09/10/08

Section V: Validation of Target Gene Knockdown with Quantitative PCR

Introduction:

This section contains protocols to use quantitative PCR (qPCR) for:

- pre-validation of gene expression in different cell lines and
- high-throughput validation of target gene knockdown following lentiviral delivery of TRC shRNA constructs.

We recommend first determining gene expression levels in cultured cells to establish that a gene is expressed and can be quantified by qPCR ("pre-screen") prior to validating target gene knockdown with TRC shRNA constructs. Knockdown validation consists of infecting cells with lentiviral shRNA stocks, selecting for transduced cells, isolating total RNA, synthesizing cDNA, and quantifying expression of the target gene and an endogenous control gene with qPCR. It is important to include reference infections with non-targeting constructs for comparison. This protocol describes a high-throughput qPCR method using SYBR green assays and ΔΔCt analysis to calculate target gene knockdown; other qPCR assays and analysis methods are feasible but may require some changes to the protocol below.

Knockdown validation consists of the following steps:

Pre-screen	Test qPCR assay on cDNA sample from non-transduced cells
Day 0	Seed cells
Day 1	Infect cells (see Section III: Lentiviral Infection)
Day 2	*24 hours after virus addition:* Remove media; replace with fresh media containing puromycin
Day 4^{+}	≥ *48 hours after puromycin addition:* Lyse cells for RNA isolation
	Isolate total RNA
	Synthesize cDNA
	Quantify gene expression with qPCR

All lentiviral procedures should be carried out in accordance with biosafety requirements of the host institution.

Part 1: Pre-screen: Confirmation of gene expression and assay quality

I. Materials

Human or mouse cell line and appropriate growth media
TriZol/TriReagent (e.g. Molecular Research Center, #TR-118)
Falcon tubes
Chloroform
M-MLV Reverse Transcriptase (Sigma, #M1302)
Oligo dT_{20} (ordered from oligo vendor such as IDT)
Random 9-mer oligos (ordered from oligo vendor such as IDT)
dNTPs (e.g. Stratagene, #200415-51)
RNAse-free PCR plate
Plate seal
RNase-free water (e.g. Ambion, #9922)
SYBR Green assay for target gene (e.g. selected from PrimerBank)
SYBR Green assay for endogenous control gene (e.g. selected from PrimerBank)
SYBR Green qPCR Master Mix (e.g. Sigma, # S9194)
qPCR 384-well optically clear plate and seal (e.g. Roche, # 5102430001)

II. Instructions

1. Grow cells to 80-95% confluency in a tissue culture flask. Optional: treat these cells as you would for TRC shRNA knockdown (e.g. infect with control virus, select with puromycin) to generate a representative sample to pre-validate target gene expression.
2. Isolate total RNA according to the TriReagent protocol, with the following recommendations and changes:
   a. Remove media completely. Optional: wash cells gently with PBS; remove completely. Lyse cells with 1 mL TriReagent per cm^2 flask surface area. Gently shake or rock the flask for 5 minutes at room temperature to promote lysis. Pipet up and down to mix well, then transfer TriReagent to a Falcon tube.
      Note: for suspension cells, use TriReagent LS (Mol. Research Center, #TS-120) as per protocol. Samples may be stored in TriReagent at -80 °C.
   b. Add 0.2mL of chloroform for every 1mL of TriReagent. Mix by pipetting, then shake vigorously for 45-60 seconds, or until the mixture is pink and homogeneous. Incubate at room temperature for 2-3 min.
   c. Pre-chill a centrifuge to 4 ºC. Centrifuge for 15 minutes at 10,000g (or as fast as possible) at 4 ºC. The sample should resolve into a clear aqueous layer above a pink organic layer; there may be white material at the interface. Be careful not to shake or invert the sample or disturb the interface.
   d. Transfer the top aqueous phase to a fresh Falcon tube, being extremely careful to not disrupt or disturb the interface, or to transfer any white or pink material. It is much better to sacrifice yield than to get the final drops of the aqueous layer and risk contamination with the interface or the organic layer. Dispose of the pink organic layer as phenol waste.
   e. Option 1: proceed with RNeasy column (recommended).
      Option 2: precipitate the RNA by adding 0.5 volume isopropanol and mix well. Centrifuge for 15 minutes at 10,000g (or as fast as possible) at 4 ºC (mark the tube for centrifuge orientation to be able to locate a pellet). Carefully pour off the supernatant. Wash pellet with 70% ethanol and spin again (same orientation). Carefully pour off the supernatant and remove residual liquid. Allow the pellet to dry for at least 5 minutes, then resuspend in RNAse-free water. After the RNA pellet is resuspended, keep the RNA sample on ice at all times. Store at -80 ºC.

3. Recommended: Purify the RNA sample on an RNeasy column, as described in the protocol.
   a. Add 1 volume (<9mL) 70% ethanol to the lysate and mix thoroughly by vortexing.
   b. Use a pipette to transfer up to 15 mL of the sample, including any precipitate, to an RNeasy maxi column and place in a 50mL collection tube (included with RNeasy kit). Close the screw-top collection tube and centrifuge for 5 minutes at 5,000g at 4 ºC and discard flow-through. If there is remaining sample, repeat this step until the sample is fully loaded on the column.
      Note: The maximum binding capacity for the RNeasy maxi columns is 6 mg, or 10^7-10^8 cells.
   c. Wash the column with 15 mL Buffer RW1. Close the collection tube and centrifuge for 5 minutes at 5,000g at 4 ºC. Discard the flow-through from the collection tube.
   d. Wash the column with 10 mL Buffer RPE. Close the collection tube and for centrifuge 5 minutes at 5,000g at 4 ºC. Discard the flow-through from the collection tube. (Add Ethanol to Buffer RPE as per RNeasy protocol.)
   e. Repeat the wash with another 10mL Buffer RPE. Close the collection tube and centrifuge for 10 minutes at 5,000g at 4 ºC. Discard the flow-through from the collection tube. Repeat the spin for 10 minutes at 5,000g at 4 ºC without closing the collection tube. Let the column sit open at room temperature in a chemical fume hood for approximately 10 minutes or until membrane is visibly dry.
   f. Elution: Transfer the RNeasy column to a new 50 mL collection tube. Pipet 1.0 mL RNase-free water directly onto the RNeasy membrane. Close the collection tube and incubate for 2 minutes at room temperature before centrifuging for 3 minutes at 5,000g at 4 ºC.
   g. Store at -80ºC.

4. Analyze the purity and concentration of the RNA sample on a spectrophotometer. Minimum purity requirements:
   $A_{260}/A_{280} > 1.8$
   $A_{260}/A_{230} > 2$, ideally absorption minimum at 230 λ.

5. Synthesize cDNA according to the M-MLV Reverse Transcriptase protocol, with the following recommendations and changes:
   a. Combine the following reagents in a reaction tube:
      0.5 µl 25 mM dNTPs
      0.33 µl 50 µM oligo dT_{20} primers
      0.66 µl 50 µM random 9mer primers
      up to 4 µg total RNA
      bring vol to 15uL RNAse-free water
      Note: for more than one RNA sample, prepare a master mix of dNTPs, primers, and water.
      Recommended: In parallel, set up a NTC (No Template Control) sample consisting of a cDNA reaction with RNA template and withoutM-MLV enzyme or a mock cDNA reaction consisting of buffers and primers only.
   b. Incubate at 70 ºC for 1 minutes to denature RNA. Immediately transfer to wet ice.
   c. Add the following reagents (in order), and mix:
      2 µl 10x RT buffer
      1 µl M-MLV Reverse Transcriptase
      Note: for more than one RNA sample, prepare a master mix of buffer, DTT, and enzyme.
   d. Incubate at 37 ºC for 50 minutes, then at 75 ºC for 10 minutes to inactivate the enzyme.
   e. The cDNA reaction may be stored at -20 ºC.

6. Serially dilute the cDNA sample to create a standard curve for qPCR. First, add 160 µl molecular biology-grade water to the cDNA reaction and mix by pipetting up and down. Make 2 further serial dilutions by combining 175µL water with 25 µL diluted cDNA to create a total of 3 standard curve samples. Dilute the NTC sample with 160 µl molecular biology-grade water (no serial dilution is required).
   Note: RNAse free water is not necessary at this step.

7. Set up qPCR reactions in 384-well optically clear PCR plates as follows:
   a. For each assay, prepare an Assay Master Mix for all reaction wells,
      e.g. all cDNA samples/assay * # dilutions * # replicate wells, plus excess:
      2.50 µl 2X SYBR Green Master Mix (contains enzyme, buffer and dNTPs)
      0.06 µl 80X SYBR Green assay (2 primers each at 20 µM)
      0.44 µl molecular biology-grade water
      We recommend running 3 replicate qPCR reactions for each cDNA dilution for each assay.
   b. On ice, aliquot 3 µl Assay Master Mix to appropriate wells in the 384-well qPCR plate.*
   c. On ice, aliquot 2 µl diluted cDNA sample to appropriate wells in the 384-well qPCR plate.*
   d. Seal the 384-well qPCR plate with an optically-clear seal. Keep on ice or at 4 ºC until running qPCR.
      Notes on qPCR setup: It is important that all samples being quantified with the same assay be on the same qPCR plate in order to calculate relative levels of gene expression.
      ** Recommended: set up 384-well qPCR reactions with a robotic liquid handling robot such as the MultiProbe II (Perkin Elmer). For manual setup, larger reaction volumes may be required for consistent data quality.*

8. Program a 384-well real-time PCR machine (e.g. Roche LightCycler480 or ABI 7900HT) to define the appropriate samples, assays, and detector (SYBR Green) for the qPCR plate layout. Cycling parameters:
   - incubate 2 minutes at 95 °C (enzyme activation)
   - cycle 15 seconds at 95 °C / 10 seconds at 60 °C / 20 seconds at 72 °C x40^{+} cycles
   - melt curve: ramp from 60 °C to 95 °C at 1 °C per second.

   Run time is ~ 1 hour on the LightCycler480 instrument.

9. Analyze target gene expression for both detection (e.g. Ct value of highest cDNA input) and for quantification (e.g. linearity and slope of standard curve). Only continue with high-throughput knockdown validation (Part 2) if gene expression is detectable and the assay is quantitative. The ideal assay will also have no signal in the NTC sample, or a sufficient shift in Ct or in the melt curve data to be able to distinguish specific from non-specific signal.

Part 2: HT Knockdown Validation

I. Materials

Viral infections:
96-well tissue culture plates
Human or mouse cell line and appropriate growth media
Polybrene (Hexadimethrine bromide; Sigma H 9268) or Protamine sulfate (MP Biomedicals #194729)
Puromycin Dihydrochloride (Sigma #P8833)

Quantitative PCR:
RNeasy 96 kit (Qiagen, #74182)
RNase-free water (e.g. Ambion, #9922)
M-MLV Reverse Transcriptase (Sigma, #M1302)
Oligo dT_{20} (ordered from oligo vendor such as IDT)
Random 9-mer oligos (ordered from oligo vendor such as IDT)
dNTPs (e.g. Stratagene, #200415-51)
RNAse-free PCR plate
Plate seal
SYBR Green assay for target gene (e.g. selected from PrimerBank)
SYBR Green assay for endogenous control gene (e.g. selected from PrimerBank)
SYBR Green qPCR Master Mix (e.g. Sigma, # S9194)
qPCR 384-well optically clear plate and seal (e.g. Roche, # 5102430001)

Note: this protocol requires a 384-well real-time PCR machine, such as the LightCycler480 (Roche) or the 7900HT (ABI), and SYBR Green PCR assays. Use of an alternate real-time PCR machine or an alternate qPCR assay system may require changes to the protocol, for example an alternative qPCR enzyme mix, a larger reaction volume for a 96-well format, etc.

II. Instructions

1. Infect cells in 96-well tissue culture plates as described in Section III: Lentiviral Infections. Infections should be optimized for the following conditions:
   a. Seed cells such that the density at the time of lysis (RNA isolation) is 80-95% confluent.
   b. Infect cells with desired viral MOI.
      Note that low-MOI (<1) infections will lead to significant cell death following selection.
   c. Select cells with appropriate dose of puromycin for at least 48 hours.
   d. Include reference infections with non-targeting constructs for comparison to knockdown constructs.

2. Isolate total RNA according to the Qiagen RNeasy 96 protocol, with the following recommendations and changes:
   a. Remove all media prior to adding lysis buffer. Add lysis buffer and shake back and forth on a flat surface for 30 seconds, then rotate the plate 180 degrees and shake again for 30 seconds.
      Note: some cell lines may require additional physical scraping in lysis buffer for efficient lysis.
   b. (Optional) Cell lysates may be stored at -80 °C prior to RNA isolation. Thaw plates at room temperature (~10 mintues) and pipette up and down gently to mix before proceeding.
   c. Use the centrifugation protocol rather than using a vacuum manifold.
      Note: we recommend using a high-speed 96-well plate centrifuge such as the 4K15C centrifuge (Qiagen #81210).
   d. After the final wash step, empty the waste collection plate and spin again for 4 minutes to ensure that the columns are dry. Then let the plate sit at room temp for an additional 4 minutes, ideally in a chemical fume hood with high air flow, for a final drying step.
   e. Elute total RNA with one addition of RNAse-free water. The volume of RNA that will be recovered is less than the volume of RNAse free water used to elute. Eluting with 60 µl will usually return at least 45 µl, enough for multiple cDNA reactions.

f. If possible, move directly from elution to cDNA synthesis without freezing the RNA. Freeze/thaw cycles may decrease RNA quality. RNA should be frozen at -80 °C for long term storage.

3. Synthesize cDNA according to the M-MLV Reverse Transcriptase protocol, with the following recommendations and changes:
   a. Add 7 µL total RNA per sample to separate wells in an RNAse-free 96-well PCR plate. Store any remaining RNA at at -80 °C.
   b. Seal the cDNA plate and incubate at 70 ºC for 1 minute to denature RNA. Immediately transfer to wet ice.
   c. Prepare sufficient Master Mix for all samples (plus excess):

0.5 µl	25 mM dNTPs
0.33 µl	50 µM oligo dT_{20} primers
0.66 µl	50 µM random 9mer primers
2 µl	10x RT buffer
1 µl	M-MLV Reverse Transcriptase
8.5 µl	RNAse-free water

   d. Unseal the PCR plate and add 13 µL Master Mix per well. Mix.
   e. Seal the plate and incubate at 37 ºC for 50 minutes, then at 75 ºC for 10 minutes to inactivate the enzyme.
   f. The cDNA reactions may be stored at -20 ºC.

4. Dilute the cDNA samples prior to setting up qPCR reactions. Add 160 µl molecular biology-grade water to each reaction and mix by pipetting up and down. Be careful of cross contamination.
   Note: RNAse free water is not necessary at this step.

5. Set up qPCR reactions in 384-well optically clear PCR plates as follows:
   a. For each assay (both target gene and endogenous control gene), prepare an Assay Master Mix for all reaction wells (e.g. all cDNA samples/assay * # replicate wells, plus excess):

2.50 µl	2X SYBR Green Master Mix (contains enzyme, buffer and dNTPs)
0.06 µl	80X SYBR Green assay (2 primers each at 20 µM)
0.44 µl	molecular biology-grade water

   We recommend running 3 replicate qPCR reactions for each cDNA sample for each assay.
   b. On ice, aliquot 3 µl Assay Master Mix to appropriate wells in the 384-well qPCR plate.*
   c. On ice, aliquot 2 µl diluted cDNA sample to appropriate wells in the 384-well qPCR plate.*
   d. Seal the 384-well qPCR plate with an optically-clear seal.
   Notes on qPCR setup: In addition to target gene reactions, endogenous control gene reactions are required for each sample. It is not necessary for these reactions be on the same plate as the target gene reactions. It is important that all samples being quantified with the same assay be on the same qPCR plate in order to calculate relative levels of gene expression.
   ** Recommended: set up 384-well qPCR reactions with a robotic liquid handling robot such as the MultiProbe II (Perkin Elmer). When manual setup is required, larger reaction volumes may be required for consistent data quality.*

6. Program a 384-well real-time PCR machine (e.g. Roche LightCycler480 or ABI 7900HT) to define the appropriate samples, assays, and detector (SYBR Green) for the qPCR plate layout. Cycling parameters:
   - incubate 2 minutes at 95 °C (enzyme activation)
   - cycle 15 seconds at 95 °C / 10 seconds at 60 °C / 20 seconds at 72 °C x40^{+} cycles
   - melt curve: ramp from 60 °C to 95 °C at 1 °C per second.

   Run time is ~ 1 hour on the LightCycler480 instrument.

7. Analyze target gene knockdown using the ΔΔCt analysis method (see ABI User Bulletin #2 and/or Current Protocols in Molecular Biology, Unit 15.8: High-Throughput Real-Time Quantitative Reverse Transcription PCR). Use the reference infections with non-targeting constructs as reference samples to define 100% expression. *Note that only samples that are quantified on the same plate with the same qPCR assay may be directly compared for relative gene expression. Data quality indicators include the melt curve data (amplicon T_m) and technical replicate consistency.*

Version Notes:

September 2008

cDNA Synthesis – Improvements to the cDNA synthesis step include use of a cocktail of oligo-dT and random 9mer primers and use of Sigma's M-MLV reverse transcriptase. We have found that, for most target genes, oligo-dT is sufficient for cDNA priming, but for some transcripts adding random 9mer primers can improve qPCR detection. We have also compared panels of different reverse transcriptase enzymes and vendors across a variety of target genes and have found that M-MLV from Sigma often outperforms other reverse transcriptases, including SuperScript II.

SYBR Green – Since the first version of this protocol, we have switched from Taqman assays to SYBR Green assays. This requires slight protocol modifications including a different 2x Master Mix formulation (with SYBR Green dye), 3-temperature cycling conditions for better SYBR Green fluorescence, and addition of a melt curve to measure amplicon T_m. We have tested a panel of different 2x SYBR Green Master Mixed and have found very comparable performance from different vendors. There are a variety of sources for SYBR Green assays, including software packages, pre-designed assay collections, and commercial sources. We select SYBR Green assays from PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html), a collection of pre-designed SYBR Green assays for human and mouse genes. We additionally confirm assay specificity for the target gene by BLAST, and we determine whether the assay amplifies all Entrez transcripts for the target gene and whether the assay crosses an intron, both of which are preferred.

qPCR Reaction Volume – We have optimized our robotic liquid handling systems to allow the qPCR reaction volume to decrease to 5 µL total volume. We recommend using this small reaction volume only with robotic qPCR reaction setup and only after careful optimization.

LightCycler480 – We now routinely use a LightCycler480 384w qPCR instrument from Roche for high-throughput qPCR. We have found that the LightCycler480 generates equivalent SYBR Green data as the ABI 7900HT, and offers other advantages for our high-throughput process (faster cycling times, better data management options).