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**Making MSCV-PGK-hCD25 GFP microRNA sensors**

## 1) Constructing Sensors

### a) Order primers from IDT carrying sensor sequence + enzymatic restriction sites:

Not1 EcoRI EcoRV

GGCCGC(REV.COMP.SEQ)CGAT(REV.COMP.SEQ)G AATTC(REV.COMP.SEQ)TCAC(REV.COMP.SEQ)GAT

GC( SEQ.U→T )GCTA( SEQ.U→T )CTTAA G( SEQ.U→T )AGTG( SEQ.U→T )CTA

Example:

**Mmu-miR-24 -3p**

ACTUAL mature SEQ: uggcucaguucagcaggaacag

REPLACE U→ T SEQ: tggctcagttcagcaggaacag

REVERSE COMP SEQ: CTGTTCCTGCTGAACTGAGCCA

Add enzymatic sites: miR-24 sensor primers: A+B; C+D

A) GGCCGCCTGTTCCTGCTGAACTGAGCCACGATCTGTTCCTGCTGAACTGAGCCAG

B) AATTCtggctcagttcagcaggaacagATCGtggctcagttcagcaggaacagGC

C) AATTCCTGTTCCTGCTGAACTGAGCCATCACCTGTTCCTGCTGAACTGAGCCAGAT

D) ATCtggctcagttcagcaggaacagGTGAtggctcagttcagcaggaacagG

Strategy:

4 oligos per construct (A,B,C,D): A anneals with B, C anneals with D.

### b) Annealing step:

Annealing Buffer: 50mM Tris pH 8.0, 70mM NaCl

(or: 100mM PotassiµM acetate, 2mM Magnesium acetate, 3mM HEPES, pH7.4)

1) Resuspend primers at 100µM in $H_2O$ or TE (10mM Tris, pH8.0; 1mM EDTA)
2) Make primer set 1: 2µL oligo A (100µM), 2µL oligo B (100µM) + 46 µL Annealing Buffer.
   Primer set 2: 2µL oligo C (100µM), 2µL oligo D (100µM) + 46 µL Annealing Buffer.

   Concentration of each resulting dsDNA set:

$$(approx)600g \Big/ mol * bp * (approx)60bp = \frac{36000g}{mol} * 4\mu mol/L = 144ng/\mu L$$

3) Incubate at 95C heat block for 5min.
4) Remove entire heat block from heat source and allow to cool down to RT slowly (~3hrs).

**c) Phosphorylate annealed oligo sets:** (annealing rxn in 50µL→ 144ng/µL each set)

1) Mix 5µL set 1 with 5µL set 2 (10µL) + 16µL MQ water → 26µL total
2) PNK phosphorylate oligo mix for ligation:
   - add 3µL 10x buffer for T4 DNA ligase (w/ATP) (NEB)
   - add 1µL T4 Polynucleotide Kinase.
   - Incubate at 37C for 30min

-30 µL rxn is now at 24ng/µL (of EACH "set" of phosphorylated oligo [2 sets])
- Dilute phosphorylated oligo mix: 5µL phosphorylated mix + 15µL MQ water; → 6ng/µL of each oligo

**e) Not1/EcoRV digest & dephosphorylate pEntr-GFP vector** (If you already have a cut/dephosphorylated pEntrGFP vector, skip to step f)

1) Digest pEntr-GFP vector with Not1 and EcoRV. Example rxn (change based on how much DNA you have):
    - 2.5uL EcoRV (20,000U/mL)
    - 5 uL Not I (10,000U/mL)
    - 7.5 uL 10x NEBuffer 3.1
    - X uL pEntr-GFP (5ug)
    - add H20 to 75uL
2) Run digestion mix in 1% agarose gel.
3) Gel extract the digested band (~2.8kb). (we use Sigma Gel extract kit)
4) Dephosphorylate the cut/purified vector with Antarctic Phosphatase (NEB) so it doesn't ligate to itself, 30min-1hr at 37C. Example rxn:
    - 25 uL of cut/purified vector
    - 2uL Antartic Phosphotase
    - 5uL 10X antarctic phosphotase buffer
    - 18uL H20
5) PCR clean up the dephosphorylated vector (Sigma kit) to get rid of buffer, enzymes and elute the DNA in about 40µL.
6) Nanodrop to measure concentration. Dilute/concentrate cut vector to 50ng/µL

**f) Ligate sensor sequence into pEntr-GFP vector**

- We use Quick Ligation kit from NEB (M2200)

1) Combine 50ng of vector with a 3-fold and 6-fold molar excess of insert to a final volume of 10uL. Include a "no insert" control reaction. Concept:
    - -pEntr: ~2800bp
    - -inserts: ~60bp each = approx 50x smaller than vector
    - -Vector's MW is approx 50x as much as the insert's because it's approx 50x as long, so you need 50x more vector than insert for a 1:1 molar ratio (to get the same number of "ends" to ligate). For a 1:1 molar ratio with 50ng vector, you would need 1ng of insert. For a 3:1 ratio, you need 3ng of insert. 6:1 = 6ng of insert.

Example 6-fold molar excess reaction:
    - 1 uL pEntr-GFP cut vector (50ng/uL)
    - 1 uL of phosphorylated sensor oligos (6 ng/uL)
    - 8 uL MQ $H_2O$

2) Add 10µL of 2x Quick ligation Reaction buffer
3) Add 1µL of Quick T4 DNA ligase and mix thoroughly. Quick vortex.
4) Centrifuge briefly and incubate at RT for 5min.
5) Chill on ice, then transform bacteria or store at -20C.

**g) Transform ligated pEntr-GFP-sensor plasmid into competent cells.**

1) Thaw competent cells on ice. (We use NEB Top10 competent cells).
2) Each vial has ~50µL of bacteria. Split 25uL of bacteria into eppies per ligation reaction, plus one extra for a "No Insert" control.

3) Add 2μL of ligation mix into 25μL of bacteria.
4) Incubate on ice for 15-30min.
5) Heat shock for 2 min at 37C, or 45 sec on 42C; chill on ice for 5min.
6) Add SOC media or LB broth at 9:1, next to flame. (180μL per 25uL of bacteria)
7) Incubate in shaker or water bath at 37C for 1hr. (For Kanamycin-resistant, make sure to incubate for at least 1 hr; when doing Ampicillin-selection can cut to 30-45 min.)
8) Pre-heat and dry KANAMYCIN-resistant LB plate to 37C, while bacteria is incubating.
9) Spread 25-30% (~50uL) of total bacteria mix onto KAN-plate.
10) Incubate over-night in 37C bacterial incubator.

**h) Got colonies? Screen them by bacterial PCR.** (If your "no insert" plate has few to no colonies relative to the other plates, skip to h-4, but pick multiple colonies [~3-5] to miniprep and sequence)

Add 100μL of MQ water into 96-well plate (I prefer U-bottom plates with lids). I usually screen at least 12 colonies from each plate. And 3-4 colonies from control "No insert".

1. With a 20μL pipette tip, poke each colony and transfer a small amount of bacteria into each well. Set the pipette at 20μL and pipette up/down several times to flush out the bacteria into the well. Label each colony with the well # it goes to in the 96-well plate.
2. Keep plate on ice. Bacteria will lyse by itself eventually. If not, PCR denaturation step will take care of it. Plate can be stored at -20C.
3. Make a PCR mix:
   a. 10 uL 2x MyTaq Red
   b. 0.2 uL eGFP F (25uM)
   c. 0.2 uL mature miRNA seq (25uM)
   d. 4.6 uL H20

   **Primers:** we use Forward primer for GFP and the microRNA mature DNA sequence as reverse primer. The PCR band is ~750bp.

   EGFP Fwd primer: AGCTGACCCTGAAGTTCATCTG

   - If your pEntr-GFP vector came from digested pEntr-GFP-sensor plasmid, include a separate PCR using the GFP Forward primer + primer for that specific sensor. There might be some undigested sensor in your DNA mix.
   - Aliquot 15μL of mix into PCR plate
   - With a multi-channel, add 5μL of Bacterial prep into PCR mix.

   e. PCR program:
      i. 94C 3min
      ii. 35 cycles: 94C 15sec, 58C 25sec, 72C 45sec
      iii. 72C 10min
      iv. Hold at 4C
   f. Load most of PCR mix (maybe 17μL) in 1% agarose gel.
   g. If you get a band for YOUR sensor sequence and not for the other sensor (where pEntr came from)→ miniprep and sequence that colony. Do more than 1 if you have multiple positive clones.
4. Grow the right colony, by dipping a 200μL pipette tip into the colony and dropping it into 3mL of LB broth + KANAMYCIN. Grow O/N in shaker at 37C.
5. Miniprep using Sigma kit, have DNA eluted at least at 100ng/μL for sequencing (if less, it's okay, try to sequence anyway).
6. Send for sequencing using Sequetech, select "Plasmid" and use their own cNew eGFP primers.
   a. When get results back, make sure all 4 microRNA binding sequence are there. Also check it's in right direction and enzymatic sites are correct.
   b. Got right pEntr-GFP-sensor plasmid? Clonase it into MSCV-hCD25+ccdb vector

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**i) Gateway LR clonase reaction:** the whole rxn can be halved (75ng + 75ng in 4µL + 1µL enzyme)

Gateway LR Clonase II: Invitrogen 11791-020

1. Add 50-150ng of entry clone + 150ng MSCV-hCD25+ccdb (150ng). Adjust volume to 8uL with TE buffer, pH8.0. Include a "no entry clone" control reaction.
2. Thaw the LR clonase II enzyme mix on ice for about 2 minutes. Vortex the LR clonase II enzyme, mix briefly twice (2 sec each time).
3. Add 2µL of LR clonase enzyme II to the reaction and mix well by vortexing twice. Quick spin down. Return enzyme to -20C
5. Incubate the clonase reaction at 25C for 1 hr.
6. Add 1µL of Proteinase K solution to terminate reaction. Vortex briefly. Incubate samples at 37C for 10min.
7. Can store in -20C, or transform into bacteria and spread it on AMPICILLIN LB plates.
8. Pick 2 clones→ miniprep→ diagnostic digest of ~1ug with EcoRV → run on a 1% agarose gel to confirm clonase rxn worked (you should see 3 large bands: >3kb, >2kb, >1kb)

**j) Maxiprep!**

1. Pick the colony confirmed by the EcoRV digest and make a starter culture: drop tip in 3ml LB broth+ AMP. Do this in AM.
2. Incubate in 37C shaker for 8hrs.
3. Add 150-250mL LB broth + ampicillin into Erlenmeyer flask that is 10x larger than the volume of your starter culture
4. Add starter culture into broth at 1:750.
5. Incubate O/N in shaker at 37C.
6. Maxiprep culture following instructions of Sigma Maxiprep kit. Select elution option for maximum recovery of plasmid, and then complete the DNA concentration step.
7. Use a sharpie to mark the location of the DNA pellet, as it will turn clear while the EtOH dries. Resuspend in ~500uL TE. Let sit on ice for 10min to dissolve completely. Pipette over the location of the pellet - if liquid is sticking to the tube there, the DNA has not fully gone into solution.
9. Nanodrop. Dilute to 1ug/µL. Store in -20C.

You can create sensor-bearing virus through Calcium Phosphate Transfection of phoenix cells, and then transduce mouse CD4+ T cells with the resulting virus.