Quantitative real-time PCR (qRT-PCR)

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INTRODUCTION

To compare *kdpFABC* expression between the *E. coli* strains MG1655 (wild-type) and MG1655 (*kdpA4*), a KdpFABC-inactive mutant after a shift to K^+ limitation quantitative real-time PCR was used. Real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses. This is made possible by including a fluorescent DNA-binding dye. This binding-dye has little fluorescence when free in solution, but when it is bound to double stranded DNA the fluorescent signal rises up to 1000-fold. Therefore, the fluorescent signal is proportional to the amount of double stranded DNA present in a reaction. The threshold cycle (C_T) is the cycle number when enough amplified product accumulated to yield a detectable fluorescent signal. The C_T value of a reaction is determined by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background. Thus, the reaction will have a low, or early, C_T. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. Thus, the reaction will have a high, or late, C_T.

MATERIALS

cDNA from the *E. coli* strains MG1655 and MG1655 (*kdpA4*) after a shift to K^+ limitation

REAGENTS

- iQ™ SYBR® Green Supermix (BIORAD, Munich)
- DEPC (Diethyl pyrocarbonate) (Roth, Karlsruhe)
- Set of specific primers
- Genomic DNA (for assay optimization)
- Phire (hot-start polymerase, New England Biolabs)
- 5x Phire reaction buffer (New England Biolabs)
- Agarose (Serva)
- dNTP´s
- dH_2O

REAGENT SET-UP

- **DEPC-H_2O:** Add 1 ml DEPC to 1 l dH_2O, stir over night under the hood and autoclave
- **dH20:** autoclave prior to use

EQUIPMENT

- iQ™5-cycler Multicolor real-time PCR detection system (BIORAD, Munich)
- 96-well 0.2 ml thin-wall PCR plates (BIORAD, Munich)
- Optical film sealing kit (BIORAD, Munich)
- Cycler (Mastercycler, Eppendorf)
- Agarose gel electrophoresis
- NanoDrop Spectrophotometer (Peqlab)
- QIAquick-gelextraction Kit (Qiagen)
- Reaction tubes 1.5 ml (RNase-free)
- Reaction tubes 0.5 ml (RNase-free)

PROCEDURE

TIMING ~ 3 days. This includes primer design and assay optimization

1. Primer and amplicon design

NOTE: A successful real-time PCR reaction requires efficient and specific amplification of the product. Both primers and target sequence can affect this efficiency. Therefore, care must be taken when choosing a target sequence and designing primers

For amplicon design, follow these guidelines:

- Design amplicon to be 75–200 bp. Shorter amplicons are typically amplified with higher efficiency. An amplicon should be at least 75 bp to easily distinguish it from any primer-dimers that might form

- Avoid secondary structure if possible
- Avoid templates with long (>4) repeats of single bases
- Maintain a GC content of 50–60%

For primer design, follow these parameters:

- Design primers with a GC content of 50–60%
- Maintain a melting temperature (T_m) between 50ºC and 65ºC. We calculate Tm values using the nearest-neighbor method with values of 50 mM for salt concentration and 300 nM for oligonucleotide concentration
- Avoid secondary structure; adjust primer locations outside of the target sequence secondary structure if required

Primers used: (product size: 89 bp)

KdpA-qPCR-1 sense: 5´-CCAACCGCACTGACCAACTTC-3´
KdpA-qPCR-1 antisense: 5´-TCGCCCATCACTTCACCAAAG-3´

2. Assay validation and optimization

Identification of the optimal annealing temperature and primer concentration

NOTE: Genomic DNA is used for assay optimization as cDNA is too expensive for this purpose. Prepare a premix containing SYBR-Green, primers and DEPC-treated H_2O

- Test a range of annealing temperatures above and below the calculated T_m of the primers (use temperature gradient feature of the cycler)
- Test a range of primer concentrations for example 50 nM, 100 nM, 200 nM, 250 nM
- Use 12.5 µl iQ SYBR-Green Supermix, 1 µl genomic DNA and different primer concentrations (50 nM-250 nM) and add them up to a total volume of 25 µl with DEPC-treated H_2O for each reaction
  (a no template control (NTC) was carried along, too)

Construction of a standard curve to evaluate assay performance

- Prepare serial dilutions (1:10) of genomic DNA.
- Use 12.5 µl iQ SYBR-Green Supermix, 1 µl of the serial diluted genomic DNA and different primer concentrations (50 nM-250 nM) and add them up to a total volume of 25 µl with DEPC-treated H_2O for each reaction

  The efficiency of the assay should be between **80-100%** and the C_T values of the replicates should be similar

- Cycler protocol:
  95℃, 3 min; [95℃, 10 s; X℃ (Gradient), 30s] x40

During the second step (annealing, extension) the increase in fluorescence was recorded as the SYBR-Green bound to the DNA and the C_T-value was determined for each sample

- Check the specifity of the qPCR assay by using the melt-curve function after the qPCR-run:

  95℃, 30 s (Denaturation of the double stranded PC R product)
  70℃, 30 s (Formation of the double stranded PCR p roduct)
  Increase in temperature up to 95℃ (51 cycles with a temperature increase of 0.5℃ each cycle) (Detection of the melt point)
- Also run products on an agarose gel

3. qRT-PCR run

- Use 12.5 µl iQ SYBR-Green Supermix, 2 µl cDNA and 250 nM primers (or the determined optimal primer concentration) and add them up to a total volume of 25 µl with DEPC-treated H_2O
  (a no template control (NTC) was carried along, too)
- Cycler protocol:
  95℃, 3 min; [95℃, 10 s; 65℃ (or optimized temper ature), 30s] x40
- Check for the specifity of the qPCR assay by running a melt-curve and also run products on an agarose gel

4. Generation of a standard

To determine the amount of *kdpA* in each sample a *kdpA*-DNA standard was carried along with each qPCR run

Template: *E. coli* MG1655 genomic DNA
Primer: KdpA-qPCR-1 sense, KdpA-qPCR-1 antisense
DNA-Polymerase: Phire (hot-start polymerase, New England Biolabs)

Template	2 µl
Primer s [10µM]	2,5 µl
Primer as [10 µM]	2,5 µl
dNTP´s	1 µl
5x Phire reaction buffer	10 µl
Phire polymerase	1 µl
dH_2O	to 50 µl

98℃, 30 s; [98℃, 5 s; 66℃, 5 s; 72℃, 10 s] x30; 72℃, 1 min

- The product was run on an agarose-gel (1%)
- Specific products were extracted with a QIAquick-gelextraction Kit (Qiagen)
- Determination of the DNA concentration with NanoDrop Spectrophotometer

- For each qPCR reaction 12.5 µl iQ SYBR-Green Supermix 1 µl DNA standard in different dilutions (10^{-2}-10^{-7}) and 250 nM primer were added up to a total volume of 25 µl with DEPC-treated H_2O

Each dilution of the *kdpA*-DNA standard gave a certain C_T-value. As the concentration of the standard was known, the concentration of *kdpA* (cDNA) in each sample could be calculated via the obtained C_T-value

REFERENCES

Application guide: Real-Time PCR (BIORAD)