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**Protocol status:** In development
We are still developing and optimizing this protocol

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# GENERATING THE POOLED SINGLE GUIDE RNA LIBRARIES

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## ABSTRACT

This protocol outlines the designing, cloning, and next-generation sequencing (NGS) of a pooled single guide RNA (sgRNA) library used with CRISPRi technology to knock down (KD) genes within midbrain dopaminergic (mDA) neurons. Dual guides1 have been designed to target 30 candidate genes that regulate cytoskeletal function, Ca2+ homeostasis, and mRNA metabolism. The causative role of these candidate genes in post-mitotic mDA neurons is yet to be characterised transcriptomically through single-cell RNA sequencing (scRNA-seq) Our objective is to elucidate the impact of our chosen gene perturbations in post-mitotic mDA neurons due to CRISPR inactivation with scRNA-seq.

## ATTACHMENTS

GENERATING THE POOLED SINGLE GUIDE RNA LIBRARIES.docx

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**Keywords:** ASAPCRN, Perturb - Seq, CRISPRi, NGS sequencing

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Grant ID: ASAP-000472

## MATERIALS

| | A | B | C |
|---|---|---|---|
| | MATERIAL | COMPANY | CATALOG |
| | A base Lentiviral Vector: pJR85 | Addgene | 140095 |
| | An insert from: pJR89 | Addgene | 140096 |
| | Library Oligo Pool | Twist Bioscience | Custom Ordered |
| | FastDigest Bpu 1102I | Thermo Fisher Scientific | FD0094 |
| | FastDigest BstXI | Thermo Fisher Scientific | FD1024 |
| | BsmBI-v2 | New England Biolabs (NEB) | R0739S |
| | T4 DNA Ligase | New England Biolabs (NEB) | M0202S |
| | PvuI | New England Biolabs (NEB) | R0150S |
| | NEB Stable Competent E. coli (High Efficiency) | New England Biolabs (NEB) | C3040H |
| | KAPA HiFi Hotstart PCR Kit | Roche | KK2502 |

BEFORE START INSTRUCTIONS

1. **Introduction:**

For our dual gRNA libraries, we use the following three elements:

A base Lentiviral Vector: pJR85

- Lentiviral CRISPR guide vector expressing an eGFP-NT2 sgRNA with cs1 incorporated in the loop of the sgRNA constant region.
- The BsmBI sites were removed to allow for programmed dual sgRNA library cloning.

A insert from: pJR89

- This plasmid contains sgRNA constant region and hU6 insert for programmed dual sgRNA cloning.
- The sgRNA constant region contains a capture sequence (cs1) in the stem loop for direct capture Perturb-seq

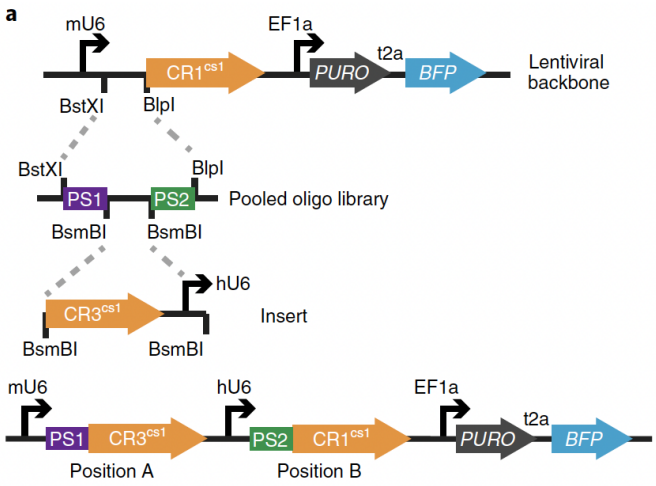


Figure 1: Figure  illustrating the programmed dual-guide library cloning strategy. Paired sgRNA targeting sequences are synthesized on individual oligos and inserted into a direct-capture Perturb-seq vector through ligation. Subsequently, an sgRNA constant region and hU6 promoter are introduced between the sgRNA targeting sequences, resulting in the completion of the final vector. This example depicts the design of a CR3cs1/CR1cs1 library. Repogle et al. 1

Library oligo pools:

- The 10x Genomics compatible sgRNA library oligo pool was designed in silico by Weatherhitt Lab (Garvan) and was ordered from Twist Bioscience.

## Synthesizing sgRNA oligonucleotides

1 The library oligo pool was designed in silico by the Weatheritt Lab (Garvan) and was ordered from Twist Bioscience. We utilised two in-silico tools FlashFry4 and CRISPRDO2, for guide design. Per gene, we designed two dual guides containing two sgRNAs targeting the interest gene. These are the protospacer,

PS1, and PS2 sequences found in Figure 1 above. We ordered a pool of oligo with PS1-constant region as per Supplementary in Repogle et al. 1 and an example to target TP53 is below:

sgTP53
TCACAACTACACCAGAAGCCACCTTGTTGGAGGGAAGCGTGTCACCGTCGGTTTCAGAGCGAGACGTGTTTGAT
CTCGGGCCGTCTCAGAAACATGGAAGTCTAGAGCCACCGTCCAGTTTAAGAGCTAAGCTGGCAACACTTTGACG
AAGA

where:
PCR Adapters 002:
Fwd -TCACAACTACACCAGAAG
Rev - GCAACACTTTGACGAAGA
Red rest. enzyme sites
Photospacer A - AGGGAAGCGTGTCACCGTCG
Photospacer B - AAGTCTAGAGCCACCGTCCA
CR3/cs1 paper sequence between photospacers-
GTTTCAGAGCGAGACGTGTTTGATCTCGGGCCGTCTCAGAAACATG

2

- Prioritised guides that are found to be reported in hCRISPRi3 .
- Prioritised guides that are found replicated across CRISPRDO2, FlashFry4 and with high specificity score (Moreno-Mateos2015 On Target) and low off-target score (Hsu2013).
- Do not target known common variants found across the Parkinson's Progression Markers Initiative (PPMI) cohort.
- Do not contain enzyme restriction sites BstXI/Blp that are used within the dual guide strategy reported.
- Paired dual guides that maximised the distance between respective guides.

## Generating pooled sgRNA plasmid library using the sgRNA oligonucleotides

3 The oligo Pools were delivered as a lyophilised product pooled in a single tube with a yield of 76.7ng.

- Preparation of stock solution of the lyophilised oligo pool:
- The oligo pool, ordered from Twist bioscience, contained 30 hit genes with 70 oligos at 152bps.
- Prepare a stock solution of the Oligo Pool by resuspending in 10 mM Tris buffer, pH 8.0 to a concentration of at least 20 ng/µl.
- Stock solution concentration (ng/ul)=[Total Yield (ng)/ Resuspension volume (ul)]
- Use the KAPA HiFi HotStart PCR Kit to perform PCR. Since the oligo pool comes under the 2-100 oligos at 151-200nt, it will require 12-14 PCR cycles according to the manufacturer's protocol.
- Detailed protocol for reconstitution of oligo pool and PCR amplification step can be found in the following link: https://www.twistbioscience.com/sites/default/files/resources/2019-09/Guidelines_OligoPools_%20Amplification_29Aug19_Rev5.1.pdf

4 The pooled sgRNA mDA Neuron Library was cloned according to the following protocol by Weatheritt Lab Cloning of pooled sgRNAs into lentiviral vector.

## Production of lentiviral supernatant

5 Viral supernatant was produced in accordance with the following protocol:

CITATION

Renuka Ravi Gupta, Nona Farbehi, Helaine Graziele Santos Vieira, Helen Elizabeth King, hendersa, Vikram Khurana, Gist Croft, Robert J Weatheritt, Lorenz Studer, Joseph Powell. LENTIVIRAL PRODUCTION FOR PCRISPRi DUAL GUIDE mDA NEURON LIBRARY. protocols.io.
LINK
https://protocols.io/view/lentiviral-production-for-pcrispri-dual-guide-mda-c9uez6te

## Titration of lentiviral supernatant

6 Lentiviral titration was done on H9 dCAS9 CRISPRi cells to determine the amount of virus required for an MOI of 0.1-0.3 (10% - 30% BFP positive cells).

CITATION

Renuka Ravi Gupta, Nona Farbehi, hendersa, Vikram Khurana, Gist Croft, Lorenz Studer, Joseph Powell. LENTIVIRAL TITRATION FOR HUMAN PLURIPOTENT STEM CELLS. protocols.io.
LINK
https://protocols.io/view/lentiviral-titration-for-human-pluripotent-stem-ce-c9wnz7de

## Transduction of H9 dCAS9 CRISPRi

7 H9 dCAS9 CRISPRi were transduced with the pooled CRISPRi library to obtain the gDNA (genomic DNA) after lentiviral transduction.

CITATION

Renuka Ravi Gupta, Nona Farbehi, hendersa, Vikram Khurana, Gist Croft, Lorenz Studer, Joseph Powell. LENTIVIRAL TRANSDUCTION OF HUMAN PLURIPOTENT STEM CELLS. protocols.io.
LINK
https://protocols.io/view/lentiviral-transduction-of-human-pluripotent-stem-c9wtz7en

## sgRNA pool NGS sequencing library preparation

8 The genomic DNA was sample prepped for Illumina sequencing

CITATION

Renuka Ravi Gupta, Helaine Graziele Santos Vieira, Helen Elizabeth King, Nona Farbehi, hendersa, Vikram Khurana, Gist Croft, Robert J Weatheritt, Lorenz Studer, Joseph Powell. gRNA POOL NGS SEQUENCING LIBRARY PREPARATION. protocols.io.
LINK
https://protocols.io/view/grna-pool-ngs-sequencing-library-preparation-c9wgz7bw