal candidates for targeted therapy with CAR-T cells. Most of the approved CAR constructs are targeting CD19 which is not present in most normal tissues with the exception of B cells [221]. These CAR receptors show good clinical results and high safety profile. However, their use requires careful consideration of therapy-associated side effects resulting from strong immune activation and the underlying mechanisms of action. Cytokine release syndrome (CRS) is a significant side effect, which is now largely controllable through close monitoring. At the time of writing, all FDA-approved CAR-T cells are second-generation [221], which may reflect both regulatory constraints and intrinsic design-related limitations of later CAR generations, including tonic signaling-associated adverse effects [230]. Despite remarkable clinical successes in hematological malignancies, the transfer of this therapeutic approach to solid tumors remains a significantly greater challenge. A total of 158 solid cancer indications were approved by the European Medicines Agency (EMA) between 2009 and 2021 [231]. Lung cancer, breast cancer and melanoma are tumors with the highest number of new indications, with non-small-cell lung cancer leading [232]. The specific biological properties of solid tumors present several hurdles that have so far limited the effectiveness of CAR-T cell therapies. This prompts an important question: how can CAR-T cells be optimized to achieve effective responses against solid tumors? Does the selection of a specific phenotype together with hyperoxic culture conditions inherently limit the ability of these cells to function in solid tumor environments?

2 Aim and Perspective

T cells not only make up a large proportion of peripheral lymphocytes, they are also essential for defending against a variety of diseases, including cancer. Cancer itself remains one of the major causes of death globally, with an incidence that did not change between 1990 and 2019 [1]. The development of advanced therapy medicinal products (ATMPs), such as CAR-T cells, represents a major milestone in addressing this cause of mortality. The demand for such therapies will increase dramatically over the next years [233]. In the long term, this will not only lead to a bottleneck in production and production facilities, but also lead to high financial burden on the healthcare system. As these therapies become more widely used, affordable and scalable strategies must be developed to enable broader clinical implementation. Considerable cost savings can be achieved through decentralized production of CAR-T cells in academic or clinical settings [234].

To date, starting materials, especially viral vectors, have to be sourced from industry partners and abroad. This introduces dependencies, both financial and in terms of intellectual property and marketability. For novel CAR constructs, especially for smaller batches, lentiviral vectors therefore represent a bottleneck. To enable early access for clinics and orphan diseases, it is crucial to implement LV manufacturing. A comprehensive understanding of the structural and biological determinants of the final LV product is therefore required. Achieving pharmaceutical-grade vector production requires accounting for the vector's intrinsic fragility and optimizing all process steps within the manufacturing platform.

The next step would be the implementation of the vector in an automated CAR-T manufacturing process. Despite the technological sophistication of cultivation devices such as the CliniMACS Prodigy®, the molecular and cellular mechanisms during the generation of CAR-T cells from T cells have remained largely unaddressed. Advancing our understanding of T cell biology in this therapeutic context may guide the rational optimization of cell-based therapies. This could enable patient-oriented optimization strategies tailored to individual needs. Moreover, efforts to shorten manufacturing timelines highlight the need for a deeper understanding of this complex biological process [235, 236].

Taken together, this work aims to establish a platform enabling the generation of novel CAR-T cell constructs and the development of T-cell-biology-guided manufacturing processes for clinical use.

3 Materials and Methods

3.1 Equipment and devices

Devices	Manufacturer	Serial No.
Laboratory incubator 3032	Lauda-GFL Gesellschaft für Labortechnik	10129598C
Laboratory incubator B 28	Binder GmbH	83215
Laboratory thermostat WB 7	Memmert GmbH & Co. KG	1298.0309
Odyssey	LiCor Biosciences	OFC-0321
Schüttelgerät ES-20	Grant Instruments Ltd.	01011115090144
Biostat® RM 20 230V Custom Design	Sartorius	4143300213
CliniMACS Prodigy	Miltenyi Biotec	627
laboratory thermostat HERAcell	Heraeus Med	98111578
CO2 resistant shaker (orbital)	Fisher Scientific	L4CT81104044
Safety workbench Herasafe HSP 18	Heraeus Med	98109915
EVO Tangential Flow Filtration System	Cytiva (Pall)	4906695531
Centrifuge Rotixa 50 RS	Hettich	632
FACS Canto II	BD Biosciences	V33896202161
FACS Fortessa X-20 Special Research Order	BD Biosciences	H656385J4001
Spark 10M	Tecan	189000545

Equipment	Manufacturer	Reference No.
Sticky pads, 140 × 140 mm	Fisher Scientific	88881126
CoolCell™ LX cell freezer container	Corning	CLS432003-1EA
Counting chamber Neubauer	Carl Roth	T735.1
QuadroMACS™ Starting Kit (LS)	Miltenyi Biotec	130-091-051

3.2 Materials

3.2.1 Chemicals and Reagents

Chemical / Reagent	Manufacturer	Reference No.
Sodium butyrate	Sigma-Aldrich	B5887-1G
HEPES buffer, 1 M solution (pH 7.3)	Fisher Scientific	10648593
Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich	H8264-500ml
Invitrogen™ SYBR™ Gold nucleic acid staining solution	Invitrogen	S11494
Polybrene Infection / Transfection Reagent	Sigma-Aldrich	TR-1003-G
Ethanol absolute	AppliChem	A1613
Benzonase® Nuclease, Purity >90 %	Merck Millipore	70746-3
Calibration solution (pH 7.01)	Hanna Instruments	HI70007C
Calibration solution (pH 4.01)	Hanna Instruments	HI70004C
TritonX-100	Sigma-Aldrich	X100RS-5G
Tris-Acetate EDTA-Buffer (10×)	Sigma-Aldrich	T9650-1L

3.2.2 Filters and Plastic Consumables

Filter / Consumable	Manufacturer	Reference No.
Flexsafe® RM 2L optical	Sartorius	DFO002L
Cellstar® Cell Culture Microplate, 96-Well, Flat Bottom, White,	Greiner Bio-One	655083
Greiner culture flasks, T175	Greiner Bio-One	C7356
Greiner culture flasks, T75	Greiner Bio-One	C7231
Greiner culture flasks, T25	Greiner Bio-One	C6481
Masterflex® single-use gamma irradiated Sampling Bottle, PETG Bottle, 125 mL	VWR International	MFLX95644-20
Masterflex® single-use gamma irradiated Sampling Bottle, PETG Bottle, 250 mL	VWR International	MFLX95644-22
Polycarbonate Erlenmeyer Flask with Vent Cap, 125 mL	Corning	431143
Polycarbonate Erlenmeyer Flask with Vent Cap, 250 mL	Corning	431144
Polycarbonate Erlenmeyer Flask with Vent Cap, 500 mL	Corning	431145
Polycarbonate Erlenmeyer Flask with Vent Cap, 1 L	Corning	431147
Shake flask, 250ml	Fisher Scientific	11765253
Shake flask, 500ml	Fisher Scientific	11725263
Bottle top vacuum filtration system, 500 mL	Corning	513-3355
Reagent reservoirs	VWR International	613-1184
Protein LoBind® Tubes 50mL	Eppendorf	30122240
Tuflux® Silicone Tubing, 1/8" × 1/4", 100 m	Sartorius	FSA117425
Luer-Lock female	Carl Roth	CT60.1
Luer-Lock male	Carl Roth	CT64.1
Luer plug male	Carl Roth	CT70.1
Luer plug female	Carl Roth	CT69.1
Syringe filter, Puradisc™ Aqua 30	Whatman products (Cytiva)	514-0288
AcroPrep™ 24-well Supor EKV filtration plate, 0.65/0.2 µm	Cytiva (Pall)	97017
Filtration capsules, AcroPak™ 200, Membrane Filter Capsules Fluorodyne A	Cytiva (Pall Lab)	514-4086
MiniMate™ 100K Omega™	Cytiva (Pall)	516-6881
MiniMate™ 300K Omega™	Cytiva (Pall)	515-0121
Sartocon® Slice 50, Hydrosart®, 300kDa, 50cm²	Sartorius	3D91447950ELLPU
Sartocon® Slice 200, Hydrosart®	Sartorius	3081447902E-SW
LS Columns	Miltenyi Biotec	130-042-401
CliniMACS Prodigy® TS 520	Miltenyi Biotec	200-073-613

3.2.3 Plasmids

Plasmid	Source	Reference No.
pCAG-VSVG	Addgene	35616
psPAX2	Addgene	12260
pCDH-2ndCar_CD276HL-NLuc-T2A-mCherry	Previously prepared in lab	#119
pCDH-2ndCar_CD276HL-NLuc-T2A-GFP	Previously prepared in lab	#248
2ndCAR_CD276Dotti-Myc-T2A-CD19t	Previously prepared in lab	#305
2ndCAR_GD2-scFvHL-Myc-T2A-CD19t	Previously prepared in lab	#313

3.2.4 Cell Culture Supplies

Cell Culture Supply	Manufacturer	Reference No.
Dulbecco's phosphate buffered solution (DPBS)	Gibco	14190250
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	12491015
RPMI 1640	Gibco	31870025
Poly-L- glutamic acid sodium salt	Sigma-Aldrich	P4761-100MG
Pierce™ bovine serum albumin, 2 mg/ml	Fisher Scientific	23209
LV-MAX™ Production Medium	Thermo Fisher Scientific	A3583402
LV-MAX™ Supplement	Thermo Fisher Scientific	A35348
LV-MAX™ Transfection Reagent	Thermo Fisher Scientific	A35348
LV-MAX™ Enhancer	Thermo Fisher Scientific	A35348
Opti-MEM™ reduced serum medium, 100mL	Thermo Fisher Scientific	10149832
BioColl® cell separating solution	Bio&Sell	L6115
CliniMACS® PBS/EDTA Buffer CR/GMP	Miltenyi Biotec	200-070-022
TexMACS™ GMP Medium	Miltenyi Biotec	170-076-306
TexMACS™ Medium	Miltenyi Biotec	130-097-196
Recombinant human IL-7 (interleukin 7)	Miltenyi Biotec	130-095-362
Recombinant human IL-15 (interleukin 15)	Miltenyi Biotec	130-095-760
MACS® GMP Recombinant Human IL-7	Miltenyi Biotec	170-076-111
MACS® GMP Recombinant Human IL-15	Miltenyi Biotec	170-076-186
CD4 MicroBeads, human	Miltenyi Biotec	130-045-101
CD8 MicroBeads, human	Miltenyi Biotec	130-045-201
CliniMACS® CD4 Reagent CR/GMP	Miltenyi Biotec	200-070-213
CliniMACS® CD8 Reagent CR/GMP	Miltenyi Biotec	200-070-215
T Cell TransAct™, human	Miltenyi Biotec	130-128-758
MACS® GMP T Cell TransAct™ Large Scale CR/GMP	Miltenyi Biotec	200-076-204
CryoSure-DMSO	WAK-Chemie	WAK-DMSO-10

3.2.5 Cell Lines

Gibco™ Viral Production Cells

Gibco Viral Production Cells are suspension-adapted derivatives of the HEK 293F cell line, optimized for high-yield lentiviral production using the LV-MAX Lentiviral Production System. Grown in a chemically defined, serum- and protein-free LV-MAX Production Medium (Thermo Fisher Scientific, Cat. No. A35684).

Burkitt's lymphoma*

The Raji cell line was isolated in 1963 by R.J.V. Pulvertaft from a Burkitt lymphoma in the left upper jaw of an 11-year-old boy [237, 238]. It expresses B cell antigens (DSMZ, Cat. No. ACC 319).

Alveolar rhabdomyosarcoma*

The RH-30 cell line was established from bone marrow cells of a child with rhabdomyosarcoma [239] (DSMZ, Cat. No. ACC 489).

Neuroblastoma*

The IMR-32 cell line was established by W.W. Nichols, J. Lee and S. Dwight in April, 1967 from an abdominal mass occurring in a 13-month-old Caucasian male. The tumor was diagnosed as a neuroblastoma with rare areas of organoid differentiation [240] (generously donated by AG Lang).

Neuroblastoma*

The LS (ACC675) cell line was established in 1991 from a stage III abdominal tumor of a 16-month-old girl with neuroblastoma[241] (generously donated by Karin Schilbach)

Fibrosarcoma

The HT-1080 cell line was established from a biopsy of a fibrosarcoma of a 35-year-old man in 1972. The patient did not receive any chemo- or radiotherapy (generously donated by AG Sonanini).

T cell leukemia

The Jurkat cell line was established from the peripheral blood of a 14-year-old boy with acute lymphoblastic leukemia (ALL) at first relapse in 1976 (in the original paper by Schneider et al. (DSMZ, Cat. No. ACC 282).

*Knock-out or transduction to express Luciferase for cell lines Raji, RH-30, IMR-30 and LS had previously been done in the AG Seitz lab [242].

3.2.6 Flow Cytometry Reagents

Reagent	Manufacturer	Reference No.
CliniMACS® PBS/EDTA buffer	Miltenyi Biotec	200-070-025
FcR Blocking Reagent, human	Miltenyi Biotec	130-059-901
CD3 Antibody, anti-human, Vio® Bright R720	Miltenyi Biotec	130-127-377
CD4 Antibody, anti-human, VioGreen®	Miltenyi Biotec	130-113-259
CD8 Antibody, anti-human, PE	Miltenyi Biotec	130-110-678
CD19 Antibody, anti-human, APC	Miltenyi Biotec	130-113-642
CD25 Antibody, anti-human, Vio® Bright B515	Miltenyi Biotec	130-115-536
CD27 Antibody, anti-Human, BUV496	BD OptiBuild™	741145
CD45RA Antibody, anti-human, VioBlue®	Miltenyi Biotec	130-117-743
CD45RO Antibody, anti-human, APC-Vio® 770	Miltenyi Biotec	130-113-557
CD69 Antibody, anti-human, Brilliant Violet 650™	BioLegend	310933
CD95 Antibody BUV737, Anti-Human	BD Horizon™	612790
CD152 (CTLA-4) Antibody, anti-human, BUV805	BD Horizon™	569655
CD279 (PD1) Antibody, anti-human, APC	Miltenyi Biotec	130-120-389
CD223 (LAG-3) Antibody, anti-human, BV786	BD OptiBuild™	744727
CD366 (TIM-3) Antibody, anti-human, PE-Vio® 770	Miltenyi Biotec	130-121-334
HLA-DR Antibody, anti-human, Spark UV™ 387	BioLegend	307689
c-myc Antibody, anti-human/mouse/rat, biotin	Miltenyi Biotec	130-124-877
CD19 CAR Detection Reagent, biotin	Miltenyi Biotec	130-115-965
Biotin Antibody, APC	Miltenyi Biotec	130-110-952
Biotin Antibody, FITC	Miltenyi Biotec	130-113-290
Biotin Antibody, PE	Miltenyi Biotec	130-110-951

3.2.7 Kits

Kit	Manufacturer	Reference No.
HEK293T Host Cell Protein ELISA Kit	Enzo Life Science	ENZ-KIT 162-0001
Lenti-X™ p24 Rapid Titer Kit	Takara Bio	632200

3.3 Lentivirus Manufacturing

3.3.1 Master Cell Bank Generation

A vial of Gibco™ viral production cells was thawed and cultivated for three passages in shake flasks. On day of cell bank procedure, the cell concentration and cell viability were determined. A solution of 10 % DMSO in LV MAX™ Production Medium (PM) was

generated. Cells were centrifuged at -9°C at $300 \times g$ for 5 min, then resuspended at a concentration of 1×10^7 cells/mL. Aliquots of 1 mL were generated in cryo-vials. Freezing took place in Corning CoolCells overnight. Long-term storage was in liquid N_2 .

3.3.2 Suspension HEK293 Cell Culture

The appropriate volume of PM for a subculture was preheated in a shake flask for at least 20 minutes in an incubator. The incubator was set to 37°C and 8 % CO_2 with an orbital shaker at 125 rpm (19 mm shaking diameter). A vial was thawed in a water bath at 37°C . 1 mL of the cells was transferred to 30 mL pre-incubated PM in a 125 mL shaking flask. After three days, 1 mL of sample was removed with a serological pipette. Viable cell concentration (VCC) and viability were determined using a Neubauer cell counter. Cells were sub cultured at a VCC of $>1 \times 10^6$ viable cells/mL and viability $\geq 90\%$. A seeding density of 0.5×10^6 cells/ml was specified for sub-culturing for 3 days and 0.3×10^6 cells/ml for sub-culturing after 4 days. Cells were cultured until they reached a density of $3.5 - 5.5 \times 10^6$ viable cells/mL, after which the next sub culture was performed. Culture volumes can be found in Table 8.

Table 8 Cultivation volumes for LV MAX™ HEK293 cells.

Well plate / Flask size	Culture Volume
6-well	2.5 mL
125 mL	30 mL
250 mL	60 mL
500 mL	120 mL
1 L	240 mL
2 L	480 mL

3.3.3 Cell Cycle Analysis

Cells were centrifuged at $500 \times g$ for 5 min at 4°C . Washing was done twice with 5 mL cold $1\times$ DPBS and centrifuged at $500 \times g$ for 5 min at 4°C . Resuspension was done carefully in 500 μL $1\times$ DPBS. Cells were transferred to a 15 mL centrifuge tube containing 4.5 mL of ice-cold 70 % EtOH while constantly vortexing slightly. Incubation was done at 4°C for 2 hours. Cells were centrifuged at $1000 \times g$ for 5 minutes. The supernatant was removed carefully. Cells were resuspended in 5 mL $1\times$ DPBS and incubated for 15 min at RT. Cell count was performed during incubation again to adjust the cell concentration later ($1 - 2 \times 10^6$ cells per staining). Cells were again centrifuged at $1000 \times g$ for 5 minutes. Resuspension was done in 300 μL of 1 $\mu\text{g/mL}$ DAPI at 0.1 % (v/v) TritonX-100 in $1\times$ DPBS. Incubation was done for 30 min at RT, protected from light. Cells were transferred into a FACS tube for acquisition at the cytometer. DAPI signal was read in channel 405 – 450/50, linear mode.

3.3.4 Transfection

For each transfection, 2.5 μg total plasmid DNA per ml of culture volume were used. The ratio of lentiviral packaging plasmid to lentiviral transfer plasmid was 3:2, corresponding

to 1.5 µg packaging plasmid to 1 µg transfer plasmid. Plasmid A (containing VSVg) to Plasmid B (containing gag, pol and rev) were added in a ratio of 1:2, corresponding to 0.5 µg and 1 µg per mL culture volume. Cells were cultured for at least 3 passages after thaw. One day before transfection, after reaching a cell density of $3.5 - 5.5 \times 10^6$ viable cells/mL, cell density was adjusted to 3.5×10^6 viable cells/mL. On the day of transfection, DNA was thawed on ice in the morning. VCC should have reached between $5.0 - 7.0 \times 10^6$ living cells/ml. Viability should be $\geq 95\%$, to proceed with transfection. VCC was adjusted to 4.7×10^6 viable cells/mL by adding fresh PM. For a transfection of 60 mL cell culture volume, 3 mL of Supplement were added. 360 µL Transfection Reagent and plasmid DNA were each diluted separately in 3 mL Opti-MEM. Both were then mixed and incubated for 10 minutes to allow complex formation. The solution was added to the cells. Final concentration after transfection was 4×10^6 viable cells/mL. 5 to 6 hours after transfection, 2.4 mL Enhancer were added to the cells. Harvesting took place 48 hours after transfection.

3.3.5 Bioreactor Setup

On the control tower a "New Batch" was initiated. The packaging of a Flexsafe® 2 L optical bag was opened under laminar airflow. A long sterile tube was connected to the inoculum / harvest port A (Figure 4).

Bag Configurations – Flexsafe® RM Optical Bags

Flexsafe® RM 2 L optical

DFO002L

Ports	
Port A	Dip Tube Silicone, C-Flex® 374 tubing $\frac{1}{8}$ " $\times$ $\frac{1}{4}$ " (1000 mm), FM-Luer
Port B	DO sensor
Port C	pH sensor
Port D	Septum for needle-free sampling, FM-Luer
Port E; H	Silicone tubing $\frac{3}{16}$ " $\times$ $\frac{5}{16}$ " (50 mm), FM-Luer
Port F	Gas Outlet Filter with Check Valve, M-Luer
Port G	Gas Inlet Filter $\frac{1}{4}$ " Hose barb

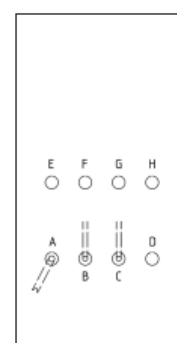


Figure 4 Bag configuration of Sartorius Flexsafe® RM 2 L optical.

The bag was installed on the rocker tableau and tightened with metal clamps. Probes for pH and DO were installed on the bag. Calibration was performed on the control tower according to the bag's configuration (Table 9). Tubes for gassing were connected to the sterile filter (port G) on the bag. The bag was filled with air at 0.5 L/minute until 1 mbar was reached. The exhaust heater was connected to vent filter (port F). The cover on for the tableau was installed and the balance was set to zero.

Table 9 Bag configuration of optical probes of pH and dissolved oxygen (DO).

pH		DO	
Temperature	36.6 °C	Temperature	36.8 °C
f(max)	26.16 °	0% sat	58.31 °
f(min)	54.98 °	100% sat	24.86 °
dPH	0.51 pH		
pH0	7.26 pH		

The bag was filled with PM via the connected inoculation tube from within the laminar air flow up until the tableau's balance reached 0.3 kg. Temperature control was started and set to 37 °C. DO control was turned off and basal air gassing of 0.15 L/minute was initiated. Wave motion was initiated with a rocking angle of 4 ° and 30 rocks per min.

Control for pH was turned off and basal CO₂ gassing at 0.015 L/minute was initiated. After incubation for 6 hours DO, pH and temperature had stabilized. Re-calibration of DO and pH was performed. DO was set to 100 % saturation. For pH a sample was taken via the sample port (port D) and measured externally on a Radiometer. The external value was entered at the control tower. Gassing regime was then initiated with DO set-point 40 % and pH control via CO₂ at 7.25.

3.3.6 Bioreactor Inoculation and Cultivation

A seed density of 0.3×10^6 cells/mL in 300 mL for cultivation of 4 days was prepared. The according medium was removed from the bag via the inoculation tube within the laminar air flow. The respective volume of the cell suspension was transferred into the bag via the same tube. The cell concentration was determined after 1 hour of incubation. A sample was taken every following day to check for cell concentration, viability and re-calibrate the pH externally.

One day before transfection, cell concentration was adjusted to 3.5×10^6 cells/mL by removing the according amount of cell suspension from the bag and replacing it with fresh PM.

On day of transfection, reagents were prepared as previously describe in section 3.3.4. The solution of DNA and transfection reagent was transferred to the bag via the inoculation port.

3.3.7 Lentivirus Concentration by Precipitation

30 mL of the culture supernatant was transferred to a low-bind 50 mL centrifuge tube and centrifuged at $1300 \times g$ at 4 °C for 10 min. The supernatant was transferred to a new low-bind tube and centrifuged again. 10 mL Takara LentiX Concentrator were added to

the 30 mL supernatant and mixed by inversion. Incubation on ice for 1 hour followed. The solution was then centrifuged at $1500 \times g$ for 45 minutes at 4°C . The supernatant was discarded and the pellet was resuspended in the desired buffer at a volume reduction of 1:50. 50 μL aliquots were prepared in cryo-vials and snap-frozen on dry ice. Long-term storage was at -80°C .

3.3.8 Clarification

Syringe Membrane Filter

Prior to filtration, the cell suspension was centrifuged twice at $1300 \times g$ for 5 minutes at RT. A Puradisc™ Aqua 30 syringe filter was then flushed with 5 mL PM before use. Each filter was capable of filtering 13 mL cell-free supernatant before clogging.

Depth-Filtration Plate

AcroPrep™ 24-well Supor EKV was filled with 6 mL of cell suspension per well. The plate was centrifuged in swing-bucket at $1000 \times g$ for 5 minutes at RT.

Depth-Filtration Capsule

Flushed a filtration capsule AcroPak™ 200 with 50 mL 0.2 M HEPES. Slowly increased pump starting at 6 to a maximum 30 mL/min. Cell suspension was centrifuged twice at $1300 \times g$ for 10 minutes at RT. Transferred the supernatant to the reservoir and set pump to 6 mL/min and slowly increased to a maximum 40 mL/min.

3.3.9 Transmembrane Pressure Scouting

Transmembrane Pressure Scouting (TMP) scouting was performed as previously described in Agrawal et al. [243].

3.3.10 Normalized Water Permeability Test

Normalized water permeability (NWP) tests serve as a reference value before the first use of the process and after each cleaning to verify cleaning effectiveness. The tank is filled with filtered water (ideally at 25°C). The membrane is then rinsed with water from the tank. Then the retentate valve is closed completely, while the permeate valve remains open. Next, the TMP is set, and three values are recorded: 5, 10 and 15 PSI. It must be waited until the pressure stabilizes, at least 5 minutes, only then can the data be recorded. It is documented how long (in minutes) it takes to filter 10 mL of water.

3.3.11 Ultrafiltration / Diafiltration

Ultrafiltration / Diafiltration was either performed on a MiniMate Evo™ System or the Sartoflow® Smart. The respective filter was flushed with 0.22 μm filtrated osmotic water before use. After flush, water was drained as low as the minimum working volume of the system. The respective buffer for use was circulated for 30 minutes at 20 mL/minute, for at least 20 mL with filtrate clamp closed and 20 mL with filtrate clamp open. When 10 mL

of buffer remained in the reservoir, the system was set to circulation with filtrate open until start.

After adding the viral solution, the filtrate clamp was closed. Circulation was initiated for 5 minutes at pump speed of 30 mL/minute. The retentate clamp would be closed to reach a specified TMP. Ultrafiltration was stopped when reaching the desired target volume. Diafiltration was performed with the reservoir lid closed to enable vacuum generation. The lid was connected to a bottle containing the desired buffer. Filtration was continued at the same TMP as used for ultrafiltration until 5 diafiltration volumes (DVs) were processed.

3.4 Lentivirus Quality Control

3.4.1 Cell Titration

Jurkat Cell Cultivation

A vial of cells was thawed in a water bath heated to 37 °C, until a small ice crystal remained. The content was then transferred to 10 mL cold RPMI +10 % FCS +L-Glutamine in a 15 mL centrifugation tube. Centrifugation was performed at 190 × g for 5 minutes. The supernatant was discarded and the pellet was resuspended in warm 10 mL RPMI +10 % FCS +L-Glutamine in a T75 cell culture flask. The flask was transferred to an incubator at 37 °C with 5 % CO₂.

HT-1080 Cell Cultivation

A vial of cells was thawed in a water bath heated to 37 °C, until a small ice crystal remained. The content was then transferred to 10 mL cold DMEM +20 % FCS in a 15 mL centrifugation tube. Centrifugation was performed at 190 × g for 5 minutes. The supernatant was discarded and the pellet was resuspended in warm 10 mL DMEM + 20 % FCS in a 10 cm cell culture dish. The dish was transferred to an incubator at 37 °C with 5 % CO₂. For split, cells were washed with 5 mL 1× DPBS twice and then detached with 3 mL trypsin for 5 minutes. Cells were then seeded at the desired target cell density.

Transduction and Evaluation

Prior to titration, adherent were trypsinized. A predefined concentration of cells/well in 100 µL were prepared in a 96-well plate. Cells were then incubated for 2 – 4 hours at 37 °C. For serial dilution, Polybrene was diluted to a final concentration of 8 µg/mL in DMEM with 20 % FCS and penicillin (100 units/mL) / streptomycin (100 µg/mL) (P/S). 135 µL were added to each well of a U-bottom 96-well plate. The sample would be sequentially diluted by adding 15 µL in the first well, then continuing to the next well. The plate would be stored at 4 °C.

After 2 – 4 hours incubation of the cells, medium was removed. 100 µL of the diluted virus from the prepared U-bottom 96-well plate would be transferred to the cells. Then

cells were centrifuged at $900 \times g$ for 30 minutes at RT. Cells were then incubated for 24 hours. Medium was then exchanged with fresh one.

72 hours later, medium was removed and cells were be trypsinized in 150 μ L Trypsin. Detached cells could be transferred to 5 mL round button tubes for antibody staining and flow cytometry analysis. Cells were washed with 3 mL of CliniMACS PBS at $450 \times g$ for 8 minutes at RT. A master mix for antibody staining was prepared with 1 μ L antibody per 100 μ L sample. Incubation for 20 minutes at 4 °C. Cells were again washed and then processed on a flow cytometry system.

3.4.2 Host Cell DNA

SYBRGold was employed as a sensitive method for fluorescent staining and detection of DNA using standard ultraviolet transilluminators. 2 $\times$ SYBRGold solution was prepared by mixing 5 mL 1 $\times$ TAE-buffer and 1 μ L SYBRGold. A serial dilution of plasmid #305 in triplicates was prepared. 25 μ L of 2 $\times$ SYBRGold buffer were transferred into wells of a 96-well cell culture plate. Then 25 μ L of standard or diluted samples were added. Detection was done at a Spark 10M Plate Reader at 535 nm.

3.4.3 Host Cell Proteins

HEK293T Host Cell Protein ELISA Kit was employed for this analysis. Reporting Antibody was diluted with 1 $\times$ dilution buffer. The HCP standard dilution was prepared in 1:3 dilution steps. Samples were diluted in 1 $\times$ dilution buffer in duplicates with a target concentration between 3 – 27 μ g/mL. 8-well strips were prepared for standard dilution (14 $\times$), negative control (2 $\times$) and sample duplicates and placed into the plate holder. 100 μ L of each standard, negative control and samples were added into the wells of the plate, then incubated and sealed for 1.5 hours at RT. Wells were washed with 250 μ L of 1 $\times$ PBS-T wash buffer three times. 100 μ L of Reporting Antibody was added to each well and incubated and sealed for 45 minutes at RT. Streptavidin-HRP conjugate was prepared and added. Then TMB substrate was added to each well. Incubation with a sealed plate at RT for 12 minutes followed. 100 μ L of Stop Solution was added into each well. The plate was then placed in the plate reader. The absorbance at 450 nm at RT was measured.

3.4.4 HEK Cell Cycle

Cells were centrifuged at $500 \times g$ for 5 minutes at 4 °C, then washed with 5 mL cold 1 $\times$ DPBS. The pellet was carefully resuspended in 500 μ L of 1 $\times$ DPBS. Cell suspension was transferred to a 15 mL centrifugation tube containing 4.5 mL of ice-cold 70 % ethanol while vortexing slightly. This was followed by incubation at 4 °C for 2 hours. Cells were centrifuged at $1000 \times g$ for 5 minutes at RT. The supernatant was removed. The cell pellet was resuspended in 5 mL 1 $\times$ DPBS, and incubated for 15 minutes at RT. Cells were centrifuged at $1000 \times g$ for 5 minutes at RT. The pellet was resuspended in 300 μ L of 1 μ g/mL DAPI, 0.1 % (v/v) TritonX-100 in PBS and incubated for 30 minutes at RT

protected from light. The sample was transferred to a round-bottom 5 mL tube for acquisition at a flow cytometer. The DAPI signal was analyzed in 405 – 450/50 laser/filter in linear mode.

3.5 CAR-T Cell Manufacturing

3.5.1 Manual T cell Isolation and Transduction

Blood or buffy coat was diluted 1:1 with of 1× DPBS. Peripheral Blood Mononuclear Cells (PBMCs) were separated by addition of 35 mL cells to 15 mL Biocoll separating solution in a 50 mL centrifugation tube. After centrifugation at $400 \times g$ without break for 30 minutes at RT, plasma was aspirated leaving the mononuclear cell at the interphase. The layer was carefully transferred to a new tube. The interlayer was diluted in 1× DPBS to a total volume of 50 mL. This was followed by centrifugation at $300 \times g$ for 10 minutes at RT. The supernatant was removed and cells were washed two more times.

Magnetic labeling was done with CD8 and CD4 MicroBeads at cold conditions. Cells were resuspended in 80 μ L of CliniMACS® PBS per 10^7 cells. 20 μ L of CD4 and 20 μ L CD8 MicroBeads per 10^7 cells were added. Cells were incubated for 15 minutes at 4 °C, then centrifuged at $300 \times g$ for 10 minutes at RT. Cells were resuspended in 3 mL CliniMACS PBS. LS columns were placed in a QuadroMACS™ magnetic rack and then activated with 5 mL CliniMACS PBS. Cells were applied onto the column. The column was washed 3-times, unlabeled cells were discarded. The column was removed from the rack and cells were eluted with 5 mL CliniMACS PBS. Cells were washed in 50 mL 1× DPBS at $300 \times g$ for 10 minutes at RT. Cells were resuspended in TexMACS® medium with IL-7 (10 ng/ml), IL-15 (5 ng/ml), P/S. 1×10^6 cells/mL were distributed in a 24-well plate with 2 mL per well. 20 μ L of TransAct™ were added to each well. Activated cells were then incubated at 37 °C with 5 % CO₂.

24 hours after application of T Cell TransAct™, Lentivirus was thawed on ice. TexMACS medium in the 24 hours after application of TransAct™, Lentivirus was thawed on ice. Medium in the well was reduced to roughly 250 μ L per well. The lentivirus was added to each well. Spin-oculation was performed at $600 \times g$ for 90 minutes without brake at 34 °C. Cells were then incubated for 4.5 – 6 hours at 37 °C at 5 % CO₂. Then, fresh fully supplemented TexMACS® medium with 3 % human serum albumin was added to reach a cell density of 1×10^6 cells/mL. In the following days, cells were maintained at a cell density between $0.5 - 2 \times 10^6$ cells/mL and a lactate concentration of ≤ 5 mmol/L by addition of supplemented TexMACS® medium to the culture. TexMACS medium, supplemented with human serum albumin, was used until day 5 of cultivation. Afterwards, TexMACS medium containing only interleukins and P/S, was used.

3.5.2 CliniMACS Prodigy® Manufacturing

Leukapheresis was analyzed on MACSQuant® for cell content. A CliniMACS Prodigy® TS 520 tubing-set was installed and primed according to the device's GUI (Figure 3).

CliniMACS Prodigy® TS 520 setup

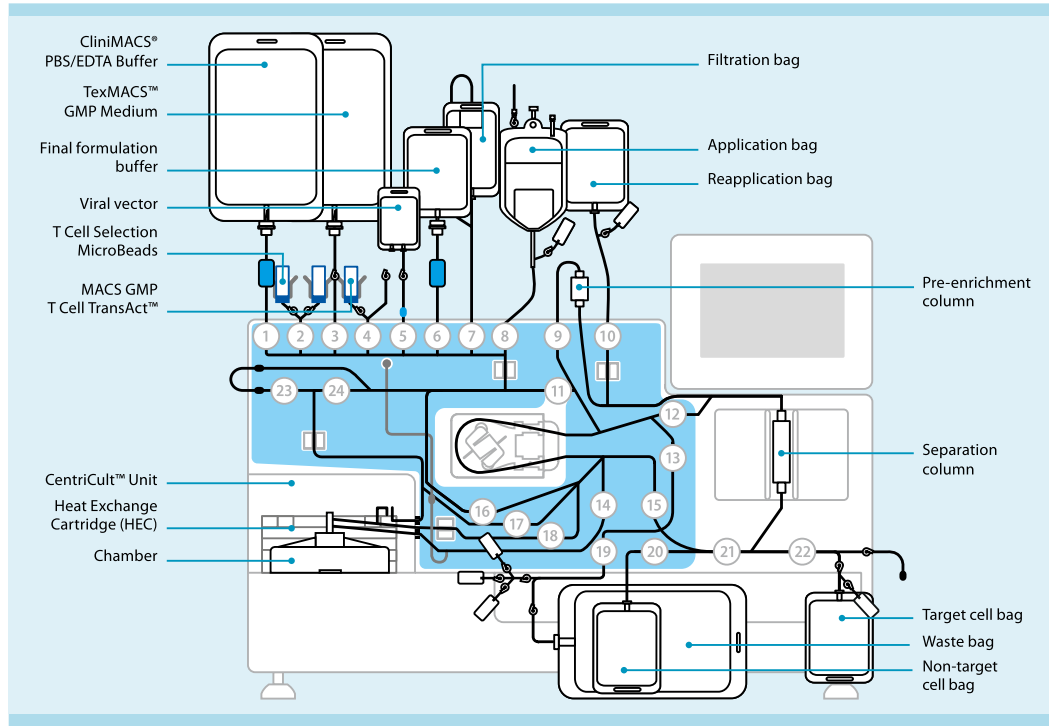


Figure 5 Official CliniMACS Prodigy® TS 520 setup [244].

CliniMACS PBS/EDTA (0.5 % human serum albumin) and supplemented TexMACS (IL-7, IL-15 and 3 % pooled human serum) bags were spiked. Then, bags were placed in the right position for waste, target, non-target and formulation. Leak-test and priming were initiated. Starting material was welded to the application bag and the content was then transferred into it. Vials of CD4 and CD8 MicroBeads were spiked to the system. T cell enrichment and automated red blood count reduction were initiated. After determining concentration of CD3⁺ cells, adjustment to cell count of 1×10^8 CD3⁺ cells in 100 mL was done by CliniMACS Prodigy, after entering the data. Finally, activation of T cells was started after spike of TransAct™.

24 hours after activation, viral transduction was performed. A CD19-CAR Lentivirus bag was thawed at RT. The bag was then welded to the tube set. Transduction was initiated on the system.

Cultivation volume was increased to 200 mL on day 3 and remained constant. Medium exchange of 130 mL was performed every day and two-times a day from process day 9 on. Cultivation was done until day 12. Samples were collected every day, except day 1.

3.6 Phenotyping, Functional and Intracellular Analysis

3.6.1 Cytotox Assay

The functional assay was based on the conversion of luciferin as a substrate by luciferase-producing tumor cell lines (Raji, IMR-32 or RH30 were employer). The absence of a luminescence signal compared to a control indicates the lysis of the tumor cells.

The tumor cell lines were seeded at a density of 30 000 cells per well in 50 μ L RPMI (with L-glutamine and P/S) in a white 96-well cell culture plate. The effector cells (CAR-T cells or control T cells) were added in various concentrations in 50 μ L supp. RPMI (Table 10).

Table 10 Two different dilution series used on effector to target ratios (CAR-T to tumor cell). Values for both serial dilutions shown both as fraction and decimal representation.

Effector to Target Ratio (E:T)								
Initial 5:1	5:1	5:2	5:4	5:8	5:16	5:32	5:64	5:128
	5:1	2.5:1	1.25:1	0.63:1	0.31:1	0.16:1	0.08:1	0.04:1
Initial 4:1	4:1	2:1	1:1	1:2	1:4	1:8	1:16	1:32
	4:1	2:1	1:1	0.5:1	0.25:1	0.13:1	0.06:1	0.03:1

Tumor cells in RPMI dilution served as the standard series: 100 %, 75 %, 50 %, 25 %, 10 %, and cell-free. Finally, 50 μ L of luciferin was added with a concentration of 1 μ g/mL. Depending on the condition, the wells had to be filled to a total volume of 200 μ L. The cells were incubated at 37 °C and 5 % CO₂ for 72 hours. After 24, 48 and 72 hours, the luminescence signal was measured at 37 °C.

3.6.2 Re-Challenge

CAR-T cells were repeatedly brought into contact with fresh tumor cells. Raji or RH30 cells were used. The assay was performed in 6-well cell culture plates. Raji cells were seeded at 0.5×10^6 cells per well in 1 mL supplemented RPMI. Then, 2×10^6 CAR-T cells were added in 2 mL of supplemented RPMI, resulting in an E:T of 4:1. The cells were incubated for 48 hours at 37 °C and 5 % CO₂. Subsequently, cells were either prepared in a new re-challenge setup, used as a sample for DigiWest analysis or examined in a flow cytometry analysis.

3.6.3 Flow Cytometry Analysis

Two panels were set up for flow cytometry analysis. Each sample was prepared twice. The Core panel was used in both staining approaches and then combined with either the Phenotype or Condition panel. All markers and their antibody-fluorochrome conjugates are listed in Table 11.

3 Materials and Methods

Table 11 Flow cytometry panel combination for activation and exhaustion profiling and phenotyping of CAR-T cells. Antibodies of the Core Panel were either combined with Phenotype or Condition panel.

	Antibody Target	Fluorochrome
Core Panel	CD3	Vio® Bright R720
	CD4	BV510
	CD8	PE
	CD27	BUV496
	CD19-CAR Biotin	Biotin-APC/-FITC
	Live-Dead	7-AAD
Phenotype Panel	CD45RA	Vioblue®
	CD45RO	APC-Vio® 770
	CD25	Vio® Bright B515
	CD69	BV650
	Ki-67	BV711
	CD95	BUV737
Condition Panel	PD-1	APC
	CTLA-4	BUV805
	TIM-3	PE-Vio® 770
	LAG-3	BV786
	HLA-DR	Spark UV™ 387

Of each sample, 0.3×10^6 cells were prepared in 3 mL cold CliniMACS PBS in a round-bottom centrifuge tube. Cells were vortexed and briefly centrifuged at $450 \times g$ for 5 minutes at RT. The supernatant was discarded and the cells were resuspended in 300 μ L CliniMACS PBS. A master mix of FcR blocking reagent was prepared so that 0.6 μ L was added to 300 μ L of cell suspension, followed by incubation for 10 minutes at 4 °C. The cells were then washed in 3 mL CliniMACS PBS at $450 \times g$ for 5 minutes at RT. Antibodies for Core Panel + Phenotype Panel or Core Panel + Condition Panel were then added and incubated for 30 minutes at 4 °C. Cells were then washed again and resuspended in 300 μ L CliniMACS PBS. This was followed by secondary anti-biotin antibody staining and incubation for 30 minutes at 4 °C. After a final washing step, cells were resuspended in 300 μ L CliniMACS PBS and combined with 3 μ L 7-AAD. After incubation for 5 minutes at RT, cells were processed on a FACS Fortessa X-20.

Evaluation was done with FlowJo v10.10.1 (Becton Dickinson, USA) employing gating strategies described in Table 12 and Table 13.

Table 12 Gating Strategy for T cell activation.

	Non-activated	Early activated	Robust activated	Late activated
CD25	-	-	+	+
CD69	-	+	+	-

Table 13 Gating strategy for T cell subtypes.

	T _N	T _{SCM}	T _{CM}	T _{EM}	T _{EFF}	T _{EMRA}
CD45RA	+	+	-	-	+	+
CD45RO	-	-	+	+	-	-
CD27	+	+	+	-	-	-
CD95	-	+	+	+	+	-

3.6.4 DigiWest Sample Preparation

2×10^6 cells were centrifuged at $450 \times g$ for 5 minutes at 4°C in a 50 mL low-bind centrifuge tube. The supernatant was aspirated carefully. The pellet was then resuspended in 5 mL cold $1\times$ DPBS, the suspension was transferred to a new 50 mL low-bind centrifuge tube. Cells were spun down again at $450 \times g$ for 5 minutes at 4°C , the supernatant was again aspirated carefully. This step was repeated one more time. The dry pellet was then snap-frozen on dry ice and stored at -80°C .

Samples were processed by the Naturwissenschaftliches und Medizinisches Institut (NMI) as previously described [245].

3.6.5 Statistical Methods

Graphs and corresponding statistical analyses were generated using Prism v10.1.1 (GraphPad Software, USA). Unless otherwise stated, the mean value was calculated and presented with the standard deviation of the mean (SD). The number of independent experiments and the total number of repetitions (n) are indicated in the respective figure legend.

To compare two dependent samples, two-tailed paired t-tests were performed, normal distribution could be verified in all represented cases using the Shapiro-Wilk test. The significance level was set at $\alpha = 0.05$.

To investigate several groups, an ordinary two-way analysis of variance (ANOVA) was performed. Subsequently, pair comparisons of each test subject were examined using the Dunnett test, whereby significance level was set at $\alpha = 0.05$.

For the correlation analysis between two parameters, simple linear regression was first performed. Based on the given normality of the measurements examined using D'Agostino tests, two-tailed Pearson correlations were then calculated. The statistical test is indicated in the respective image caption.

The standard notation was used to indicate the significance level (***: $0.0001 \leq p \text{ value} < 0.001$, **: $0.001 \leq p \text{ value} < 0.01$, *: $0.01 \leq p \text{ value} < 0.05$, n.s.: $p \text{ value} \leq 0.05$).

4 Results

4.1 Lentiviral Vector Manufacturing Platform

4.1.1 Master Cell Bank Generation and Evaluation

A single vial of LV MAX™ HEK293 cells was used for expansion and master cell bank generation. Cells were expanded in suspension at 37 °C and 8 % CO₂ for 4 passages to a volume of 500 mL (Figure 6). Seeding concentration in each split was 0.5×10^6 viable cell/mL. On passage 4 cells reached 3.3×10^6 viable cell/mL and a viability of 95.9 %. During expansion, the cell-specific growth rate was between 0.3 and 0.7 cells/cell/day. 105 vials each containing 1×10^7 cells in 1 mL were prepared for cell banking. Each round of cell banking was done for 35 vials in parallel.

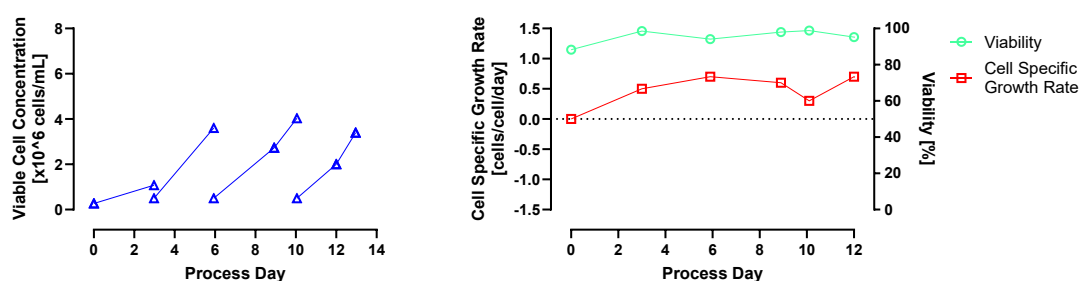


Figure 6 Cultivation data for master cell bank generation. **Left:** Subculture x-y plot of viable cell concentration for each process day. **Right:** x-y plot of cell-specific growth rate and viability of respective process days. Viability was determined as ratio of viable cells to total cells per mL. Cell-specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. Data shown for single Master cell bank generation ($n=1$).

Stability of the cell bank was evaluated by comparing three expansions span over three years. Data represents cultivations shortly after MCB generation, one year and three years after MCB generation. In passage 4, after cell thaw, cells ranged between 0.5 and 1.1×10^6 cells/mL after dilution in 30 mL cultivation volume. On passage 5, viable cell concentration was between 1.6 and 2.4×10^6 cells/mL. In passage 6, cells reached between 3.4×10^6 cells/mL and 5.1×10^6 cells/mL. Passage 7 had a range between 3.2×10^6 cells/mL and 3.9×10^6 cells/mL. The viability in all passages was above 94 %. Cell-specific growth rate was between 0.3 and 0.7 cells/cell/day. These data indicate a highly reproducible cultivation and stable cell line regarding 4 passages for expansion to production volume.

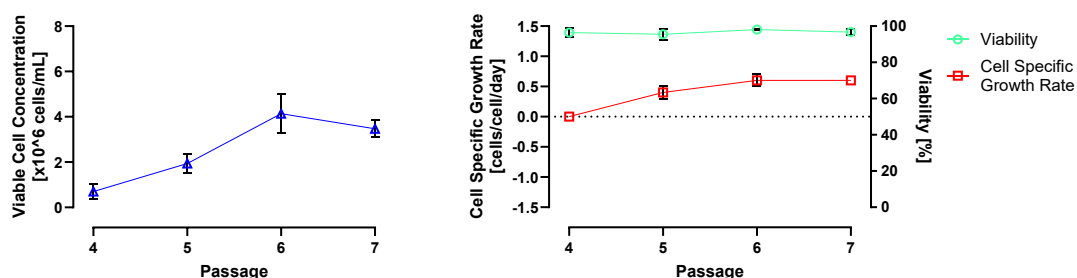


Figure 7 Cultivation data plot of three cultivations compared. **Left:** x-y plot of viable cell concentration in each cultivation passage (subculture). **Right:** x-y plot of cell-specific growth rate and viability of respective process days. Viability was determined as ratio of viable cells to total cells per mL. Cell-specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. Mean data shown for the 3 separate cultivations ($n=3$). 1. Cultivation shortly after master cell bank (MCB) generation, 2. one year after MCB-generation and 3. 3 years after MCB-generation.

4.1.2 Analysis of Cellular Biology for Optimum Transfection Time

Figure 8 shows cell culture data from the 30 mL (only first passage) to 60 mL volume in shake flask cultivation. It was shown that both cultures exhibited similar growth. A maximum cell density of 8.4×10^6 cells/mL (30 mL) and 8.1×10^6 cells/mL (60 mL) was reached on day 6. Viability remained constant and over 90 %. The cell-specific growth rate showed a slight decline after day 4 for both volumes. Glucose decreased steadily and reached a value below the measurement limit on day 6. This indicated that glucose was almost completely depleted from the medium. Cultivation was stopped at day 6. Lactate concentration rose until day 3, remained stable until day 5 and decreased towards day 6. The pH decreased until day 4 or 5 and increased strongly towards day 6, to 7.36 (30 mL) and 7.29 (60 mL).

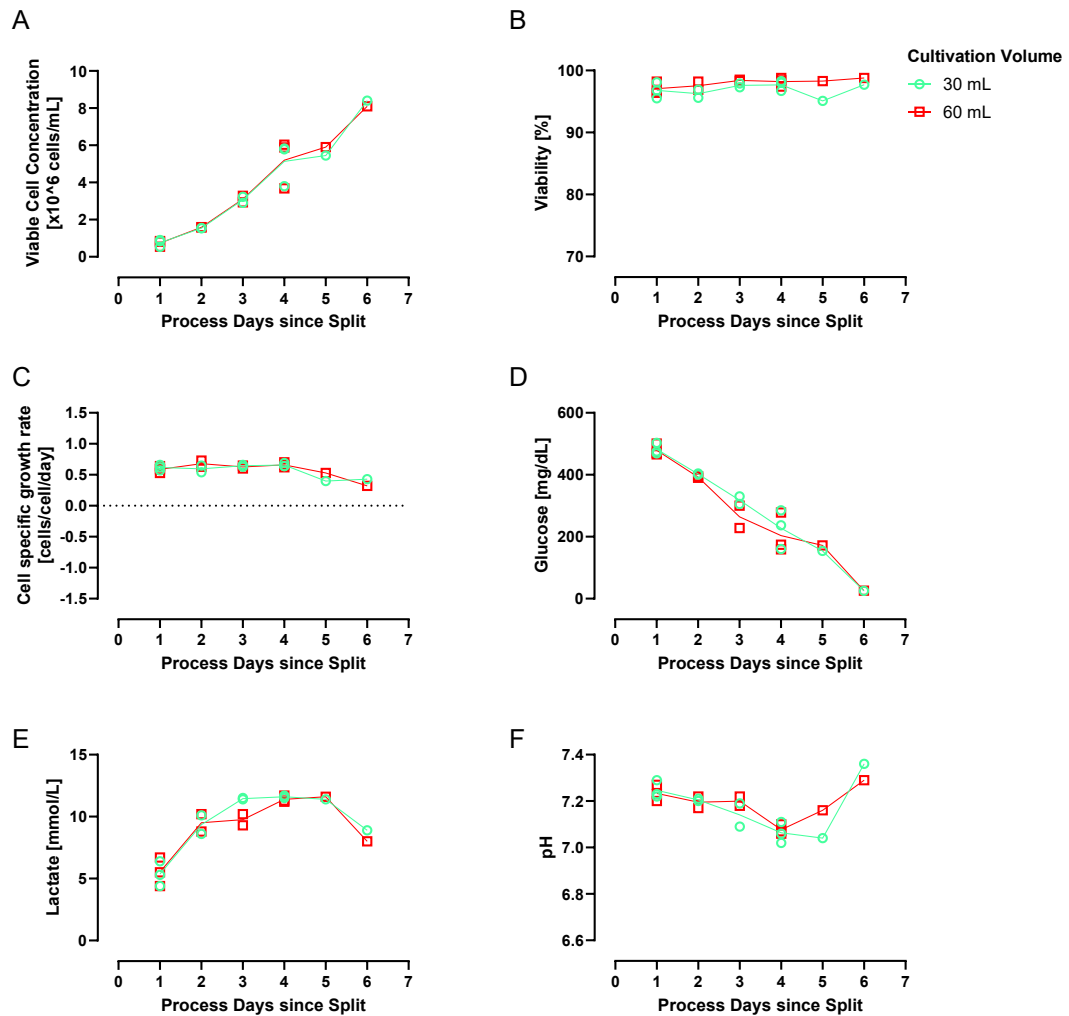


Figure 8 Cultivation data plots for 6-day-cultivation. The scale was either 30 mL volume (green) or 60 mL volume (red). **A:** x-y plot of viable cell concentration. **B:** x-y plot of viability determined as ratio of viable cells to total cells per mL. **C:** x-y plot of cell-specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. **D:** x-y plot of glucose concentration (determined via Radiometer). **E:** x-y plot of lactate concentration (determined via Radiometer). **F:** x-y plot of pH (determined via Radiometer). Three cultivations were compared for each, with samples collected on selected days; individual data for each cultivation are shown ($n=6$).

The cell cycle data were generated via DAPI staining and subsequent flow cytometry analysis. The data showed a constant decrease in the proportion of cells in the G2- and M-phases, as well as an increase in the G0- and G1-phases over the course of 6 process days (Figure 9).

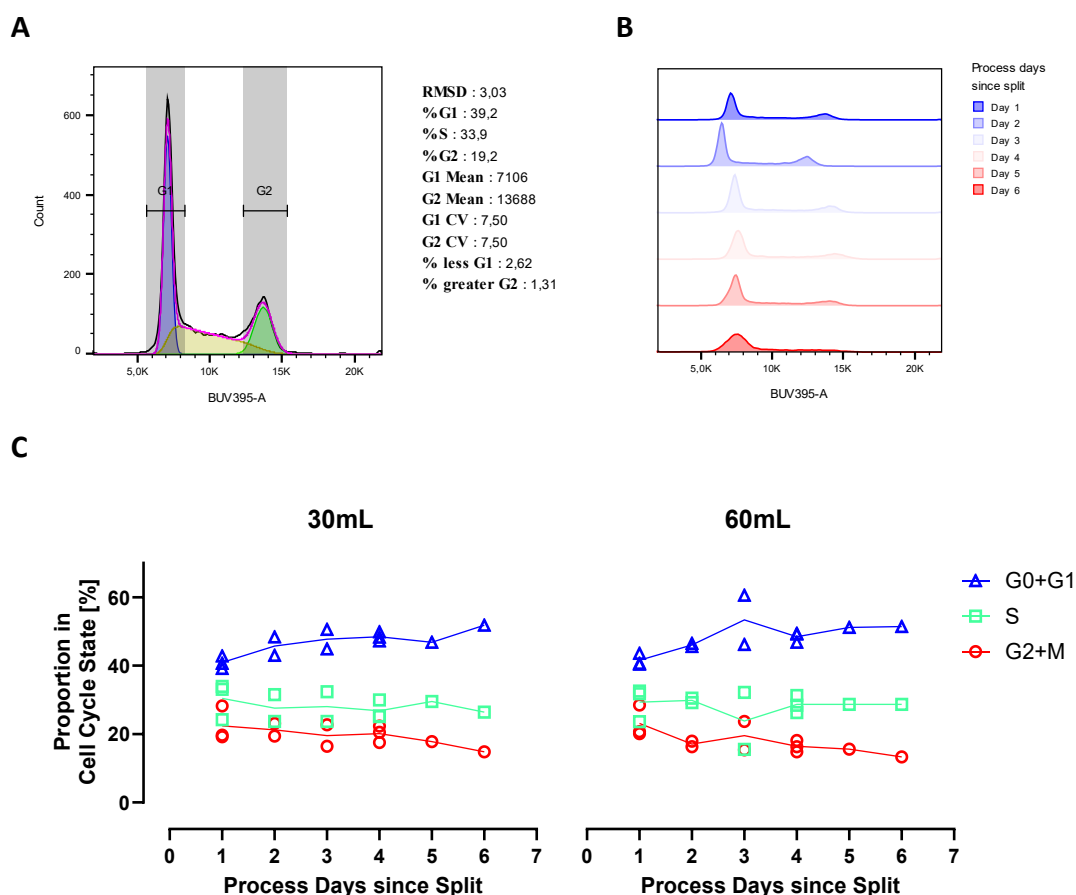


Figure 9 Cell cycle data collected after split of LV MAX™ HEK293 cells and over the course of 6 days. **A:** Histogram of analysis for cell cycle states into G1-, S- and G2-phase based on BUV-395 signal employing Watson-model. **B:** Histogram of process days 1 to 6 of BUV-396 channel. **C:** cell cycle proportions over the course of 6 days. Three cultivations were compared for each scale, with samples collected on selected days; individual data for each cultivation are shown (n=6). Cell cycle analysis see Appendix Supplementary Figure 1.

4.1.3 Influence of Supplementation on Cell Cultivation

To investigate the influence of the reagents in the LV MAX™ system, cell data was examined 24 hours after the addition of Enhancer and Supplement. As control, 5 mmol/L sodium butyrate addition was examined as a control for cell cycle arrest. The cell concentration 4 days after last split was 4.3×10^6 cells/mL. To simulate transfection, a cell density of 3.5×10^6 cells/mL was set in 30 mL on the day before the reagents were added. The next day, 0.05 mL of Supplement per mL culture volume and 0.05 mL of Enhancer per mL culture volume were prepared in duplicates.

The cell concentration and cell-specific growth rate were slightly reduced one day after Supplement-treatment. Viability was not affected in all conditions. Glucose was higher for Supplement-treatment and slightly increased with Enhancer addition. Lactate concentration for Supplement-treatment was greatly increased and was very low for Enhancer-treatment. The untreated control and sodium butyrate treatment were in between. The pH was much lower for supplement treatment with only around 7.0 compared to mean of 7.4 for the other treatments. Cell data can be found in Figure 10.

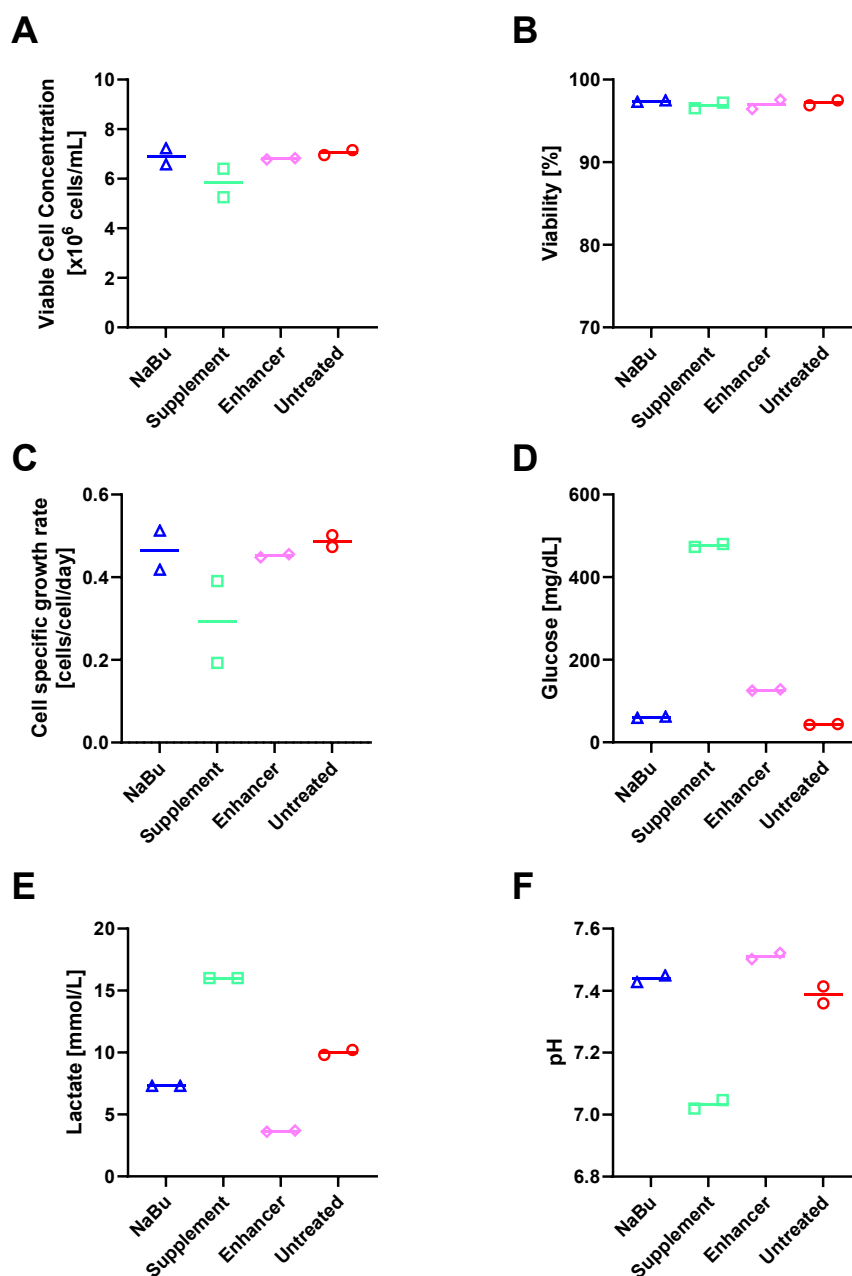


Figure 10 Cell cultivation of LV MAX™ HEK293 data one day after treatment with either sodium butyrate (NaBu), LV MAX™ Supplement, LV MAX™ Enhancer or untreated. **A:** dot plot of viable cell concentration. **B:** dot plot of viability determined as ratio of viable cells to total cells per mL. **C:** dot plot of cell-specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. **D:** dot plot of glucose concentration (determined via Radiometer). **E:** dot plot of lactate concentration (determined via Radiometer). **F:** dot plot of measured pH (determined via Radiometer). Data of replicates shown individually ($n=2$).

Enhancer and sodium butyrate showed an increased proportion of cells in G0- and G1-phase and decreased proportion for S- and G2/M-phase. Supplement had an increased G2/M-population with reduction of cells in G0/G1-phase, comparable to the untreated control.

Data from cell culture and cell cycle analysis indicate that LV MAX™ Enhancer causes cell cycle arrest. Supplement on the other hand has a strong inhibiting impact on glucose consumption and leads to increased lactate accumulation.

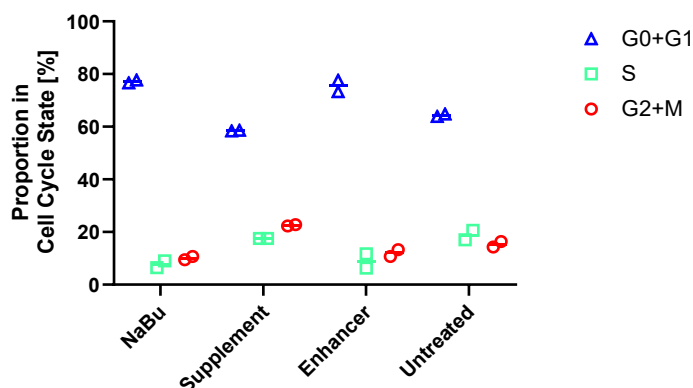


Figure 11 Dot plot of cell cycle data of LV MAX™ HEK293 cells treated with either sodium butyrate (NaBu), LV MAX™ Supplement, LV MAX™ Enhancer or untreated. Cells were cultivated 4 days, then incubated for one day with each reagent. Analysis of cell cycle states into G0/G1-, S- and G2/M-phase based on BUV-510 signal. Data of replicates shown individually (n=2). Cell cycle analysis see Appendix Supplementary Figure 2.

4.1.4 Transfection and Initial Functional Test of Lentivirus Production

Transfection of plasmid #119 (CD276-CAR with mCherry co-expression) was performed in a 60 ml LV MAX HEK293™ cell culture in passage 7. The suspension was centrifuged and concentrated to 50:1 using LentiX concentrator. Transduction was done on Jurkat cells (immortalized T cells). Previously produced lentivirus of the same construct in adherent HEK293T cells (100:1 concentration) was used for comparison. Transduction of the Jurkat cells was done at 1×10^6 cells/ml in 1 ml in 12-well. LV MAX™ system was compared to the Lentivirus from adherent cell culture by addition of 75 µl lentivirus, respectively. The control was untreated. The function to transduce efficiently was determined by the expression of mCherry 9 days after adding the virus (Figure 12). The control showed some autofluorescence with 3.1 % mCherry-positive cells. In the adherent production, an expression of 83.4 % was detected (100× concentrated). For LV MAX™, expression was comparable with 81.1 % mCherry-positive cells (50× concentrated).

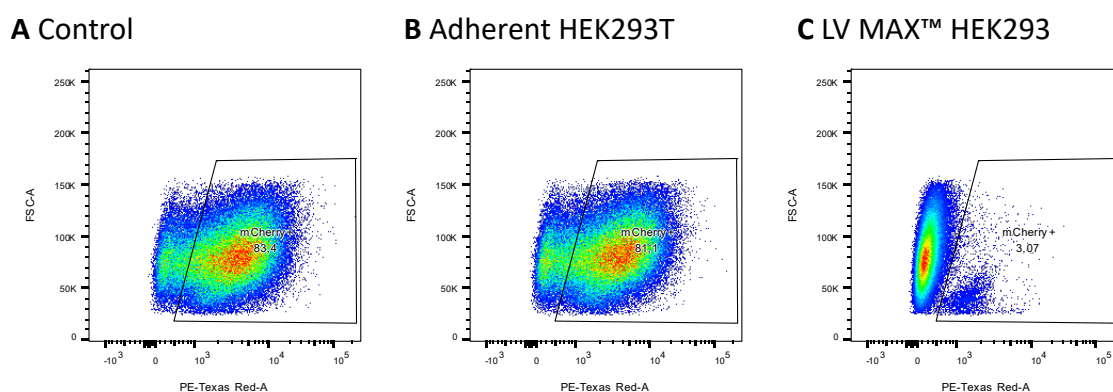


Figure 12 Flow cytometry plot of Jurkat cell line 9 days after transduction with construct #119 (CD276-CAR with co-expression of mCherry). **A:** Jurkat cells cultivated without transduction. **B:** previously produced Lentivirus employing adherent HEK293T cell line. **C:** Lentivirus produced with LV MAX™ HEK293 cell line. Three separate transductions with lentivirus shown ($n=3$). See gating strategy in Supplementary Figure 3.

4.1.5 Establishment of a Flow Cytometry-based Lentivirus Titration Method for the Determination of Transducing Units

Target Cell Line

Two cell lines were considered for titration of lentiviral vectors. Jurkat cells, are immortalized T cells and therefore actually closest to the target cells. HT-1080 cells, which are well suited for titrations due to their adherent and planar growth (increased cell surface area for virus targeting) at very low cell densities.

First, the cultivation process was standardized to achieve high reproducibility and minimize cultivation-related variability which would impact efficiency of transduction of the lentivirus. Stable cultivation was more readily achieved for Jurkat cells due to their growth in suspension. Cells were cultivated in motionless T75-flask cultivation at 37 °C with 5 % CO₂. The highest cell-specific growth rate was achieved with an inoculation between 0.17 and 0.3 ×10⁶ cells/mL and a split every 2 to 3 days (Figure 13).

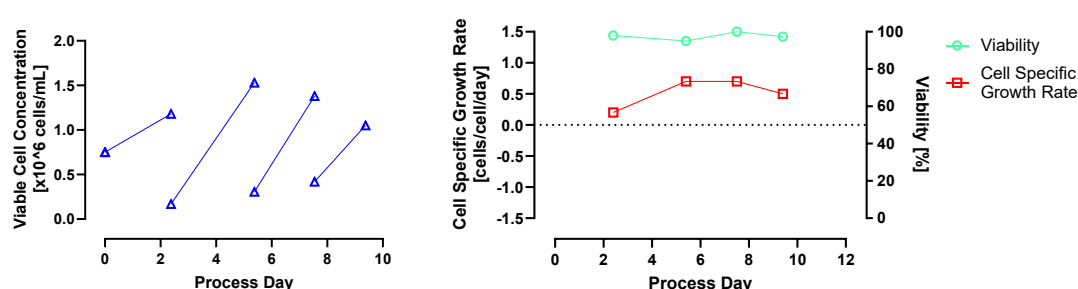


Figure 13 Jurkat cultivation optimization for reproducibility. **Left:** Subculture x-y plot of viable cell concentration for each process day. **Right:** x-y plot of cell-specific growth rate and viability of respective process days. Viability was determined as ratio of viable cells to total cells per mL. Cell-specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. Data shown for single cultivation ($n=1$).

HT-1080 cells were complicated by their adherent growth. Cells were cultivated in 10 cm dishes at 37 °C and 5 % CO₂. An optimum cell-specific growth rate was achieved at reduced seeding concentrations of only 0.011 to 0.035 ×10⁶ cells/mL and a split rhythm

of 2 to 3 days. Only at these low seeding densities a confluence of 60 % could be achieved (Figure 14).

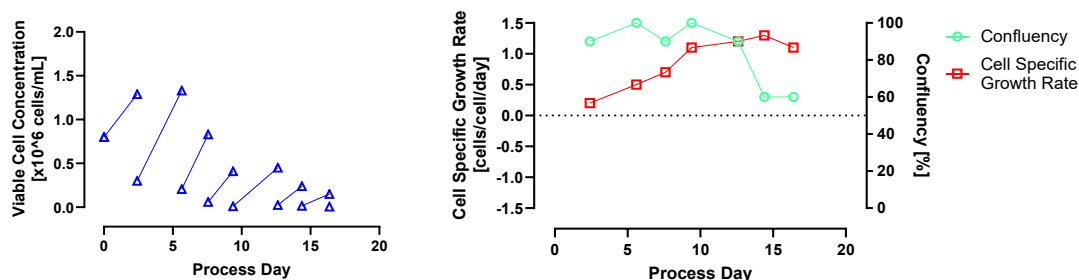


Figure 14 HT1080 cultivation optimization for reproducibility. **Left:** Subculture x-y plot of viable cell concentration for each process day. **Right:** x-y plot of cell-specific growth rate and viability of respective process days. Confluency was determined by estimation with a confocal microscope. Cell specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. Data shown for single cultivation ($n=1$).

Viral Titer Determination

Establishing the linear range is critical for reliable titer quantification and prevent over- or underestimation of the titer. Overestimation occurs at the limit of detection where background noise is mixing with actual signal. Underestimation happens when several viruses transduce one cell, while being only counted as single positive event. The objective was to determine a broad linear range with high reproducibility. Seeding densities 8 000, 30000 and 100 000 cells per well in 0,1 mL (96-well plate) were tested (Figure 15). In each of these three approaches, the vector was diluted in 10-log steps from 1:10 to 1:1 000 000. Previously generated virus of construct #248 (CD276-CAR with co-expression of GFP), produced with LV MAX™ HEK293 cells and concentrated to 50×, was used to estimate linear operating range for titration. The cells were transduced according to chapter 3.4.1. Expression was determined by flow cytometry of the GFP signal. To determine the linear range, the proportion of infectious units was determined using Equation 1.

$$TUs/mL = \frac{\text{cells per well} \times \text{virus dilution} \times \% \text{ expression}}{0,1 \text{ mL (volume per well)}} \quad \text{Equation 1}$$

TUs/mL = transducing units per mL

For a seeding density of 8 000 cells per well, a nearly linear range for viral dilution between 1:10 000 and 1:100 000 was observed. The range was detected in the calculated TUs plot, indicated by plateau of more than on dilution (Figure 15). For seeding densities with more than 8 000 cells per well, no linear range was found. Reducing the seeding density further to increase the range was not possible. Cell loss during medium change due to suspension growth was a limiting factor for this approach.

In addition, Jurkat cells grow poorly at lower cell densities. Further cell loss would lead to insufficient concentrations for flowcytometry analysis.

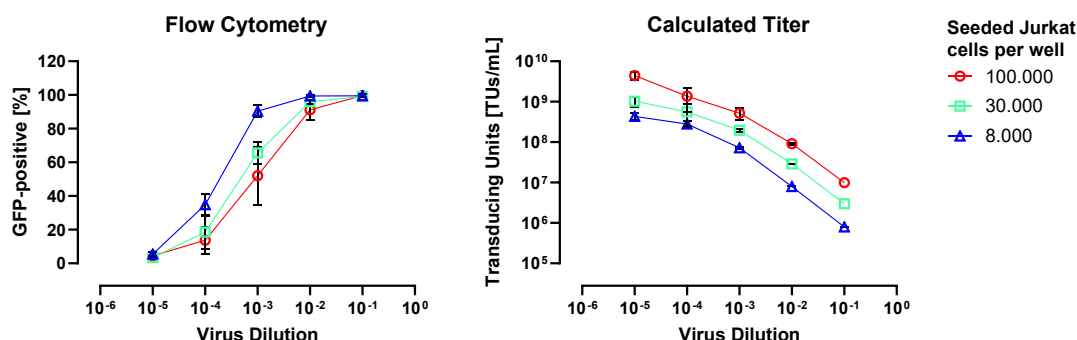


Figure 15 Lentivirus titration on Jurkat cells via GFP-expression. Virus construct #248 (CD276-CAR with co-expression of GFP), 50× concentrated and non-concentrated (1×). **Left:** x-y plot of detected GFP co-expression via flow cytometry analysis. **Right:** calculated transducing units (TU) based on proportion of GFP-positive cells calculated via Equation 1. Three seeding densities with mean data shown for viral transduction performed in quadruplicates (n=12). Individual flow cytometry plots see Supplementary Figure 4, Supplementary Figure 5 and Supplementary Figure 6.

For the Jurkat cell line, a seeding density of 8 000 cells per well produced measurements approaching a linear range. Cell concentration could not be reduced beyond this level, although lower concentrations were achievable with HT-1080 cells. Here, the cell count was reduced slightly further to 7 000 cells per well. For the 50× sample, a linear range between dilution 1:10 000 and 1:100 000 was found (Figure 16). A batch of non-concentrated vector was additionally tested. For the 1× sample, a broader was found plateau ranging between 1:100 and 1:10 000.

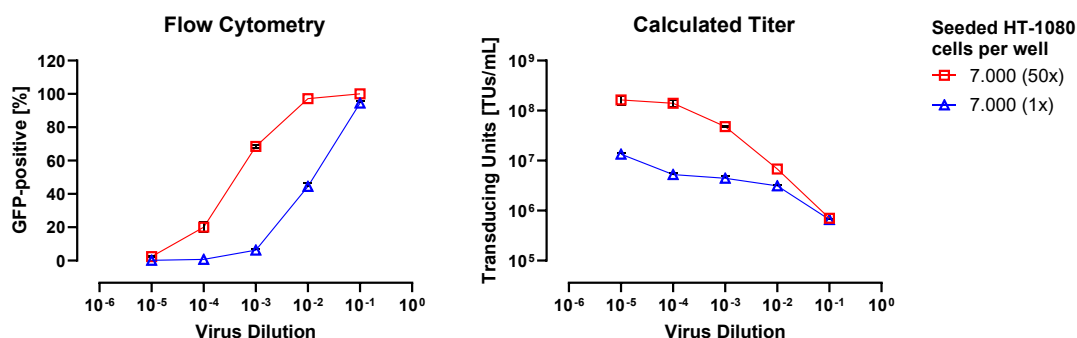


Figure 16 Lentivirus titration on HT-1080 cells via GFP-expression. Virus construct #248 (CD276-CAR with co-expression of GFP), 50× concentrated and non-concentrated (1×). **Left:** x-y plot of detected GFP co-expression via flow cytometry analysis. **Right:** calculated transducing units (TU) based on proportion of GFP-positive cells calculated via Equation 1. Two viral batches tested with mean data shown for viral transduction performed in quadruplicates (n=8). Individual flow cytometry plots see Supplementary Figure 7.

To determine lentiviral titers of clinically relevant constructs, a titration method independent of fluorescent reporter expression was established. Construct #305 was utilized, a CD276-CAR with myc-tag and co-expression of a CD19 tag. Integration of the CAR construct could therefore be tested either via myc (fused to the CAR) or via CD19 expression. Staining of myc was achieved with primary antibody anti-myc biotin and

secondary anti-biotin PE. Anti-CD19 PE conjugate was used for the co-expressed tag . In flow cytometry analysis, both CAR and CD19 positive populations could be well separated (Figure 18).

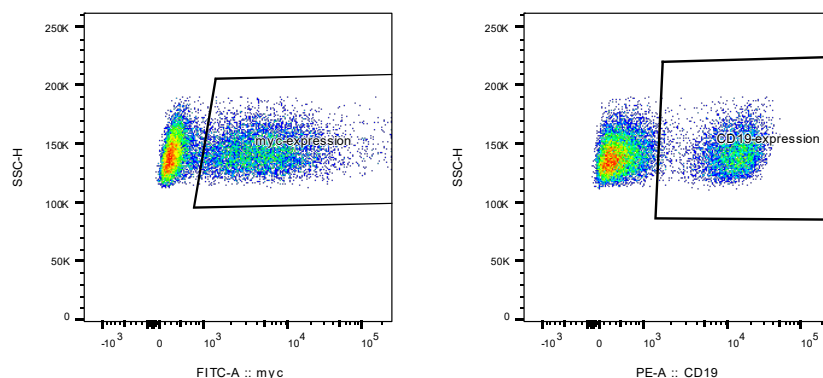


Figure 17 Flow cytometry analysis for detection of CAR and co-expressed CD19 tag. **Left:** staining of myc tag fused to CAR via myc-FITC. **Right:** staining of co-expressed CD19 tag via CD19-PE staining. HT-1080 cells were previously transduced with lentiviral construct #305 (CD276-CAR with myc-tag and co-expression of CD19).

Antibody-staining with washing steps resulted in loss of cells prior to flow cytometry measurement. Titration had to be further optimized to reduce cell loss. Centrifugation speed was increased (350 to $450 \times g$) and prolonged (5 to 8 minutes). CliniMACS PBS buffer for washing steps was supplemented with 0.5% human serum album (HSA) to reduce cell loss during washing and prepared at RT (lower viscosity). These changes reduced cell loss significantly. Dilution series was changed from 10 -log to 5 -log, which further broadened the linear range. Titration for the CD19 tag found a linear range between $1:5^5$ to $1:5^9$ (Figure 18). Same results were observed for $1:5^6$ and $1:5^7$ measurements for CAR-myc staining.

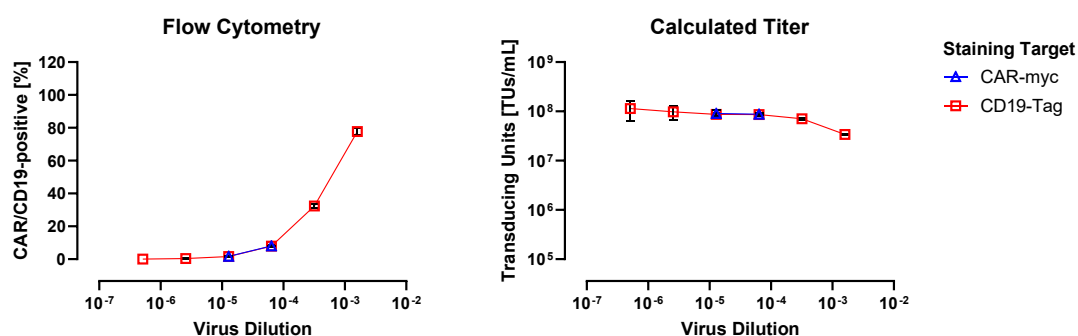


Figure 18 Lentivirus titration on HT-1080 cells via antibody-fluorochrome detection. Virus construct #305 (CD276-CAR with myc-tag and co-expression of CD19), $10\times$ concentrated. **Left:** x-y plot of detected tags via antibody-based flow cytometry analysis (either anti-myc or anti-CD19). **Right:** calculated transducing units (TU) based on proportion of positive cells calculated via Equation 1. Two viral batch tested with mean data shown for viral transduction performed in triplicates ($n=36$ for CD19 tag and $n=12$ for CAR-myc). Individual flow cytometry plots see Supplementary Figure 8.

4.1.6 Cell Cultivation-dependent Vector Stability in Varying Buffers

A key challenge of lentiviral vector purification is its inherent fragility. Building on the methods described in the previous chapter, it was possible to investigate the vector stability. Again, construct #305 (CD276-CAR with myc-tag and co-expression of CD19) was used as a model lentiviral CAR vector. Four harvest procedures were performed. Preparatory steps prior to stability assays included centrifugation of the viral supernatant and filtration with a low-bind 0.45 μm syringe filter. The clarified supernatant was then stored in low-bind 1.5 mL tubes at RT. Duration of initial downstream processing steps was estimated at 4 hours. Subsequent stability tests were assessed within this time span.

Harvests 1, 3 and 4 were highly comparable. The TU over time showed slight decrease of infectious particles, from a mean initial titer of 1.4×10^7 TU/mL to 1.1×10^7 TU/mL. This corresponds to a mean recovery of 79 % of infectious particles within 4 hours (Figure 19). Harvest 2 showed a very steep decay of infectious particles. Here, the initial titer was already lower at 0.8×10^7 TUs/mL and declined to 0.2×10^7 TUs/mL, corresponding to a loss of 25 % from the initial TUs.

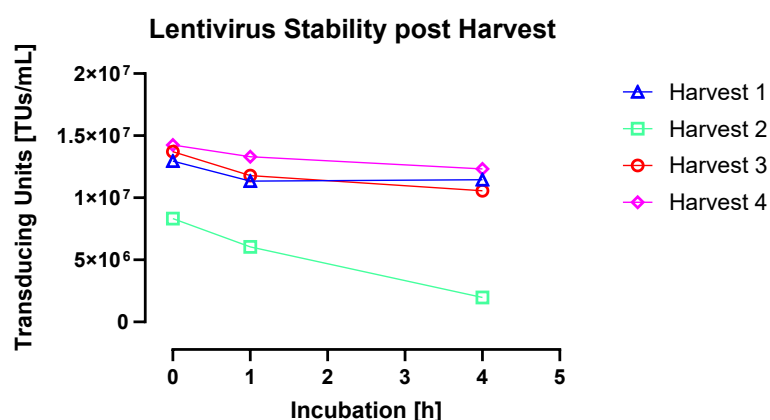


Figure 19 Lentivirus stability of clarified supernatant over time. x-y plot of transducing units per ml (TUs/ml) over 4 hours. Viral dilution before transduction 1:1 000. 4 Harvests performed with 3 time-points (0, 1, mean data of titration in duplicates based on CD19-expression ($n=24$)).

Cell culture condition was examined as a key parameter to explore potential explanations for Harvest 2. An increased cell concentration during cultivation was observed at two passages (Figure 23 E). These passages were found to be second split and the day of transfection. The differences among the other harvests were less pronounced.

However, dilution in different buffers differed significantly between these harvests. Cell culture medium PM, HBSS which is used to maintain cell culture for short processes through osmotic balance and physiological pH, and HEPES buffer, which maintains pH correctly even outside incubators (is CO_2 -independent) and is suitable for protein purification, were selected (Figure 20). For HEPES, varying osmolarities were compared (0.05 M, 0.2 M and 1 M). Clarified supernatant was diluted 1:10 in each buffer and then incubated at RT for up to 4 hours. PM and HBSS displayed similar trends: a steep decline

in TUs was observed for Harvests (except for Harvest 1). HEPES at 0.05 M and 0.2 M concentration performed best, titers remained relatively stable after 4 hours. With a mean initial titer (excluding Harvest 2) of 1.3×10^6 TUs/mL, a good 0.9×10^6 TUs/mL were recovered 4 hours post clarification.

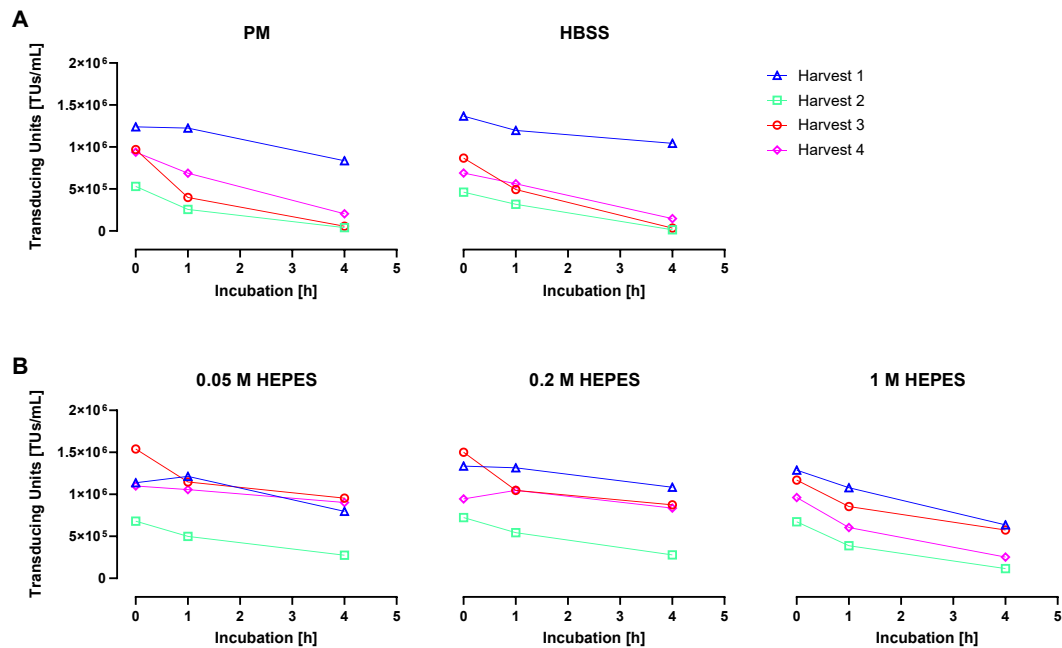


Figure 20 Lentivirus stability in varying buffer conditions over time. x-y plot of transducing units per ml (TUs/ml) over 4 hours. Viral dilution before transduction 1:100. 4 Harvests performed with 3 time-points (0, 1, 4h) mean data of titration in duplicates based on CD19-expression. **A:** Clarified supernatant was diluted 1:10 in LV MAX™ Production Medium (PM, n=24) or Hanks' Balanced Salt Solution (HBSS, n=24). **B:** Clarified supernatant was diluted 1:10 in either 0.05 M (n=24), 0.2 M (n=24) or 1 M HEPES (n=24).

Data were analyzed using an ordinary two-way ANOVA with harvest (1 – 4) and selected buffer (PM, HBSS, 0.05 M HEPES, 0.2 M HEPES, 1 M HEPES) and recovery in percent as dependent variable. There was a significant main effect for the selection of the buffer, $F(4, 20) = 65.79$, $p < 0.0001$, and harvest: $F(3, 20) = 83.31$, $p < 0.0001$. The interaction between buffer and harvest was significant, buffer $\times$ harvest: $F(12, 20) = 10.44$, $p < 0.0001$. Dunnett-adjusted post hoc comparisons (Figure 21) with 0.2 M HEPES showed significant difference to PM, HBSS and 1 M HEPES ($p < 0.0001$).

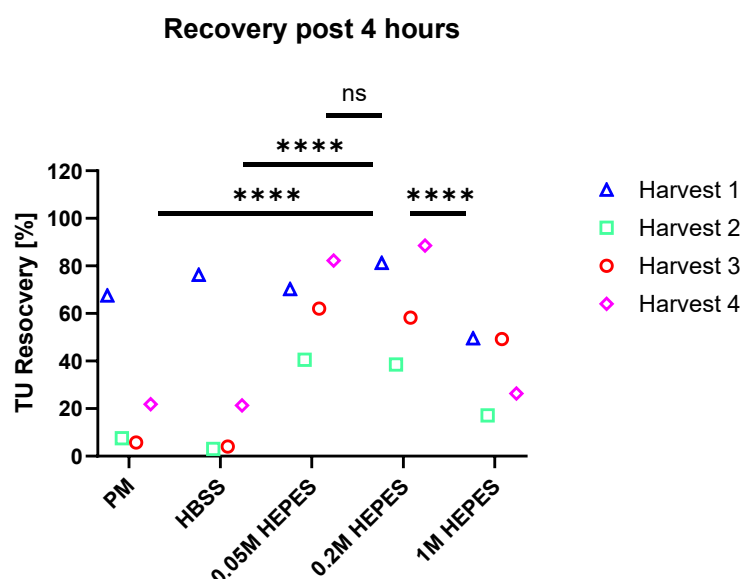


Figure 21 Lentivirus recovery in varying buffer conditions after 4 hours. Dot chart of recovered transducing units per ml (TUs/ml) in percent (initial / 4 hours). Mean data of duplicates shown ($n=40$). Data were analyzed using two-way ANOVA with Dunnet multiple-comparisons test. Exact p values and interaction effects are reported in the text.

Additional tests were conducted to determine effects of freeze-thaw cycle. Samples of Harvest 1 – 3 were frozen on dry ice immediately after dilution in the respective buffers (snap-freeze), no sample could be taken from Harvest 4. Harvest 1 was particularly prominent, with a strikingly higher recovery compared to Harvests 2 and 3 (Figure 22). Only HEPES with concentrations of 0.2 M and 1 M achieved notable values exceeding 2 %. Data were again analyzed using an ordinary two-way ANOVA with harvest (1 – 3) and selected buffer (PM, HBSS, 0.05 M HEPES, 0.2 M HEPES, 1 M HEPES) and recovery in percent as dependent variable. There was a significant main effect for the selection of the buffer, $F(4, 15) = 12.23$, $p < 0.0001$, and harvest: $F(2, 15) = 414.3$, $p < 0.0001$. The interaction between buffer and harvest was significant, buffer $\times$ harvest: $F(8, 15) = 3.853$, $p < 0.0118$. Dunnet-adjusted post hoc comparisons (Figure 21) with 0.2 M HEPES showed significant difference to HBSS and 1 M HEPES.

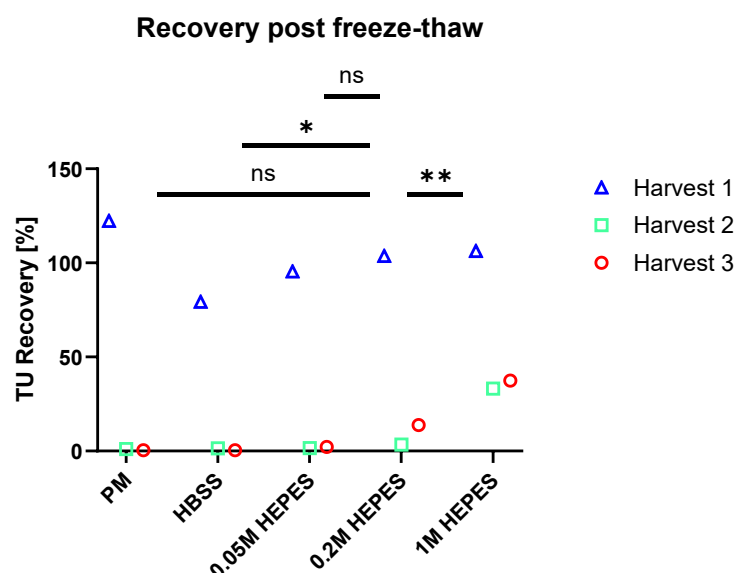


Figure 22 Lentivirus recovery in varying buffer conditions after one freeze-thaw cycle. Dot chart of recovered transducing units per ml (TUs/ml) in percent (initial / after thaw). Mean data of duplicates shown (n=30). Data were analyzed using two-way ANOVA with Dunnett multiple-comparisons test. Exact p values and interaction effects are reported in the text.

The statistical data indicated varying initial conditions in Harvest 1 – 4, namely cell culture, had a massive influence that would also be in interaction with selected buffer. This effect would influence both recovery over time and freeze-thaw cycles. For this reason, cell culture data were examined closely and differences are summarized in the following.

Harvest 1

Cell concentration one day before transfection was just above 4×10^6 viable cells/ml (Figure 23 A). The desired overgrowth was achieved by transfection day, over 6×10^6 viable cells/ml. More than 30 % of culture medium in fresh PM was added. Cell concentration decreased between transfection and harvest day.

Harvest 2

In the second passage, the split had to be carried out one day later due to low cell concentration (Figure 23 B). The cell count at the time of measurement was below the limit of at least 3.5×10^6 viable cells/ml. This was most likely a measurement error, as the calculated growth rate rose very sharply afterwards. Accordingly, the split should not have been postponed by one day. The desired overgrowth on the day of transfection was unexpectedly high, close to 8×10^6 viable cells/ml. In the time between transfection and harvest cell count decreased slightly.

Harvest 3

Only 2.5×10^6 viable cells/mL on the day before transfection and 4.5×10^6 viable cells/mL on the day of transfection (Figure 23 C). No overgrowth was observed on the day of

transfection, therefore, no fresh medium was added on this day. A slight increase in cell count was observed between transfection and harvest day.

Harvest 4

Cultivation was most similar to Harvest 1. A cell concentration of 3.4×10^6 viable cells/mL was reached on the day before transfection and 4.8×10^6 viable cells/mL transfection day (Figure 23 D). Cells were slightly overgrown, but hardly any addition of fresh medium before transfection had to be done. A drop in cell count between transfection and harvest was observed.

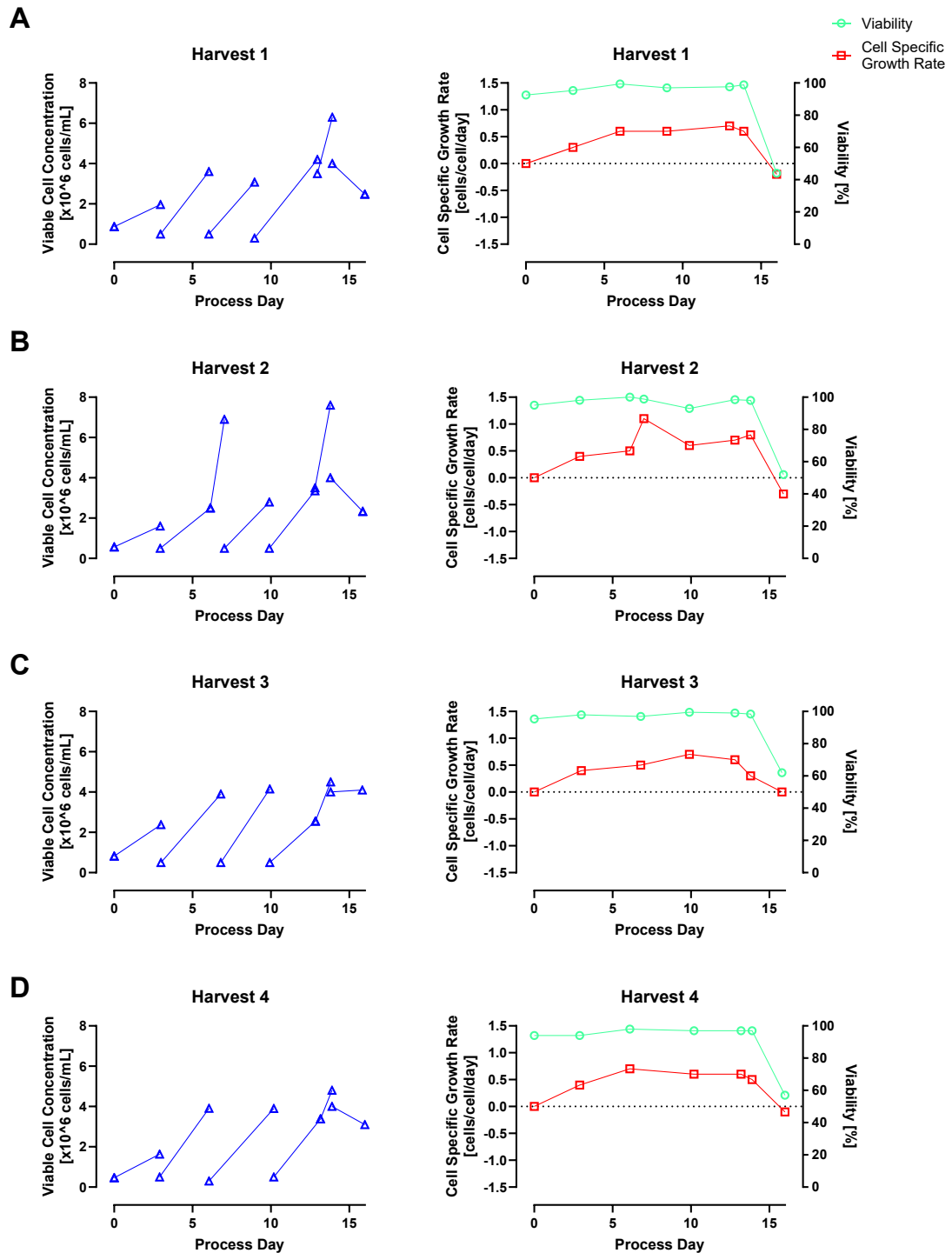


Figure 23 Cultivation data of prior to stability assay (Harvest 1 to 4). Left, subculture x-y plot of viable cell concentration for each process day. Right, x-y plot of cell-specific growth rate and viability of respective process days. Viability was determined as ratio of viable cells to total cells per mL. Cell specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. Data shown respectively for single expansion ($n=1$).

4.1.7 Adapting Seed Density to Accommodate Vector Stability

Based on the findings in the previous chapter 4.1.6, cell culture was identified as a decisive factor influencing vector stability. Cell concentration shortly before transfection as well as on the same day appeared to be particularly important. To confirm this,

different seeding densities were investigated one subculture prior to transfection (Figure 24). Again, construct #305 (CD276-CAR with myc-tag and co-expression of CD19) was used as a model lentiviral CAR vector. Four days prior to transfection (day 7), cells were seeded at 0.5×10^6 cells/mL, 0.65×10^6 cells/mL and 0.75×10^6 cells/mL. Transfection was performed on day 11, cells were adjusted to 4×10^6 cells/mL during transfection procedure (according to chapter 3.3.4). Additional environmental parameters that had not been examined previously were included: glucose, lactate and pH. DAPI staining-based cell cycle data were also collected, as well as data on titer and stability.

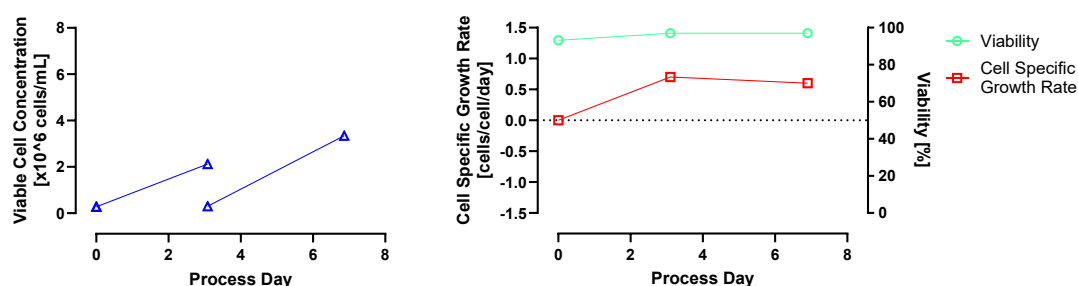


Figure 24 Cultivation data of prior to different seed strategies. **Left:** subculture x-y plot of viable cell concentration for each process day. **Right:** x-y plot of cell-specific growth rate and viability of respective process days. Viability was determined as ratio of viable cells to total cells per mL. Cell specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. Data shown respectively for single expansion ($n=1$).

On day 10, 3 days after seed, increased seeding density had led to a slightly higher cell count for 0.75×10^6 cells/mL (Figure 25 A). Viability data between different seeds were highly comparable. Cell specific growth rates were initially similar on day 10, but dropped for 0.75×10^6 cells/mL on day 11, while increasing for the lower seed of 0.5×10^6 cells/mL. Glucose was consumed between days 10 and 11, with lower values for 0.75×10^6 cells/mL (Figure 25 B). Lactate concentration was similar on day 10, decreased for increased seed on day 11 and remained constant for lower seed. Prior to day 12, there was a pH difference between 0.1 – 0.2. For each seed, pH increased after adjusting to target concentration of 4×10^6 cells/mL (day 11), but fell over the course of 6 hours. One day after transfection, for each pH was comparable with 6.86 (0.5×10^6 cells/mL) and 6.87 (0.75×10^6 cells/mL). Difference in proportions of cell cycle phases were small (Figure 26 A). Cells in G2 and M phase were less present for 0.75×10^6 cells/mL (Figure 26 B).

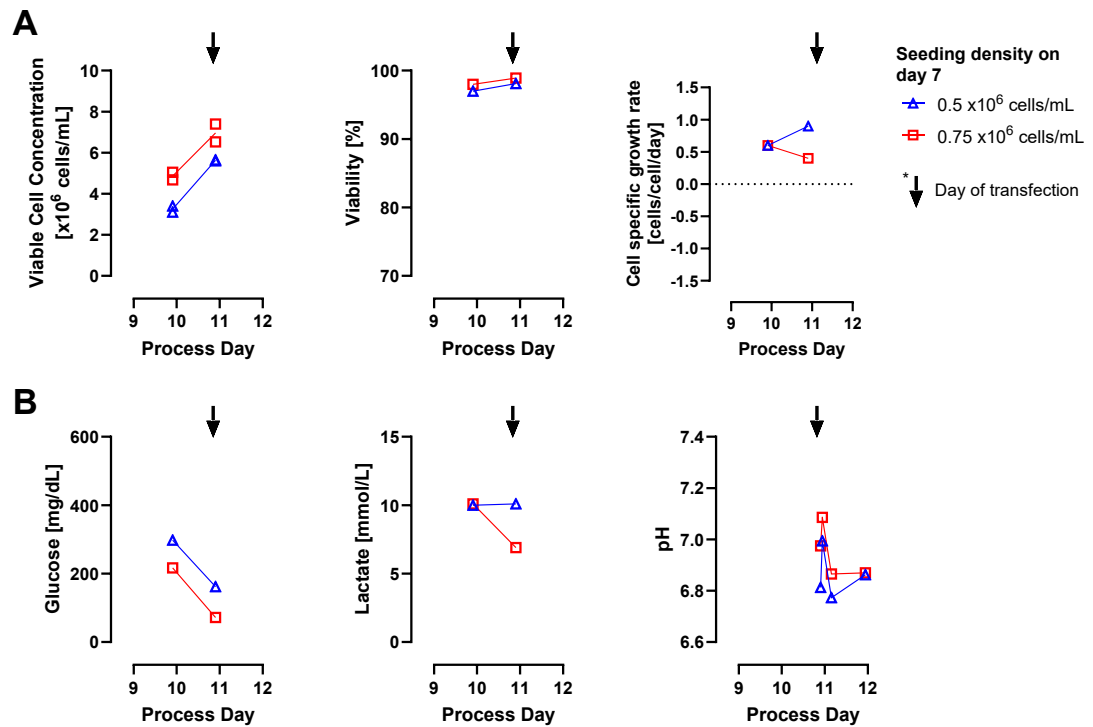


Figure 25 Cultivation data of after different seed strategies. Data shown respectively for single expansion with different seeding strategies on day 7: 0.5 or 0.76 $\times 10^6$ cells/mL ($n=2$). **A:** cell culture data in x-y plot of viable cell concentration for each process day, viability and cell-specific growth rate and of respective process days. Viability was determined as ratio of viable cells to total cells per mL. Cell specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. **B:** cell culture environmental conditions, glucose concentration (Radiometer), lactate concentration (Radiometer), pH (measured via ÅKTA Go before transfection process, immediately after, before addition of supplement and one day after transfection)

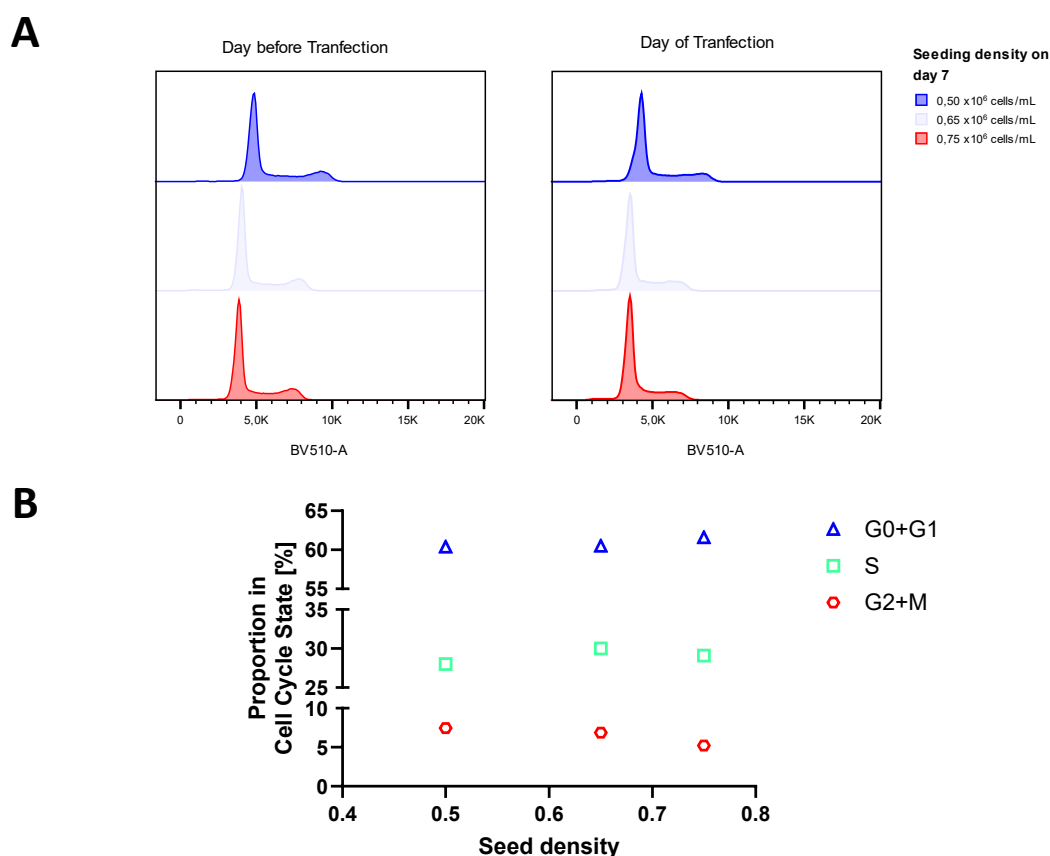


Figure 26 Cell cycle data collected between day 10 (day before transfection) and 11 (day of transfection). Cell cycle proportions over the different seeding densities 0.5×10^6 cells/mL, 0.65×10^6 cells/mL and 0.75×10^6 cells/mL ($n=3$), flow cytometry analysis see appendix Supplementary Figure 9 Cell cycle analysis of HEK293 cells with different seeding densities. Seed of 0.5×10^6 cells/mL, 0.65×10^6 cells/mL or 0.75×10^6 cells/mL. **A**: day before transfection. **B**: day of transfection.. **A**: histogram of flow cytometry analysis of BV510 channel DAPI stained cells. **B**: dot plot of analysis of cell cycle states G1-, S- and G2-phase.

Seed 0.75×10^6 cells/mL resulted in a pronounced increase in harvest titer with 1.8×10^7 TUs/mL (Figure 27) compared with the baseline titer of 1.0×10^7 TUs/mL for 0.5×10^6 cells/mL commonly observed in this process (chapter 4.1.5). Consistent with previous observations, vector without further processing (clarification only) was relatively stable for 4 hours. Recoveries ranged from 88.5 % to 93.9 %. Titer values were corrected for buffer dilution to allow comparison with the initial titers after clarification. Dilution in PM was again associated with significant losses. Immediately following dilution, titers were reduced to 0.7×10^7 TUs/mL (seed: 0.5×10^6 cells/mL) and 1.6×10^7 TUs/mL (seed: 0.75×10^6 cells/mL). After 4 hours, lower seeding density resulted in a recovery of only 8.2% TUs/mL while increased seeding density resulted in 23.0 %. 0.2 M HEPES exhibited greater stabilization of the lentivirus. Recoveries of 59.8 % were obtained in both cases despite differing initial titers of 0.8×10^7 TUs/mL (seed: 0.5×10^6 cells/mL) and 1.9×10^7 TUs/mL (seed: 0.75×10^6 cells/mL).

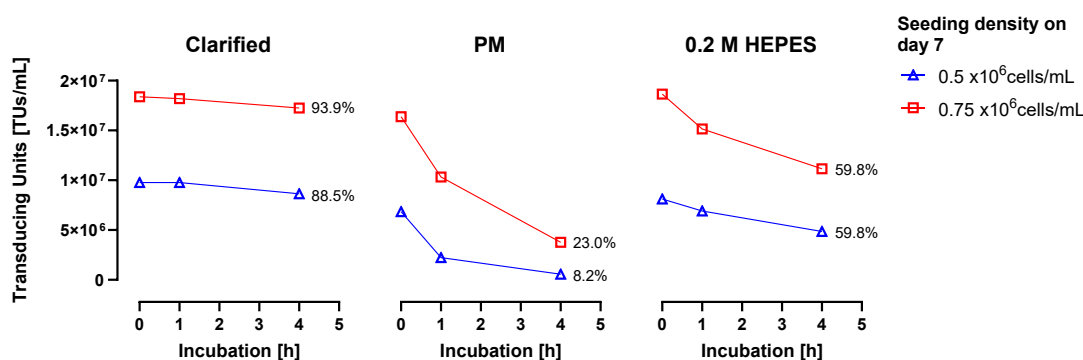


Figure 27 Varying seed density and the effect on lentivirus stability in different buffer conditions over time with. x-y plot of transducing units per ml (TUs/ml) over 4 hours with recovery in percent. 3 buffer conditions with 3 time-points (0, 1, 4) lean data of titration in duplicates based on CD19-expression (n=36). Viral dilution before transduction 1:1 000 (Clarified) or 1:100 (PM and 0.2 M HEPES). Titer data of PM and 0.2 M HEPES were corrected for 1:10 dilution in buffer.

4.1.8 Scale-Up to Bioreactor Manufacturing

Subsequently, an experiment was conducted at a larger scale. A GMP production scale would be above 5 L of cell culture volume. These volumes cannot be handled well in manual cell culture. A wave bag bioreactor system was employed which would allow for improved scaling compared to conventional stirred tank reactors with small laboratory footprint. This allows a working range from 0.3 – 25 L with sterile connection to transfer the cells into the reactor and later reagents for transfection.

Medium Conditioning and Sterility Test

A Sartorius Flexsafe® RM 2L optical bag for cultivation was prepared. This bag had a cell culture working volume between 0.25 – 1 L. The bag was filled with PM. Dissolved oxygen (DO) and pH probes were calibrated according to the instructions on the bag. A basal air flow of 0.15 L/min was set. Basal air flow of CO₂ was 0.05 L/minute to accommodate for conditions expected in an incubator. The temperature control via the base plate was set to 37 °C. After 6 hours incubation of the medium in the bag, values for DO and pH were stable. A sample was taken and external pH measurement was carried out on the radiometer for re-calibration. Calibration was performed from pH 6.78 to 7.26. A 24-hour incubation of medium without cells served as a sterility control. PM remained clear, online-pH and DO were unchanged. Data glucose and lactate were checked and remained unchanged (Table 14). The bioreactor was therefore declared as not contaminated.

Table 14 Analysis with Radiometer of samples taken from bioreactor, 6 and 24 hours after initiation of medium conditioning.

	6 hours post media fill	24 hours post media fill
pH	7.255	7.276
Dissolved CO ₂	44.7 mmHg	43.3 mmHg
Dissolved O ₂	127 mmHg	77.3 mmHg
Potassium	5.7 mmol/L	6.1 mmol/L
Glucose	616 mg/dL	653 mg/dL
Lactate	0 mmol/L	0 mmol/L

Bioreactor Inoculation

After medium conditioning, DO levels were defined as saturated, the probe was therefore recalibrated from 86.9 % to 100 %. Automated control of DO was activated to a set point of 40 % via a cascade of air, O₂ and N₂. Active pH-control was initiated to pH 7.25 with automated CO₂-gassing. Rocking motion of the bioreactor was set to 30 rpm at an angle of 10°. Inoculation of the bioreactor was done on process day 20 (Figure 28). Inoculation of the bioreactor with cells was prepared for a target cell density of 0.3×10^6 cells/mL at 300 mL. Before inoculation, excess volume was removed from the bioreactor with peristaltic pumps. Then 90×10^6 cells were fed into the system via the same tube. After 1 hour of incubation, a sample was taken from the bioreactor and the cell concentration was determined with 0.26×10^6 cells/mL which was close to the desired 0.3×10^6 cells/mL. The following day, a sample was taken for daily pH recalibration.

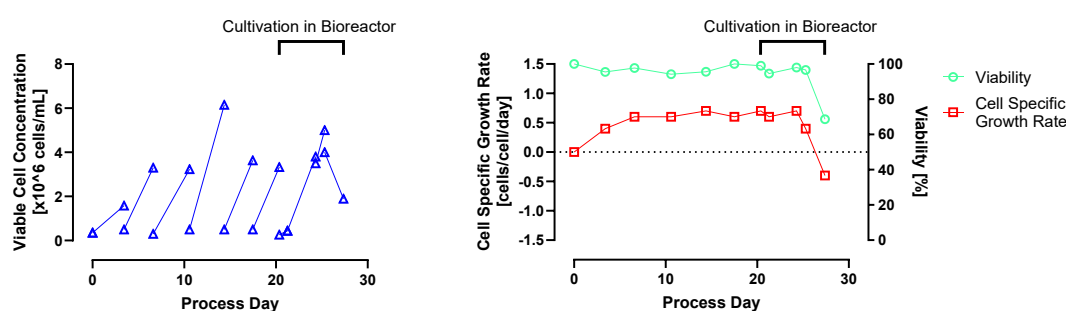


Figure 28 Cultivation data prior and during bioreactor cultivation. **Left:** Subculture x-y plot of viable cell concentration for each process day. **Right:** x-y plot of cell-specific growth rate and viability of respective process days. Viability was determined as ratio of viable cells to total cells per mL. Cell specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. Data shown for single expansion ($n=1$).

Transfection

Transfection of model construct #305 (CD276-CAR with myc-tag and co-expression of CD19) took place on day 25. The DO level was actively controlled to the set point of 40 % via N₂, air and O₂ gassing (Figure 29 A). Spikes of increased DO level were observed on

the day of inoculation, transfection day and after the start of harvesting. From day 22 onwards (3 days before transfection), pH started to passively decrease from 7.25 to below 6.9 (Figure 29 B). The pH could only be controlled via CO₂ (acidic). On day of transfection, active pH control was changed from 7.25 to 6.8 (pH-shift). Prior to transfection, the pH had almost approached the defined set point.

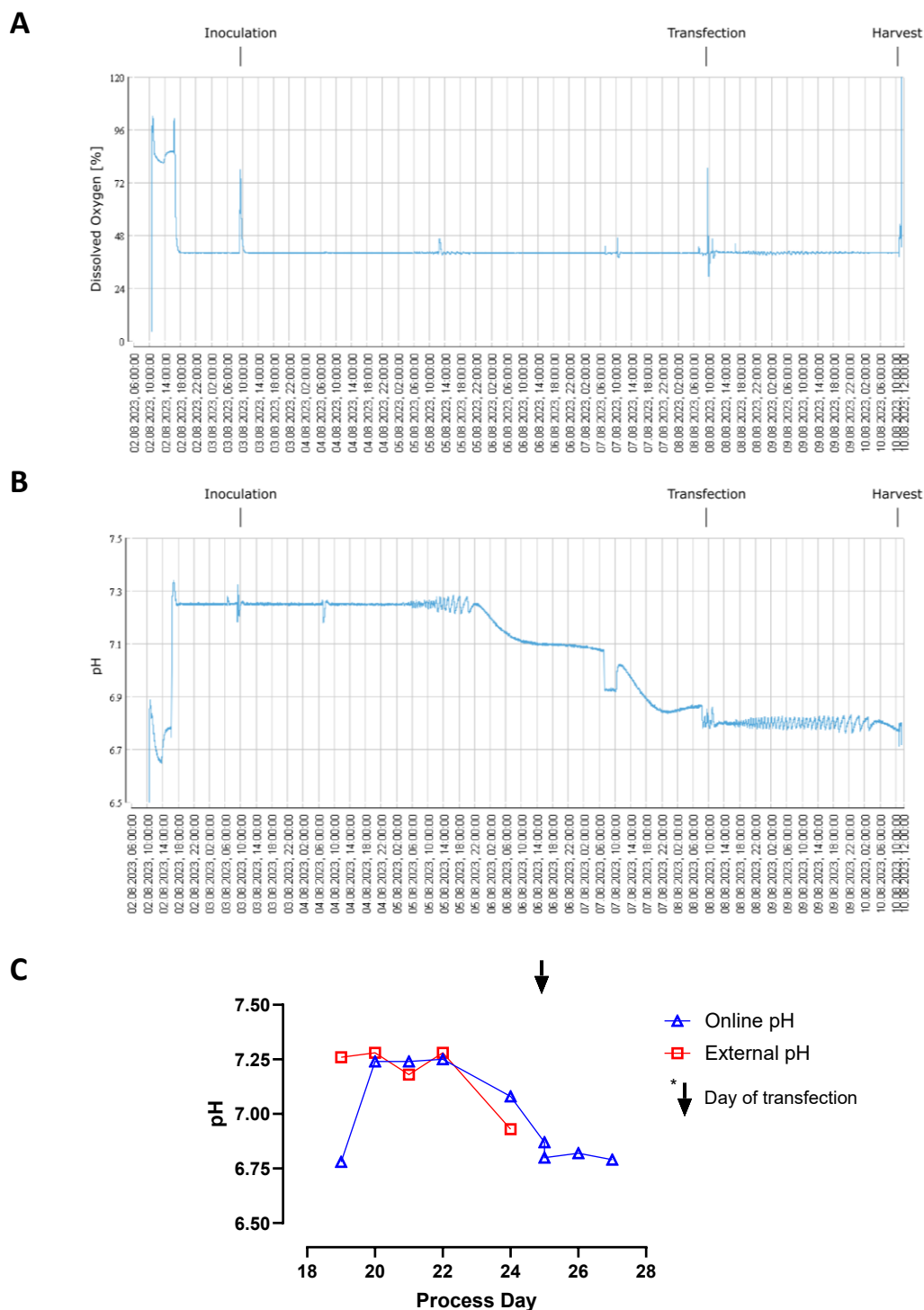


Figure 29 Bioreactor data on dissolved oxygen (DO) and pH. **A:** x-y plot of recorded online data on DO based on the optical sensors. **B:** x-y plot of recorded online data on pH based on the optical sensors. **C:** denoted data of pH from bioreactor display every day and externally measured pH for re-calibration via Radiometer. Data on gassing regime for air, O₂, N₂ and O₂ can be found in Appendix Supplementary Figure 10.

DNase Treatment and Harvest

An early purification step can already be integrated into the bioreactor process. The enzymatic breakdown of DNA by DNase treatment. 60 µL Benzonase were diluted in 5 mL PM. 30 minutes prior to harvesting, DNase treatment was carried out by injection (syringe port) into the bioreactor bag. Agarose gel electrophoresis was performed to

assess for DNA reduction. It was demonstrated that the amount of DNA could be strongly reduced (Figure 30). This was particularly evident for DNA with a very short number of base pairs <0.1 kb.

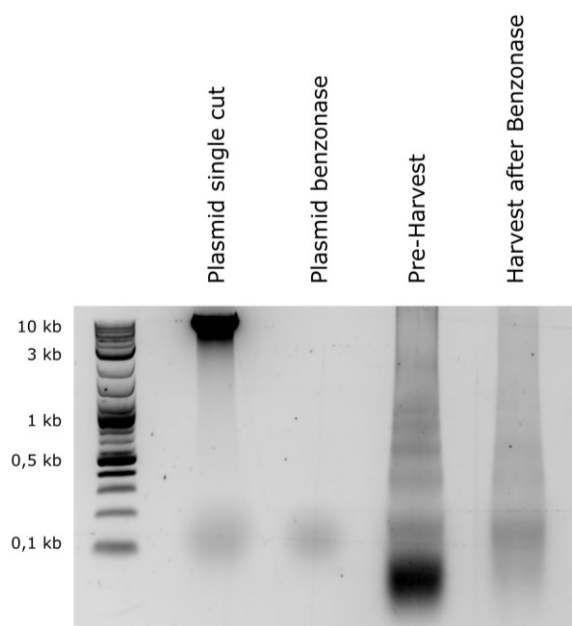


Figure 30 Agarose gel electrophoresis before and after DNase treatment in the bioreactor. 1. lane: control plasmid after restriction enzyme digest, 2. lane: control plasmid after Benzonase treatment, 3. lane: sample taken from bioreactor before Benzonase treatment, 4. lane: sample taken after Benzonase treatment.

A titer of 1.0×10^7 TUs/mL was achieved in the bioreactor. DNase treatment lowered the final yield to 0.8×10^7 TUs/mL (85.5 %).

4.1.9 Ultrafiltration/Diafiltration Procedure for Lentiviral Vectors

As noted in the previous chapter 4.1.6, freeze-thaw cycles had a significant impact on the titer. This effect also interacts with the choice of buffer. This suggests that freezing the sample compromises accurate measurement of the true in situ value. The titer of frozen samples varies significantly between the supernatant and subsequent rebuffing steps. Furthermore, prolonged incubation (6 hours) of HT-1080 cells due processing resulted in a decrease in measured titer (Appendix Supp 8). Accurate determination of infectious titers throughout the process necessitated extensive processing steps, significantly increasing labor and time requirements.

Process Pressures

The feed pressure (P_F) is defined as the pressure measured immediately upstream of the filter. The retentate pressure (P_R) is defined as the pressure measured downstream of the filter in the retentate line. From these two pressures, the delta pressure ($P_F - P_R$) and the transmembrane pressure (TMP, the average of P_F and P_R) can be calculated (Figure 31). These parameters reflect system stress conditions and enable the detection of pressure drops indicative of leaks. TMP was maintained constant by regulating the

retentate flow. Optimal pressure conditions for both membrane types were identified via TMP scouting (MiniMate and Sartocan see Appendix Supplementary Figure 11 and Supplementary Figure 12).

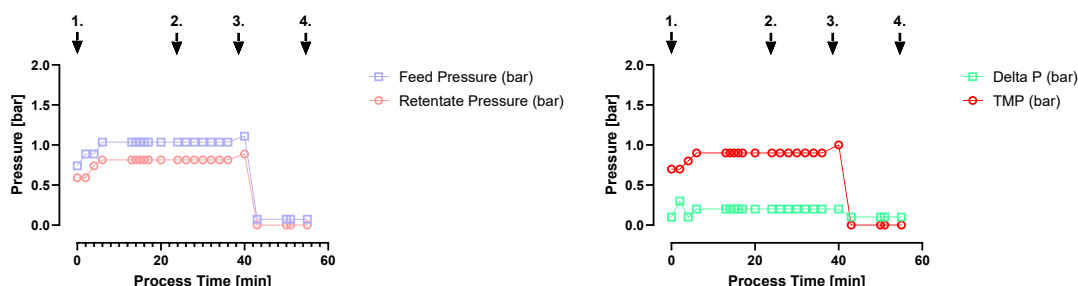


Figure 31 Pressure process data of Harvest 6. **Left:** Feed pressure (P_F) and retentate pressure (P_R). **Right:** delta pressure ($P_F - P_R$) and transmembrane pressure (TMP, $(P_F + P_R)/2$). 1. initiation of diafiltration (buffer exchange), 2. Initiation of ultrafiltration (concentration), 3. end of ultrafiltration and start of circulation, 4. end of process.

Volumetric Data

Volumes were recorded, with the retentate volume defined as the volume contained within the system reservoir (Figure 32). The permeate volume, representing waste in this case, corresponds to the fraction passing through the filter and removed from the system. The retentate flow rate, determined by the pump speed, reflects the circulation flow within the system. From these parameters, the permeate flux (J) can be calculated (Equation 2), enabling detection of membrane fouling and blockage.

$$J = \frac{V_p}{t \cdot A}$$

Equation 2

J = Permeate Flux [LMH]
 V_p = Permeate Volume [L]
 t = Time [hours]
 A = Membrane area [m^2]

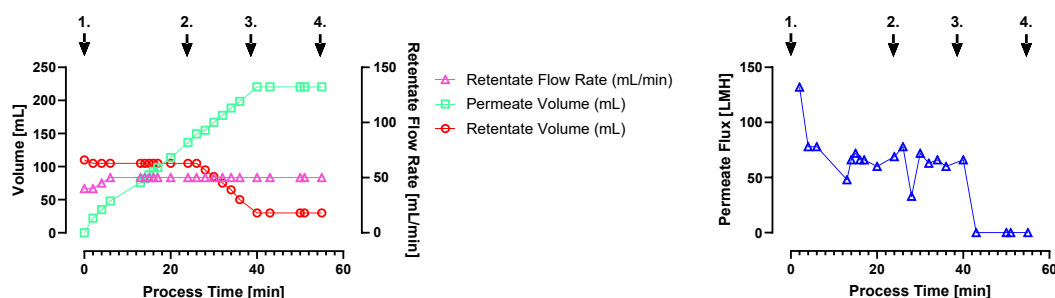


Figure 32 Volumetric process data of Harvest 6. **Left:** retentate flow rate is the pump speed, permeate flow rate is the volume collected passing the filter, retentate volume is the retained volume in the system. **Right:** permeate flux is the filtrate flow rate over the membrane area (Equation 2). 1. initiation of diafiltration (buffer exchange), 2. Initiation of ultrafiltration (concentration), 3. end of ultrafiltration and start of circulation, 4. end of process.

4.1.10 Comparative Analysis of Clarification and TFF Setups

Filters of by Cytiva (formerly PALL) and Sartorius were tested (Table 15 and Table 16). Syringe filters, filter plates and capsules were tested for initial clarification. For tangential flow filtration (TFF), flat sheet systems were employed. Filtrations were performed on the MiniMate Evo™ System, except for Harvest 8 (performed on Sartoflow® Smart). The filter areas ranged from 50 – 200 cm². Pore size cut-off was 100 kDa or 300 kDa. Diafiltration took place in buffers PM, 0.2 M HEPES, 1 M HEPES and HBSS.

Table 15 Experimental design for the initial purification step clarification. 6 harvests were freshly processed and tested in various combinations.

Har-vest	Manu-facturer	Filter	Pore Size	Conditioning
5			None	
6	Cytiva	AcroPrep™ 24-well Supor EKV filtration plate	0.65 + 0.2 µm	PM
7	Cytiva	Integra Biosciences™ filter	0.45 µm	PM
8	Cytiva	Integra Biosciences™ filter	0.45 µm	PM
9	Cytiva	Integra Biosciences™ filter	0.45 µm	PM
10	Cytiva	Fluorodynce Acropak 200	0.2 µm	0.2 M HEPES

Table 16 Experimental design for the second purification steps ultra-/diafiltration. 6 harvests were freshly processed and tested in various combinations.

Har-vest	Manu-facturer	Filter	Area	Cut-Off	Conditioning	Storage Buffer
5	Cytiva	MiniMate™ TFF Capsule	50 cm ²	100 kDa	1 M HEPES +50 mM NaCl (pH 7.5)	
6	Cytiva	MiniMate™ TFF Capsule	50 cm ²	100 kDa	1 M HEPES +50 mM NaCl (pH 7.5)	
7	Cytiva	MiniMate™ TFF Capsule	50 cm ²	300 kDa	PM	
8	Sartorius	Sartocon® Slice 50	50 cm ²	300 kDa	PM	1 M HEPES
9	Sartorius	Sartocon® Slice 200	200 cm ²	300 kDa	PM	HBSS
10	Sartorius	Sartocon® Slice 50	50 cm ²	300 kDa	0.2 M HEPES	

Purification Yields

Harvest 5 to 7 were performed with MiniMate TFF cassettes. For Harvest 5, the initial clarification step was omitted. Both Harvest 5 and 6 were performed with initial diafiltration, followed by ultrafiltration. A concentration factor of 2.4× for Harvest 5 and 1.3× for Harvest 6 was achieved. Yields between Harvest 5 and 6 were highly comparable but low, with a significant loss of TUs throughout the purification, resulting in 14.2 % (Harvest 5) and 10.8 % (Harvest 6) of the initial TUs (Figure 33). Inclusion of a clarification step via depth filtration did not significantly impact yield of TUs for Harvest 6. For Harvest 7 depth filtration (filter plates) was exchanged by a simple membrane filter (syringe) for initial clarification. A 300 kDa filter with a larger pore size was used instead of the 100 kDa filter. Furthermore, ultrafiltration was performed first followed by diafiltration, a method that reduces buffer consumption and process time (Appendix Supplementary Figure 13 and Supplementary Figure 14). The concentration factor was 2.4×. This shortened approach, despite employing PM as buffer (chapter 4.1.7), contributed to an improved final yield of 38.5 %.

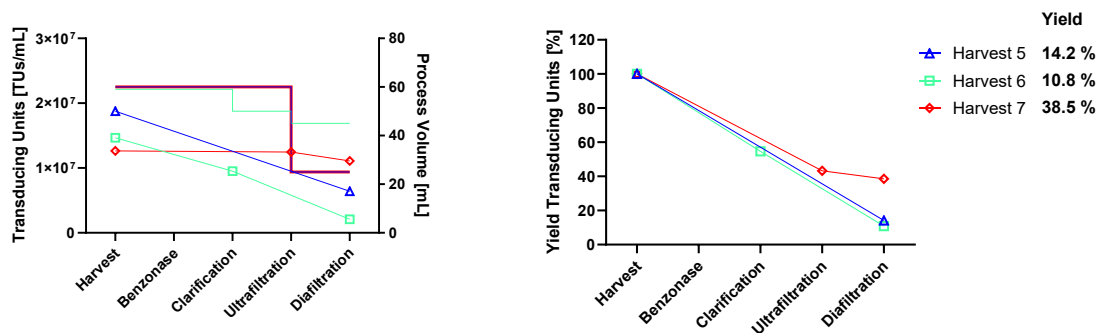


Figure 33 Lentivirus yield during purification steps Benzonase (DNase) treatment, clarification, ultrafiltration and diafiltration for Harvest 5 to 7. **Left:** x-y plot of Transducing Units (TU) per mL and process volume. **Right:** x-y plot of yield calculated based on process volume in subsequent downstream processing steps. The volume reduction was as follows: Harvest 5: 60 to 25 mL (2.4×), Harvest 6: 60 to 45 mL (1.3×), and Harvest 7: 60 to 25 mL (2.4×). 25 mL was the minimum hold-up volume of the system.

Harvest 8 was performed on a demo device provided by Sartorius (Sartoflow® Smart). Initial filtration had been done with syringe filters. TFF was conducted with an initial ultrafiltration step, followed by diafiltration using a Sartocan filter (Figure 34). A concentration factor of 6.3× was achieved. The final yield was only 11.7 %. Harvest 9 was performed on the MiniMate EVO™ system using a flat sheet Sartocan filter with a significantly larger surface area of 200 cm². The filter was not encased in a capsule, instead, it was clamped into a metal holder. HBSS was used as the buffer system. A concentration factor of 1.7× was applied. The setup with a large membrane area significantly reduced processing time, enabling completion of the entire procedure in under 25 minutes. A final yield of 45.5 % was recovered. For Harvest 10, TFF was performed using a Sartocan capsule filter equipped with the smaller membrane area. The buffer system was 0.2 M HEPES. Here, an increased starting volume of 283 mL and

a final volume of 25 mL, resulted in a concentration factor of 11.3×. A final yield of 20.2 % was achieved.

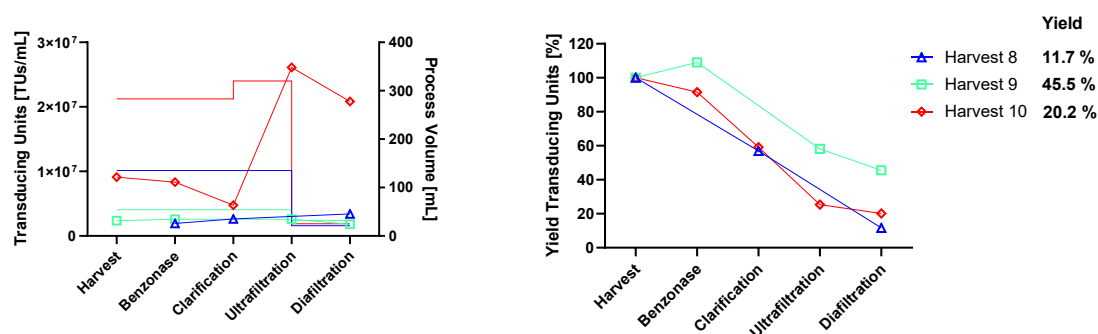


Figure 34 Lentivirus yield during purification steps Benzonase (DNase) treatment, clarification, ultrafiltration and diafiltration for Harvest 8 to 10. **Left:** x-y plot of Transducing Units (TU) per mL and process volume. **Right:** x-y plot of yield calculated based on process volume in subsequent downstream processing steps. The volume reduction was as follows: Harvest 8: 135 to 21 mL (6.3×), Harvest 9: 54 to 32 mL (1.7×), and Harvest 10: 283 to 25 mL (11.3×). 25 mL was the minimum hold-up volume of the MiniMate EVO™ system (Harvest 10), it was lower for Sartorius Sartoflow® Smart (Harvest 8).

4.1.11 Evaluation of Cell Culture-related Impurities

Establishment of a Fast and Sensitive DNA-Impurities-Detection Method

To quantify DNA contamination, a new method was established that would be suitable for fast process screening. The method should be significantly more sensitive than pure UV absorption spectrophotometry (e.g. Nanodrop) and faster than qPCR. The plasmid DNA of construct #305, which is present in the harvest and should be removed during purification, was used to generate a standard curve. DNA was detected after SYBR™ Gold staining and UV detection at 535 nm emission. A dilution series of DNA for plasmid for construct #305 was employed to calculate the concentration of DNA in a given sample (Figure 35).

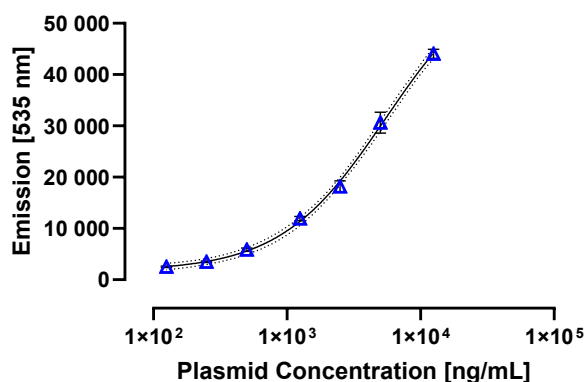


Figure 35 Standard curve of UV signal after SYBR Gold staining of a dilution series of the plasmid for plasmid DNA. Emission at 535 nm was fitted to a four-parameter logistic model (4-PL) against DNA concentration using GraphPad Prism. The best-fit parameters were Bottom = 1670, Top = 61334, $IC_{50} = 5.496$, and Hill slope = 1.7067 ($R^2 = 0.9961$). Error bars represent SD of triplicates. Confidence interval of 95 %, (n=24).

A dilution series of plasmid DNA from the gag/pol packaging plasmid was used as a control (Figure 36). The linearity of the control was demonstrated for all three samples between 2 and 10 ng/ μ L.

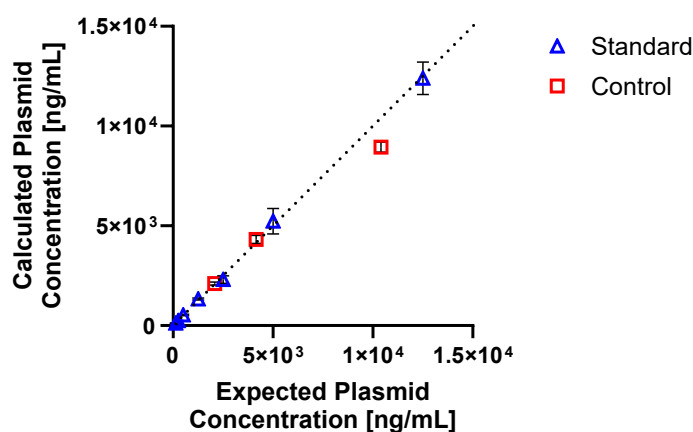


Figure 36 x-y plot of SYBR Gold staining linearity by comparison of the expected concentration (according to UV absorption spectrophotometry) to the calculated concentration based on the dilution factor.

To validate the method, samples from Harvest 10 were measured and spiked with the control (4 ng/ μ L of gag/pol packaging plasmid per sample). High recovery rates were demonstrated for the samples Harvest, Ultrafiltration, Ultrafiltration flow-through (FT), Diafiltration and Diafiltration flow-through (FT). Benzonase and Clarified were slightly below the expected value (Figure 37). The values were found at the expected levels, the method was thus confirmed as successful.

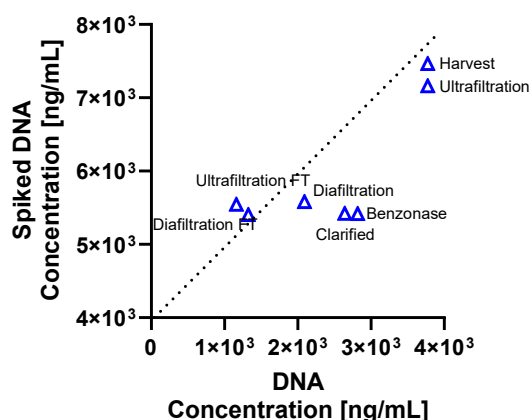


Figure 37 x-y plot of control measurement with samples from Harvest 10 measured against spiked samples (added 4 ng/μL of gag/pol packaging plasmid per sample).

Process Screening for DNA Impurities

Harvest 8 and 10 were examined. In both harvests, an elevation of DNA concentration was observed after volume reduction in ultrafiltration, the concentration did not exceed the initial DNA concentration before purification (Figure 38). For Harvest 10, a clear reduction in DNA concentration was observed after diafiltration. The DNA clearance for Harvest 8 was 81 % and for Harvest 10 by 95 % from bulk supernatant.

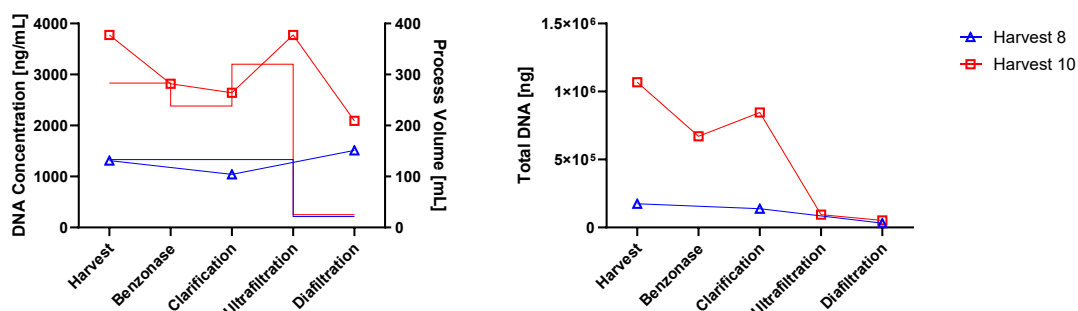


Figure 38 DNA clearance during purification steps Benzoinase (DNase) treatment, clarification, ultrafiltration and diafiltration for Harvest 8 to 10. **Left:** x-y plot of DNA concentration per and process volume. **Right:** x-y plot of clearance calculated based on process volume in subsequent downstream processing steps. Harvest 8: 135 to 21 mL (6.3×), and Harvest 10: 283 to 25 mL (11.3×). 25 mL was the minimum hold-up volume of the MiniMate EVO™ system (Harvest 10), it was lower for Sartorius Sartoflow® Smart (Harvest 8).

To determine the cause of the unexpectedly low decrease in DNA concentration during purification, fragment lengths were analyzed. Contrary to what was observed in chapter 4.1.8 (agarose gel), DNA could still be detected after DNase treatment with this new sensitive method. Agarose gel electrophoresis was again performed to determine the proportion of DNA fragments whose length remained unaffected by purification (Figure 39).

DNA fragments with <0.1 kB were found in the Harvest sample. These were no longer found in the Benzoinase sample. In the Diafiltration sample, a band between 0.1 – 0.2 kB

was found. This suggests that smaller fragments <0.1 kDa were reduced by the DNase step. To estimate the molar mass of double-stranded DNA, an average value of ≈ 650 Da per base pair can be estimated. At 0.1 kDa = 100 bp, this results in 65 kDa ($100 \text{ bp} \times 0.65 \text{ kDa}$). The concentration step (factor $11.3\times$) increased the density of larger DNA fragments (>0.1 kDa), presumably retained by the 300 kDa cut-off of the filter membrane.

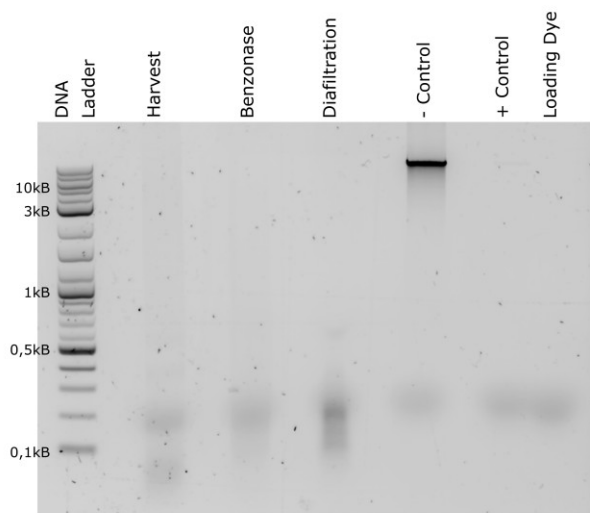


Figure 39 Agarose Gel of different purification steps. Harvest, Benzonase (DNase) treatment and diafiltration for Harvest 10. Volume reduction from 283 to 25 mL ($11.3\times$).

HEK293 Host Cell Proteins Detection

A commercial kit was used to detect HEK293 host cell proteins. A standard curve was generated (Figure 40). The concentrations of HEK293 proteins during various purification steps for Harvest 8 and 10 were then calculated.

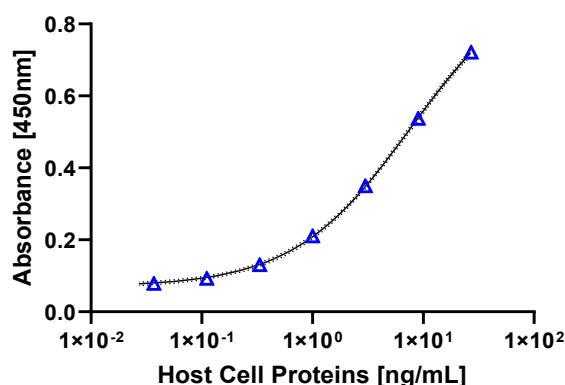


Figure 40 Standard curve of dilution series of HEK293 host cell protein standard. Absorbance at 405 nm was fitted to a four-parameter logistic model (4-PL) against a standard of HEK293 host cell protein concentration using GraphPad Prism. The best-fit parameters were Bottom = 0.06890 , Top = 0.9536 , $IC_{50} = 7711$ and Hill slope = 0.8197 ($R^2 = 0.9995$). Confidence interval of 95% , ($n = 16$). Example of standard curve for Harvest 10.

The concentration of host cell proteins (HCPs) increased significantly during the ultrafiltration step (Figure 41). This shows that a large proportion of the host proteins

were retained by the filter. For Harvest 10, a reduction in HCP concentration was observed after diafiltration. The total amount of host cell proteins was reduced from 132 mg (after harvest) to 50 mg (after diafiltration), with a concentration factor of 11.3× (HCP clearance: 62 %). No sample was taken directly after the ultrafiltration step for Harvest 8, but it can be assumed that the value here is higher compared to diafiltration. No reduction in total HCPs was observed here (concentration factor 6.3×).

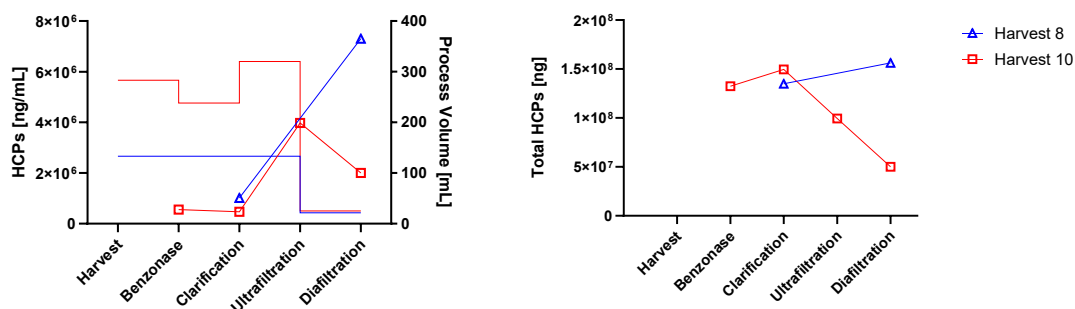


Figure 41 Host cell protein (HCP) clearance during purification steps Benzonase (DNase) treatment, clarification, ultrafiltration and diafiltration for Harvest 8 to 10. **Left:** x-y plot of HCP concentration per and process volume. **Right:** x-y plot of clearance calculated based on process volume in subsequent downstream processing steps. Harvest 8: 135 to 21 mL (6.3×), and Harvest 10: 283 to 25 mL (11.3×). 25 mL was the minimum hold-up volume of the MiniMate EVO™ system (Harvest 10), it was lower for Sartorius Sartoflow® Smart (Harvest 8).

4.2 Method Development and Panel Design for the Prodigy Process Analysis

The objective was to analyze tumor-triggered activation and examine exhaustion mechanisms. This chapter explains the development of an assay and establishment of an intracellular protein and phosphorylation panel based on a new Re-Challenge model. This enables improved characterization of the different stages of CAR-T cell expansion.

4.2.1 Re-Challenge Model

The cytotoxic activity of CAR-T cells on tumor cells was investigated (no previous tumor contact = TC 0). Tumor lysis at effector to target ratios (E:T) between 5:1 and 0.04:1 (log-2 levels) was analyzed via luciferase activity of tumor cells. Measurement took place 24, 48 and 72 hours after the start of the assay. CAR-T cells from different expansions were used. For 3 days of expansion, cells are referred to as “early” (Figure 42 A) and after 7 days of expansion as “late” CAR-T cells (Figure 42 B and C). In parallel to cytotoxicity assays, a re-challenge model was established. This combination enabled analysis of tumor-dependent activation in combination with a CAR-T cell expansion profile. Re-challenges were performed at E:T of 4:1. CAR-T cells were brought into contact with tumor cells for 48 hours. After incubation, the supernatant was completely removed and the cells were placed in fresh medium and tested in a separate cytotoxicity assay. The re-challenge was repeated two times (TC 1 and TC 2).

It was noted that CAR-T cells from early expansion exhibited immediate lytic activity. After tumor contact, the lytic activity decreased. CAR-T cells of late expansion showed poor initial toxicity and an increased lytic activity upon second tumor contact. The initial tumor lysis (TC 0) of late CAR-T cells also appears in cells without a corresponding target epitope for the CAR (CD276KO). This indicates unspecific lysis of tumor cells which is reduced in TC 1 and TC 2.

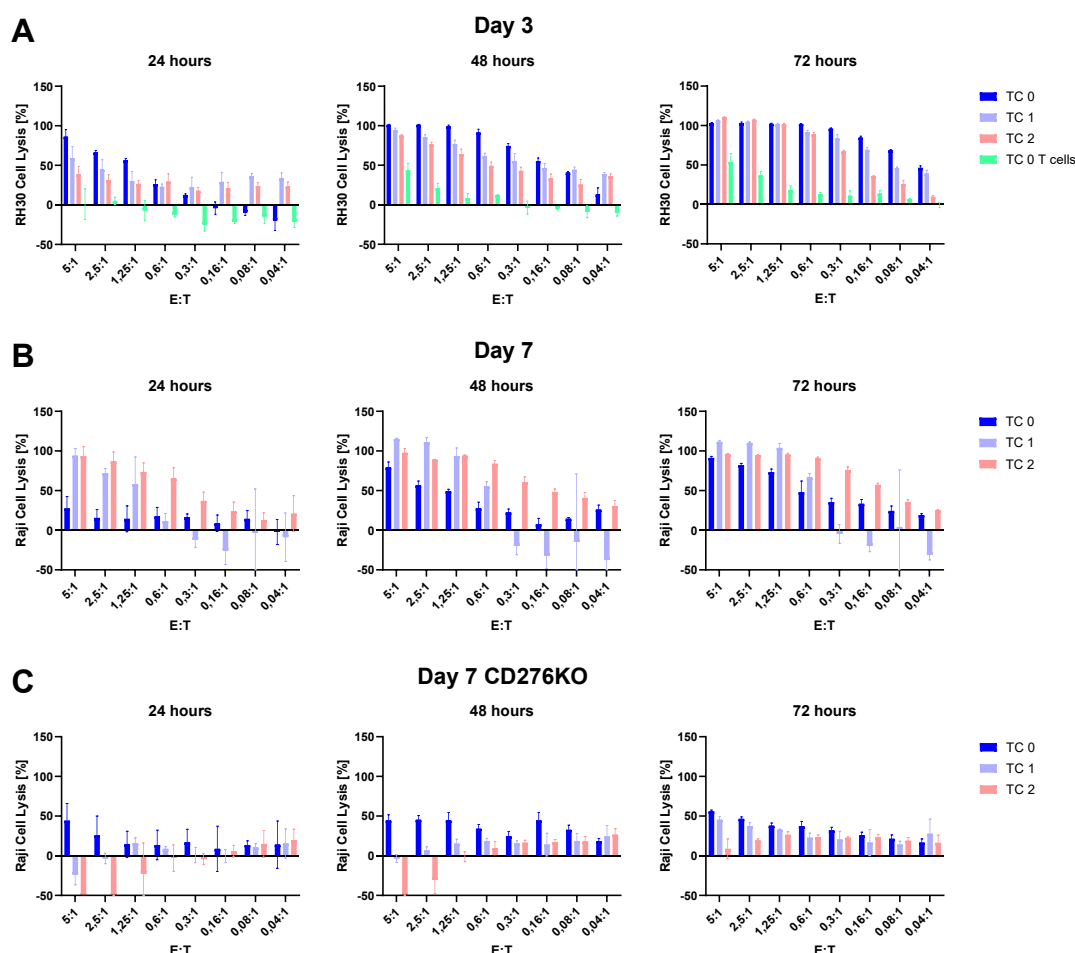


Figure 42 Re-challenge combined with cytotoxicity assay on tumor cell lysis. Measured every 24 hours for each sample an effector to target ratio between of 5:1 – 0.04:1 (2-log steps). For each sampling day, repeated tumor contacts (TC) every 48 hours were performed. TCs are shown between TC 0 (no tumor contact) and TC 2. Each point represents the mean of an analytical triplicate ($n=3$). Negative values are truncated from the plot; “negative lysis” was not considered quantitatively. **A:** bar chart of samples taken from day 3 of CAR-T expansion, target cell line was RH30 cell line. **B:** bar chart of samples taken from day 7 of CAR-T expansion, target cell line was Raji cell line. **C:** bar chart of samples taken from day 7 of CAR-T expansion, target cell line was CD276KO Raji cell line.

4.2.2 Cellular Marker Panel Setup

Three CAR-T cell expansions were analyzed employing DigiWest™ technology (Expansion 1 – 3). A wide variety of proteins and phosphorylation sites (analytes) was selected. The relevance of an analyte was defined by the variability of its signal over the course of CAR-T manufacturing (days of expansion). Samples covered a range between 0 – 18 days (Table 17).

Table 17 Sampling pattern for three CAR-T or T cell expansion. "X" marks the day when a sample was taken for further analysis via DigiWest.

Day of Expansion	Expansion 1		Expansion 2	Expansion 3	
	CAR-T cells	T cells	CAR-T cells	CAR-T cells	T cells
0					X
1					X
3	X	X	X	X	
7			X	X	X
9	X	X			
10			X		
11	X	X			
14	X	X			
18	X	X			

For each sample, 2×10^6 cells were collected for DigiWest™ analysis. The number and type of analytes that could be examined per sample depended on the amount of protein obtained and the quality of the collection. The sample collection process had been optimized to ensure maximum quality (chapter 3.6.4). For the three expansions, a wide variety of analytes in different combinations was tested. To increase the number of analytes and given the upper limit of analytes per sample, repeated testing of all analytes was not feasible. Analytes that showed no signal were excluded. The sample variance per analyte was then calculated separately for each expansion over the respective days (Equation 3 and Equation 4).

$$s^2 = \frac{1}{n-1} \sum_{i=1}^n (x_i - \bar{x})^2 \quad \text{Equation 3}$$

$$\bar{x} = \frac{1}{n} \sum_{i=1}^n x_i \quad \text{Equation 4}$$

s^2 = sample variance
 $\bar{x}$ = sample mean
 n = sample size
 $n - 1$ = degrees of freedom
 i = index

The top 15 analytes with the highest variance were found to be CD8β, PI3Kβ, LYN/LCK/HCK/BLK phosphorylation at Tyr397/Tyr394/Tyr411/Tyr389 (conserved site), c-Jun, c-Jun phosphorylation at Ser73, Ox40, GITR, IRAK1 phosphorylation at Thr387, TRAF 1, Granzyme B, LAG-3, SLAMF4, PD-1, IFITM1 and CTLA-4 (Figure 43).

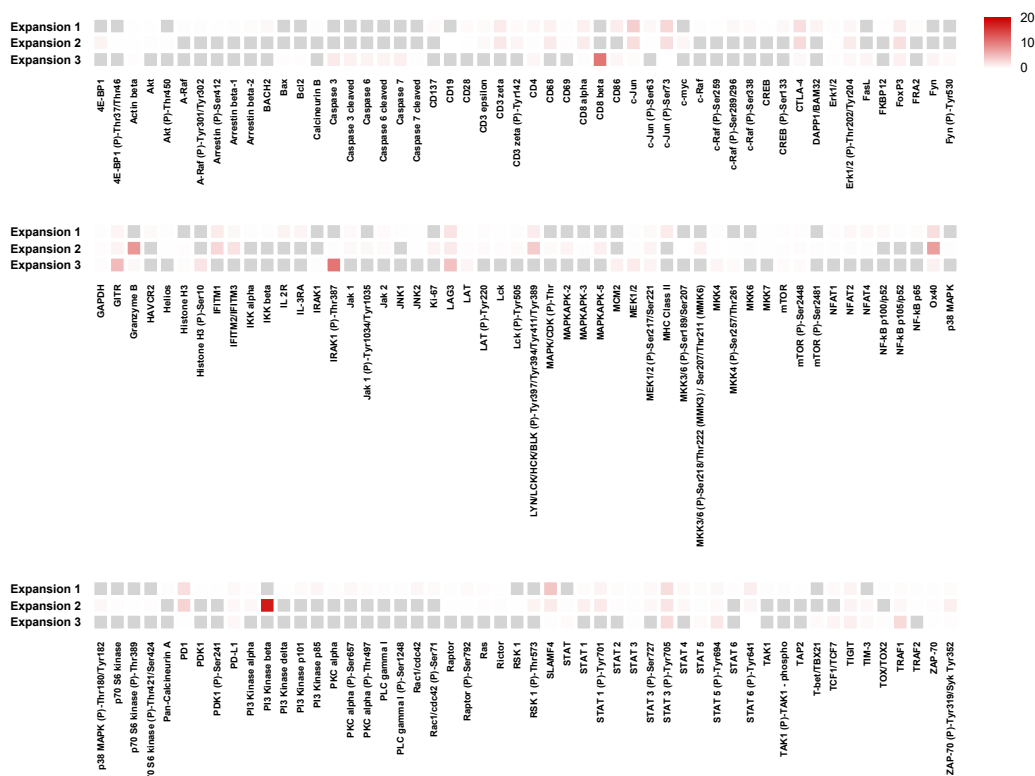


Figure 43 Matrix plot of DigiWest data on variance during CAR-T and T cell expansion. Sample variance was calculated across the respective expansion days (sampling Table 17) for expansion no. 1 ($n = 10$), expansion no. 2 ($n = 3$) and expansion no. 3 ($n = 5$) on 156 different analytes. Phosphorylation site indicated by "(P)". Data was log-2 normalized to mean signal of all proteins per expansion respectively.

4.2.3 Combining DigiWest with Re-Challenge

Additional analyses were performed on samples from Expansion 3 where a re-challenge model was applied. Here, CAR-T cells were exposed to three 72-hour-cycles of contact with RH30 tumor cells (tumor contact = TC). The E:T ratio was set to 4:1 in each re-challenge. Cells were sampled from expansion days 3 and 10. DigiWest analyses were then performed on each re-challenge (TC 1 – TC 3) for both expansion days. Finally, sample variance was calculated each analyte. Of the previous top of 15 analytes with highest variance, 11 were analyzed Expansion 3 (Figure 44).

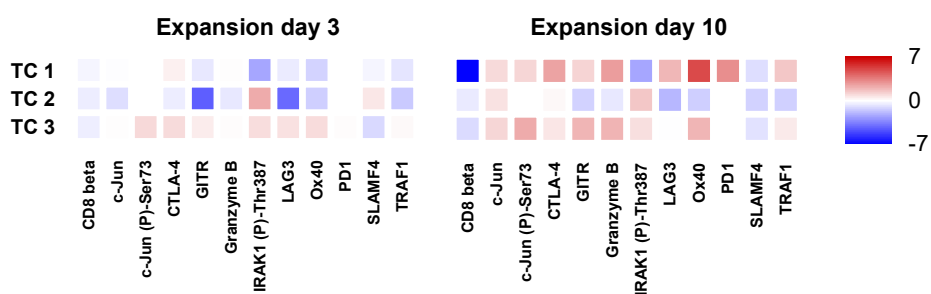


Figure 44 Matrix plot of DigiWest analytes signals of expansion no. 3. Samples of expansion day 3 and 10 were processed in a re-challenge model with three consecutive rounds of tumor contact (TC 1 to 3) with RH30 cells. Data was log-2 normalized to mean signal of all proteins.

A clear discrepancy between the two expansion days could be identified, with tendencies and trends also observable within the re-challenges. Associated intracellular cascades and pathways were therefore considered relevant for T cell expansion in order to establish a corresponding panel.

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CD19-CAR-T phenotyping during Prodigy manufacturing uncovers critical T cell transition

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Additional Declarations: There is **NO** Competing Interest.

1 **CD19-CAR-T phenotyping during Prodigy**
2 **manufacturing uncovers critical T cell transition**

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24 **Contributions**

25 F.H.S. and D.A. conceived and designed the study. F.H.S., I.K., F.S., S.F. and M.F.
26 established assay models and panel designs. F.H.S. and I.K. performed the
27 experiments with the assistance of S.K., K.L. and K.W. F.S. performed the DigiWest
28 analysis. Data collection and analysis was done by F.H.S., I.K. and F.S.. F.H.S. and
29 F.R. performed the statistical analyses. Figures were generated by F.H.S.. F.H.S.,
30 D.A. and K.S contributed to writing of the manuscript. D.A., C.M.S., M.F.T., T.B.,
31 C.L. and P.L. provided scientific oversight. D.A., C.M.S., M.F.T. and P.L. supervised
32 the study.

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35 **Abstract**

36 Advances in cell therapy engineering have driven chimeric antigen receptor T cell
37 manufacturing from manual procedures to highly automated and technically
38 sophisticated production systems. To meet the increasing demand for high-
39 throughput manufacturing, current efforts focus on accelerating production while
40 maintaining/increasing product quality. Yet, to date, the precise daily progression
41 of the T cell states during manufacturing have not been examined in detail.
42 Here we characterize anti-CD19 CAR-T cell manufacturing on a CliniMACS Prodigy
43 platform under current good manufacturing practice (cGMP) conditions,
44 integrating cytotoxic activity profiling with comprehensive phenotyping across a
45 complete production cycle. Using over 130 extra- and intracellular markers related
46 to T cell activation, CAR signaling, metabolism, apoptosis, and hypoxia, we identify
47 a pivotal transition point between process days 5 and 7 marking the shift from
48 early activation to a stable/durable, functionally more specific T cell phenotype.

49 These data shed light on cellular remodeling throughout semi-automated CAR-T
50 manufacturing and provide a mechanistic framework for modulating T cell
51 phenotypes across distinct production phases.

52

53 **Introduction**

54 A decade after their clinical debut, chimeric antigen receptor (CAR)-T cells
55 continue to redefine the limits of engineered immunity, revealing both
56 unprecedented therapeutic durability and a complex landscape of resistance that
57 now shapes the field's most urgent research questions. The CAR is a chimeric
58 molecule of a T cell receptor (TCR) fused with an antigen recognition domain, such
59 as a single-chain fragment (scFv) of a monoclonal antibody [1-4]. This approach
60 enables long-term disease control and offers the additional advantage of rapid
61 onset of action [5, 6]. Building on their remarkable clinical successes, ranging from
62 durable remissions in refractory B-cell malignancies to expanding indications in
63 myeloma and beyond, CAR-T therapies now face a pivotal bottleneck in
64 manufacturing, where the biological constraints of T cell activation meet the
65 practical realities of scalable production [7, 8].

66 An outstanding question is how the inherently artificial process of CAR-T cell
67 manufacturing affects the functional properties and phenotypic characteristics of
68 the generated cells. From a metabolic perspective, the manufacturing process
69 dramatically diverges from physiological T cell expansion. When cultivated in
70 standard medium (Iscove's modified Dulbecco medium), cells receive 25 mM
71 glucose and 4 mM glutamine. Without knowing the exact amounts in TexMACS, it
72 can be assumed that the values are also significantly higher than physiological

73 levels. T cells cultivated in cell culture medium (TexMACS™) are then exposed to
74 5-times times the standard blood sugar level, 8-times the standard glutamine level,
75 and two to four times the oxygen partial pressure compared to the conditions in
76 the blood [9]. Additionally, these cells undergo supraphysiological activation via
77 CD3/CD28 stimulation and are exposed to cytokine concentrations (IL-7 and IL-15)
78 that far exceed physiological levels [10]. These additives primarily aim to enhance
79 longevity, proliferative capacity, and antitumor efficacy of the final CAR-T cell
80 product, thereby promoting increased stemness [11, 12]. CAR-T cell products
81 enriched with a high proportion of stem cell memory (TSCM), naïve (TN), or
82 central memory (TCM) T cells exhibit superior antitumor responses and in vivo
83 persistence [13]. However, conventional protocols require extended ex vivo culture
84 periods (7 to 14 days) resulting in the reduction/depletion of some of these subsets
85 [14].

86 Shortened processes are promising approaches to increase stemness and there are
87 already some trends in this direction [14, 15]. In these studies, cell pools at the
88 midpoint of cell culture (day 5), where characterized by the enrichment of TSCM
89 and TCM. In contrast, prolonged culture (day 10) resulted in a more terminally
90 differentiated phenotype dominated by terminal effector function cells (TEFF) [16].
91 Furthermore, approaches of 24-hour processes seem to preserve a significantly
92 higher percentage of naïve and stem cell-like T cells eliminating the ex vivo
93 expansion step [14]. There is a prevailing assumption that increased speed is
94 inherently beneficial. However, the exact temporal ordering of events and a full
95 combinatorial analysis of changes to the phenotype, metabolism, and functionality
96 of the cells have remained largely unexplored. Accordingly, the potential gains and
97 compromises inherent to early CAR-T cells remain unresolved.

98 In this study, we dissect the molecular chronology of a GMP-compliant anti-CD19
99 CAR-T cell manufacturing process by interrogating each individual day, rather
100 than an abbreviated process. Using an in vitro tumor re-challenging model we
101 investigated potential cellular triggers and markers of senescence and exhaustion
102 through multicolor flow cytometry and DigiWest™-based phosphoproteomic
103 profiling (>130 surface/intracellular markers). This comprehensive approach
104 illuminates T cell activation trajectories, CAR-T signaling dynamics during/after
105 tumor engagement and the underlying metabolic/ stress adaptations. Critically we
106 define key inflection points that govern phenotypic trade-offs between rapid
107 cytolytic potency and sustained effector durability, offering actionable control
108 points for bespoke CAR-T engineering.

109 **Results**

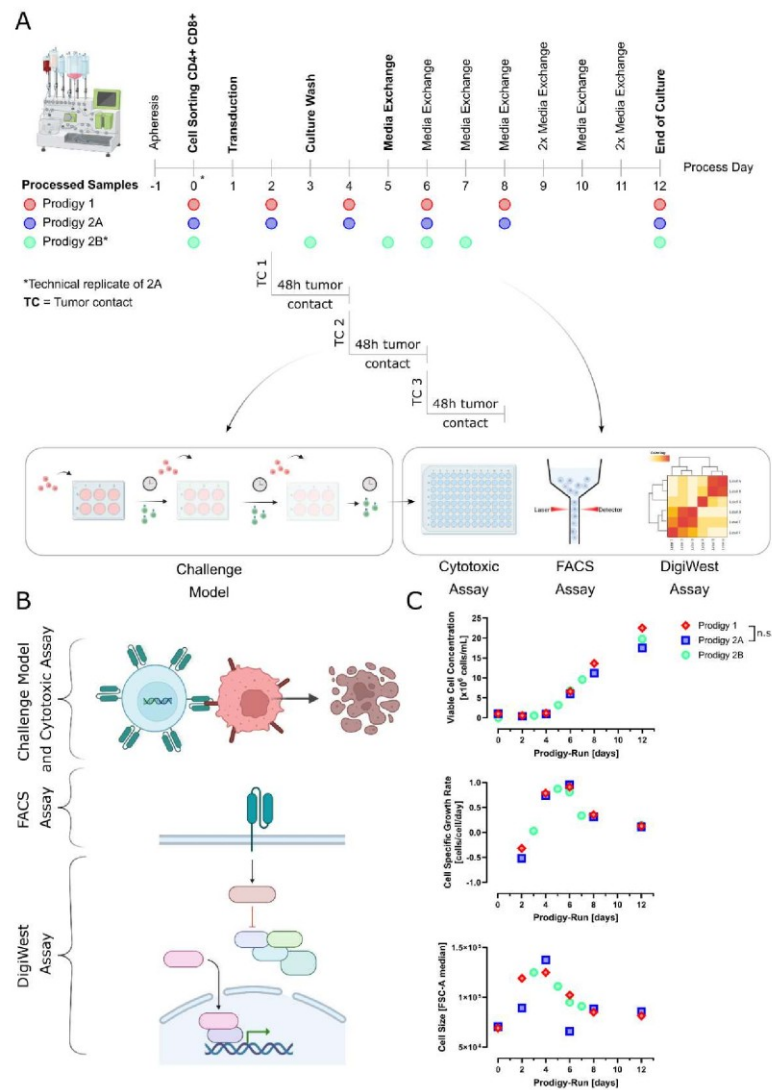
110 Standardized GMP manufacturing and structured in-process 111 sampling design

112 Three CliniMACS Prodigy® manufacturing runs were performed. Prodigy 1 and
113 Prodigy 2 were initiated with apheresis collections from distinct donors as
114 biological replicates. Bystander cells were depleted through T cell enrichment
115 using CD4- and CD8-labeled magnetic beads. Following enrichment, cultures were
116 initiated at a density of 1×10^8 CD4+/CD8+ cells/mL in 100 mL culture volume. A
117 technical replicate for Prodigy 2 was generated by allocating excess cells on day 0
118 resulting in Prodigy 2A and Prodigy 2B. A defined sampling scheme was chosen to
119 capture the entire manufacturing process, while minimizing volume depletion from
120 any single run (Fig. 1 A). All subsequent steps, such as T cell activation using
121 Miltenyi TransAct™ reagent, were executed independently on each device.

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122 Paired analysis between Prodigy 1 and Prodigy 2A for viable cell concentration,
123 cell specific growth rate and cell size were performed using a two-sided paired t
124 test and revealed no significant differences ($p > 0.05$, Supplementary Table 1).
125 Under the tightly controlled cGMP conditions, combined with the statistical
126 analysis between runs, the replicates were considered highly comparable and were
127 therefore pooled for subsequent data analyses.

128 **Fig. 1: CliniMACS Prodigy® process run and sampling pattern for follow-up**
129 **analytical procedures.**



130

131 **A** Overview of the 12-day manufacturing process for three process runs (Prodigy 1:
132 biological replicate no. 1, Prodigy 2: biological replicate no. 2, which branches into
133 technical replicates Prodigy 2A and 2B). **B** Schematic interpretation of all
134 analytical methods used to comprehensively capture the cellular phenotype. **C** Cell
135 culture data showing total cell concentration, specific growth rate (logarithm of
136 (current total cell count / total cell count on the previous day) / time interval
137 between measurements) and average cell size based on forward scatter from flow
138 cytometry measurements. Statistical analysis indicated no significant differences
139 (n.s.) between Prodigy 1 and Prodigy 2A, Supplementary Table 1.
140

141 Early and late manufacturing stages yield functionally distinct 142 CAR-T cytotoxic profiles

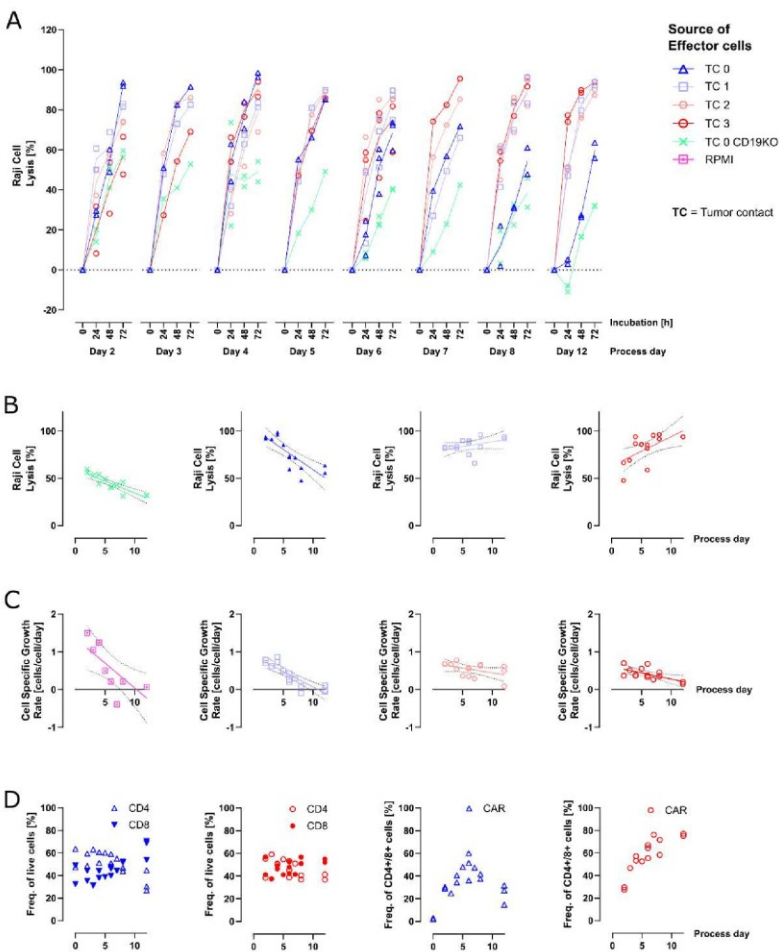
143 A luciferase-expressing Burkitt's lymphoma (Raji) cell line was used as the CD19-
144 CAR target. This line has been widely employed as a benchmark model for CD19-
145 CAR responsiveness in previous studies [10, 17, 18]. Raji cells underwent three
146 sequential rounds of co-culture with CAR-T cells (tumor contacts, TC 1 - TC 3) at a
147 fixed effector to target (E:T) ratio of 4:1. To prevent carryover of soluble factors
148 between challenges, culture supernatant was removed prior to each re-stimulation.
149 Flow cytometry analysis of Raji cells confirmed that incomplete lysis between
150 rounds did not result in substantial transfer of viable tumor cells with fewer than
151 1 % of Raji cells traceable at TC 3 (Supplementary Figure 1). Cytotoxicity assays
152 across a range of E:T ratios (4:1, 2:1, 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32) were
153 performed before the first and after each subsequent tumor contact.

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154 In addition, a cytotoxicity assay was performed on a CD19 knock-out Raji cell line
155 (CD19KO) to determine non-specific cytotoxic activity. Cytotox data (Fig. 2 A) of
156 samples taken directly from the CliniMACS Prodigy® (referred to as TC 0) showed
157 a significant negative correlation between increasing process days and tumor cell
158 lysis after 72 hours of incubation with CAR-T cells at E:T 1:1 (Fig. 2 B and
159 Supplementary Table 2). Additional evaluation of E:T 1:4 and 4:1 can be found in
160 Supplementary Figure 2. Process days closer to harvest (day 6 to 12) did not reach
161 100 % cell lysis making them less potent than early cells. On Raji CD19 KO, CAR-T
162 cells showed a similar negative trend, but were significantly lower than TC 0
163 (Supplementary Table 3). Thereby showing specificity towards the target antigen.
164 Contrary to these observations, CAR-T cells that had encountered tumor cells in
165 three cycles (TC 3), showed a positive correlation between increasing process days
166 a tumor cell lysis, showing more durability. To evaluate the effect of the effector
167 cell count and its capacity for proliferation, cell specific growth rate was analyzed
168 between each TC (48 hours). Additional data was collected for cells cultivated in
169 medium without tumor cells (RPMI) prior to TC 1 (Fig. 2 C). The cell specific
170 growth rate decreased significantly in later process days (Supplementary Table 4).
171 However, the negative slope was flattening with increasing tumor cycles (TC 2 to
172 TC 3). Interestingly, in samples of harvest day 12, the cell specific growth rates in
173 all TCs were close to 0. CD4+ to CD8+ ratios were examined via flow cytometry
174 analysis (Fig. 2 D). Towards harvest day 12, the ratio was favoring CD8+ cells for
175 both TC 0 and TC 3. No CAR expression was observed on day 0 due to lentiviral
176 transduction on process day 1. CAR-expression could be detected by day 2. The
177 proportion of CAR+ cells reached a peak on day 6, followed by a slight decline

178 towards day 12. In samples taken after TC 3, the proportion of CAR+ cells steadily
179 increased in correlation with increasing process days.

180 **Fig. 2: Functional characterization through sequential repeated tumor**
181 **contacts performed on each process day.**



182

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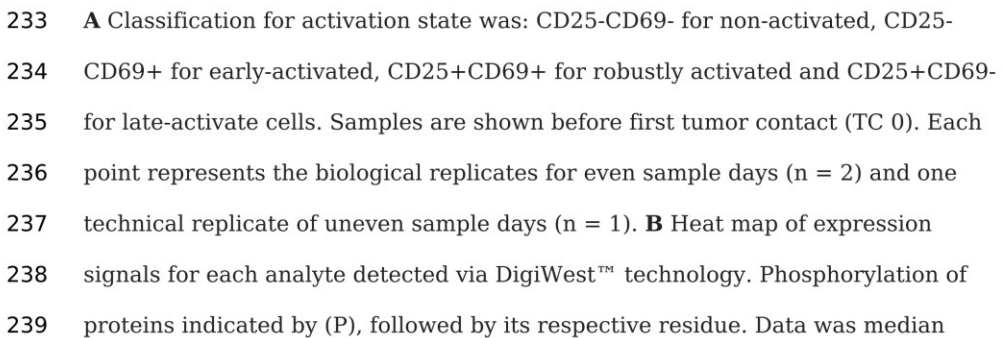
183 **A** Cytotoxic assay plot on Raji cell lysis measured every 24 hours for each process
184 day (lower x-axis) at an effector to target ratio of 1:1. For each sampling day,
185 repeated tumor contacts (TC) every 48 hour were performed. TCs are shown
186 between TC 0 (no tumor contact, sampled taken directly from CliniMACS
187 Prodigy®) and TC 3. Unspecific lysis measured on a CD19KO cell line of Raji (TC 0
188 CD19KO). Each point represents the mean of an analytical triplicate (n = 3). **B**
189 Scatter plots for cytotoxic assay on Raji cell lysis separated for each TC. Simple
190 linear regression was fitted independently for each group. Dotted areas indicate
191 95% confidence intervals. Data (except for TC 2) met the assumption of normality
192 (Shapiro-Wilk normality test, $p > 0.05$). Slopes differed significantly from zero, $p <$
193 0.05 (except TC 1, $p = 0.15$). Exact slope estimates, 95% CIs, and model
194 parameters for each group are provided in Supplementary Table 2. Significant
195 differences based on t test between TC 0 CD19KO and TC 0 (Supplementary Table
196 3). **C** Scatter plots for cell specific growth rate between each TC and control
197 (culture medium RPMI). Simple linear regression was fitted independently for each
198 group. Dotted areas indicate 95% confidence intervals. Data met the assumption of
199 normality (Shapiro-Wilk normality test, $p > 0.05$). Slopes differed significantly from
200 zero, $p < 0.05$. Exact slope estimates, 95% CIs, and model parameters for each
201 group are provided in Supplementary Table 4. **D** Scatter plots for CD4, CD8 and
202 CD19-CAR expression.
203 Each point represents the mean of analytical triplicates from the biological
204 replicates for even sample days $n = 2$ and one technical replicate of uneven sample
205 days $n = 1$. Day 6 was sampled for all process runs. Day 12 had to be omitted for
206 Prodigy 1.
207

208 Manufacturing stage-dependent dynamics shape CAR-T
209 activation profiles

210 Both extracellular and intracellular data were examined to capture T cell activation
211 dynamics continuously over the course of the process. Extracellular activation
212 marker expression was evaluated via flow cytometry of CD4+ or CD8+ cells
213 expressing CD25 and/or CD69 (Fig. 3 A). A high content of early (CD25-/CD69+)
214 and robustly activated (CD25+/CD69+) cells was found on day 2. We expected an
215 increased abundance of early-activated cells on day 1, however, due to the
216 transduction process no sample could be taken. A predominance of cells in late
217 activation (CD25+/CD69-) was found between day 4 and 6. At process day 12, cells
218 almost exclusively comprised inactive cells (CD25-/CD69-).

219 Evaluation of T cell activation at the intracellular level, was evaluated by
220 DigiWest™ analysis [19]. Normalization to process sample day 0 (respective for
221 Prodigy 1 and Prodigy 2) was chosen for all analytes of T cell activation. Markers
222 and their phosphorylations, involved early in the activation cascade were found
223 with reduced signals on process days 2 to 5 (Fig. 3 B). Across the 12-day process,
224 markers CD3ε, CD8α, Lck, Ras, phosphorylation of LAT at tyrosine-191 and PLCγ1
225 showed a distinct biphasic pattern with a critical transition between day 5 and 7, a
226 period coinciding with the described pattern described by flow cytometry. Further
227 downstream, markers such as JNK2, ERK1/2, mTOR, NFκB1 and NFκB p65,
228 exhibited a lasting reduction from day 2 up until day 12 (Fig. 3 C).





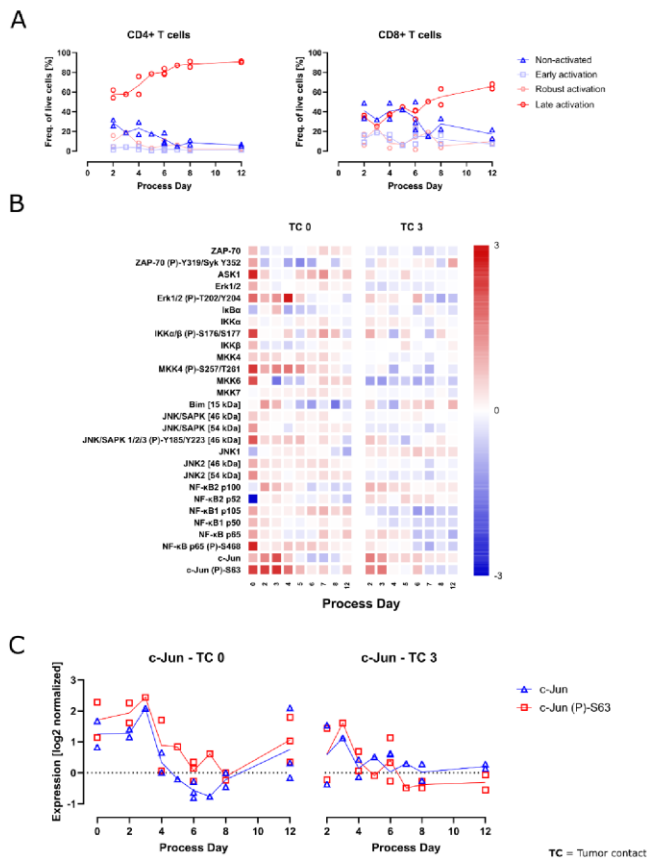
240 centered and log-2 transformed to the respective day 0 signal of each analyte.
241 Samples are shown for Prodigy 2 (n = 1) and its technical replicate (n = 1), mean
242 values represented for day 6 and 12 (n = 2). Data of Prodigy 1 can be found in
243 Supplementary Figure 3. **C** T-cell activation signaling cascade [20-27] with each
244 node color-coded to reflect corresponding DigiWest™ median centered and log-2
245 transformed data. Data shown for Prodigy 2A.

246

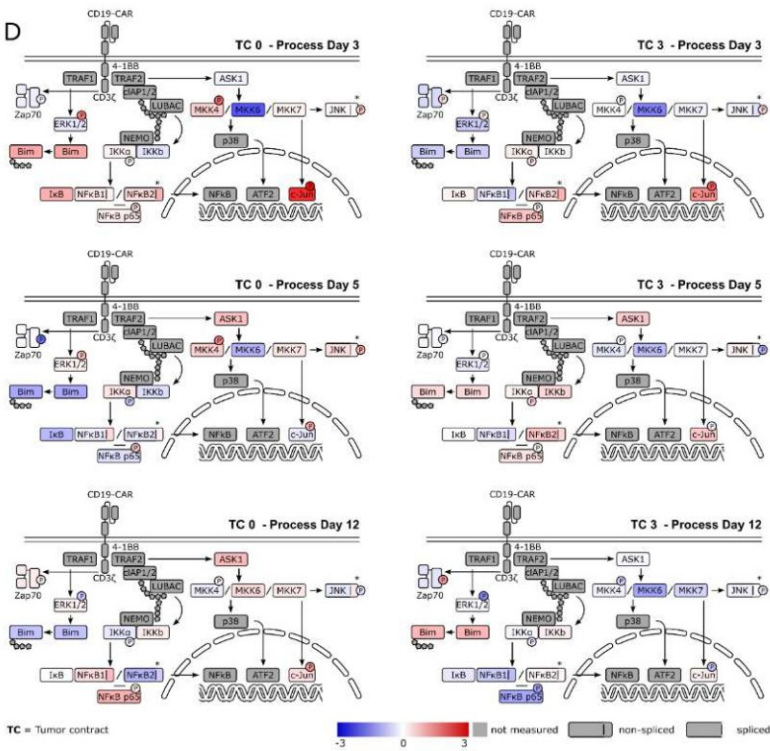
247 Tumour-induced CAR-signaling profiles

248 Following TC 3, a high proportion of inactive cells was detected in early process
249 samples, particularly prominently among CD8+ cells (Fig. 4 A). In contrast,
250 harvest day 12, activated cells constituted the majority of the population. In
251 comparison to Fig. 3 A, these findings suggest effective reactivation of CAR-T cells
252 after repeated tumor contact. Proteins such as ZAP-70, ASK1, or ERK1, key
253 components of the initial signaling hub downstream (Fig. 4 D) of the of T cell
254 receptor [20-22] as well as CAR signaling [28-30], displayed activated forms,
255 though upregulation or phosphorylation between day 3 to 5, prior to tumor
256 contact/engagement (Supplementary Figure 4). The signals for c-Jun, a core
257 component of the AP-1 transcription factor complex and thus a critical regulator of
258 proliferation under stress conditions [31], showed a distinctly phase-dependent
259 pattern throughout the process (Fig. 4 C). Unlike the aforementioned upstream
260 proteins, c-Jun activation was strongly reflected in TC 3 samples, showing a strong
261 signal early in the process and an attenuation towards the end of the process.

262 Fig. 4: Activation and CAR-signaling dynamics before and after tumor



263 engagement.



264

265 **A** Flow cytometry-based scatter plot of activation profile. Classification for

266 activation state was: CD25-CD69- for non-activated, CD25-CD69+ for early-

267 activated, CD25+CD69+ for robustly activated and CD25+CD69- for late-activate

268 cells. Samples taken after three consecutive tumor contacts (TC 3). Each point

269 represents the biological replicates for even sample days (n = 2) and one technical

270 replicate of uneven sample days (n = 1). Day 6 was sampled for all process runs (n

271 = 3). Process day 12 sample of Prodigy 1 had to be omitted for TC 3 (n = 2). **B**

272 Heat map of expression signals for each analyte detected via DigiWest™

273 technology. Phosphorylation for analytes indicated by (P) followed by its respective

274 residue. Data was median centered and log-2 transformed, separately for samples
275 of Prodigy 1 or Prodigy 2. Data are shown for Prodigy 2A, for even sample days (n
276 = 1) and Prodigy 2B for uneven sample days (n = 1). Day 6 and 12 were sampled
277 for both process runs, mean values represented (n = 2). Data of Prodigy 1 can be
278 found in Supplementary Figure 5. **C** Scatter plot of DigiWest™ data for c-Jun (TC
279 0) and after tumor contact (TC 3). Each point represents the biological replicates
280 for even sample days n = 2 and one technical replicate of uneven sample days n =
281 1. Day 6 was sampled for all process runs (n = 3). **D** CAR-signaling cascade [28-
282 30], with each node color-coded to reflect corresponding DigiWest™ data
283 expression levels. Data shown for Prodigy 2B (n = 1).

284

285 Transient stress and exhaustion-related signatures characterize 286 early expansion phase

287 Flow cytometry analyses were performed employing markers LAG-3, PD-1 and
288 TIM-3 (Fig. 5 A). LAG-3 expression for Prodigy 1 was consistently low. Prodigy 2,
289 showed an increase in expression between day 2 and day 8, then a decline towards
290 day 12 (TC 0). In TC 3 both Prodigy 1 and Prodigy 2 displayed a transient increase
291 in LAG-3 expression before declining. Prodigy 2 showed a decreasing curve, while
292 Prodigy 1, being at a lower level, increased towards day 8. PD-1-expression was
293 aligned among Prodigy 1 and Prodigy 2. TC 0 showed a peak between days 2 and 3
294 for both CD4+ and CD8+ cells. In TC 3 for CD4+ cells, PD-1 showed an initial peak
295 at day 3 and a subsequent second peak on day 7, subsequently falling again. For
296 CD8+ cells, values remained consistently low. TIM-3-expression rose sharply for
297 both CD4+ and CD8+ cells between days 2 and 3 (TC 0). The expression remained

298 relatively stable, with singular variations between Prodigy 1 and Prodigy 2 on
299 individual days. On day 12, Prodigy 1 had significantly lower TIM-3 expression
300 compared to Prodigy 2. For CD4+ cells in TC 3, TIM-3 showed a similar expression
301 pattern to PD-1. In contrast, CD8+ cells of TC 3 showed two peaks of high TIM-3
302 expression on day 3 and on day 7.

303 Intracellularly, the Caspase 3 (35 kDa) inactive form [32] remained stable between
304 day 0 and 3, increased moderately from day 4 onwards, reflecting the renewed
305 availability of the proenzyme (Fig. 5 B Apoptosis). At TC 3, the inactive form was
306 present in low levels, with negative peaks for both day 0 and 3. Activated 19 kDa
307 Caspase 3 [32] displayed a short-lived yet pronounced peak (days 2 to 4) in TC 0
308 and then returned to baseline levels. While in TC 3, this form was constantly
309 elevated. The Caspase 3-generated 89 kDa cleavage product PARP [33] diverged
310 between Prodigy 1 and 2, with a steep reduction on day 4 up to day 12 for Prodigy
311 2. While in Prodigy 1, levels sank from day 2 to 6 and then elevated again to 12. Its
312 uncleaved form (116 kDa) shows two peaks on day 0 and between day 6 and 7.
313 Both Bim isoforms (15 kDa and 23 kDa) have a strictly proapoptotic effect [34].
314 Bim showed early activation induction (day 2 to 3), followed by a reduced signal,
315 consistent with an acute proapoptotic stress response that is dampened again after
316 initial T cell activation. In TC 3, both isoforms showed a low signal until day 3,
317 followed by a continuous increase until day 12. Bcl2, the key anti-apoptotic
318 antagonist of Bim [34], showed reduced signal intensity by day 4, returning to
319 baseline level by day 12, indicating an early attenuation of survival signaling
320 during the activation phase followed by late re-stabilization.

321 In TC 3, Bcl2 showed a pronounced negative trend (Fig. 5 B Apoptosis). Survivin, a
322 key protein for proliferation and mitotic stability [35], was strongly elevated until

323 day 4 and then dropped significantly. In TC 3, Survivin remained largely constant,
324 suggesting subdued proliferative reactivation in the tumor context. The protein
325 p53, with pro- and anti-apoptotic influence [36], increased until day 4 and then
326 declined. Its phosphorylation at serine-37 was low until day 3, but then showed a
327 marked increase until day 8. After repetitive tumor contact, p53 phosphorylation
328 peaked between days 4 and 6.

329 Analytes Lactate dehydrogenase A (LDHA), Hypoxia-inducible factor 1 (HIF1),
330 Carboanhydrase IX (CA IX), and Forkhead box protein O3 (FoxO3a) were selected
331 to assess hypoxia during CliniMACS Prodigy® manufacturing (Fig. 5 B Hypoxia).
332 LDHA plays a key role in energy production through the metabolism of lactate to
333 pyruvate and vice versa [37]. LDHA showed a strong early increase of
334 approximately two log₂-steps between day 0 and day 2. HIF1 α is a protein unit
335 that controls cellular response in low oxygen conditions. It is part of the
336 transcription factor HIF-1, which stabilizes in the cell nucleus during oxygen
337 deficiency and activates genes together with another subunit HIF1 β (ARNT) [38].
338 Both peaks overlapped between day 2 and 6. Data on HIF1 were available
339 exclusively for Prodigy 2. CA IX, a canonical HIF1 target and pH regulator in
340 tumor cells [39], initially declined in signal strength from day 0 to day 2. The signal
341 then returned to baseline levels by day 4, followed by another decline and
342 subsequent continuous increase until day 12. The phosphorylation of FoxO3a at
343 Serine-318/321, key marker of FoxO3a inactivation [40], decreased by
344 approximately three log₂ steps between day 0 and day 5. This strong
345 dephosphorylation event indicated functional reactivation and potential nuclear
346 translocation. Subsequently, phosphorylation increased, peaking on day 7,

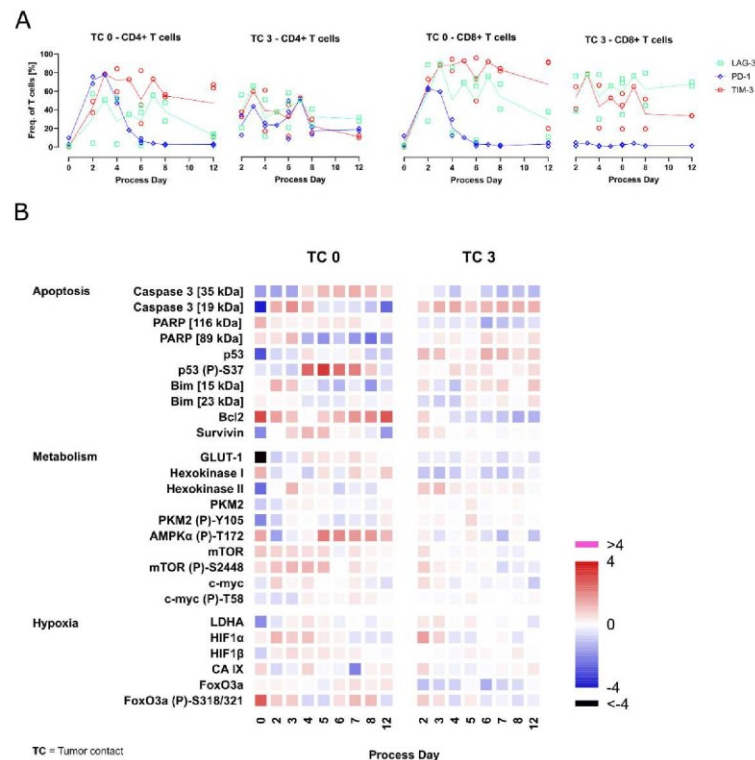
347 followed by a decrease until day 12. The opposing regulation of total protein and
348 phosphorylation signal underscored the relevance of its posttranslational control.

349 Metabolic activity was examined by several key players. A limiting transporter for
350 glucose-dependent energy supply GLUT-1 [41] showed an abrupt increase of four
351 log-2 levels between day 0 to day 2, indicating early, massive upregulation of
352 glucose uptake after cell activation (Fig. 5 B Metabolism). This was followed by a
353 slight further increase until day 5, followed by stabilization, reflecting a transition
354 from the initial activation phase to a metabolically established state. Hexokinase I,
355 which is responsible for the first irreversible glycolysis reaction [42], showed an
356 early decline on day 2, suggesting a temporary reduction in basal glycolysis flux.

357 From day 4 onward, a continuous re-induction was observed until initial levels
358 were restored on day 12. Hexokinase II exhibited a pronounced increase between
359 day 0 and day 2, indicating early metabolic reprogramming. A peak on day 3
360 pointed to the phase of maximum glycolytic induction, followed by a slight decline
361 until day 5, as is typical during transitions from acute activation to stabilized
362 metabolic states. A renewed slight increase until day 12 suggests persistent or
363 renewed metabolic demands, possibly in the course of continued expansion. PKM2,
364 the pyruvate kinase isoform, typical of proliferating and activated cells [43],
365 showed a temporal course that closely correlated with the pattern of Hexokinase
366 II. This underscored the coordinated upregulation of two central enzymes of
367 glycolysis during metabolic reprogramming. Between day 0 and day 2, there was a
368 marked increase, suggesting an early enhancement of glycolytic throughput. The
369 moderate decline until day 5, followed by a slight increase again until day 12.

370 AMPK α phosphorylation at threonine-172 indicates its activated form as a
371 mediator for metabolism of lipids [44]. It highlighted a pronounced decline

372 between day 0 and day 2, indicating an early phase of metabolic relief or sufficient
373 energy reserves. Subsequently, phosphorylation increased strongly until day 5.
374
375 **Fig. 5: Expression profiles of inhibitory immune checkpoints, metabolism,**
376 **hypoxia, and apoptosis.**



377
378 **A** Flow cytometry-based scatter plot of markers for inhibitory immune checkpoints
379 LAG-3, PD-1 and TIM-3. Samples taken before tumor contact (TC 0) and after three
380 consecutive tumor contacts (TC 3). Each point represents the biological replicates

381 for even sample days (n = 1) and one technical replicate of uneven sample days (n
382 = 1). Day 6 was sampled for all process runs (n = 3). Process day 12 sample of
383 Prodigy 1 had to be omitted for TC 3 (n = 2). **B** Heat map of expression signals for
384 each analyte detected via DigiWest™ technology. Phosphorylation for analytes
385 indicated by (P) followed by its respective residue. Data was median centered and
386 log-2 transformed, separately for samples of Prodigy 1 or Prodigy 2. Data are
387 shown for Prodigy 2A, for even sample days (n = 1) and Prodigy 2B for uneven
388 sample days (n = 1). Day 6 and 12 were sampled for both process runs, mean
389 values represented (n = 2). Data of Prodigy 1 can be found in Supplementary
390 Figure 6.

391

392 **Discussion**

393 Semi-automated CAR-T cell manufacturing is a huge step forward in decentralized
394 point-of-care manufacturing. Several strategies for processes shortening have
395 already been proposed [10, 14, 45, 46]. The present study characterizes
396 manufacturing on the CliniMACS Prodigy®, delineating distinct phenotypic
397 phases, and highlighting options for process optimization and rational process
398 shortening.

399 **Cytotoxic anti-tumor activity reveals temporal shift**

400 To identify critical shifts during manufacturing, a combination of complementary
401 high-resolution analytical approaches was employed. A cytotox-re-challenge model
402 served as an analytical approach for functionality of CAR-T cells, which allowed for
403 canonical sampling of all essential process days (Fig. 6).

404 First tumor encounter revealed a significant negative temporal correlation.
405 Complete tumor cell lysis (E:T at 1:1) was only detected in samples collected in
406 earlier days of the manufacturing process (early potency). It has been reported
407 that early CARs induced non-specific lysis, indicative of NKG2D-mediated, target-
408 independent tumor cell killing [47, 48], however, tumor lysis in our study was
409 significantly lower in the controls where targets were CD19 negative. This
410 suggests, enhanced early tumor lysis cannot be explained by activation alone; and
411 that CAR function already contributes early, driving superior efficacy versus day
412 12. This means that the CAR is responsible for the early high lytic activity.
413 Interestingly, following repeated tumor contact, samples from early process phases
414 exhibited markedly poorer lysis than those from late phases, despite reduced
415 proliferative capacity late phase cells exhibited increased durability. This day-
416 dependent reversal of functional properties prompted comprehensive profiling of
417 activation state, metabolic programs and overall phenotype to delineate the timing
418 and mechanistic basis of this shift.

419 **CD4/CD8 ratio during manufacturing**

420 To identify the reasons for the dramatic loss of tumorolytic activity during CAR
421 manufacturing, we also analyzed the CD4/CD8 ratio. A conspicuous observation
422 was a shift of the CD4:CD8 ratio, favoring CD8⁺ cells in the later process day
423 samples, a trend that has also been described in other studies on CliniMACS
424 Prodigy® cultivation [46, 49, 50]. A shift that has been accounted for supportive
425 influence of CD4⁺ T cells on CD8⁺ T cell expansion [51] or might result from
426 fundamentally different biology of CD4⁺ T cells vs CD8⁺ T cells regarding
427 proliferation and cell division. Although both CD4⁺ and CD8⁺ T cells undergo an
428 autonomous program of differentiation, the kinetics and efficiency of CD8⁺ T cell

429 proliferation differ substantially from those of CD4+ T cell proliferation. The time
430 of antigen exposure required to launch the proliferative program for naive CD8+ T
431 cells seems to be less than that required for naive CD4+ T cells [52-55]. CD8+ T
432 cells also divide sooner and have a faster rate of cell division than do CD4+ T cells
433 [51, 52, 56-58]. For clinical translation a 1:1 CD4:CD8 T cell ratio is favored,
434 preclinical and clinical data show that a balanced mix promotes expansion in
435 patients and persistent antitumor activity [59]. However, retrospective analysis of
436 clinical data has shown that a higher proportion of CD8+ cells is beneficial for the
437 clinical outcome, with higher response rates [60]. Consequently, the observed
438 CD8+ dominance appears to be a direct result of the inherent proliferative
439 advantage of this subset, posing a structural challenge for maintaining a strictly
440 balanced 1:1 ratio during extended automated manufacturing.

441 **T cell Activation State**

442 Following T cell enrichment, cells were activated using polymers targeting both
443 CD3 and CD28 in the presence of cytokines. Upon recognition of antigen-MHC
444 complexes, the TCR-CD3 complex underwent rapid internalization (Supplementary
445 Figure 7) followed by swift degradation [61-63]. Flow cytometry data confirmed
446 robust activation of both CD4+ and CD8+ cells 48 hours post stimulation with over
447 half exhibiting late state activation markers, a pattern consistent with prior
448 reports [64]. This late-activated fraction remained predominant in both subsets
449 until day 6 after which an inactive phenotype progressively emerged with CD8+
450 cells showing faster exhaustion by day 12. Notably, TCR-proximal signaling
451 proteins (e.g. Lck, PLC γ , PKC θ , RAS) displayed biphasic dynamics (Fig. 3),
452 featuring early depletion and re-expression to baseline by days 5-6, most
453 pronounced for Lck, a key regulator of TCR signaling and trafficking [65, 66]. In

454 contrast nuclear and transcriptional effectors (e.g. JNK2, ERK1/2, mTOR, NF- κ B1,
455 NF- κ B p65) [20, 21, 24], showed profound, sustained depletion through day 12,
456 except for c-Jun, which peaked sharply on day 2 before declining, as typical for
457 immediate-early genes [67].

458 Compared to the endogenous TCR, CAR-T cell stimulation yields different
459 functional outcomes in sensitivity, effector function, and exhaustion. CAR and TCR
460 form separate immunological synapses without direct cross-phosphorylation of
461 their CD3 ζ chains [68] with CAR triggering faster cytotoxicity and IFN- γ release
462 [68, 69] but reduced proliferative capacity upon prolonged exposure [70, 71]. At
463 the molecular level, CAR induces weaker LAT phosphorylation than TCR [72]
464 (Supplementary Figure 8) rendering TCR up to 100 times more antigen-sensitive
465 [72]. While TCR-driven activation efficiently generates potent effector cells [71],
466 these are short-lived due to activation-induced cell death (AICD) [73]. Here,
467 transient CD3/CD28 hyperactivation upregulated PD-1 by day 4 post tumor-
468 contact, curbing late stage effector function in re-challenge assays. Surviving cells,
469 acting as a selective filter, transitioned into a durable, long-lived pool. Subset
470 analysis further revealed an early TEM predominance (days 2-3) a transient TCM
471 peak (days 4-5), followed by sustained CD8+ cells dominance and TCM
472 reacquisition in CD4+ cells (see Supplementary Figure 9). Upon tumor engagement
473 TEM cells overwhelmingly prevailed (with minor TEFF) while TCM remained as a
474 small, transient fraction (days 2-5).

475 These findings substantially advance our understanding of the observed
476 tumorolytic activity. Incorporating more quiescent yet pre-selected cells at late
477 manufacturing stages ensures sustained cytotoxicity. Conversely, early-stage cells

478 characterized by heightened proliferation and full activation exhibit superior
479 cytolytic potency but diminished durability.

480 **Metabolic reprogramming during CAR-T differentiation**

481 The CAR T manufacturing process demands precise control of multiple parameters
482 to yield sufficient numbers of activated CAR-T cells. The Prodigy system handling
483 high cell densities, particularly late in culture, necessitates frequent medium
484 exchanges. This environment profoundly impacts cellular metabolism, stress
485 responses, and apoptosis – unexplored aspects systematically profiled here.

486 Post-activation (days 0–2), GLUT-1 exhibited the most pronounced upregulation
487 among all analytes examined, reflecting robust glucose (and hexose/pentose)
488 import critical for metabolic rewiring in the early phase of the manufacturing
489 process. Hexokinase II surged steeply by day 2, peaked on day 3, then plateaued,
490 while PKM2, the dominant glycolytic isoform in proliferating immune cells,
491 mirrored this trajectory [43]. AMPK α – amongst other important metabolic
492 pathways controlling lipid metabolism through phosphorylation [44] rose sharply
493 from day 2, plateauing by day 5 to signal waning activation and lipid metabolism
494 restraint via phosphorylation; this aligned precisely with the proliferation curve.

495 Through their multifunctional activities, c-myc and mTOR serve as key regulators
496 of cellular metabolic programs. c-myc acts as the primary switch for metabolic
497 reprogramming after antigen recognition [74]. A major function of c-myc in T cells
498 is to control the expression of amino acid transporters [75]. Without c-myc, T cells
499 cannot increase their biomass because they lack the building blocks for protein
500 production [75]. In addition, it controls glutamine metabolism, which is essential
501 for the production of nucleotides and polyamines [74]. Furthermore, c-myc induces

502 genes for glucose metabolism to provide energy and intermediates for cell growth
503 [74]. mTOR acts as a central integrator of environmental signals, linking nutrient
504 availability to immune functions [76]. The subunit mTORC1 promotes aerobic
505 glycolysis (Warburg effect) and protein biosynthesis [77]. High activity leads to the
506 formation of potent effector cells but prevents the development of memory cells
507 [77]. While mTORC1 drives glycolysis, its inhibition promotes fatty acid oxidation
508 and mitochondrial capacity, which supports the survival of long-term memory cells
509 [77].

510 Collectively, these findings indicate that metabolic rewiring drives a shift from a
511 purely proliferative toward a specialized effector phenotype. This confirms that the
512 observed transition in cytotoxic activity is fundamentally rooted in the cells'
513 changing energetic and biosynthetic requirements across different manufacturing
514 stages.

515 **LDHA upregulation confirms glycolytic adaptation under hypoxia**

516 Additionally, cellular stress responses were evaluated with particular emphasis on
517 hypoxia. Under hypoxic or anaerobic conditions, lactate dehydrogenase A (LDHA)
518 preferentially converts pyruvate to lactate, thereby regenerating NAD⁺ from
519 NADH to maintain glycolytic flux [37].

520 LDHA upregulation is hypoxia-inducible via HIF-1 α , enhancing lactate production
521 in low-oxygen environments typical of tumors or inflamed tissues. This maintains
522 glycolytic flux when oxidative phosphorylation is limited. LDHA signals peaked
523 between days 3 and 5, preceding daily media changes and indicating hypoxia.
524 More definitively, HIF-1 α , continuously synthesized and degraded under normoxia
525 – stabilizes under low oxygen, accumulates in the cytoplasm, translocates to the

526 nucleus. dimerizes with HIF1 β for an active transcription factor which drives
527 hypoxic gene transcription [38]. HIF1 α peaked between days 2 and 4, followed by
528 elevated HIF-1 β levels on days 4 and 8. Carbonic anhydrase IX (CA IX) a canonical
529 HIF-1 target, localizes on the cell surface under hypoxia where it regulates
530 extracellular pH through electrolyte secretion and acid-base homeostasis and was
531 detected by days 4–6 [39]. These temporal synchronized dynamics, alongside
532 LDHA upregulation confirm transient hypoxic conditions during the middle phase
533 of the process (days 4 to 6).

534 FoxO3a which serves as a key transcription factor that senses and responds to
535 diverse cellular stresses such as oxidative damage, nutrient deprivation, and
536 hypoxia [78], further supports this mid-phase stress response. FoxO3a declined
537 sharply by day 5, peaked thereafter and fell again by day 12, its inhibitory
538 phosphorylation at Ser318/321 followed an comparable, more amplified pattern
539 [79].

540 Daily media exchange starts on day 5, coinciding with peaks of hypoxia markers
541 like LDHA/HIF-1 α , followed shortly thereafter by the expression of exhaustion
542 markers post-tumor contact (Fig. 5 A), suggesting cumulative O₂ depletion mid-
543 culture. However, some stress-related responses such as LAG-3 expression and
544 FoxO3a phosphorylation varied across biological replicates (Fig. 5 A and B),
545 implying some donor-specific T cell sensitivity to transient hypoxia rather than a
546 uniform process flaw. While the early rise of hypoxia markers suggests that an
547 earlier initiation of media exchanges could mitigate metabolic stress, such an
548 intervention requires a careful trade-off. It might prolong the proliferative phase, it
549 also risks diluting essential autocrine cytokines and bypassing the “natural
550 selection” that yields a more robust and mature cell product. Consequently, this

551 transient hypoxic stress likely acts as a key physiological trigger that drives the
552 observed shift toward a less proliferative, yet more durable T cell phenotype.

553 **Apoptosis, Proliferation Marker and anti-Apoptotic Dynamics**

554 To integrate these findings on a specific type of stress, markers of apoptosis were
555 assessed to determine whether the above-conditions promoted a proapoptotic
556 state. The 35 kDa full length Caspase 3 increased markedly from day 4 onwards,
557 consistent with proenzyme accumulation. In contrast, the activated 19 kDa
558 Caspase 3 displayed a brief but pronounced peak between days 2 and 4 and
559 remained persistently elevated after tumor contact. The 89 kDa PARP cleavage
560 product generated by Caspase 3 declined sharply from day 4 supporting sustained
561 caspase activity. p53 whose predominant biologic function is pro-apoptotic, was
562 upregulated between days 3 and 7 with strong phosphorylation at serine-37 from
563 days 4 and 8, indicating robust p53 activation and severe cellular stress, typically
564 linked to DNA damage [80]. After tumor contact, p53 phosphorylation peaked on
565 days 4 - 6 and on day 12. Both Bim isoforms (15 kDa and 23 kDa), strictly pro-
566 apoptotic, showed dynamically distinct peaks on days 2 - 3, before declining,
567 reflecting acute stress post activation. After tumor contact, Bim rebounded
568 strongly from day 5 onwards.

569 Bcl-2, Bim's key antagonist, decreased by day 4, hit a nadir on day 5, but reached
570 its baseline value by day 12. In tumor coculture TC 3, it showed a sustained
571 negative trend. Survivin, essential in mitosis, peaked on days 3 - 5, typical of early
572 proliferative expansion, before normalizing but remained stable in TC 3, indicating
573 blunted proliferation upon tumor engagement. proliferative reactivation in the
574 tumor context.

575 In contrast to a terminal pro-apoptotic shift, the observed dynamics of Bcl-2,
576 Survivin, and Caspase-3 suggest a transient phase of cellular stress mid-
577 manufacturing that is successfully resolved. Ultimately, the 12-day process yields a
578 product that closely resembles the initial cellular state in terms of viability, rather
579 than showing signs of accumulated apoptotic damage.

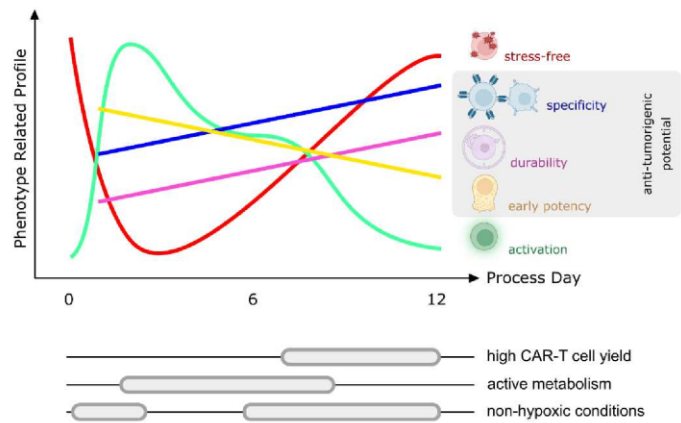
580 **Summary**

581 The manufacturing process resolves into distinct early and late phases
582 (Supplementary Fig. 10) demarcated by a critical functional and metabolic
583 inflection point. Shortened protocols generate a phenotype distinct from the
584 standard 12-day process, featuring rapid tumor lysis but compromised persistence
585 (Fig. 6). Conversely, late-stage cells display delayed yet more specific and durable
586 effector function.

587 Unexpectedly, exhaustion markers were enriched in early samples, owing to
588 hyperacute initial activation, whereas day 12 cells exhibited metabolic profiles
589 resembling a naïve-like state, aligning with recent observations from CliniMACS
590 Prodigy® processes [50]. While abbreviated manufacturing accelerates therapy
591 delivery [14, 16], our data reveal associated phenotypic trade-offs in yield and
592 quality that can be strategically exploited: fast-acting, high-yield profiles for
593 aggressive tumors versus the sustained specificity of late-stage cells for minimal
594 residual disease.

595

596 **Fig. 6: Process characterisation and phase-specific profiling**



597

598 Approximation of the 12-day-cellular stress response profile, target specificity,
599 cytotoxic durability (post three tumor contacts), early cytolytic potency upon initial
600 tumor engagement and T cell activation dynamics. Additionally, process-related
601 metrics including CAR-T cell yield, metabolic reprogramming and adaption to
602 hypoxic conditions.

603

604 **Methods**

605 **CAR-T Manufacturing**

606 CliniMACS Prodigy® TS 520 (200073-613), CliniMACS® PBS/EDTA Buffer
607 CR/GMP (200070-022), TexMACS™ GMP Medium (170076-306), MACS® GMP
608 Recombinant Human IL-7 (170076-111), MACS® GMP Recombinant Human IL-15
609 (170076-186), CliniMACS® CD4 Reagent CR/GMP (200070-213), CliniMACS®
610 CD8 Reagent CR/GMP (200070-215) and MACS® GMP T Cell TransAct™ Large

611 Scale CR/GMP (200076-204) were derived from Miltenyi Biotec. Bags were placed
612 in the right position for waste, target, non-target and formulation. Leak-test and
613 priming were initiated. Starting material was welded to the application bag and
614 the content was then transferred into it. Vials of CD4 and CD8 MicroBeads were
615 spiked to the system. T cell enrichment and automated red blood count reduction
616 were initiated. After determining concentration of CD4+/8+ cells, adjustment to
617 cell count of 1×10^8 cells in 100 mL were done by CliniMACS Prodigy®, after
618 entering the data. Finally, activation of T cells was started after spike of
619 TransAct™. 24 hours after activation, viral transduction was performed, a CD19-
620 CAR Lentivirus bag (Miltenyi Biotec, 200-072-102), was thawed at RT. The bag was
621 then welded to the tube set. Transduction was initiated on the system. Cultivation
622 volume was increased to 200 mL on day 3 and remained constant. Medium
623 exchange of 50 mL on day 5 and 130 mL was performed every day starting day 6
624 and two-times a day from process day 9 on. Cultivation was done until day 12.
625 Samples were collected for day 0, between day 2 to 8 and harvest day 12.

626 Cytotoxic Assay

627 Luciferase-transduced Raji cells (DSMZ, ACC 319) had been previously transduced
628 with luciferase gene in our lab [81]. The tumor cell lines were seeded at a density
629 of 30,000 cells per well in 50 μ L complete RPMI medium in a flat white 96-well cell
630 culture plate (Greiner Bio-One, 655083). Dissolved D-luciferin potassium salt
631 (PerkinElmer, 122799-5) was added to each well at a concentration of 4 μ g/mL.
632 Effector cells were seeded at the specified effector-target cell ratios (E:T). The
633 total volume per well was 200 μ L. The plates were incubated in a HERAcell
634 incubator (Heraeus Med, 98111578) at 37 °C and 5% CO₂. Bioluminescence was
635 measured with the Tecan Spark 10M (Tecan, 189000545) at 37 °C at the specified

636 time points. Lysis was calculated based on the relative luminescence of the test
637 conditions using a lysis formula based on a standard dilution series.

638 Re-Challenge

639 Raji cells (DSMZ, ACC 319) were seeded at 0.5×10^6 cells per well in 1 mL
640 complete RPMI in 6-well cell culture plates. 2×10^6 effector cells in 2 mL of
641 complete RPMI. The cells were incubated in a HERAcell incubator (Heraeus Med,
642 98111578) for 48 hours at 37 °C and 5 % CO₂.

643 Flow Cytometry Analysis

644 Anti-human antibodies used include: CD3 Antibody, anti-human, Vio® Bright R720
645 (Miltenyi Biotec, 130-127-377), CD4-VioGreen® (Miltenyi Biotec, 130-113-259),
646 CD8-PE (Miltenyi Biotec, 130-110-678), CD19-APC (Miltenyi Biotec, 130-113-642),
647 CD25-Vio® Bright B515 (Miltenyi Biotec, 130-115-536), CD27-BUV496 (BD
648 OptiBuild™, 741145), CD45RA-VioBlue® (Miltenyi Biotec, 130-117-743), CD45RO-
649 APC-Vio® 770 (Miltenyi Biotec, 130-113-557), CD69-Brilliant Violet 650™
650 (BioLegend, 310933), CD95 Antibody BUV737-Anti-Human (BD Horizon™,
651 612790), CD279 (PD1)-APC (Miltenyi Biotec, 130-120-389), CD223 (LAG-3)-BV786
652 (BD OptiBuild™, 744727) and CD366 (TIM-3)-PE-Vio® 770 (Miltenyi Biotec, 130-
653 121-334).

654 0.3×10^6 cells were prepared in 3 mL cold CliniMACS® PBS/EDTA buffer (Miltenyi
655 Biotec, 200-070-025) in a round-bottom centrifuge tube. After washing, cells were
656 prepared with FcR Blocking Reagent, human (Miltenyi Biotec, 130-059-901) at
657 0.6 µL 300 µL of cell suspension, followed by incubation for 10 minutes at 4 °C.
658 After second wash, antibodies were added with two panels (Panel 1: CD3, CD4,
659 CD8, CD27, CD19-CAR Biotin, CD45RA, CD45RO, CD25, CD69, CD95 and Panel 2:

660 CD3, CD4, CD8, CD27, CD19-CAR Biotin, PD-1, TIM-3, LAG-3) and incubated for
661 30 minutes at 4 °C. Followed by washing and secondary anti-biotin antibody
662 staining with incubation for 30 minutes at 4 °C (Panel 1: Biotin-APC and Panel 2:
663 Biotin-FITC). Final 7-AAD live-dead staining was performed. Cells were processed
664 on a FACS Fortessa X-20 (BD Biosciences, H656385J4001).

665 Intracellular Analysis

666 The DigiWest™ approach is a bead-based Western blot method that enables highly
667 parallel analysis of protein expression and modification status with molecular
668 weight resolution [19]. For each sample, 2×10^6 cells were collected and
669 centrifuged at 450 x g for 5 minutes at 4 °C in a 50 mL low-bind centrifuge tube
670 (Eppendorf, 0030122240). The supernatant was aspirated carefully. The pellet was
671 then resuspended in 5 mL cold 1x DPBS, the suspension was transferred to a new
672 50 mL low-bind centrifuge tube. Cells were spun down again at 450 x g for
673 5 minutes at 4 °C, the supernatant was again aspirated carefully. This step was
674 repeated one more time. The dry pellet was then snap-frozen on dry ice and stored
675 at -80 °C.

676 Samples were lysed with 50 µL lysis buffer (2% LDS, 25mM DTT, PhosSTOP™
677 Phosphatase Inhibitor, cOmplete™ Protease Inhibitor) for 20 minutes at 95 °C.
678 Then spun 5 minutes at 16,000 x g in a QIAshredder (Qiagen, 79656) column.
679 Proteins were separated by size using SDS-polyacrylamide gel electrophoresis
680 (SDS-PAGE), transferred to a membrane, and biotinylated. The sample lane was
681 cut into 96 horizontal strips representing molecular weight fractions, and the
682 proteins are eluted. The eluted proteins were then immobilized on neutravidin-
683 coated Luminex beads, with each of the 96 bead sets representing a defined

684 molecular weight fraction. The pooled beads, which now correspond to a digitized
685 Western blot trace, were incubated with specific antibodies in the immunoassay
686 and the signals are read out using a Flexmap 3D instrument (Luminex Corp.
687 Austin, TX). Intrinsic marker proteins were used to accurately assign the molecular
688 weight to the 96 bead populations. Quantitative evaluation was performed by an
689 algorithm that visualizes the protein bands as peaks and integrates their area
690 above a local baseline, yielding numerical output values.

691

692 Statistical information

693 Unless otherwise stated, the individual values were represented in each plot. The
694 number of independent experiments and the total number of repetitions (n) are
695 indicated in the respective figure description. Analytical replicates (n = 3) done in
696 cytotoxic assays are represented by their mean values.

697 Normality was tested prior to linear regression analyses two-tailed paired t-tests
698 using a Shapiro-Wilk test.

699 Paired comparisons between Prodigy 1 and Prodigy 2A for viable cell
700 concentration, cell specific growth rate and cell size were analyzed using a two-
701 sided paired t test. The number of paired observations (n) was 5 (process day 2, 4,
702 6, 8 and 12). Data are presented as the mean paired difference (Prodigy 2A -
703 Prodigy 1) together with the 95% confidence interval (CI). The assumptions of
704 normality were assessed on the paired differences using the Shapiro-Wilk test.
705 Test statistics (t and degrees of freedom) and exact P values are reported in
706 Supplementary Table 1. A p value < 0.05 was considered statistically significant.

707 To analyze unspecific lysis, the same type of t test was applied, with n = 14 paired
708 comparisons. The assumptions of normality were assessed on the paired
709 differences using the Shapiro-Wilk test.

710 To examine whether process day predicts cytotoxicity, a simple linear regression
711 was performed, with time as the independent variable and cytotoxicity as the
712 dependent variable. The strength and significance of the relationship were
713 assessed using the regression slope, its 95% confidence interval, and the
714 coefficient of determination (R^2). All tests were two-sided with $\alpha = 0.05$.

715 Statistical analyses were conducted in GraphPad Prism software (version 10.1.1,
716 GraphPad Software, Inc., USA).

717 Two-dimensional hierarchical clustering (analyte x samples) was performed using
718 MultiExperiment Viewer (MeV) v4.9. Prior to clustering, the data were median-
719 centered and log2-transformed per analyte to make different signal strengths
720 comparable. Distances were calculated using Euclidean distance; clustering was
721 performed agglomeratively with complete linkage. Leaf order optimization was
722 done for improved visualization for analytes and samples.

723 **Data Availability**

724 The datasets generated and/or analysed during the current study are available
725 from the corresponding author on reasonable request. For original data, please
726 contact florian.schinle@med.uni-tuebingen.de.

727

728 **References**

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949

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955

956 **Ethics declarations**

957 Competing interests

958 The authors declare no competing financial interests.

959

960 Ethics

961 Only apheresis from healthy donors was used as the starting material for
962 production. This was approved by the Ethics Committee of the University Hospital
963 Tübingen (ethics approval No. 507/2017B01)

6 Discussion

Applied CAR-T cell immunotherapy is currently at a critical juncture, still facing major challenges. Chief among these is the successful translation of novel constructs from research settings into clinical practice. To date, all FDA-approved CAR-T cell therapies are second-generation constructs, predominantly targeting CD19 [221], although numerous alternative targets have been investigated.

As described in chapter 1.3.4, manufacturing of GMP-grade lentiviral vectors for transformation of T cells into CAR-T cells represent a major bottleneck. There are five key factors that drive cost and complexity. 1. Cytotoxicity of viral components: In particular, the widely used envelope protein VSVg limits the development of stable production cell lines [131, 178]. 2. Variability of transfection: mostly due to transient transfection with multiple plasmids [131]. 3. Stability: high sensitivity of the lipid-particles to factors such as temperature, pH, salt concentration and shear forces [178]. 4. Scalability: transition from small laboratory scale to production scale, such as bioreactors, require difficult adaptation of the cells [180]. 5. Yield after downstream processing: fluctuating yield between different production batches (batch-to-batch variability), optimization of filters and chromatographic processes is a balancing act to remove contaminants (such as host cell DNA) without damaging the sensitive vectors [189, 246].

Beyond lentiviral vector considerations, a major limitation lies in the insufficient understanding of CAR-T manufacturing itself. While key aspects of CAR-T manufacturing have been extensively studied [85, 89, 247, 248], day-resolved changes across individual process days remain insufficiently characterized.

The following sections summarize the key advances achieved in this work, which are centered on the biological characterization of the process. These findings highlight progress toward overcoming the identified barriers and provide a foundation for further development.

6.1 Establishment of a Comprehensively Characterized Master Cell Bank

The first step to overcome one of the aforementioned challenges was the use of a commercial serum-free grown suspension HEK293 cell line for transfection of the target construct. A stable cell line as an alternative would have entailed cytotoxicity due to VSVg. A constant release of lentiviral vectors by a stable cell line would impair growth during expansion phase [131, 178]. Limiting vector production by the cell to 48 hours (between transfection and harvest) increases cellular yield and therefore titer. Additionally, cells can be handled in S1 environment prior to transfection, reducing risk and regulatory requirements. On top, a transfection protocol allows for exchange of the gene of interest, enabling a switch to other CAR constructs. This approach meant that only the packaging plasmid needs to be adapted in a different manufacturing target, rather than re-establishing the whole cell line.

The ICH Q5D guideline describes the derivation, establishment, characterization, and use of cell banks for biotechnological / biological medicinal products and explicitly addresses the system consisting of a master cell bank (MCB). The guideline aims to reduce variability and maximize reproducibility. This was addressed in this work with the establishment and investigation of a MCB (chapter 4.1.1). Precise data on the number of living and dead cells was collected to assess proliferation or cytotoxicity between expansion and harvest [249]. Doubling time, or in its converted form the cell-specific growth rate, is a critical acceptance criterion, the monitoring of which indicates trends that may point to underlying biological problems [250]. Additional cell culture parameters were included, such as the amount of glucose (indirect glucose consumption), lactate, pH and cell cycle data. Long-term cultivation exerts selection pressure on cells, which can lead to genetic and phenotypic changes after a few passages [251]. Therefore, the relevance of these data depends on the duration from cell thaw to end of cell cultivation (harvest). Data collection was designed to map the steps up to a production in a bioreactor system. Repeated expansion measurements demonstrated a high degree of consistency for the aforementioned cellular data.

Comparability was evaluated across two different scales (30 – 60mL), revealing again high degree of consistency. Reproducibility of the cell culture derived from the previously established master cell bank was confirmed.

6.2 Development of a Robust LV Titration Assay

Accurate determination of the multiplicity of infection (MOI) is essential for successful target cell transduction [252]. This parameter, corresponding to the LV-to-target cell ratio, serves to balance efficiency and safety considerations. The value is calculated based on the measured titer of infectious genomes (IG)/mL or transducing units (TU)/mL. The titers achieved in bulk harvests or culture supernatants in optimized

suspension cultures may range between 10^7 and 10^8 TU/mL [131, 187, 253, 254]. In comparison, productivity of adherent cell systems ranges between 10^6 and 10^8 TU/ml [131, 188, 255, 256]. Notably, general comparisons of different studies are difficult due to the employment of different analytical methods. Only a few comparative studies have been conducted using the same vector. Adding to this, it has been shown that the size of the construct to be transduced determines the bulk titer, the larger the construct, the lower the titer [257]. This can lead to an overestimation of the productivity of the system when using small constructs such as GFP with a base pair length of only 730 [258]. Several studies on LV manufacturing platforms are based on GFP-LV constructs. Changing the construct to a more complex one is expected to yield in lower titers [180, 189, 202].

For ensuring reproducibility in a GMP-compliant manufacturing process, infectious titer of lentiviral vectors is a key parameter. To this end, the measurement of this value must be reproducible and measurable at different dilution factors. The method must cover a linear working range where samples such as supernatant, intermediate steps and the final highly concentrated and purified lentiviral vector can be accurately determined. Titration can be particle-based, either ELISA or RT-qPCR [178]. The advantage is the relatively rapid determination of the total viral particles. However, this is not sufficient to determine the number of infectious particles. The active portion of vectors can only be verified via transduction itself. The number of functional particles might be determined via qPCR of integrated genes. This is a robust method, however, titration by flow cytometry offers some advantages. By determining the actual expression of the target gene, translation of the gene into the CAR receptor can be directly assessed. This allows the function to be directly inferred, as complex or large integrated genes correlate with the number of functional particles [257]. Published methods are predominantly fluorescence-based and do not address how the required degree of reproducibility can be achieved [179, 259, 260].

This work shows the establishment of highly reproducible analytical method for determination of transducing units per mL. Two cell lines with different morphology were compared. Jurkat cells, an immortalized T cell line, which resembles the target cell type and, HT-1080 cells, a fibroblast cell line, which offers advantages over Jurkat in terms of morphology and cultivation. HT-1080 cell grow adherently, can be seeded at very low concentrations and offer large surface area for viral transduction with their planar growth. Optimization of cell culture conditions aimed to substantial improvement in reproducibility across experimental runs, ensuring consistent and reliable outcomes. In a direct comparison of the two cell lines, the HT-1080 cell line clearly emerged as the superior option.

By determining the linear range, accurate and reproducible measurement of the ratio between target cells and viral concentration was achieved. Within the linear range, signals are above background noise while remaining below the threshold at which

multiple viral infections per cell lead to underestimation due to single-event detection. A serial dilution approach enabled highly reproducible assessment of infectivity across a broad range of lentiviral concentrations. This formed the basis for subsequent evaluation of purification steps and stability across different buffer conditions.

In summary, a cell-based, flow cytometry–assisted analytical method for determining infectious lentiviral titers was successfully established with high precision and reproducibility.

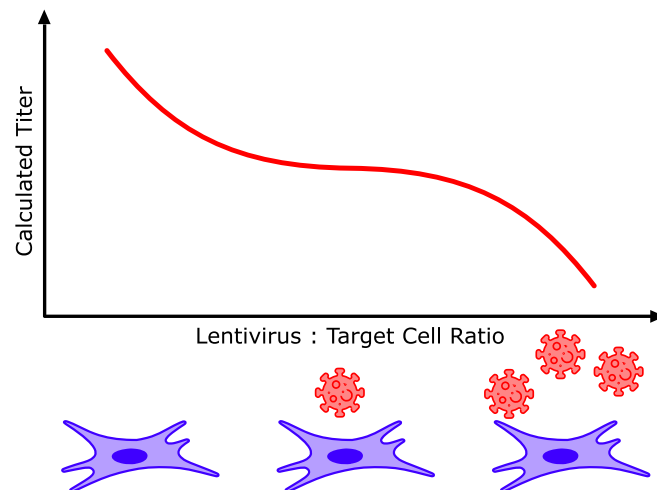


Figure 45 Linear titration range for calculation of Transducing Units (TUs). Calculated titer is underestimated if the of transducing units exceed the number of target cells. Overestimation happens close to background noise of non-transduced cells.

6.3 Pre-Transfection Seeding Density Drives LV Titer and Stability

Lentiviral vector production requires a balance between efficient transfection and effective expression and packaging of viral components, processes that are strongly influenced by the cell cycle of the producer cells.

A range of transfection reagents is available for efficient gene delivery. Electroporation is one option for transfection of producer cell lines [253]. However, this method falls short on scalability. It is difficult to transfer to an industrial scale due to current protocols involving a centrifugation step prior to electroporation. On a larger scale, such manipulation would require special equipment such as continuous centrifuges. While calcium phosphate was historically the standard transfection method, polyethylenimine (PEI) has become the preferred approach for lentiviral vector production in current research [261, 262]. PEI transfection is characterized by its independence from culture conditions, no need for medium change after transfection and applicability for transfection in suspension culture [263]. Notably, DNA precipitation of suspension cells with calcium phosphate is likely to be less effective due to the constant mixing of the culture. Therefore, other transfection agents such as cationic polymers are generally better applicable such as Lipofectamine, Fugen or 293fectin.

These alternatives have achieved high transfection levels [264]. Lipofectamine reagents are chemically defined and consist of a few low-molecular-weight cationic lipids (e.g. DOSPA, DOTMA, DOTAP) plus one or two neutral “helper lipids”, usually 1,2-Dioleoyl-sn-glycerophosphoethanolamine (DOPE) or cholesterol [181, 265]. The molecules are easily extractable from organic phases, show characteristic retention times in reversed-phase high performance liquid chromatography (RP-HPLC), and provide clear mass spectrometry signatures. Furthermore, there are validated methods for detection in the final product [265, 266]. In contrast, for PEI there is currently no comparable validated routine method due to the analytical complexity of the polymer [131]. That poses a major problem for translation into clinical practice. Therefore, a lipofectamine solution was opted for with reagents from commercial supplier to simplify the transfer to GMP. By relying on company-provided materials and specifications, the process of obtaining manufacturing authorization becomes simpler than validating each reagent individually.

For the cellular production of lentiviral vectors, the optimal phase is not a proliferative one [267]. To reduce vial re-transduction of the production cell line, cell cycle arrest in G0 is advantageous [268]. This means, initially cells should be in a more proliferative state for the transfection of packaging and transfer plasmids, followed by a G0-phase arrest. Supplementation of specific reagents helps to tailor cells to this phenotype. Sodium butyrate is commonly used as a transfection enhancer to improve transfection efficiency. Ansorge et al. showed a more than tenfold increase in titer in LV production when employing 5 mmol/L sodium butyrate [187]. Sodium butyrate is an inhibitor of histone deacetylases, which prevents DNA compaction and thus allows better access to promoters. This in turn improves RNA transcription and thus vector production [269]. However, the effect of sodium butyrate has not been demonstrated in some other studies [255]. The variable results of different studies could be due to the plasmid constructs themselves, as their possible interactions with histones theoretically depend on the DNA sequences and promoter types. This study examined the effect of the supplemented reagent on the producer HEK293 cell line and demonstrated how these reagents modulate cell cycle-related factors.

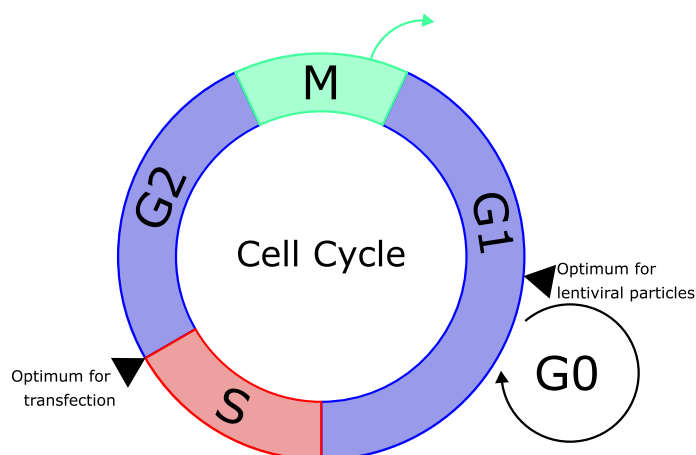


Figure 46 Schematic overview for a cell cycle. The M phase is when the cell divides through mitosis and cytokinesis, while G1 is a growth phase in which the cell increases in size and synthesizes proteins and organelles. During S phase the DNA is replicated, G2 serves as a final preparation and quality-control phase before mitosis, and G0 is a quiescent state where cells exit the cycle temporarily or permanently and do not divide [270].

One factor that has not been mentioned so far is cell culture. As seen in this study, the cellular history leading up to the day of the transfection has a massive impact on the yield of lentiviral vectors, and moreover, it even influences the stability. Stability remains a key challenge in the manufacturing of LV. Their sensitivity to various external factors makes processing complex. This can be attributed to the inherent structure of LVs, their lipid-based envelope and the absence of an outer protein shell. Hence, they are sensitive to external factors such as shear stress [158], salt concentrations [179] or pH [271, 272]. The decay of LVs represents a biphasic inactivation kinetic [271], where half-lives between 4 and 35 hours at 37 °C have been observed [158, 271, 273]. At room temperature (20 °C) half-life of up to 50 hours have been observed, but data availability on such prolonged stabilities is limited [271]. Even at 4 °C, the half-lives vary considerably, with values between 18 – 250 hours [271, 274]. Sensitivity to freeze thaw cycles further add to the delicate handling of LV [158, 275]. The time-dependent measurement of TUs, can therefore provide crucial information on quality of the source and losses not attributable to storage buffer or purification steps.

In this study multiple cultivation procedures were investigated. It was found that cell density prior to transfection had a major influence on vector stability. This even overshadowed varying buffer conditions. It was found that growth over a cell density of 6×10^6 cells/mL prior to transfection was a key driver. When overgrowth happened on the day of transfection, it led to increased titers and improved stability even in unfavorable buffers such as PM. However, overgrowth was detrimental when it occurred in any prior process day. An active change of seeding density to initiate this in a timed manner, led to a 2-fold increase in titer, furthermore, it enhanced stability. It was found that this adjustment shifted the portion of cells from G2/M to G0/G1 phase. This also changed the pH in a lactate-independent manner. This means that the increased cell density did not lead to oxidative metabolism being overloaded and more lactate being

excreted [276]. Lower pH therefore appears to be driven by an additional factor that favors production. This underlines that stability is dependent not only on how the vector is further processed or stored in [206] but also on the conditions in which the virus is produced [277]. And even the cell culture medium and its osmolality during production play into this [277].

In summary, there is a reproducible cellular pattern that can be controlled by the cell concentration which mitigates not only the viral titer but its stability as well. This finding has important implications for downstream purification processes.

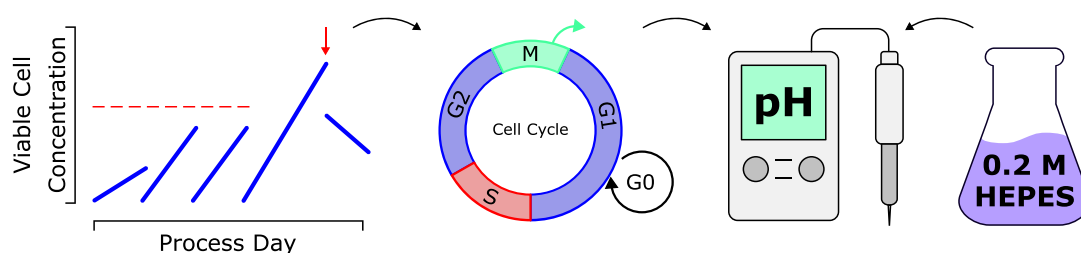


Figure 47 Insights gained from investigating the key factors influencing factors for lentiviral vector stability. Cell concentration on the day of transfection the changed cell cycle and increased pH of the cell culture which must be attributed by choice of buffering system (ideally 0.2 M HEPES).

6.4 Bioreactor Cultivation Enables LV Production Scale-Up

Upstream processing (USP) in bioreactors plays a central role in modern biotechnology by enabling the controlled cultivation of cells for the production of valuable biological products. Through precise regulation of environmental parameters such as temperature, pH, dissolved oxygen and nutrient supply, bioreactors provide optimal conditions for cellular growth and metabolic activity. Stirred tank bioreactors offer the simplest scaling method in USP. In development, these systems may be used in scales ranging from 15 mL for development [278], to 5 – 10 L vessels [279]. Production typically begins at scales of 200, however, scale-up extends well beyond this, with these systems supporting production volumes of up to 10 000 L. Optimization for higher scaling of stirred tank bioreactors is based on typical scaling parameters such as volumetric mass transfer coefficients (kLa), power-to-volume ratios (P/V) and tip speeds [280, 281]. This already limits the use of PEI in transient transfection production strategies (chapter 6.3). Wave-motion bioreactors provide a gentle environment that supports efficient producer cell expansion. Here, cultivation is performed in inflated single-use bags that are gently rocked on a platform to ensure efficient mixing while maintaining low shear conditions. The advantage of this method is the high gas mass transfer with significantly lower shear stress on the cells due to the absence of stirrers. Attempts in implementing these bioreactors have already been done for the production of vectors from stable producer cell lines, in which case adherent cells were used employing Fibra-Cel discs [256].

In this study, a successful scale-up was achieved in a single-use, bag-operated bioreactor, enabling controlled cultivation under scalable and reproducible conditions. This strategy

allows manufacturing to be conducted in a closed system, thereby minimizing handling steps and reducing the risk of contamination. Processing would otherwise have to take place in a highly controlled class A cleanroom when translating to GMP [282]. By contrast, handling of single-use bag systems via welding or sterile connectors can be performed in class C, reducing the need for costly and more complicated higher-grade environments [282]. Process safety is further enhanced through separation of steps between safety class S1 and S2. Expansion of the cell culture can be done in lower class S1, while transfection and subsequent virus-containing steps would be handled in a closed system in S2.

Bioreactor culture conditions were optimized to closely replicate manual manufacturing conditions. For the first time, the pH profile was monitored in real time during cultivation. Shortly before transfection, a decline in pH was observed, closely mirroring the pattern seen in manual cultivation (chapter 4.1.7). As part of the purification strategy, an initial DNase digestion step was implemented to eliminate residual DNA and support subsequent filtration processes. This step was successful and led to a significant reduction in measurable DNA fragments. The resulting titers matched those obtained in manual production, suggesting that the process was successfully adapted to the semi-automated bioreactor system. A total yield of 2.4×10^9 TUs was achieved in a production volume of 300 mL. At an MOI of 10, 240×10^6 T cells could be transduced to CAR-T cells. This translates to at least 2 full CliniMACS Prodigy® runs (each 100×10^6 T cells, see Chapter 5). Following the previous establishment of MCB, transfection protocols and now bioreactor production, a full seed train had now been established. This marks important milestones towards full pharmaceutical-grade manufacturing.

A seed train covering the entire process from cell bank expansion to bioreactor production and initial downstream processing was established, enabling the generation of titers sufficient for two CAR-T cell batches.

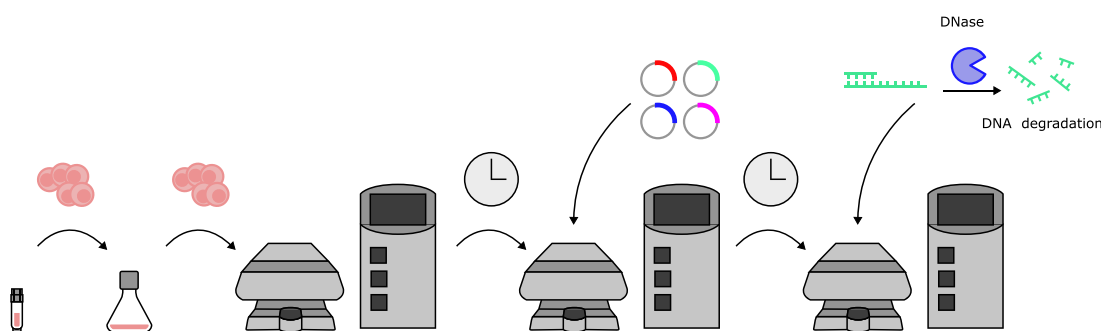


Figure 48 Upstream processing (USP) seed train. Steps from cell thaw and expansion, bioreactor inoculation, transfection with packaging and transfer plasmid and final DNase treatment.

6.5 Combinatorial Testing Enables the Identification of an Optimized Purification Strategy

Downstream processing refers to all steps that take place after the production of a biological target and is crucial for the quality, safety, and cost-effectiveness of biopharmaceutical products. Purification is indispensable for pharmaceutical products and typically includes cell separation, product capture, purification and formulation. It is often the most cost- and time-intensive part of a biotechnological manufacturing process. The selection and combination of suitable separation and purification processes, such as chromatography, filtration or precipitation, requires precise adaptation to the physicochemical properties of the target product and the process matrix. Particularly for recombinant proteins and modern biologics, a rational, quality-driven downstream design is essential to meet regulatory requirements and enable robust industrial scaling [283].

The steps involved in purifying lentiviruses can be carried out in various combinations and must be adapted to the specific requirements of the product [184]. There are differences between the applications of lentiviruses, for e.g. material for cellular therapy or for intravenous administration. Either way, purification usually begins with clarification, in which cells and cell debris are efficiently removed from the harvest material using methods such as depth filtration or microfiltration [178, 184, 195]. Notably, in this case the use of suspension cultures increased the particle load compared to adherent cultures.

As a subsequent step, UF/DF should be implemented for initial bulk purification, involving buffer exchange and concentration. It might as well be used in a subsequent polishing. UF/DF via TFF allows for high throughput and thus offers great potential for use in large-scale LV productions [284]. The process is driven by the pressure difference across the membrane. This forces smaller particles, such as proteins and DNA fragments, through the pores, while larger particles, such as viruses, are retained. The virus-containing retentate continues to circulate across the membrane, leading to a steady increase in concentration [196]. The efficiency of clarification in TFF depends on the

retention coefficients of the feed and the individual components. Unlike conventional filters, the retentate does not form a layer on the membrane, which significantly reduces fouling. Nevertheless, this factor should not be neglected. Even in UF/DF, membrane fouling is a key limitation that leads to a decrease in permeate flow rate and an increase in TMP [184]. The sensitivity of lentiviruses to shear forces and pressure changes during UF/DF requires precise control to prevent particle inactivation [131].

The polishing step follows concentration and buffer exchange. Here, the vectors are selectively isolated using chromatography (e.g., anion exchange) to separate remaining proteins and nucleic acids from the host cells [131, 195]. For the establishment of a clinical-grade vector purification process, depth filtration and UF/DF for concentration and buffer exchange were identified as critical steps. Depth filtration as means of clarification results in higher losses compared other methods such as centrifugation, however, it remains indispensable for subsequent transfer to GMP-compliant manufacturing [285]. UF/DF is essential for direct processing and performed multiple times throughout the workflow. Furthermore, an intermediate storage step may be implemented following the buffer exchange, serving as a controlled process pause. Purification of Lentiviruses is associated with substantial losses of functional vectors [189], primary focus was therefore placed on TU recovery. Stability tests were performed in parallel. A total of 6 purification runs with varying combinations were compared.

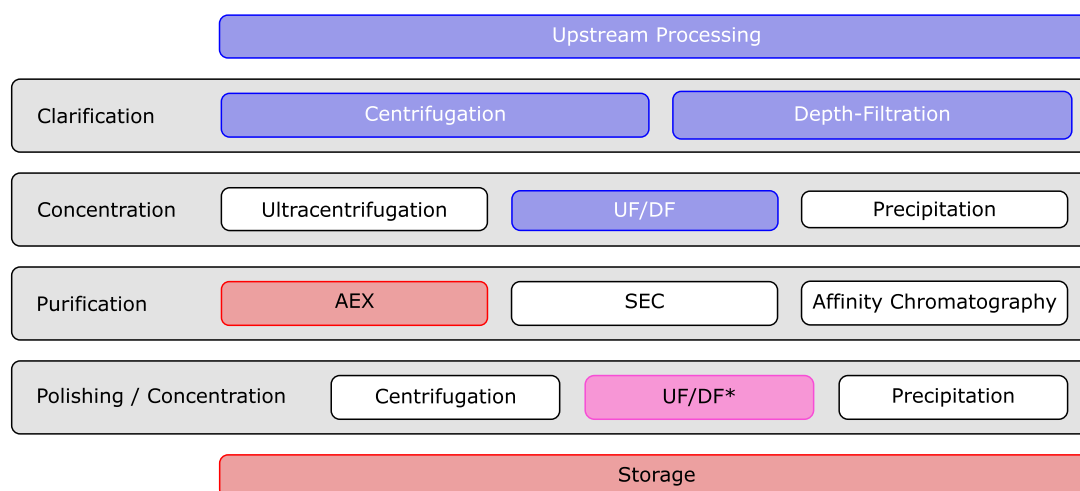


Figure 49 Selected strategy for Downstream processing of lentiviral vectors. Adapted from Bandeira et al. [207]. Blue: established steps, red: planned processes. *Ultrafiltration/diafiltration (UF/DF) is highly comparable between steps concentration and polishing, therefore already partly established.

As anticipated, substantial losses in functional titer were observed during purification. It was demonstrated that switching the sequence, and beginning with ultrafiltration then diafiltration, represents a significant step toward shortening the process. In conjunction with the stability data in various buffers, this also contributed to significantly improved yields. However, this had an impact on the membrane. A higher initial concentration due to this sequence of purification steps in the solution caused the permeate flux to decrease gradually (Appendix Supplementary Figure 13 and Supplementary Figure 14).

Pressures and flow rates had been kept within acceptable ranges according to TMP scouting (Appendix Supplementary Figure 11 and Supplementary Figure 12), the process was not performed in a pressure-independent gel layer-controlled region [243]. However, poor regenerability of the membranes in NWP tests [243] confirmed that this was a sign of strong membrane fouling. This suggests that a significant amount of material had blocked the pores of the membrane. Most likely a combination of lentiviruses and larger particles. Reduction of pressures might help to reduce membrane fouling (Harvest 10), however, process times were then extended and yields were reduced as well. This also appeared to have only partially delayed the decline of permeate flux. The membrane with large surface area most likely benefits here.

It was interesting to note that PM was used in the cases with the highest recoveries. However, since the processes had always lasted well under 4 hours, this could explain the lower loss in TUs observed here (Chapters 4.1.6 and 4.1.7). The same applied to HBSS, which also achieved good results. Harvest 10, with a volume of approximately 300 mL and a duration of 2 hours benefitted most likely from the use of 0.2 M HEPES, which had been identified as the optimal buffer. Harvest 9 showed high recoveries of functional lentiviral vector, despite poor cell culture (Appendix Supplementary Figure 17) with very comparable overgrowth as observed in Harvest 2 and having a similarly low initial titer. These findings strongly support this setup and can be attributed, in part, to the reduced process duration, when stability is not immediately affecting the outcome. The increased recovery observed in Harvest 7 may be attributable to differences in the cell culture conditions. Here, the desired overgrowth as described in chapter 6.3 had been observed.

Best-performing runs employed a fivefold diafiltration volume and filters with a pore size of 300 kDa. The two best runs had very short processing times. The highest-performing purification setup (Table 18, Harvest 9) achieved a yield of 45.5 % of the original functional vector and utilized a membrane surface area of 200 cm². The reduced processing time due to the large membrane was a major influencing factor, as cell culture conditions indicated poor stability of the vector. For longer processes, cell culture optimization according to Chapter 6.3 again becomes critical, as the shorter process durations observed here were a consequence of smaller batch sizes. Processing should be conducted in the clarified supernatant for as long as possible, which was shown in stability testing as the optimum condition for increased recovery. Furthermore, strong membrane fouling was observed, which was mitigated by increasing the membrane surface area. Compared to literature, employing membranes with an area of 200 cm² for these small batches would be significantly disproportionate [286, 287]. This may reflect the intrinsic biological constraints of lentiviral production and thus be unavoidable. It should also be noted that each titration was initiated at the same time as the sample collection. In addition, it was found that prolonged incubation of the titration cell line also leads to a decrease in the measured titer. A slight effect was observed after 6 hours

of incubation (Appendix Supplementary Figure 18). This illustrates the immense effort required just for this single step in the process of performing lentiviral purification.

Overall, significant membrane fouling was observed. Although this phenomenon is described in the literature, it remained unclear how rapidly this occurs as shown in this study. Buffer effects were less pronounced than anticipated, likely due to the short process duration, whereas cell history remained a major determinant of final viral yields.

Table 18 Purification setup in direct comparison. **Blue highlight:** top performing combination.

Har-vest	Filter	Cut-Off	Area	Conditioning	Storage Buffer	Volume Re-duction	Diafiltration Volume
5	MiniMate™ TFF Capsule	100 kDa	50cm ²	1 M HEPES +50 mM NaCl (pH 7.5)		2.4×	6
6	MiniMate™ TFF Capsule	100 kDa	50cm ²	1 M HEPES +50 mM NaCl (pH 7.5)		1.3×	none
7	MiniMate™ TFF Capsule	300 kDa	50cm ²	PM		2.4×	5
8	Sartocon® Slice 50	300 kDa	50cm ²	PM	1 M HEPES	6.3×	5
9	Sartocon® Slice 200	300 kDa	200cm ²	PM	HBSS	1.7×	5
10	Sartocon® Slice 50	300 kDa	50cm ²	0.2 M HEPES		11.3×	5

6.6 Host Cell-Derived Impurities: Analysis and Risk Considerations

Residues from the host cell, in particular proteins (HCPs) and DNA, pose significant risks to patient safety and product stability. HCPs are proteins produced by the production cell line during growth and protein biosynthesis. Even after several purification steps, traces of HCPs may remain in the final product. The main risk of HCPs is their ability to trigger an immune response in the patient. Since HCPs are foreign proteins, the patient's immune system can produce antibodies against them [3]. HCPs can act as adjuvants and enhance the immune response against the actual therapeutic protein. This can lead to mild allergic reactions or even anaphylactic shock [288]. Some HCPs have enzymatic activity that can attack the therapeutic protein or excipients in the formulation. Proteases can cleave the active molecule, thereby eliminating its effectiveness. A well-known example is the degradation of polysorbates (common stabilizers in biopharmaceuticals) by residual amounts of lipases. This leads to the formation of particles and reduces shelf life [289]. The amount of residual host cell DNA must be strictly controlled. The authorities set tight limits here, usually <10 ng per dose and a fragment length of <200 base pairs [208, 209] (Table 6). The biggest concern with DNA is the potential integration of DNA fragments into the patient's genome. If DNA

fragments containing oncogenes or infectious viral sequences are integrated into the genome of a human cell, they could theoretically cause cancer or activate latent viruses [290]. In addition, free DNA can lead to unwanted inflammatory reactions and activation of the innate immune system [3]. Removal is essential for approval, not only as a matter of patient safety, but also of reproducibility.

The use of qPCR-based assays was considered too resource-intensive to enable real-time monitoring and optimization across the many individual steps of the process. Therefore, a new DNA assay was established that performs similarly to an ELISA (chapter 4.1.11). The assay enabled the generation of reliable data within one hour. The resulting data were subsequently used for further evaluation.

In-line sampling and subsequent impurity analyses were conducted for Harvest 8 and 10. Across both runs, total DNA clearance ranged from 81 % to 95 %, indicating effective reduction of residual nucleic acids. In Harvest 8, no measurable reduction of HCPs was achieved, indicating insufficient clearance under the applied process conditions. In contrast, Harvest 10 demonstrated a 62 % clearance in HCP levels, reflecting a low to moderate improvement in impurity removal efficiency.

Overall, the impurity clearance data were consistent with other findings of an initial UF/DF step [189]. Regulatory and quality requirements for ex vivo products are expected to be distinct from those applicable to in vivo products. Nevertheless, implementation of an anion exchange chromatography (AEX) step is required, as the reduction of HCPs and DNA was insufficient to meet the specified acceptance criteria.

6.7 Development of a Tailored Intracellular Marker Panel to Monitor CAR-T Cell Expansion and Functional Dynamics

The previously described establishment of a GMP vector platform was carried out employing an anti-CD276 CAR as a model construct (construct #305, with myc-tag and co-expression of CD19). In clinical practice, such vectors are employed in semi-automated CAR-T cell manufacturing systems. These systems and cultivation processes have been extensively developed and optimized over the years. While early CAR-T approaches emphasized highly differentiated effector T cells, more recent research and clinical practice increasingly favor T_{SCM} and T_{CM} subsets [89]. In contrast to T_{EFF}, these cells show increased persistence and anti-tumor efficacy and perform more powerful expansion. Studies have shown that the T_{SCM} phenotype exhibits even greater persistence and is more effective against tumors compared to T_{CM}. The gene expression signature and functional phenotype define this population as a distinct subset of memory T lymphocytes that lies between T_N and T_{CM} cells [248]. The use of specific cytokine cocktails favors this phenotype [85]. Cultivation in medium containing IL-7 and IL-15 together with binding of CD3/CD28 shifts differentiation and prefers expansion of more T_{SCM} lymphocytes [85].

From a biological systems perspective, cultivation with these additives in automated systems differs substantially from the physiological environment *in vivo*. For instance, activated T cells cultivated in standard medium (Iscoves modified Dulbecco medium plus 10 % serum) receive 25 mM glucose (5× as much standard blood sugar as physiological 5 mM) and 4 mM glutamine (8× as much as standard blood sugar at 0.5 mM) [291]. Most *in vitro* expansions are performed at significantly higher nutrient and O₂ concentrations than in normal physiology, these conditions simulate a hyperglycemic and hyperoxic environment that never occurs *in vivo* [292]. This raises the central question of how this combination modulates cellular behavior and function over time.

DigiWest™ enables multiplex analysis of more than 100 analytes per sample via antigen-antibody interactions [245]. This allows to track intracellular protein expression and phosphorylations, data which is not available in next generation sequencing. In this work, a specifically designed panel for intracellular markers for CAR-T cell production was developed. This shall provide mechanistic insight into how CAR-T manufacturing shapes intracellular signaling pathways. Various analytes were tested on T cells and CAR-T cells from three manual expansions, from initial day 0 up to 18 days. Variance analysis was used to detect analytes whose expression strongly depended on the day of expansion. These were assigned to their interaction cluster and pathway association.

Early molecular events downstream of the TCR initiate and orchestrate proximal signaling cascades driving T cell activation. CD8 beta is a co-receptor that stabilizes the TCR-MHC I interaction. LYN/LCK/HCK/BLK (P)-Tyr397/Tyr394/Tyr411/Tyr389 are phosphorylated Src-family kinases that initiate the signaling cascade. PI3 Kinase beta is

essential for metabolic reprogramming and survival during early activation. c-Jun is a component of the AP-1 transcription factor complex, its phosphorylation at Serine-73 activates it, triggered by JNK signaling to drive proliferation [23, 293].

Co-stimulatory receptors and adaptor proteins integrate secondary signals to modulate and amplify T cell activation pathways. Ox40 (CD134) is a TNFR-family member that promotes expansion and long-term memory. GITR (TNFRSF18) enhances effector T cell functions and can abrogate Treg suppression [294]. Co-inhibitory Receptors (Immune Checkpoints). PD-1 limits T cell activity in peripheral tissues during inflammatory responses. CTLA-4 competes with co-stimulatory receptors to inhibit early T cell activation in lymph nodes. LAG-3 negatively regulates T cell proliferation and homeostasis. SLAMF4 (2B4), while context-dependent, it often acts as an inhibitory receptor in chronic viral infections and T cell exhaustion [295, 296].

Cytokine-mediated and inflammatory signaling pathways shape the magnitude and functional polarization of immune responses. IRAK1 (P)-Thr387 is a key kinase in the IL-1 Receptor and Toll-like Receptor pathways, bridging innate and adaptive signals [297]. Effector specialization and fate determination. Granzyme B is a cytotoxic serine protease used by CD8⁺ T cells to induce apoptosis in target cells. IFITM1 is an interferon-induced protein that plays a role in antiviral defense and cell-cycle regulation [298, 299].

The antibody panel was strategically designed to monitor immune cell activation and functional dynamics. The new panel was based proximal TCR signaling, co-stimulatory receptors and adapters (Figure 50). Furthermore, CAR signaling-specific associated proteins and cytokine signals. Considering the automated nature of clinical CAR-T manufacturing in the CliniMACS Prodigy®, the panel was supplemented with case-specific analytes to ensure comprehensive coverage of cellular metabolism, indicators for hypoxia and markers associated with apoptosis. Co-inhibitory receptors and effector specialization were chosen to be analyzed via flow cytometry. The panel was used in the analysis of a full GMP production process

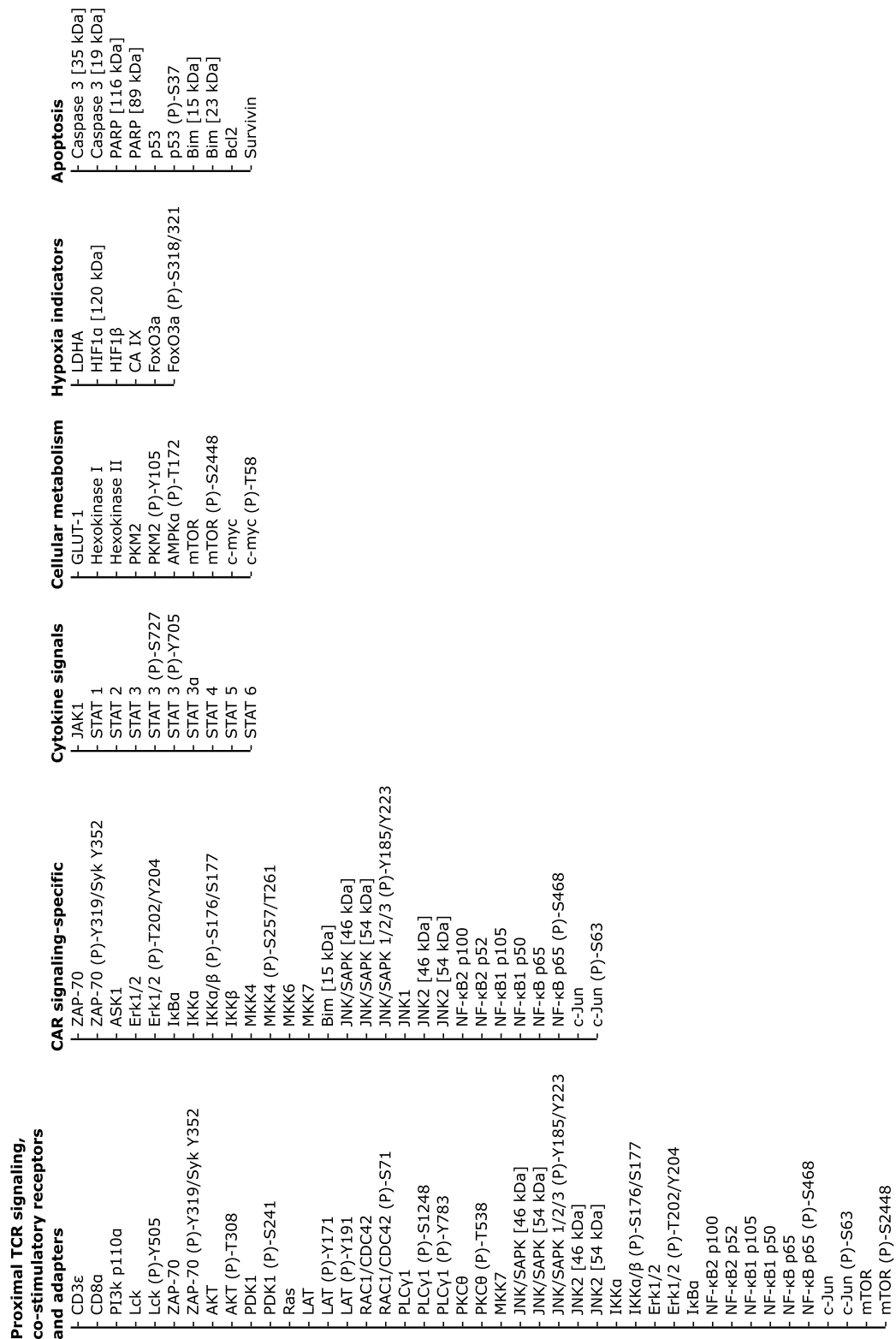


Figure 50 Panel design for DigiWest analysis. Pathway affiliations of proximal TCR signaling, costimulatory receptors and adapters, CAR signaling-specific associated proteins, cytokine signaling, cell metabolism, indicators of hypoxia, and markers associated with apoptosis.

6.8 Conclusion and Outlook

Lentiviral vector manufacturing is inherently linked to the CAR-T cell manufacturing workflow and cannot be optimized in isolation. Accordingly, both processes should be co-optimized. This approach was implemented in the present study.

Critical vector quality attributes strongly influence transduction efficiency, vector copy number and ultimately CAR expression in T cells. Functional titer, purity, infectivity, genome integrity, and lot-to-lot consistency. Safety testing is a regulatory necessity and a clinical risk-control strategy. Variability here can propagate into inconsistent CAR-T expansion, phenotype, potency and safety. For clinical translation, this means the vector must be treated as a critical starting material with tightly controlled specifications linked to downstream CAR-T performance. Clinical-grade lentiviral vector manufacturing must minimize risks associated with replication-competent lentivirus (RCL), insertional mutagenesis, residual plasmid DNA, host cell proteins, and process-related impurities [175, 178, 184, 188, 208, 209]. Purification of lentiviral vectors remains particularly challenging due to their intrinsic sensitivity to process conditions, including pH fluctuations, temperature stress, and shear forces. A next step in development would be anion exchange chromatography (AEX). This is particularly difficult due to high salt concentrations during elution that can cause osmotic stress, which damages the viral membrane and can lead to massive losses in activity of up to 50 % [300]. Furthermore, the considerable size of the viral particles and the limited binding capacity of conventional resin-based media restrict mass transport, as the particles cannot penetrate the pores [300]. Here, newer developments such as VSVg-binding columns could be employed [301]. Future development will be pursued through collaborative efforts within a larger multidisciplinary team. This next phase will focus on translating these developments into a scalable framework.

Clinical manufacturing processes are inherently dynamic. Academic medical centers are central to the development and translation of novel therapeutic approaches involved preclinical work, early-phase trials and later-stage development. By integrating the lentiviral vector platform for novel CAR-constructs with the established panel and improved understanding of intracellular processes during CAR-T manufacturing, future therapies can be more precisely tailored to patient needs. This is addressing a clear and growing clinical need. The global ATMP market was valued at USD 27.04 billion in 2022 and projected to reach USD 90.98 billion by 2030 [302]. This platform is designed to enhance the development and accessibility of improved CAR-T cell therapies, which is especially important for the treatment of rare diseases.

7 Summary

The development of so-called Advanced Therapy Medicinal Products (ATMPs), particularly CAR-T cells, represents a significant advance in the treatment of certain forms of blood and lymphoid cancers. However, the sharp increase in demand for such therapies expected in the coming years is accompanied by considerable challenges, including bottlenecks in manufacturing capacity and infrastructure, as well as a growing financial burden on healthcare systems. To enable broader clinical application, cost-effective, scalable and clinically relevant strategies are therefore required. Against this backdrop, this thesis is clearly application-oriented and addresses key questions regarding clinical feasibility. In terms of content, it can be divided into two main topics: first, the CAR-T cell manufacturing procedure and second, the genetic tool, the lentiviral vector. Both aspects are investigated under Good Manufacturing Practice (GMP) to improve the cellular biological understanding and clinically feasible manufacturing strategies for CAR-T cells in academic and clinical settings.

The starting point for Chapter 1 of this thesis is the observation that the production of lentiviral vectors of sufficient quality for research purposes is widespread, whereas significantly higher regulatory and quality requirements apply for clinical use. To date, these vectors have typically been sourced from industrial partners, whose manufacturing processes are optimized for large-scale production to ensure economic viability. To establish an in-house production platform, a thorough understanding of the structural and biological determinants of the final virus solution is required. This thesis therefore pursued an integrated approach that combines the development of upstream processing (USP) in cell culture with downstream processing (DSP) for purification. The focus was on identifying the combination of cellular effects of the HEK293 cell line, protocols and materials that would highly stable and pure Lentivirus, while remaining feasible on a small scale and allowing for subsequent scaling as needed. This included the establishment of analytical methods, as well as scaling up to a bioreactor and supplementary analytical procedures such quantitative cell-based detection of infectious particles. In particular, it became apparent that the adaptation of the cell culture has a massive influence on processability. The results show that both the titer and the stability of the lentivirus depend on the exact cellular state on the day of transfection. Targeted overgrowth on the day of transfection alters the cell cycle and pH, and consequently the stability during subsequent processing. Through this optimization, the titer was doubled and a suitable buffer system was identified. Furthermore, various purification steps were tested in combination, resulting in the establishment of a filtration process without centrifugation in combination with tangential flow filtration.

Chapter 2 focuses on the manufacturing of CAR-T cells employing semi-automated processes. Although such production methods are becoming increasingly important in translational and clinical research, their temporal dynamics and process-related effects

on the cells remain insufficiently characterized in the current literature. In particular, it remains unclear how individual phases within the respective process days can be characterized and which cellular and technical dynamics play a role in this. To answer these questions, a comprehensive methodological approach was established that combines phenotypic, intracellular and functional analyses. The experimental design comprised three Prodigy GMP runs conducted in a cleanroom using cell material from healthy donors, accompanied by daily sampling throughout the entire process. A specific flow cytometry panel was developed to investigate extracellular markers, while intracellular changes were detected using a panel of more than 130 analytes using DigiWest™ technology. In addition, differences in cytotoxicity were recorded and correlated with the underlying cellular states to assess their functional relevance at the cellular level. To this end, a combination of cytotoxicity and re-challenge models was established, allowing the effector function of the cells and their responsiveness under repeated stimulation to be analyzed. Key biological processes, such as T-cell activation, changes in CAR-signaling before and after repeated tumor contact, and metabolic reprogramming processes, could be tracked in detail throughout the entire CAR-T cell production process. The results demonstrate that the manufacturing process is characterized by clearly distinguishable functional phases, with a critical phase transition occurring between days 5 and 7. The hypoxia observed on day 5 may have contributed to cell selection.

The results presented in Chapter 1 underscore the importance of carefully selecting and combining appropriate cell culture and purification steps and deepen our understanding of the relevant factors influencing the production of clinical-grade lentiviral vectors. The results from Chapter 2 show that the manufacturing process is characterized by day-specific phenotypes and that shortening the process would result in a biologically different cell preparation. These results from GMP-related work are particularly relevant for clinical centers that manufacture CAR-T cells or have multiple CAR constructs in their development pipeline. Thus, this work contributes to the further development of decentralized manufacturing strategies for ATMPs and establishes a practical foundation for patient-oriented application and implementation in academic and clinical production environments.

8 Zusammenfassung

Die Entwicklung sogenannter Advanced Therapy Medicinal Products (ATMPs), insbesondere von CAR-T-Zellen, stellt einen bedeutenden Fortschritt in der Behandlung bestimmter Formen von Blut- und Lymphdrüsenkrebs dar. Mit der in den kommenden Jahren erwarteten stark zunehmenden Nachfrage nach solchen Therapien gehen jedoch erhebliche Herausforderungen einher, darunter Engpässe in Herstellungskapazitäten und Infrastruktur sowie eine wachsende finanzielle Belastung der Gesundheitssysteme. Um eine breitere klinische Anwendung zu ermöglichen, sind daher kosteneffiziente, skalierbare und kliniknahe Strategien erforderlich. Vor diesem Hintergrund ist die vorliegende Arbeit klar anwendungsorientiert und adressiert zentrale Fragestellungen der klinischen Umsetzbarkeit. Inhaltlich lässt sie sich in zwei Hauptthemen gliedern: Zum einen den CAR-T-Herstellungsprozess und zum anderen die des genetischen Werkzeugs, dem lentiviralen Vektor. Beide Aspekte werden unter Good Manufacturing Practice (GMP) untersucht, um das zellbiologische Verständnis und klinisch umsetzbare Herstellungsstrategien für CAR-T-Zellen in akademischen und klinischen Einrichtungen zu verbessern.

Ausgangspunkt für Kapitel 1 dieser Arbeit ist die Beobachtung, dass die Herstellung lentiviraler Vektoren in einer für Forschungszwecke ausreichenden Qualität weit verbreitet ist, während für den klinischen Einsatz deutlich höhere regulatorische und qualitative Anforderungen gelten. Bislang müssen diese dann in der Regel von industriellen Partnern bezogen werden, welche Herstellungsprozesse primär auf große für die Firmen wirtschaftlichere Produktionsmengen ausgerichtet haben. Um eine eigene Herstellplattform aufzubauen, ist ein fundiertes Verständnis der strukturellen und biologischen Determinanten der finalen Viruslösung erforderlich. Die Arbeit verfolgte daher einen integrierten Ansatz, der die kombinierte Entwicklung des Upstream Processings (USP) in der Zellkultur mit dem Downstream Processing (DSP) zur Aufreinigung verbindet. Im Mittelpunkt stand dabei die Untersuchung der HEK293-Zelllinie, Protokollen und Materialien, die hochstabile und reine Lentiviren ermöglichen, welche im kleinen Maßstab praktikabel bleiben und eine bedarfsgerechte spätere Skalierung ermöglichen. Hierzu gehörte die Etablierung analytischer Methoden, darüber hinaus eine Skalierung in einen Bioreaktor und ergänzende analytische Verfahren wie der quantitative zellbasierte Nachweis infektiöser Partikel. Dabei zeigte sich insbesondere, dass die Anpassung der Zellkultur massiven Einfluss auf die Verarbeitbarkeit hat. Die Ergebnisse zeigen, dass sowohl Titer als auch Stabilität des Lentivirus vom exakten zellulären Zustand am Tag der Transfektion abhängen. Das gezielte Überwachsen am Tag der Transfektion verändert Zellzyklus und pH und in der Folge die Stabilität bei der späteren Verarbeitung. Mit dieser Optimierung konnte der Titer verdoppelt und ein geeignetes Puffersystem gefunden werden. Ferner wurden kombinatorisch Aufreinigungsschritte getestet, wobei eine Filtration ohne Zentrifugation in Kombination mit Tangentialstromfiltration etabliert werden konnte.

Ausgangspunkt des Kapitel 2 ist die Herstellung von CAR-T-Zellen unter Verwendung semi-automatisierter Prozesse. Obwohl solche Herstellungsverfahren in der translationalen und klinischen Forschung zunehmend an Bedeutung gewinnen, sind sie in der bisherigen Literatur in ihrem zeitlichen und prozessualen Ablauf bislang nicht detailliert beschrieben. Insbesondere bleibt unklar, wie sich einzelne Phasen innerhalb der jeweiligen Prozesstage charakterisieren lassen und welche zellulären und technischen Dynamiken dabei eine Rolle spielen. Zur Beantwortung dieser Fragestellungen wurde ein umfassender methodischer Ansatz etabliert, der phänotypische, intrazelluläre und funktionelle Analysen miteinander verbindet. Das experimentelle Design umfasste drei im Reinraum durchgeführte Prodigy-GMP-Läufe unter Verwendung von Zellmaterial gesunder Spender, begleitet von einer täglichen Probennahme über den gesamten Prozessverlauf. Zur Untersuchung extrazellulärer Marker wurde ein spezifisches Durchflusszytometrie-Panel entwickelt, während intrazelluläre Veränderungen mithilfe eines Panels von mehr als 130 Analyten mittels DigiWest™ Technologie erfasst wurden. Darüber hinaus wurden Unterschiede in der Zytotoxizität erfasst und mit den zugrunde liegenden zellulären Zuständen in Beziehung gesetzt, um deren funktionelle Relevanz auf zellulärer Ebene zu bewerten. Dafür wurde eine Kombination aus Zytotoxizitäts- und Re-Challenge-Modell etabliert, sodass die Effektorfunktion der Zellen und ihre Reaktionsfähigkeit unter wiederholter Stimulation analysiert werden konnten. Wesentliche biologische Prozesse, wie T-Zell-Aktivierung, Veränderungen in der CAR-Signalgebung vor und nach wiederholtem Tumorkontakt, sowie Prozesse der metabolischen Umprogrammierung konnten über die gesamte CAR-T-Zell-Herstellung detailliert verfolgt werden. Die Ergebnisse belegen, dass der Herstellungsprozess durch klar abgrenzbare funktionelle Phasen geprägt ist, mit einem kritischen Phasenübergang zwischen Tag 5 und 7. Die an Tag 5 festgestellte Hypoxie könnte zu einer Selektion der Zellen beigetragen haben.

Die Ergebnisse von Kapitel 1 unterstreichen die Wichtigkeit einer gezielten Auswahl und Kombination geeigneter Zellkultur- und Aufreinigungsschritten und vertiefen das Verständnis relevanter Einflussfaktoren bei der Herstellung lentiviraler Vektoren in klinischer Qualität. Die Ergebnisse aus Kapitel 2 zeigen, dass der Herstellungsprozess durch prozesstagabhängige Phänotypen geprägt ist und eine Prozessverkürzung zu einem biologisch anderen Zellpräparat führen würde. Diese Resultate aus GMP-bezogener Arbeit sind insbesondere für klinische Zentren relevant, die CAR-T-Zellen herstellen oder mehrere CAR-Konstrukte in ihrer Entwicklungspipeline haben. Damit leistet diese Arbeit einen Beitrag zur Weiterentwicklung dezentralisierter Herstellungsstrategien für ATMPs und schafft eine praxisnahe Grundlage für patientenorientierte Anwendung Implementierung in akademischen und klinischen Produktionsumgebungen.

9 Statement

Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit mit dem Titel „Advancing CAR-T Manufacturing Through Comprehensive Cellular Analysis and the Development of a Lentiviral Vector Platform“ selbständig verfasst, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet habe.

Ich erkläre, dass die Richtlinien zur Sicherung guter wissenschaftlicher Praxis der Universität Tübingen (Beschluss des Senats vom 25.05.2001) beachtet wurden.

Ich versichere Eides statt, dass diese Angaben wahr sind und dass ich nichts verschwiegen habe.

Mir ist bekannt, dass die falsche Abgabe einer Versicherung an Eides statt mit Freiheitsstrafe bis zu drei Jahren oder mit Geldstrafe bestraft wird.

Tübingen, 2026

10 Declaration of Contribution

Lentiviral production including cell culture, transfections, virus production, transductions, analytical procedures (ELISA, flow cytometry, WB analysis, functional luciferase activity measurements were performed by me). Respectively, experiments were designed and planned by me. The general project idea and the project outline have been generated by me. Further I planned, analyzed, prepared, and interpreted the data in this thesis. Reagent and assay assets were furthermore selected by me.

Isa-Maria Klink was a master intern enrolled in Molecular Cell Biology and Immunology at the University of Tübingen and was supervised by me. She supported on Prodigy process sampling and analytical methods.

Elisabeth Pezzuto was master intern enrolled in Molecular Medicine at the University of Tübingen and was supervised by me. She supported analysis for vector stability.

Tim Bradl was a master intern enrolled in Biochemistry at the University of Tübingen. He helped to develop the cell cycle analyses method.

DigiWest analysis were performed by Dr. Felix Ruoff and Simon Fink of the NMI Natural and Medical Sciences Institute at the University of Tübingen.

Dr. Daniel Atar of Children's Hospital Tübingen supported on planning, access and coordination of CAR-T cell GMP manufacturing.

11 List of Publications

11.1 Scientific journals

In Submission: **Florian Harald Schinle**, Isa-Maria Klink, Dr. Felix Schäfer, Sylvia Klein, Katrin Lutz, Maximilian Feige, Kathrin Wolsing, Simon Fink, Prof. Dr. med. Tamam Bakchoul, Prof. Dr. med. Claudia Lengerke, Prof. Dr. Karin Schilbach, Prof. Dr. Peter Lang, Dr. Markus Felix Templin, Dr. med. Christian Martin Seitz, Dr. Daniel Atar. CD19-CAR-T phenotyping during Prodigy manufacturing uncovers critical T cell transition. 2025. DOI:10.21203/rs.3.rs-9071948/v1

Accepted: Anna-Sophia Mast, Peter Lang, Patrick Schlegel, Friso G Calkoen, Daniel Atar, Sophia Scheuermann, Sylvia Klein, Christiane Braun, **Florian Schinle**, Marina Schmidt, Luca Hensen, Martin Ebinger, Michaela Döring, Jürgen Schäfer, Johannes H Schulte, Bader Alahmari, Peirong Hu, Dina Schneider, Rimas Orentas, Rupert Handgretinger, Christian M. Seitz. Human CART22.19 Therapy in Refractory Pediatric B-ALL: Insights from a Named-Patient Cohort. *Journal for ImmunoTherapy of Cancer*. 2025. DOI:10.1101/2025.09.09.25335341

11.2 Contribution for scientific Meetings

Florian Schinle, Dr. med. Christian Seitz, Daniel Atar. Modular Viral Vector Platform for Mid-Scale Production. *GRTC Retreat*. Tübingen, Germany. 04.07.2024

Florian Schinle, Prof. Dr. med. Peter Lang, Dr. med. Christian Seitz, Daniel Atar. Session 5: GMP-Production. *Symposium on "Advanced Gene & RNA Therapy - future clinical perspectives"*, Tübingen, Germany. 14.04.2023.

F. Schinle, F. Schmitt, P. Lang, N. Malek, C. M. Seitz. Solid CAR-T: Modulare Minifabriken zur autonomen Produktion von CAR-T Zellen. *Projekttreffen V*. Fraunhofer IPA, Stuttgart, Germany. 06.10.2023

11.3 Presentation, Poster

Florian Schinle, Daniel Atar, Kathrin Wolsing, Fee Schmitt, Veit Scheble, Peter Lang, Nisar Malek, Christian M. Seitz. Establishment of an innovate closed vector production platform. *1st International iFIT Conference 2023*. Zell am See, Austria. 21.03.-23.03.2023.

F. Schinle, K. Wolsing, F. Schmitt, P. Lang, N. Malek, C. M. Seitz, D. Atar. Modular Viral Vector Platform for Mid-Scale Production. *GRTC Retreat*. Tübingen, Germany. 04.07.2024.

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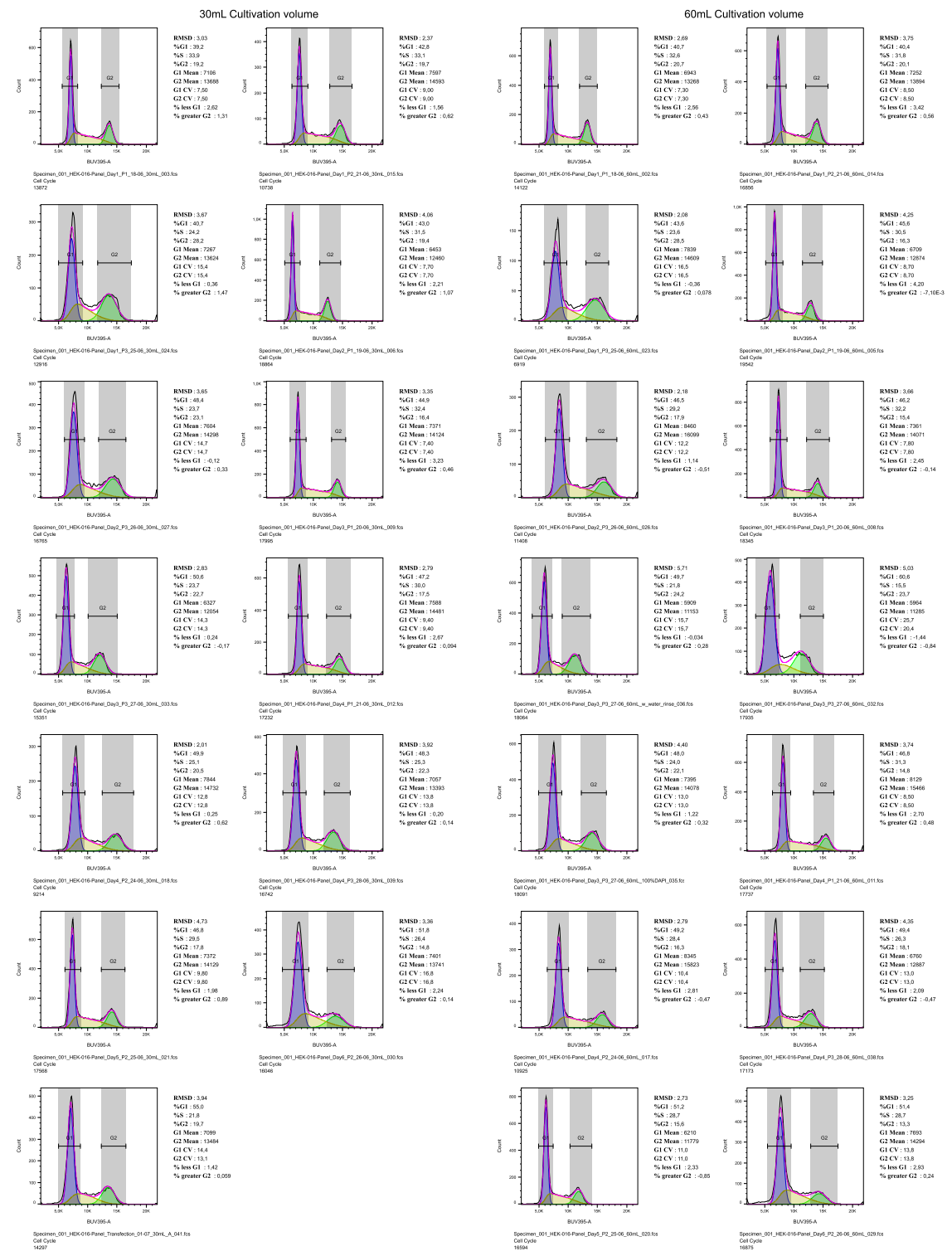
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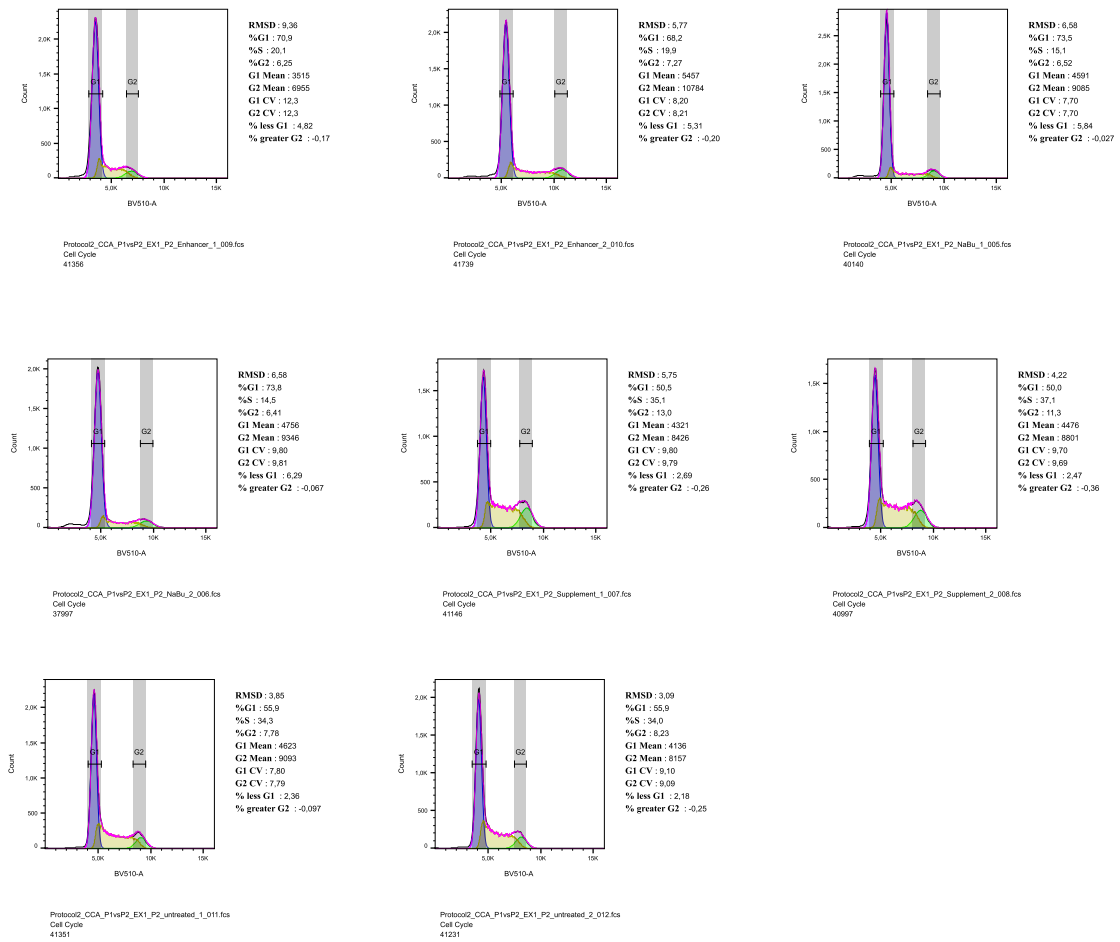
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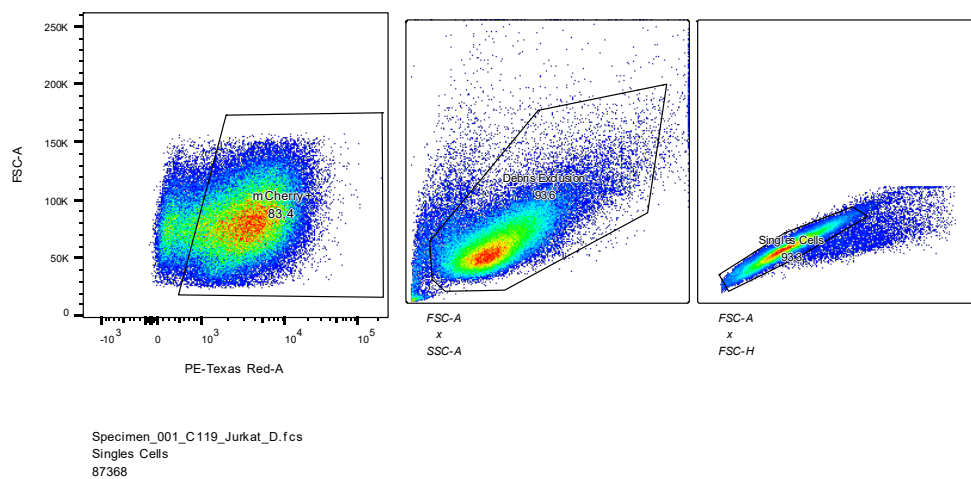
13 Appendix



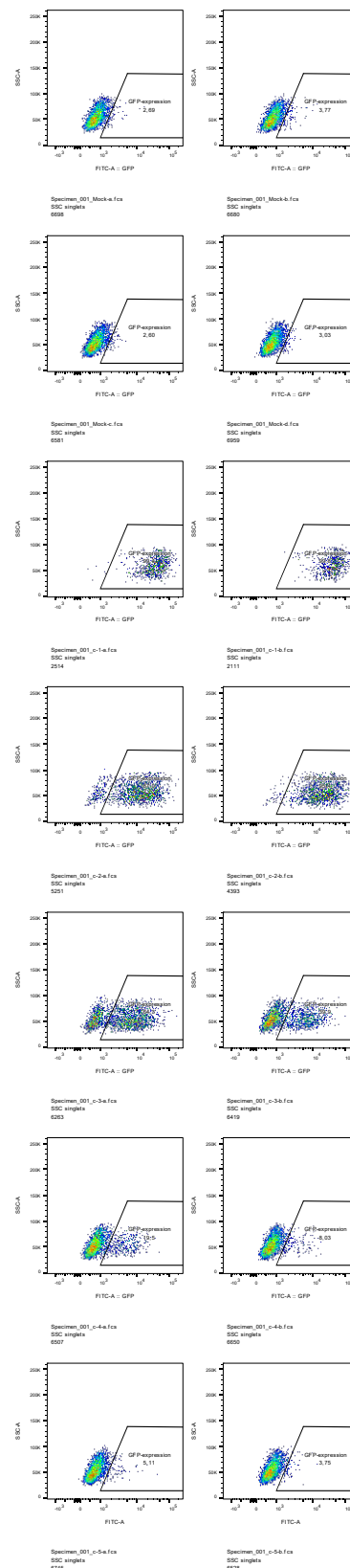
Supplementary Figure 1 Cell cycle analysis of HEK293 LV MAX™ cells throughout 6 process days post-split cultivated in 30mL or 60mL cultivation volume.



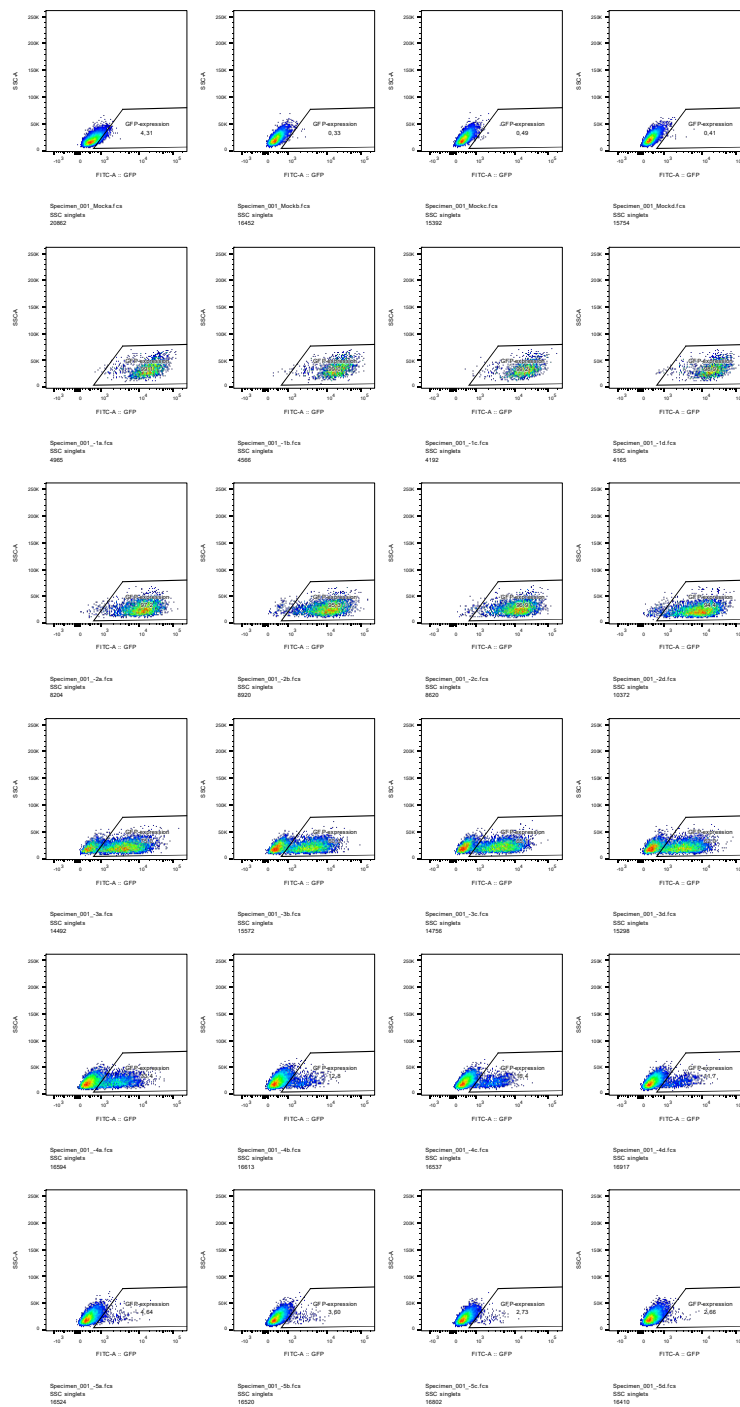
Supplementary Figure 2 Cell cycle analysis of HEK293 cells with conditions of either Sodium butyrate, LV MAX Enhancer, LV MAX Supplement or untreated.



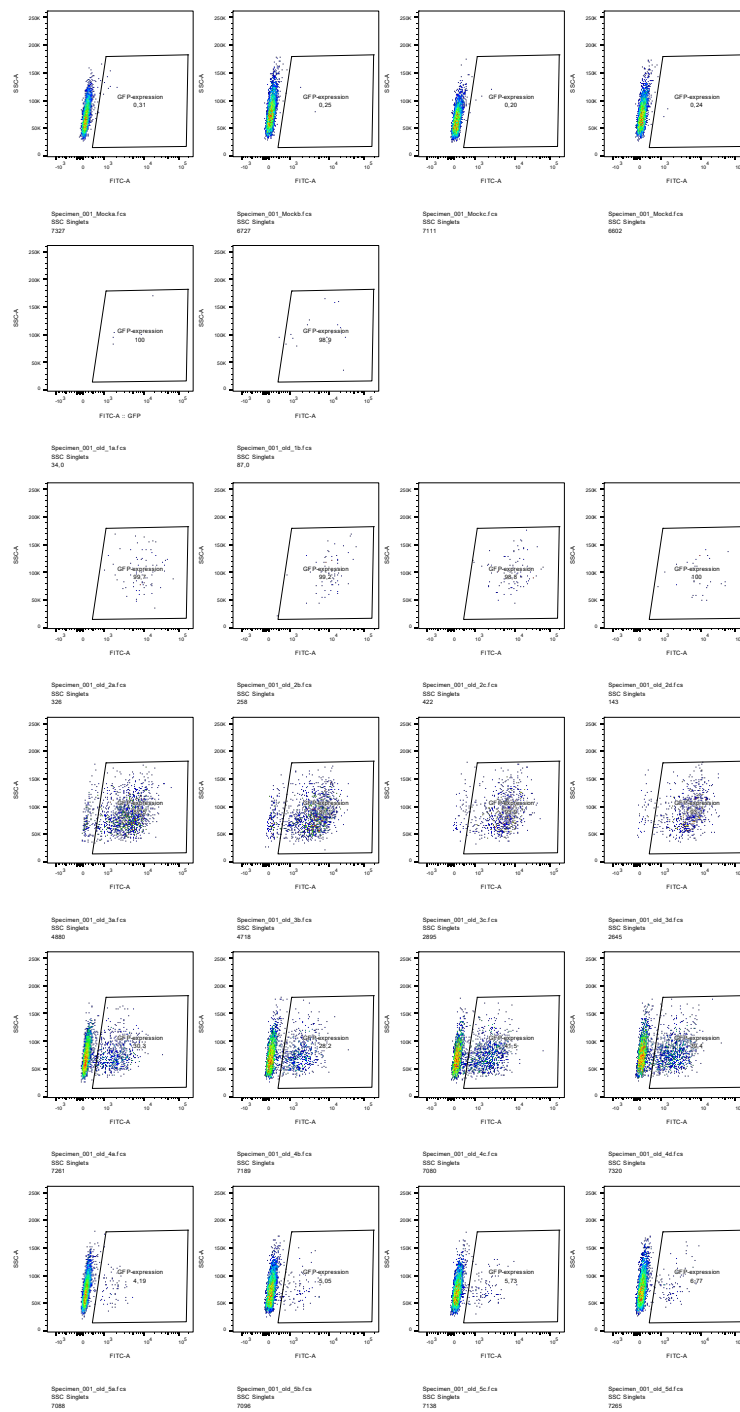
Supplementary Figure 3 Example of gating strategy for flow cytometry analysis to exclude cell debris and duplets.



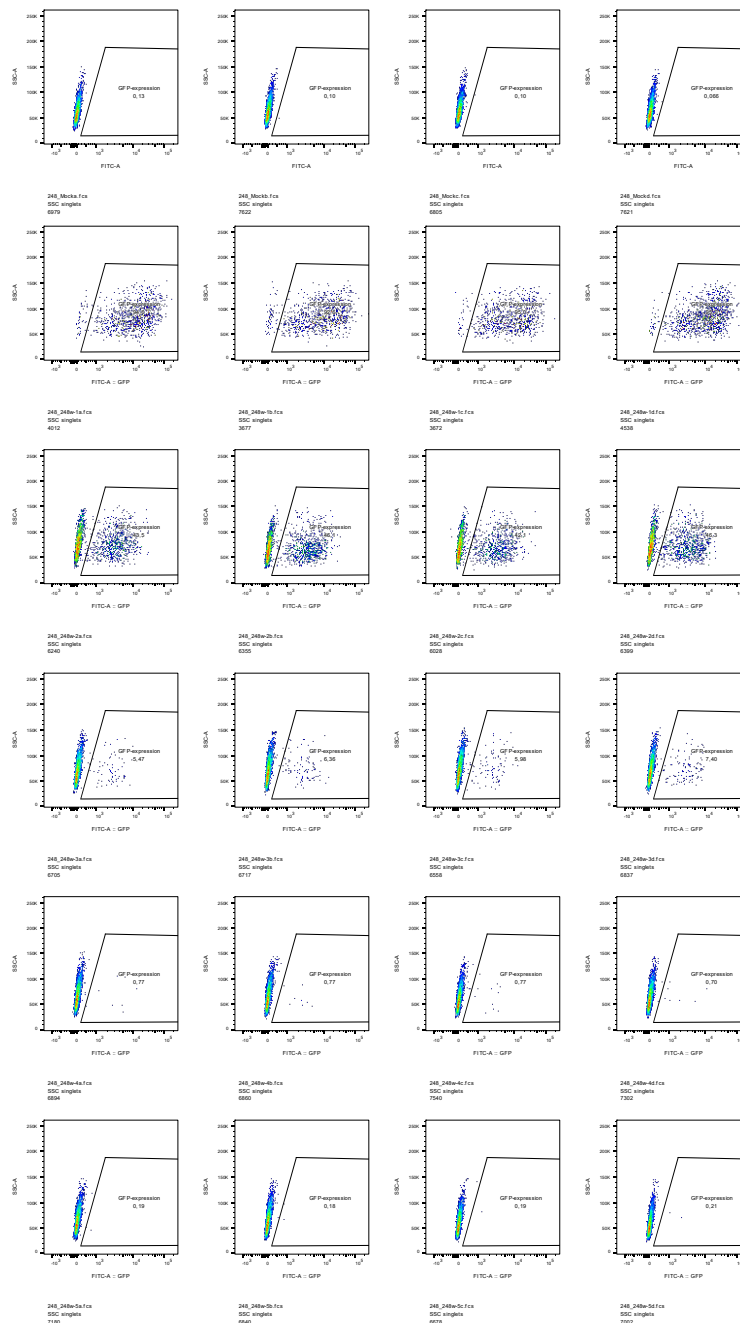
Supplementary Figure 4 Titration of lentiviral vector on 100 000 Jurkat cells. Detection of CAR construct via co-expression of GFP. Each plot corresponds to one measurement, 5×10 -log dilution steps in duplicates.



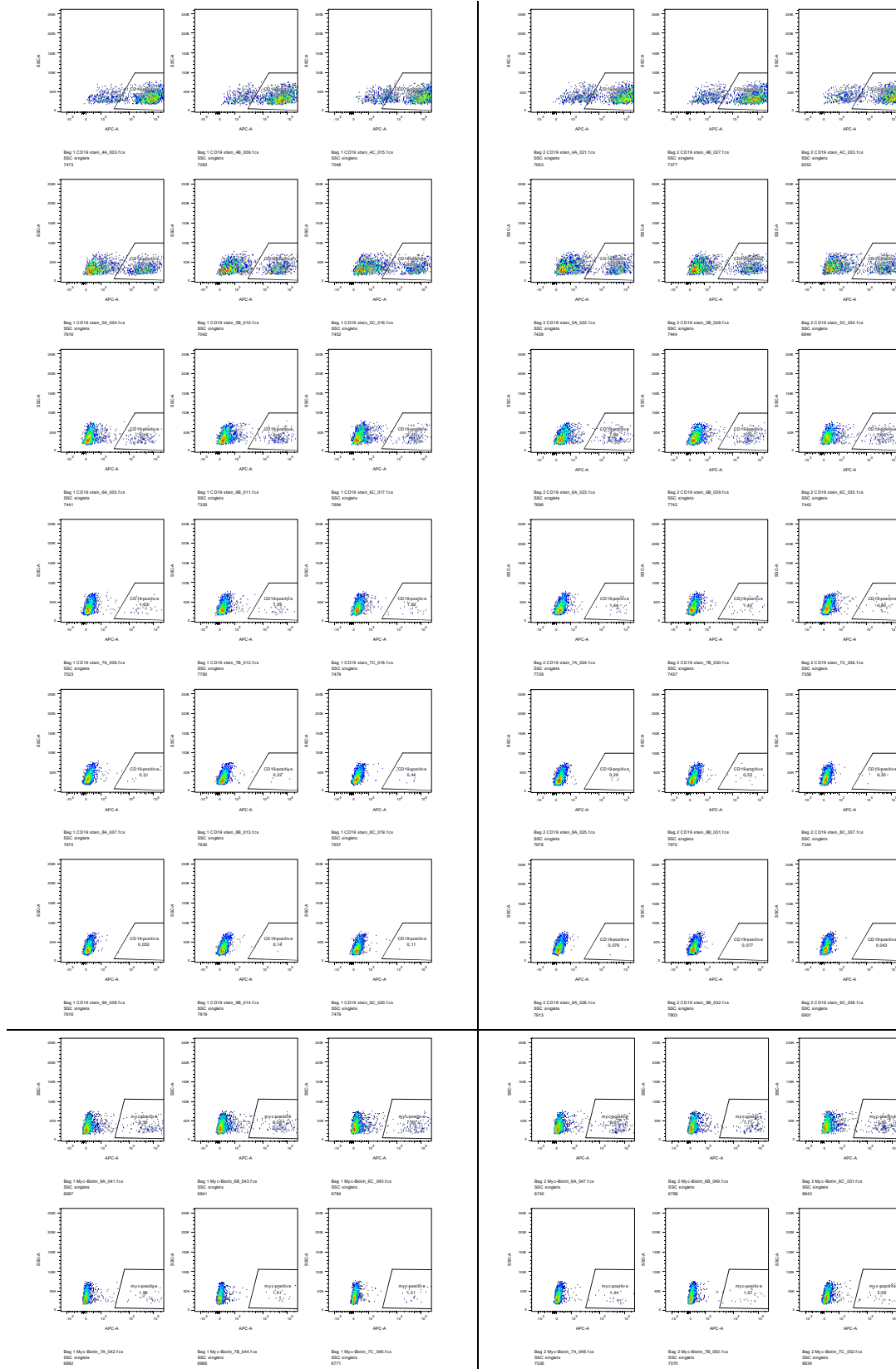
Supplementary Figure 5 Titration of lentiviral vector on 30 000 Jurkat cells. Detection of CAR construct via co-expression of GFP. Each plot corresponds to one measurement, 5×10 -log dilution steps in quadruplicates.



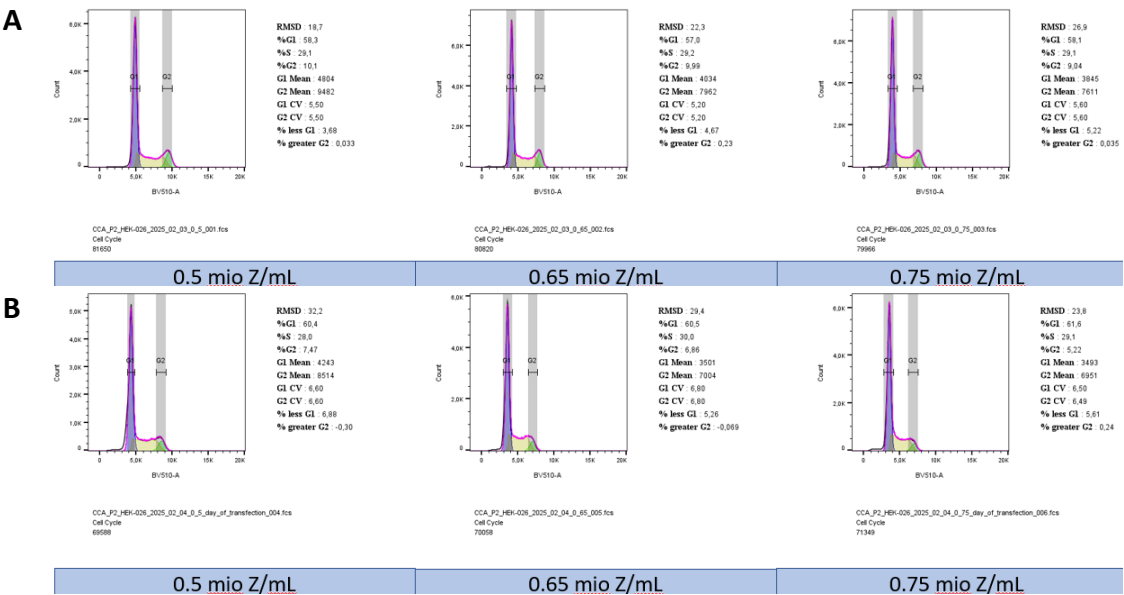
Supplementary Figure 6 Titration of lentiviral vector on 8 000 Jurkat cells. Detection of CAR construct via co-expression of GFP. Each plot corresponds to one measurement, 5× 10-log dilution steps in quadruplicates.



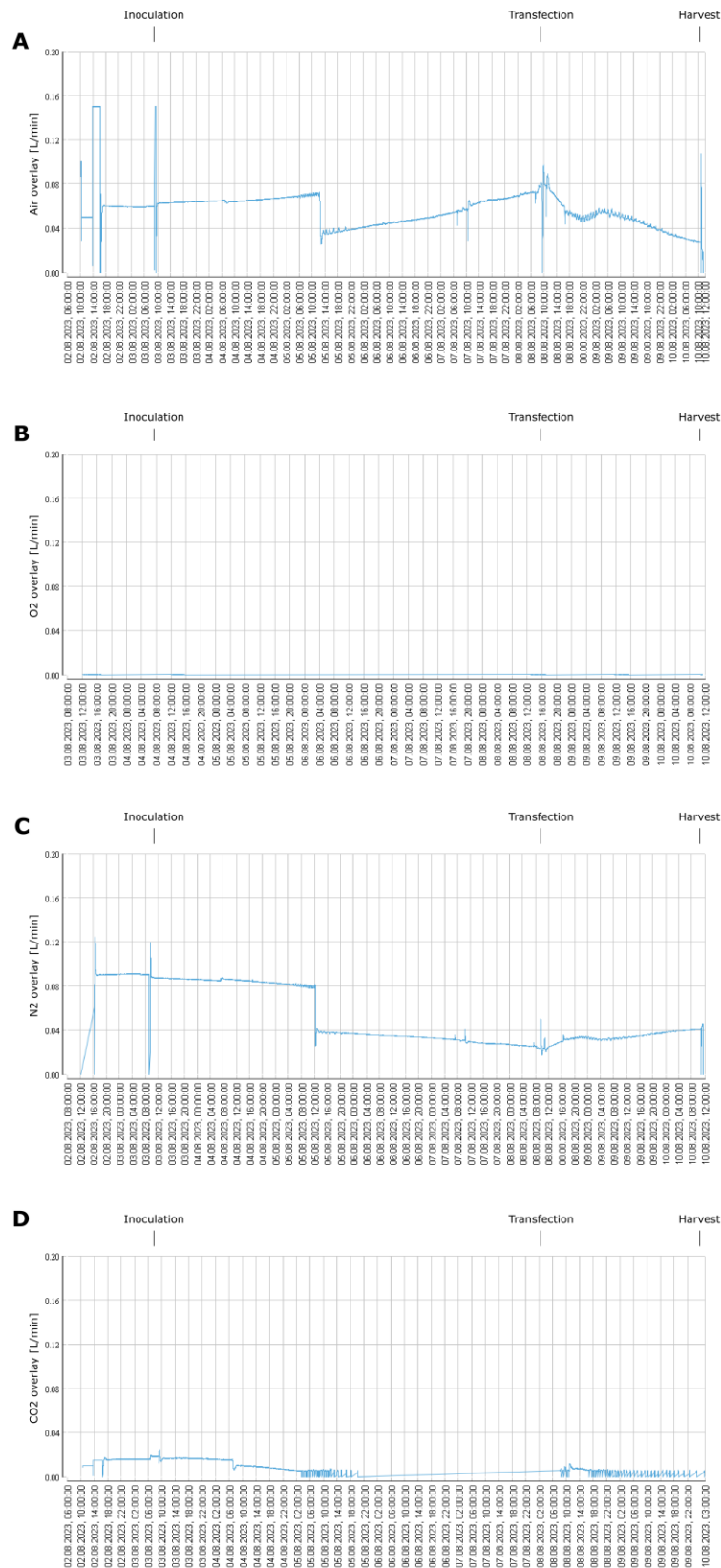
Supplementary Figure 7 Titration of lentiviral vector on 7 000 HT-1080 cells. Detection of CAR construct via co-expression of GFP. Each plot corresponds to one measurement, 5×10 -log dilution steps in quadruplicates.



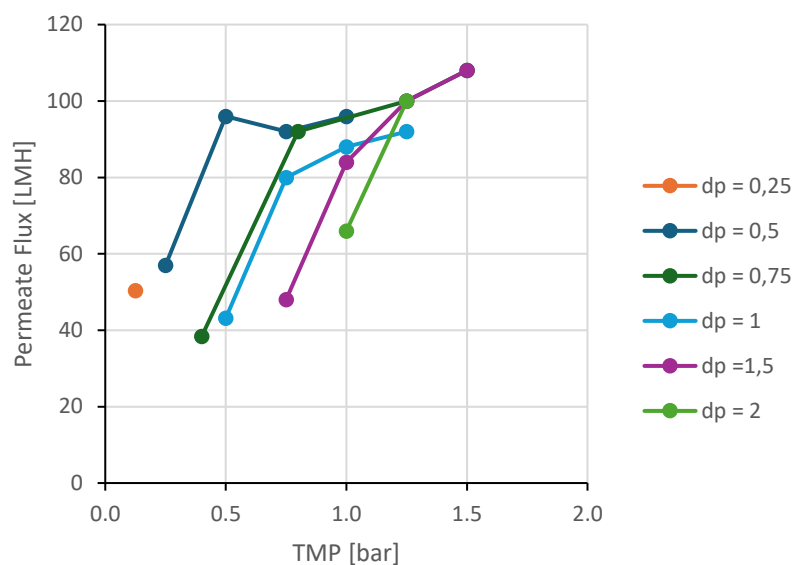
Supplementary Figure 8 Titration with antibody staining of lentiviral vector on HT-1080 Jurkat cells. Detection of CAR construct via myc-tag and co-expression of CD19. Each plot corresponds to one measurement, 5-log dilution steps in triplicates of two batches.



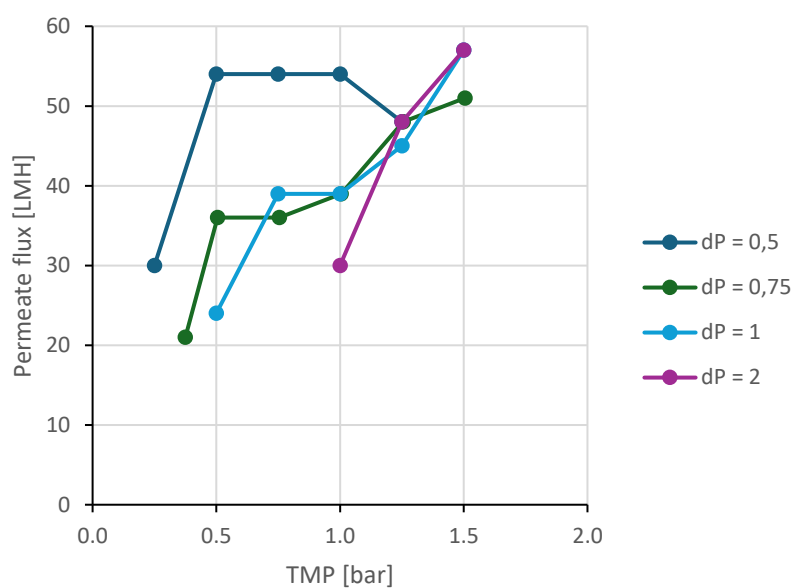
Supplementary Figure 9 Cell cycle analysis of HEK293 cells with different seeding densities. Seed of 0.5×10^6 cells/mL, 0.65×10^6 cells/mL or 0.75×10^6 cells/mL. **A:** day before transfection. **B:** day of transfection.



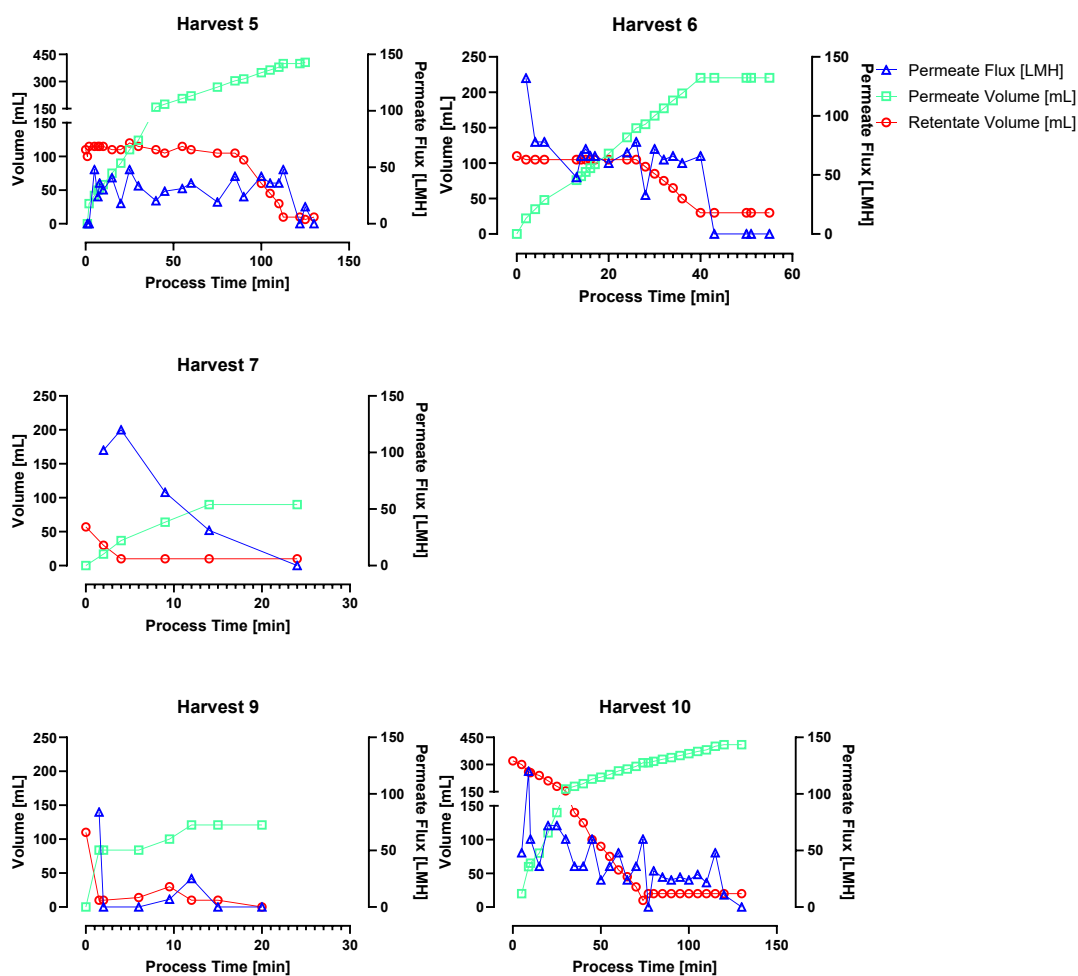
Supplementary Figure 10 Online-data during bioreactor cultivation. **A:** flow of air overlay. **B:** O₂ overlay. **C:** flow of N₂ overlay. **D:** CO₂ overlay.



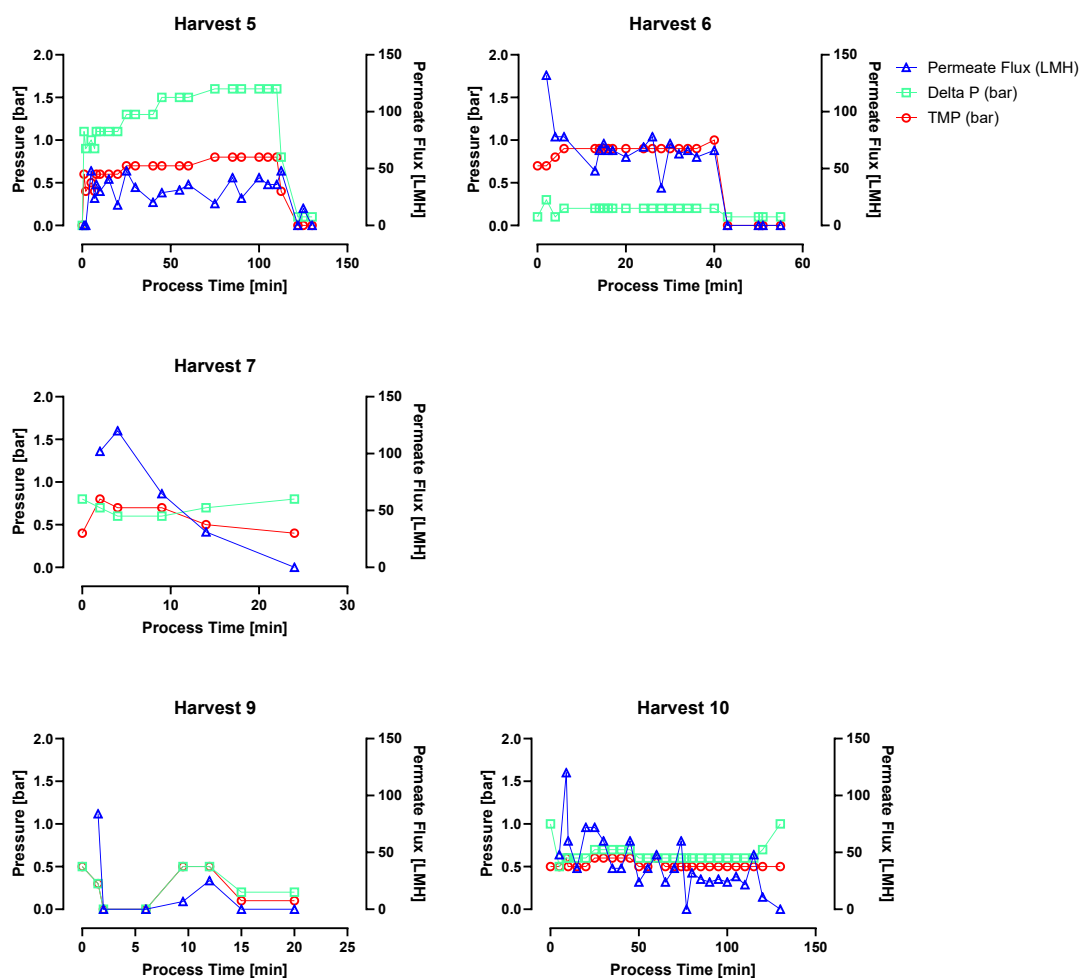
Supplementary Figure 11 Transmembrane pressure (TMP) scouting for MiniMate cassette. To detect optimum pressure for highest Filtrate Flux (LMH), before membrane fouling appears.



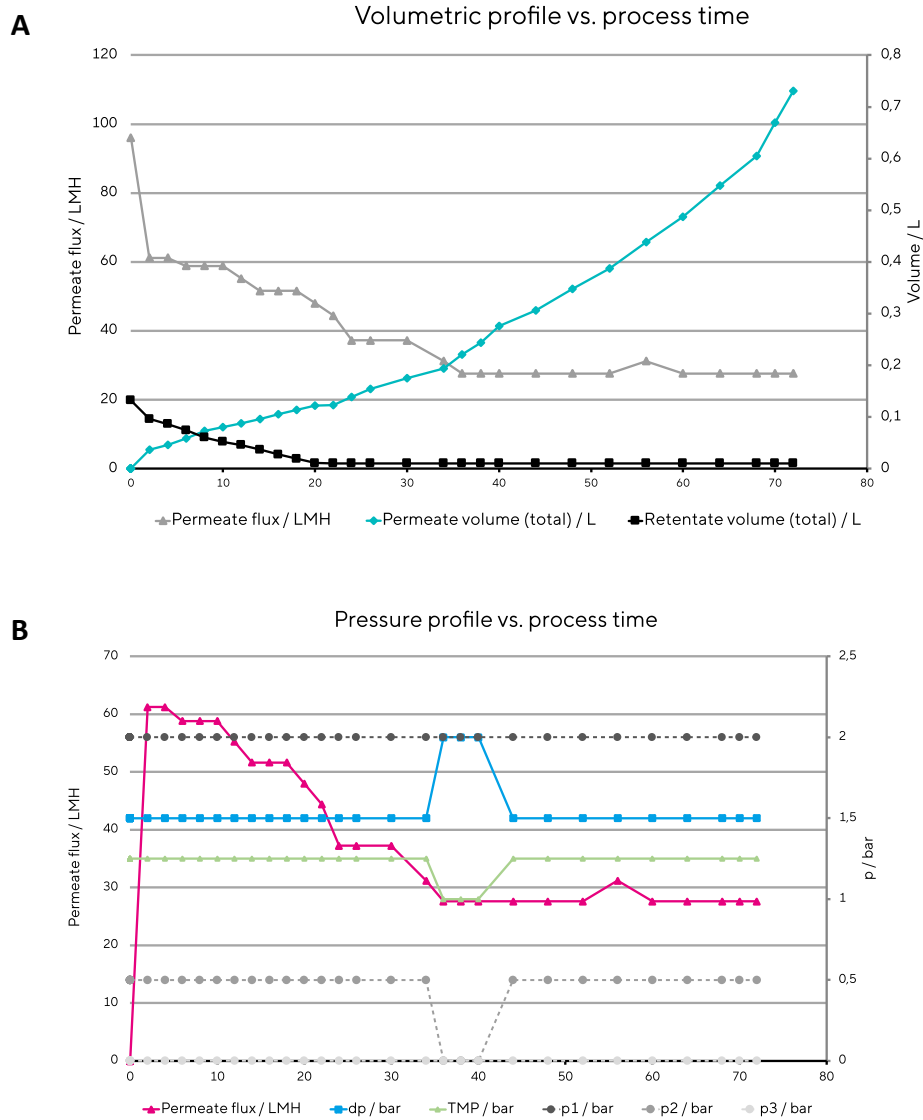
Supplementary Figure 12 Transmembrane pressure (TMP) scouting for Sartocon cassette. To detect optimum pressure for highest Filtrate Flux (LMH), before membrane fouling appears.



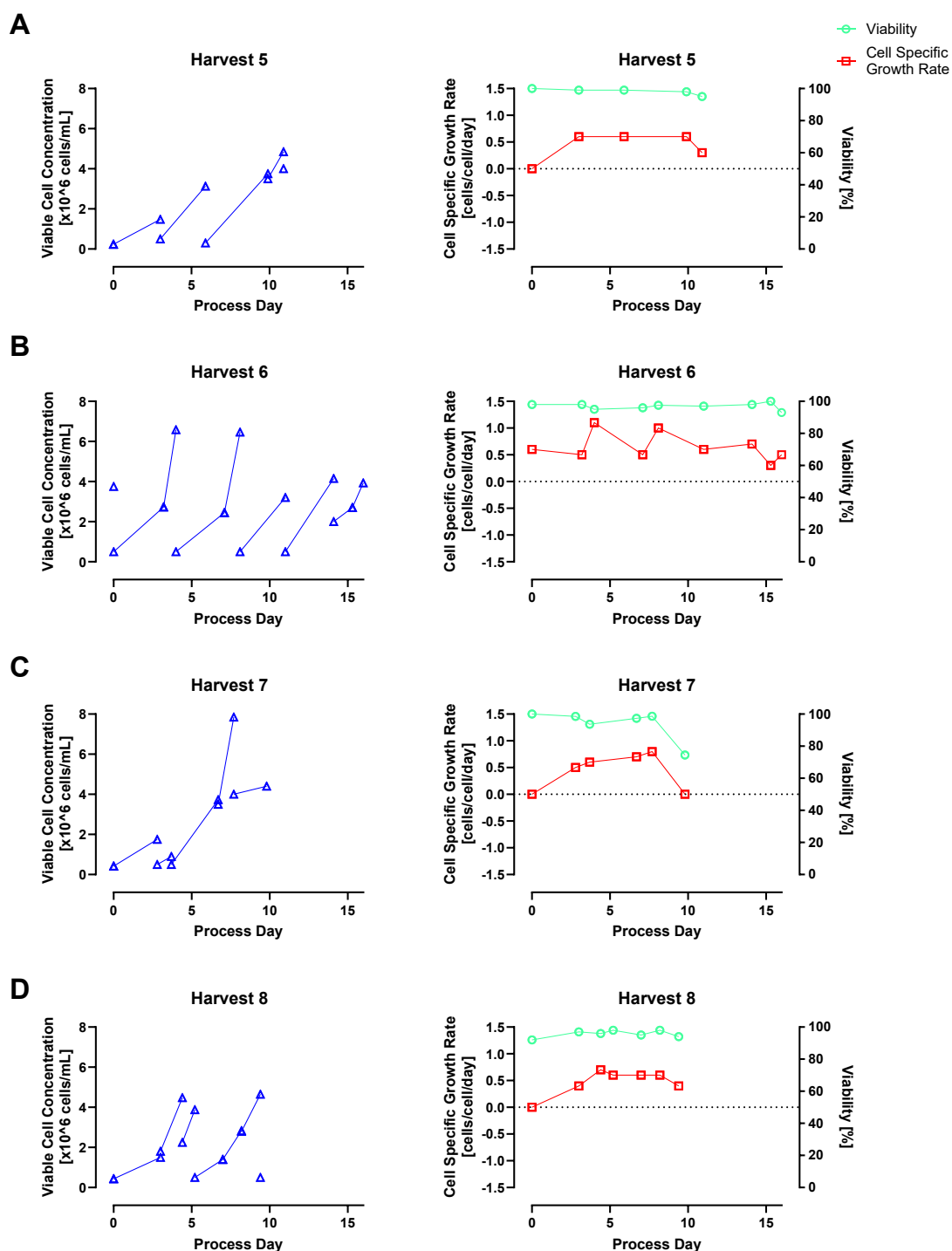
Supplementary Figure 13 Volumetric process data of ultra-/diafiltration. x-y plots of process volume and permeate flux ($L/m^2/hour$) over time of Harvest 5 to 10 (except Harvest 8).



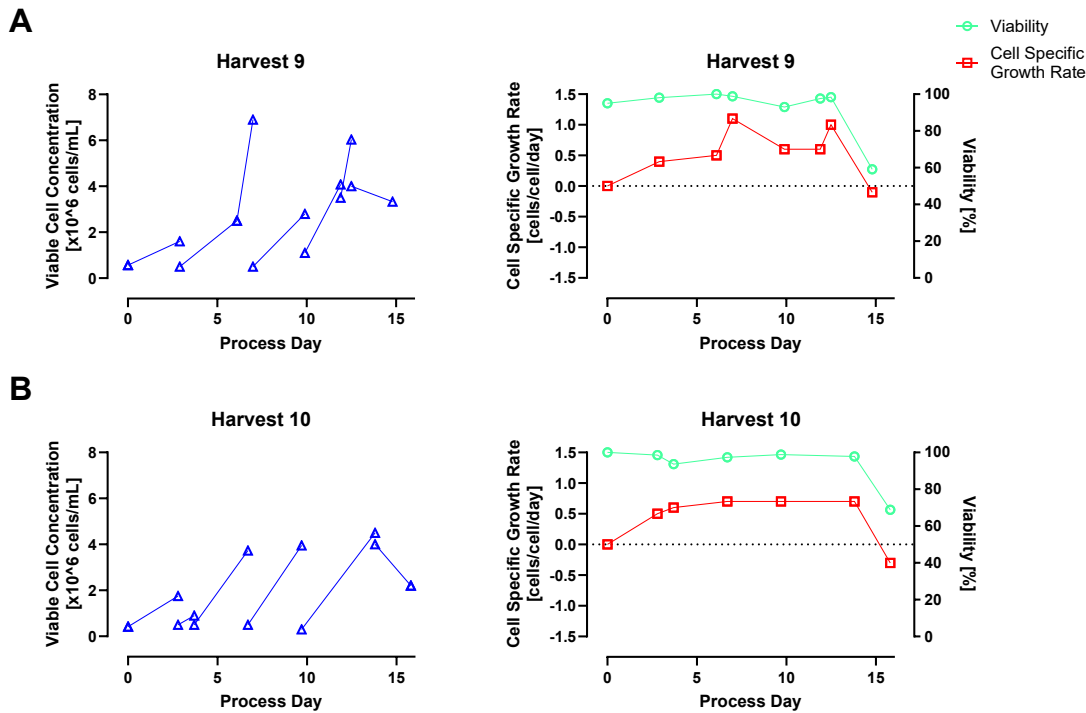
Supplementary Figure 14 Pressure process data of ultra-/diafiltration. x-y plots of delta and transmembrane pressure as well as permeate flux ($L/m^2/hour$) over time of Harvest 5 to 10 (except Harvest 8).



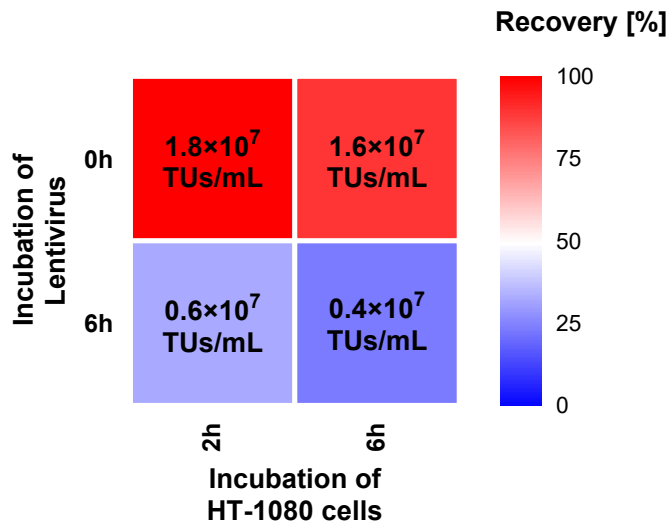
Supplementary Figure 15 Pressure and volumetric process data of ultra-/diafiltration. **A:** x-y plot of process volume (retentate volume) and permeate volume as well as permeate flux ($L/m^2/hour$) over time of Harvest 8. **B:** x-y plots of delta, transmembrane pressure and singular pressure data as well as permeate flux ($L/m^2/hour$) over time of Harvest 8



Supplementary Figure 16 Cultivation data of prior to stability assay (Harvest 5 to 8). Left: subculture x-y plot of viable cell concentration for each process day. Right: x-y plot of cell-specific growth rate and viability of respective process days. Viability was determined as ratio of viable cells to total cells per mL. Cell specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. Data shown respectively for single expansion ($n=1$).



Supplementary Figure 17 Cultivation data of prior to stability assay (Harvest 9 and 10). Left: subculture x-y plot of viable cell concentration for each process day. Right: x-y plot of cell-specific growth rate and viability of respective process days. Viability was determined as ratio of viable cells to total cells per mL. Cell specific growth rate based on $\ln(\text{current total cell count}/\text{prior total cell count})/\text{time interval}$. Data shown respectively for single expansion ($n=1$).



Supplementary Figure 18 Matrix plot of recovery of transducing units depending on incubation of HT-1080 cells and Lentivirus over time. x-axis is incubation of HT-1080 cells between 2 and 6 hours, y-axis incubation of virus at room temperature for 6 hours or immediate titration.