



PATROLS

Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Reverse Transcriptase PCR for lung models

**This is a a) SOP used by members of
PATROLS only or (b) SOP
recommended for external use by
PATROLS**

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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1 Introduction:

DOMAIN: *Quantitative Reverse Transcriptase qPCR*

Due to the rapid development and implementation of a diverse array of engineered nanomaterials (ENM), it is becoming increasingly evident that humans will inevitably be exposed to ENM on a regular basis. There is a rising concern regarding the related toxicity following ENM exposure. In an effort to reduce the reliance on animal experimentation, and lower costs and resources several approaches are being investigated to optimise ENM hazard assessment (Ates et al., 2018). An increasingly popular approach is the application of gene expression analysis, which can be used to discriminate transcriptional changes following exposure to ENM (Mo et al., 2012).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is considered the 'gold standard' for gene expression quantification (Schmittgen et al., 2008). RT-qPCR is a relatively simple, inexpensive, sensitive and specific tool used to determine the expression level of target genes (Alvarez and Doné, 2014). There are four principle techniques employed when performing RT-qPCR; the isolation of RNA from cellular material, reverse transcription (RT) of RNA to complimentary DNA (cDNA), and real-time polymerase chain reaction (qPCR).

PCR arrays can be supplied as 96 or 384 well plates that are predesigned with functionally related genes in disease-associated and/or known cell signalling pathways. They offer a semi high-through put approach that provides real-time information about activation or inhibition of key pathways. This SOP focuses on the use of the personalised predesigned Bio-Rad RT-qPCR array. It contained 16 genes associated with various published adverse outcome pathways (AOPs) (inflammatory markers, apoptosis, epithelial and monocyte/macrophage cellular markers), 3 housekeeping genes and 5 different assay controls. This SOP has been established to support the identification of an endpoint biomarker panel in lung cell models that can be used as an effective screening approach for implications to human health following ENM exposure.

1.1 Scope and limits of the protocol

This SOP was established with the intention to be used for the evaluation of transcriptional profiles changes associated with inflammatory responses in lung models following ENM exposure over both an acute or long-term, and repeated dose regime. This method allows for the identification of a biomarker panel that can be used as an effective screening approach.

Limitations of the Protocol

1. Pre-defined disease pathway PCR array plates can be costly and therefore it may not be feasible to run each sample in triplicate.

1.2 Validation state of protocol

Level of advancement towards standardization	Level reached (please mark only one with “X”)
Stage 1: Internal laboratory method under development	
Stage 2: Validated internal laboratory method	x
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	N
Has the method been submitted for standardisation (to OECD, CEN, ISO,...) in its own right or as part of another standardisation project?	N
Is the method included in an existing standard (or ongoing	N

standardisation work)

If yes, specify

[standard reference
number, eg. EN
17199-4]

2 Terms and Definitions:

Agglomerate

Collection of weakly or medium strongly bound *particles* where the resulting external surface area is similar to the sum of the surface areas of the individual components.

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.2]

Aggregate

Particle comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components.

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent or ionic bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former primary particles.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.3, modified — Note 1 adapted.]

Nanoscale

Length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE : ISO/TS 80004-1: 2016, definition 2.1]

Nanotechnology

Application of scientific knowledge to manipulate and control matter predominantly in the *nanoscale* to make use of size- and structure-dependent properties and phenomena distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material.

Note 1 to entry: Manipulation and control includes material synthesis.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.3]

Nanomaterial

Material with any external dimension in the *nanoscale* or having internal structure or surface structure in the nanoscale.

Note 1 to entry: This generic term is inclusive of *nano-object* and *nanostuctured material*.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.4]

Nano-object

Discrete piece of material with one, two or three external dimensions in the *nanoscale*.

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.5]

Nanostructure

Composition of inter-related constituent parts in which one or more of those parts is a *nanoscale* region.

Note 1 to entry: A region is defined by a boundary representing a discontinuity in properties.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.6]

Nanostructured material

Material having internal *nanostructure* or surface nanostructure.

Note 1 to entry: This definition does not exclude the possibility for a *nano-object* to have internal structure or surface structure. If external dimension(s) are in the *nanoscale*, the term nano-object is recommended.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.7]

Engineered nanomaterial

Nanomaterial designed for specific purpose or function

[SOURCE: ISO/TS 80004-1: 2016, definition 2.8]

Manufactured nanomaterial

Nanomaterial intentionally produced to have selected properties or composition.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.9]

Incidental nanomaterial

Nanomaterial generated as an unintentional by-product of a process.

Note 1 to entry: The process includes manufacturing, bio-technological or other processes.

Note 2 to entry: See “ultrafine particle” in ISO/TR 27628:2007, 2.21

Particle

Minute piece of matter with defined physical boundaries.

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to *nano-objects*.

[SOURCE: ISO 26824:2013, 1.1]

Substance

Single chemical element or compound, or a complex structure of compounds.

[SOURCE: ISO 10993-9:2009, definition 3.6]

3 Abbreviations:

cDNA - complementary DNA

Cq – Quantification cycle (Cq) values

PCR – Polymerase Chain Reaction

RT-qPCR – Reverse transcription real-time PCR/ Quantitative reverse transcription PCR

PBS – Phosphate Buffered Saline

PPE – Personal Protective Equipment

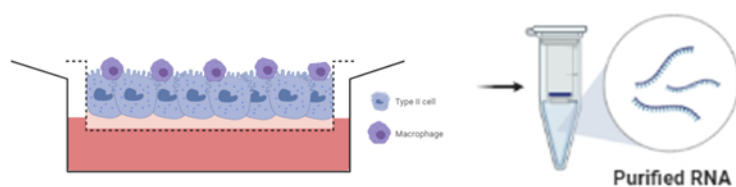
4 Principle of the Method:

Quantitative Reverse Transcription PCR (RT-qPCR) is used when the starting material is RNA. RNA is first transcribed from total RNA into complementary DNA (cDNA) by reverse transcriptase. The cDNA is then used as the template for the qPCR reaction. RT-qPCR is considered the ‘gold standard’ for gene expression analysis. The aim of this SOP is to systematically demonstrate how to extract RNA

from lung models grown on transwell inserts (PATROLS_SOP_A549 for example), reverse transcribe RNA into cDNA templates, perform qPCR of the cDNA transcripts using a PCR array. This protocol can be broken down into 4 key stages as described in Figure 1 below:

RT-qPCR Array Workflow

1 Extract RNA



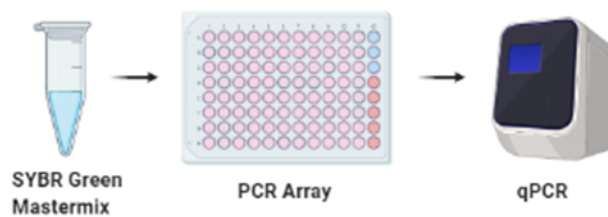
2 Evaluate RNA quality



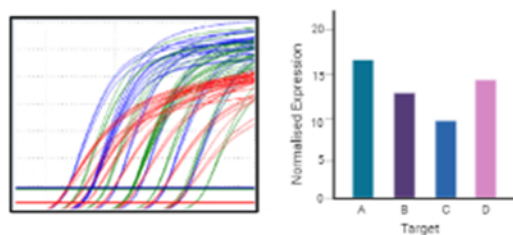
3 Reverse transcribe RNA to cDNA



4 Real-time PCR using PCR array



5 Gene Expression analysis



Created in BioRender.com bio

Figure 1: Schematic Diagram of RT-qPCR Workflow (Created with BioRender.com).

5 Description of the Method:

5.1 Biological setting & test system used:

The cultivation and exposure of the lung model to ENM should be carried out under sterile conditions in a Class 2 Laminar Tissue Culture Hood as per (PATROLS_SOP_A549).

After the RNA has been harvested the remainder of the procedure will be performed in a HEPA-filtered vertical laminar flow PCR hood.

5.2 Chemicals and reagents used:

- PBS pH 7.4 1X, MgCl₂ and CaCl₂ Free (14190-094, GIBCO®, Paisley, UK),
- Ethanol (10048291, Fisher Scientific)
- QIAGEN RNeasy mini kit (74106, Qiagen)
- QIAGEN QIAshredders (79656, Qiagen)
- iTaq Universal SYBR green Supermix (1725120, Bio-Rad)
- iScript gDNA Clear cDNA Synthesis Kit (172-5034, Bio-Rad)
- RNase Zap (Merck, R2020)

5.3 Apparatus and equipment used:

All tissue culture equipment was sourced from Greiner Bio-One, UK unless stated otherwise. Equivalent equipment purchased from alternative suppliers can be used if desired, although the products listed below are recommended for this SOP.

- Laminar Class II Tissue Culture Hood (e.g. Scanlaf Mars)
- Centrifuge (Eppendorf 5810 R)
- T-25 (690175) and T-75 (660175) Tissue Culture Flask
- P1000 and P200 micropipettes
- Non-Filtered, Sterile 200µl and 1000µl Pipette tips
- 15ml Centrifuge Tubes
- 50ml Falcon Tube
- 3ml Pasteur pipette
- Bio-Rad Prime PCR Custom plate 96W (10025217)

- Bio-Rad Microseal 'B' Film (MSB1001)
- PCR hood (e.g. Labconco Purifier PCR Enclosure)
- Microcentrifuge (Star Lab)
- NanoDrop ND-100 spectrophotometer
- NanoDrop Software (at least version 3.1.2)
- Bio-Rad CFX Connect Real-Time PCR system
- Thermo Cycler T-100, Bio-Rad
- CFX Manager software (at least version 3.1)

5.4 Reporting of protected elements:

This SOP does not have any associated patent restrictions, specific licenses, material transfer agreements or commercial purchase requirements to perform the protocol described.

5.5 Health and safety precautions:

Prior to any use of this SOP a full risk assessment should be completed, considering all potential risks associated with chemicals equipment and use, in compliance with national regulation. Training of personnel should be completed before any person is working with the SOP.

Standard health and safety precautions associated with working within a laboratory environment and performing mammalian cell culture, as described by the European Agency for Safety and Health at Work (<https://osha.europa.eu/en/safety-and-health-legislation/european-guidelines>), should be adopted when conducting this SOP. In addition, all health and safety precautions outlined in the MSDS data sheets associated with the specific chemicals required must be followed.

5.6 Reagent preparation:

In preparation for the SOP to be performed, it is advised that the following solutions summarised below are prepared:

- Add the kit specific volume of ethanol (96–100%) directly to the RPE buffer (supplied as part of the Qiagen RNA easy Mini Kit) as indicated on the bottle to obtain a working solution.

5.7 Procedure:

5.7.1 Lung cell harvest

1. Remove the transwell companion plates (in which the transwell membranes are inside) from the incubator.
2. Using a 1000 µL pipette, aspirate the cell culture medium from the basal side and pipette into 1.5ml ependorfs (N.B. *if the supernatants are to be used for additional biological endpoints then they must be stored appropriately at this time*). Ensure there is no cell culture medium is left on the apical side of the transwell membrane insert.
3. Wash the transwell membrane inserts with in 1 mL of sterile PBS (1x) at room temperature on both the apical and the basal side.
4. Disgard the PBS to waste.

5.7.2 RNA Extraction

RNA extraction is performed as per the QIAGEN RNeasy mini kit manufacturers instructions (using QIAGEN QIAshredders) outlined at: <https://www.qiagen.com/us/shop/automated-solutions/quality-assurance/rneasy-mini-kit/#productdetails>. All centrifugation steps are carried out at room temperature.

1. Clean down bench area completely with RNase Zap.
2. Add 300µl Buffer RLT (to an insert (of any size. 300µl of Buffer RLT is per sample), pipetting vigorously to ensure lysis of the cells.
3. Pipette the lysate directly into a QIAshredder spin column (purple column) that is in a 2 mL collection tube and centrifuge for 2 minutes at 8000 x g.
4. Add 300µl of 70% ethanol to lysate and mix well by pipetting.
5. Transfer a maximum of 700µl sample into RNeasy spin column (pink column) that is in a 2 mL collection tube.

6. Centrifuge for 15 seconds at 8000 x g and discard the flow-through.
7. If sample exceeds 700µl, reuse the empty collection tube from step 5 and repeat until all of the sample has been centrifuged, discarding flow-through after each centrifugation.
8. Add 700µl Buffer RW1 to RNeasy spin column and centrifuge for 15 seconds at 8000 x g, then discard flow-through.

Note: When removing spin column from collection tube make sure the tube does not come into contact with the flow-through.

9. Add 500µl Buffer RPE to RNeasy spin column.
10. Centrifuge for 15 seconds at 8000 x g and discard flow-through and reuse collection tube.
11. Add 500µl buffer RPE to RNeasy spin column.
12. Centrifuge for 2 minutes at 8000 x g and discard flow-through.
13. Place RNeasy spin column into new 2 mL collection tube.
14. Centrifuge for 1 minute at 17000 xg and discard flow-through
15. Place RNeasy spin column into new 1.5 mL collection tube.
16. Add 40µl RNase free water to the membrane of the RNeasy spin column - centrifuge for 1 minute at 8000 x g to elute RNA into the 1.5ml collection tube.
17. The RNA is then tested for its quality and yield using the Nanodrop as follows;
18. Ensure the nanodrop software is set to estimate RNA.
19. Blank the Nanodrop with 1µl of RNase free water and press measure (an expected output is outlined in step 22).
20. Wipe the Nanodrop probe clean with a lint free wipe (such as a kimwipe).
21. Add 1µl of RNA sample to Nanodrop and press measure.
22. Record the purity and yield as indicated by the Nanodrop software;
 - i. $A_{260}/A_{280} > 2.0$ for pure RNA (assess DNA contamination)
 - ii. $A_{260}/A_{230} \sim 2.0$ for pure RNA (assess protein contamination)
23. When reading multiple samples, repeat step 18 in between each sample. Each sample will take round 45-60seconds to measure.

24. RNA samples, at this stage, can then be stored at -80 °C freezer in single aliquots until further experimentation can occur. Otherwise, they can be used immediately for cDNA synthesis (see section 5.7.3).

5.7.3 cDNA Synthesis

The synthesis of cDNA is achieved as per manufacturers instructions, using the iScript Reverse Transcription Supermix for RT-PCR - Bio-Rad (1708840).

1. Turn on the laminar flow PCR hood. Thaw kit contents (stored at -20) on ice.
2. Thaw RNA samples on ice. This is to keep the samples cold and to ensure they don't reach room temperature.
3. Place all items needed into the PCR hood. All steps going forward should be carried out in a PCR hood.
4. Mix the contents of all tubes by inverting the tube and shaking gently, and centrifuge briefly before placing back onto ice.
5. Use the same amount of RNA in each cDNA synthesis reaction (please see example below).

Worked example:

1000ng (1µg) (commonly used) of RNA.

$$\text{Volume of RNA (}\mu\text{l)} = \frac{1000\text{ng}}{\text{Concentrations (ng/}\mu\text{l)}}$$

$$\text{Volume of RNA (}\mu\text{l)} = 1000\text{ng} / 295 \text{ ng/ } \mu\text{l}$$

$$\text{Volume of RNA (}\mu\text{l)} = 3.389 \mu\text{l}$$

6. For each RNA sample prepare a cDNA synthesis reaction on ice in a 200µl PCR tube.
7. The reaction is then set up as described in Table 2 for a single cDNA synthesis reaction. It is common practice to make one extra reaction mix beyond that which is required to ensure that after pipetting or human errors there will still be enough of the reaction mix.

Table 2: RT master mix set-up

Component	Volume per reaction (µl)
iScript RT Supermix	4
RNA template (1µg)	Variable
Nuclease free water	Variable
Total	20µl

Note: Prepare enough to include pipetting error as suggested above.

8. Prepare the RT master mix as indicated in Table 2 per each sample to be tested.
9. Mix all samples thoroughly *via* pipetting.
10. For every RNA sample prepare a no-RT (no reverse-transcriptase control means that transcription to cDNA should not have occurred) control reaction. Set-up up the no-RT control in the same way as described in Table 2 above expect replace the RT supermix with the no-RT control supermix. This will allow the detection of genomic DNA amplicons (*please refere to section 5.8.2*).
11. Prepare Reverse Transcription Control assay. For each RNA sample, include 1µl of the control RNA template in each 20µl cDNA synthesis reaction as described in Table 2 above (*please refere to section 5.8.2*).
12. Incubate the complete reaction mix tubes in a thermal cycler (Bio-rad, T100) using the protocol below:

Table 3: cDNA Synthesis thermal cycler Protocol

Thermal Cycler protocol	
Priming	5 minutes at 25°C
Reverse Transcription	20 minutes at 46°C
RT inactivation	1 minute at 95°C

13. The cDNA generated can be used immediately for qPCR on the PCR array plates or frozen at -80°C.

Real-Time PCR (qPCR) using the Bio-Rad Custom PCR Array Plates qPCR is conducted as per manufactures instructions using the iTaq Universal SYBR Green Supermix (172-5120) with custom PCR Array plates (Bio-Rad 10025217).

1. Turn on the PCR hood (if not already on from previous step) and wipe down with 70% ethanol. The PCR hood can be used straight after turning on and wiping down.
2. Remove PCR Array plates from the 4°C fridge and allow to come to room temperature.
3. Thaw the iTaq Universal SYBR Green Supermix, cDNA samples (Sample, no-RT control & RT control) and Positive PCR control RNA template on ice in the PCR hood.
4. Store all tubes (SYBR Green Supermix and cDNA samples) away from direct light. **All steps going forward should be carried out in the PCR hood.**
5. Mix all tubes SYBR Green Supermix and cDNA samples by inverting gently shaking the tube, and centrifuge briefly using the microcentrifuge.
6. Remove the seal of the array plate.
7. Dilute each cDNA sample to a total of 100ul using nuclease free water.

Worked example:

1000ng RNA/ 20µl RT reaction = 50ng/µl cDNA

Dilute to 100µl = 10ng/µl

Use 1µl diluted cDNA per 20µl qPCR reaction = 10ng
cDNA

8. To account for pipetting error, prepare enough qPCR reaction mix for all one PCR array plate on ice at a time (for one 96 well plate – make 25 reactions) as per Table 4 below.

Table 4: qPCR reaction master mix

Component	Volume per reaction (µl)	Final Concentration
iTaq Universal SYBR Green Supermix	10	1x
PCR Primer assay	Lyophilised on plate	-
cDNA sample	1µl	10ng
Nuclease Free Water	Variable	-
Total Volume	20µl	-

9. Transfer 20µl of the qPCR reaction mix into each well of the PCR array plate except the gDNA and RT control wells (see *Appendix 1 for example plate layout*).
10. Prepare the qPCR reaction mix for the gDNA and RT control well as per table 4 above, using the no-RT control and RT control cDNA generated in *section 5.7.3*, add 20µl of the controls to the appropriate wells.
11. Add 1µl of the PCR control assay into the PCR control well (total volume in this well will be 21µl in total (qPCR reaction master mix and PCR control assay)).

5.7.4 Performing Real-time PCR using the Bio-Rad CFX Connect qPCR System

1. Seal the plate with an optical seal (specified in section 5.3).
2. Centrifuge to remove bubbles at 4,000rpm for 2 minutes at room temperature.
3. Load the PCR plate into the Bio-Rad CFX Connect instrument.
4. Run the plate using the settings identified in Table 5 below

Table 5: PrimerPCR Cycling Protocol

Bio-Rad CFX qPCR protocol	Temperature	Time	Number of Cycles
Activation	95°C	2 minutes	1
Denaturation	95°C	5 seconds	40
Annealing/Extension	60°C	30 seconds	
Melt Curve	65°C -95°C	5 seconds/step	1

(0.5°C
increments)

5.8 Quality control & acceptance criteria:

To ensure specificity and sensitivity of the method, at each step there are a number of measures in place to ensure a high standard of quality control.

5.8.1 RNA Extraction Controls

When performing RNA extractions, the following purity criteria should be met:

A260/A280 > 2.0 for pure RNA (assess DNA contamination)

A260/A230 ~2.0 for pure RNA (assess protein contamination)

5.8.2 PCR Array Quality Controls

Interpretation of the qPCR assay controls was adapted from the Bio-Rad '*PrimePCR Assays, Arrays, and Controls for Real-Time PCR*' instruction manual (<https://www.bio-rad.com/webroot/web/pdf/lsr/global/english/primePCR/10000088666.pdf>). Positive PCR control Assay

The positive PCR control assay targets a synthetic DNA template to determine if samples contain inhibitors or other factors that may negatively affect gene expression within your sample. The synthetic DNA template is not present in the human genome.

- Primers are lyophilised on the PCR Array plate.
- DNA template is provided in a separate tube, and is added to the appropriate well as described above in *section 5.7.4, point. 10*.
- For each PCR array, prepare a positive PCR control reaction mix (cDNA template and positive PCR control assay). Add 1µl PCR control assay into the appropriate control well (total volume in this well will be 21µl in total (qPCR reaction master mix and PCR control assay) as described above in *section 5.7.4, point 9*.

Interpretation of the Positive PCR control

From the PCR machine we are given Cq values for each well – this indicates the number of amplification cycles the machine has completed before detecting the gene of interest.

Cq \geq 30 indicates poor PCR performance and gene expression results are likely to be compromised.

1. Reverse Transcription (RT) Control

The RT control assay introduces a synthetic RNA template into the cDNA synthesis reaction to evaluate RT performance. The sequence of the synthetic RNA template is not present in the human transcriptome.

- Primers are lyophilised on the PCR Array plate.
- RNA template is provided in a separate tube, resuspend in 200 μ l nuclease-free water on ice.
- For each RNA sample, prepare a RT control sample by adding 1 μ l of the control RNA template into the 20 μ l cDNA synthesis reaction (as described in Table 2 above), and proceed with the reverse transcription.
- Once RT control cDNA is synthesized (as per *section 5.7.4, point. 10*), prepare a qPCR control reaction mix as described in Table 4 above.
- Add this into the appropriate well on the plate.

Interpretation of the RT control

Cq \geq 30 indicates poor reverse transcription and gene expression results are likely to be compromised.

2. DNA contamination Control (gDNA)

The DNA contamination control assay is a species-specific control assay that targets a non-transcribed region of the genome. It is used to determine

whether genomic DNA (gDNA) is present in a sample at a level that might affect qPCR results.

- This SOP is designed for samples without pre-amplification.
- Primers are lyophilised on the PCR Array plate.
- Add qPCR reaction master mix (containing the No-RT control cDNA sample) to the appropriate well as per the rest of the plate.

Interpretation of gDNA control

$Cq \geq 35$ indicates below single copy detection; therefore, no gDNA present.

$Cq < 35$ indicates the sample is contaminated with genomic DNA (gDNA) and gene expression results are likely to be compromised. The relative contribution of gDNA contamination to a samples signal can be determined by comparing the Cq value for a given gene of interest to the Cq value for the DNA contamination control assay using the following calculation and table 6 below:

$$(\text{gene of interest } Cq) - (\text{gDNA } Cq) = \Delta Cq$$

Table 6: Interpretation of gDNA contamination

ΔCq	Percentage contribution
1	50
2	25
3	12.5
4	6.25
5	3.13
6	1.56
7	0.78

3. RNA quality Assay (RQ1 & RQ2)

The RNA quality assay is a pair of assays (RQ1 & RQ2) that target the same transcript at different locations with different amplicon sizes. The RQ1 & RQ2

assays must be used as a pair for each cDNA sample. Differences in Cq values can indicate whether RNA degradation may be negatively impacting genes expression.

- The RQ1 & RQ2 primers are lyophilised on the PCR Array plate.
- Add 20µl of the qPCR reaction master mix (including cDNA sample) to the appropriate well as per the rest of the plate.

Interpretation of RQ1 & RQ2

To identify the ΔCq between the RQ1 and RQ2, use the following equation:

$$(RQ2\ Cq) - (RQ1\ Cq) = \Delta Cq$$

$\Delta Cq \leq 3.0$ RNA degradation is minimal and will likely have little to no effect on gene expression

$\Delta Cq > 3.0$ RNA gene expression results may be compromised

4. Housekeeping / Reference genes

Housekeeping (HK) genes correct for loading differences and other variations present in each sample. The expression level of a HK gene should not vary across the biological condition being studied. It is important to confirm the HK gene you will be using is appropriate for the cells within your model using previously published literature.

- Primers for TBP, HPRT1 and GAPDH are lyophilised on the PCR Array plate.
- Add 20µl qPCR reaction master mix (including cDNA sample) to the appropriate well as per the rest of the plate.

Interpretation of Housekeeping genes

HK genes are used to normalise for variation in the amount of input RNA with your samples for accurate gene expression analysis, this is done using the Bio-Rad CFX analysis software.

6 Data Analysis and Reporting of Data:

The delta-delta Ct method was used to analyse the data for this SOP (Livak and Schmittgen, 2001). It is a method used in order to calculate the relative gene expression fold over control.

First the Ct (cycle threshold) of the house keeping gene is subtracted from the Ct value of the gene of interest. This gives ΔCt (Δ =difference between) for your sample.

$$\Delta Ct = Ct (\text{gene of interest}) - Ct (\text{housekeeping gene})$$

Then using the average Ct value of the negative control wells, and using this to find the $\Delta\Delta Ct$ value for each sample.

$$\Delta\Delta Ct = \Delta Ct (\text{Sample}) - \Delta Ct (\text{control average})$$

Using the $\Delta\Delta Ct$ we can calculate the fold change of the gene expression.

$$\text{Fold change} = 2^{-(\Delta\Delta Ct)}$$

This fold change is then graphed using the software of choice.

7 Publications:

Not applicable for this SOP.

8 References

ALVAREZ, M. L. & DONÉ, S. C. 2014. SYBR® Green and TaqMan® quantitative PCR arrays: expression profile of genes relevant to a pathway or a disease state. *Methods Mol Biol*, 1182, 321-59.

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8.0 Appendix I

Bio-Rad custom PCR array plate:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BCL2	CCL2	BCL2	CCL2	BCL2	CCL2	BCL2	CCL2	TBP	TBP	TBP	TBP
B	CASP3	SOD2	CASP3	SOD2	CASP3	SOD2	CASP3	SOD2	GAPDH	GAPDH	GAPDH	GAPDH
C	COL1A1	IL33	COL1A1	IL33	COL1A1	IL33	COL1A1	IL33	HPRT1	HPRT1	HPRT1	HPRT1
D	IL10	CCL5	IL10	CCL5	IL10	CCL5	IL10	CCL5	gDNA	gDNA	gDNA	gDNA
E	IL1B	CEBPB	IL1B	CEBPB	IL1B	CEBPB	IL1B	CEBPB	PCR	PCR	PCR	PCR
F	IL6	TLR4	IL6	TLR4	IL6	TLR4	IL6	TLR4	RQ1	RQ1	RQ1	RQ1
G	TNF	IL15	TNF	IL15	TNF	IL15	TNF	IL15	RQ2	RQ2	RQ2	RQ2
H	IL18	IL5	IL18	IL5	IL18	IL5	IL18	IL5	RT	RT	RT	RT
		Sample 1	Sample 2	Sample 3	Sample 4							