Time: 2024.06.16-2024.06.18

1. **Experiment:** Construction of the plasmids for CAR
2. **Time:** 2024.06.16-2024.06.18
3. **Member:** Xiaoyuan Chen, Xudong Tang, Hanyue Liu
4. **Material:** NKR constructs (GenePharma), pRRLSIN.cPPT.PGK-GFP.WPRE plasmids, Rapid DNA Ligation Kit (Genstar), NucleoBond Xtra Midi Maxi EF plasmid purification kit (Macherey-Nagel 740420.10).
5. **Method:**

NKR constructs were cloned into the pRRLSIN.cPPT.PGK-GFP.WPRE plasmids. Both the NKR target fragments and vectors were double-digested using specific restriction enzymes according to the protocol described in the subsequent table and figure.

Reagent	Volume (1×)
10×NEB Smartcut	2 μL
Target fragment CARs	1 μg
Xbal	1 μL
BamHI	1 μL
ddH_2O	Up to 20 μL
10×NEB Smartcut	2 μL
Vector pRRLSIN-GMI	2 μg
Xbal	1 μL
BamHI	1 μL
ddH_2O	Up to 20 μL

The reactions were maintained at 37°C 1 hr, inactivated at 65°C for 5 mins, and then stored at 4°C. The products were analyzed using 1.5% agarose gel electrophoresis at 120V for 45 mins, and target bands were subsequently recovered.

The linear vectors and the target segments were recycled and then connected as follows:

Reagent	Volume (1×)
5×T4 ligase buffer	4 μL
Cleaved CARs	20 ng
Linear pRRLSIN-GMI	3 ng
T4 ligase	1 μL
ddH_2O	Up to 20 μL

The whole system was incubated at 16°C for 30 mins, inactivate at 65°C for 10 mins, and then stored at 4°C. The connection products were transformed into Stbl3 by T4 ligase (Rapid DNA Ligation Kit, Genstar). Following clone verification by sequencing, bacterial cultures (500 mL) were grown in 2L flasks, and plasmids were extracted using the NucleoBond Xtra Midi Maxi EF plasmid purification kit (Macherey-Nagel 740420.10).