

## Quantitative (real-time) reverse transcription polymerase chain reaction (qRT-PCR)

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There are many different techniques available for measuring the amount of a specific mRNA species in a sample of total RNA. One method that has become increasingly popular in recent years is quantitative (real time) reverse transcription polymerase chain reaction (qRT-PCR). Because qRT-PCR is very sensitive and quantitative, it is now commonly used by many researchers who study gene expression.

During qRT-PCR, each mRNA molecule is first converted into cDNA by the enzyme reverse transcriptase. Then the cDNA molecules are amplified (via PCR) using primers that are specific for the mRNA/cDNA sequence that the researcher wishes to measure. The amount of PCR product that is produced from each sample should be proportional to the amount of the targeted mRNA species that was in the original sample.

However, in order to make sure that the amount of PCR product really is proportional to the amount of template mRNA in the original sample, the amount of PCR product must be measured during the exponential phase of the amplification. To ensure that this happens, a special PCR instrument is used that can measure the amount of PCR product after each cycle of amplification (instead of just at the end of the last cycle).

For “standard” PCR it is typical to measure the amount of PCR product (after all the cycles are complete) by running the reaction products on a gel and seeing how big of a band you get. For qRT-PCR, however, a dye (SYBR green) is included in the reaction tube during the PCR amplification. When SYBR green is bound to double-stranded DNA it gives off a green fluorescence. The qRT-PCR instrument takes a measurement of the amount of green fluorescence (which represents the amount of PCR product) in each tube after every cycle of PCR. This gives you a report (in real time) of how the amplification proceeded.

In any type of mRNA quantitation, it is common to use some sort of internal standard to ensure that the same amount of total RNA is present in all of the samples. In the case of qRT-PCR, it is common to measure the expression of a “housekeeping gene” (eg. *CBP20*) in each of the samples being tested. In our case we will assume that *CBP20* is expressed equally in all cells of Arabidopsis under all environmental conditions. We will then express *RBCS* gene expression in terms of the ratio of *RBCS* mRNA/*CBP20* mRNA in any given tissue.

**Important details.** To amplify *RBCS* cDNA you will be using a pair of primers that is designed to produce a PCR product that is 99 bp long. To amplify *CBP20* cDNA you will be using a pair of primers that is designed to produce a PCR product that is 130 bp long. The reagents that you will use for reverse transcription and PCR come from the Quantifast SYBR Green RT-PCR kit (Qiagen catalog # 204154).

## EXPERIMENTAL PROCEDURE

**I. Create a new “Experiment”.** This “experiment” will include two groups, 24 wells for each group

**1.** Open the StepOne software. Choose **Set Up**, then **Design Wizard**.

**2.** Define the “Experiment Properties”

Experiment Name

User Name

For the Instrument, select StepOne (48 wells)

For the type of experiment, select Quantitation

**3.** Define the “Methods and Materials”

For the Quantitation method, select Comparative C<sub>T</sub>

For reagents, select SYBR Green Reagents

For template, select RNA and 1-Step RT-PCR

**4.** Set up the “Targets”

For the number of targets enter 2

The first target will be the mRNA you want to measure (*RBCS*)

The second target will be the endogenous control (*CBP20*)

For Reporter, enter SYBR

For Quencher, enter None

**5.** Set up the “Samples”

Enter the number of samples that you have (presumably 12)

For the number of replicates enter 2

For the number of negative controls enter 0

Enter names for the samples (Reference, 1, 2, 3, etc.)

Ask to set up All Sample/Target Reactions

Print (or write out) the Plate Layout

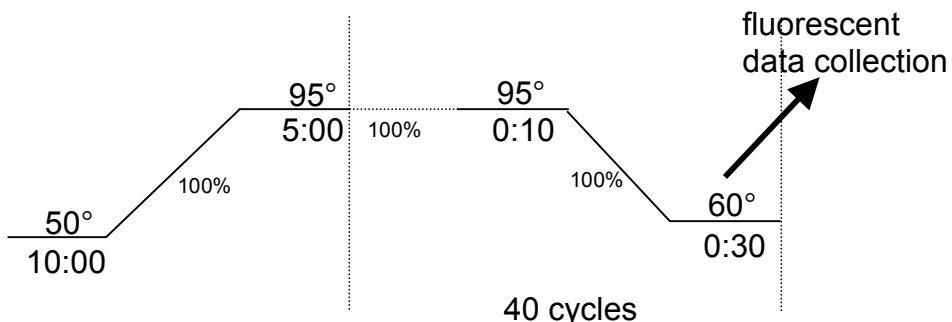
**6.** Set up the “Relative Quantitation Settings”

Select the desired reference sample

Select the desired endogenous control target

**7.** Set up the “Run Method”

reaction volume per well = 20  $\mu$ L



click on **Finish Designing Experiment**

click **Save Experiment**

## II. Set up the reactions.

1. Prepare a separate reaction mix for each target (one mix for *RBCS* and one mix for *RBP20*). For each target/sample combination, 60  $\mu\text{L}$  will be needed.

<u>Reaction mix</u>	<u>x1</u>	<u>x7</u>
2x QFSGR master mix	25.0 $\mu\text{L}$	175 $\mu\text{L}$
primer mix (10 $\mu\text{M}$ each)	5.0 $\mu\text{L}$	35
diluted QF RT mix	5.0 $\mu\text{L}$	35
RNase free water	<u>5.0 <math>\mu\text{L}</math></u>	<u>35</u>
	40.0 $\mu\text{L}$	280

2. Label a 500  $\mu\text{L}$  tube for each target/sample combination. Add 40  $\mu\text{L}$  of the correct reaction mix to each tube (on ice).

3. Add 10.0  $\mu\text{L}$  of the appropriate RNA sample (20  $\text{ng}/\mu\text{L}$ ) to each of the tubes. Mix gently by pipetting up and down.

4. Following the plate layout generated by the StepOne software, add 20  $\mu\text{L}$  of the appropriate mix/RNA sample to each well of a reaction plate (on ice).

5. Seal the reaction plate with optical adhesive film. Centrifuge the plate briefly to remove any air bubbles. Store the plate on ice in the dark until you are ready to perform the qPCR run.

## III. Run the reactions.

1. Click on the **Run Experiment** icon. Select your desired experiment and open it.

2. Open the drawer of the StepOne instrument and put your reaction plate into the sample block.

3. Click **Run** in the navigation pane. Click **START RUN**.

4. The progress of the run may be viewed by selecting **Run Method** or **Amplification Plot**. The run will take about one hour.

5. After the reaction has finished, remove your reaction plate from the sample block and store it at  $-20^\circ$ .