**Real Time PCR (rtPCR)**

**Workflow**: *Students will isolate mRNA from 1 35 mM dish, convert the RNA to cDNA and then perform rtPCR using Sybrgreen. Students will work to determine the expression of NHE1 or CHP1 in cells and human tumor samples.*

*There are three phases or steps to the experiment. 1) mRNA isolation, 2) cDNA preparation from mRNA and 3) rtPCR and post-experiment analysis.*  The experimental design – YOU will do using the instructions for each step, is described at the end of the handout. Use this handout (for THIS TIME ONLY) for the protocol you CAN use the steps shown here verbatim. YOU must decide the DESIGN of the overall experiment. The GOAL of the experiment is to determine the change in expression of human NHE1 between two cell lines; PS120 lung fibroblasts and PSN lung fibroblasts. YOU must determine, using the protocol below, how to set up the experiment and include the controls shown below! Each lab section will do this in a total of 4 groups.

***Step 1 mRNA isolation***

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**Principle and procedure**: The RNeasy Mini Kit from Qiagen (look up for more details) is used for purification of total RNA. Biological samples are first lysed and homogenized in a highly denaturing guanidine-isothiocyanate–containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to the flow-through to provide appropriate binding conditions for RNA, and the sample is then applied to an RNeasy spin column, where total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30 μl, or more, of water. With the RNeasy Plus procedure, all RNA molecules longer than 200 nucleotides are isolated. The procedure provides an enrichment for mRNA, since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded. The size distribution of the purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently.

**Handling RNA**: Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the isolation procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

**General handling** Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep isolated RNA on ice when aliquots are pipetted for downstream applications. To remove RNase contamination from bench surfaces, nondisposable plasticware, and laboratory equipment (e.g., pipets and electrophoresis tanks), use of RNaseKiller or RNAseAway. RNase contamination can alternatively be removed using general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA\* followed by RNase-free water, or rinse with chloroform if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS), rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry

A 35 mm dish, when 100% confluent will contain 1-2 x 106 cells yielding 10-30 ug of RNA for most cultured cell types. A full guide for RNA isolation using the Qiagen RNAeaze Mini kit is linked on the class website. These instructions are the short version without background found in the full handout.

*The* [*QIAshredder*](http://www.qiagen.com/FAQ/ProductLineLink.aspx?ProductLineId=1000256)*is a unique biopolymer shredding system in a microcentrifuge spin-column format. It homogenizes cell or tissue lysates to reduce viscosity. Homogenization shears the high molecular weight genomic DNA and other high-molecular-weight cellular components to create a homogenous lysate. The QIAshredder is chemically inert and will not bind nucleic acids.*

**mRNA Isolation Protocol**: Cells grown in a monolayer in cell-culture vessels are lysed directly in the vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet prior to lysis. ***Ensure thatß-ME is added to Buffer RLT Plus before use.***

1. **Completely aspirate the cell-culture medium and rinse with PBS**. Remove ALL PBS as incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy Mini spin column membrane.
2. **Add 350 µl of Buffer RLT Plus to the dish and using a rubber policeman, scrape the cells**, and transfer to a clean microfuge tube. THEN pipette up and down several times with the tip of the pipet pushed down against the bottom of the tube to sheer/disrupt cells.
3. **Pipet the lysate directly into a QIAshredder spin column** placed in a 2 ml collection tube, and centrifuge for 2 min at max speed.
4. **Transfer the homogenized lysate to a gDNA Eliminator spin column** placed in a 2 ml collection tube. Centrifuge for 30 s at ≥8000 x *g* (≥10,000 rpm). Discard the column, and save the flow-through.

**Note**: *Make sure that no liquid remains on the column membrane after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membrane*.

1. **Add 350 μl of 70% ethanol to the flow-through**, and mix well by pipetting. Do not centrifuge. If some lysate was lost during homogenization and DNA removal, adjust the volume of ethanol accordingly.

**Note**: *When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure*.

1. **Transfer up to 700 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. Reuse the collection tube for the next step.

If the sample volume exceeds 700 μl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

1. **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. Reuse the collection tube in step 8.

**Note**: *After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.*

1. **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. Reuse the collection tube in step 9.

**Note**: *Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.*

1. **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.
* The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

**Note**: *After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur*.

1. **Place the RNeasy spin column in a new 1.5 ml collection tube**. Add 50 µl RNase-free water directly to the spin column membrane and centrifuge for 1 min at >8,000 x g ***to elute the RNA***.
2. **Place the RNAeasy spin column** in a new 2 ml collection tube, and discard the old collection tube with the flow-through. Centrifuge at 1 min. This will help to eliminate possible carryover of buffer RPE.
3. **Measure RNA using the NanoDrop**. An initial suggestion is to use a 1:5 dilution (prepare 10 µl) in TE buffer pH 7.0.

**Quantification of RNA**  To ensure significance, A260 readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 μg of RNA per ml (A260=1→44 μg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 μl

Dilution = 10 μl of RNA sample + 490 μl of 10 mM Tris·Cl,\* pH 7.0 (1/50 dilution)

Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free)

A260 = 0.2

Concentration of RNA sample = 44 μg/ml x A260 x dilution factor

= 44 μg/ml x 0.2 x 50

= 440 μg/ml

Total amount = concentration x volume in milliliters

= 440 μg/ml x 0.1 ml

= 44 μg of RNA

**Purity of RNA**: The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A260/A280 ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A260/A280 ratio can vary greatly. Lower pH results in a lower A260/A280 ratio and reduced sensitivity to protein contamination. For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A260/A280 ratio of 1.9–2.1 in 10 mM Tris·Cl, pH 7.5. The A260/A230 ratio should be 1.7 and the concentration determined by A260 should be >40µg/ml. Always be sure to calibrate the spectrophotometer with the same solution used for dilution.

For determination of RNA concentration, however, the recommend dilution of the sample in a buffer (TE) with neutral pH since the relationship between absorbance and concentration (A260 reading of 1 = 44 μg/ml RNA) is based on an extinction coefficient

calculated for RNA at neutral pH.

***Part 2 - cDNA Preparation***: We will be using BioRad’s iScript Reverse transcription supermix to create cDNA from the mRNA. All of the mRNA must be converted to cDNA ONE TIME (one copy of cDNA for each copy of mRNA). This is done using a reverse transcriptase. The mastermix (supermix in this case) will contain the nucleotides to make the cDNA, reverse transcriptase (RT), salts, and buffers needed for the reaction.

The reaction starts with a priming step followed by a single extension step before a step to kill (denature) the reverse transcriptase. BioRad’s supermix contains reverse transcriptase, RNase inhibitors, dNTPs, primers MgCl and stabilizers.

Assemble all reactions on ice in a PCR tube (not a plate) until we start the reaction.

**CONTROL 1** – See the control section below when preparing cDNA from your RNA sample.

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| ***BioRad iScript real time PCR reagents*** |
| Prepare the reverse-transcription mix according to the following for 1 reaction* + 5X iScript Biorad Supermix 4 µl
	+ 0.5 µg RNA (1 pg-1µg) x µl
	+ RNase-free water x µl (assay tube total = 20 µl)
* Primer the reverse transcriptase five min at 25oC
* Extension reaction for 20 min at 46oC
* Kill the transcriptase at 95oC for 1 min.
* Place the reaction in the freezer to store until rtPCR.
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***Part 3 – rtPCR***

**Designing rtPCR Primers.**

* Find the sequence NHE1 (SL9A1) and beta actin (ACTB) both human. Use this information to design real time PCR sequences.
* Find a how to design real time PCR primer website. Remember we are using Sybr green not a probe PCR assay. Also remember this is the expressed gene from a plasmid, thus there are no intron-exon regions to consider.
* Design a real time (qPCR) primer for our project. In a simple handout write the primer with its information (Tm GC content…) AND the key points to consider when designing a qPCR primer for Sybr green real time PCR. NOT a sequencing or general PCR primer…

**FOR THE LAB – I will provide a rtPCR primer pair for both. Use your lab book to indicate your design and HOW you got there.**

 **For each gene analyzed perform the following***.*

* Prepare the PCR components in a PCR PLATE
	+ SYBR Green Master Mix 12.5 µl
	+ cDNA synthesis reaction 1 µl
	+ Gene Primer (10 µM stock) 1 µl \*\*\*\* ONLY ONE GENE PRIMER PER REACTION!!!
	+ RNase-free water 10.5 µl
* Briefly mix and then centrifuge the plate and place into the cycler.
* Program the thermocycler as follows:
	+ Cycles Duration Temp Comments
	+ 1 10 min 95oC HotStart DNA is activated at this step
	+ 40 15 s 95oC

 1 min 60oC Perform fluorescence data collection

* Perform a dissociation (melting) curve analysis to verify PCR specificity. Run a melting curve program and generate a first derivative dissociation curve. A single peak should appear in each reaction at temperatures greater than 80oC.
* Determine the CT value for each sample. Calculate the CT for your experiment.

**Experimental Design**. You will be given two flasks of cells. PS120 and PSN. Determine using the expression level of NHE1 using BACT as a normalized control. You will also prepare PCR controls.

1. A no template control (NTC) omits any DNA or RNA template from a rtPCR reaction, and serves as a general control for extraneous nucleic acid contamination. When using SYBR Green chemistry, this also serves as an important control for primer dimer formation.

2. A no reverse transcriptase control (NRT) involves carrying out the reverse transcription step (preparation of cDNA) in the absence of reverse transcriptase. This control assesses the amount of DNA contamination present in an RNA preparation.

**Write up**: Include the lab book – one or two sentence introduction for the entire experiment. Include a section for results (observation of fluorescence of cells, nanodrop information, concentration calculations and so forth…). Calculate the results of your rtPCR and comment on the melt curve. Record and analyze your Ct value. Discuss your negative controls. Using both the fluorescence data and the ddCT calculations explain HOW you know NHE is differentially expressed in one cell line over the other.