Integrating Cell Culture and Gene Expression Analysis by QRT PCR

Jim Cooper January 2021

Relative Gene Expression by QRT-PCR is a powerful technique combining cell culture and molecular biology to enable a researcher to determine if a gene of interest (GOI) is expressed in a population of cells and whether its level of expression can be regulated through external physical or chemical treatment[1].

The technique is accessible to labs with tissue culture facilities and an appropriate thermocycler. Experiments may be carried out rapidly with relatively high throughput. The main limitation is that it only indicates the expression of transcriptionally regulated genes at the mRNA level and provides no evidence as to whether the specific protein is expressed. The accuracy and value of the method relies on the adoption of good cell culture and molecular biology practices to avoid confounding factors that might affect results.

Typically, a cell culture representing a relevant tissue, is treated with a compound (for example a dilution series to generate dose-response data) in parallel with an untreated control of the same cell line. The experiment will usually be set up in triplicate in multi-well culture plates with a single well representing a replicate. It is important to ensure that an adequate stock of authentic and contamination free cells is prepared before the experiment and the timing of cell culture coincides with the experimental set up. The size of well and number of cells in each replicate must be pre-determined and suitable to provide an adequate amount of RNA. RNA is innately unstable and readily degraded, so it is imperative that the integrity of the RNA is preserved and assessed in the culture and extraction process.

During the exposure of cells to the experimental conditions it is advised that any changes in morphology are recorded and observations supported by good quality photographs to reinforce gene expression data. For example, Figure 1A and B show differences in morphology in A549 epithelial lung cells (ECACC 86012804) treated with dexamethasone in a gene expression experiment for Surfactant Protein B (SFTPB). Treated cells (B) adopted a flattened and elongated morphology compared to the untreated controls. In addition, evidence of adverse effects such as cell

detachment and appearance of large vacuoles might indicate toxic effects or induction of apoptosis. It may be worth considering the set-up of additional wells of controls and treatments for cells count and viability assessment.

Once the experiment is complete, biological activity in the cells must be halted to minimise any unwanted impact of the cell harvest process on gene expression. This is one of the most critical steps. The RNA must be exposed to stabilising agents such as strong denaturants like guanidine salts, sodium dodecylsulfate (SDS) or phenol-based compounds to lower the risk of RNA degradation. It is recommended that the culture plates are placed on ice at 4°C during harvest. At ECACC we remove the culture medium by aspiration, wash the cell monolayer briefly with chilled PBS solution followed by addition of chilled homogenization buffer (Promega®) supplemented with thioglycerol to disrupt cells and stabilise the RNA. The cell homogenate is either immediately processed for RNA extraction or rapidly frozen in dry-ice and stored at -80 for extraction later.

Once RNA is extracted and in its final elution buffer it must be kept chilled at 4°C or frozen at -80°C or below. RNAse inhibitors may be added. Extracted RNA should be quantified using a spectrophotometer (e.g. Nanodrop™) to determine the absorbance at 260nm and 280nm. RNA has a A260/A280 ratio near 2.

A small volume of each eluted RNA sample should be assessed for structural integrity. This can be achieved through electrophoresis in a 1.4% agarose gel with ethidium bromide to visualise intact ribosomal 18S and 28S bands. Alternatively, the RNA Integrity Number (RIN) can be determined using a bio-analyzer[2]. RIN scores range from 1 (low integrity) to 10 high integrity. Low RIN scores indicate RNA degradation and a score of 8 or more is generally considered to be desirable for gene expression studies.

RNA cannot be directly amplified by PCR so the next step is to convert the mRNA to complementary DNA (cDNA) using reverse transcription. A fixed quantity of each of the RNA samples is reverse transcribed, usually using an oligo dT primer to bind to the poly A tail of mRNA and reverse transcriptase. It is important to try to process samples from one experiment together and with the same batches of reagents and the same conditions. Each sample should also have a “Reverse Transcriptase Free” (RT-

free) control that will be run in parallel with the cDNA to insure against non-specific amplification. Reserve any of the remaining RNA at -80 or below for the generation of repeat batches of cDNA if required. Once cDNA is prepared, QRT PCR can commence. cDNA is more stable than RNA however it is recommended to work on ice.

PCR reactions from experiments should be run in duplicate together on the same assay plate using well designed primers for the GOI. Ideally, the primers and probes should be designed to bridge intron/exon boundaries to ensure that the amplification is specific to transcribed cDNA. Fluorescence, proportional to the amount of amplified product, is read at the end of each PCR cycle. The critical read-out is the cycle number at which fluorescence crosses a baseline threshold (the 'Ct' value). Low copy numbers of the GOI will generate high Ct values and higher levels of expression will generate low Ct values. Little or no amplification (very high Ct values) should be seen in water and RT-free controls.

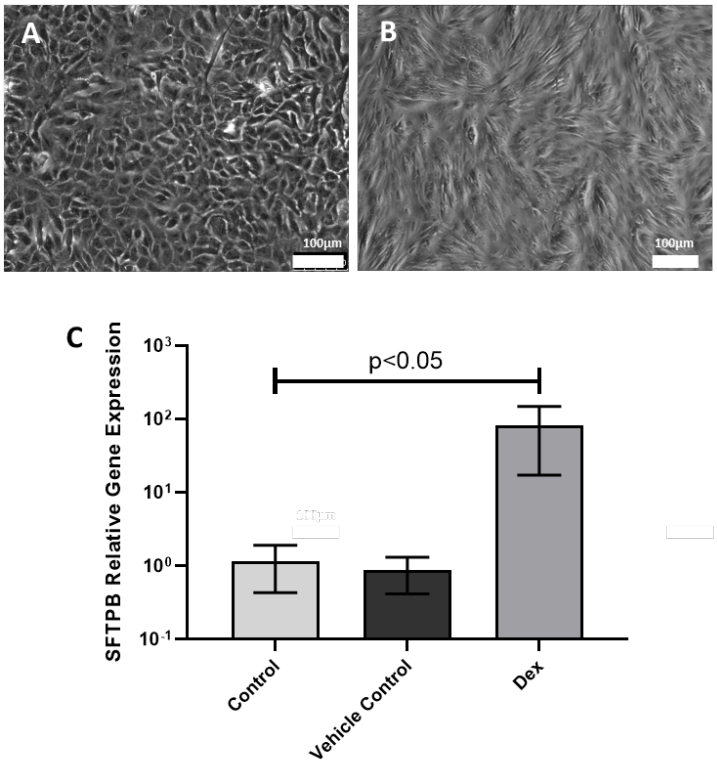

Figure 1. Effect of dexamethasone on SFTPB gene expression in A549 epithelial lung cells (ECACC 86012804). Phase contrast images of untreated control (A) and dexamethasone treated cells (B). The graph (C) shows the QRT-PCR relative gene expression of untreated cells (Control), ethanol vehicle control and dexamethasone treated A549 cells (Dex) using TaqMan® primers. Gene expression was calculated using the ΔΔCt method with GAPDH and ATP5B as H-KGs.

At this point, there may be an indication of the relative gene expression of the GOI as compared to the control, however for accurate and valid assessment, the amplification data must be normalised to a reference or "house-keeping" gene (H-KG). This is a constitutive gene that is uniformly expressed by cells at consistent level, and importantly, not perturbed by the experimental conditions. Typical H-KGs might include Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Beta Actin (ACTB), there are however many other genes that can be used. H-KG selection is dependent on both the experimental conditions and the choice of cell line. H-KGs can be used singularly or, to increase validity, in combinations of two or more genes. Before the GOI is assessed by QRT-PCR it is good practice to ensure the selected H-KGs are stably expressed at similar levels in all the cDNA samples from the experiment. This is particularly important in the case of a new cell line or new experimental treatment. If there is evidence that the conditions have affected H-KG expression or that the H-KG may not be appropriate for the cell line (low expression for example), then the use of a geNORM assay may be warranted to determine optimum H-KGs. In geNORM, QRT-PCR reactions are run on the experimental cDNAs against a dozen or so potential H-KG. Mathematical analysis of the Ct values will indicate the genes with least variation that may then be used in future experiments. To assist with geNORM analysis, commercial kits containing primers and computer software to run the calculations are available (Primer Design, UK). If the expression of all H-KGs assessed decline with treatment this may indicate widespread gene down regulation or of a toxic effect.

To calculate relative gene expression, the mean Ct values of biological and technical replicates of each of the H-KG are determined and the geometric mean deduced for each sample[3]. This value is then subtracted from the mean values of the replicate Ct values of the GOI to provide the first "delta" (Δ) value. The mean delta values of the control samples are then subtracted from the delta value of each of the samples to provide the "delta-delta Ct" (ΔΔCt) value for each sample (as a check, the ΔΔCt for the mean of the controls should be zero). To convert the ΔΔCt to a meaningful value of expression and to reflect the exponential doubling of amplified product in the PCR reaction, 'relative expression' is calculated by raising two to the power of the negative ΔΔCt value ($2^{-\Delta\Delta Ct}$) giving the control a value of "1" and all other samples a value of

expression relative to the control[4]. Figure 1C shows the relative gene expression of SFTPB (calculated by the ΔΔCt method) in A549 cells treated with dexamethasone compared to untreated control cells. Dexamethasone was dissolved in ethanol, hence the inclusion of a vehicle control).

Ensuring Success in QRT-PCR Experiments:

- Cell lines used in experiments should accurately represent the tissue under investigation as much as possible. They should be well maintained, checked for identity and free from contamination and mycoplasma. Avoid using over-passaged cells and follow good practice cell culture guidelines.
- Ensure adequate quantities of cells are cultured and schedule cell growth to coincide with experiment timelines. Calculate the number of cells required from the numbers of experimental treatments, controls and replicate wells, the surface area of the wells and the required seeding density. Over-estimate the number of cells required by ~20-30% to provide contingency.
- Check the treated cells under the microscope during the experiment. Note any morphological changes or evidence of toxicity and take photographs if possible.
- Set-up additional wells of treated and untreated cells for cell count and viability assessment at time harvest for RNA extraction.
- Harvest cells quickly for RNA extraction, on ice if possible to halt biological activity. Add homogenisation / stabilisation solutions directly to the cells to prevent RNA degradation.
- Quantify and qualify extracted RNA and only use high quality, pure RNA for reverse transcription to cDNA. Prepare RT-free controls.
- Ensure primers and probes are well designed, and if possible, span intron/exon boundaries.
- Check that selected H-KG are stably expressed and suitable for the experimental conditions and chosen cell line. Consider geNORM analysis to select appropriate H-KGs.

References:

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