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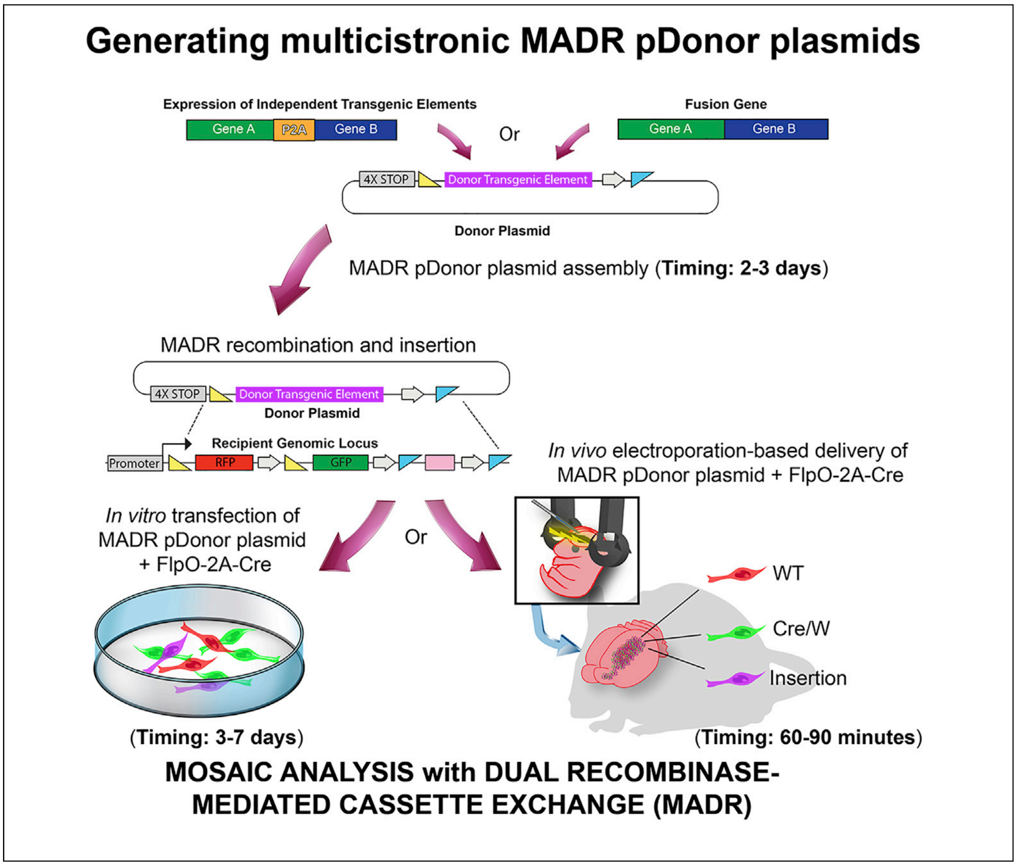
Protocol

Preparation, Assembly, and Transduction of Transgenic Elements Using Mosaic Analysis with Dual Recombinases (MADR)


Generating multicistronic MADR pDonor plasmids
Expression of Independent Transgenic Elements
Gene A
P2A
Gene B
Or
Fusion Gene
Gene A
Gene B
4X STOP
Donor Transgenic Element
Donor Plasmid
MADR pDonor plasmid assembly (Timing: 2-3 days)
MADR recombination and insertion
4X STOP
Donor Transgenic Element
Donor Plasmid
Recipient Genomic Locus
Promoter
RFP
GFP
In vivo electroporation-based delivery of MADR pDonor plasmid + FlpO-2A-Cre
In vitro transfection of MADR pDonor plasmid + FlpO-2A-Cre
Or
WT
Cre/W
Insertion
(Timing: 3-7 days)
(Timing: 60-90 minutes)
MOSAIC ANALYSIS with DUAL RECOMBINASE-MEDIATED CASSETTE EXCHANGE (MADR)

This protocol focuses on the cloning and stable integration of sequences of interest by the integration of a mosaic analysis with dual recombinases (MADR) plasmid that includes fusion proteins or independent proteins under the control of 2A peptide or IRES elements. Additionally, we describe how to generate a neural stem cell culture from Gt(ROSA)26Sort$^{m4(ACTB\text{-}tdTomato,\ EGFP)Luo/}$J mice, and validate the MADR plasmids *in vitro* and *in vivo* by neonatal mouse brain electroporation. This protocol can be generalized to analyze any transgenic element using MADR technology.

David Rincon Fernandez Pacheco, Sara Sabet, Joshua J. Breunig

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HIGHLIGHTS

MADR allows single or dual copy somatic transgenesis in cells and tissues

Details of the cloning of sequences of interest into a MADR plasmid

Outlines MADR plasmid insertion into primary cell lines by nucleofection (*in vitro*)

Details procedure for MADR plasmid insertion via brain electroporation (*in vivo*)

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Protocol

Preparation, Assembly, and Transduction of Transgenic Elements Using Mosaic Analysis with Dual Recombinases (MADR)

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SUMMARY

This protocol focuses on the cloning and stable integration of sequences of interest by the use of a mosaic analysis with dual recombinases (MADR) plasmid that includes fusion proteins or independent proteins under the control of 2A peptide or IRES elements. Additionally, we describe how to generate a neural stem cell culture from Gt(ROSA)26Sor$^{tm4(ACTB\text{-}tdTomato,\ EGFP)Luo/}$J mice, and validate the MADR plasmids *in vitro* and *in vivo* by neonatal mouse brain electroporation. This protocol can be generalized to analyze any transgenic element using MADR technology.

For complete details on the use and execution of this protocol, please refer to Kim et al. (2019).

BEFORE YOU BEGIN

Neural Stem Cell Primary Culture Generation

Timing: 1–2 weeks

Any primary line or self-renewing cell line that is amenable to transfection or electrofection (i.e., nucleofection or electroporation) can be employed as a platform for *in vitro* validation of MADR pDonor plasmids or experimental development. On this protocol we focused on neonatal brain neural stem cell primary culture:

1. Prepare in a 50 mL conical tube fresh Collagenase IV dissociation buffer, filter with a Steriflip and maintain in ice until use:

Collagenase IV	32 mg
Trypsin inhibitor	20 mg
DNase I	10 mg
PBS	10 mL

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2. Prepare Basal cell culture media, with the following recipe and filter:

Neurobasal media	500 mL
50× B27 supplement without vitamin A	10 mL
100× Penicillin-streptomycin-amphotericin	5 mL
100× Glutamax	5 mL

3. Prepare working media by adding to 50 mL of Basal media: EGF to a final concentration of 20 ng/mL), FGF (20 ng/mL) and heparin (2 μg/mL) and filter.
4. Prepare a CELLstart coated flask by diluting CELLstart in dPBS at a 1:50 ratio and incubating at 37°C for 2 h prior to plating cells. CELLstart can be aspirated off and replaced with media right before plating cells.

   Note: Collagenase IV cocktail and Basal media can be stored at 4°C for 1 month in sterile conditions. Working media can be stored at 4°C for a week in sterile conditions.

5. After euthanization of neonatal (P0-P7) heterozygous mice Gt(ROSA)26Sort$^{m4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J (or any other MADR acceptor mice), harvest the brain (5 min):
   a. Gently peel the skin and open the skull to expose the brain
   b. Detach the brain from the skull sectioning the optic nerve and carefully releasing the olfactory bulbs from the meninges (which tend to get attached to them)
   c. Wash the brain with cold PBS to eliminate blood
   d. Discard the cerebellum by cutting it with a scalpel or razor blade

6. Dissociate the cerebrum by physical and enzymatic methods:
   a. With 2 scalpels using a scissor-like motion cut the tissue in pieces <1 mm^3
   b. Collect the tissue in 4–5 mL of PBS and gently pellet the fragments in a 5 mL conical tube (300 × *g*, 5 min)

   Note: While ice cold PBS and prechilled centrifuge can be recommended, when there is concern about the viability of the cells during the procedure, we have not found any differences compared with room temperature (20°C–25°C) processing until this point.

   c. Eliminate the supernatant and add 3 mL of Collagenase IV cocktail

Incubate at 37°C in an orbital shaker for 45 min or until a high degree of dissociation is achieved (the dissociation become cloudy and tissue fragments disappear). Incubations with the Collagenase IV cocktail should not be increased over 45 min in order to avoid excessive damage to the dissociated cells.

7. Pre-wet a 40 μm cell strainer on a 50 mL conical tube with 1 mL of PBS.
8. Filter the dissociation through the 40 μm cell strainer, and wash the strainer with 10 mL of PBS.
9. Centrifuge at 500 × *g* for 5 min, discard supernatant, and resuspend cells in 5 mL working media.
10. Count the cells with a cell counter or hematocytometer, and seed cells in a CELLstart coated flask or plate at a cell density of 20,000–40,000 cells/cm^2.
11. Change media after 24 h, washing with PBS to eliminate debris and dead cells.
12. After a couple of passages (where it is recommended to use gentle cell detaching reagent such as Tryp-LE, or non-enzymatic cell detaching solutions) or when high viability is achieved, the cells can be used for MADR reaction.

Note: The cell culture generated with this method will be composed of a mixture of neural stem cells and differentiated cells. However, after a couple of passages, differentiated cells (astrocytes, microglia, neurons, etc.) will be washed out due to the expansion of neural stem cells.

Before You Begin *In Vivo* Electroporation

Timing: 5–20 min

13. Prepare electroporation (EP) mix
    a. Prepare electroporation mix in a PCR tube. Mix should be composed of a MADR donor plasmid and pCAG FlpO-2A-Cre plasmids for integration (we recommend plasmid preparation using a molar ratio of 0.1:1 of pCAG FlpO-2A-Cre : MADR pDonor plasmid). Mix should also include 5% fast green as a visual aid during injection
    b. Quick spin down the mix to remove any bubbles
14. Prepare Electroporator
    a. Plug in and turn on electroporator. Make sure the current is set at desired voltage (for postnatal ventricular EP we use 120 mV with 5 pulses at 50 ms)
    b. Plug in respective diode size for your mice. Prepare the diodes by adding a small amount of electrode gel onto the respective paddles
15. Prepare the microinjector
    a. Plug in and turn on the microinjector. Check that tubing is properly fitted in port and there are no kinks.
    b. Make sure the pressure settings are set at Hold:0, Injection Pressure (middle) 140 hPA efflux (this number will vary depending on how short you trim your needle tip and, thus, the diameter of the opening).
16. Prepare Glass Tips
    a. Using Sutter Instrument Co. tip puller, set heat settings at Heat#1 at 880, Heat#2 at 680 and pull at 800.
    b. Fit capillary tube in holder and pull needle tip. Discard the top pulled portion, keep bottom tip taking care to not break tip.
    c. Trim tips: Tips need to be trimmed prior to injection. We recommend the tips are trimmed just before injection to prevent breakage (see Troubleshooting).
    d. Insert tip into micropipette holder taking care securing it by twisting the tip in.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Bacterial and Virus Strains		
Stellar chemically competent cells for cloning	Clontech	Cat# 636766
Chemicals, Peptides, and Recombinant Proteins		
Human epidermal growth factor	Sigma-Aldrich	Cat# E9644
Heparin solution 0.2%	STEMCELL Technologies	Cat# 07980
Basic fibroblast growth factor (bFGF)	Millipore	Cat# GF003
Collagenase IV	Worthington Biochemical	Cat# LS004189
DNase I	Worthington Biochemical	Cat# LS002007
Neurobasal media	Thermo Fisher Scientific	Cat# 21103049
Trypsin inhibitor	N/A	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Penicillin-streptomycin-amphotericin	Thermo Fisher Scientific	Cat# 15240096
B-27 supplement without vitamin A	Thermo Fisher Scientific	Cat# A3353501
Glutamax	Thermo Fisher Scientific	Cat# 35050061
CELLstart CST	Thermo Fisher Scientific	Cat# A10142-01
Tris-EDTA buffer	Sigma-Aldrich	Cat# E8008-100ML
Fast green dye	Sigma-Aldrich	Cat# F7258-25 g
SignaGel electrode gel	Medline Industries	Cat# PLI1525CSZ
PBS 1×	N/A	N/A
SOC media	N/A	N/A
Critical Commercial Assays		
NEBuilder HiFi DNA Assembly Master Mix	New England Biolabs	Cat# E2621L
EcoRI-HF	New England Biolabs	Cat# R3101S
EagI-HF	New England Biolabs	Cat# R3505S
NotI-HF	New England Biolabs	Cat# R3189S
KAPA HiFi PCR reagents	Kapabiosystems	Cat# KR0368
Mouse Neural Stem Cell Nucleofector Kit	Lonza	Cat# VPG-1004
NucleoSpin Gel and PCR Clean-up	Takara Bio	Cat# 740609.250
Experimental Models: Organisms/Strains		
Mouse: Heterozygous Gt(ROSA)26Sortm4 (ACTB-tdTomato,-EGFP)Luo/J	The Jackson Laboratory	JAX: 007676
Oligonucleotides		
RMCEseq-F primer (TCGACCTGCAGCCCAAGCTA)	N/A	N/A
Addgene WPRE-R primer (CATAGCGTAAAAGGAGCAACA)	N/A	N/A
Recombinant DNA		
MADR pDonor TagBFP2-3xFlag (cyto) WPRE	Addgene	Cat# 129421
pCAG FlpO-2A-Cre EV	Addgene	Cat# 129419
Other		
MilliporeSigma Steriflip sterile disposable vacuum filter	Fisher Scientific	Cat# SCGP00525
Sutter Instrument	Zenoworks Digital Microinjector	Cat# BRE
Borosilicate glass capillaries	Kwik-Fil	Lot# 2010322
ECM 830 Square Wave Electroporation system	Hardvard Apparatus	W3 45-0052
Nucleofector 2B	Lonza	Cat# AAB-1001
Ice bucket	N/A	N/A
Heat lamp or heat pad	N/A	N/A

MATERIALS AND EQUIPMENT

Synthetized 2A or IRES sequences in case of multiple sequences cloning. Alternatively, sources for PCR of these sequences are:

Element to Clone	Plasmid Name	Source	Identifier
IRES element	pEF1a-IRES-NEO	Addgene	#28019
T2A element	pCAG FlpO-2A-Cre EV	Addgene	#129419
P2A element (includes codon alternated variants to avoid recombination)	MADR pDonor-H3F3A-K27M-EGFP pTV1 Pdgfra D842V COTv1 Trp53-V5 WPRE	Addgene	#131462

1. Ampicillin-agar plates (100 μg/mL)
2. Ice
3. Pipettors
4. Dissection scissors
5. Scalpel
6. Razor blade

Alternatives: "Proxy" cell lines genetically engineered to be MADR acceptor can be used as an alternative of primary cell cultures for *in vitro* validation (see (Ayala-Sarmiento et al., 2020) accompanying STAR Protocol for methods and discussion).

Alternatives: A heat pad can be used instead of a heat lamp; any sharp scissors can be used to trim the needle tips.

STEP-BY-STEP METHOD DETAILS

MADR Plasmid Cloning: Primer Design for Cloning of Sequences of Interest

⏲ Timing: 1–5 days (contingent on time necessary for some of the steps)

This step describes the specific steps for primer design.

1. Determine the order of the sequences to be cloned together in MADR pDonor TagBFP2-3xFlag (cyto) WPRE (Figure 1A).

   Note: Different IRES elements can promote different ratios of translation of the proteins in the bicistronic mRNA (Koh et al., 2013). On the other hand, 2A elements while maintaining a similar ratio of translation add a 19 amino acid tag in the C-terminal region of the 5′ protein and a proline residue in the N-terminal region of the 3′ protein (Liu et al., 2017). Therefore, possible artifacts resulting from the 19 amino acid tag, N-terminal proline, or the effects of a differential ratio must be case by case studied to determine the best sequences order and selection of 2A/IRES elements. This is often a key consideration when choosing ordering of elements in tandem. For example, proteins recalcitrant to effects of the addition of 19 amino acids on the C-terminus should be first in a P2A-linked series, while sensitive proteins should be last as the additional proline is often cleaved without altering function.

2. Design primers for the 5′ sequence to be cloned in the MADR pDonor plasmid:
   a. Forward primer (**Fw1**) should add an overhang which overlap with the MADR pDonor TagBFP2-3xFlag (cyto) WPRE sequence immediately upstream of EcoRI restriction enzyme sequence (CTTATCGATACCGTCGACCTCGAGGGGGGG) (Figure 1B).
   b. Reverse primer should add an overhang homologous to the 5′ sequence of the downstream sequence (the following fusion protein (**Rv1**) or the 2A/IRES element (**Rv1′**)) (Figure 1B).

   ⚠ CRITICAL: In case of cloning fusion protein or using 2A elements with protein-coding sequences, the 5′ cloned sequence must lack the stop codon to allow for the translation of downstream sequences. On the other hand, while using IRES elements, stop codons must be maintained in place to ensure proper translation.

3. Design primers for the following 3′ sequence to be cloned in the MADR pDonor plasmid:
   a. Forward primer should add an overhang which overlap with the upstream sequence (5′ sequence to clone in the MADR pDonor plasmid (**Fw2**) or 3′ sequence of the 2A/IRES element (**Fw2′**)) (Figure 1B).

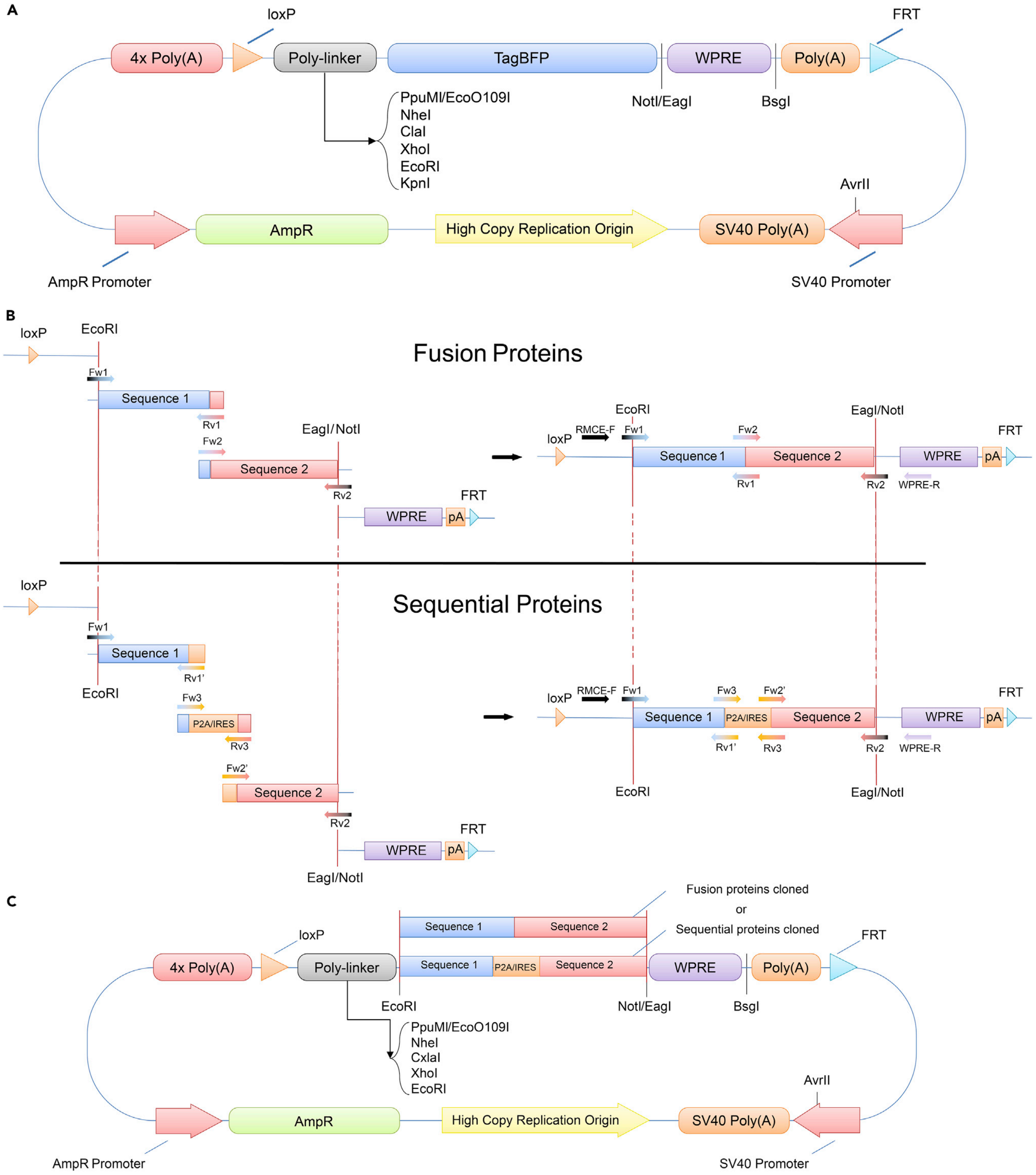
A
loxP
FRT
4x Poly(A)
Poly-linker
TagBFP
WPRE
Poly(A)
PpuMI/EcoO109I
NheI
ClaI
XhoI
EcoRI
KpnI
NotI/EagI
BsgI
AvrII
AmpR
High Copy Replication Origin
SV40 Poly(A)
AmpR Promoter
SV40 Promoter
B
loxP
EcoRI
Fusion Proteins
Fw1
Sequence 1
Rv1
Fw2
EagI/NotI
Sequence 2
Rv2
WPRE
pA
FRT
RMCE-F
Fw1
Fw2
Sequence 1
Sequence 2
EagI/NotI
Rv1
Rv2
WPRE-R
loxP
Sequential Proteins
Fw1
Sequence 1
Rv1'
EcoRI
Fw3
P2A/IRES
Rv3
Fw2'
Sequence 2
Rv2
WPRE
pA
FRT
EagI/NotI
RMCE-F
Fw1
Fw3
Fw2'
Sequence 1
P2A/IRES
Sequence 2
Rv1'
Rv3
Rv2
WPRE-R
EcoRI
EagI/NotI
C
Fusion proteins cloned
or
Sequential proteins cloned
loxP
FRT
Sequence 1
Sequence 2
4x Poly(A)
Poly-linker
Sequence 1
P2A/IRES
Sequence 2
WPRE
Poly(A)
EcoRI
NotI/EagI
BsgI
PpuMI/EcoO109I
NheI
CxlaI
XhoI
EcoRI
AvrII
AmpR
High Copy Replication Origin
SV40 Poly(A)
AmpR Promoter
SV40 Promoter

Figure 1. pDonor Cloning Strategy

(A) MADR pDonor TagBFP2-3xFlag (cyto) WPRE schematic representation.

(B) Cloning strategy for fusion and sequential proteins.

(C) Final MADR pDonor plasmid resultant of the cloning process

  b. Reverse primer (**Rv2**) should add an overhang which overlap with the MADR pDonor TagBFP2-3xFlag (cyto) WPRE sequence immediately downstream of EagI/NotI restriction enzymes targets sequence (GAGGTTGATTAGCACCTGAGGAGTGCGGCC) (Figure 1B).

Note: **Rv1** and **Fw2** primer can be complementary sequences in case of fusion protein cloning.

4. Design primers for amplifying 2A/IRES elements, 2A and IRES sequences can be synthetized or subcloned from other plasmids using overlapping primers with each sequence to be included:
   a. Forward primer (**Fw3**) must be comprised of an overhang of ~15 bp corresponding to the 3′ end of the upstream sequence to be cloned (not including the stop codon in case of using 2A elements) followed by the sequence corresponding to the 5′ region of the 2A or IRES element (Figure 1B).
   b. Reverse primer (**Rv3**) must have an overhang of ~15 bp homologous to the 5′ region of the downstream sequence ending to be cloned followed by the complementary sequence to the 3′ region of the 2A or IRES element (Figure 1B).

Note: **Fw3** and **Rv1**′ as well as **Rv3** and **Fw2**′ primers can be complementary sequences. Table 1 contains primer designed to amplify 2A and IRES elements with a Tm of ~50°C. This sequences can be combined with specific overhangs to generate Fw3 and Rv3 primers.

Note: Differences in cleavage efficiency between P2A and T2A elements have been noted in the bibliography. In our hands, P2A sequences showed higher cleavage efficiency as reported by others (Kim et al., 2011). However, these results are inconsistent with findings in other laboratories (Liu et al., 2017). Moreover, it is important to note that in case of using more than one P2A element in the cloning design (e.g., cloning three or more protein sequentially), codon alternated variants should be employed to avoid recombination (Szymczak-Workman et al., 2012). Within the suggested source for P2A elements (Addgene #131462), two P2A sequences with codon alternation can be found.

⚠ CRITICAL: Cloned sequences must be promoter-less since MADR technology relies in an endogenous promoter to induce the expression in only recombined cells avoiding episomal expression. Fusion proteins and 2A elements must be in frame to ensure expression of downstream proteins.

PCR of Sequences of Interest from cDNA or Commercially Available Plasmids

Timing: >3 h (time depends on the PCR fragments sizes to be amplified)

5. Using a cDNA as a template, reverse transcription of an RNA extraction of a sample expressing the sequence of interest, or a plasmid containing the sequences of interest (mRNA, lncRNA, miRNA, 2A, or IRES elements, etc.) (Figure 2) prepare each PCR reaction in 200 μL tubes as follows:
   a. 12.5 μL of KAPA HiFi Mastermix in each PCR reaction
   b. 1 μL of each primer (10 μM stock)
   c. 5–100 ng of sequence template
   d. Complete the volume with nuclease free water until 25 μL
6. Setup the PCR reaction in the thermocycler using as general guidelines the following scheme:

Step	Temperature	Time
DNA denaturing	98°C	1–5 min (depending on the template size)
Annealing*	Primer dependent	30 s

(*Continued on next page*)

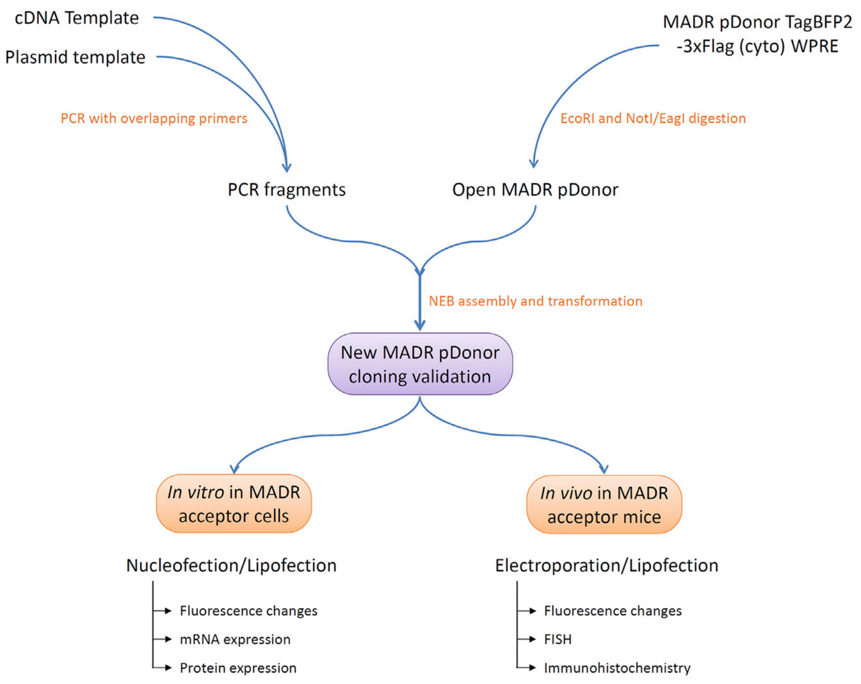
cDNA Template
Plasmid template
PCR with overlapping primers
MADR pDonor TagBFP2 -3xFlag (cyto) WPRE
EcoRI and NotI/EagI digestion
PCR fragments
Open MADR pDonor
NEB assembly and transformation
New MADR pDonor cloning validation
In vitro in MADR acceptor cells
In vivo in MADR acceptor mice
Nucleofection/Lipofection
Fluorescence changes
mRNA expression
Protein expression
Electroporation/Lipofection
Fluorescence changes
FISH
Immunohistochemistry

Figure 2. pDonor Plasmid Generation and General Schematic

Protocol steps schematic: MADR pDonor TagBFP2-3Flag (cyto) WPRE plasmid can be opened by EcoRI and NotI digestion and used as a backbone to clone the sequences of interest which can be obtain by PCR from a cDNA from a source of expression of the sequence of interest or from a plasmid where the sequence was already cloned. By NEB assembly the new MADR pDonor containing the sequences of interest can be generated and validated both *in vitro* and *in vivo*

Continued

Step	Temperature	Time
Elongation*	72°C	1 min/kb of sequence to amplify
DNA denaturing*	98°C	30 s
Final elongation	72°C	5 min
Cool down	4°C	Until recover of samples

*35–40 cycles.

7. Confirm by agarose gel electrophoresis the presence of the bands at the specific sizes.
8. Purify bands with NucleoSpin Gel & PCR Clean-up kit following manufacturer instructions (Figure 2)

Note: Overhangs in the primers can interfere with PCR reaction; to avoid inefficient reactions, annealing temperature must be carefully set (usually by increasing the annealing temperature 1°C–5°C).The setting of this temperature is especially important when amplifying from cDNA samples where similar sequences can promote unspecific amplifications. In these cases, a two-step PCR reaction can be tried to correct PCR amplification. Alternatively, in highly complex sequence structures or complex gene rearrangements it would be advisable to turn to commercial services of DNA synthesis to sped up the generation of those fragments.

Digestion of MADR pDonor TagBFP2-3xFlag (cyto) WPRE

Timing: 2 h

9. Digest 3 μg of MADR pDonor TagBFP2-3xFlag (cyto) WPRE with EcoRI and NotI/EagI at 37°C for 1.5 h.
10. Confirm by agarose gel electrophoresis the proper digestion. Two distinct bands should appear
    a. TagBFP digested sequence band of 805 bp
    b. MADR pDonor plasmid of 5,107 bp
11. Isolate and purify MADR pDonor band (5,107 bp) with NucleoSpin Gel and PCR Clean-up following manufacturer instructions (Figure 2).

Note: Alternative restriction enzymes can be used to linearize the MADR pDonor plasmid (Figure 1A), but to simplify the protocol we have focused on EcoRI and EagI/NotI restriction enzyme sites which flank the TagBFP sequence. In case of using other restriction enzymes, overhangs in the primers **Fw1** and **Rv2** must be modified to ensure the proper overlap with the digested MADR pDonor plasmid.

HiFi Assembly and Transformation of MADR pDonor Plasmid

Timing: 2–3 days

Alternatives: While a number of different cloning methods can be applied, this protocol recommends the use of HiFi DNA Assembly or Gibson assembly technologies to avoid problems derived from the use of other classical methods (such as restriction enzymes directed cloning), where only restriction enzymes present in the plasmid poly-linkers that do not cut the sequences of interest can be used while digesting both the plasmid and the sequences of interest. Moreover, using restriction enzymes becomes increasingly difficult when building fusion proteins or including a high number of sequences within the same plasmid. Gibson assembly technology simplifies the process of cloning and is highly recommended in this case.

12. Following manufacturer instruction, perform HiFi DNA Assembly of the digested plasmid and PCRs of the sequences to be cloned together with the sequences of IRES or 2A elements. In brief:
    a. Prepare assembly reaction in a 200 μL tube:
        - 10 μL of HiFi DNA assembly mastermix
        - 50–100 ng of purified MADR pDonor band
        - 1:2 molar ratio of each of the sequences to clone (or a 1:5 ratio if there are more than 5 sequence to insert in the plasmid at the same time)
        - Complete volume with nuclease free water to 20 μL

Note: To calculate the quantity of PCRs sequences to use you must consider the size of each fragment compared with the plasmid to clone into. For example, our digested plasmid has a size of ~5,000 bp, if the sequence to be inserted has a size of ~100 bp, to fit a ratio of 1:2 plasmid:sequence, you must add 4 ng of your PCR sequence for each 100 ng of digested plasmid. See following equation:

$$\text{Sequence}_{\text{mass}} = \text{Plasmid}_{\text{mass}} \times \frac{\text{Sequence}_{\text{ratio}}}{\text{Plasmid}_{\text{ratio}}} \times \frac{\text{Sequence}_{\text{size}}}{\text{Plasmid}_{\text{size}}}$$

    b. Incubate the assembly reaction at 50°C for 1 h and store at 4°C until use

13. Transform competent bacteria with the assembly:
    a. Thaw one aliquot of Stellar competent bacteria on ice
    b. Mix 50 μL of stellar competent bacteria with 2 μL of the assembly reaction and incubate 20 min on ice

   c. Heat shock at 42°C for 45 s
   d. Incubate 2 min on ice
   e. Dilute transformation with 500 μL of SOC media and incubate with shaking for 45 min at 37°C
   f. Seed 100 μL of bacteria culture in an ampicillin-agar plate and incubate O/N at 37°C
14. Select two or more colonies of the agar plate avoiding overgrowth or mixed colonies and miniprep following manufacturer instructions or validate by PCR the presence of the inserts in the plasmid and then miniprep (Figure 2).
15. Validate the cloned sequence integrity with specific primers for the inserted sequences (Figure 1C).
    a. **RMCE-F primer** (TCGACCTGCAGCCCAAGCTA) can be used to validate the inserted sequences from the 5′-end (Figure 1B).
    b. **Addgene WPRE-R primer** (CATAGCGTAAAAGGAGCAACA) can be used to validate the inserted sequences from the 3′ end (Figure 1B).
    c. In case the inserted sequences cannot be completely validated with RCMEseq-F and Addgene WPRE-R primers internal primers must be designed to complete the sequencing.
16. Validation of the proper expression of the cloned sequences can be done *in vitro* in MADR acceptor cell lines by lipofection or nucleofection or *in vivo* by electroporation.

MADR Reaction *In Vitro* in a Primary Cell Culture

Timing: 3–7 days

17. Incubate the neural stem cells primary culture until reach 90% confluence in a T25 CELLstart CST coated flask.

Note: Alternatively, proxy cell lines can be used. Primary cell cultures are especially refractory to lipofection, therefore nucleofection is recommended to achieve a successful MADR reaction. Original MADR pDonor TagBFP2-3xFlag (cyto) WPRE plasmid can be used in parallel as positive control of MADR reaction.

18. Collect cells:
    a) Remove media and incubate cell culture with 1.5 mL of Tryp-LE solution until detachment.
    b) Add 6 mL of PBS and wash flask surface collecting the cells in a 15 mL conical tube
    c) Centrifuge at 500 × *g* for 4 min.
    d) Discard supernatant.
19. Create a plasmid mix with 2–5 μg of the cloned MADR pDonor plasmid and pCAG FlpO-2A-Cre EV with a ratio of 1:0.1.
20. Nucleofect neural stem cells with the plasmid mix using the Lonza mouse neural stem cell nucleofector kit in a Lonza nucleofector with the program A-033 (''Mouse NSC'') following manufacturer instruction.
21. After nucleofection add 0.5 mL of cell culture media to the nucleofection cuvette and incubate at 20-25°C for 30 min
22. Seed nucleofected cells in a freshly CELLstart CST coated T25 flask and incubate cells for 48 h.

Note: Nucleofection can induce strong cell death; it is recommended to change the media after 24 h if there is a significant cell death to avoid a second wave of death in the cell culture.

23. Depending of the MADR pDonor plasmid cloned, confirm recombination by the expression of new fluorescent markers and the lack of TdTomato and EGFP membrane fluorescence in recombining cells (Figures 3A and 3B).
24. Purify recombined cells by FAC sort against red and green fluorescence and/or by the fluorescent protein cloned in the MADR pDonor plasmid. Alternatively, antibiotic resistance cassettes can be included in the MADR pDonor plasmid cloning for culture selection.

Note: MADR reaction with MADR pDonor plasmids which codify for green or red fluorescent reporters can be validated in TagBFP2 or dark proxy cell lines which have been engineered to lack both TdTomato and EGFP expression (Ayala-Sarmiento et al., 2020).

25. Quantitative and/or real time PCR and/or Western blot, as well as any other quantitative or semi-quantitative technique such as immunohistochemistry (IHC), single cell western blot or fluorescent *in situ* hybridization (FISH), can be used to validate and quantify proper expression levels of the cloned proteins. While western blot or IHC must rely on antibodies against the specific cloned proteins, RNA based detections can be directed against the WPRE sequence, which would be common to any cloning following the present guidelines.

MADR Reaction *In Vivo* through Brain Electroporation

⏲ Timing: 20–60 min (depending on the number of mice and efficiency)

Mouse Pups Preparation for Electroporation

⏲ Timing: 5–10 min

26. Mouse pups need to be properly anesthetized on ice
    a) Place the number of P0-P2 mice you would like to electroporate in a small box on ice. Placing them prone on their stomachs speeds up the anesthesia process.
    b) Mice will be fully anesthetized when tail tip pinch elicits no response.

⚠ CRITICAL: Avoid maintaining the pups for extended periods of time on ice. No more than 10 min should be necessary to ensure pup anesthesia.

Prepare Glass Pipette for Injection

⏲ Timing: 1–5 min

27. Using the pulled needle fitted into the microinjector system, lay PCR tube with EP mix on side and guide the needle into the tube taking care to fully immerse the tip into solution, but not allowing the tip to touch the end of the tube (which causes needle breakage). ***Note:*** Tips should be trimmed (see Before You Begin In Vivo Electroporation) to guarantee enough pressure for successful injection. The closer the tip is trimmed to the base, the lower pressure needed to inject (see Troubleshooting). A representative trimmed tip is showed in Figure 4A.
28. Aspirate EP mix into tip **slowly** by turning the pressure gauge (right-most) so the numbers become more negative (range will be −10 to −75 depending on how viscous EP mix is). Using your eyes as guidance, monitor the mix being aspirated up into the pulled needle, making sure it does not go past the needle into the microinjector.
    a. Be careful not to include any bubbles as those can damage the microinjector, and can be fatal for pups being injected
29. Check that one microinjection pulse emits 1 μL. This can be done by injecting a small amount of liquid into the negative space of a "6" or "9" on a piece of parafilm (Figure 4B). Change pressure in iterations of 20 hPA on injector until 1 μL of solution is ejected.

⚠ CRITICAL: If using this moment as a pause point, it is important to make sure the tip does not dry out, causing blockage in injection later on. This can be done by either swirling the tip of the glass needle in the EP mix periodically, or periodically injecting. If you have waited a significant period of time (>10 min) check there is no blockage in the tip by test-injecting onto parafilm.

⏸ **Pause Point:** This moment can be a pause point in case the mice are still not anesthetized

Inject Mice with Pulled Pipette Tip

⏲ Timing: 1–3 min

30. Insert needle targeting one of the two lateral ventricles, using lambda as a guideline for injection, and keeping needle perpendicular to skull, penetrate skull with needle ~2.5 mm, until there is little resistance (Figure 4C)
    c) Inject EP mix into the ventricle by pressing the corresponding foot paddle.
    d) Check ventricle under light to see that there is fast green that can be seen in the ventricle.

Electroporate Mice

⏲ Timing: 1–3 min

31. Set Signa gel coated paddles on the outside of the pup's skull, sitting just behind its eyes (Figure 4D), make sure the positive electrode is on the outside of the ventricle you are electroporating (IE if you are electroporating the outside of the left ventricle, the positive electrode should be on the left side of the mouse).

⚠ CRITICAL: Make sure to keep your hands clear of the electrode, as well as keep the pup's paws clear of its face. This prevents any short circuit in the electrical pulse for the user as well as the pup.

32. Press corresponding foot paddle to start the electroporation, sweeping the positive paddle laterally along the skull (or in the direction desired to capture the region of interest). Continue to sweep paddle(s) until the pulses have completed.

Note: You will feel slight pulsing in the mouse's body as you are electroporating, this guarantees that you are getting current to the region of interest.

Recover Mice

⏲ Timing: 5–10 min

33. Move pups under a heat lamp for recovery
34. Pups are ready to be returned to their cage when their coloring has returned back to a healthy pink, and there is reaction to tail tip pinching.

EXPECTED OUTCOMES

***In Vitro* MADR Reaction by Nucleofection**

Nucleofection of Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J Neural stem cells primary culture with MADR pDonor in combination with pCAG FlpO-2A-Cre EV promote the recombination of the cell genomic ROSA26 locus region with the sequences flanked by LoxP and FRT elements in the MADR pDonor plasmid.

The efficiency of the recombination measured as a percentage of the total number of cells nucleofected can change widely between experiments, but usually falls between 0.3 to 10% a week after nucleofection. Therefore, selection of cells by FAC sort or by the inclusion of antibiotic resistance and culture selection is necessary to obtain pure populations.

***In Vivo* MADR Reaction by Brain Ventricle Electroporation**

The number of MADR transduced cells is largely dictated by the concentration of the MADR donor, the concentration of FlpO and Cre recombinases, and the proliferation rate of the targeted populations (Kim et al., 2019). Specifically, the number of MADR cells versus Cre recombined cells can be titrated in a defined population by varying the ratio of donor plasmid to recombinase plasmid.

Under the conditions suggested in this protocol (0.1 μg/μL of FlpO-2A-Cre EV: 1 μg/μL of donor plasmid), a pattern emerges whereby MADR transduction inversely correlates with the initial mitotic activity of the cells. Specifically, striatal glial cells are readily Cre recombined but are more rarely MADR transduced. Conversely, the radial glial populations, which are relatively more quiescent given their role as bona fide neural stem cells, make up a major population of MADR cells. Notably, ependymal cells, which have been recently reported to be the result of terminal asymmetric or symmetric divisions (Ortiz-Álvarez et al., 2019) tend to be readily targeted by MADR -presumably due to the fact that they do not dilute the plasmids after the initial cell division targeted by electroporation.

The cell cycle of the CNS lengthens over development, and postnatal cells are relatively more quiescent than their embryonic counterparts so smaller initial populations are typically transduced by postnatal electroporation. Thus, if large numbers of parenchymal glia or embryonically generated neurons are desired, *in utero* electroporation should be performed targeting the local region.

We have noted that the perdurance of FlpO and Cre leads to a transient potential for "hopping" in and out of the genome by the donor cargo until the FlpO and Cre are diluted by subsequent cell divisions. Because the donor DNA copy numbers are presumably more substantial than the single-copy recipient transgene locus, insertion is typically favored. However, this necessitates that the titration of FlpO-2A-Cre and Donor plasmids and their relative ratios be determined empirically based on the desired ratio of starting populations (recombination only versus donor insertions). To reduce this "hopping," and reduce the percentage of double labeled cells we would advise using a self-excising FlpO-2A-Cre (Addgene Plasmid #130986). This construct presents FlpO and Cre surrounded by FRT sites, to avoid bacterial self-recombination during DNA production the FlpO contains an intron blocking its expression in prokaryotic cells. In this case, it must be taken in to account that the lower half-life of the self-excising FlpO-2A-Cre will lead to a lower number of recombined cells compared with the standard FlpO-2A-Cre but will greatly reduce the number of double marked cells.

Transient reporter ambiguities can be seen in more quiescent populations such as ependymal cells. Specifically, double positive (Cre reporter and MADR reporter) cells are more frequent over the initial days and weeks post electroporation. We infer that this is potentially due to several factors including 1) ependymal cells are generated by terminal divisions, thus, trapping higher initial amounts of plasmids, and 2) the slower metabolism intrinsic to ependymal cells (Llorens-Bobadilla et al., 2015). Notably, though we have not empirically determined if this is the case, we observe a slow diminishment of mG EGFP over time (2 weeks) indicative of a much longer half-life compared with standard cytoplasmic EGFP. Again, such reporter ambiguities due to protein perdurance in different compartments are often intrinsic to fluorescent proteins as was observed in the initial report of the mT/mG mice (Muzumdar et al., 2007).

LIMITATIONS

Plasmid Size and Transformation

While no size limitation has been found in our experience for MADR recombination in mice and cell lines, the increase in the size and complexity of the sequences cloned in the MADR pDonor can reduce the efficiency of transformation in bacteria or promote recombination. This is a general issue in bacteria transformation and can be reduced by traditional methods such as:

1. Using electro-competent bacteria stocks
2. Using low recombinant bacteria stocks such as Stbl3

3. Incubating bacteria transformation at 30°C O/N instead of 37°C to reduce bacterial recombination

MADR Acceptor Cells and Animals

MADR technology relies in the recombination with endogenous elements of the acceptor cells. Therefore, only cells containing an acceptor cassette are suitable for this technology. The minimal acceptor cassette must be comprised of:

1. **Endogenous promoter**: MADR pDonor plasmids are promoter-less to limit expression to recombined cells.
2. **LoxP and FRT sites**: While recombinases can be modified, the basic MADR tool relies in Cre and FlpO activity to promote recombination (Figure 3A). These sequences must be in the correct direction to ensure recombination. We have tested inverted LoxP sites as negative control, yielding virtually no transgene expression.
   a) LoxP sequence: 5′-ATAACTTCGTATAGCATACATTATACGAAGTTAT-3′
   b) FRT sequence: 5′-GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC-3′
3. Optional: **Fluorescent reporters** within LoxP and FRT sequences as a method to visually check MADR reaction and FAC sort recombined cells.

The cassette presents in our basic MADR pDonor is developed to work with Gt(ROSA)26Sort$^{m4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J mice cells cassette. In case of using mice or cell lines with altered recombination sites those changes must be paired in the MADR pDonor plasmid. Generation of MADR technology acceptor Proxy Cell lines as well as its optimization and adjustment to different experimental designs are detailed in (Ayala-Sarmiento et al., 2020) accompanying STAR Protocol.

Electroporation

It should be noted that electroporation is viable in different regions of dividing cells in both the CNS and the rest of the body. This protocol outlines specifically targeting a proliferating niche within the neonatal ventricular zone, and thus is limited to the cell types that present active mitosis in the moment of electroporation. Nuclear pore dilatators can be used to electroporate post-mitotic cell. Alternatively, *in utero* electroporation can be used to target cells which typically are post-mitotic at P0-P2 by transducing their progenitor cells.

Finally, electroporation is focal and is best for mosaic generation from a defined population of cells. For cell or tissue-wide expression or broader mosaic control, the use of more traditional Cre lineage tracing (Breunig et al., 2007) or mosaic methods such as MADM (Beattie et al., 2020) are advised.

TROUBLESHOOTING

Problem 1

Low ratio of MADR recombination vs Cre activity

Potential Solution

Ratio between MADR pDonor and FlpO and Cre recombinases must be carefully adjusted. *In vivo* experiments show a higher efficiency of insertion in a ratio of 0.1:1 FlpO-2A-Cre:MADR pDonor (Kim et al., 2019). However, total number of cells recombined gets reduced while reducing the total mass of FlpO-2A-Cre plasmid on the mix.

Problem 2

Inconsistent expression of the MADR acceptor cassette

Potential Solution

The expression of the acceptor cassette recombined in proxy cells and presents in primary cultures of cells from heterozygous Gt(ROSA)26Sort$^{m4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J mice is under the control of a

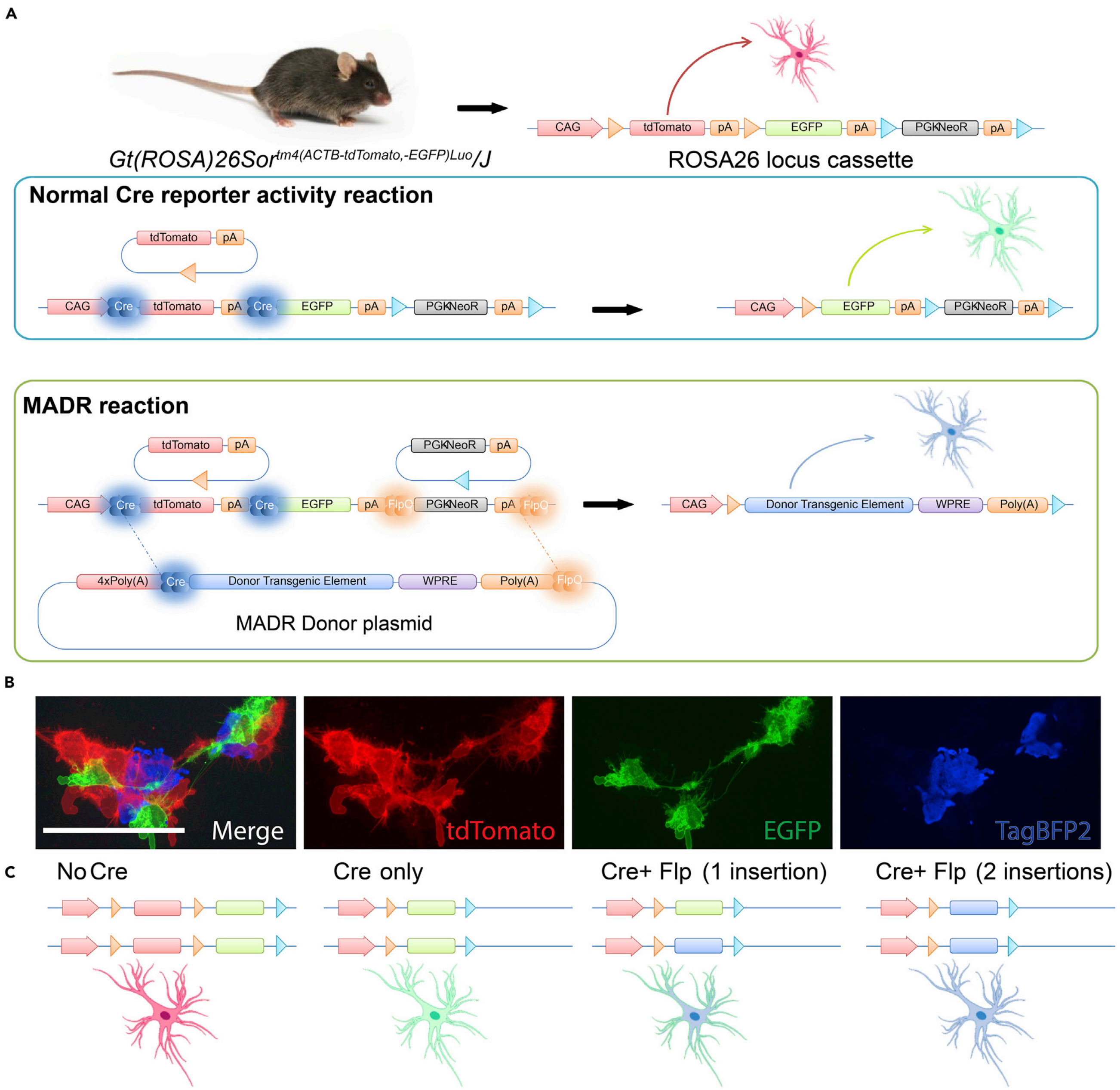
A
Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J
CAG
tdTomato
pA
EGFP
pA
PGKNeoR
pA
ROSA26 locus cassette
Normal Cre reporter activity reaction
tdTomato
pA
CAG
Cre
tdTomato
pA
Cre
EGFP
pA
PGKNeoR
pA
CAG
EGFP
pA
PGKNeoR
pA
MADR reaction
tdTomato
pA
PGKNeoR
pA
CAG
Cre
tdTomato
pA
Cre
EGFP
pA
FlpO
PGKNeoR
pA
FlpO
CAG
Donor Transgenic Element
WPRE
Poly(A)
4xPoly(A)
Cre
Donor Transgenic Element
WPRE
Poly(A)
FlpO
MADR Donor plasmid
B
Merge
tdTomato
EGFP
TagBFP2
C
No Cre
Cre only
Cre+ Flp (1 insertion)
Cre+ Flp (2 insertions)

Figure 3. MADR Reaction and Expected Results

(A) Gt(ROSA)26Sor$^{tm4(ACTB-td-tomato,-EGFP)Luo/J}$ mouse cassette structure during normal Cre reporter activity and MADR-mediated recombination reactions.

(B) Representative results of MADR recombination with MADR pDonor TagBFP2-3xFlag (cyto) WPRE plasmid *in vitro* in heterozygous MADR acceptor cells, resulting in only TdTomato, EGFP or TagBFP2 (heterozygous insertion) expression. Adapted with permission from Kim et al. (2019); copyright 2019 Elsevier.

(C) MADR reaction and expected results in homozygous MADR acceptor cells after MADR transfection with MADR pDonor TagBFP2-3xFlag (cyto) WPRE, resulting in TdTomato, EGFP or TagBFP (homozygous insertion) expression as well as EGFP+TagBFP2 combined expression (heterozygous insertion).

CAG promoter. This promoter has been reported to be altered in some cell types such as Lymphocytes (Baup et al., 2009). Moreover, methylation of CAG promoter can reduce the cassette expression (Zhou et al., 2014). To avoid this issue in specific cell types or mice, alternative promoters can be used to drive the expression of the cassette.

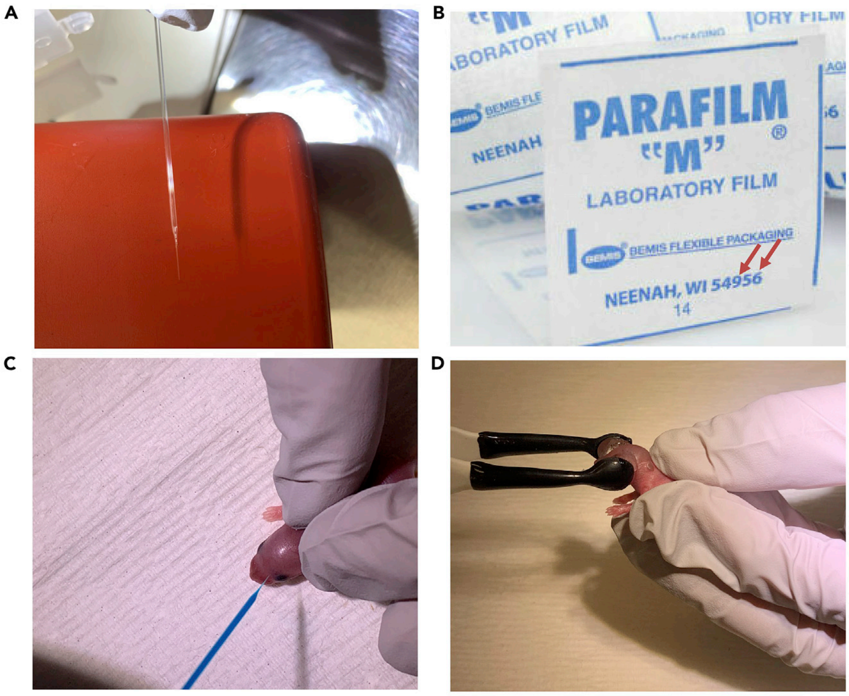

Figure 4. ***In Vivo* Electroporation Strategy**

(A) Tip should be trimmed with sufficient length to allow access to ventricle, but not so long that you risk it breaking during injection.

(B) 1 μL of plasmid preparation mixture should fit within the circular part of the "6" or "9" on a segment of Parafilm "M." (Creating a reference drop by pipetting this volume of the DNA mix with a P2 Pipette is recommended.)

(C) During injection, tip should be injected lateral to the skull.

(D) When setting EP paddles, paddles should rest slightly behind eyes in order to access the whole ventricle.

Alternative promoters driving FlpO-2A-Cre and/or the MADR cistron can also be used to ensure or avoid expression in specific cell types in experiments involving mixed populations (i.e., *in vitro* co-culture of different cell types or *in vivo* electroporation)

In some cases, high or supraphysiological expression of a given transgenic element may be desired. Addition of mRNA stabilization elements such as WPRE will increase expression of mRNA from the MADR locus. Further, in cases where gene amplification is to be modeled (e.g., cancer), one can multiplex MADR with piggyBac elements (Breunig et al., 2015; Kim et al., 2019).

Inconsistency between animals in a group all treated with the same mix—in the absence of gross neuropathological abnormality (e.g., hydrocephalus)—is most likely due to inconsistent handling by the electroporator. As with any method, practitioner consistency will increase with increased experience with the procedure. An additional means of increasing consistency within and across groups is to create an "assembly line" where several members of the lab perform the electroporation procedure together—each performing only one aspect. This also has the added benefit of increasing the numbers of animals that can be electroporated. By combining this with in vitro fertilization by a Rodent Genome Targeting core to synchronize the generation of pups, a small lab can readily electroporate hundreds of mice within a few hours.

Problem 3

Dual presence of MADR recombination and EGFP expression

Table 1. Primer Homologous Sequences for 2A and IRES Elements with Annealing Temperature Adjusted to ~50°C

Genetic Element	Plasmid	5′ primer sequence	3′ primer sequence
T2A	pCAG FlpO-2A-Cre EV	GAGGGCAGGGGAAGT	GGGCCCGGGATTTTCCT
P2A sequence 1	MADR pDonor-H3F3A-K27M-EGFP pTV1 Pdgfra D842V COTv1 Trp53-V5 WPRE	GCCACGAACTTCTCT	AGGACCGGGGTTTTC
P2A sequence 2		GCAACCAATTTTTCACTC	AGGCCCAGGATTCTC
IRES	pEF1a-IRES-NEO	ACGTTACTGGCCGAA	CTTTGAAAAACACGATGATAAT

These sequences can be combined with specific overhangs to design the primers **Fw3 and Rv3.**

Potential Solution

Validate that recipient cells carry a single copy of the MADR acceptor cassette. The experiments detailed in this protocol use heterozygous cells for the MADR acceptor cassette. The presence of a single copy of this acceptor cassette is required to ensure the fluorescence changes as explained below.

MADR recombination eliminates the cassette responsible of TdTomato and EGFP expression from the ROSA26 locus of heterozygous Gt(ROSA)26Sort$^{m4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J mice/cells. Therefore, after transfection or electroporation *in vivo* or *in vitro* (Figures 3A and 3B), the expected results are:

1. **Membrane TdTomato positive cells**: Non-recombinant cells which have not recombined with MADR donor cassette nor have Cre activity.
2. **Membrane EGFP positive cells**: Cells which have undergo a Cre recombination eliminating the TdTomato cassette and leaving EGFP cassette under the control of the endogenous CAG promoter
3. **MADR-recombinant cells**: Cells that has completed MADR recombination eliminating both TdTomato and EGFP cassettes and integrating under the control of the endogenous CAG promoter the sequences cloned in the MADR pDonor plasmid.

However, in case of using homozygous Gt(ROSA)26Sort$^{m4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J mice/cells, two ROSA26 loci would be available for recombination allowing for two additional combinatorial results:

1. **Heterozygous MADR-recombinant cells**: Only one of the ROSA26 loci recombine with the MADR pDonor plasmid while the remaining suffers only Cre activity. These cells present membrane EGFP expression as well as MADR recombination. This cells would be homologous to the MADR-recombinant cells in heterozygous Gt(ROSA)26Sort$^{m4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J cells (Figures 3A and 3B).
2. **Homozygous MADR recombination**: Both ROSA26 loci perform MADR recombination, which eliminates the endogenous expression of both membrane TdTomato and membrane EGFP. The expression of the sequences inserted by MADR recombination will be duplicated comparing with heterozygous MADR-recombinant cells which only express one copy (Figure 3C).

Problem 4

MADR pDonor plasmids expression lacking FlpO-2a-Cre co-transfection

Potential Solution

MADR pDonor plasmid lack promoter and contain three SV40 poly(A) signals 5′ of the cloned sequences to promote expression only in recombining cells under the regulation of the endogenous CAG promoter, avoiding episomal expression. However, in any plasmid transfection, there is a low possibility of promoter-less expression or random integration of MADR pDonor plasmids. We have detected low expression levels of MADR plasmids in experiments *in vitro* with secondary recombinases that enhance secondary signals. However, no expression of MADR pDonor electroporated without FlpO-2A-Cre has been detected in our hands by *in vivo* electroporation.

Transfection *in vitro* promotes the presence of a high number of molecules of the plasmid in the cells, which could be related with a higher rate of random integration or a low episomal expression when lipofecting/nucleofecting MADR plasmids. This becomes further relevant in studies involving enzymatic processes with irreversible and strong reactions or intrinsically amplifying signaling pathways (for example, secondary recombinases which induce expression of other proteins such as a Flex system) (Schnütgen et al., 2003).

Problem 5

Unbalanced expression of the different sequences cloned in the MADR pDonor plasmid

Potential Solution

MADR technology allows for the generation of multicistronic mRNA under the control of the CAG promoter, which ensure a similar ratio of expression of all the sequences cloned. However, it is possible that sequences prone to generate secondary structures due to a high number of repetitions or internal sequence homology can interfere with the transcription and translation of the mRNA. This effect could lead to a differential expression ratio between the sequences included in the cassette. However, in our experience at least 5 protein-coding sequences can be cloned and integrated with MADR technology without a significant loss of expression in any of them. But we cannot ensure that more complex sequences would not interfere with the normal expression of the integrated cassette.

Problem 6

During electroporation, pressure is too low to properly inject into ventricle

Potential Solution

Sometimes if during injection process, there is no injection of EP mix into the ventricle, or there is significant passive efflux of EP mix after needle is removed from skull. If this is the case, use a new pulled glass needle and cut the tip so that it is slightly longer, and modify the pressure to be higher. The closer the tip cut is to the base of the needle, the lower the pressure needs to be in order to efflux the EP mix (due to a lower resistance in the needle).

Problem 7

While electroporating the DNA mix clogs the tip

Potential Solution

This usually happens due to a few reasons, the first of which is outlined in the troubleshooting comment above. Usually there is no DNA mix efflux because the tip has dried out. In this case it is always prudent to either keep the tip wet by periodically swirling it in the DNA mix or increase the pressure and inject a test round of the mix onto parafilm. This removes any clogs in the tip.

Problem 8

Mice do not get fully anesthetized in a reasonable time

Potential Solution

If the anesthetizing process is taking too long, it is best to check that the mice pups are cooling on ice with their bellies fully prone to the ice. Adding water to the ice bath increases conductance. However, ensure that mice pups are not getting wet by the water of ice to avoid damage.

Problem 9

How do I know that the electroporation worked?

Potential Solution

There are a few indicators of a good electroporation (1) the injection delivered the DNA mix to the correct ventricle (2) there was little time between the injection and the electroporation to allow DNA to be washed out of the ventricle (3) you could feel the pulses of the current during the electroporation. As with any model optimization, you can sacrifice a pup after 24 h to confirm that the electroporation worked

Problem 10

I had issues injecting mice the first time around… can I re-inject them?

Potential Solution

While it is not *recommended*, you can inject a mouse a second time if your tip broke, or if there was a clog. If this is the case, it is imperative to do this before you electroporate the mouse. Once a current has been passed, you cannot repeat.

Problem 11

My needle broke!

Potential Solution

It is prudent to keep a pair of tweezers on hand in case a tip breaks. This usually happens when you trim the needle too long. It is safe to remove the needle tip with tweezers and proceed on with the experiment.

Problem 12

Mice pups stop breathing after electroporation

Potential Solution

This is usually a sign that paddles were set too posterior on the skull: paddles set too posteriorly target lower brain functions. Mice can be resuscitated by gently massaging their chests until their breathing has returned.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Josh Breunig (joshua.breunig@cshs.org).

Materials Availability

Selected plasmids are available from Addgene: http://www.addgene.org/browse/article/28203812/

Data and Code Availability

No data or code are necessary or included.

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AUTHOR CONTRIBUTIONS

J.J.B. conceived the *in vivo* recombination strategy. D.R.F.P. and S.S. wrote the manuscript and prepared the figures under the supervision of J.J.B. All authors read and edited the final version of this manuscript.

DECLARATION OF INTERESTS

Cedars-Sinai has filed for patent protection for *in vivo* dual recombinase-mediated cassette exchange and disease models thereof.

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