Real-time qPCR FAQ's

Real-time qPCR: Placing an order

What real-time qPCR services does the facility provide?

The facility provides three levels of service for real-time qPCR. In all cases it is the responsibility of the user to provide the primers, or probe/primer sets used in the assay.
Full Service- In this service, the user supplies the facility with the cDNA and the facility sets up the real-time assay, performs the run and analyzes the data. The SDS files and the text data files are uploaded to the user's BioDesktop account. Printouts are available upon request.
Self Service- In this service the facility will provide the user with the consumables necessary to set up the real-time qPCR run. The user will set up the assay and then will be trained by the facility staff to use the 7500 Fast instrument in 307 HSRF. The staff will train the user to analyze the data. Once the user has been trained, they can sign up for blocks of time to use the instrument through their BioDesktop account. This room now has evening and weekend access. To gain access during these off-hour times fill out the appropriate paper work and return to a staff member.
½ hour Rental- The 7500 Fast instrument can be "rented" by half hour blocks of time. This is particularly useful for applications such as SNP analysis that just require an end point read of the plate.

How do I place an order for full service real-time qPCR?

After you login to your account, click on "DNA Depot" in the left-hand menu of your BioDesktop home page. You will see links for the online order forms. Fill out all information, and then click OK at the bottom of the page. You will receive an email confirmation of your order.

What information will I need to provide when I place an order?

Your name, Principle Investigator of lab, Phone, and Budget number.

You will NEED to know the following about your AOD (Assay on Demand):

Species- human, rat, mouse, etc
Target- name of your gene of interest
Assay ID suffix (g/m/s)-
The gene expression Assay ID suffix indicates the assay placement. This information is provided by Applied Biosystems when you select your AOD.
* "_m" indicates an assay whose probe spans an exon junction and will not detect genomic DNA. No DNase treatment needed.
* "_s" indicates an assay whose primers and probes are designed within a single exon, such assays will, by definition detect genomic DNA. DNase treatment needed.
* "_g" indicates an assay that may detect genomic DNA. DNase treatment needed

Dye- usually Fam or Vic for AOD's.
Quencher- usually non-F (non-Fluorescent) for AOD's.

You will NEED to know the following about your <u>dual-labeled probe/primer sets</u> or primer sets for <u>SYBR Green</u>:

Species- human, rat, mouse, etc
Target- name of your gene of interest

Special conditions- Put "default" if you want us to use the default 200nM probe /900nM primer conditions. If we have developed special conditions for your probe/primer set, put them here. SYBR Green assays: put "use 50nM primers" in this box.
Dye- Options include Fam, Joe, Tet, Vic, Ned, or SYBR Green.
Quencher- Options include tamra, non-F, (non-fluorescent) BHQ (blackhole quencher), and you can put N/A for SYBR Green.

You will NEED to know the following information about your experiment:

Endogenous control- You need to know which target gene is your endogenous control.
Standard Curve or Comparative CT Analysis- You will need to know if your assay has passed the validation and can be run using the Comparative Ct analysis method or if it didn't pass and needs to be run using a standard curve. Validations are required with designed probe/primer sets. Assay's on Demand (from Applied Biosystems and others) are ready for Comparative Ct analysis. It is up to the investigator to decide if further validation is needed for their samples.
Calibrator Sample- Our software can generate relative expression of targets if using the Comparative Ct analysis method. To do this, we need to know which sample is the calibrator, (the sample that you want all others to be compared to). If you are combining a number of samples for a control treatment we can show you how to do the calculations yourself. If you are doing a standard curve analysis we will teach you how to perform the calculations and you will need to calculate the relative expression data yourself.

How do I print a copy of my order form?

See the BioDesktop FAQ.

Where do I put my samples?

Probes/primers and AOD's: These are purchased by the investigator. Probe/primer sets and primer sets for SYBR Green should be brought to the facility at 10uM. AOD's can be brought in the original tube sent from Applied Biosystems (or any other company). Please make sure the species and target name are on the tube. We will make 50uL aliquots into 0.5ml tubes and they will be kept in the Investigator's box in the –20 freezer.

Samples- Samples should come in their own tray or box and be clearly labeled with the USER'S NAME, the DATE, and PHONE NUMBER. Put in the –20 freezer on the same shelf as the Investigator's box. After we have set up the run, the samples will be moved to the door shelves of the freezer. When you get your data please REMOVE the sample box from the freezer door. We will occasionally throw away old sample boxes if the door gets full, so if you want your samples make sure to take them!

** When you bring your samples to the facility, LOOK in the Investigator's box to make sure you have the probe/primer sets or AOD's requested.

When will my samples be run and how will I be notified?

Turn-around is usually 2-3 days. We occasionally experience periods of high demand and it may take more time. Cancer-qualifying projects will receive priority during these times. If you need data quickly, please feel free to talk to Tim or Mary Lou.

You will receive an email from the BioDesktop when your data is ready. We sometimes edit this message to have specific remarks about how your samples ran, so please look at this message!

Real-time qPCR: Sample information

Template

What method of RNA extraction do you recommend?

For cell culture, we recommend the Qiagen RNeasy Mini Kit (cat# 74104) and TriZol followed by Qiagen RNeasy (with DNase treatment on column if necessary) for tissue.

Do I need to DNase treat my RNA before continuing on?

If you have not eliminated the chance of genomic amplification in your probe and primer design you will need to do a DNase treatment. We recommend you DNase treat the RNA on a column versus post RNA recovery. We have found the RNase free DNase set from Qiagen to perform well (cat#79254). RNA must be eluted in water (RNase and DNase free).

Should I check the integrity of my RNA?

RNA integrity or intactness is critical for a successful and accurate qPCR assay. We recommend checking your RNA on a gel or submitting your RNA to the Microarray facility for Bioanalyzer analysis. Concentration is essential since the dynamic range of the Bioanalyzer is 5-500ng/ul for the NanoChip and 200pg/ul to 5ng/ul for the PicoChip.

How can I check the concentration of my RNA?

RNA concentration of samples can be determined in the DNA Facility using the NanoDrop. This will use 2uL of your sample and provides accurate quantitation from a

range of 3ng/uL to 3ug/uL. If you suspect your concentration is lower, quantitation can be assessed by the Agilent Bioanalyzer. Please see Tim or Scott if this is the case.

How much RNA should I use in my cDNA reaction?

We recommend using between 100-1000 ng of RNA in your cDNA reaction. You **MUST** start with the **same amount** of total RNA in each sample. A first strand cDNA sysnthesis reaction is sufficient. You can buy cDNA reaction kits from Invitrogen, Applied Biosystems, Qiagen, Bio-Rad, amongst many others. Oligo d(T), Random hexamers, or a combination of the two can be used to prime the reverse transcription reaction EXCEPT if you choose to use 18s rRNA as an endogenous control. 18s rRNA requires the use of random hexamers since it lacks a poly A tail. To ensure all genomic DNA has been removed, a (-)RT reaction should be run.

Probe/primers

SYBR Green

SYBR Green: SYBR Green requires primer sets specific to the target(s) and endogenous control of interest. We recommend amplicon sizes from 200-400bp. The SYBR Green dye binds to double-stranded DNA and detection is monitored by measuring the increase in fluorescence during cycling. SYBR Green reactions will detect all double-stranded DNA including non-specific products. Primer design and concentration are critical in this assay as they can falsely contribute to the measurements. A dissociation curve will ALWAYS be run with this type of assay to determine if any other amplicon or primer-dimer formations are contributing to the overall SYBR Green fluorescence measurements.

Dual-labeled Probe/primer sets

Dual-labeled probe: This assay requires a primer set and a dual-labeled probe specific to the target(s) and endogenous control of interest to determine expression. This assay adds an extra layer of specificity since the probe also has to bind and measures the release of the fluorescent dye attached to the 5' end of the probe.

Probe/Primer Design: The facility has two software packages (Primer Express, Primer Select) to assist in primer and probe design for development of a dual-labeled or a SYBR Green assay. There is also Primer and Probe design freeware available that allows you to perform design from your local setting:

We recommended you design the dual-labeled probe to span intron/exon boundaries to eliminate amplification of genomic DNA. If you need assistance or training with design, please contact a DNA staff member to set up an appt.

Assay on Demand or TaqMan® Gene Expression Assays

Applied Biosystems has more than 500,000 pre-designed, gene-specific TaqMan® probe and primer sets for quantitative gene expression studies of human, mouse, and rat genes.

They have also just added probe and primer sets for Arabadopsis thaliana and Drosophila melanogaster genes. These assays are validated for use with comparative C_T studies.

To see if they have an assay designed for your target go to:
http://myscience.appliedbiosystems.com/cdsEntry/Form/gene_expression_keyword.jsp

Assay by Design
A number of companies are now offering a design service when you choose them to purchase your probe/primer sets.

Applied Biosystems:
http://www.appliedbiosystems.com/catalog/myab/StoreCatalog/products/CategoryDetails.jsp?ID=1542

Sigma Genosys: http://orders.sigma-genosys.eu.com/probedesign/

Qiagen: http://www1.qiagen.com/Products/Pcr/QuantiTect/GeneExpressionAssays.aspx

Assay development

How do I go about choosing my endogenous control?
Every assay requires an endogenous control to normalize the target(s) for differences in total RNA added to each reaction. This should be a gene that does not change expression due to treatments or exposures. Please consider the following document to ascertain the acceptable changes in expression levels of an endogenous control target (pages 5-20).
http://docs.appliedbiosystems.com/pebiodocs/04308134.pdf

Do I need to test for genomic contamination and if so, how do I do this?
If you have not eliminated the chance of genomic amplification in your probe and primer design you will need to do a DNase treatment. You should do a test to make sure this treatment was complete. You can provide a –RT reaction for each sample. qPCR amplification of these samples will indicate genomic contamination. Although many AOD's are designed not to amplify genomic DNA, we have seen cases where they did amplify genomic DNA so it might be a good practice to try one with each new lot of AOD.

What is a validation test?
In order to use the comparative C_T method of calculation, a validation test must be run. The validation will determine if the efficiencies of the target and the endogenous control amplifications are approximately equal. A validation test requires a serial dilution of a sample that expresses the target(s) of interest to be run along with the endogenous control. The endogenous control C_T will be subtracted from the C_T of the target at each dilution. The ΔC_T's will be plotted against the log input amount of the serial dilution. If the slope is less then 0.20, the Comparative C_T method can be used. If the slope is greater than this, the standard curve method must be used.

Comparative C_T method

COMPARATIVE C_T METHOD: This method can be used ONLY if you have proven that the target(s) and the endogenous control PCR are at similar efficiencies. Applied Biosystems Assays on Demand come validated and therefore researchers can use this method. If you are using designed probe and primer sets you need to first VALIDATE the targets with the endogenous control before using this method. If they do not pass the validation test, a standard curve should be applied. This method calculates changes in gene expression in samples relative to a control or calibrator sample. Refer to "Relative Quantitation Of Gene Expression: ABI PRISM 7700 Sequence Detection System: User Bulletin #2: Rev B " from Applied Biosystems to see the calculations involved in this method. You can download this document from Applied Biosystems at: http://docs.appliedbiosystems.com/search.taf?_UserReference=6AC24E8AF20AB96E42C1569A

Absolute Standard Curve

ABSOLUTE QUANTITATION: This is an assay that requires a standard curve of known copy or molecule numbers be run to apply unknown samples. An example where this assay would be appropriate is in quantifying bacterial or viral load in an unknown sample. Refer to "Relative Quantitation Of Gene Expression: ABI PRISM 7700 Sequence Detection System: User Bulletin #2: Rev B " from Applied Biosystems to see the calculations involved in this method. You can download this document from Applied Biosystems at: http://docs.appliedbiosystems.com/search.taf?_UserReference=6AC24E8AF20AB96E42C1569A

Relative Standard Curve

RELATIVE QUANTITATION: This is an assay that requires a relative standard curve be run at the same time as your samples. This standard curve can be generated by using a sample that is proven to be a high expressor of the target, a PCR product of the target, or a plasmid with a PCR insert. This method calculates changes in gene expression in samples relative to a control or calibrator sample. Refer to "Relative Quantitation Of Gene Expression: ABI PRISM 7700 Sequence Detection System: User Bulletin #2: Rev B " from Applied Biosystems to see the calculations involved in this method. You can download this document from Applied Biosystems at: http://docs.appliedbiosystems.com/search.taf?_UserReference=6AC24E8AF20AB96E42C1569A

Real-time qPCR: Format of data

What format will my real-time qPCR data be in?

The Sequence Detection System (SDS2.1) software generates a .sds file for each run. If the comparative Ct method is being used a .sdm study file will also be created. If your

order takes more than one 96 well plate, we will wait to upload the data all at once, so you will see multiple .sds files. The data is exported to a .txt file. All files will be uploaded to your BioDesktop account.

How do I download my data from the BioDesktop?

Please see the BioDesktop FAQ page.

Real-time qPCR: How to view data

How do I view my data?

You will need the Sequence Detection System software to open the .sds or .sdm files. This software is available to you from the facility if you have a PC. This software is also available on two computers in our workstation. We would be happy to train you in the use of this software, just ask Tim or Mary Lou to set up an appointment.
The .txt file can be opened in notepad or Excel.

How do I open the data report?

The data report is a .txt file that can be opened in Excel.