Rapid Development of Real-Time RT-PCR Assays Using Universal ProbeLibrary: Applications for Dissecting Signaling Pathways by RNA Interference

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Introduction

To characterize gene function on a genome-wide scale, reliable and rapid methods to quantify gene expression levels are required. A key advance in functional genomics has been the use of RNA interference (RNAi), which allows the silencing of genes in metazoans through the introduction of short, double-stranded (ds) RNAs homologous to endogenous target mRNAs [1]. RNAi has been successfully used to silence gene expression in invertebrate and mammalian cultured cells, thereby enabling loss-of-function studies that were previously not feasible. RNAi has become a widely used approach to study the phenotypic effects of knocked down components of many pathways implicated in physiology and disease. Stringent quality-control procedures to monitor RNAi experiments are essential. Requirements for such a methodology are (a) high flexibility in assay design if many different genes need to be examined, (b) precision in assessing gene expression levels, and (c) ability to measure expression levels of multiple genes in parallel.

RT-PCR has become a popular technology for quantifying gene expression levels on both relative and absolute scales. Several techniques have been developed to measure the amount of amplified products and determine template concentration. Such techniques include the use of SYBR Green I, a dye which intercalates into dsDNA and hence provides a good estimate of the amount of amplicon produced. SYBR Green I, however, also detects potential primer dimers and unspecific PCR products. Therefore, a stringent optimization of primers and PCR conditions is often required. A second technique makes use of fluorescently-labeled probes that bind to a specific nucleotide sequence within the amplicon, excluding recognition of potential PCR by-products. Hydrolysis probes, for example, consist of a fluorogenic group and a quencher to reduce background fluorescence when not bound to the specific target sequence. Upon binding to the PCR product, the 5´-3´ exonuclease activity of the Taq polymerase cleaves the probe and releases the fluorogenic group, which then emits light.

A versatile design of RT-PCR assays and the necessary experimental optimization remain a significant technical challenge. To perform large-scale RT-PCR experiments, it would be desirable to combine the flexibility of an "out-of-the-box" SYBR Green I assay with the specificity of hydrolysis probes. We used signal transduction systems in *Drosophila* as a model and developed assays that measure the mRNA concentration of target genes using the *Drosophila* Universal ProbeLibrary [2]. These assays allowed us to predict models of pathway topologies.

Materials and Methods

Universal ProbeLibrary probes

The *Drosophila* Universal ProbeLibrary consists of 90 probes of 8–9 nucleotides (nt) in length that are shorter than commonly used hydrolysis probes (20–25 nt). By inclusion of the high-affinity nucleotide analogue LNA (locked nucleic acid), probe-DNA duplexes have a melting temperature similar to those of nonmodified probe-DNA duplexes and can therefore be used with standard quantitative PCR methods. Due to its length, each Universal ProbeLibrary probe can detect sequences in the transcriptome that recur several times. A set of 90 probes can cover the complete transcriptome. Careful selection of probe and corresponding primer pair is necessary to ensure specificity. An online assay design software (ProbeFinder; http://www.universalprobelibrary.com) was used to identify suitable primer pairs and the matching probe.

Cell culture and RNAi experiments

Drosophila SL2 cells were cultured in Schneider's medium (Invitrogen) supplemented with 10% fetal bovine serum (PAA) and penicillin-streptomycin (Invitrogen) at 25°C. Twenty-four hours prior to immune challenge, 5 million cells were seeded in a 35-mm tissue culture dish (Greiner) in

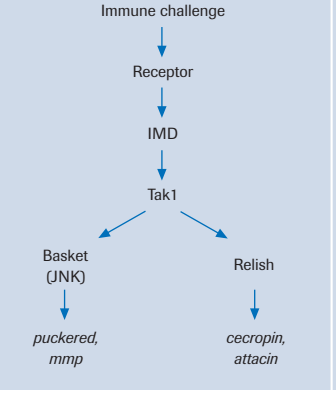

Figure 1: IMD pathway. Upon immune challenge with gram-negative bacteria the IMD pathway is activated. Downstream of Tak1 it branches into the Relish and the JNK pathways, each with its specific target genes.

Table 1: Primers used in quantitative real-time PCR

Transcript	Primer	forward	reverse	Probe #
cecA2 (CG1367)		ggacaatcggaagctggtt	tgtgctgaccaacacgttc	78
puc (CG7850)		gccacatcagaacatcaagc	ccgttttccgtgcatctt	23
rp49 (CG7939)		cggatcgatatgctaagctgt	gcgcttgttcgatccgta	10
imd (CG5576)		cctttcgagaaggcacagtt	tgcctttgtgtttctttgctc	2
attA (CG10146)		cacaatgtggtgggtcagg	ggcaccatgaccagcatt	65
mtk (CG8175)		ccaccgagctaagatgcaa	tctgccagcactgatgtagc	90

1.5 ml serum-free medium. For RNAi experiments, 15 µg dsRNA was added to the cell culture medium. After the one-hour incubation, 2 ml serum-containing medium was added and cells were transferred into a 25 cm^2 tissue culture flask (Greiner) after 24 hours. Cells were incubated for 84 hours to ensure protein depletion. To induce the immune response, cells were stimulated for 10, 30, 60, 120, or 180 minutes by exposure to heat inactivated *E. coli* (200 µg/ml).

RNA synthesis

DsRNA was generated using gene-specific primers (including T7 promoter sequences) to amplify fragments from genomic DNA by PCR. Subsequently, amplicons were used in an *in vitro* transcription reaction to generate dsRNA, which was purified and quality checked by gel electrophoresis.The following primer pair was used to amplify PCR templates for dsRNAs synthesis to silence *imd*: forward 5´-TAATACGACTCACTATAGGATGTCAAAGCTCAGGAACCT, reverse 5´-TAATACGACTCACTATAGGATGCTGACCGTTTTGCGCG. Full information on primers targeting every gene in the *Drosophila* genome is also available at http://rnai.dkfz.de.

Reverse transcription

Total RNA was isolated from cells according to standard methods; 4 µg of total RNA was treated with DNase I for 30 minutes and reverse transcribed.

Quantitative real-time PCR

In a 20-µl RT-PCR reaction, 2 µl of the 1:10 diluted cDNA was used according to a standard protocol. RT-PCR was performed using the LightCycler® 2.0 Instrument, LightCycler® FastStart DNA Master HybProbe, and the *Drosophila* Universal Probe-Library (for primer information see Table 1). Levels of the ribosomal protein Rp49 were used to normalize the data.

Results

Signaling pathways that control innate immune responses have been studied in various organisms to understand the principal mechanisms governing host defence responses. In recent years, it was shown that many molecular mechanisms controlling immune responses in humans have well-conserved counterparts in genetically tractable model organisms such as *Drosophila* [3]. Genetic studies have examined two distinct NFκB-dependent pathways commonly referred to as Toll and IMD/Rel. These two pathways control the synthesis of small secreted peptides which have potent antimicrobial activity [4].

We have previously used SYBR Green I RT-PCR assays with the LightCycler® 2.0 Instrument to examine changes in signaling pathway activity by means of their target genes [5]. Each NFκB pathway activates the expression of a specific set of target genes. Measuring changes in expression levels of target genes provides a method to monitor pathway activation. Upon recognition of surface structures of gram-negative bacteria, the IMD pathway is activated. This pathway controls expression of antimicrobial peptides such as Cecropin (Cec), Attacin (Att) and Metchnikowin (Mtk). The kinase Tak1 is not only a component of the IMD pathway, but can also activate the JNK signaling pathway and the subsequent expression of specific target genes such as *puckered* (*puc*) and *matrix metalloprotease* (*mmp*) (Figure1) [6].

We first designed RT-PCR assays for a series of target genes. Assays designed included *mtk*, *attA*, *cecA2*, and *puc* that respond to both IMD branches. The ProbeFinder software was used to identify primer pairs and corresponding probes. As a first step, we tested the performance of the assays by determining primer efficiencies using a serial dilution of target gene cDNA. As shown in Figure 2a, *rp49* primers performed in a range between 0.052 pg and 52 pg of cDNA, with a primer efficiency of 2.0. Similar results were

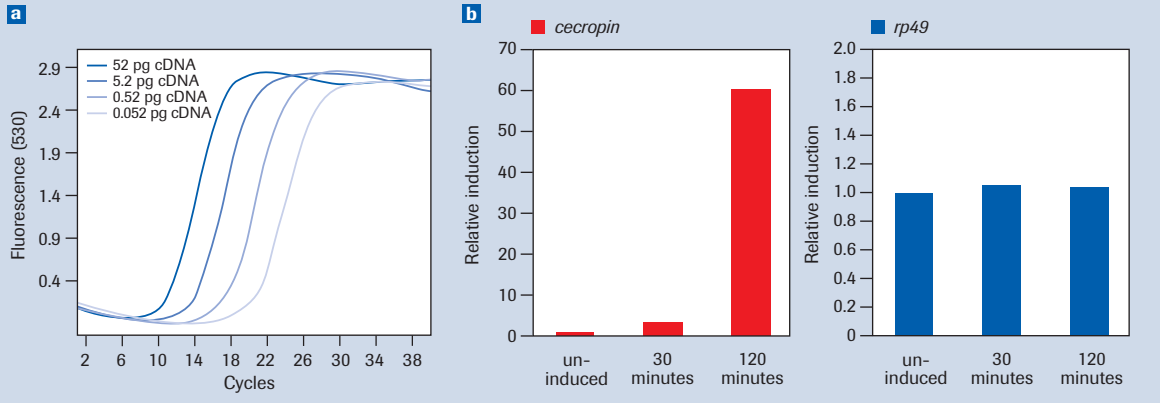

Figure 2: (a) Assessing the quality of the designed assay. *Rp49* primer efficiency was determined using four tenfold cDNA dilutions. **(b) Monitoring IMD pathway activation.** Activation was measured by quantifying Relish target gene expression (*cec*). *Rp49* levels remained constant.

obtained for all other designed RT-PCR assays which had an expected efficiency of 1.9–2.0 (data not shown).

We then analyzed pathway induction by performing a time-course of pathway activation after an immune stimulus by gram-negative bacteria. To this end, a suspension of heat-inactivated *E. coli* was added to the medium of cultured *Drosophila* SL2 cells for 10, 30, 60, 120, and 180 minutes. Subsequently, expression levels of target genes were examined using the established RT-PCR assays. Pathway induction was observed as soon as 10 minutes after immune challenge, with *cec* being 2-fold induced and *puc* 10-fold (data not shown). *Puc* transcript levels remained constant (data not shown) whereas *cec* went further up: After 30 minutes *cec* transcripts increased 3-fold and after 120 minutes we observed a 60-fold induction which did not further increase after 180 minutes (Figure 2b and data not shown). As expected, *rp49*, a ribosomal gene used for normalization purposes, did not show changes in expression during the induction time course (Figure 2b). These experiments showed that the Universal ProbeLibrary assays accurately quantified gene expression levels similar to previously obtained results without the need for optimizing real-time PCR assays.

Next, we examined whether expression of pathway components and targets is reduced after knockdown of genes by RNAi. RNAi in invertebrates is highly efficient due to a lack of an interferon response to long dsRNAs. To perform RNAi in cultured *Drosophila* cells, 500–700 bp dsRNAs are synthesized from DNA templates containing terminal T7 promoters, and the resulting molecules are simply added to the culture medium [7]. These dsRNA molecules are autonomously taken up by cells through an unknown transport mechanism and are intracellularly processed into functional 21mer siRNAs. Using this experimental approach, we monitored changes in target gene expression after depletion of the essential component IMD. Cells incubated with dsRNA against *imd* were stimulated with *E. coli* overnight. RNAi against *green fluorescent protein* (*gfp*) was used as a negative control. Following reverse transcription of isolated total RNA, levels of *cecA2* transcripts were assessed using the Universal ProbeLibrary. *CecA2* expression levels were significantly reduced after RNAi against *imd*, whereas *rp49* levels were not affected (Figure 3). These results clearly demonstrate the requirement of IMD to induce target gene expression. Additionally, we tested whether the knockdown of the *imd* transcript is observed using a RT-PCR assay. As shown in Figure 4, RNAi against *imd* effectively reduced its mRNA level by 91%.

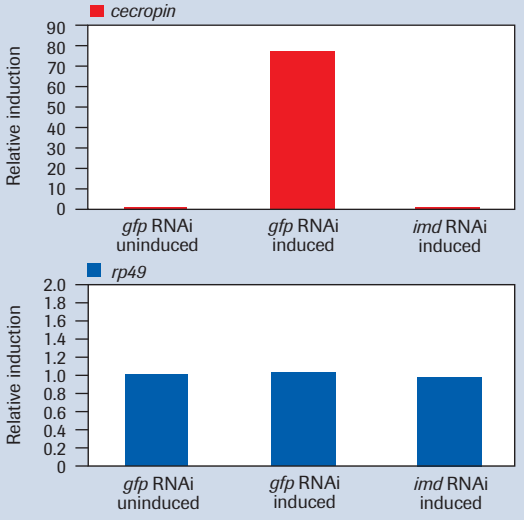

Figure 3: Effect of knocking down pathway components. RNAi was used to deplete *imd*. Altered target gene induction of *cec* is shown. *Rp49* was not affected.

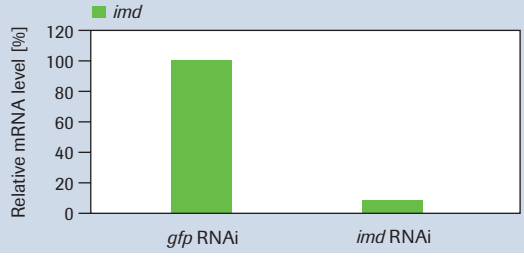

Figure 4: Knockdown of *imd*. RNAi against *imd* effectively reduces its transcript level.

Conclusion

The systematic analysis of signaling pathways is a key question in modern biology. Developing quantitative and manifold tools to measure dynamic changes in gene expression levels remains a major challenge. RNAi approaches are widely used to study the role of proteins in cellular pathways. To test whether a gene plays a role in a specific signaling pathway, its expression is silenced by RNAi and the loss-of-function phenotype is assessed by observing the modulation of target gene expression of the signaling pathway. The experiments demonstrate that using a set of pre-designed probes provide the versatility to develop quantitative assays based on gene expression signatures. Thus, combining RNAi and RT-PCR may provide a very powerful tool for investigating the involvement of specific genes in signal transduction pathways. Pre-designed probes such as the Universal ProbeLibrary could significantly shorten the lengthy and expensive assay development phase required in conventional RT-PCR approaches. ■

References

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