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Yeast Functional Analysis Report

A QPCR-based reporter system to study post-transcriptional regulation via the 3′ untranslated region of mRNA in *Saccharomyces cerevisiae*

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Abstract

Post-transcriptional regulation via the 3′ untranslated region (3′ UTR) of mRNA is an important factor in governing eukaryotic gene expression. Achieving detailed understanding of these processes requires highly quantitative systems in which comparative studies can be performed. To this end, we have developed a plasmid reporter system for *Saccharomyces cerevisiae*, in which the 3′ UTR can be easily replaced and modified. Accurate quantification of the tandem affinity purification tag (TAP)-reporter protein and of TAP-mRNA is achieved by immuno-QPCR and by RT-QPCR, respectively. We have used our reporter system to evaluate the consequences on gene expression from varying the 3′ UTR, a problem often encountered during C-terminal tagging of proteins. It was clear that the choice of 3′ UTR was a strong determinant of the reporter expression, in a manner dependent on the growth conditions used. Mutations affecting either decapping (*lsm1* Δ) or deadenylation (*pop2* Δ) were also found to affect reporter gene expression in a highly 3′ UTR-dependent manner. Our results using this set-up clearly indicate that the common strategy used for C-terminal tagging, with concomitant replacement of the native 3′ UTR, will very likely provide incorrect conclusions on gene expression. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: **3′ untranslated region; post-transcriptional regulation; reporter gene; QPCR; C-terminal tagging**

Introduction

According to the central dogma of molecular biology, genes encoded by DNA are transcribed to RNA, which is in turn translated to proteins. The initiation of transcription is the first and possibly also the most important level of gene expression regulation (Orphanides and Reinberg, 2002). However, it is also well established that, especially in eukaryotes, the nascent transcripts undergo extensive processing, e.g. 5′ capping, 3′ polyadenylation and splicing prior to their export from the nucleus (Hieronymus and Silver, 2004; Moore, 2005). Furthermore, the stability, localization and translational state of mRNA can be modified by binding of proteins to both translated and untranslated regions of mRNA (Dreyfuss *et al.*, 2002; Keene, 2007; Mazumder *et al.*, 2003; Parker and Song, 2004), and especially the 3′ untranslated region (3′ UTR) seems to be a target of many regulatory proteins (Mazumder *et al.*, 2003). It also appears that the mechanisms and proteins involved

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in post-transcriptional regulation are to a large extent conserved throughout evolution. In fact, many of the involved proteins and mechanisms have first been identified in unicellular eukaryotes such as *Saccharomyces cerevisiae* (Parker and Song, 2004), which has thus proved to be an excellent system to elucidate mechanisms pertinent to 3′ UTR-dependent regulation. Furthermore, the sequencing of the genome of *S. cerevisiae* (Goffeau *et al*., 1996) has spawned the development of methods which enable a truly global understanding of regulatory processes in cells from this species, on all levels, from DNA, via RNA to proteins and metabolites (Suter *et al*., 2006). A common strategy for the study of proteins is to utilize tagging domains (e.g. green fluorescent protein, antibody epitopes or affinity purification tags). Tagging of proteins at the C-terminal is often preferred to tagging on the N-terminal, since the latter is more likely to interfere with protein localization and, in addition, frequently removes the native promotor. In *S. cerevisiae*, tag collections have been developed to enable a wide variety of C-terminal fusion domains which have subsequently been used to construct genome-wide libraries of TAP tagged and GFP-tagged yeast strains for global studies on the localization and expression of essentially all yeast proteins (Ghaemmaghami *et al*., 2003; Huh *et al*., 2003; Newman *et al*., 2006). The resulting collections are widely used; the papers describing them are cited hundreds of times. However, the common C-terminal tagging strategy will also lead to a concomitant replacement of the gene's native 3′ UTR (in the case of the genome-wide collections with that from the *ADH1* gene), which is problematic, since it will in effect remove an entire layer of post-transcriptional regulation acting via the 3′ UTR.

Since gene regulation is such a highly complex process, which can be influenced by many factors not directly related to the process under study, it is often advantageous to utilize a non-native reporter gene to obtain quantitative data pertinent to a specific regulatory element or factor (von der Haar *et al*., 2007). Therefore, in order to facilitate studying the regulatory mechanisms acting via specific 3′ UTRs in isolation, we have developed a plasmid reporter system in which the 3′ UTR constitutes the variable region. The reporter gene we have chosen is the tandem affinity purification tag (TAP; Rigaut *et al*., 1999), mainly due to the availability of highly accurate quantification of the reporter protein via immuno-qPCR (Lind and Norbeck, 2007) and mRNA via RT-qPCR. In a 'proof of principle' application, we find that the identity of the 3′ UTR is an important determinant for gene expression pattern, in relation to both growth conditions and mutants involved in decapping and deadenylation. Our findings highlight the need for keeping a native 3′ UTR when a correct quantification of tagged proteins is desired.

Materials and methods

Cloning and transformation of the 3′ UTR-reporter plasmids

A fragment containing 380 bp of the *COX17* promotor was cloned *Xho*I/*Bam*HI in the centromeric plasmid pRS315 (Sikorski and Hieter, 1989) to produce p315–COX17p. The 3′ UTR of COX17 and HSP12 (333 and 592 bp downstream of the last amino acid codon, respectively) was cloned *Bam*HI/*Sac*I in p315–COX17p, while the 3′ UTR of *RPL18B* (554 bp downstream of the last amino acid codon) was cloned *Bam*HI/*Not*I in p315–COX17p to produce p315–COX17p–*Bam*HI–XXXUTR (with XXX denoting either *RPL18B, HSP12* or *COX17*). Finally, a TAP tag fragment with an added start codon and a C-terminal stop codon was ligated in the *Bam*HI sites to make p315–COX17p–TAP–XXXUTR (with XXX denoting either *RPL18B, HSP12* or *COX17*). A fragment containing the TAP tag (Rigaut *et al*., 1999) followed by a stop-codon and the 237 bp *ADH1*– 3′ UTR (as found in the TAP tagged genomic DNA from the *NOT5*–TAP strain; Ghaemmaghami *et al*., 2003) was ligated *Bam*HI/*Sac*I into p315–COX17p to produce p315–COX17p–TAP–ADH1UTR (Figure 1A). Plasmids were subsequently transformed into strains (BY4742wt, *lsm1*Δ and *pop2*Δ; Brachmann *et al*., 1998; Winzeler *et al*., 1999) as indicated with selection on SC (−leu) 2% w/v glucose.

The plasmids 316–COX17–HA–COX17UTR and 316–COX17p–HA–RPL18BUTR, which were used for Figure 2B, contain the native COX17 promotor and open reading frame followed by a 2×HA-tag and either COX17 or RPL18B 3′ UTR. The backbone vector was pRS316 (Sikorski and Hieter, 1989).

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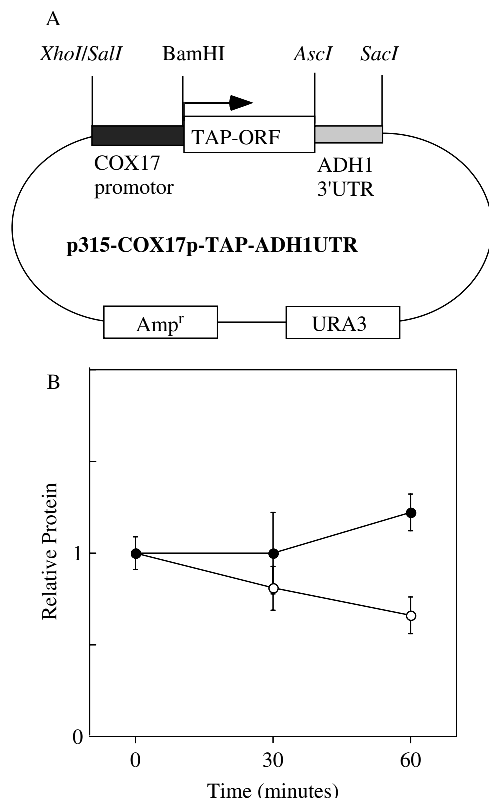

Figure 1. The 3′ UTR reporter plasmid and stability of the TAP-protein reporter. (A) Map of the plasmid p315–COX17p–TAP–ADH1UTR, which can be used to integrate any 3′ UTR between the unique *Asc*I and *Sac*I sites. The complete sequence of the *Xho*I/*Sac*I insert can be found in the web supplement. (B) The relative protein levels of TAP-reporter from the plasmid p315–COX17p–TAP–ADH1UTR following addition of cycloheximide (5 mM final concentration) or water. All values are normalized against the expression at the time 0 min, which is set to a value of 1 for each treatment. Standard deviations of biological triplicates are shown as error bars

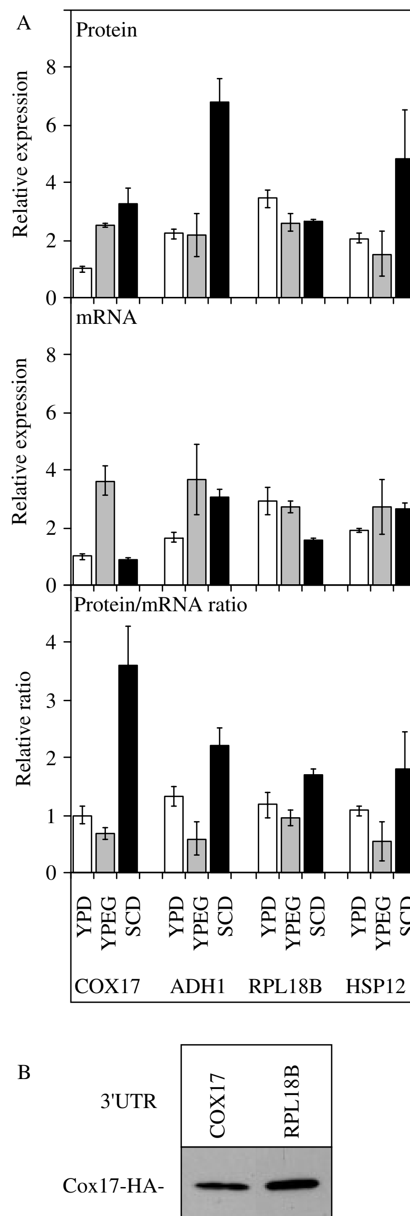

Figure 2. Each 3′ UTR mediates a growth condition-specific expression profile. (A) Regulation of a TAP-reporter construct dependent on either of the four different 3′ UTRs, as indicated. YPD growth, white bars; YPEG growth, grey bars; SCD growth, black bars. Relative reporter protein levels (upper panel), relative reporter mRNA (centre panel) and relative protein : mRNA-ratio (lower panel) are shown. All values are normalized against the *COX17* 3′ UTR-dependent expression on YPD. Standard deviations of biological triplicates are shown as error bars. (B) Western blot of cells grown in YPD and expressing full-length COX17–2×HA with 3′ UTR from either COX17 or RPL18B as indicated. Detection with mouse monoclonal anti HA-antibody

A detailed description of the cloning procedure for the above plasmids is given in the supporting information, together with nucleotide sequences of the inserts from the four TAP-reporter plasmids, all of which have been verified by sequencing.

Growth conditions

Strains were precultured in synthetic complete medium (−Leu or −Ura, as applicable) with 2% w/v glucose (henceforth referred to as SCD), and this medium was also used for the SCD-grown

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cells. Complex medium (YP) was also used, containing 2% peptone and 1% yeast extract, and supplemented with either 2% glucose (YPD) or 3% ethanol/2% glycerol (YPEG). The cultures were then inoculated to an initial OD of approximately 0.1. Cultures were performed in triplicate in either 6 ml culture (YPD) in 50 ml Falcon tubes or in 10 ml cultures (YPEG or YNB) in 100 ml e-flasks. The cells were harvested at OD = 0.5–1.0. 5 ml samples were harvested to be used for protein quantification and 1 ml samples were harvested for the mRNA analysis. Both sets of samples were stored at −80 °C until extraction.

Protein extraction and analysis

Proteins were extracted by vortexing together with glass beads and 100 μl MilliQ water using a Fastprep (FP120, Savant), followed by the addition of 100 μl 2× SDS-mix (0.1 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 4% β-mercaptoethanol) and 5 min incubation at 100 °C. The extract was centrifuged for 5 min at 14 000 × g and the supernatant was transferred to a new tube. The total protein concentration was determined using Bio-Rad Dc Protein Assay (Bio-Rad Laboratories, Sweden), preceded by a TCA precipitation step, but otherwise according to the manufacturer's instructions. The TAP tag was quantified with immuno-qPCR with anti-protein A IgY as described (Lind and Norbeck, 2007). The samples were normalized by total protein amount added to the immuno-qPCR. Western blotting for detection of HA-tagged Cox17p was performed essentially as previously described (Lind and Norbeck, 2007), with the mouse monoclonal antibody (clone 12CA5; Roche Diagnostics, USA).

RNA extraction

The RNA was extracted using the EZNA Yeast RNA Kit (Omega Bio-tek) according to the manufacturer's instructions, except that the lyticase step was omitted and 200 mg glass beads were used instead of 50 mg. The samples were DNase-treated on-column during extraction using the EZNA RNase-Free DNase I Set (Omega Bio-tek). Total RNA concentration was determined using a NanoDrop ND-1000 (NanoDrop Technologies) and representative RNA samples were quality-checked on an Agilent 2100 Bioanalyser according to the manufacturer's instructions.

The RNA was subsequently converted to cDNA by reverse transcription, using iScript cDNA Synthesis Kit (BioRad), following the manufacturer's instructions except that each reaction was made in duplicate in a 10 μl volume.

Quantitative PCR for the determination of TAP tag mRNA

The qPCR to analyse the mRNA was run on a BioRad iQ5 real-time PCR instrument. The 20 μl PCR contained 1× PCR buffer (Sigma-Aldrich), 4 mM $MgCl_2$ (Sigma-Aldrich), 0.2 mM of each dNTP (Sigma-Aldrich), 0.3 μM of each primer (MWG Biotech), 10 nM fluorescein, 0.5× SYBR Green I (Molecular Probes), 1 U Taq polymerase (Sigma-Aldrich) and 1 μl cDNA. Primers for the TAP tag were TTCATAGCCGTCTCAGCAG and AATTTGTTGTCCACGGCTTC. The expression was normalized using the reference genes *IPP1* (primers GACACCCCAACCTACTCCAA and GAACCGGAGATGAAGAACCA) and *ACT1* (primers CTGCCGGTATTGACCAAACT and CG GTGATTTCCTTTTGCATT). The cycling conditions were: 3 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. Calculations of real-time PCR data were made using GenEx (MultiD, Sweden).

Results and discussion

In studying 3′ UTR-mediated regulatory processes involving Puf proteins, we have realized the need for highly accurate methods to quantify the gene expression changes, and also for a system allowing for comparative studies on many different 3′ UTR sequences. Therefore, in order to facilitate the study of the 3′ UTR effect on gene/protein expression, we have made use of the TAP tag as a reporter gene. Choosing the TAP tag enables us to perform a highly quantitative analysis both on the level of TAP-protein by immuno-QPCR (Lind and Norbeck, 2007) and by RT-QPCR on the level of the corresponding mRNA, the latter normalized to genomic *ACT1* and *IPP1* expression. We constructed a set of plasmids in which the TAP tag is expressed under the control of the *COX17* promotor and in which the 3′ UTR constitutes the variable domain (Figure 1A). To our knowledge, the TAP tag has not previously been used on its own as

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a reporter, even though the protein A constituent of the TAP tag has been utilized as a reporter in analysis of non-stop mRNA decay, with quantification of protein A via Western blotting (Wilson *et al*., 2007). We have therefore verified that a TAP tag under the control of *COX17* promotor and the *ADH1* 3′ UTR was expressed at an easily detected level. It was furthermore not known whether a TAP protein would be stable as an independent protein and we therefore performed a cycloheximide treatment experiment, in which we found the TAP tag protein, as detected by immuno-QPCR (Lind and Norbeck, 2007), to have an approximate half-life of 80 min (Figure 1B). Thus, the TAP protein is a useful reporter gene for monitoring gene expression with high precision during steady-state conditions of growth as well as during increases in reporter expression. However, in common with many other reporter proteins, the relative stability precludes its use in following rapidly diminished gene expression. The reporter plasmid is constructed in a modular fashion, the main feature being that the 3′ UTR can be easily exchanged by ligation between the *Asc*I and *Sac*I sites (Figure 1A). It should be noted that a UGA-stop codon followed by a G might give somewhat higher read-through (Bonetti *et al*., 1995), which in turn suggests that users should consider adding an additional downstream stop codon after the *Asc*I site when cloning new 3′ UTRs in our reporter plasmid. In addition, if required, the reporter itself can also easily be exchanged by cutting/ligation with the *Bam*HI and *Asc*I sites flanking the TAP tag. Alternatively, the 3′ UTR can be linked to any gene via ligation of an engineered PCR fragment into the upstream *Xho*I or *Sal*I sites in combination with the *Asc*I site preceding the 3′ UTR (Figure 1A). However, the main objective of the study was to demonstrate the use of our reporter system in monitoring the effect of varying the 3′ UTR on gene expression. We therefore constructed plasmids with four different 3′ UTR sequences (from *ADH1, COX17, HSP12* or *RPL18B*), which corresponds to genes representing a wide range of protein classes. The *COX17* 3′ UTR was chosen to represent proteins with mitochondrial function and also due to its known regulation by Puf3p (Foat *et al*., 2005; Olivas and Parker, 2000). *ADH1* 3′ UTR was chosen as a representative of the glycolytic pathway proteins and also due to its being used in both the genome wide TAP tag and GFP tag collections in yeast (Ghaemmaghami *et al*., 2003; Huh *et al*., 2003). *RPL18B* 3′ UTR was chosen as a representative of the ribosomal protein-coding genes, which are subject to regulation by mRNA decay and translation initiation (Foat *et al*., 2005; Grigull *et al*., 2004). *HSP12* 3′ UTR was chosen as a typical representative of the genes in the environmental stress response (ESR; Gasch *et al*., 2000).

As an initial experiment, we set out to verify that the reporter plasmid with a *COX17* 3′ UTR displayed the previously reported increase in expression in a *puf3*-deletion strain (Olivas and Parker, 2000). In a *puf3*-deletion strain growing on glucose-based rich medium (YPD), the *COX17* 3′ UTR gave 2.00 ± 0.26-fold higher expression on the level of mRNA and a 3.00 ± 0.39-fold higher expression of the TAP protein compared to expression in the wild-type. On rich medium with ethanol/glycerol as carbon/energy source (YPEG), the *COX17*-dependent TAP protein and mRNA expression was higher than on YPD medium, by factors of 2.50 ± 0.08 and 3.63 ± 0.53, respectively, and no significant increase was seen in a *puf3*-deletion strain. This observation fits well with the suggestion that Puf3p is inactive on a non-fermentable carbon source (Foat *et al*., 2005). We conclude that results using our system correlate well with previous studies.

Next, cells expressing the TAP reporter under the control of one of the four 3′ UTRs were grown to steady state in three different media (YPD, YPEG and SCD), reflecting the difference in rich (YPD) vs. defined (SCD) media as well as a difference between fermentative (YPD) and respiratory (YPEG) growth. An expression profile, in relation to growth conditions, was obtained for each 3′ UTR, at the level of both TAP protein and TAP mRNA, and it was immediately evident that each tested 3′ UTR mediates a unique expression profile in relation to the three growth conditions (Figure 2A). Thus, on YPD *COX17* 3′ UTR mediated the lowest relative expression, while the 3′ UTRs of *ADH1* and *RPL18B* gave a two- and three-fold increase in expression, respectively. Comparison of HA-tagged native Cox17 protein under the control of *COX17* or *RPL18B* 3′ UTR gave similar results for expression, as evaluated by Western blotting (Figure 2B). The expression of a reporter dependent on the 3′ UTR from *RPL18B* was largely non-responsive to the culture medium, whereas all the other three 3′ UTRs showed a clear regulation in at least one condition (Figure 1A).

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We can also calculate the protein : mRNA ratio and, since we are looking at steady-state growing cultures of yeast, this will allow for an estimate of translational efficiency, i.e. how much protein is produced from each mRNA. The most prominent difference in this parameter was seen for the *COX17* 3′ UTR, which displayed a three- to fourfold higher protein : mRNA ratio on SCD compared to that on YPD (Figure 2A, lower panel), indicative of a clearly higher translational efficiency.

We also decided to investigate whether the 3′ UTR constructs would respond differently to gene deletions in pathways affecting the processing of transcripts. The normal pathway for degradation of mRNA in eukaryotes starts with removal of the 3′ poly(A) tail and proceeds either with decapping and 5′ exonucleolytic degradation or by the exosome-mediated 3′ exonuclease activity (Parker and Song, 2004). At least in yeast, the deadenylation activity in the cytoplasm is mainly dependent on both Pop2p and Ccr4p (Tucker *et al.*, 2001) and we therefore set out to test whether a *pop2* Δ strain would display 3′ UTR-dependent changes in expression of the TAP reporter. Loss of the decapping enzyme core subunits is lethal in our strain background, BY4742 (Giaever *et al.*, 2002) and we have therefore chosen to study the *lsm1* Δ strain, previously reported to inhibit decapping (Tharun *et al.*, 2000). We expressed the reporter constructs dependent on either *COX17, ADH1* or *RPL18B* 3′ UTRs in the *pop2* Δ and the *lsm1* Δ strains and compared the expression in YPD-grown cells to that of the corresponding wild-type strain. Both the *pop2* Δ and the *lsm1* Δ strains showed a clear 3′ UTR-dependent change in expression of the TAP protein reporter (Figure 3). An increased protein expression from the *COX17* 3′ UTR was observed in both the *pop2* Δ and, especially prominently, the *lsm1* Δ strain, the *ADH1* 3′ UTR was most affected by the *lsm1* Δ strain, while the *RPL18B* 3′ UTR showed only a minor effect on protein expression from either deletion. TAP reporter mRNA levels were in good agreement with protein levels, but the protein : mRNA ratio was generally lower for the *pop2* Δ and *lsm1* Δ strains (Figure 2). Thus, using our reporter system, we can clearly verify that the behaviour of a transcript in relation to decapping or deadenylation will to a large extent depend on the 3′ UTR used.

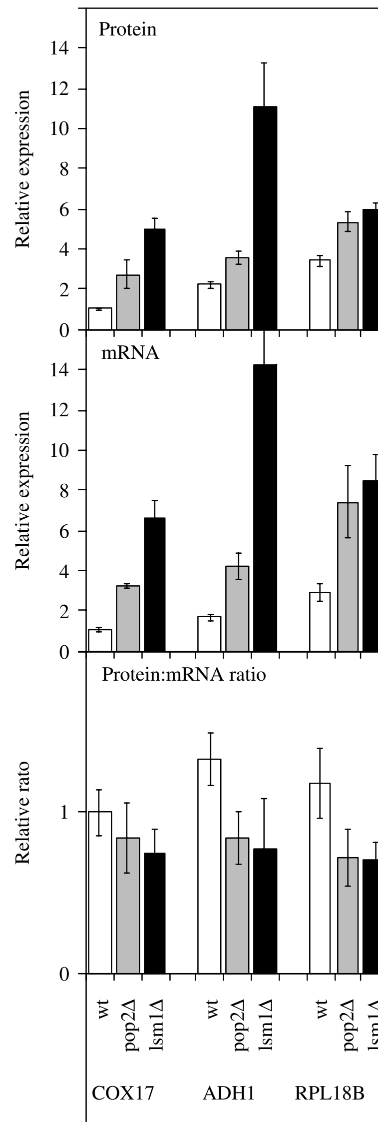

Figure 3. Regulation of a TAP-reporter construct by deadenylation and decapping. Three different reporter constructs dependent on 3′ UTRs from *COX17, ADH1* and *RPL18B* were tested during growth on YPD-medium. Three different strains were compared, wild-type (wt), white bars; *pop2*Δ, grey bars; *lsm1*Δ, black bars. Relative reporter protein levels (upper panel), relative reporter mRNA (centre panel) and relative protein : mRNA-ratio (lower panel) are shown. All values are normalized against the *COX17* 3′ UTR-dependent expression on YPD. Standard deviations of biological triplicates are shown as error bars

In conclusion, we have set up a QPCR-based reporter system which is suitable for highly quantitative studies on 3′ UTR-dependent regulatory mechanisms in *S. cerevisiae*. We have used this

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system to illustrate the strong impact from the choice of 3′ UTR on condition-specific regulation as well as on the effects of mutations involved in post-transcriptional regulation. Our results clearly indicate that care has to be taken in interpreting quantitative results from studies using the normal C-terminal tagging procedure, due to the omission of the important regulatory mechanisms acting via the 3′ UTR. We believe that our system will also enable large-scale analysis of either many different 3′ UTRs and many different conditions and/or mutants, due to the possibility of robotization on the level of strain handling and the QPCR-based steps.

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Supporting information

Supporting information may be found in the online version of this article.

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