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Efficiency testing for quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

$\Delta\Delta C_T$ method for qRT-PCR data analysis

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1 Introduction

There are three main methods to evaluate quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) results: (i) standard curve method (see e.g. Livak and Schmittgen, 2001 or Morrison et al., 1998) , (ii) Pfaffl method (see e.g. Pfaffl, 2001) and (iii) the *ddCt* method (see e.g. Livak and Schmittgen, 2001).

Here we refer to the *ddCt* method only. In contrast to other methods the *ddCt* method is an approximation method and makes various assumptions (Zhang et al., 2013). The main advantages of the *ddCt* method lie in the reduction of experimental effort and the easiness of implementation. Beyond that in many cases results are similar to other non-approximation methods (Livak and Schmittgen, 2001).

Amplification efficiencies can influence the measured C_T values. The same target concentration of the same sample will result in different C_T values when amplified under low vs high efficiency conditions. Thus a prerequisite for the assumptions of the *ddCt* method to work is that the **amplification efficiencies** of the housekeeping gene and the gene(s) of interest are approximately equal (Zhang et al., 2013).

2 Principle of the Method

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is used to amplify and simultaneously quantify a DNA molecule (gene) of interest. The procedure relies on the general PCR principle which will not be further detailed here (for basic textbook knowledge see e.g. "Molekulare Genetik, Rolf Knippers"). To assess the amount of an actively transcribed gene of interest its level of messenger RNA (mRNA) is analyzed. Therefore total RNA is isolated and purified. Using reverse transcriptase copy DNA (cDNA) is synthesized and serves as the template for PCR analysis.

To assess the amplification efficiency of a primer pair of interest a 5-log dilution series of the template is performed and measured in **triplicates**. For PCR reaction and DNA detection an iCycler (BioRad) and Sybr Green are used, respectively.

3 Applicability and Limitations

This is only a brief description of how to assess amplification efficiencies. We do not describe in detail how to culture cells that serve as the source of RNA but rather start with the description of total RNA extraction. We use the RNeasy Micro kit (Qiagen) for RNA extraction and purification, the iScript cDNA synthesis kit (BioRad) for cDNA synthesis and SYBR Green to detect DNA amplification in an iCycler (BioRad). Any other mRNA extraction method and cDNA synthesis procedure revealing comparable purity and yield of mRNA can be used instead.

4 Related Documents

Table 1: Documents needed to proceed according to this SOP and additional NM-related interference control protocols.

Document ID	Document Title
	RNeasy® Micro Handbook - Qiagen

5 Equipment and Reagents

5.1 Equipment

- iCycler (or comparable real-time PCR device)
- Microcentrifuge (with rotor for 2 ml tubes)
- Micro-pipettes (0.5 µl – 1 ml)
- Microreaction tubes (1.5 ml; e.g. from Eppendorf)
- optically clear heat seal (e.g. BioRad)
- qRT-PCR suitable 96-well plates (e.g.Multiplate™ low-profile 96-well unskirted PCR plates, BioRad)
- RNase-free pipet tips
- spectrophotometer (to assess RNA concentration and purity; e.g. Nanodrop ND-1000)
- Vortex®

5.2 Reagents

- DNase I *[Qiagen #79254]*
- Ethanol (70% and 100%) *[CAS number: 64-17-5]*
- iQ™ SYBR® Green Supermix *[BioRad#170-8882]*
- iScript™ cDNA Synthesis Kit *[BioRad #170-8891]*
- Nuclease-free ddH_2O
- QIAshredder homogenizer *[Qiagen #79656]*
- RNeasy® Micro Kit *[Qiagen #74004]*
- specific primer pairs for the respective genes of interest
- β-mercaptoethanol *[CAS number: 60-24-2]*

6 Procedure

6.1 Flow chart

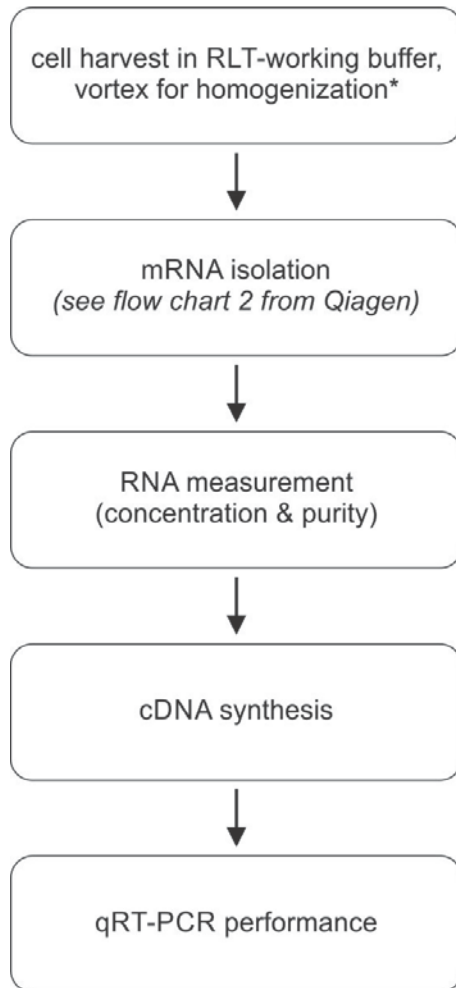

Figure 1: Brief outline of the workflow: from cell harvest to qRT-PCR performance.

*RLT-working buffer is specific for the RNeasy Micro kit (Qiagen). Use the sampling buffer suitable for you RNA extraction method. Samples can be stored in RLT-working buffer at -20°C for several weeks or even months.

6.2 RNA isolation

Proceed according to the RNeasy® Micro Handbook provided by Qiagen. Only cell harvest is described briefly below.

- Prepare RLT-working buffer by adding 10 µl β-mercaptoethanol per 1 ml of RLT buffer. 350 µl RLT-working buffer is needed per well of a 24-well plate.
- Remove supernatant and add 350 µl RLT-working buffer per well.
- Remove cells from the bottom of the cell culture well using a sterile pipet tip. The RLT-working buffer – cell mixture is viscous and clear.
- Make sure to scratch around the well long and rigorous enough to remove all cells from the well.
- Transfer everything into a 1.5 ml microreaction tube.
- Vortex to homogenize samples.
- Samples in RLT-working buffer can be frozen at -20°C till further processing.
- Proceed according to the RNeasy® Micro Handbook (attached to this SOP). For a brief overview see Figure 2.

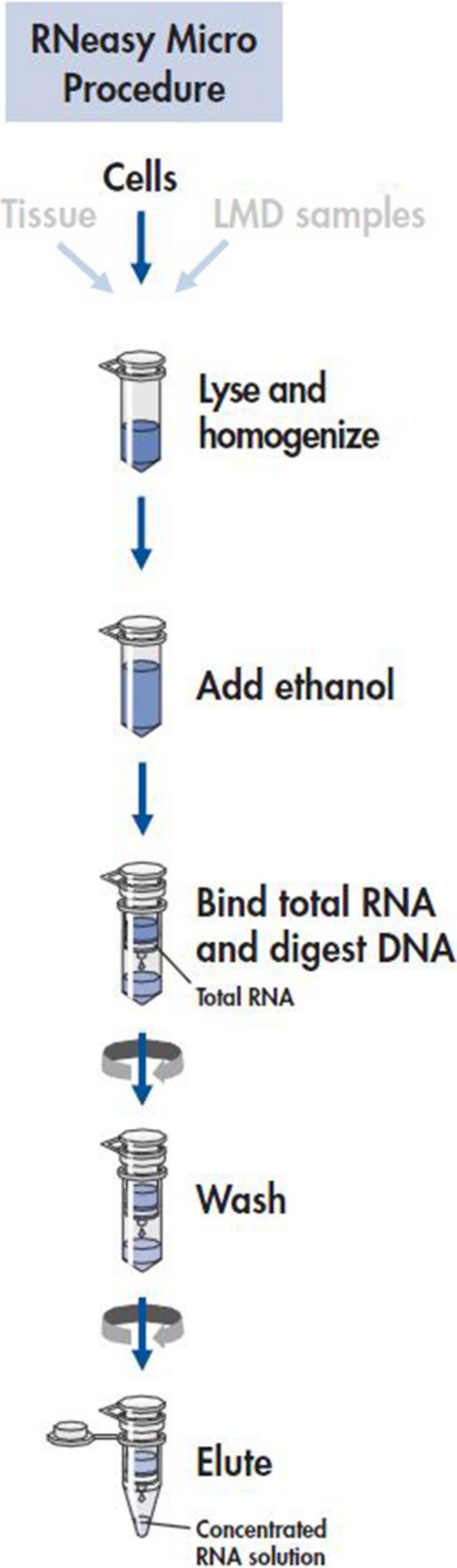

Figure 2: Workflow of total RNA isolation. Picture source: RNeasy Micro Handbook 12/2007. (Modified to illustrate, that we start from cell material only, but that other biological sources (tissue, LMD samples) could also be processed.) To extract RNA stick closely to the protocol of the manufacturer.

6.3 RNA measurement

Here we describe only the computer assisted optical density (OD) measurement using the Nanodrop ND-1000 spectrophotometer. When using any other equipment please refer to the corresponding protocol/SOP.

Make sure to mix every sample properly before measurement. All samples are measured in duplicates.

- Start computer and nanodrop software.
- Clean sensor with ddH_2O and tissue.
- Choose “Nucleic Acid” as the measurement parameter.
- Initialization:
    - Put 1 µl ddH_2O on top of the sensor.
    - Close lid.
    - Press “o.k.” (software).
- Choose sample type “RNA40”.
- Clean sensor with ddH_2O and tissue.
- Blank measurement:
    - Put 1 µl of solvent (in our case RNA is dissolved in ddH_2O) on top of the sensor.
    - Close lid.
    - Press “blank” (software).
- Clean sensor with ddH_2O and tissue.
- Sample measurement:
    - Put 1 µl per sample on top of the sensor.
    - Close lid.
    - Press “measurement” (software).
    - Measure every sample twice.
    - Clean sensor with ddH_2O and tissue after each sample.
- Proceed with subsequent sample.
- Safe report and transfer values to excel for further calculations.

Report will contain the following parameters:

- Concentration of RNA (ng/µl)
- A260: OD measured at 260 nm
  (used to calculate RNA concentration according to equation (1))

$$\textit{RNA conc. (ng/µl) = OD(260 nm) x dilution factor x 40} \qquad (1)$$

- A280: OD measured at 280 nm
  (used to assess contamination by proteins or phenols which absorb at 280 nm)
- Ratio 260/280
  (used to assess the purity of the RNA preparation; a ratio ~2 is considered as “pure RNA”)

6.4 cDNA synthesis using iScript™ (BioRad)

Volumes given are for one single reaction only. Make sure to prepare enough iScript master mix for all samples to be processed.

iScript master mix:

- 5x iScript reaction mix 4 µl
- Add iScript reverse transcriptase 1 µl

Note: Prepare this mix shortly before usage. If necessary keep it on ice (at 4°C) until needed.

Note: As it is almost impossible to completely eliminate genomic DNA from RNA samples, it is important to run a so called "no amplification control" (NAC). This reaction contains all reagents except the reverse transcriptase. If later on in the qRT-PCR reaction a product is formed, it indicates the presence of contaminating DNA in the sample. Make sure to include these mock samples into the calculation for the iScript master mix.

Calculate the volumes needed per sample for a final amount of **200 ng RNA.**

All further steps are performed in a qRT-PCR 96-well plate.

- Add **200 ng RNA** of each sample into one 96-well.
- Ad up with **ddH$_2$O** to a final volume of **15 µl**.
- Add **5 µl iScript** master mix per well.
  Results in a total reaction volume of **20 µl** (per well).

- Run reverse transcription program on iCycler:
  5 minutes 25°C
  30 minutes 42°C
  5 min 85°C
  hold at 4°C

- After reaction is completed add **80 µl ddH$_2$O** to the reaction mix.
  Results in a total volume of 100 µl that contain an equivalent of 200 ng RNA. Assuming a complete transcription into cDNA this corresponds to a final concentration of **2 ng/µl cDNA**.

6.5 Real-time PCR using iQ™ SYBR® Green Supermix and iCycler

Volumes given are for one single reaction only. Working stock concentration for all primers used is 10 µM. All reactions are performed in **triplicates** in a qRT-PCR 96-well plate and in a total volume of 12 µl.

6.5.1 Controls to be run

- NAC (see 6.7): these wells receive 5 µl mock cDNA samples (as described above) instead of template. A signal here indicates contamination of samples with genomic DNA.
- No template control (NTC): these wells receive 5 µl ddH$_2$O instead of template. A signal here indicates that one or more of the qRT-PCR reagents is contaminated with the amplicon.

6.5.2 Dilution of template

The cDNA template (resulting from the cDNA synthesis step described in 6.4) is serially diluted over 5 orders of magnitude as shown in Table 2.

Table 2: **Serial dilution of the cDNA template in ddH_2O.** *5µl of each sample (1-5) are used in the qRT-PCR reaction. Thus the starting quantity of cDNA per reaction is given by the volume of 5 µl and the concentration of the respective sample.

No.	dilution	concentration (ng/µl cDNA)	starting quantity per reaction (ng cDNA*)
1	undiluted	2	10
2	1:10	0.2	1
3	1:100	0.02	0.1
4	1:1000	0.002	0.01
5	1:10000	0.0002	0.001

To assess the efficiency of one primer pair in triplicate measurements the following volumes are needed:

1. 30 µl of cDNA template (undiluted) → 2 ng/µl (1)
2. 3 µl of cDNA template (1) (2 ng/µl) are mixed with 27 µl of ddH_2O → 0.2 ng/µl (2)
3. 3 µl of sub-dilution 2 (0.2 ng/µl) are mixed with 27 µl of ddH_2O → 0.02 ng/µl (3)
4. 3 µl of sub-dilution 3 (0.02 ng/µl) are mixed with 27 µl of ddH_2O → 0.002 ng/µl (4)
5. 3 µl of sub-dilution 4 (0.002 ng/µl) are mixed with 27 µl of ddH_2O → 0.0002 ng/µl (5)

6.5.3 qRT-PCR reaction

- Make sure to prepare enough **iQ SYBR Green master mix** for all samples to be processed. Volumes given here are enough for one sample only:

Reagent	Volume	
iQ SYBR Green Supermix	6.00	µl
primer_sense (10 µM)	0.24	µl
primer_antisense (10 µM)	0.24	µl
ddH_2O	0.52	µl
total volume	7.00	µl

- Add 5 µl of the respective dilutions (1-5 Table 2) of the cDNA template, the NAC template or ddH_2O (NTC) to the appropriate wells of a 96-well qRT-PCR plate.
  Note: This small volume has to be pipetted as a compact drop directly to the wall of the well.
- Add 7 µl of the iQ SYBR Green master mix. Avoid bubble formation.
- Seal the plate with an optically clear heat seal (e.g. BioRad). Avoid finger prints on top of the foil. These would impair fluorescence readout.
- Run appropriate PCR program. On the iCylcer used here: *2Step60+Melt* (see Table 3 and Figure 3)

Table 3: iCycler qRT-PCR program *2Step60+Melt*. *60°C corresponds to the annealing temperature of the primers and has to be adapted to the specifications of the respective primer pair.

step no.	temperature (°C)	duration (min.)
1	95	3:00
2	95	0:10
3	60*	0:30
4	GO TO 2	39 more times
5	95	0:10
6 (melt curve)	65 to 95 (increment 0.5°C)	
END	4	∞

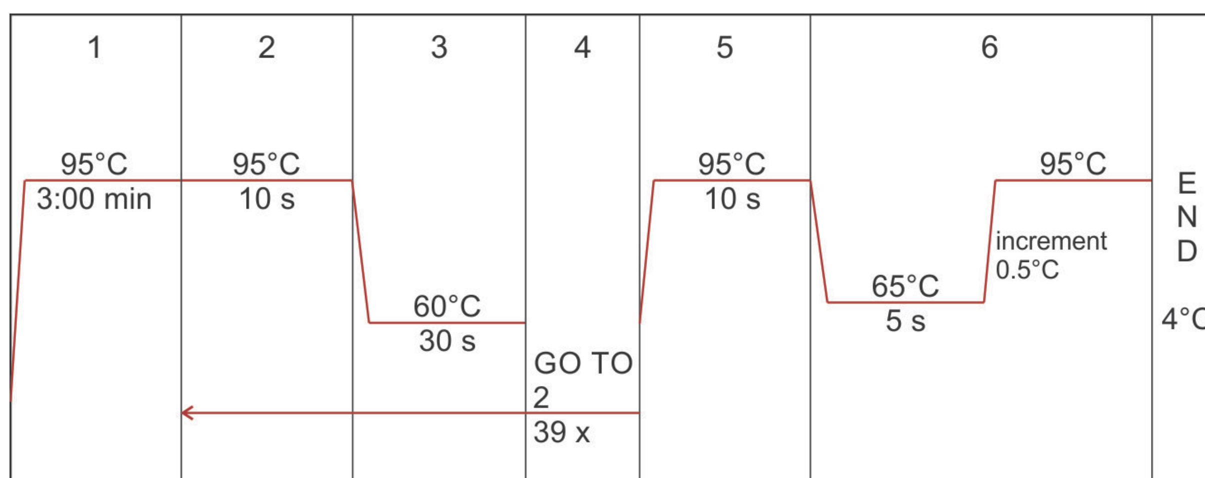

Figure 3: iCycler Program *2Step60+Melt*. 60 refers to the annealing temperature of the primers (60°C) in step 3. This temperature has to be adapted to primer specifications.

6.6 Data evaluation

The iCycler software allows defining "standards" that correspond to the 5 serial dilutions defined in Table 2 and their corresponding "starting quantity". With this information and the measured C_T values a standard curve is generated (as an example see figure 4). The **slope** of this standard curve is necessary to calculate the **PCR efficiency**. A slope of -3.3 ± 10% reflects an efficiency of 100% ± 10%.

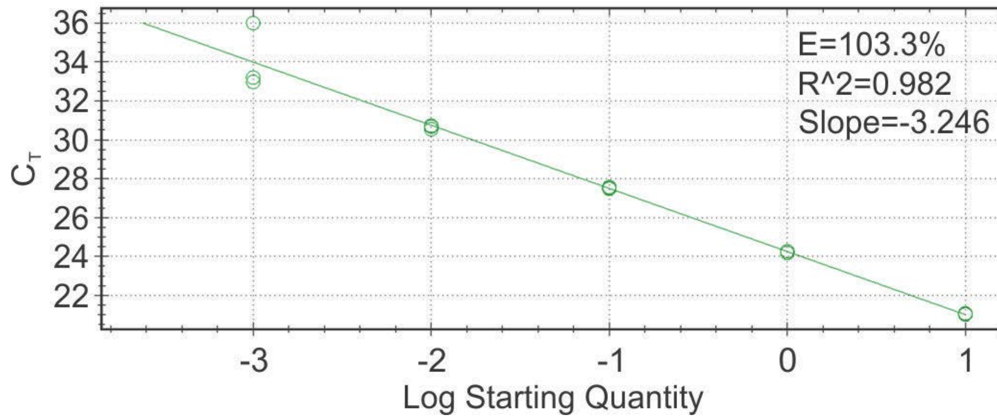

Figure 4: Example of PCR efficiency determination using Nrf2 primers, SYBR Green and an iCycler (BioRad). Template concentrations from 10 to 0.001 ng (see also Table 2) were used and measured in triplicates. Every green open circle resembles one of these measurements. The resulting C_T values are plotted against the LOG of the starting quantity. The slope, and with that the PCR efficiency, as well as the correlation coefficient R^2 fulfill the acceptance criteria (see chapter 7 below). One problem occurs for the lowest concentration tested were triplicate measurements differ considerably.

The **correlation coefficient R^2** is another critical parameter that can be extracted from the standard curve. It is a measure for the strength of the relationship of two variables. A R^2 of 1 would be the perfect correlation (all values exactly on the curve). A R^2 of 0 would be no correlation at all (random distribution of the measured values). A R^2 value >0.9 provides good confidence in correlating two values.

7 Quality Control, Quality Assurance, Acceptance Criteria

RNA purity: A ratio of absorbance at 260 nm and 280 nm of ~2 is generally accepted.

PCR efficiency: Generally, a PCR efficiency between 90 and 110% is considered acceptable (*Lifetechnolgie)*.

Negative controls (NAC and NTC) have to be negative (no C_T value detectable).

The standard deviation of the triplicate measurements has to be as small as possible. To be able to measure 2-fold concentration changes in more than 95% of all cases, the standard deviation has to be ≤0.25 (for more explanation see: http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_053906.pdf)

R^2 should be above 0.9.

Melt curves of each amplicon yield a sharp peak at the melting temperature of the respective amplicon. This indicates that the products are specific. As a conclusion SYBR Green fluorescence is directly correlated to the accumulation of the product of interest.

8 Health and Safety Warnings, Cautions and Waste Treatment

Cell seeding has to be carried out under sterile conditions in a laminar flow cabinet (biological hazard standard). For this only sterile equipment must be used and operators should wear laboratory coat and gloves (according to laboratory internal standards).

Discard all materials used to handle cells (including remaining cells themselves) according to the appropriate procedure for special biological waste (i.e. by autoclaving).

9 Abbreviations

cDNA	copy deoxyribonucleic acid
ddH_2O	double-distilled water
DNA	deoxyribonucleic acid
mRNA	massenger ribonucleic acid
NAC	no amplification control
NTC	no template control
OD	Optical density
qRT-PCR	quantitative real-time reverse transcription polymerase chain reaction

10 References

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