PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Reverse Transcriptase PCR for Hepatocarcinogenicity Biomarkers in 3D HepG2 Liver Spheroids

This is a 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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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	17/04/2020	Initial Document	Gillian Conway

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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 [1]. An increasingly popular approach is the application of gene expression analysis, which can be used to discriminate transcriptional changes following exposure to ENM [2].

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is considered the 'gold standard' for gene expression quantification [3]. RT-qPCR is a relatively simple, inexpensive, sensitive and specific tool used to determine the expression level of target genes [4]. 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 364 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 predesigned Bio-Rad Hepatocellular Carcinoma RT-qPCR array. It contains 88 genes associated with hepato-carcinoma, 3 housekeeping genes and 5 different assay controls. This SOP has been established to support the identification of an endpoint biomarker panel in HepG2 liver spheroids 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 carcinogenesis in 3D HepG2 liver models following ENM exposure over both an acute or long-term, and repeated dose regime. This method allows for the identification of an advanced endpoint liver associated biomarker panel that can be used as an effective screening approach.

Limitations of the Protocol

1. Predefined 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 standardisation work)	N

2 Terms and Definitions:

Engineered Nanomaterial

Nanomaterial designed for specific purpose or function

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

Spheroid

A spherical, heterogeneous cluster of proliferating, quiescent, and necrotic cells in

Culture that retain three-dimensional architecture and tissue-specific functions

[SOURCE: Spheroid Culture in Cancer Research (Bjerkvig, 1992)]

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 HepG2 spheroids, reverse transcribe RNA into cDNA templates, perform qPCR of the cDNA transcripts using a predefined hepatocellular carcinoma PCR array. This protocol can be broken down into 4 key stages as described in Figure 1 below:

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

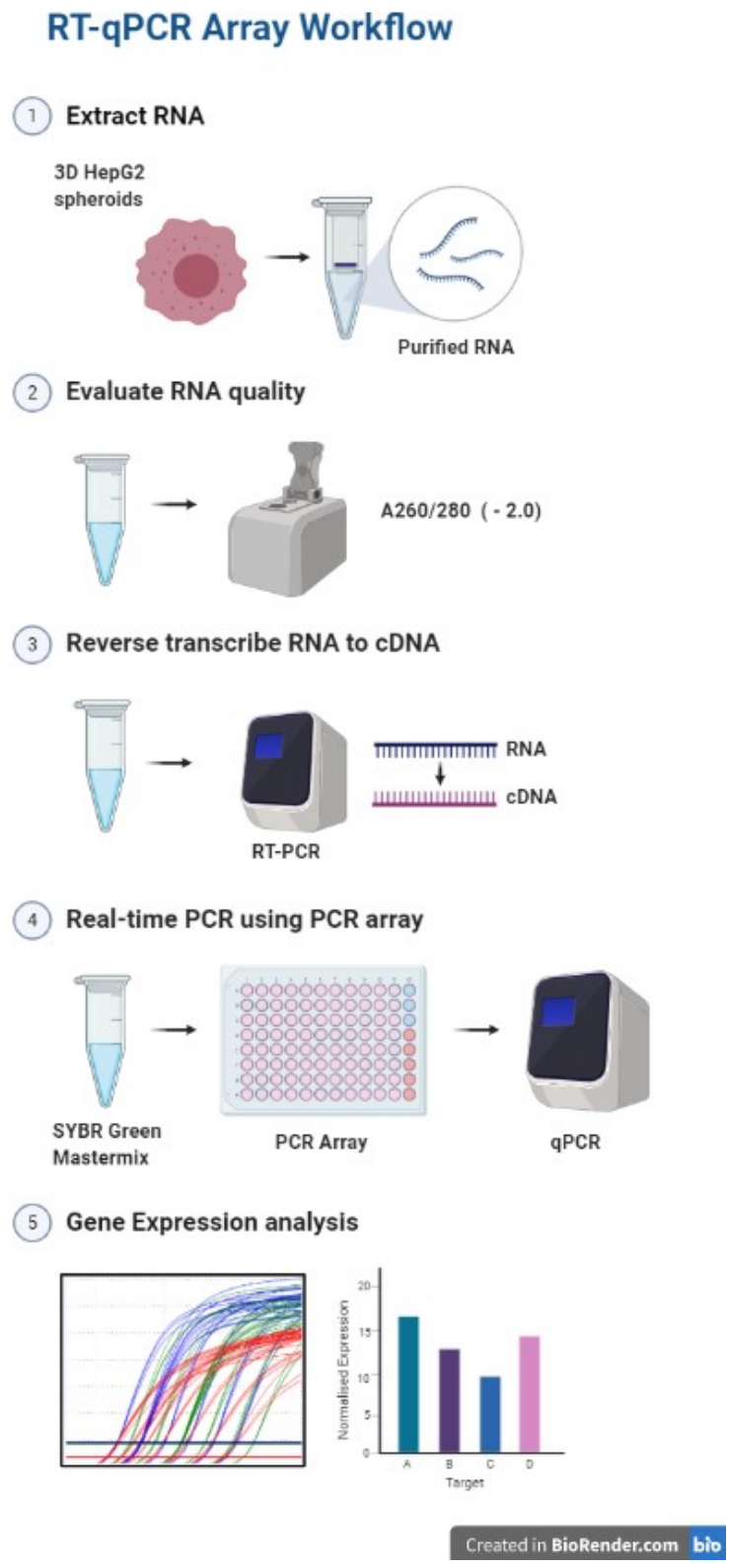

***Figure 2**: Schematic Diagram of RT-qPCR Workflow (Created with BioRender.com).*

5 Description of the Method:

5.1 Biological setting & test system used:

The cultivation and exposure of 3D HepG2 spheroids to ENM should be carried out under sterile conditions in a Class 2 Laminar Tissue Culture Hood as per (PATROLS Deliverable 4.1 SOP '3D In Vitro HepG2 Spheroid Model' and '*NanoGenoTox Dispersion Protocol (Grant Agreement No. 20092101, 2018)* respectively).

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

- The advanced *in vitro* 3D model utilised in this SOP is the Human Caucasian Hepatocellular Carcinoma derived epithelial cell line, HepG2 (ATCC®-HB-8065TM, American Type Culture Collection) spheroid model, established and described in PATROLS Deliverable 4.1 SOP '3D In Vitro HepG2 Spheroid Model'.

5.2 Chemicals and reagents used:

PBS pH 7.4 1X, $MgCl_2$ and $CaCl_2$ 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)
PrimePCR Hepatocellular Carcinoma Tier 1 Pathway Plate, 96 well (10034966, Bio-Rad)

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
- Centrifuge
- T-25 and T-75 Tissue Culture Flask
- Micropipettes 1-1000µl
- Non-Filtered, Sterile 200µl and 1000µl Pipette tips

- 15ml Tubes
- 50ml Tubes
- PCR plate seals
- PCR hood
- Microcentrifuge
- NanoDrop
- Real-Time PCR system
- Thermo Cyler

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 in below are prepared:

- Add 4 volumes 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:

This SOP is to be used following the culture and exposure of HepG2 spheroids to engineered nanomaterials (ENMs) as per the following protocols: PATROLS Deliverable 4.1 SOP '*3D In Vitro HepG2 Spheroid Model'*, and '*NanoGenoTox Dispersion Protocol (Grant Agreement No. 20092101, 2018)* respectively.

5.7.1 HepG2 Spheroid Harvesting

1. Remove the 96-well plate from the incubator.
2. Using a 200 µL pipette, aspirate the 100 µL of cell culture medium including the spheroid tissue from each well and collect in a sterile, 15 mL centrifuge tube. Take care to avoid contact with the agarose.
3. Once collected, centrifuge the spheroid suspension at 230 x *g* for 5 min. Remove the supernatant and store at -80 °C for further endpoint analysis (e.g., liver function tests) later.
4. Re-suspend the pellet of spheroids in 1 mL of sterile, room temperature PBS (1x).
5. Once washed, centrifuge the spheroid suspension again at 230 x *g* for 3 min. Discard the supernatant, re-suspend in re-suspend cell pellet in 2mL of room temperature PBS (1X).

5.7.2 RNA Extraction

RNA extraction is performed as per the QIAGEN RNeasy mini kit manufacturer's instructions (using QIAGEN QIAshredders).

1. Clean down bench area with RNase Zap.
2. Discard the PBS supernatant into disinfectant.
3. Re-suspend the pellet in 600µl Buffer RLT, pipetting vigorously to ensure lysis of the spheroid.
4. 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*.
5. Add 1 volume (600µl) of 70% ethanol to lysate and mix well by pipetting.
6. Transfer 700µl sample into RNeasy spin column (pink column) that is in a 2 mL collection tube.
7. Centrifuge for 15 seconds at 8000 *x g* and discard the flow-through.

8. If sample exceeds 700µl, reuse empty collection tube from step nine and repeat until all of the sample has been centrifuged, discarding flow-through after each centrifugation.
9. 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

10. Add 500µl Buffer RPE to RNeasy spin column.
11. Centrifuge for 15 seconds at 8000 *x g* and discard flow-through and reuse collection tube.
12. Add 500µl buffer RPE to RNeasy spin column.
13. Centrifuge for 2 minutes at 8000 *x g* and discard flow-through.
14. Place RNeasy spin column into new 1.5 mL collection tube.
15. Add 30-50µl RNase free water to the membrane of the RNeasy spin column - centrifuge for 1 minute at 8000 *x g* to elute RNA.
16. The RNA is the tested for quality and yield on the Nanodrop as follows.
17. Ensure the nanodrop software is set to estimate RNA.
18. Blank the Nanodrop with 1µl of RNAse free water and press measure.
19. Wipe the Nanodrop probe clean with a lint free wipe
20. Add 1µl of RNA sample to Nanodrop and press measure.
21. Record the purity and yield;
    i. A260/A280 > 2.0 for pure RNA (assess DNA contamination)
    ii. A260/A230 ~2.0 for pure RNA (assess protein contamination)
22. When reading multiple samples, repeat step18 in between each sample.
23. Samples at this stage can be stored at-80 °C freezer in single aliquots until needed or can be used immediately for cDNA synthesis.

5.7.3 cDNA Synthesis

- The synthesis of cDNA is achieved as per manufactures instructions, using the iScript gDNA Clear cDNA Synthesis Kit (172-5034, Bio-Rad).

1. Turn on the laminar flow PCR laminar flow hood and wipe down with 70% ethanol.
2. Thaw the contents of the gDNA Clear cDNA Synthesis Kit (stored at -20°C) and RNA samples on ice.

3. Place all items needed into the PCR hood, all steps going forward should be carried out in the PCR hood where possible.
4. Make a DNase master mix by combining the iScript DNase and iScript DNase Buffer as per the table 1 below. Scale up as appropriate.
5. Mix thoroughly by pipetting.

Table 1: DNase Master Mix

Component	Volume per reaction (µl)
iScript DNase	0.5
iScript DNase Buffer	1.5
Total	2µl

6. Use the same amount of RNA in each cDNA synthesis reaction.

Worked example:

1000ng (1µg) (commonly used) of RNA.

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

Volume of RNA (µl) = 1000ng/ 295 ng/µl

Volume of RNA (µl) = 3.389 µl

7. For each RNA sample, prepare an RNA/DNase reaction mix on ice in a 200µl PCR tube as described in table 2 below. To account for pipetting error, it is advised to make one extra reaction mix than required).
8. Prepare a Reverse Transcription Control assay. For each RNA sample, include 1µl of the control RNA template (provided with the PCR array plates) in each 20µl cDNA synthesis reaction as described in Table 2 below (*please refer to section 5.8.2*).

Table 2: RNA/DNase reaction mix.

Component	Volume per reaction (µl)	Volume per reaction (µl)
		*RT control assay**
DNase master mix	2	2
RNA template (1µg)	Variable	Variable

control RNA template	/	1
Nuclease free water	Variable	Variable
Total	16µl	16µl

9. Mix thoroughly by pipetting and centrifuge briefly to remove bubbles and collect the contents at the base of the tube.
10. Place tube into the Thermal Cycler and set the DNase reaction protocol as described in table 3 below.

Table 3: DNase Reaction Protocol

Step	Temperature (°C)	Time (minutes)
DNase Digestion	25	5
DNase inactivation	75	5
Storage	4;ice	Until RT step

11. Prepare the reverse transcription master mix by adding iScript Reverse Transcription (RT) Supermix (5x)/No RT Control to the DNase treated RNA template as described in table 4 below.
12. For every RNA sample prepare a no-RT 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 refer to section 5.8.2*).

Table 4: RT master mix set-up

Component	Volume per reaction (µl)
iScript RT Supermix/No RT control	4
DNase treated RNA	16
Total	20µl

13. Mix thoroughly by pipetting and briefly centrifuge using the microcentrifuge to remove bubbles and collect the contents at the base of the tube.
14. Incubate the complete reaction mix tubes in a thermal cycler using the protocol described in table 5 below:

Table 5: 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
Hold	4°C

15. The cDNA generated can be used immediately for qPCR on the PCR array plates or frozen at -80°C for up to three weeks.

5.7.4 Real-Time PCR (qPCR) using the Hepatocellular Carcinoma PCR Array

qPCR is conducted as per manufactures instructions using the iTaq Universal SYBR Green Supermix (172-5120) with Hepato-carcinoma PCR Array plates (Bio-Rad 10034966).

1. Turn on the PCR hood (if not already on from previous step) and wipe down with 70% ethanol.
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 where possible.
5. Mix all tubes SYBR Green Supermix and cDNA samples by inversion 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 99 reactions) as per Table 6 below.

Table 6: qPCR reaction master mix

Component	Volume per reaction (µl)	Final Concertation
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 the plate layout*).
10. Prepare the qPCR reaction mix for the gDNA and RT control well as per table 6 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).

5.7.5 Performing Real-time PCR

1. Seal the plate with an optical seal.
2. Centrifuge to remove bubbles at 2600 *x g* for 2 minutes at room temperature.
3. Load the PCR plate into the real-time PCR instrument.
4. Run the plate using the settings identified in Table 7 below

Table 7: PrimerPCR Cycling Protocol

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 (0.5°C increments)	5 seconds/step	1

5.8 Quality control & acceptance criteria:

To ensure specificity and sensitive of the methods, 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.

1. 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 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 pt. 9.*

Interpretation of the Positive PCR control

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

2. 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 200µl nuclease-free water on ice.

- For each RNA sample, prepare a RT control sample by adding 1µl of the control RNA template into the 20µl cDNA synthesis reaction (as described in Table 2 above), and proceed with the reverse transcription.
- Once RT control cDNA is synthesized (as per 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 ≥ 30 indicates poor reverse transcription and gene expression results are likely to be compromised.

3. 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 ≥ 35 indicates below single copy detection; therefore, no gDNA present.

Cq < 35 indicates the sample is contaminated with 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:

(gene of interest Cq) – (gDNA Cq) = ΔCq

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**

4. 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 od 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:

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

ΔCq ≤ 3.0 RNA degradation is minimal and will likely have little to no effect on gene expression

ΔCq > 3.0 RNA gene expression results may be compromised

5. 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.

- 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 using the ΔΔCq method.

6 Data Analysis and Reporting of Data:

Data analysis was performed as per manufactures instructions using the gene study function in the real time PCR software (i.e. Bio-Rad CFX Manager software, version 3.1), which is covered in point 1-7. Alternatively, the normalised gene expression using the ΔΔCq method can be calculated manually in excel which is covered in point 8.

1. Each qPCR data file is individually analysed to determine if the internal plate controls were met. Any outlier wells were excluded from analysis at this point.
2. A gene study is then created using the CFX manager software.
3. Individual qPCR plate data file are added to the gene study using the 'add files' function.
4. As more than one housekeeping gene was used, the software calculates the Target Stability Value using two parameters
    a. The coefficient of variation (CV)
    b. M-value

    As per the Bio-Rad PCR array manual the acceptable values for stably expressed genes are stated below in table 7.

Table 7. Housekeeping gene stability

Sample	CV	M
Homogenous	<0.25	<0.5
Heterogeneous	<0.5	<1

5. The software calculates the normalised gene expression (ΔΔCq) using the negative untreated control. Normalised gene expression is the relative quantity of the gene of interest normalised to the quantities of the negative control, also known as Fold Change. An example of the data output is demonstrated below in table 8.
6. The data is then exported to excel.
7. Genes that have been down regulated are identified as a negative number. To converted genes that have been down regulated as a measure of fold change they need to be plugged into the following equation:

1/x = - (fold change)

Table 8. Example of CFX Manager data output

Target	Sample	Mean Cq	Mean Efficiency Corrected Cq	Normalized Expression	Relative Normalized Expression	Regulation	Compared to Regulation Threshold	P-Value
MMP3	NEG	29.87	29.87	0.01026	1.00000	1.00000	No change	
MMP3	TiO2 1.0	33.25	33.25	0.00531	0.51723	-1.93336	No change	0.572702
MMP3	AFB1	31.93	31.93	0.00111	0.10813	-9.24794	Down regulated	0.419056

8. To calculate ΔΔCt in excel, the following step are performed:
    a. Average the Ct values (ΔCt) for the technical replicates of each sample
    b. Using the average ΔCt values for each sample, calculate the ΔCt for each sample using the following equation:

        ΔCt test sample = Ct (gene of interest) – Ct (housekeeping gene)

    c. Calculate the ΔΔCt values for each sample using the following equation:

        ΔΔCt = Ct (gene of interest) – Ct (control average)

    d. Calculate the fold change in expression by doing '2 to the power of negative ΔΔCt' as demonstrated in the equation below:

        Fold gene expression = 2^-(ΔΔCt)

9. The data is plotted on a graph as a measure of Fold change to the negative control.

7 Publications

Not applicable for this SOP.

8 References

1. Ates, G., Mertens, B., Heymans, A., et al. (2018) A novel genotoxin-specific qPCR array based on the metabolically competent human HepaRG™ cell line as a rapid and reliable tool for improved in vitro hazard assessment. *Arch. Toxicol.*, **92**,1593–1608. doi:10.1007/s00204-018-2172-5.

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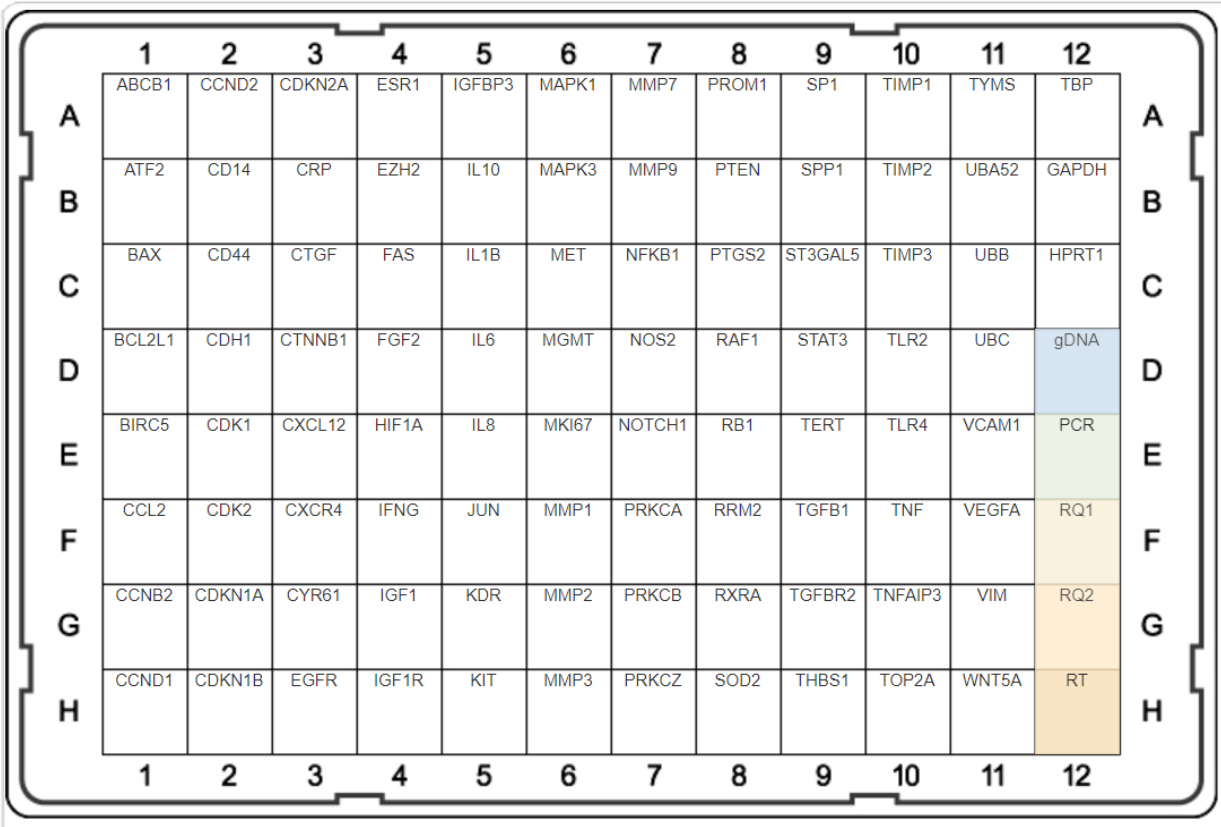
3. Schmittgen, T.D., Lee, E.J., and Jiang, J. (2008) High-throughput real-time PCR. *Methods Mol. Biol.*, **429**,89–98. doi:10.1007/978-1-60327-040-3_7.

4. Alvarez, M.L. and 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**,321–359. doi:10.1007/978-1-4939-1062-5_27.

9 Appendix I

Bio-Rad Hepatocellular Carcinoma predefined PCR array plate.



	1	2	3	4	5	6	7	8	9	10	11	12
A	ABCB1	CCND2	CDKN2A	ESR1	IGFBP3	MAPK1	MMP7	PROM1	SP1	TIMP1	TYMS	TBP
B	ATF2	CD14	CRP	EZH2	IL10	MAPK3	MMP9	PTEN	SPP1	TIMP2	UBA52	GAPDH
C	BAX	CD44	CTGF	FAS	IL1B	MET	NFKB1	PTGS2	ST3GAL5	TIMP3	UBB	HPRT1
D	BCL2L1	CDH1	CTNNB1	FGF2	IL6	MGMT	NOS2	RAF1	STAT3	TLR2	UBC	gDNA
E	BIRC5	CDK1	CXCL12	HIF1A	IL8	MKI67	NOTCH1	RB1	TERT	TLR4	VCAM1	PCR
F	CCL2	CDK2	CXCR4	IFNG	JUN	MMP1	PRKCA	RRM2	TGFB1	TNF	VEGFA	RQ1
G	CCNB2	CDKN1A	CYR61	IGF1	KDR	MMP2	PRKCB	RXRA	TGFBR2	TNFAIP3	VIM	RQ2
H	CCND1	CDKN1B	EGFR	IGF1R	KIT	MMP3	PRKCZ	SOD2	THBS1	TOP2A	WNT5A	RT