Table 1: Antibodies to determine humanization level (Panel 1)

Target	Fluorochrome	Clone[1]	Catalogue No.	Company
Anti-human CD45	BV510	2D1	368526	BioLegend
Anti-human CD3	PerCp	BW264/56	130-113-131	Miltenyi Biotec
Anti-human CD19	Alexa Fluor700	HIB19	56-0199-42	Thermofisher
Anti-human CD14	VioGreen	REA599	130-110-525	Miltenyi Biotec

[1]Of each antibody clone 1.5 µl is used in 50 µl FACS staining buffer

Table 2: Antibodies for detection and characterization of *in vivo* generated CAR T cells (Panel 2)

Target	Fluorochrome	Clone[1]	Catalogue No.	Company
Anti-human CD45	BV510	2D1	368526	BioLegend
Anti-human CD3	PerCp	BW264/56	130-113-131	Miltenyi Biotec
Anti-human CD4	PE-CF594	RPA-T4	562281	BD
Anti-human CD8	FITC	BW135/80	130-113-157	Miltenyi Biotec
Myc-Tag	PE	9B11	3739	Cell Signaling
Anti-human CD19	Alexa Fluor700	HIB19	56-0199-42	Thermofisher
Anti-human CD45RA	BV421	HI100	304130	BioLegend
Anti-human CD62L	BV605	DREG-56	304833	BioLegend
Anti-human PD-1	PE-Vio770	PD1.3.1.3	130-117-698	Miltenyi Biotec
Anti-human Lag-3	Alexa 647	T47-530	565716	BD
Mouse isotype control	BV510	MOPC-21	400171	BioLegend
Mouse isotype control	PerCp	S43.10	130-099-190	Miltenyi Biotec
Mouse isotype control	PE-CF594	X40	562292	BD
Mouse isotype control	FITC	S43.10	130-113-271	Miltenyi Biotec
Mouse isotype control	PE	S43.10	130-113-272	Miltenyi Biotec
Mouse isotype control	Alexa Fluor700	P3.6.2.8.1	56-4714-80	Thermofisher
Mouse isotype control	BV421	MPC-11	400342	BioLegend
Mouse isotype control	BV605	MOPC-21	400162	BioLegend
Mouse isotype control	PE-Vio770	IS6-11E5.11	130-098-562	Miltenyi Biotech
Mouse isotype control	Alexa 647	J606	560803	BD Pharmingen

[1] Of each antibody clone 1 µl is used for staining.

Table 3: Trouble shooting

Steps	Problem	Possible Causes	Potential solution
1-14	Packaging cells and/or vector stock contaminated with microorganisms	Wrong handling of material	Take care not to move your hand or material above an opened cell culture plate. Be very cautious with your materials like tips, media bottles, etc. Exchange all materials required for transfection.
1-2	Low transfection efficiency (<80% determined with GFP encoding transfer vector plasmid)	Condition of Lenti-X 293T	<u>Culture conditions:</u> Monitor the condition and health of the cells daily. Identify the best source (and lot) of FBS experimentally. Cells must not be cultivated for more than 20 passages. Cells may not be grown to over-confluence. Split cells twice a week and seed at a 1:8-1:10 dilution.
			<u>Seeding of Lenti-X 293T:</u> Cells must be distributed uniformly over the plate.
2		Suboptimal handling during transfection	<u>Time of transfection:</u> Cells should be at least 75% confluent by the time of performing the transfection, but still should not grow in 3-dimensional clusters.
3-6			<u>During transfection:</u> Avoid that cells get dry when kept in little media. Do not extend to more than 1h 30 min. Avoid detaching cells when adding the transfection mix onto the cells.
4		Poor plasmid quality, e.g. too high level of endotoxin and RNA	Check plasmids for absence of RNA by agarose gel analysis. Use endotoxin-free plasmid preparation systems.
		Wrong plasmid ratios	Make sure to determine the plasmid concentration correctly. If using different plasmids than those in the current protocol adapt ratios in the transfection mix based on molecular ratios.
15	Low gene transfer activity on target cells	Inefficient particle formation and release from packaging cells.	Determine particle number in vector stock. If below $5x10^{11}$/ml, improve transfection efficiency as explained above.
16		Absence/low level of target receptor	Check expression of target receptor via flow cytometry. See Fig. 4a as an example for sufficient receptor density. Use alternative cell line for quantification of gene transfer activity.
15-16		Sufficient number of vector particles (above $5x10^{11}$/ml) inactive in gene transfer (below $2x10^{6}$ t.u./ml).	Check concentration step. Determine gene transfer activity in unconcentrated harvest. Loss of gene transfer activity during concentration should not be more than two-fold. Check identity and functionality of all plasmids used, especially surface expression and receptor binding activity of the MV-H or NiV-G encoding plasmids[61]. Check target-receptor binding of the vector particles[32].
16-17		Insufficient contact between vector particles and target cells under given culture conditions	Transduction enhancers like Vectofusin [32] substantially increase transduction of target cells *in vitro*. Also spinfection enhances gene transfer[32].
		Inactivation of vector particles through high endocytosis rate of target receptor	Block endocytosis e.g. by NH_4Cl or bafilomycin to enhance gene delivery activity [53].
21	Low and/or too slow humanization	Busulfex conditioning is not strong enough.	Calibrate the Busulfex dose carefully according to the weight of the mice and do not inject the hCD34+ cells earlier than 30-36h after Busulfex injection otherwise these hCD34+ will be wiped out.
24-34		Insufficient purity of human CD34+ cells used for injection	Use human CD34+ cells with a purity greater than 90% containing less than 3% T cells and B cells.
		Too few living human CD34+ cells injected	Make sure that a minimum of $7x10^{4}$ living CD34+ cells is injected. Better inject at least $1x10^{5}$ cells to speed up humanization.

98-105	No signal detected by FACS for *in vivo* generated CAR T cells	Inactive vector stock	Improve production conditions as described above.
		Inadequate FACS staining and analysis	Low signal intensity either due to insufficient antibody or quenching of fluorochrome. Perform antibody titration and use optimal amount of antibody. To avoid quenching of fluorochrome, measure samples within three days post staining. For storage longer than one day resuspend samples in FACS Wash buffer post fixing. Set up a positive control, ideally a mixture of CAR+ and CAR- T lymphocytes in serial dilutions. 1% CAR+ should be readily detectable with the staining protocol.
106-107	No detection of CAR T cells by qPCR	Inadequate qPCR, poor DNA quality	DNA might have been lost in preparation. Determine DNA concentration and be sure to use the accurate amount of DNA for qPCR. Make sure that DNA is of sufficient quality by gel electrophoresis.
108		Inadequate qPCR, poor design of primers and probe	Redesign primers and probe using appropriate software.
111		When positive signal from FACS, this could be a false positive signal.	Compensate FACS settings using beads as well as single stainings to avoid spillovers.
		When negative signal from FACS, inactive vector stock	Improve production conditions as described above.