Quantitative Real-Time RT-PCR for Gene Expression Assays

Background

Real-time PCR is a powerful new tool in the study of gene expression. As with each tool used in differential gene expression determination (e.g. Northern blotting, competitive rt-PCR, and differential display-PCR), it is necessary to qualify the standardization method. These studies require the thoroughness and awareness of the researcher from the onset to perform an accurate and significant experiment. Factors such as - sample collection time (circadian influences), collection method (intactness of the RNA), and accurate quantification of starting material (small errors are amplified) - can and will influence the data. There are several approaches for the determination of gene expression levels but the information gathered falls into one of two categories: relative or absolute levels.

Target Selection

Housekeeping Gene (HKG):

The correct selection of the HKG for your quantitative rt-PCR reactions is essential to a successful assay. Due to the complexity of biological systems, there is not a single HKG that will serve the purpose of normalization for all assays. The HKG selected must have consistent expression in the experimental system being studied. A thorough investigation of previously published literature corresponding to your experimental system (i.e., cell type and/or treatment) is the easiest method for making this determination.

Three common housekeeping genes used in gene expression studies today are:

1) β-actin
2) 18s rRNA
3) GAPDH
As with any PCR for quantification or diagnostics, the HKG should be cloned into a high copy number plasmid for the purposes of having a positive control. Once cloned into an appropriate vector, it can be isolated in high concentrations for storage and use throughout your experiments. This positive control will serve as a check to ensure the proper performance of the DNA amplification portion of your reaction and as a means of troubleshooting potential problems during experimentation.

**Gene of Interest (GOI)**

The GOI amplicon should be under 150 bp in length for quantitative real-time PCR. This amplicon should also be cloned using standard techniques as a means of generating a sample to be used as a positive control. This will serve as your check for the proper performance of the PCR portion of the experiment.

**Quantification Approaches**

Two main approaches can be used for the performance of your quantification reactions. The first format will require you to prepare standard curves and will allow you to generate quantitative values for each sample. The second format does not require the generation of a standard curve. However, the information generated with this format will be for comparison of induction/repression levels between experimental groups.

**Quantitative Levels:**

Prepare a dilution series in duplicate of your purified plasmid for the GOI and from the positive controls that you generated. Perform your optimized real-time PCR assay on the rt-PCR products from your unknown samples and determine the starting concentration based on the threshold cycle (C\(_T\)) relative to the standard curve.

Although this method can be successful, there are major caveats to this approach. First, you must assure that each of your unknown RNA samples are of high purity and at the same starting concentration. In addition, an assumption must be made that the RNA integrity and rt-PCR process is identical for each of your unknown samples.

**Relative Levels**

As an alternative, purified RNA can be generated through the use of an *in vitro* transcription system followed by accurate quantification. This quantification can be done using a fluorometer and a sensitive assay for RNA detection (Versafluor and RiboGreen, Molecular Probes). Through the use of RNA as the standard for your assays, you will take into account the reverse transcription step of your assay.

This approach is less complicated. However, the data generated can only be used for the comparative analysis between unknown samples within the same experiment. First, you
will perform your rt-PCR reactions targeting both your HKG and your GOI. This is followed by the optimized quantitative PCR. By subtracting the difference of the \(C_{T}\) levels between the GOI and HKG, you can normalize your data. The difference in \(C_{T}\) calculated can then be compared to the expression levels of the control sample. Shown below is an example data set and their relative expression levels derived.

<table>
<thead>
<tr>
<th>Sample</th>
<th>GOI (C_{T})</th>
<th>HKG (C_{T})</th>
<th>(\Delta C_{T})</th>
<th>(\Delta C_{T}^{2})</th>
<th>Expression(^{1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Control)</td>
<td>28</td>
<td>25</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>23</td>
<td>2</td>
<td>-1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>26</td>
<td>-2</td>
<td>-5</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>24</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^{1}\) \(\Delta C_{T}\) is the difference in \(C_{T}\) values between the GOI and the HKG as calculated by \((\text{GOI }C_{T}) - (\text{HKG }C_{T})\).

\(^{2}\) \(\Delta C_{T}^{2}\) is the difference between the Sample \(\Delta C_{T}\) and the Control \(\Delta C_{T}\).

\(^{3}\) Relative Expression is calculated as \(2^{\Delta C_{T}^{2}}\).

*A major caveat of this method is that both the GOI and HKG must amplify at the same efficiency in the PCR. You can determine if this is the case by performing amplifications on different starting amounts of RNA then finding the threshold cycle for both the GOI and HKG for these various starting amounts. At this point, calculate the difference between the \(C_{T}\)s for each starting amount. As the starting amount of RNA is varied, the differences between the \(C_{T}\)s should remain essentially constant. If they do not, then this approach is not valid for this set of parameters (primers and probes). You can try optimizing this with different primer sets to get the same amplification rate, thereby obtaining a useful assay for this approach.*