**Protocol RT-qPCR for analyzing viral positive- and negative-sense RNA abundance during Reovirus infection**

**Primer design**

Design a set of primers for qPCR to amplify a region of our gene of interest.

* Open Primer 3: <http://bioinfo.ut.ee/primer3-0.4.0/>
  + Paste your sequence into the search box
  + Give a name in the “Sequence ID” box
  + Enter the following numbers in the appropriate slots. For anything not specifically stated below, leave as the default:
    - Product Size Ranges: 75-150
    - Number to return: 25
    - Primer Size: Min: 18; Opt: 20; Max: 23
    - Primer TM: Min: 58; Opt: 60; Max: 62
    - Product TM: Min: blank; Opt: blank; Max: 86
    - Primer GC%: Min: 50; Opt: blank; Max: 60
    - Max Poly-x: 3
    - GC Clamp: 1
  + Click “Pick Primers”
* Primer 3 Output definitions- These give information about the actual primers themselves
  + Start= start position- the position of the 5’ base of the primer
  + Len= oligo length- the length of the primer
  + Tm= melting temperature- melting temperature of the primer
  + Gc%= percent of g or c bases in the primer
  + Any\_th= self-complementarity- the self-complementarity score of the primer, taken as a measure of its tendency to anneal to itself or form secondary structures
  + 3’\_th= 3’ self-complementarity- taken as a measure of its tendency to form a primer-dimer with itself
  + Seq= the actual sequence of the primer
* How to pick a primer set:
  + Want “pair any comp”= 3
  + Want “pair 3’ comp”= 0, or as low as possible
  + Go through these numbers for the various outputs and find some that seem reasonable.
* Resuspend primers:
  + Make 500 μM stock:
    - Add MQ (volume = nmol primer x 2 in μl)
    - Vortex
    - Store at -20°C
  + Make 10 μM working dilution:
    - 2 μl of 500 μM stock + 98 μl MQ
    - Stare at -20°C

**RNA extraction**

RNA was extracted from 50 μl of thawed lysate using the RNeasy mini kit (Qiagen)

* Add 350 μl Buffer RLT to lysate
* Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed
* Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.
* Transfer up to 800 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through
* Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
* Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
* Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane
* Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.
* Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 20 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.

For more information, check <http://www.bea.ki.se/documents/EN-RNeasy%20handbook.pdf>

**cDNA Synthesis**

* Add the following components to a nuclease-free 1.5 microcentrifuge tube:
  + 2 pmol of gene-specific primer (**2 μl of a 10 μM stock solution**)
    - Forward primer for negative-sense RNA
    - Reverse primer for positive-sense RNA
  + 10 pg–5 μg total RNA (**4μl of extracted RNA**)
  + 1 μl 10 mM dNTP Mix
  + 6 μl of DEPC-treated water
* Heat mixture to 95°C for 3 minutes1 (heatblock) and incubate on ice for 5 minute
* Collect the contents of the tube by brief centrifugation and add:
  + 4 μl 5X First-Strand Buffer
  + 1 μl 0.1 M DTT
  + 1 μl RNaseOUT™
  + 1 μl of SuperScript™ III RT (200 units/μl)

|  |  |
| --- | --- |
| Component | Volume (µL) |
| RNA + dNTP + RT primer | 13 |
| 5X buffer | 4 |
| DTT | 1 |
| RNaseOUT | 1 |
| SuperScript III | 1 |
| Total | 20 |

* Collect the contents of the tube by brief centrifugation
* Incubate at 55°C for 50 minutes (heatblock)
* Inactivate the reaction by heating at 70°C for 15 minutes (heatblock)
* The cDNA can now be used as a template for amplification in qPCR

For more information, check <https://tools.thermofisher.com/content/sfs/manuals/superscriptIII_man.pdf>

**Fast SYBR Green qPCR for Relative Quantification**

7500 Fast Real-Time PCR System in Antczak Lab (room 215)

All users MUST be trained by Don Miller

Sign up for instrument in advance using Localendar website ([www.localendar.com](http://www.localendar.com)) login: antczak\_realtime, password: antczakqpcr

Work at designated qPCR bench, wear gloves, use filter tips. Keep all reagents on ice.

Always first add negative controls. Add plasmid positive controls last with separate pipettors.

* Remove reagents from -20°C and place on ice:
  + RT product
  + 10 μM forward and reverse primers
  + Fast SYBR green
* Place microamp fast optical 96-well reaction plate with barcode in holder on ice, do not let plate wells come into contact with ice.
* Make up master mix(es). For 0.3 μM primer concentration, 1x is as follows:
  + SYBR Green 10 μl
  + 10 μM Forward Primer 0.6 μl
  + 10 μM Reverse Primer 0.6 μl
  + MQ Water 6.8 μl
* Mix master mix(es) by gently flicking tubes. Do not vortex.
* Add 18 μl master mix to each well, changing pipette tip for each well.
* Add 2.0 μl water to each NTC well, changing pipette tip for each well.
* Add 2.0 μl template DNA to each sample well, changing pipette tip for each well.
* Cover plate with optical adhesive cover.
* Spin plate briefly in table top centrifuge at about 200 x g, 4°C.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| a | Mock + sense | | | Mock - sense | | |  |  |  | 10-4 plasmid control | | |
| b | 0 hpi + sense | | | 0 hpi - sense | | |  |  |  | 10-5 plasmid control | | |
| c | 4 hpi + sense | | | 4 hpi - sense | | |  |  |  | 10-6 plasmid control | | |
| d | 8 hpi + sense | | | 8 hpi - sense | | |  |  |  |  |  |  |
| e | 12 hpi + sense | | | 12 hpi - sense | | |  |  |  |  |  |  |
| f | 16 hpi + sense | | | 16 hpi - sense | | |  |  |  |  |  |  |
| g | 24 hpi + sense | | | 24 hpi - sense | | |  |  |  |  |  |  |
| h |  |  |  |  |  |  |  |  |  | No Template Control | | |

Example of plate lay-out

* Record run information in thermal cycler log book.
* Set up thermal cycler and start run as instructed by Don Miller or Matt Pennington
* Software settings thermal cycler
  + New Experiment
    - 7500 Fast
      * Quantitation – Comparative Ct (ΔΔCt)
      * SYBR Green Reagents
      * Fast
      * Add primer Target
      * Add sample names
      * Passive reference set at “ROX”
      * Check if Melting curve is on

**Fast SYBR Green qPCR with Standards**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| a | Mock + sense | | | Mock - sense | | |  |  |  |  | 10-4 Std | |
| b | 0 hpi + sense | | | 0 hpi - sense | | |  |  |  |  | 10-5 Std | |
| c | 4 hpi + sense | | | 4 hpi - sense | | |  |  |  |  | 10-6 Std | |
| d | 8 hpi + sense | | | 8 hpi - sense | | |  |  |  |  | 10-7 Std | |
| e | 12 hpi + sense | | | 12 hpi - sense | | |  |  |  |  | 10-8 Std | |
| f | 16 hpi + sense | | | 16 hpi - sense | | |  |  |  |  |  |  |
| g | 24 hpi + sense | | | 24 hpi - sense | | |  |  |  |  |  |  |
| h |  |  |  |  |  |  |  |  |  | No Template Control | | |

* Plasmid used for standards during qPCR with S3 primers
  + pT7-M2-S2-S3-S4T3D (Plasmid #33302)
  + <https://www.addgene.org/33302/>
  + 9688bp
* Calculate copy number of standard DNA molecules
  + <http://cels.uri.edu/gsc/cndna.html>
  + Y molecules/μl= [(Xg/μl DNA)/(plasmid length in basepairsx650g/mol)]x6.022x1023 molecules/mol
  + Calculation for the 10-4 plasmid dilution
    - [(3441x10-13g/μl DNA)/(9688 bpx 650 g/mol)]x6.022x1023 = 3.29x107 copies/μl
    - 2 μl added so 6.58x107 copies in the 10-4 plasmid standard
* Software settings thermal cycler
  + New Experiment
    - 7500 Fast
    - Quantitation – standard curve
    - Ask Don Miller for specifics on settings in program
      * Define and set up Standards

1. Ooms, L. S., Kobayashi, T., Dermody, T. S. & Chappell, J. D. A post-entry step in the mammalian orthoreovirus replication cycle is a determinant of cell tropism. *J. Biol. Chem.* **285,** 41604–13 (2010).