Interferences of PCR Effectivity: Importance for Quantitative Analyses

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Abstract: Importance of the Polymerase chain reaction (PCR) have already crossed the border of mere target DNA sequence present or absence analysis. For number analyses e.g. Genetically Modified Organisms (GMOs) or gene expression assesment the DNA quantification is demanded. Real-time (or quantitative) PCR is the most used tool for nucleic acids quantification. PCR efficiency has relevant importance on DNA quantification – it should be almost same for each PCR and its value should varied between 90–100%. There are a lot of PCR enhancers and inhibitors well known. We described impact of used DNA solvent and used laboratory plastic on real-time PCR efficiency.

Keywords: real-time PCR; DNA quantification; PCR efficiency; GMO analysis

Deoxyribonucleic acid (DNA) is the fundamental molecule of the living cellular organisms. The main role of DNA molecules is the storage of genetic information, which is encoded by nucleotide sequence. DNA molecules are present in each cell of organism and are sequentially specific. That enable effectual usage of DNA as a target molecule for several analysis.

The basic properties of DNA was at first proved by Watson and Crick (1953), who first suggested structure of double-stranded DNA. The second important finding for establishing of molecular techniques was a discovery of DNA denaturation and renaturation ability (Marmur & Doty 1961).

The discovery of DNA polymerase chain reaction principle made possible that DNA has been universally used as a target molecule in several analyses.

Recently, PCR and another PCR-based methods have been probably most used analytical tools in molecular-biology laboratories worldwide.

PCR – historical overview, the principle

PCR is *in vitro* technique, which allows to amplify a part of DNA – the reaction may generate as many as millions copies of a particular DNA sequence. The process of specific DNA target amplification was at first decribed by Kleppe and Khorana (Kleppe *et al.* 1971). They use repair replication, which duplicated and then quadrupled a small synthetic molecule with the help of two primers and DNA polymerase.

However, the man, who improved PCR and made it the central technique in molecular biology was Kary B. Mullis. In year 1993 became Nobel Prize in Chemistry for his development of the Polymerase Chain Reaction. The main improvement was the incorporation of thermal cycling and using thermostable polymerase. The thermal cycling allowed the rapid and exponential amplification of large quantities of any desired DNA sequence from an extremely complex template. Using of thermostable Taq polymerase made the PCR relatively easy to done (Mullis *et al.* 1986; Saiki *et al.* 1988).

Thus, PCR relies on thermal cycling with three necessary steps – denaturation, annealing and extension. Denaturation step causes separation of DNA template by disrupting the hydrogen bonds between complementary bases of the DNA strands. Annealing step allows annealing of the primers to the single-stranded DNA template and in extension step the DNA polymerase synthesises a new DNA

strand complementary to the DNA template by adding dNTPs in 5' to 3' direction. Every another cycle is analog. PCR product of expected lenght is firstly produced at the 3rd PCR cycle. After *n*-cycles is reaction finished by adding some inhibitor or by cooling the reaction mixture. Schema of the PCR is shown in Figure 1.

Theoretically, the final number of PCR products is given by formula:

$$N = 2^n \tag{1}$$

where:
N – initial number of template DNA molecules
n – number of PCR cycles

This relation is valid in case of 100% efficiency of PCR. The basic components for PCR are DNA template that contains the DNA region to be amplified, two primers that are complementary to the 3' ends of each of the sense and anti-sense strand of the target on DNA template, deoxynucleoside triphosphates (dNTPs), which are the building blocks for new DNA strand, Taq polymerase or another thermostable DNA polymerase, Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase and divalent cation magnesium ions and monovalent cation potassium ions as a DNA polymerase activators.

Application of PCR

From its beginning, PCR shows how strong instrument is in very different parts of DNA analysis.

In routine laboratory practise is probably most used PCR for selective amplification of a specific DNA region. In diagnostic analyses positive amplification signal confirms presence of the target region in sample DNA. Otherwise, products of amplification could be used for subsequent work- as a hybridisation probes for Southern or Northern hybridisation or for DNA Microarrays (Southern 1975; Alwine *et al.* 1977; Vora *et al.* 2004; Leimanis *et al.* 2006; Xu *et al.* 2007), or as an object of Sanger sequencing (Sanger *et al.* 1977; Ovesná *et al.* 2000). Methods as AFPL (Amplified Fragment Length Polymorphism) or RFLP (Restriction Fragment Length Polymorphism), coming up from PCR, serve for genetic fingerprint generating, which is used in several techniques based on unknown DNA analysis, such a forensic techniques or in genome mapping, localisation of genetic disease genes, determination of risk for a disease and for paternity testing (Vos *et al.* 1995; Powell *et al.* 1996; Mueller & Wolfenbarger 1999; Leišová *et al.* 2007).

Real-time PCR

Quantitative or real-time PCR is another upgrade of classical PCR. Real-time PCR is used in a lot of applications, e.g. for diagnostics of infectious diseases, cancer or genetic abnormalities, for GMO quantification or for gene expression quantification (Heid *et al.* 1996; Vaitilingom *et al.* 1999; Bustin 2000; Peiris *et al.* 2003; Holst-Jensen *et al.* 2003).

By real-time PCR the increase of quantity of a PCR products during the thermal cycling could be measured. Increase of PCR product is visualised by using fluorescent dyes, such as Sybr Green, or fluorophore containing DNA probes, such as TaqMan.

Principle of fluorescent signal generation by TaqMan probes and Sybr Green demonstrate Figure 2.

Whole PCR process can be divided into three stages (Figure 3).

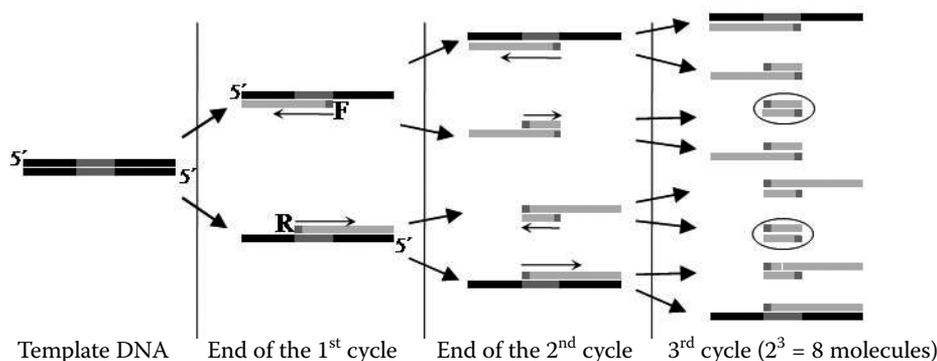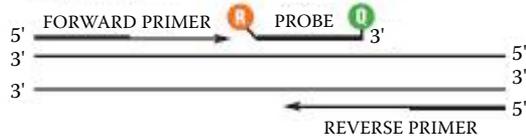Template DNA   End of the 1st cycle   End of the 2nd cycle   3rd cycle (2^3 = 8 molecules)

Figure 1. Schema of PCR. Target PCR product is indicated by circle
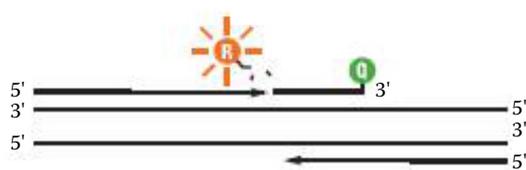
TaqMan® probe-based assay chemistry

1. **Polymerisation**: A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan® probe, respectively.
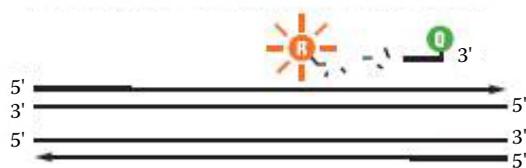
2. **Strand displacement**: When the probe is intact, the reporter dye emission is quenched.
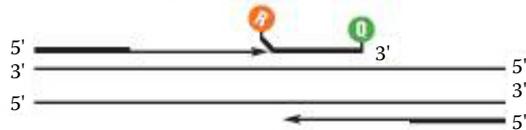
3. **Cleavege**: During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.
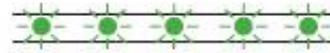
3. **Polymerisation completed**: Once separated from the quencher, the reporter dye emits its characteristic fluorescence.
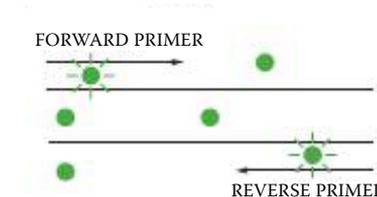
SYBR® green I Dye assay chemistry

1. **Reaction setup**: The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.

2. **Denaturation**: When the DNA is denatured, the SYBR® Green I Dye is released and the fluorescence is drastically reduced.

3. **Polymerisation**: During extension, primers anneal and PCR product is generated.

4. **Polymerisation completed**: When polymerisation is complete, SYBR® Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.
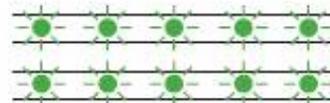

Figure 2. Principle of fluorescent signal generation using SYBR® Green or TaqMan® chemistry (source Applied Biosystems)

In the first exponential stage the PCR efficiency nears to 100% – it means that at every cycle the amount of products is doubled. This stage is used for DNA quantification. In the second linear stage the yield of PCR is highly variable. In the third stage, plateau phase, there no more products accumulate. PCR products are analysed using agarose gel upon this stage.

Efficiency of PCR drops from the exponential to the plateau stage – as the main mechanism for the attainment of PCR plateau phase is highly probable the inhibitory effect of endogenous amplicon DNA on the activity of DNA polymerase (Kainz 2000).

The starting amount of sample DNA is evaluated on the basis of quantity PCR product in definite part
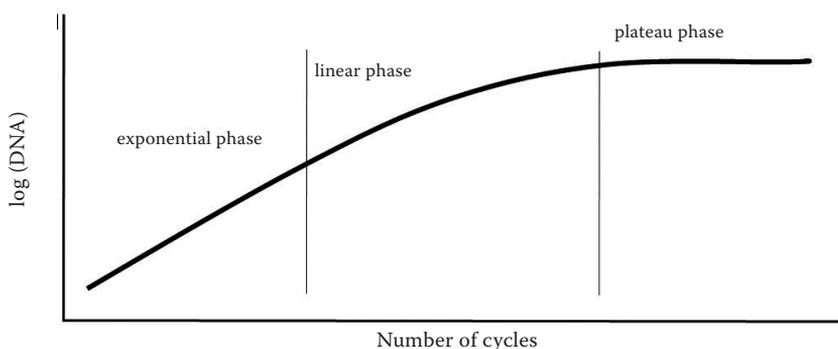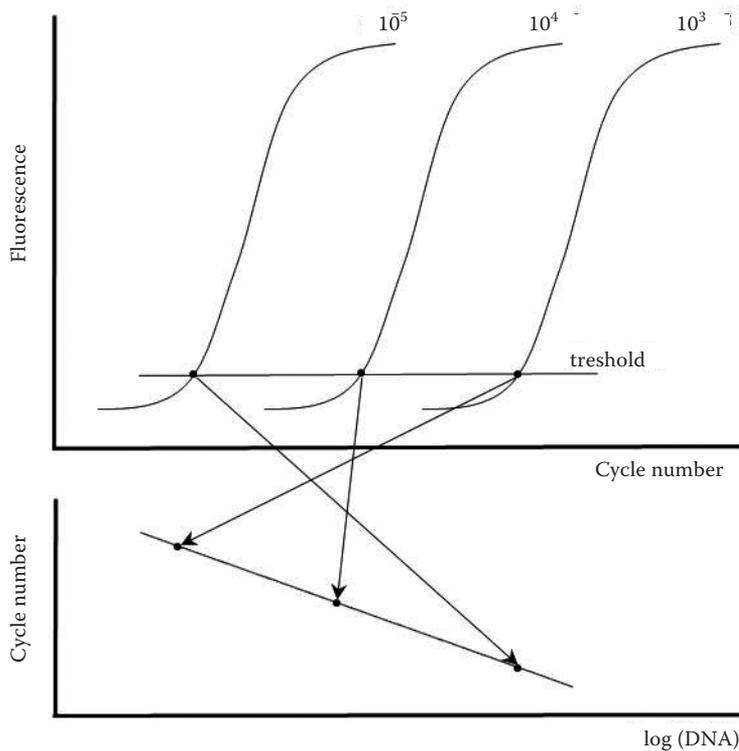

Figure 3. Diagram of PCR stages

Figure 4. Calibration curve creation. Curves represent starting copy number (10^5, 10^4 and 10^3 DNA copies) of the target DNA sequence in the reaction

of exponential amplification. A fixed fluorescence threshold is set significantly above the baseline. An intersection of treshold and increased fluorescent signal is defined as Cycle Treshold (Ct) and its value is used for DNA quantity interpolation. Diagram of calibration curve creation is shown in Figure 4.

For DNA quantification by real-time PCR it is very important to perform all reaction at the almost same efficiency. The calibration curve get the slope value from –3.6 to –3.1 when the efficiency of reaction was between 90–100%. When the efficiency of reaction was 100%, the slope of calibration curve equals – 3.323. Any problem with PCR efficiency may lead to inaccurate quantification of unknown sample.

Formula for calculation of PCR efficiency comes from the slope of calibration curve and is given by relation:

$$\text{Efficiency} = 100 - (10^{-1/\text{slope}}) \times 100 \qquad (2)$$

PCR inhibitors

PCR is characterised by high sensitivity. Thus, presence of contamination and inhibitors in samples or in laboratory backround represent a big problem. That is why the strict anticontamination conditions during PCR performance should be kept.

PCR inhibitors could originated from sample and its surrounding or from pre-PCR reagents. Among well-known PCR inhibitors belong e.g. agar, EDTA (ethylenediaminetetraacetic acid), ethanol, SDS (sodium dodecyl sulfate), DMSO (dimethyl sulfoxide), NaCl, phenol, proteinase K, cellulose, powder from gloves, calcium and metal cations.

MATERIAL AND METHODS

DNA isolation. DNA isolation from the certified reference material 10% MON863 (CRM IRMM

Table 1. Dilution of calibration curve from 10% MON863

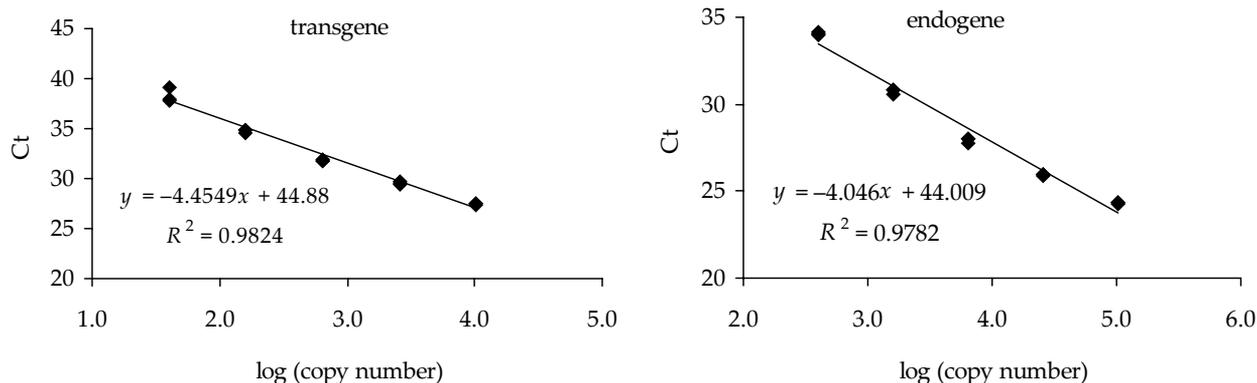| Sample code	S1	S2	S3	S4	S5
Maize genome copies	102 752	25 688	6 422	1 606	401
MON863 GM copies	10 275	2 569	642	161	40

Figure 5. Calibration curve with DNA solved by TE buffer

416-3) was carried out by CTAB method (EN ISO 21571:2002). DNA was dissolved in 0.1 × TE buffer.

Calibration curve construction. A calibration curve was produced by plotting Ct-values against the logarithm of the target copy number for the calibration points. The calibration curves consist of five dilutions of DNA extracted from the 10% candidate CRM IRMM-416-3. A series of one to four dilution intervals at a starting concentration of 102 752 maize genome copies was used. This corresponds to 280 ng of DNA with one maize genome assumed to correlate to 2.725 pg of haploid maize genomic DNA (Arumuganathan & Earle 1991).

In the first real-time PCR run was used 0.1 × TE buffer for series of DNA dilution, in the second real-time PCR run was for DNA dilution used water. Dilution scheme is shown in Table 1.

Real-time PCR run. Amplification reactions for real-time PCR of maize transgenic line MON863 (Joint Research Centre 2005) were performed using TaqMan PCR core reagents (Applied Biosystems, USA). Coctail PCR mixtures were prepared sepa-

rately for the maize endogenous *adh1* reference system and for transgene MON863 system. End concentrations of PCR components were follow: TaqMan Universal PCR Master Mix 1×; for the reference endogenous system were used primers *adh1*-F (CCAGCCTCATGGCCAAAG) 150nM; *adh1*-R (CCTTCTTGGCGGCTTATCTG) 150nM; probe *adh1* (6-FAM-CTTAGGGGCAGACTCCCGT-GTTCCCT-TAMRA) 50nM; for the GMO target system MON863 were used primers MON863-F (GTAGGATCGGAAAGCTTGGTAC) 150nM; MON863-R (TGTTACGGCCTAAATGCTGAACT) 150nM; probe MON863 (6-FAM-TGAACAC-CCATCCGAACAAGTAGGGTCA-TAMRA) 50nM. The volume was adjusted with water for PCR to 45 µl. Real-time PCRs were performed using 5 µl of template DNA.

Amplification was performed in the real-time PCR equipment ABI 7900 HT (Applied Biosystems, USA) and consisted of: 2 min at 50°C; 10 min at 95°C; 45 cycles with 15 s at 95°C, 1 min at 60°C. The fluorescent signal was measured at 60°C, which is step of annealing and extension.
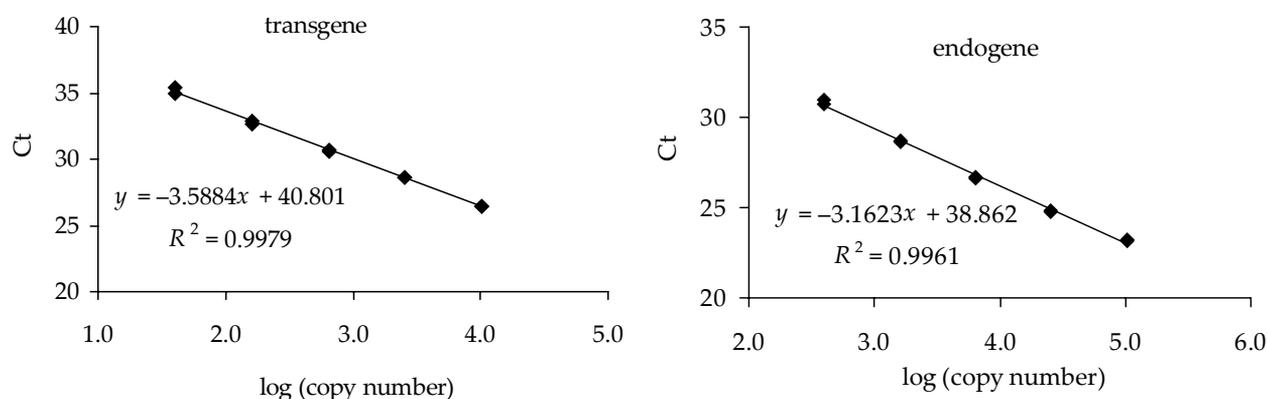

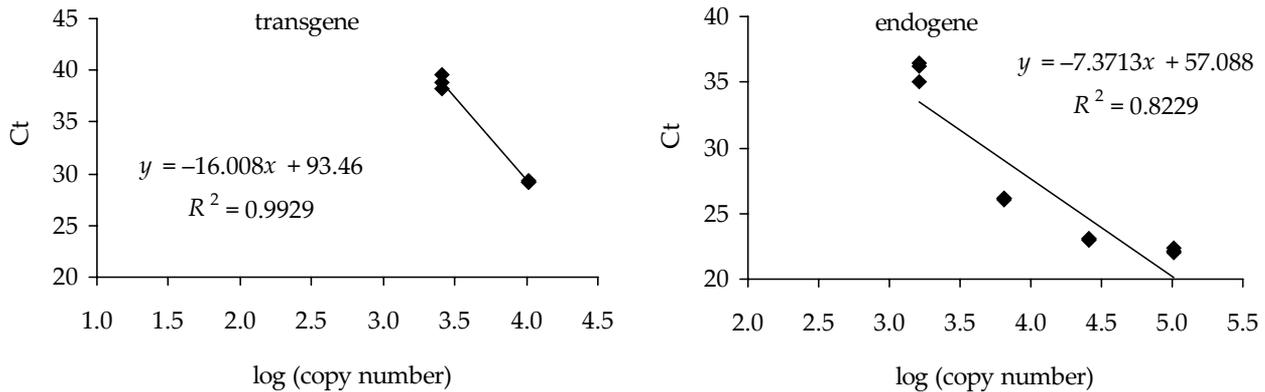Figure 6. Calibration curve with DNA solved by water

Figure 7. Calibration curve with using of colored microtubes

RESULTS AND DISCUSSION

As was mentioned above, efficiency of real-time PCRs have fundamental impact on DNA quantification. In case of poor efficiency, the calibration curve had a bad slope and the interpolation of sample quantity could be encubered by error.

We have found two various cases when the calibration curve was affected by at first look common events. Both cases were discovered during the quantification of transgenic maze line MON863.

In the first real-time PCR calibration curves of reference endogenous and target GMO real-time PCR system seem well for the first look (Figure 5). But the slopes of both calibration curves indicate, that the PCR efficiencies are unsufficient. PCR efficiencies for endogenous reference and transgenic systems were 76.7% and 67.7%, respectively.

Even new diluted calibration curve, new chemistry and another operator do not lead to better results. We used to dissolved DNA samples in 0.1 × TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5). At the Skirball Institute for Biomolecular Medi-

cine (2004) was found, that although TE buffer is often used as DNA solvent, the presence of excess EDTA can result in poor sequencing results. In their experimente was shown, that one molecule EDTA chelates two molecules of Mg. The final concentration of Mg^{2+} cations have influence on the process of PCR.

On the basis of this findings we decided to change DNA solvent and use water instead TE buffer. Real-time runs with calibration curve prepared by dilution with water are shown in Figure 6.

After that, PCR efficiencies for endogenous reference and transgenic systems equal 92.9% and 90.0%, respectively.

Results of other problematic real-time PCR run for MON863 quantification is shown in Figure 7. The efficiency of PCRs is so heterogenic, that it is impossible to create calibration curve.

There was recapitulated all work procedure. At first was excluded the possibility of faulty chemistry and the fail of operator. After then we focused on used plastics and tips. We found that the samples for calibration curve were prepared in colored
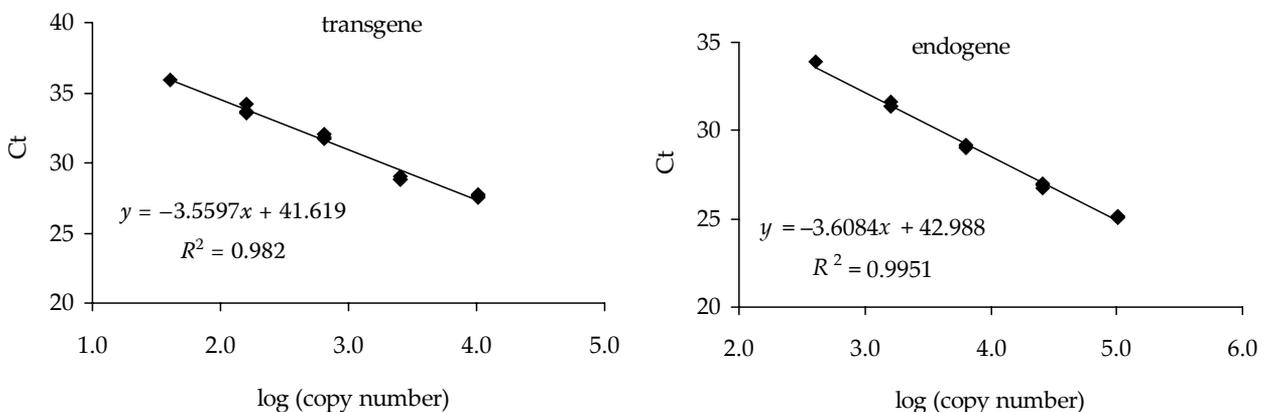

Figure 8. Calibration curve without using of colored microtubes

microtubes. These microtubes were supplied as a equivalent to non-colored tubes from a established provider. For coloration of microtubes are used several metals and there is a possibility, that in tubes some of their residues could occured. That could affect the PCR efficiency because of the inhibitory activity of metals on PCR.

We repeated the real-time run without using of any colored microtube and the calibration curve was satisfactory as is shown in Figure 8. The real-time PCR efficiency was calculated as 89.3% for the endogenous reference system and as 90.6% transgenic system, which we rated as satisfactory.

CONCLUSIONS

The process of DNA quantification using real-time PCR is affected by several factors, starting with sample type, method of DNA extraction, quality of extracted DNA, used chemistry and good optimalised reaction and ending with well prepared calibration curve. Even if it seems, that all processes and used chemistry are verified and reliable, problems could happened.

We described two accidents in real-time PCR quantification of GM maize MON863 and their solutions.

The first problem was connected with using of 0.1 × TE buffer as a solvent for DNA. It was shown, that perhaps EDTA presented in TE influences PCR effectivity and due that self DNA quantification. The problem was solved by change of DNA solvent.

The second problem was probably caused by using of colored microtubes, which are in molecular-biology laboratories commonly used. However, it was demonstrate, that perhaps residues of metals in these tubes can caused PCR inhibition.

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