UMGC Q-RT-PCR Project Submission Guidelines

(Document version: 05/20/16)

Please read these guidelines carefully. Incorrect submissions will delay your project.

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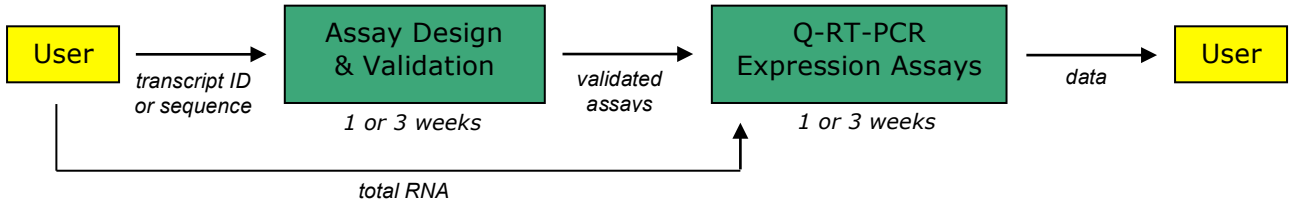
I. UMGC Contacts

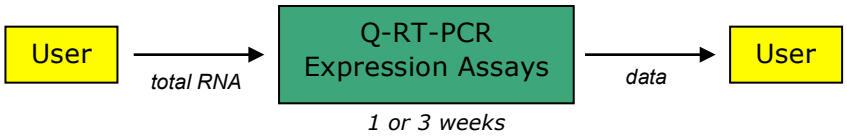
To submit assay and project requests, E-mail completed forms to qpcr@umn.edu. To contact the UMGC with questions ***that are not answered below***, E-mail qpcr@umn.edu or call Darrell Johnson at 612-624-4736.

II. Q-RT-PCR Project Workflows

There are two basic workflows for a Q-RT-PCR project, depending on whether or not primer-probe sets have already been designed and validated. If validated primer-probe designs are *not* yet in hand, the workflow involves two phases, as shown a) assay design/validation and b) Q-RT-PCR analysis (user = yellow, UMGC = green).



Alternatively, if assays have already been designed and validated, the project will only involve Q-RT-PCR itself:



Because the two phases of Q-RT-PCR are independent of each other, and also have different requirements, the UMGC manages these two phases separately, using different submission forms and project reports. Note, however, that for the most rapid turnaround, you will want to coordinate the two phases of your project, by submitting assay requests and RNA samples simultaneously. This will ensure that as soon as your assays are validated, you can submit an expression project request and Q-RT-PCR can commence without delay.

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III. Assay Design and Validation

The Roche Universal Probe Library (UPL) Technology provides hydrolysis-probe ("Taqman") performance at an affordable price, as the acquisition of a new UPL assay merely requires the purchase of two plain oligonucleotides. The design and validation of a UPL expression assay at the UMGC involves the following steps, which are described in detail on the following pages:

A. user submits assay request(s);

B. if the species is one for which we do not possess a universal reference RNA pool, user submits RNA for assay validation;

C. UMGC performs design, orders oligos, assembles assays, reverse-transcribes sample RNA, generates standard curve of cDNA, and runs Q-PCR in order to determine efficiency of amplification;

D. UMGC returns to the user an assay validation report.

NOTE: unless the assay design and validation process result in a working assay, users are not charged.

A. Submission of assay request. To request the design/validation of an assay for your gene-of-interest, use the two-page Excel form entitled *UMGC_Q-RT-PCR_Assay_Request_Form.xls*.

On **PAGE 1**, you are instructed to enter personal and billing information. Fields include:

- Personal: **Name**, **Phone**, **E-mail**, **PI** (the PI who is funding the work)
- Billing: **Fund**, **Dept ID**, **Program/Project** (required) and **Employee ID**, **Chart Field 1**, **Chart Field 2** (if required by your department)

In addition, you will be asked to choose between two different turnaround times:

1-week = $45 per gene
3-week = $25 per gene

The difference in price reflects the fact that a single set of primers does not always guarantee a successful outcome. For 3-week turnaround, then, a single pair of primers is ordered and tested per week, with up to three serial attempts made (≈ 1 week per attempt). For the 1-week turnaround, three different primer pairs are ordered and tested in parallel, reducing the time required to determine whether or not a validated design is possible. Although faster, this approach is wasteful and hence more expensive, as most of the time it is not necessary to order more than 1 pair of primers in order to achieve a successful assay.

On **PAGE 2**, you are instructed to enter up to 96 genes of interest. The following fields are required:

- **Species**. Use the proper taxonomic name for the species of interest, i.e. *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Drosophila melanogaster, Caenorhabditis elegans*, etc.

- **Gene Symbol**. (If the gene has not been annotated in a public database, submit a custom name.) When entering the gene symbol, **use the official gene symbol spelled and formatted exactly as specified by Entrez Gene (http://www.ncbi.nlm.nih.gov/gene)**. For instance, the human beta-actin gene symbol is ACTB, whereas for mouse and rat it is Actb.

- **Sequence identifier and/*or* sequence**. If available, submit a sequence identifier (transcript accession number) that defines a specific transcript in a sequence database, rather than pasting in a sequence *per se*. Our preferred identifier is a RefSeq transcript identifier (or "NM#"), described more fully at http://www.ncbi.nlm.nih.gov/refseq/key.html. Transcript accession numbers are easy to find by starting with a gene symbol- or gene name-based search at Entrez Gene (http://www.ncbi.nlm.nih.gov/gene). If the sequence of interest is not available in any database, you can submit a sequence directly. If you know where exon-intron boundaries are in your transcript, they should be marked NNNN[]NNNN, where NNNN represent the sequences of the 3' and 5' ends of neighboring exons, and [] replaces the entire intronic sequence.

- **Specify if the assay design is to be shared with the UMN community** once validated ("Yes" or "No"). The default setting for this field is "Yes", as it will be in the interest of all UMN researchers to

share UMGC-validated assays. Once validated, an assay will be available to everyone without an up-front validation fee. However, we understand that in rare circumstances, the mere identity of genes under study may be information that you want to protect; under such circumstances, we will respect your request for confidentiality, and we will exclude these assays from the list of pre-validated assays.

B. RNA submission. For human, mouse, and rat UPL assay requests, assay validation will be achieved using universal reference RNA samples (users do not have to provide RNA). For all other species, provide 5 ug of total RNA, preferably a pool of a diversity of tissues and/or experimental conditions. A diverse RNA pool will increase the probability that any give gene transcript is present, thereby facilitating validation. **Failure to submit a total RNA sample for validation—if required—will delay design/validation.**

C. Validation. The UMGC will perform design and validation. Default parameters are to design intron-spanning primer pairs where possible, i.e. primer pairs that preferentially amplify cDNA over gDNA, which helps to prevent artifacts from contaminating DNA in RNA preps. All designs are built to a single set of PCR cycling conditions. **Note: design will occur once per week, on Wednesday morning, and users must therefore submit sequences no later than 10:00 a.m. each Wednesday in order to receive results the following Wednesday (for 1-week turnaround) or the third Wednesday (for 3-week turnaround).** Assay submissions received after 10:00 a.m. on Wednesday morning will not be designed until the following week.

D. Validation Report. The UMGC will return an assay validation report for successful assays, which will include raw data points from the standard curve, and a calculation of the resulting efficiency. Our procedure for validation is to run a 5-point dilution series of cDNA in duplicate, and to calculate efficiencies over all dilutions with a reasonable Ct (i.e. earlier than 32-33 cycles). No-template controls are also run, and must be negative for an assay to be considered validated.

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IV. Q-RT-PCR Expression Assays

Once assays are available, it is possible to proceed to expression analysis. There are two different types of expression project, depending on whether or not the RNA samples have been previously submitted to the UMGC. The majority of projects will involve new RNA samples. However, if the samples in question have been used in a prior project, residual RNA or cDNA from that project exists, and additional analyses of these samples are desired, then re-submission of samples and reverse transcription may not be necessary, and the project will be termed a *continuation* project. There are consequently two slightly different forms for a Q-RT-PCR expression project, one for new samples, and one for continuation projects.

No matter which type of expression project you undertake, however, there are three steps involved:

A. user submits project request(s) and RNA samples;

B. UMGC performs reverse transcription, assembles Q-RT-PCR run plates, runs Q-RT-PCR, and analyzes data;

C. UMGC returns to the user the raw data (Ct values), a performance report (reproducibility metrics), and an expression analysis report.

Steps A-C are here explained in detail.

A. Submission of project request. To request a Q-RT-PCR expression project for new samples, use the Excel-based form entitled *UMGC_Q-RT-PCR_Project_Request_Form.xls* document. For a continuation project, use *UMGC_Q-RT-PCR_Project_Continuation_Form.xls*. These forms are largely the same, except where noted below. Throughput the forms, user input fields are shaded light yellow (these are the *only* fields in which information can be entered).

Important Note: These forms contain formulas linked to input cells, which can be corrupted if the form is used improperly, so please follow these two simple rules: First, when pasting into the form from some other spreadsheet, **use the PASTE SPECIAL—VALUES function in Excel**. If you are unsure how to use PASTE SPECIAL and cannot find someone in your lab to help you, please contact Darrell Johnson. Second, **don't cut and paste within the form itself**. When you cut and paste within the forms, you move the referenced cells, thereby disrupting the relationships between sheets.

On **PAGE 1**, you are instructed to enter personal and billing information. Fields include:

- Personal: **Name**, **Phone**, **E-mail**, **PI** (the PI who is funding the work)
- Billing: **Fund**, **Dept ID**, **Program/Project** (required) and **Employee ID**, **Chart Field 1**, **Chart Field 2** (if required by your department)

In addition, you will be asked to choose between two different turnaround times:

1-week (faster but more expensive)
3-week (slower but less expensive)

In addition to the yellow-shaded input fields, there are also a number of summary fields that reflect entries made on pages 2-4. These include:

- *Samples Submitted* (summarizes the number of samples submitted on PAGE 2);
- *Samples Charged for Q-PCR* (rounds up the number of samples submitted to the nearest multiple of 8 samples, as Q-PCR charges are incurred in units of 8 samples);
- *Genes Submitted* (summarizes the number of assays submitted on PAGE 3);
- *Cost Per Data Point* (reports the pricing tier of your project, a function of the number of samples, the number of genes, and the turnaround time);
- *Number of Data Points* (totals the number of sample x assay Cts to be reported (each of which is a mean of two technical replicates);
- *Reverse Transcription Cost* (totals the charge for reverse transcription, a function of the number of non-blank, +RT samples, and whether or not you have normalized your RNA samples to 200 ng/ul);
- *–RT Control Cost* (totals the charge for –RT control samples, which because no reverse transcriptase enzyme is consumed, cost less to bring through a mock RT reaction);

- *Q-PCR Cost* (totals the charge for all Q-PCR reactions, a function of the number of samples, number of assays, and turnaround time)
- *Total Cost* (sums *Reverse Transcription Cost*, *–RT Control Cost*, and *Q-PCR Cost*; this is what you will be charged for the completed project).

On **PAGE 2**, you are instructed to enter up to 384 sample names, corresponding to up to four 96-well plates of samples submitted. Sample IDs in this submission form must correspond exactly to the orientation of samples submitted in 96-well plates. A mistake in the identification of samples on this form will, of course, render your Q-RT-PCR results meaningless. Due to the importance of correct sample submission, detailed instructions are provided on the forms themselves. **Please take special care to read and understand these instructions, as it is during sample submission that errors are most often committed**. Please contact Darrell Johnson at the UMGC if anything is unclear.

It is on PAGE 2 that the differences between the two forms (new samples versus continuation projects) are evident. For new projects, there are six pieces of information required: 1) Sample ID, 2) Normalization State, 3) Volume, 4) Mass, 5) Mass of cDNA For Q-PCR, 6) –RT Control State. For continuation projects, only three of these pieces of information are required: 1) Sample ID, 2) Mass of cDNA For Q-PCR, 3) –RT Control State. However, in addition, the **UMGC Project Name**, **Sample Plate Name**, **Sample Plate Well** of the *original* submission are required. Lastly, you can elect to **use existing cDNA** from a prior project.

On **PAGE 3**, you are instructed enter the IDs of up to 96 validated assays whose expression you wish to measure. The submitted assays can be:

- assays that you have had recently validated by the UMGC;
- assays from the lists of pre-validated assays shown on the form.

Currently, the list of pre-validated assays is limited to our house-keeping assays, but we will periodically issue updated version of the forms that will include a growing list of pre-validated assays.

Note: You must refer to the assay by its Assay Name, provided to you at the time of assay validation, or as written in the lists of pre-validated assays below. Assay names take the format GeneSymbol(##), e.g. ACTB(01) or Alas1(02). Do NOT simply enter the name of the gene or a gene symbol. A validated assay name is required. Also, do NOT enter the name of an assay that you have *just* submitted for validation until you have received official notification that the assay has been validated.

B. UMGC Project Execution. The UMGC will carry out your Q-RT-PCR project, at a pace determined by your turnaround time request. Projects are started on Fridays and results returned on Fridays; stated turnaround times *are from these dates*. In other words, a sample submitted early (e.g., on a Wednesday) will not enter the pipeline until Friday. **Note: users must submit samples no later than 5:00 p.m. each Friday in order to receive results the following Friday (for 1-week turnaround) or the third Friday (for 3-week turnaround).** Sample submissions received after 5:00 p.m. on Friday afternoon will not be entered into the process until the following Friday.

C. Data & Reports. The UMGC will provide data reports, including raw values, performance metrics, and differential expression results.

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V. Sample Requirements and Extraction

A. Sample requirements for Q-RT-PCR are as follows:

- total RNA (mRNA purification is not required) dissolved in WATER, Tris pH 8.0 (the elution buffer in many commercial RNA isolation kits), or *low-EDTA* TE (TE pH 8.0 with EDTA at 0.1 mM instead of 1 mM)
- a minimum of 2 µg (discuss with UMGC staff if this is not possible)
- a minimum of 10 ul volume
- all samples normalized to 200 ng/ul (or we will normalize samples for $4 per sample during RT)
- significantly free of contaminating DNA (treatment with DNase may be required—see below)
- 260/280 ratio ≈ 2.0 (acceptable range ≈1.75-2.25)
- 260/230 ratio ≥ 2.0 (acceptable minimum ≈ 1.00)

B. Total RNA isolation can be achieved with a wide variety of commercial kits. Best results will be achieved with reagents designed to deactivate contaminating RNase enzymes, and best practices for working with RNA should be followed. The company Ambion is our recommended provider for RNA-isolation reagents, due to their extensive experience with RNA and excellent technical documentation, although there are many other excellent sources for reagents and kits (e.g., QIAGEN). Ambion provides a useful online "wizard" for selecting RNA isolation reagents at

http://www.ambion.com/techlib/trees/RNA/index.html,

and also maintains a reference library of technical documents pertaining to RNA, including an excellent guide and top ten list for those unfamiliar with working with RNA:

http://www.ambion.com/techlib/basics/rnaisol/index.html
http://www.ambion.com/techlib/tn/91/9113.html

C. A modest amount of DNA contamination is common in RNA samples prepared without DNase treatment. For genes expressed a relatively high level, DNA contamination is not a problem, as the copy number of RNA per cell typically dwarfs that of DNA for transcribed genes. Moreover, we often manage to design intron-spanning primer sets that will not amplify contaminating gDNA. However, in some cases, for instance where low-level expression is of interest and cDNA-specific primers are not available, DNA contamination may be a problem, as it will result in late Q-PCR amplification signals that will interfere with the detection of a cDNA-derived amplicon. Under such circumstances, you should assess the presence of contaminating DNA in your RNA samples by including –RTase controls in your project. Additionally, you may want to include DNase treatment as an integral part of your RNA isolation. For additional information, read Ambion's guide to DNA contamination (http://www.ambion.com/techlib/tb/tb_176.html). A convenient reagent for removal of DNA is Ambion's TURBO DNA-*free* (http://www.ambion.com/catalog/CatNum.php?1907).

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VI. Pricing

Our pricing model is tiered, with cost per data point dropping in inverse proportion to the number of samples and number of genes per project. Rates are lower in cases where users are wiling to wait a little longer for their assay validation and expression assays. Note, a "data point" is a single sample *x* a single gene-specific assay, run in duplicate reaction wells. A table of Q-PCR rates is provided in the document, *UMGC_Q-RT-PCR_2011_Rates.pdf*.

The following details are worth noting:

A. The cost for assay design and validation is

- $25 per gene (3-week turnaround) or
- $45 per gene (1-week turnaround)

and is described in more detail in Section II above. **NOTE: unless the assay design and validation process result in a working assay, users are not charged.**

B. The cost for reverse-transcription (RT) is charged per sample *submitted*, and is

- $5.00 per normalized sample (submitted at 200 ng/ul);
- $9.00 per un-normalized sample (submitted at unknown concentration);
- $2.50 per –RTase control (carried through reverse transcription, without addition of RTase).

Typically, a single RT reaction is adequate for several dozen Q-PCR reactions, hence one RT reaction per sample is all that will be required for an entire Q-RT-PCR project. For genes expressed at low copy number, where a greater load of cDNA per Q-PCR reactions may be required, additional RT reactions may be required, and UMGC staff will consult with you if this is the case.

C. The cost for Q-PCR is calculated in multiples of 8 samples, as our work is performed using multi-channel pipetting heads with 8 fixed tips. Consequently, if you submit fewer than a multiple of 8 samples, your number of submitted samples will be rounded up to the nearest multiple of 8 for the purpose of calculating your re-charge.

Our assay and project submission forms include built-in cost calculators.

VII. Turnaround and Timing

A. Q-RT-PCR assay design/validation turnaround options are 1-week or 3-weeks. Design/validation cycles begin each Wednesday at 10:00 a.m., which is the deadline for submission of assay request forms. The deadline for the UMGC to return validation results to you is the end of the working day on Wednesday, 1 or 3 weeks later.

B. Q-RT-PCR expression assay turnaround options are also 1-week or 3-weeks. Expression analysis cycles begin each Friday at 5 p.m., which is the deadline for submission of project request forms and RNA. The deadline for the UMGC to return validation results to you is the end of the working day on Friday, 1 or 3 weeks later.

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VIII. Frequently Asked Questions (FAQs).

1. *"How many technical replicates are run for each sample x assay combination?"*

   We run two replicates (technical duplicates) for each "data point," which is calculated as the mean of the duplicate reaction wells. Raw data returned include 1) the Ct of each duplicate, 2) the mean Ct of the duplicates, and 3) the standard deviation of the duplicates.

2. *"Is the 'rate' per data point the rate* per replicate*, or the rate* per pair *of technical replicates?"*

   The rate per data point refers to the pair of technical replicates. You are not charged for each replicate independently, but for the mean value that *is* the "data point."

3. *"Do you sequence amplicons during assay validation?"*

   No, we do not routinely perform sequence validation, although this can be arranged and performed in the UMGC if so required.

4. *"How can I quantitate and QC my total RNA sample?"*

   Simple UV spectrophotometry is adequate, and can be carried out on a standard UV spectrophotometer or a specialized instrument such as the Nanodrop. The UMGC also provides a quantitation service that you can use, if you do not have convenient access to a Nanodrop instrument. In addition to using the A260 to calculate RNA concentration, also record the 260/280 and 260/230 ratios in order to ensure that your samples are of high quality.

5. *"Do I need to perform QC on my sample using an Agilent Bioanalyzer?"*

   We do not *require* electrophoretic QC of RNA. However, it is good practice, particularly when beginning a project using a new tissue source or extraction protocol, to validate that the quality of RNA is acceptable. Although the requirement for non-degraded RNA is not as strict for Q-RT-PCR as for microarray analysis, serious RNA degradation may interference with effective Q-PCR. The UMGC provides a QC service on the Agilent Bioanalyzer, if you do not have convenient access to such an instrument.