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Generation of CRISPR constructs

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ABSTRACT

This protocol details the procedure of generation of CRISPR constructs.

ATTACHMENTS

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MATERIALS TEXT

Buffers and reagents:

- pSpCas9(BB)-2A-GFP
- (PX458) **addgene Catalog #48138**
- Qiagen miniprep kit (Qiagen, #28104)
- BbsI **NEB Catalog #3539**
- Alkaline Phosphatase, Calf Intestinal (CIP) **New England Biolabs Catalog #M0290**
- NEBuilder HiFi DNA Assembly Master Mix - 10 rxns **New England Biolabs Catalog #E2621S**
- NEB® 5-alpha Competent E. coli (NEB #C2987)
- **Growth broth:** a mixture of LB broth and Super broth with 1:1 ratio

Procedure 17h 5m

1 Designing gRNAs using https://chopchop.cbu.uib.no.

I prefer this website because it also gives you the primer sequences for sequencing analysis.

1.1 **"Target":** Put in the gene name/"In": choose the species.

For human cell lines, I choose "Homo sapiens (hg38/GRCh38)/"Using": for knockout I choose "CRISPR/Cas9"/"For": I choose "knock-out".

1.2 Do not change anything in "General" tab.

Make sure in "target specific region of gene", "Coding region" is chosen.

1.3 In the "Cas9" tab, make sure you choose "No requirements" for "5' requirements for

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sgRNA" and tick "I intend to replace the leading nucleotides with "GG"" (3 options of the "Sef-complementarity (Thyme et al.)" should be ticked).

1.4 In "Primers" tab, I choose product size from 200 to 500 and minimum distance from primer to target site at least 100.

1.5 Click "Find target sites".

2 Choose the top-ranking gRNA sequences that target the earliest exon possible.

Make sure that the targeted exon is shared between the isoforms (check on https://asia.ensembl.org/index.html).
If the protein is too big or it's not possible to choose a target common in all the isoforms, you can use two different gRNAs.

3 Click on the chosen target sequence, another window with all the information related to this gRNA sequence will appear.

- In this window, you can also find a table with primer pairs to amplify the targeted region for sequencing analysis.
- You can copy and paste these sequences into a word document and order them.
- If no primers appear, go back to "Primers" tab from step one and change the parameters.

4 Copy the target sequence without the PAM into the highlighted region of the below sequence:

ATCTTGTGGAAAGGACGAAACACCG **Copy the target sequence without the PAM here** GTTTTAGAGCTAGAAATAGCAAGTT.

5 Order the above sequence as a primer for Gibson assembly.

6 Preparing cut pSpCas9(BB)-2A-GFP:

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6.1 

Cut the vector with BbsI:

- **10 µg** of vectors
- **2 µL** of BbsI
- **3 µL** of NEBuffer™ r1.1
- Add sterile milliQ water to **30 µL**
- Incubate for 6-8 hours at **37 °C**

6.2  1h

After that, add **1 µL** of CIP and incubate for no longer than **01:00:00** .

6.3 Heat de-activate at **60 °C** for **00:05:00** . 5m

6.4 Run the reaction on a 0.5 % DNA agarose gel.

6.5 Extract the cut vector, determine the concentration, dilute it to **10 ng/µl** and aliquot to **1 µL** aliquots and store at **-20 °C** .

7  2h

Dilute the primer from steps 4 and 5 to the final concentration of **0.8 micromolar (µM)** (1/125 dilution of the **100 micromolar (µM)** stock). Set up a Gibson assembly reaction as following:

- **1 µL** of the diluted primers
- **1 µL** of BbsI-linearised pSpCas9(BB)-2A-GFP
- **2 µL** of HiFi DNA Assembly Master Mix
- Incubate at **50 °C** for **02:00:00** .

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Transform **1.8 µL** of the mix from step 7 (the rest can be stored at **-20 °C** as a backup in case the transformation does not result in any colonies) using **10 µL** of the NEB® 5-alpha Competent E. coli cells with manufacturer's instructions.

Note: The cells come in with bigger volume so make sure you make 10 µl aliquots upon thawing out.

9

The next day, pick up a few colonies and set up overnight cultures in growth broth.

10 Miniprep the cultures to purify plasmids and send them for sequencing using this primer (5' GCTCACCTCGACCATGGTAAT 3').

11 Once sequenced verified, the CRISPR constructs are now ready to be used for transfection.

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