Development of a protocol for single worm quantitative RT-PCR

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Summary

One of many advantages to use *C.elegans* as an experimental model is the availability of thousands of genetic mutants. They are a quite precious source for the characterization of the function of specific genes. Of particular interest is the identification of target genes of regulatory proteins. However, many of these genes are lethal or show maternal lethality, thus limiting the isolation of large amount of mRNA for high throughput assays, e.g. microarray analysis. Here we describe the use of different RNA isolation methods in combination with both semi-quantitative RT-PCR and real time quantitative RT-PCR to analyze the putative target genes of proteins of interest.

Introduction

1. Comparative quantitation by using Semi-quantitative RT-PCR

The transcription levels of target genes of proteins of interest can change due to different genetic backgrounds, at different environments or different drug treatment. The relative abundance of a transcript in different samples can be by semi-quantitative or relative RT (reverse transcription)-PCR. Typically, the signal from the RT-PCR product is normalized to the signal from an internal control in all samples and co-amplified at the same time as the target. Generally, the use of a house keeping gene as internal control (IC) improves the reliability of the quantitative result of the PCR reaction of putative target of interesting proteins after reverse transcription. Transcripts of housekeeping genes are frequently chosen as an internal control because they are abundantly expressed at relatively constant rates in different samples. Because of their high abundance,

relatively low internal control primer concentrations be helpful to minimize the preferential amplification of the internal controls. In this multiplex PCR, several PCR products from different primer pairs are amplified simultaneously in a single tube. The length of PCR product is generally < 500bp with a size difference of approximately 150 bp-200 bp between them to be sure that the products can be easily distinguished on an agarose gel. If possible, the designed primers or probes should bridge two exons so that they cannot amplify any contamining genomic DNA (http://www.QIAGEN.com). Furthermore, amplification efficiency should be biased minimally by size differences. The PCR amplification should end in the exponential phase for both the internal control reference and the PCR product of interest (PI). Thus, the quantity of PCR products should still increase in an exponential fashion along the last amplification cycles. Since this assay is linear only over a very short range, rare targets will possibly be below the limit of detection, while abundant targets will be past the xponential phase. In order to extend the linear range, duplicate reactions may be performed for a greater or smaller number of cycles or on serial dilutions of the sample. Once optimal conditions are settled, each reaction can be tested by checking that the PI/IC ratio is constant for PCR performed by using serial dilution of the sample. In summary, it's critical to determine the RT-PCR conditions that are suitable for both amplicons by varying the amount of template, the number of cycles, the annealing temperature, and the extension time. Finally, the normalized data from different samples can be compared and are informative for the characterization of particular target gene of interesting protein.

2.Comparative quantitation by using Real time RT-PCR

Quantitative real-time RT-PCR is based on detection of a fluorescent signal produced proportionally during the amplification of a PCR product. This allows to visualize directly the exponential part of the PCR reaction.

Principle of quantitative real time PCR

Quantitative real-time PCR is based on the detection of a fluorescent signal produced during the amplification of a PCR product. This allows to visualize directly the exponential part of the PCR reaction using TaqMan or SYBRGreen (http://www.appliedbiosystems.com/molecularbiology/about/pcr/sds/)

2.1 Detection system for the TaqMan and SYBRGreen RT-PCR (see Figure 1)

In brief, the detection system for the TaqMan and SYBRGreen RT-PCR consists of a thermal cycler connected to a laser and charge coupled device (CCD) optics system.

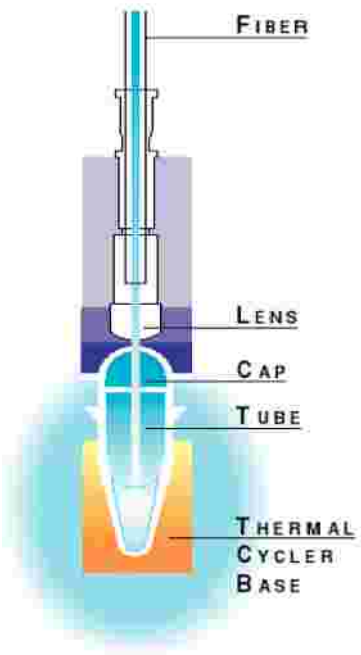


Figure 1 Scheme of the detection system

An optical fiber inserted through a lens is positioned over each well, and laser ight is directed through the fiber to excite the fluorochrome the PCR solution. Emissions are sent through the fiber to the CCD camera, where they are analyzed by the software's algorithms. Collected data are subsequently sent to the computer.

The software calculates the threshold cycle (Ct) for each reaction with which there is a linear relationship to the amount of starting DNA. Ct the threshold cycle i.e. the cycle number at which the reporter dye emission intensities rises above background noise (Figures 2-3) The Ct is determined at the most exponential phase the reaction and is more reliable than end-point measurements of accumulated PCR products used by

traditional PCR methods. The Ct is inversely proportional to the copy number of the target template; the higher the template concentration, the lower the threshold cycle measured. There are many advantages to quantifying gene sequences using this technology, foremost being precision and sensitivity. This precision exists because the quantification of the gene sequence is determined by the Ct which is calculated during the exponential phase of the reaction (http://www.QIAGEN.com).

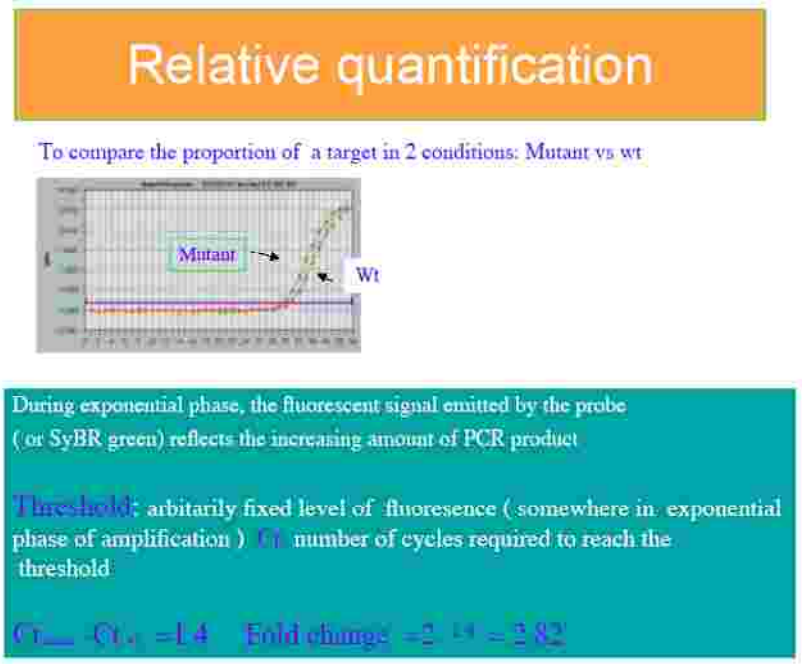


Figure 2 Principle of relative quantification

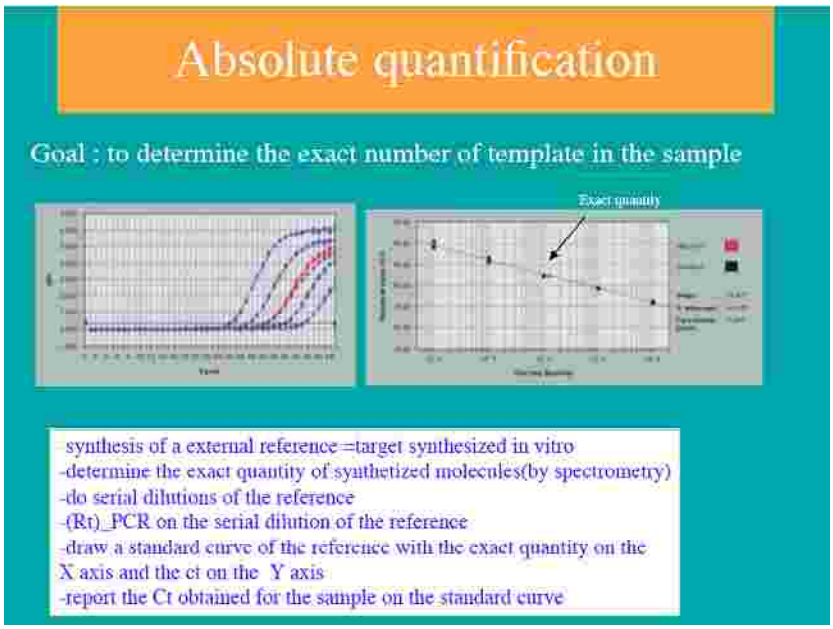


Figure 3 Principle of absolute quantification

These windows show the amount of fluorescence obtained in each amplification cycle for each reaction. The threshold cycle (Ct) is shown by the darker horizontal line.

2.2 Real-time Reporters

SYBR® Green, TaqMan®, and Molecular Beacons:

All real-time PCR systems rely upon the detection and quantification of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction.

SYBR® Green

In the simplest and most economical format, that reporter is the double-strand DNA-specific dye SYBR® Green (Molecular Probes). SYBR Green binds double-stranded DNA, and upon excitation emits light (Figure 4). Thus, as a PCR product accumulates, fluorescence increases. The advantages of SYBR Green are that it's inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction

products, which results in an overestimation of the target concentration. For single PCR product reactions with well designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles.

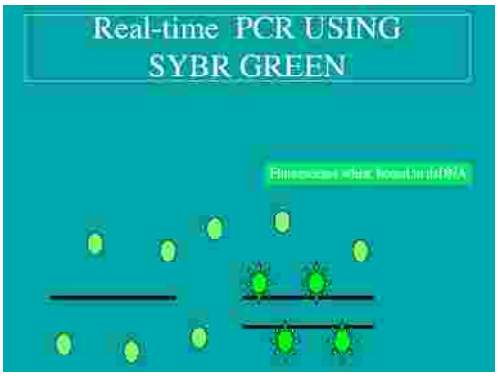


Figure 4 SYBR® Green binds double–stranded DNA

At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis. After annealing of the primers, a few dye molecules can bind to the double stranded DNA. DNA binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation. During elongation, more and more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuo an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.

TaqMan® and molecular beacons

Both TaqMan and molecular beacons are hybridization probes relying on fluorescence resonance energy transfer (FRET) for quantification. The probe is designed to anneal to the target sequence between the traditional forward and reverse primers (Figure 6). TaqMan Probes are oligonucleotides that contain a reporter fluorochrome (usually 6-carboxyfluorescein [6-FAM]) and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine [TAMRA]) added at any T position or at the 3' end. When irradiated, the

excited fluorochrome transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent substrate. probes are designed to hybridize to an internal region of a PCR product and to have a higher Tm than the primers, but during the extension phase, the probe must be 100% hybridized for success of the assay. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and FRET longer occurs. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle. Molecular beacons also contain fluorescent and quenching dyes, but FRET only occurs when the quenching dye is directly adjacent to the fluorescent dye. Molecular beacons are designed to ado a hairpin structure while free in solution, bringing the fluorescent dye and quencher in close proximity. When a molecular beacon hybridizes to a target, the fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation. Unlike TaqMan probes, molecular beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal measurement. TaqMan probes and molecular beacons allow multiple DNA species to be measured in the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to the different probes. Multiplex PCR allows internal controls to be co-amplified. These hybridization probes afford a level of discrimination to obtain with SYBR Green, since they will only hybridize to true targets in a PCR and not to primer-dimers or other spurious products.

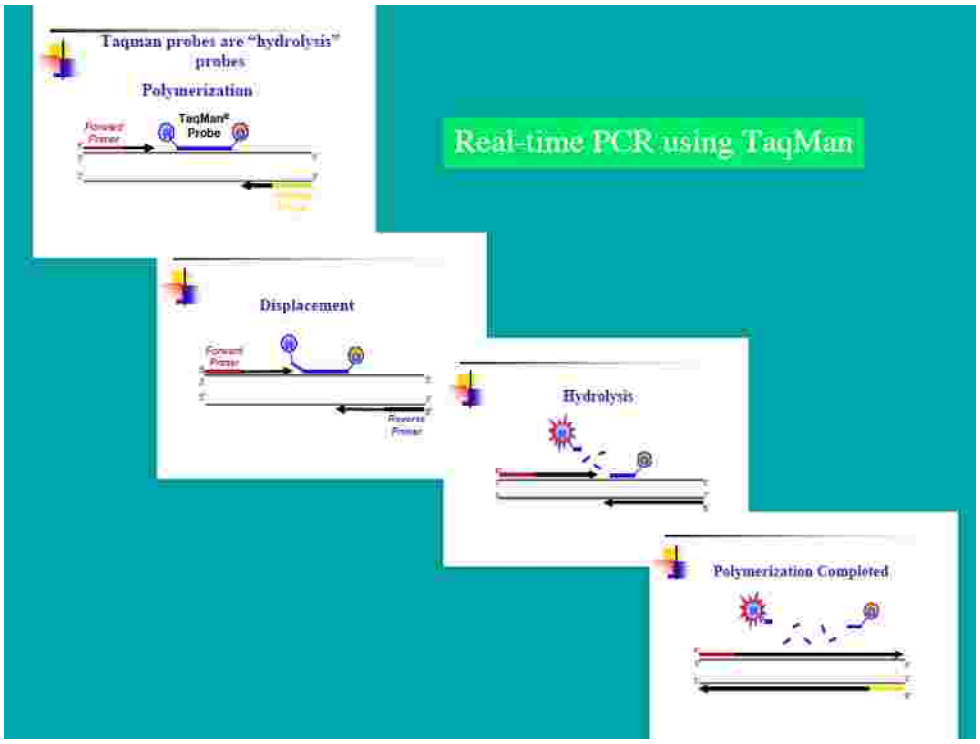


Figure 5 Fluorogenic 5' nuclease chemistry

1. Forward and reverse primers are extended with Taq merase as in a traditional PCR reaction. A probe with two fluorescent dyes attached, a reported (R) and a quencher (Q), anneals to the gene sequence between the two primers.
2. When both dyes are attached to the probe, reporter dye emission is quenched. As the polymerase extends the primer, the probe is displaced.
3. An inherent nuclease activity in the polymerase cleaves the reporter dye from the probe.
4. Once separated from the quencher, the reporter dye emits its characteristic fluorescence.

Melting curve analysis can show specificity of reactions

The peak for higher temperature shows the specificity. The non-specific primer dimer can be shown by the peak corresponding to the lower temperature (Figure 6).The different lines represent different samples with the same primers.

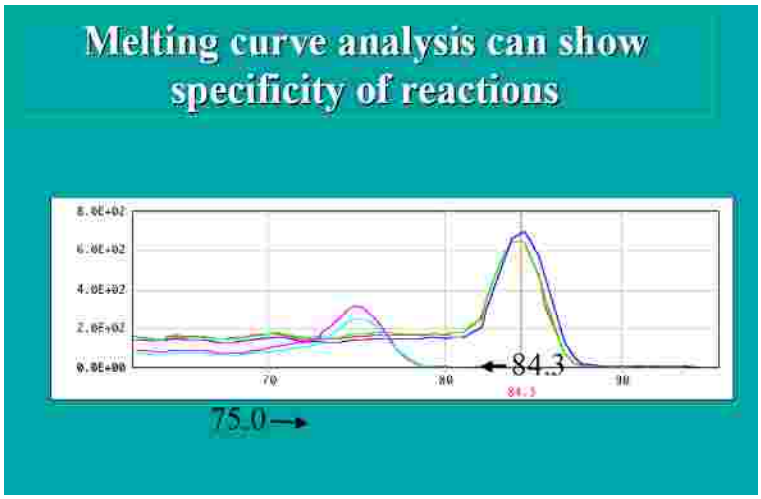


Figure 6 Melting curve analysis

2.3 Instruments

Real-time PCR requires an instrumentation platform that consists of a thermal cycler, computer, optics for fluorescence excitation and emission collection, and data acquisition and analysis software. These machines, available from several manufacturers, differ in sample capacity (some are 72-well standard format, others process fewer samples or require specialized glass capillary tubes), method of (some use lasers, others broad spectrum light sources with tunable filters), and overall sensitivity. There are also platform-specific differences in how the software processes data. SYBR is a registered trademark of Molecular Probes. TaqMan is a registered of Roche Molecular Systems. ABI PRISM is a registered trademark of PE Applied Biosystems. For cost - effective multiplex real –time PCR, Plexor technology (Promega) could be applied. Plexor™ chemistry in multiplex reactions, one of the primers for each target must have a different fluorescent label. The types and number of fluorescent labels that can be used depend upon the detection capabilities of the real-time instrument used. Besides, the Universal ProbeLibrary from Roche (https://www.roche-applied-science.com) for *C. elegans* could create a functional, highly specific, optimized assay that can be performed overnight using pre-validated universal probes.

Material and method

Development of single worm real time quantitative RT-PCR (step by step protocol)

The availability of thousands of genetic mutants is a quite precious source for the characterization of the function of specific genes. However, many of these genes are lethal or show maternal lethality in *C.elegans*, thus limiting the isolation of large amount of mRNA for high throughout assays, e.g. microarray analysis. Here we describe the use of different RNA isolation methods in combination with real time quantitative RT-PCR to analyze the putative target genes of LET-418 protein on a few worms or single worm.

Preparation of staged worms for real time quantitative RT-PCR

To characterize the function of a protein of interest, the transcriptional regulation of its downstream target genes generally needs to be studied. To assay the target genes of LET-418, we synchronized the worms (both test and reference samples) for several generations using the following treatment: Pick young and let them lay eggs. The adults are removed. Let the eggs hatch and develop to ung adults. Repeat this synchronization cycle several times. In general, we keep these worms synchronized continuously, since lack of synchronization leads to more isogenic or epigenetic variations among the worms. The changes in gene expression in *C.elegans* can result from differences in developmental time, age, environmental factors, or other experimental manipulations. Particular attention must herefore be given to the experimental design to ensure that the only difference between the samples is the one that the experimenter intends.

We synchronized worms at least twice for assaying the target genes of LET-418 by using the following precedure:

1. Pick 30-40 L3 staged wildtype (WT) and *let-418(ts)* worms;
2. After about one day at 15°C, pick 20-30 identically staged L4 or young adult worms;
3. After 5-6 hrs, most adults begin to lay eggs; Pick 20-30 egg-laying adults and transfer them on new plates for 30 min until they have produced about 150 eggs;

4. The eggs are grown to the young adult stage;
5. Repeat step1-3 until the worms reach the late L1 stage. The WT and *let-418(ts)* worms were then transferred to 25°C. After about one day, we picked 110 early L4 lavae and transferred them to lysis buffer.

Trizol based isolation of total RNA for single worm quantitative RT-PCR

By using the Trizol reagents (Chomczynski P, 1993), we can easily isolate total RNA from single or a few worms. It is powerful but care in pipeting should be taken due to the many steps. Other procedures could be basically applied, such as column chromatography based total RNA isolation or single worm in one tube RT-PCR. For low abundance transcripts, we found that the Trizol–based total RNA isolation works better than others in our hands (unpublished).

1. Put 3-30 worms into 40 µl M9 (1.7 ml eppendorf tube).
2. Add 360 µl of fresh Trizol reagent . Vortex gently, 3 x freeze – thaw.
3. Invert tube to solubilize and lyse worms. Incubate at RT for 5 min.
4. Spin at14 K at 4 ^{o}C for 10 min in a microfuge to remove insoluble material.
5. Remove the supernatant liquid to a fresh RNase free eppendorf tube, add 80 µl CHCl3 (chloroform) to each tube.
6. Invert /vortex for 15 sec. Incubate at RT for 2-3 min.
7. Spin 15 min at 14k at 4 ^{o}C to separate phases.
8. Remove upper aqueous phase and transfer into a fresh tube. Add 200 µl isopropanol and mix.
9. Incubate 10 min at RT to precipitate RNA. Recover RNA by spinning at 14 K for 10 min at 4 ^{o}C.
10. Carefully remove aqueous solution away from the pellet.
11. Wash pellet with 40 µl 75% EtOH in DEPC (diethyl pyrocarbonate) treated $H_2$0. Vortex briefly.
12. Spin at 10K for 5 min at 4 ^{o}C.
13. Remove supernatant and air dry pellets for 5-10 min.

14. Dissolve pellets in 25-50 μl DEPC H_20. To help dissolve, heat at 60°C for 10 min.
15. Take 1 μl to measure OD value to evaluate the concentration and purity.

The removal of genomic DNA

The isolated total RNA is briefly incubated in gDNA wipeout buffer (QIAGEN) at 42°C for 5 minutes to effectively eliminate contaminating genomic DNA.

In general, the classical DNase treatment can work for the removal of genomic DNA. Specifically we add a DNase treatment in chromatography column based total RNA isolation in our hands (not shown). However, the treatment using gDNA wipeout buffer is simple and fast.

The reverse transcription of total RNA

Add genomic DNA-removed template RNA to each tube containing reverse–transcription master mix (QIAGEN). Mix thoroughly by vortexing for no more than 5s. Centrifuge briefly to collect residual liquid from the sides of the tubes. Incubate for 30 min at 42°C. Then incubate for 3 min at 95°C to inactivate Quantiscript Reverse Transciptase. Add an aliquot of each finished reverse-transcription reaction to real-time PCR mix. Store reverse-transcription reactions on ice and proceed directly with real-time PCR, or for long–term storage, store reverse-transcription reactions at -20°C.

Running the real time quantitative PCR

We used the Rotor-gene 3000 real time PCR machine (Corbett Inc.) to perform the real time quantitative PCR according to the manufactor's recommendation.

Results and discussion

Application of single worm agarose gel semi-quantitative RT-PCR

The Down syndrome (DS), caused by trisomy 21, is the st common genetic cause of mental retardation, with an incidence of approximately 1 in 700 live births. There are numerous other phenotypes associated with DS, such as mental retardation and facial characteristics, heart disease, early onset Alzheimer and so on. In collaboration

with Dr. Guipponi M group, *C.elegans* was used for to study the functional/phenotypical effects of inactivation and overexpression of *C21orf80*. Here, we report on initial characterization of the *C.elegans* ortholog of *C21orf80*, a potential new protein O-fucosyltransferase that maps to Hsa21.To gain insight the biological role of *C21orf80* and its potential role in DS, *C. elegans* ortholog, *pad-2*, overexpression experiments have been done.

Analysis of *pad-2* overexpression was performed by semi-quantitative RT-PCR.
Total RNA was isolated from 30 wild-type, *hs::pad-2* transgenic, or K10G9 transgenic L4-stage larvae. *pad-2* and *ama-1* mRNAs were amplified by the Qiagen one-step RT-PCR system using the following primers 5', AAATTCGAGAAACGGAGCTG-' 3 and 5'-TACTCCTTCTCGCCTTCCAG-'3 for *pad-2*; 5'-CAGTGGCTCATGTCGAGT-'3 and 5'CGACCTTCTTTCCATCAT-'3 for *ama-1*. The PCR products were separated on an agarose gel and transgene expression levels were estimated by measuring the respective intensity of the ethidium bromide bands using the Multi-Analyst software (Bio-Rad). We performed this experiment twice and basically it gave the same results (Figure7, and Menzel O et al., 2004)

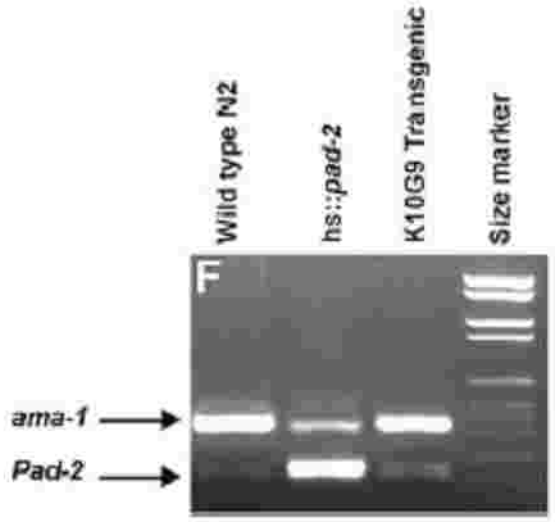


Figure 7 Agarose gel electrophoresis of *pad-2* mRNA. *ama-1* mRNA was used as internal control for semi-quantitative RT-PCR

Lane 1: Wild type animals;

Lane 2: Animal transgenic for *hs::pad-2* after heat shock (33°C, 30 minutes) ;

Lane 3: Animals transgenic for the cosmid K10G9;

Lane 4: Molecular weight marker (pBR328/*Hind*III +pBR328/*Bgl* I)

Levels of transgene expression were estimated by semiquantitative PCR that showed increased dose of *pad- 2* mRNA in both transgenic strains (*hs::pad-2* and cosmid K10G9) compared to wild-type animals. This was confirmed by the corresponding phenotype characterized by embryonic lethality associated with various morphological defects (Menzel O et al., 2004).

Application of semi-quantitative and real-time RT-PCRs of the *lag-2* mRNA levels

A recent work on the *hda-1/gon-10* mutations result in up-regulation of *lag-2* mRNA levels (Dufourcq et al., 2002). Since LET-418 and HDA-1 are members of the same nucleosome-remodelling complex, we expected that LET-418 negatively controls *lag-2* expression. Furthermore, we observed ectopic expression of *lag-2::gfp* in *let-418 (RNAi)* animals. Semi-quantitative and real-time PCR experiments were performed to test this hypothesis and we found that *lag-2 mRNA* levels indeed increase in *let-418(lf)* and *let-418(lf);chd-3(lf)* animals (Figure 8) but not *chd-3(lf)* animals (not shown).

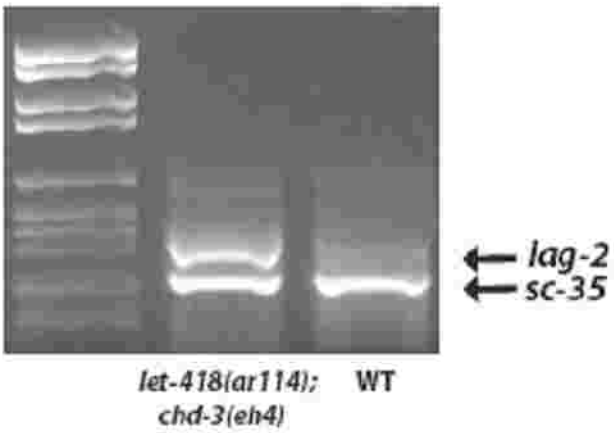
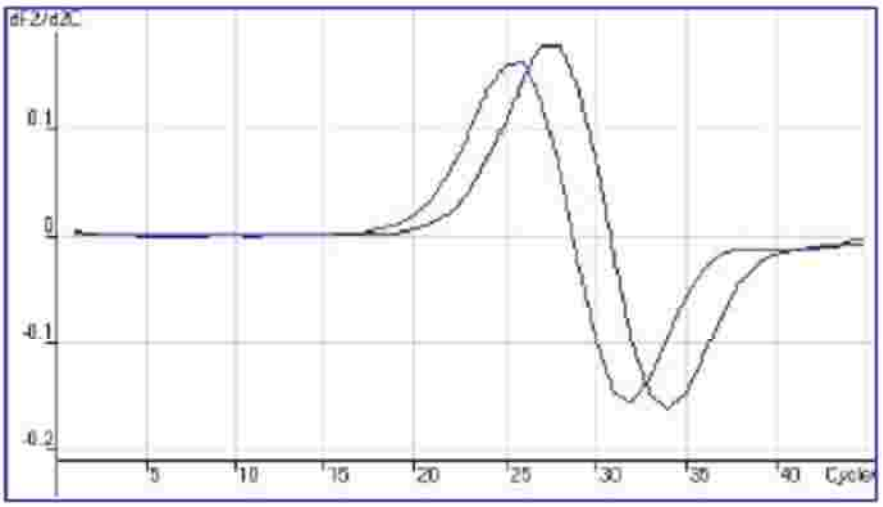


Figure 8a Semi-quantitative RT-PCRs of the *lag-2* mRNA levels

Gel analysis of the *lag-2* RT-PCR amplification products from *let-418(ar114);chd-3(eh4)* and wild-type L4 stage animals. A significantly higher amount of *lag-2* transcript is found in *let-418(ar114);chd-3(eh4)* than in wild-type animals. For comparison and normalization, the amplification of the gene *sc-35* (splicing factor) was used.



Colour	Genotype	Takeoff	Amplification
	wild-type	23. 4	1 .7 3
	let-418(ar114)	21. 5	1 .6 9

Figure 8b Real-time RT-PCRs of the *lag-2 mRNA* levels
Quantification of the *lag-2* mRNA levels by real time PCR. *lag-2* amplification was significantly higher in *let-418(ar114)* than in wild-type animals: the fold of increasement for *let-418(ar114)* against WT: $2^{\Delta Ct} = 2^{23.4--21.5} = 3.73$; repeated twice.

The increase of *lag-2 mRNA* levels in *let-418(lf)* and *let-418(lf);chd-3(lf)* animals could be proven by either agarose-gel semi-quantitive RT-PCR or real time RT-PCR method.

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