Time: 2024.05.03-2024.05.05

1. **Experiment:** Construction of the plasmids for CAR
2. **Time:** 2024.05.03-2024.05.05
3. **Member:** Yaqi Gao, Song Zhang, Xudong Tang, Xinyu Zhu
4. **Material:** 23 CAR 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:**

Twenty-three CAR constructs were cloned into the pRRLSIN.cPPT.PGK-GFP.WPRE plasmids. Both the CAR 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	2μL
Target fragment CARs	1μg
Xbal	1μL
BamHI	1μL
ddH_2O	Up to 20μL
10×NEB Smartcut	2μL
Vector pRRLSIN-GMI	2μg
Xbal	1μL
BamHI	1μL
ddH_2O	Up to 20μL

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	4μL
Cleaved CARs	20ng
Linear pRRLSIN-GMI	3ng
T4 ligase	1μL
ddH_2O	Up to 20μL

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).