Relative Quantitative RT-PCR Protocol

Wild-type and experimental plants should be planted and grown in parallel.

Wild-type and experimental plants' RNA preps and 1st strand synthesis should be done in parallel.

A suitable control should be highly expressed, preferably at equal levels in all tissues and developmental stages, but high expression in the tissue of interest is the only requirement. In Maize (*Zea maize*), Arabidopsis (*Arabidopsis thaliana*) and Petunia (*Petunia hybrida*) the glyceraldehyde-3-phosphate dehydrogenase subunit C (GAPC) transcript has been used as a control for amplification.

Dilution test

1) RT-PCR reactions are carried out using target specific primers and 0.5ul of Wild-type 1st strand template DNA at 5 different concentrations: undiluted, 1/5, 1/25, 1/125, 1/625. Using the following conditions:

0.5ul wild-type template 1st strand DNA
2.5ul 10X PCR buffer w/o MgCl
2.5ul 0.5mM dNTPs
2.0ul 25mM MgCl
2.5ul 5uM target specific primers (pre-mixed at 5uM)
14.9ul dH_2O
0.1ul Taq polymerase
25ul total reaction volume

PCR conditions:

94º 2 minutes

94º 30 seconds denaturing , 60º 30 seconds annealing , 72º 30 seconds extending
32 cycles

72º 7 minutes, 4º forever

The template used for this test is from the wild-type control plant grown in parallel for each gene of interest. (i.e. if CHR1 is to be tested, the wild-type plant grown in parallel with the CHR1 experimental plants is the one to use for dilution tests.) This test is to establish the dilution necessary to keep the target amplicon within the exponential growth phase of amplification. Conditions should be changed according to which high-temp polymerase you use, for example Hotmaster Taq requires 65º extension temperature instead of 72º. In any case, PCR conditions used during dilution tests should be identical to those used in inhibitor tests and final assays (discussed later).

2) 5ul of the PCR products amplified with target specific primers are run on an agarose gel. A dilution should be selected that appears to have produced less than 20ng of PCR product (less than 100ng in the total 25ul) and is clearly less intense than the next less dilute sample. PCR leaves the exponential phase when there are as many active polymerase molecules as there are available target templates. Experimentally this was determined to be at about 10^{12} molecules. How much this is in nanograms will vary with the size of your target. Amplification will stop altogether when dNTPs run out at about 400ng total. 100ng was chosen to be substantially less than these two limits under most conditions, so the reaction should still be in exponential growth phase in a dilution that meets this limit.

Inhibitor Test

3) Multiplex PCR is done with target specific primers, control primers and various inhibitor concentrations at the selected dilution. PCR is carried out at the selected dilution, again using wild-type control 1st strand template DNA as in step 1. This PCR is a multiplex reaction in which both the target specific primers and the control primers (in this case GAPC primers) are included. Amplification inhibitors are oligonucleotides that bind to the same sites as the control primers, but have a dideoxyC 3' modification that prevents them from serving as a template for polymerization.

Table 1 Control primers and inhibitors

Oligo	Sequence	3' modification	T_m [˚C]
F1	CACTTGAAGGGTGGTGCCAAG		61.8
F1'	CTGCAGCTCACTTGAAGGGTGGTGCCAAG	-dideoxyC	70.9
F2	CTGTCAACGACCCCTTCATC		59.4
F2'	GCTCGTCGCTGTCAACGACCCCTTCATC	-dideoxyC	71.0
F3	GCCAAGAAGGTTGTCATCTCTGC		62.4
F3'	AGGGTGGTGCCAAGAAGGTTGTCATCTCTGC	-dideoxyC	70.8
F4	CACTGCTACCCAGAAGACTGTTG		62.4
F4'	CATGCCATCACTGCTACCCAGAAGACTGTTG	-dideoxyC	69.5
R	CCTGTTGTCGCCAACGAAGTC		61.8
R'	AATGCTCGACCTGTTGTCGCCAACGAAGTC	-dideoxyC	69.5
R2	GTATCCCCACTCGTTGTCGTAC		62.1
R2'	CGGGTGCTGTATCCCCACTCGTTGTCGTAC	-dideoxyC	72.2

Table 1 Primers and inhibitors used in PCR amplification of GAPC. The melting temperatures (T_m) given are as provided by the manufacturer (MWG, ...).

Arabidopsis has worked successfully with primers F1&R (539bp) and F2&R (785bp)
Maize has worked successfully with primers F4&R2 (419bp), F3&R2 (616bp) works but not as well.
Petunia works with F1&R (~539bp) but F2&R does not work.
Inhibitors that match the primers are required in each case.

Experimental (target specific) primers

We have provided the primer sequences we designed for RT-PCR analysis of each target gene in maize (link to table). These primers were successfully used and gave consistent results throughout our quality control testing, and followed the guidelines described below. If a user designs their own primer pair to assay for mRNA abundance of the target gene, there are several important considerations for designing these primers. First, the sequence included in the inverted repeat should not include both RT-PCR primers of a pair, in case residual in tact mRNA from the transgene remains. Second, an intron should be spanned by the primer pair, or one of the primers should bridge an exon-exon junction to make genomic DNA contamination easily identified. Third, a melting temperature above 60°C is ideal, since the primers must work in a multiplex PCR with the inhibitors described above. Finally, sequence homology to other, closely related genes must be monitored to insure gene specific amplification rather than amplification of several transcripts with one primer pair.

Control primers and inhibitors are stored as premixed stocks at 50uM. Mixtures of control primers and inhibitors are made such that the total concentration of primers is 5uM but the percentage of primer vs inhibitor is measured as a percentage of the total oligonucleotide content of the solution. As such, a 50% primer vs inhibitor solution will contain 5uM of both primers and inhibitors, while a 25% primer vs inhibitor solution contains 5uM primers and only 1.25uM inhibitors (the oligos are 25% inhibitor 75% primer). This is the way the mixture has been measured in the past, but another system may be suitable.

Using this system to make primer and inhibitor mixes of 100ul, use 10ul of 50uM stock of primer. Add to this an appropriate amount of 50uM stock inhibitor from the table below:

	ul of 50uM Inhibitor	ul of dH_2O (to 100ul)
50%	10ul	80ul
45%	8.18	81.82
40%	6.67	83.33
35%	5.38	84.62
30%	4.28	85.72
25%	3.33	86.67
20%	2.5	87.5
15%	1.76	88.24

Additional values for this table may be obtained by using the formula X=(10*N)/(1-N) where N equals the amount of inhibitor as a decimal number (i.e. 15% = .15) and X will equal the number of ul of 50uM inhibitor to include in a 100ul total solution.

For relatively lowly expressed transcripts (undiluted to 1/25 dilution) 45%, 35% and 25% inhibitors are each tested. For more highly expressed transcripts we test using 35%, 25% and 15% inhibitors. A control with no control primers or inhibitors should also be run for a total of 4 PCR reactions using the following conditions (or other conditions as selected for step 1):

0.5ul wild-type template 1st strand DNA at appropriate dilution
2.5ul 10X PCR buffer w/o MgCl
2.5ul 0.5mM dNTPs
2.0ul 25mM MgCl
2.5ul 5uM target specific primers (pre-mixed at 5uM)
2.5ul 5uM control primers + Inhibitors (or H_2O)
12.4ul dH_2O
0.1ul Taq polymerase
25ul total reaction volume

PCR conditions (same as step 1):

94º 2 minutes

94º 30 seconds denaturing , 60º 30 seconds annealing , 72º 30 seconds extending
32 cycles

72º 7 minutes, 4º forever

4) Run 5ul of the PCR products on an agarose gel. Extrapolate as necessary what you think the gel would look like with intermediate concentrations such as 30%. Select an inhibitor concentration at which the control band (GAPC) is equal or slightly less intense than the experimental band. This is the amount of inhibitor to use for the final assays.

Final Assay

5) RT-PCR is done at the dilution selected in step 2 and the inhibitor concentration selected in step 4. This RT-PCR is carried out on wild-type control template and each of the experimental templates for the target of interest. A no template control should also be included to verify that none of the components have been contaminated with template or plasmid DNA. There are often wide variations in the quality of 1st strand DNA from sample to sample. It may be feasible to retroactively go back and redo PCR with less dilution in some samples as necessary. Experimental evidence gathered to date seems to indicate that when a PCR reaction leaves the log phase, the ratio of target to control has already been established and additional PCR cycles will no longer affect the results. In light of this, it may be acceptable to reduce the dilution and include 5 to 10 times more template in all reactions initially to counteract possible variations in template quality later on. Reaction conditions are the same as in step 3, but with templates as the new variable.

6) Run 5ul of the PCR products on an agarose gel. The ratio of the control band to experimental band in the wild-type control reaction is the baseline from which the experimental samples are judged. If some samples are too faint, you may have to redo the reactions using more template (less dilution) in that particular reaction. If the control band is too intense, it may interfere with judging the relative concentrations of the experimental bands and the PCR might need to be redone with more inhibitor. Absence of either the control or experimental bands in the wild-type control indicates a failed reaction. Absence of the control band in any of the experimental reactions indicates a failed reaction at least for that sample. **Relative accumulation levels of transcripts of the gene of interest may be estimated from this assay.**