Chapter 7

Analysis of Fungal Gene Expression by Real Time Quantitative PCR

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Abstract

The Real-Time quantitative PCR (qPCR) method has become central for the quantification of gene expression as well as other applications. The major advantages of qPCR are the utilization of small amount of template, high sensitivity and the ability to detect products during the reaction. After selecting qPCR among other options (northern blot, semi-quantitative PCR), one should consider several factors. The first and critical step in qPCR of fungi is the selection of an appropriate growth medium and RNA extraction method, which will avoid accumulation of inhibitors. In this chapter, we focus on detection of the accumulating product with the Syber Green dye, but other detection technologies, such as hybridization probes, might be considered as well. Accurate qPCR analysis with Syber Green depends mainly on optimal PCR reaction, and therefore it is important to design primers that will avoid formation of interfering structures. It is possible to use absolute quantification of the template in the sample, or to conduct a relative analysis, as described in this protocol. In the relative analysis method, expression of the gene of interest is compared with expression of a reference gene. According to our experience as well as according to the literature, it is recommended to use at least three reference genes in order to obtain reliable results.

Key words: qPCR, Real time, *Botrytis cinerea*, Fungi, Gene expression, Syber Green, Primer design, ddCT analysis, Relative analysis

1. Introduction

Analysis of gene expression is fundamental to biological sciences. Traditionally, northern analysis and RNAase protection assays have been used as the methods of choice, mainly in a qualitative or "semi quantitative" manner. These methods are very reliable but required large amounts of RNA, suffered from low throughput and the use of radio-labeled probes. Polymerase chain reaction (PCR) revolutionized experimental biology (1), and soon after

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its introduction it was coupled to analysis of gene expression (2). In reverse transcription (RT) PCR, cDNA is used to amplify specific products and compare their abundance with respect to reference genes to normalize the expression. This technology is robust but because of the exponential nature of the PCR reaction, end-point analysis of the amount of product, typically analyzed by gel electrophoresis, can introduce significant errors. Therefore, the development of technologies for "Real Time" detection of the amount of product that accumulates during the PCR reaction had a significant contribution to the ability to quantify the amount of nucleic acid in general, and the analysis of gene expression in particular. There are different methods, with various degree of specificity for the detection of PCR products by qPCR. The nonspecific dye Syber Green binds to double stranded DNA and is widely used, but specific hybridization probes such as the TaqMan® probes (Applied Biosystems, Carlsbad, USA) and LUX fluorescent hairpins (Invitrogen Corporation, Carlsbad, USA) can solve background problems. Central to qPCR is the question of absolute or relative analysis of gene expression. Absolute quantification is based on determination of the number of molecules in the sample, while relative quantification depends on comparison to normalize genes (3). In this chapter, we describe the methods we use for the analysis of gene expression in *Botrytis cinerea*. The methodology is based on Syber Green detection and relative gene expression analysis with three reference genes.

2. Materials

2.1. Preparation of the Fungus

1. Conidia of *B. cinerea* at a final concentration of 100/mL from 7 to 10 days old culture of the fungus grown on potato dextrose agar (PDA).
2. Four layers of sterile cheesecloth for separation of conidia from hyphae, using a spreader stick.
3. Harvest solution: 1 L of sterile double distilled water (sDDH$_2$O) 0.001% Triton X-100.

2.2. Growth Conditions

1. Gamborg B5: Prepare 800 mL of 3.16 g/L Gamborg B5 including vitamins (Duchefa Biochemie, Haarlem, the Netherlands), 8 mM $NaNO_3$, 3% (w/v) D (+)-glucose. Autoclaved for 18 min at 121°C, adjust pH to 5.7 with 1 M HCl and complete to 1 L with sDDH$_2$O.
2. Harvest of conidia: a ceramics funnel (55 mm) covered with a sterile 3 MM paper disk wetted by sterile water. Collect hyphae with a straight edge metal spoon and immediately freeze in liquid nitrogen.

2.3. Preparation of RNA and cDNA

1. Liquid nitrogen, mortar, and pestle. Clean with 70% ethanol between samples.

2.3.1. Protocol I

2. Denaturation buffer for RNA extraction: 25 mM NaCitrate, pH 7.0, 0.5% (w/v) 10% Sarkosyl, 4 M guanidine thiocyanate, 0.1 M β-mercaptoethanol. Dissolve in diethylpyrocarbonate (DEPC)-treated sDDH$_2$O to a final volume of 528 mL. Store at 4°C.
3. PCA stock solution: phenol saturated with Tris–HCl, pH 8.0, chloroform, iso-amyl alcohol (25:24:1, v/v/v). Store at 4°C in a light protected glass bottle.
4. Phenol, pH 6.7. Store at 4°C.
5. LiAcetate and LiCl stock solution: 2 M NaAcetate, pH 4.0, 6 M LiCl. Autoclaved and store at RT.
6. 2-propanol and 70% ethanol. Stored at –20°C.

2.3.2. Protocol II: Rapid Extraction

1. Use a Plant/fungi total RNA purification kit (Norgen Biotek, Thorold, Canada). The kit contains lysis solution, wash solution, elution buffer as well as mini spin columns, 2 mL collection tubes, 1.7 mL elution tubes.
2. Prepare 70 and 96% ethanol and β-mercaptoethanol to be opened in a chemical hood.

2.3.3. RNA Gel Electrophoresis

1. Agarose (e.g., SeaKem LE, Lonza, Minsk, Belarus).
2. 40 mM Tris–acetate.
3. TAE: 1 mM ethylenediaminetetraacetate (EDTA) buffer prepared as a 50× stock solution (4).
4. Formaldehyde loading dye (e.g., Ambion, Applied Biosystems, Austin, USA).
5. Safeview (Applied Biological Materials, Richmond, Canada) solution.

2.3.4. cDNA Preparation and Analysis

1. DNAse treatment: RQ1 RNase- free DNase kit (Promega, Madison, USA). Contains 10× reaction buffer, RQ1 RNase-free DNase, stop solution and DEPC-treated water.
2. cDNA synthesis: First strand cDNA synthesis kit (e.g., EZ-First strand cDNA synthesis kit, Biological industries, Bet Haemek, Israel). Contains oligo (dT) primers, random hexamer primers, 10× reaction mix, 100 mM dithiothreitol (DTT) and water.
3. Components for standard PCR amplification for cDNA analysis: specific primers, template (genomic DNA and cDNA), sterile water and components for PCR reaction (e.g., ReadyMix, ABgene, Epsom, United Kingdom). Contains 0.25 U/μl thermoprime plus DNA polymerase, 75 mM Tris–HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 mM each of the dNTP's, precipitant and red dye for electrophoresis.

4. Materials for nucleic acid analysis (see Subheading 2.3.3) with DNA size marker (e.g., GeneRuler 100 bp Plus DNA ladder, Fermentas, Burlington, Ontario).

2.4. Real Time qPCR

1. High quality cDNA.
2. Primers (see Table 1).
3. Real time qPCR reaction mix (e.g., 2×QPCR SYBR® Green Mix, ABgene, Epsom, United Kingdom). Contains SYBR® Green I dye, Thermo-Start® DNA Polymerase supplied in a proprietary reaction buffer).
4. Tubes: standard 0.2 mL tubes for qPCR or 0.1 mL tubes for qPCR (Corbett Life Science, Concorde, Australia).

Table 1
Type of primers described in different protocols

	Name	Accession	Sequence
B. cinerea	eif4A-12- F eif4A-12 R	BC1G_07971	TATTCATCGCATTGGTCGAA CAACATTCATTGGCATCTCG
	18 S rRNA-F 18 S rRNA-R	BC1G_06392.1	TTGGTTTCTAGGACCGCCG GGCAAATGCTTTCGCAGTAGT
	Actin-F Actin-R	BC1G_08198.1	CCCAATCAACCCAAAGTCCAACAG CAAATCACGACCAGCCATGTC
	β-tubulin-F β-tubulin-R	BC1G_00122.1	TTGGATTTGCTCCTTTGACCAG AGCGGCCATCATGTTCTTAGG
	HSP104-F HSP104-R	BC1G_15409.1	AAGGCTACGGAGAAGGATAAGTTG TGGTGGCGAGTTTGGGTTTG
N. crassa	L6_rRNA-F L6_rRNA-R	NCU02707.3	CAGAAATGGTACCCTGCTGAGG GCGGATGGTCTTGCGG
	ActF ActR	NCU04173.3	TCCATCATGAAGTGCGATGTC TTCTGCATACGGTCGGAGAGA
	Tub-2-F Tub-2-R	NCU04054.3	CCCGCGGTCTCAAGATGT CGCTTGAAGAGCTCCTGGAT
A. fumigatus	28 S rRNA-F 28 S rRNA-R	AB008401	GGCCCTTAAATAGCCCGGT TGAGCCGATAGTCCCCCTAA
	ActinRT1 ActinRT2	XM_746399.1	ATCGGCGGTGGTATCCTC TCTTCGTGCCATTCGTCTG
	α-tubulin-F α-tubulin-R	Afu2g14990	CGG CTAATGGAAAATACATGGC GTCTGGCCTTGAGAGATGCAA

Primer sequences are according to the following references. For the fungus *Neurospora crassa*: L6_ribosomal, (9); actin, (10), and tubulin (11). For the fungus *Aspergillus fumigatus*: 28 S rRNA, (12); actin and tubulin, (13)

3. Methods

The first stage in successful qPCR is the selection of growth conditions that will avoid subsequent interference in downstream RNA extraction such as accumulation of polysaccharides. That is why Gamborg B5, pH 5.7 medium was selected over other media for the growth of *B. cinerea* and the period of cultivation was set to 3–4 days at 22°C. The next important step is the selection of RNA extraction method. If the fungus of choice has a well established method for RNA extraction, it should be used, otherwise, selection of a reliable RNA extraction kit or procedure is a critical step and should be carefully evaluated. After considering whether qPCR is indeed the method which you should invest in (1), it is essential to figure out the scope of the work and the experimental setup. If, for example you need to analyze few genes of interest and over a long period of research, selection of hybridization probes (such as the TaqMan® probes), is likely to yield faster and more reliable results. If on the other hand, you are analyzing a gene family with high homology between member or a larger number of genes, Syber® Green technology is likely to be the method of choice. One should be aware that by choosing Syber® Green quantification, you commit yourself to rigorous optimization of the PCR reaction because if false products will be formed during the PCR reaction, they will yield erroneous results and nonoptimal efficiency will skew the mathematical models that allow correct quantification (3). There are many free or commerciality available softwares for primer design, some of which are listed below (see Note 6), but no available software is likely to solve all problems and in order to get high efficiency score, one should be prepared to design and order more than a single primer set. Next, it is important to select between relative and absolute quantification (3): in absolute quantification, one should have a plasmid containing the gene of interest (GOI) and accurate calculation of the number of plasmid molecules in the stock solution. This methodology also involves setting a calibration curve in each qPCR run, which often limits the number of experimental samples that can be accommodated in each run. Alternatively, the use of reference genes and relative quantification is sufficient, depending on the biological question. Reference genes must be carefully selected; stability of expression needs to be determined in each individual organism and experimental setup. It is strongly advised to use more than one reference gene and according to some reports, at least three reference genes are necessary. We have selected the widely used 18 S rRNA, actin, and β-tubulin genes as reference genes (Table 1), but many choices are available in the literature and the debate goes on (2, 5, 6). Last, and most importantly, the calculation method should be carefully considered.

Mathematical models suggest that the delta-delta CT method (7), which we used can yield reproducible and sufficient results. Others claim however that correction for efficiency and taking into account the presence of inhibitors in the cDNA synthesis step or the PCR steps is essential for accurate quantification by qPCR (8).

3.1. Growth Conditions

1. Harvest conidia of *B. cinerea* from a 7 to 10 days old PDA culture plate by adding 5 mL 0.001% Triton X-100 solution and scraping the spores gently with a spreader stick. Purify the conidia from hyphae by filtering it through four layers of cheesecloth. Centrifuge the conidia at 3,000 × *g* wash the pellet twice with distilled water and add 1 mL of sterile water. Count the number of conidia using a hemocytometer. Adjust to 10^7 conidia/mL.
2. Lyophilization of the hyphae was shown to be one of the most important factors in deterioration and inconsistency in the quality of RNA.
3. Prepare 60 mL of Gamborg B5 medium (see Note 1) in 250 mL Erlenmayer flasks and add conidia to a final density of 100 conidia/mL. Incubate the culture at 22°C on an orbital shaker at 120 rpm for 4 days.
4. Harvest the biomass by filtering the hyphae over a ceramic funnel with a 3 MM paper disk. Scrape hyphae from the filter paper, add to a 15 mL polypropylene tube, freeze in liquid nitrogen and store at −80°C until preparation of RNA (Note 2).

3.2. Preparation of RNA and cDNA

3.2.1. Protocol I

1. Prepare fine powder from 80 mg of dry biomass by grinding with a mortar and pestle in liquid nitrogen.
2. Immediately add the powder to 1 mL denaturation solution to prevent RNAse activity.
3. Add 1 mL PCA solution to the denaturation solution in a 2 mL tube, vortex and centrifuge at 11,000 × *g* at ambient temperature.
4. Remove the aqueous phase, add an equal volume of 2-propanol and incubated at −20°C for 1 h.
5. Centrifuge samples at 8,000 × *g* for 10 min at ambient temperature, discard the supernatant, and suspend pellet in 0.8 mL DEPC-treated water.
6. Add the following solutions sequentially, vortex samples after adding each component: 0.08 mL of 2 M $(NH_4)_2$-acetate, 0.8 mL of phenol, pH 6.7, and 0.16 mL chloroform:isoamyl alcohol (49:1, v/v).
7. Incubate samples on ice for 10 min and then centrifuge at 11,000 × *g* for 10 min. Remove the aqueous phase into a new 2 mL tube, add an equal amount of 6 M LiCl, incubate over night at −20°C.

8. Centrifuge samples at 11,000 × *g* for 10 min at 4°C and discard the supernatant. Add 1.5 mL of 70% EtOH, vortexe and incubated for 10 min at ambient temperature. Centrifuge at 8,000 × *g* for at least 5 min at 4°C and discard the supernatant.
9. Dry pellets in a laminar hood for at least 30 min, dissolve in 100 µL of DEPC-treated water, and store at –80°C until further analysis.

3.2.2. Protocol II

1. Prepare fine powder from 50 mg of dry biomass by grinding with a mortar and pestle in liquid nitrogen.
2. Process samples according to manufacturer's instructions (Norgen Biotek).
3. Determine RNA quantity and quality (1.5 µL samples from protocol I or II) using a NanoDrop 1000 instrument (see Note 3).
4. RNA stability assay: divide a 10 µl sample into two tubes: incubate one tube at 37°C and the other tube on ice for 30 min.
5. Add 2.3 µl of formaldehyde loading dye to each sample and incubate for 10 min at 65°C. Place on ice until analysis.
6. Gel electrophoresis: prepare 1% of agarose gel with 0.5×TAE and 5 µl/100 mL SafeView dye. Load samples on the gel separate by electrophorsis. Visualize RNA for the appearance of 18 S and 28 S rRNA bands. Make sure that the bands are sharp and that there are no signs of degradation before or after the stability assay.
7. Removal of DNA traces: incubate 1 µg of RNA with RQ1 DNAse according to instructions (RQ1 kit).
8. Preparation of cDNA: Convert the DNA-free RNA samples into cDNA using a mixture of olido (dT) and random hexamer primers, according to the manufacturer's instructions (see Notes 4 and 5).
9. Preliminary PCR analysis of the cDNA: this step is carried out in order to verify amplification of specific products from the obtained cDNA samples. Use 2 µL of cDNA as template and 12.5 µM of the specific primers for a PCR. Add 10 µL of reaction mixture and sDDH$_2$O to a final volume of 20 µL. Use 5–10 ng genomic DNA as a positive control and "no template control" as a negative control. Analyze PCR products on a 2% agarose gel (see Subheading 2.3.3).

3.3. Real Time qPCR

1. Primer design: Set amplicon size for the GOI between 100 and 200 bp (see Note 6). When multiple genes are analyzed, prefer a uniform melting temperature (Tm) for the different primer sets in order to be able to compare the expression of different genes in the same run. Otherwise, select primer sets by optimal parameters. Free software for primer design are Primer 3

(http://frodo.wi.mit.edu/), IDT Scitools (http://www.idtdna.com/SciTools/SciTools.aspx), or other commercially available software (see Note 7). Parameters to be considered during primer design are: the free energy for the formation of hairpins, self dimerization or heterodimer formation should be below $\Delta G \leq -9$ kcal/mol. Verify that the amplicon will not have problematic secondary structures with values of $\Delta G \leq -9$ kcal/mol using the DINAMelt Server (see Note 8).

2. Selection of reference genes: it is recommended to use three reference genes for accurate quantification of the GOI. 18 S rRNA, actin, and tubulin are commonly used reference genes for many organisms and examples for primers for *B. cinerea*, *Neurospora crassa* and *Aspergillus fumigates* are presented (Table 1) (see Note 9).
3. Preparation of the qPCR reaction: qPCR is sensitive to experimental errors. Pipettors should be calibrated routinely and accurate pipetting should be practiced. Use pipettors and tips of 10 μL volumes. The tubes should be arranged on a cooled tray. Calculate the amount of reaction mixture to be used for each primer set and add to it water and primers. Wet the pipette tips before dispensing the aliquots. Dispense the mixture to each tube and add template by applying the droplet to the wall of the tube without touching it with the pipette tip.
4. Operation of the qPCR instrument: The instructions are from the RotorGene 6000 qPCR machine (Corbett Life Science, Concorde, Australia) and should be modified according to the specific instrument or reaction mixture properties as recommended (see Note 10). Load samples into the machine and select the appropriate program (e.g., Syber Green). Define number of samples and the type of tubes. Set the acquisition point of the fluorescence signal to 74°C.
5. Reaction conditions: preactivation of the enzyme for 15 min at 95°C, strand denaturation for 10 s at 95°C, annealing of primers for 20 s at the optimal melting temperature (Tm), elongation for 20 s at 72°C, the melting stage (72–99°C) at the end of the of the program (after 40 cycles) for calculation of the Tm of the PCR product. Define gain value to a range of 0.5–2.0 (for RotorGene 3000). Sample name and type can be defined during operation or later.
6. Analysis of the quality of the results: determine the reproducibility of the triplicates. If triplicates deviate from the average by more than one cycle it indicates pipetting error and one should consider repeating the qPCR (see Subheading 3.3). The next stage is checking the quality of the product by melt curve analysis. The melt curve should appear as a single and narrow peak without shoulders. If another peak appears before the real peak, it is possible to set another acquisition

temperature (B point) between the curves, for the next run. Consider that this situation will result in nonoptimal amplification of the specific product because the nonspecific product will reduce the efficiency of the reaction.

7. Efficiency of the reaction: in the first analysis of the primers and template, run a dilution curve with genomic DNA at initial concentration of 5–10 ng, consisting of four fivefold dilutions to check the efficiency of the reaction. The optimal slope of the curve should be 3.322, and the corresponding M-value should be 1.00 ± 0.02. In case of deviation from these values, consider optimizing primer concentration, the amount of template, the annealing temperature, or ordering a new primer set.
8. Data analysis: The threshold which determines at what fluorescence level and cycle time (CT), the data will be analyzed can be set automatically or manually. The automatic threshold setup determines the point on the curve of all samples, in which deviation from technical replication (triplicates) is minimal. In a manual threshold setup, the threshold is set to a point that according to experimental experience gives reliable results (see Note 11). The qPCR data can be analyzed by the Rotor-Gene 6 software, which is part of the qPCR machine or exported to excel (Table 2). The formula which should be copied into the ddCT column is:

$$\mathrm{ddCT} = 2\text{^} - ((\mathrm{GOI\ CT_{tn}} - \mathrm{REF\ CT_{tn}}) - (\$\mathrm{GOI\ CT_{t0}} - \$\mathrm{REF\ CT_{t0}})).$$

9. Comparative analysis gene expression: data from qPCR analysis of different reference genes can be compiled in excel and reproduced as figures (see Table 3).

4. Notes

1. Acidic medium with low nitrogen content is chosen in order to minimize accumulation of polysaccharides in the growth medium and to allow efficient separation of hyphae from the medium, resulting in better RNA quality.
2. Lyophilization of the hyphae was shown to be one of the most important factors in deterioration and inconsistency in the quality of RNA.
3. Pure RNA should yield a ratio of A_{260}/A_{280} above 1.8 and the ratio of A_{260}/A_{230} should be approximately two.
4. It is recommended to use a combination of poly (dT) and random hexamer primers for first strand cDNA synthesis in

Table 2
Example of analysis of a gene of interest (GOI) and a reference gene (REF) in calculation of relative expression by the ddCT method. The specific example shows the expression of HSP104 and the 18 S rRNA after transition of *B. cinerea* strain B05.10 from 22°C to 0°C at different time points (0, 1, 4, 10, and 24 h)

	CT values		Average CT		
Sample name	GOI	REF	GOI	REF	ddCT
0	28.89	22.92			
0	28.25	24.25	27.99	23.39	1.00
0	26.84	23.00			
1	25.47	18.97			
1	26.07	21.53	25.89	20.25	0.49
1	26.14	20.24			
4	24.59	20.02			
4	24.84	21.36	24.57	20.90	1.92
4	24.27	21.33			
10	23.67	23.86			
10	23.73	22.65	23.58	22.28	9.87
10	23.34	20.33			
24	23.65	22.11			
24	23.01	21.22	23.31	21.47	6.79
24	23.26	21.07			

Table 3
Example of three genes analyzed by qPCR with three different reference genes at different time points after the beginning of the treatment. Relative expression of each of the three genes toward each of the reference genes shows a similar trend, but note that differences in expression levels may be significant

Time	Induction and repression			High induction			Repression		
h	rRNA	Actin	Tubulin	rRNA	Actin	Tubulin	rRNA	Actin	Tubulin
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1	14.7	31.5	3.5	384.1	1,236.9	26.7	0.1	0.1	0.1
4	2.6	3.0	1.1	5,199.8	2,460.9	532.9	0.1	0.1	0.1
10	0.6	0.1	0.1	2,093.8	536.9	436.0	0.3	0.2	0.1
24	0.1	0.4	0.1	76.6	1,755.4	326.1	0.1	0.0	0.0

order to increase the yield and types of RNA molecules that will be used as templates.

5. The freshly made cDNA should be divided into aliquots and stored at −20°C until analysis.
6. Amplicon size can be larger than 200 bp if it is required because of homology and primer specificity considerations or in order to facilitate the design of better primers. For further details, consult http://www.genequantification.info/.
7. Other software packages are available for primer design, e.g., *Vector NTI* (Invitrogen), *Primer Express* (Applied Biosystems), and *CLC DNA workbench* (CLC bio USA, Massachusetts, USA).
8. The DINAMelt Server application enables to check the secondary structure of the amplicon (http://www.bioinfo.rpi.edu/applications/hybrid/twostate-fold.php).
9. Expression of reference genes should be stable and correct for experimental errors. Test the stability of expression among treatments and replications and avoid the use of reference genes with inconsistent expression pattern.
10. There are many commercially available reaction mixtures. Always make sure to follow the recommended time for preheating and activation of the enzyme. For example, the ABgene Syber Green I mixture requires 15 min at 95°C, while the Takara SYBR®Premix ExTaq™ (Company details) does not require a preactivation step.
11. Threshold acquisition of the experiment can be fixed automatically or manually. In cases of one experiment analysis, the instrument automated threshold is enough, but in cases of multiple experiment analyses, we use a manually fixed threshold value of 0.05 fitted to the Rotor Gene 3,000 machine.

References

1. Kubista M, Andrade J, Bengtsson M, Forootan A, Jona'k J et al (2006) The real-time polymerase chain reaction. Mol Aspects Med 27:95–125
2. VanGuilder H, Vrana K, Freeman W (2008) Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 44: 619–626
3. Chini V, Foka A, Dimitracopoulos G, Spiliopoulou I (2007) Absolute and relative real-time PCR in the quantification of *tst* gene expression among methicillin-resistant *Staphylococcus aureus*: evaluation by two mathematical models. Lett Appl Microbiol 45:479–484
4. Sambrook F, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
5. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:1–12
6. Liu ZL, Slininger PJ (2007) Universal external RNA controls for microbial gene expression analysis using microarray and qRT-PCR. J Microbiol Methods 68:486–496
7. Schmittgen TD, Livak KJ (2001) Analysis of relative gene expression data using

real-time quantitative PCR and the 2(-delta delta C(T)) Method. Methods 25:402–408

8. Pfaffl M (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45
9. Froehlich A, Noh B, Vierstra R, Loros J, Dunlap J (2005) Genetic and molecular analysis of phytochromes from the filamentous fungus *Neurospora crassa*. Eukaryotic Cell 4:2140–2152
10. Görl M, Merrow M, Huttner B, Johnson J, Roenneberg T et al (2001) A PEST-like element in FREQUENCY determines the length of the circadian period in *Neurospora crassa*. EMBO J 20:7074–7084
11. Navarro-Sampedro L, Yanofsky C, Corrochano L (2008) A genetic selection for *Neurospora crassa* mutants altered in their light regulation of transcription. Genetics 178:171–183
12. Francesconi A, Kasai M, Harrington S, Beveridge M, Petraitiene R et al (2008) Automated and manual methods of DNA extraction for *Aspergillus fumigatus* and *Rhizopus oryzae* analyzed by quantitative real-time PCR. J Clin Microbiol 46:1978–1984
13. Semighini C, Marins M, Goldman M, Goldman G (2002) Quantitative analysis of the relative transcript levels of ABC transporter Atr genes in *Aspergillus nidulans* by real-time reverse transcription-PCR assay. Appl Environ Microbiol 68:1351–1357