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A Generalizable Design and Cloning Strategy for CAR Lentiviral Plasmids

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Payton Utzman^{1,2}, Tyler Henderson², Said Elshafae², Candelaria Deimundo Roura², Michael Pulsipher²

¹University of Utah; ²Huntsman Cancer Institute



Payton H Utzman

University of Utah

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We use this protocol and it's working

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Abstract

This protocol describes a high-efficiency strategy for cloning antibody fragments into CAR lentiviral plasmids. By incorporating Type IIS restriction sites into a parental CAR backbone, antibody domains can be inserted seamlessly using a 6 μ L HiFi DNA assembly incubated for 15 minutes. Transformation is completed in 5 minutes, and subsequent culture at 30 °C stabilizes large, toxicity-prone plasmids. This approach shortens cloning timelines, improves fidelity, and provides a reproducible framework for building CAR constructs in pre-clinical research.

Attachments



BBZ_CAR_parental_vec..



15KB

Materials

PaqCI

Hifi 2X MM

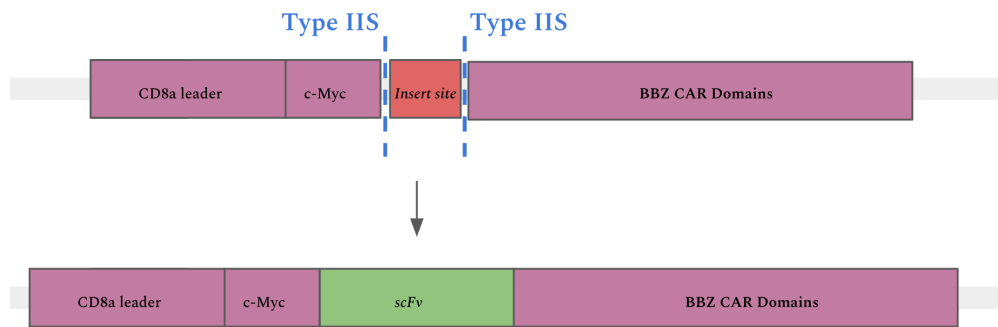
NEB Stable Transformation Kit

Introduction

- 1 Cloning CAR lentivirus plasmids can be challenging as these vectors are large, contain repetitive elements, often lack convenient unique restriction sites, and frequently display bacterial toxicity, leading to low colony recovery and unstable propagation. To address these challenges, we designed a parental CAR vector with a dedicated insertion site that simplifies antibody-fragment exchange. Linearization with a Type IIS enzyme produces a scarless backbone that can accept scFvs or nanobody domains with minimal design effort. We pair this design paradigm with a streamlined cloning protocol, 6 μ L HiFi assembly for 15 minutes, followed by a rapid 5-minute transformation and provide culture recommendations emphasizing 30 °C growth to maintain stability of toxicity-prone plasmids. Together, these elements reduce time, cost, and failure rates in CAR plasmid construction. Beyond individual CAR designs, this framework illustrates a generalizable strategy for rational plasmid design, cloning, and handling of other challenging constructs. This method has been successfully applied to the assembly of more complex constructs, including trivalent CARs.

Parental Plasmid Design

- 2 To enable rapid, high-fidelity cloning of antibody fragments into chimeric antigen receptor (CAR) plasmids, we generated a "parental" CAR plasmid. This vector was engineered with a dedicated "insertion" site positioned within the CAR reading frame at the location normally occupied by the antigen-recognition domain. The site is flanked by two Type IIS restriction enzyme recognition sequences. Type IIS enzymes (e.g., PaqCI) differ from conventional restriction endonucleases in that they cleave at a defined distance outside of their recognition site. This feature enables scarless integration of antibody fragments, preserving the reading frame. Upon digestion, the intervening sequence is excised, producing a linearized plasmid backbone optimized for seamless insertion of single-chain variable fragments (scFvs) or nanobody domains. This design streamlines CAR construction, reducing both time and complexity in generating various CAR plasmids. Additionally, this may be utilized for constructing CAR libraries with Golden Gate cloning.



The plasmid incorporates dual inward-facing Type IIS restriction sites (PacCI) strategically positioned within the chimeric antigen receptor (CAR) reading frame. Upon digestion with the enzyme, the small placeholder fragment is excised and a gene of interest can be inserted in its place.

Included in this protocol is an attached Genbank file for the second generation 4-1BB-CD3 ζ parental CAR vector we have built. This includes the annotated insertion site for an antibody fragment and can be referenced when designing future CAR parental plasmids.

Insert gene (scFv or nanobody) design

- 3 For efficient integration of antibody fragments into the Type IIS-digested vector using NEB HiFi DNA Assembly (or equivalent methods), inserts should be designed with 20–30 base pairs of homology to the plasmid sequences flanking the excision site. We recommend using 30 bp for optimal assembly efficiency. Specifically, the 5' end of the insert should share homology with the region immediately upstream of the restriction site, while the 3' end should share homology with the region immediately downstream.

Type IIS digestion of parental plasmid

- 4 Begin with the purified parental plasmid. To remove the insertion site and prepare the Parental CAR plasmid for insert assembly, digest the plasmid with the Type IIS restriction enzyme such as PacCI.
- 5 **PacCI Digestion**
 1. Reaction Setup (50 μ L)
 - DNA: 1 μ g of Parental CAR plasmid DNA.
 - PacCI Enzyme: 1 μ L
 - PacCI Activator: 1 μ L
 - CutSmart Buffer: 5 μ L
 - Total Volume: Adjust to 50 μ L with nuclease-free water.

Incubate in thermocycler at 37C for 60 minutes.

6 **PCR Purification of Digested Plasmid**

Following PaqCI digestion, the plasmid backbone can be purified using a PCR purification kit and eluted in molecular-grade water to remove residual enzymes and buffer components prior to HiFi assembly.

Note: The small excised fragment is not retained during purification, eliminating the need for gel extraction. While size differences between digested and undigested plasmids are subtle, the linearized product typically migrates more slowly than the supercoiled undigested DNA upon extended electrophoresis.

Hifi Assembly

- 7 To facilitate efficient, cost-effective, and scalable assembly reactions, we recommend using the NEB HiFi DNA Assembly Master Mix in a total reaction volume of 6 μ L.

Reaction Components:

- 2X NEBuilder HiFi DNA Assembly Master Mix: 3 μ L
- Purified, digested parental plasmid DNA: 50 ng
- Insert DNA fragment: Calculated amount to achieve a 2:1 molar ratio of insert to plasmid
- Nuclease-free water: To bring the total volume to 6 μ L Assembly Procedure:

Preparation:

- Thaw the NEBuilder HiFi DNA Assembly Master Mix on ice
- In a sterile, nuclease-free microcentrifuge tube placed on ice, combine the following:
- 50 ng of purified, digested parental plasmid DNA, Calculated ng of insert DNA fragment, 3 μ L of 2X NEBuilder HiFi DNA Assembly Master Mix, Nuclease-free water to adjust the final volume to 10 μ L
- Gently mix the reaction by pipetting up and down.

Assembly:

- Transfer the reaction tube to a thermocycler or heat block preheated to 50°C. Incubate for 15 minutes to allow assembly.

Post-Incubation:

- After incubation place on ice
- The assembled DNA is now ready for transformation into competent cells. It can be stored at 4C.



Note: We recommend including a negative control in which no insert is added (water instead). Because undigested plasmid can still yield colonies, this control helps confirm that colonies on the experimental plate result from successful assembly rather than carryover of undigested vector. With larger CAR plasmids, some background colonies may appear on the negative control plate; however, a successful assembly should yield a substantially higher number of colonies.

NEB Stable Transformation

- 8 We recommend cloning CAR T lentiviral plasmids into NEB Stable E. coli cells. Here, we have optimized the protocol to minimize the number of E. coli transformations and scaled down the reaction to be easier to process and more cost-effective.

Reaction Components:

- 15 μ L of NEB Stable Cells
- 2 μ L of HiFi Assembly product

Thaw cells

- Thaw NEB Stable Cells on ice for 10 minutes during HiFi Assembly reactions

Add HiFi Assembly

- Add 2 μ L of HiFi Assembly product
- Incubate on ice for 4 minutes

Heat Shock

- In a thermocycler set to 42 °C with hot lid off, heat shock the cells + HiFi product at 42 °C for 30 seconds
- Place back on ice for 2 minutes
- Add 150 μ L of room temperature NEB 10-beta/Stable Outgrowth Medium
- Plate 50 μ L on a half of an LB plate with antibiotic selection
- Incubate plate at 37 °C overnight

Sequence confirmation of CAR-T clones

- 9 To sequence clones, we recommend picking a colony and incubating it in LB antibiotic-selective media at 30 °C, followed by full-plasmid sequencing using services such as Plasmidsaurus.
- Confirmed clones should be glycerol stocked in 15% glycerol and stored at -80C.

Note: Due to the inherent toxicity of a CAR lentivirus in bacteria, it is important to culture the cells at 30C when in liquid media.

Maxiprep of CAR-T plasmid

- 10 To purify large quantities of transfection-grade plasmid while mitigating plasmid toxicity, we recommend growing the *E. coli* cells at 30 °C using the following protocol:

DAY 1:

- Inoculate 2mL LB culture from glycerol stock
- Incubate at 30C overnight

DAY 2-3:

- Passage saturated LB culture into 1L flask with 150mL of LB at a 1:500 dilution ratio (300uL)
- Incubate at 30C for 48 hours

DAY 4:

- Perform maxiprep according to suppliers manual.
- Yields for our plasmids >10kb when 150mL incubated at 30C for 48 hours range from 400ug to 1mg.
- We routinely sequence large plasmid purifications to confirm no de-novo mutations have accumulated, prior to HEK transfection.

Summary

- 11 In this entry, we present a plasmid design strategy for generating a parental CAR vector, enabling straightforward construction of subsequent unique functional CARs. We also provide an optimized workflow for cloning daughter constructs and propagating bacterial cultures to prepare for lentiviral production.