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Protocol for pPRIME (Provided by: Elledge Lab)

pPRIME (Potent RNA interference using microRNA expression):

Reference: Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ, *PNAS* 2005

General comments about pPRIME:

To clone shRNAs into pPRIME we used the pSM2 design algorithm that is optimized for miR30-based shRNAs to ensure correct processing of the pre-miRNA to the mature siRNA(http://katahdin.cshl.org:9331/siRNA/RNAi.cgi?type=shRNA).
A large collection of human and mouse pSM2-based shRNA sequences are available from OpenBiosystems (www.openbiosystems.com), which can be easily transferred into the pPRIME vectors (see cloning protocol below).
The pSM2-based hairpins can be transferred into pPRIME either by mating or cloning. A detailed mating protocol is attached below. For questions regarding the mating protocol please contact Mamie Li: mli@rics.bwh.harvard.edu. The cloning recipients all contain the FF3 hairpin (which targets firefly luciferase and can be used as a negative control). The FF3 hairpin can be replaced with any other hairpin by cloning into the EcoR1/Xho1 site (120 bp fragment- see cloning protocol below).

pPRIME-mating recipients

Name	Plasmid #
pPRIME-**TET-GFP**-recipient	p126
pPRIME-**TET-GIN**-recipient	p259
pPRIME-**CMV-GFP**-recipient	p191
pPRIME-**CMV-dsRed**-recipient	p209
pPRIME-**CMV-Neo**-recipient	p210
pPRIME-**CMV-LNGFR**-recipient	p211
pPRIME-**TREX-GFP**-recipient	p192

pPRIME-cloning vectors
(contain FF3 shRNA in Xho1/EcoR1 site)
- FF3 hairpin targets firefly luciferase

Name	Plasmid #
pPRIME-**TET-GFP**-FF3	p199
pPRIME-**CMV-GFP**-FF3	p201
pPRIME-**CMV-dsRed**-FF3	p232
pPRIME-**CMV-Neo**-FF3	p234
pPRIME-**CMV-LNGFR**-FF3	p236
pPRIME-**TREX-GFP**-FF3	p203

Note: We have never tested whether insertion of conventional shRNA sequences (such as for pSUPER) also work in the miR30 context. It would be important to maintain the bulges shown in Figure 1A (Stegmeier et al. PNAS 2004) to ensure correct processing of the pre-miRNA by Drosha and Dicer.

The pPRIME backbone contains an AMP resistance gene, but after mating (which introduces the Cmr marker) plasmids (including the cloning recipients) should be selected with Chloramphenicol (50ug/ml) to select against LTR-recombinants that lost the shRNA cassette.

Good luck with your experiments,

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miR30-shRNA cloning protocol (for pSM2 and pPRIME)

Note: If a pSM2-shRNA already exist in the Open Biosystems collection (http://www.openbiosystems.com/expression_arrest_shrna_libraries.php), the shRNA can be cloned into pPRIME vectors already containing firefly luciferase hairpin FF3 by transferring the Xho1/EcoR1 fragment

Design a 97mer hairpin oligo against your favorite gene (http://katahdin.cshl.org:9331/siRNA/RNAi.cgi?type=shRNA)

PCR amplify the hairpin oligo using the pSM2C forward and reverse oligos. These oligos will add XhoI and EcoRI sites to the ends of your hairpin for cloning into the pSM2C vector.

pSM2C Forward:
5'-GATGGCTG-CTCGAG-AAGGTATAT-TGCTGTTGACAGTGAGCG-3'

pSM2C Reverse:
5'-GTCTAGAG-GAATTC-CGAGGCAGTAGGCA-3'

	Stock Concentration	Final Concentration
ThermoPol Buffer	10x	1x
Mg_2SO_4	100 mM	2 mM
DMSO	100%	5%
dNTPs	2.5 mM	200 uM
Template Oligo	100 ng/uL	100 ng/rxn
Forward Primer	50 uM	0.5 uM
Reverse Primer	50 uM	0.5 uM
VENT Polymerase	2 U/uL	1 U/rxn

All components added on ice.

PCR Protocol:

Step	Temperature	Time
1	94 oC	5 min
2	94 oC	30 sec
3	54 oC	30 sec
4	75 oC	30 sec
5	Repeat steps 2-4 for 12 cycles	
6	75 oC	2 min
7	4 oC	Forever

Following PCR amplification, purify the products with PCR purification columns. Elute the PCR products with 50 uL EB.

Digest both the PCR product (insert) and the vector (pSM2C) with XhoI and EcoRI. Since the size of the PCR products are small, digest the entire volume of eluted products with the maximum amount of restriction enzymes for at least three hours

Purify digested amplified hairpin inserts with QIAquick PCR purification columns, and gel purify pSM2C vector. Note: if transferring XhoI/EcoRI hairpin fragments from one vector to another using a gel purification step, "melt" agarose at lower temperature (42 instead of 50 degrees) to reduce probability of melting hairpin (resulting in snap back s.s. DNA)

Ligate the hairpin into the pSM2C vector at a 3:1 ratio, or approximately 4.2 ng of the hairpin insert to 100 ng of the pSM2C vector, in a reaction volume of 10 uL.

After the ligation, transform half of the reaction (5 uL) into competent bacteria and plate on a chloramphenicol plate
(NOTE: pSM2 needs to be transformed into a pir1+ strain to allow for replication, whereas pPRIME vectors can replicate in conventional bacterial strains)

IMPORTANT: Sequence confirm the shRNA sequences
- add 10% DMSO to the sequencing reactions to allow opening of the shRNA secondary structure for sequencing.

Sequencing primer:

SM2C rev:
5'-GAAGTGATCTTCCGTCACAGG-3' (~170n downstream of EcoR1 site)

Protocol for pSM2 mating (Li MZ, Elledge SJ, *Nature Genetics* 2005):

Strains:

DH10βF'DOT sbcC (donor host strain, LB/Tc 5 μg/ml):
mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR recA1 endA1 araΔ139 Δ(ara, leu)7697 galU galK λ$^-$ rpsL nupG tonA umuC::pir116-frt F'(lac$^+$ pro$^+$ ΔoriT::Tc) sbcC::Frt
BW28705I /pML300 (recipient strain, LB/Sp 50 μg/ml):
lacIq rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 galU95 ΔendA9::FRT ΔrecA635::FRT umuC::ParaBAD-I-SceI-FRT + pML300(PrhaB-γβexo Ts(ori) Spectinomycin resistant), grow at 30°C

Plasmids:

pSM2-SiRNA (mir30-H2 Donor plasmid):
Cm^R Kn^R

pML382 (MSCV puro recipient plasmid)
Homology mir30 5'-PheS-homology 2 (H2) cassette in pMSCV puro.

Protocol

Transform donor plasmid into donor host and recipient plasmid into recipient host. Transformants in the recipient strain should be selected on LB/Sp^{50}/Cb^{100}/0.2% glucose plates at 30°C in order to repress the ParaBAD promoter.

1. Streak fresh donor plasmid on LB/Cm^{30}/IPTG/XGAL plate. Since the F' in donor host strain contains lac$^+$, it should be blue on XGAL plate. Also streak recipient plasmid on LB/Sp^{50}/Cb^{100}/0.2% glucose plate and grow at 30°C for overnight.
2. Start a 5 ml culture of donor from several blue colonies in LB/Cm^{30} and grow in 37°C for overnight. Also start a 5 ml culture of recipient in LB/Sp^{50}/Cb^{100}/0.2% glucose from single colony in 30°C for overnight.
3. The next day, spin down the recipient at 4000 rpm for 5 min room temperature and resuspend with 10 ml of LB. Repeat this one more time. Spin down the recipient again and resuspend in 5 ml of LB. Dilute both the donor and the recipient in LB/0.2% rhamnose(sigma R-3875). I usually do three dilution 1:100, 1:50 and 1:25. Grow in 30°C for recipient and 37 °C for donor with shaking for about 2 hours until they reach OD of 0.15-0.25.
4. Mix the donor and the recipient at equal ratio based on their OD, add 20% arabinose to final conc. of 0.2%. Mix well and incubate at 37°C without shake for 2 hrs. Then shake in 37°C for 2 hrs.
5. Dilute and plate on the selection plate (pre-warm the selection plates to 42°C). Incubate in 42°C for overnight.
6. Selection plates: 0.5% yeast extract, 1% NaCl, 0.4% glycerol, 10mM DL-p-chlorophenylalanine (Sigma C-6505), 2% agar, 0.2% L-arabinose (sigma A-3256), carbenicillin 100 μg/ml, and chloramphenicol 30 μg/ml . DL-p-chlorophenylalanine will not dissolve until after autoclaving. Glycerol, arabinose and carbenicillin and chloramphenicol are added after autoclaving. I use carbenicillin instead of ampicillin in order to cut down the background.
7. YEG media: 0.5% yeast extract, 1% NaCl, 0.4% glucose.

If you have any questions, please e-mail me at mli@rics.bwh.harvard.edu.