1. Search for the gene(s) of interest on The RNAi Consortium (TRC) portal (http://www.broadinstitute.org/rnai/public/) and select the ~five highest scoring non-overlapping targeting sequences.
   - *In general, sequences targeting the CDS are preferred because they can be rapidly screened by transient co-transfection with an expression plasmid for the gene to be knocked down*
   - *Sequences that are 100% human specific are preferred so that future addback experiments can be performed with the mouse or rat homolog*
   - *Custom targeting sequences for special applications can be designed according to the TRC design rules: http://www.broadinstitute.org/science/projects/rnai-consortium/trc-shrna-design-process*
2. Order the primers (minimum synthesis, standard desalting) listed under "Oligo design for arrayed cloning" after replacing the XhoI site in the loop (CTCGAG) with a PstI site (CTGCAG). Dissolve primers to a concentration of 50 μM in PCR-grade H_2O
   - *Unlike XhoI, PstI restriction sites are absent in pLKO.1, allowing ligated hairpins to be screened rapidly by restriction digest*
3. Anneal the shRNA primers by setting up the following reaction in a PCR tube (one annealing reaction per shRNA duplex):
   - 2.5 μl top-strand primer
   - 2.5 μl bottom-strand primer
   - 5 μl 10× annealing buffer
   - 40 μl ddH_2O

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     50 μl total volume
4. Heat the annealing reaction to 95°C for 5 min in a PCR thermocycler. Unplug the thermocycler and allow the primers to anneal by cooling to room temperature overnight.
   - *The shRNA oligos are designed to contain restriction enzyme-like cleavage ends after annealing; therefore, they do not need to be digested*
5. Phosphorylate the annealed shRNA primers by setting up the following reaction in a PCR tube:
   - 1 μl annealed primers
   - 1 μl 10 mM ATP
   - 1 μl 10× PNK reaction buffer (supplied with NEB #M0201)
   - 1 μl T4 polynucleotide kinase (NEB #M0201)
   - 6 μl ddH_2O

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     10 μl total volume
6. Incubate the phosphorylation reaction at 37°C for 30 min, then heat inactivate at 70°C for 10 min.
7. In parallel with the oligo annealing and phosphorylation, set up three 50 μl restriction digests, each containing 2 μg pLKO.1 or tet-pLKO.1, 5 μl 10× EcoRI buffer, 2.5 μl EcoRI, 2.5 μl AgeI, and 0.5 μl of 100× BSA.
   - *We have DNA stocks of pLKO.1 puro, pLKO.1 neo, pLKO.1 hygro, and tet-pLKO.1 puro which can be used depending on the combination of perturbations desired*
8. Digest pLKO.1 or tet-pLKO.1 at 37°C for 3 hr and purify the ~7 kb (if pLKO.1) or ~9 kb (if tet-pLKO.1) digested fragment on a 1% agarose gel
   - *The pLKO.1 series releases a 1.9 kb "stuffer" sequence after EcoRI-AgeI digest, which readily separates the double-cut vector from any single-cut vector*
9. Excise the digested vector, purify, and ethanol precipitate the three digests into 5 μl EB as described in Janes_PCRcloning.pdf.
10. Set up a 10 μl ligation reaction containing 7.5 μl phosphorylated-annealed shRNA oligo, 100–250 ng digested pLKO.1, 1 μl 10× T4 ligation buffer, and 0.5 μl T4 DNA ligase (New England Biolabs #M0202S).
    - *Be sure that the ligation buffer has thawed completely (no visible precipitates) and is kept at room temperature before use*
    - *Note this reaction volume is half that recommended by the manufacturer*

- *Be sure to include a control ligation that includes everything but the shRNA oligo*

11. Mix by pipetting gently, incubate at room temperature for 1 hr, and then ethanol precipitate and transform into electrocompetent bacteria as described in Janes_PCRcloning.pdf.
    - *tet-pLKO.1 cultures require incubation at 30ºC to minimize recombination of the plasmid*
12. Perform a PstI-BamHI diagnostic digest on ~four ampicillin-resistant colonies per shRNA oligo. Positive clones will yield two products at ~6.3 kb and ~700 bp (for pLKO.1 vectors) and ~6.2 kb and ~2.6 kb (for tet-pLKO.1).
13. Send three digest-positive clones out for forward shRNA sequencing at Genewiz with the "hairpin" option on the website.
14. Screen sequence-verified hairpins by transient transfection in 293T cells (if possible) or by transduction with lentiviruses as described in Janes_Virusprep.pdf. Coexpression of a V5-tagged expression construct from the ORFeome is highly recommended if there are concerns about antibody specificity.

Buffer recipes

- **10× annealing buffer** Store at room temperature
  100 mM Tris-HCl (pH 7.5)
  1 M NaCl
  10 mM EDTA