RNAi Validation System Vector Protocols

Subcloning your cDNA of interest into the validation vector

The full-length cDNA inserts in OriGene's TrueClones are flanked by two Not I sites. Not I specifically recognizes an uncommon eight base sequence; therefore, the majority of the TrueClone inserts can be released through Not I digestion without internal cutting of the insert. If an internal Not I site exists in the TrueClone insert, a complete Not I digestion will result in multiple fragments, unsuitable for subcloning directly. This situation would require either a partial digestion strategy or PCR subcloning (see instructions below).

Any cDNA clone can be used in the RNAi Validation System Vector. If the restriction sites surrounding your clone aren't compatible with Not I subcloning, then a PCR subcloning strategy utilizing the Not I, Sac II, or Sma I restriction sites will be necessary. Perform restriction analysis of your DNA or cDNA to confirm that there are no internal sites in your cDNA corresponding to your chosen subcloning sites.

Digesting the validation vector, pCMV6-Luc(V)

1. Set up a digestion reaction using 1 ug DNA with Not I using conditions recommended by the restriction enzyme provider.
2. Incubate at 37 ºC for 90 min, or as recommended by the manufacturer.
3. Add 0.5 uL calf intestinal phosphatase (CIP) to the digestion reaction, and incubate at 37ºC for an additional 30 minutes.
4. Run the digestion reaction on a 1% agarose gel. Cut out the band corresponding to the 6.2 Kb vector.
5. Use a gel purification kit to purify the linearized vector.
6. Estimate the recovered DNA quantity by spectrophotometric analysis or by running 1 uL of the purified eluate on an agarose gel and comparing the band intensity to a reference such as DNA Quanti-Ladder (cat #QLD200).
7. Contineu with the ligation and transformation protocol, below.

Not I digestion of cDNA clone (insert)

1. Digest 0.5 - 1 ug donor plasmid DNA (such as an OriGene TrueClone) with Not I using the conditions recommended by the restriction enzyme provider.
2. Run the digestion reaction on a 1% agarose gel to separate the insert and vector fragments. Cut out the band corresponding to the cDNA insert.
3. Use a gel purification kit to purify the insert fragment.
4. Estimate the recovered DNA quantity by spectrophotometric analysis or by running 1 uL of the purified eluate on an agarose gel and comparing the band intensity to a reference such as DNA Quanti-Ladder (cat #QLD200).
5. Continue with the ligation and transformation protocol, below.

Partial digestion/Not I digestion of cDNA

Partial digestion uses limiting enzyme and incubation time to produce vector/clone fragments that are not completely digested. Some of the DNA will be cut at only one of the Not I sites, some at multiple but not all Not I sites, and some will be cut at all Not I sites. In this case, you will be looking for digestion at two sites only - specifically, the two sites in the MCS but not internal to the insert.

To do a partial digestion, set up the following master mix:
10 uL 10X buffer
1 uL 100X BSA

1-4 ug DNA
up to 100 uL H_2O

1. Aliquot this master mix to five tubes, containing 30 uL, 20 uL, 20 uL, 20 uL, and 10 uL respectively.
2. To the first tube containing 30 uL, add 0.5 uL Not I enzyme (10U/uL). Pipet up and down to mix well.
3. Remove 10 uL from this tube, and add it to the next tube in the series. Pipet up and down to mix well.
4. Repeat steps 2 and 3 with all remaining tubes. Now you have 5 tubes of 20 uL digestion reactions with a serial dilution of enzyme.
5. Incubate for 15 minutes at 37ºC (instead of the usual 1-2 hours).
6. Run each digestion reaction separately on a 1% agarose gel, and look for the reaction in which you see only the 4.7 Kb vector and the full size of the insert (a partial digestion product which has left the internal Not I sites intact). Purify that fragment as the intact cDNA clone. Continue with the ligation and transformation protocol, below.

PCR Subcloning (if using enzymes other Not I to subclone)

Primer design

Design gene specific primers with restriction sequences at the ends. Consider the following recommendations when designing primers.

- Use sequence complementary to your cDNA for 18-22 bases.
- Introduce the sequence for the 5' restriction site (such as the Not I sequence GCGGCCGC) at the 5' end of the forward primer and the sequence for the 3' restriction site (such as the Sma I sequence CCCGGG) at the 5' end of the reverse primer.
- Include additional bases at the 5' end of each primer to allow for efficient digestion by the restriction enzymes. Some of these extra bases can form a G/C clamp.
- Avoid too many Gs or Cs at the 3' ends of either primer.
- Avoid long runs of any single nucleotide.
- Make sure the forward and reverse primers don't form primer dimers (especially avoid complementary sequences at the 3' ends) or significant secondary structure.

Sample primers designed using this strategy for TP53 NM_000546

Forward oligo:

GC Clamp	Not I	Gene Specific
5' GGCGC	AATA	GCGGCCGC | AAGTCTAGAGCCACCGTCCA 3'

Reverse oligo:

GC Clamp	Sma I	Gene Specific
5' GGCCG	AA	GGGCCC | AAGCGAGACCCAGTCTCAAA 3'

PCR amplification

Assemble all of the following components on ice.

50 ng cDNA clone
5 uL 10X PCR buffer with Mg^{2+}
1 uL 10 mM dNTPs
20 pmoles forward primer
20 pmoles reverse primer
1 uL Pfu polymerase

up to 50 uL water

Annealing temperatures are dictated by the primer sequences; the temperatures given below are for the sample primers indicated above. You should determine the appropriate annealing temperature for your primers, using the lowest Tm of the two primers.

When introducing new restriction sites at the end of the fragment, only the 3' part of the primer matches the target so the first few annealing cycles should be done at a lower temperature than the subsequent annealing reactions. Calculate the Tm for the **gene specific region only** of each primer and allow the primers to anneal to the template at a temperature that is 5 degrees below the calculated Tm. For the subsequent cycles, calculate the Tm for the **entire length of the primer**. If it is greater than 70°C, use an annealing temperature of 65°C. For a Tm less than 70°C, use an annealing temperature that is 5°C below the calculated Tm. The length of the extension time is dictated by the length of the expected amplification product. Use 1 minute for every 1 Kb of length.

Sample cycling conditions for TP53 primers:
94°C for 5 minutes

94°C for 1 minutes
60ºC for 1 minute
72°C for 2 minutes
Repeat once

94°C for 1 minute
65°C for 1 minute
72°C for 2 minutes
Repeat 30-33 times

72°C for 10 minutes

Ligation and transformation

Prepare a ligation according to the following protocol
1 uL 10x ligation buffer
10 ng purified digested pCMV-Luc(V) vector
20 ng* purified digested insert from donor plasmid
0.25 U ligase
up to 10 uL H_2O
*The optimal insert:vector molar ratio is about 2:1. Adjust the insert concentration accordingly.

Incubate the tube at room temperature for two hours or 16ºC overnight (according to the manufacturer's recommendation).

Transform the mixture according to the directions of the manufacturer of the competent cells. A sample protocol for chemically competent cells is listed here.

1. Add 3 to 5 uL of the ligation mixture to 50 uL of competent cells (efficiency rated > 10^8 cfu/ug DNA) on ice.
2. Mix the tube gently and incubate on ice for 20 minutes.
3. Heat shock the tube for 45 seconds at 42ºC.
4. Incbuate the tube on ice for 2 minutes, then add 500 uL SOC (recovery medium).
5. Incubate the tube at 37ºC with agitation for 1 hour.

6. Spread 50 µl of the cells on an LB ampicillin- agar plate. You may also wish to plate other dilutions of this transformation mixture on separate LB-amp plates after pelleting the cells and resuspending in a smaller volume.
7. Incubate the plates at 37ºC for 16 hour to allow colony formation.

Validating the RNAi effect: introduction of the validation construct and gene specific shRNA into mammalian cells via transfection

1. Plate HEK293 cells at 3 x 10^3 cells/ well in a 96-well plate. Grow the cells overnight in a 37°C, 5% CO_2 incubator to achieve 50% confluence.
2. In a small sterile tube, combine the following reagents in the prescribed order. (Order of reagent addition is important to achieve the optimal results.) The amounts given below are for triplicate wells.

Serum-free medium (OptiMEM I)	30 uL
TurboFectin 8.0 transfection reagent	1.8 uL
validation construct (pCMV-Luc(V) + cDNA)	0.3 ug in 1-3 uL
HuSH shRNA plasmid against the target gene	0.3 ug in 1-3 uL

   Note: Add TurboFectin 8.0 directly into the serum-free media. Do NOT let TurboFectin 8.0 touch any plastic other than the pipette tip.
3. Mix the tube contents gently. Do NOT vortex.
4. Incubate at room temperature for 15-45 minutes.
5. Add the DNA-TurboFectin mix to the triplicate wells of the tissue culture plate (10 uL in each of three wells) directly without removal of the culture media. Mix by gently rocking the plate.
6. Twenty-four to forty-eight hours post-transfection, add 15 uL of luciferase substrate (Perkin Elmer's Britelite reagent or similar) to each well. The luminescent signal can be determined by a plate reader immediately and for up to 15 minutes with virtually no loss in sensitivity.