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RESEARCH ARTICLE

Immuno-qPCR detection of the tandem affinity purification (TAP)-tag as a sensitive and accurate tool suitable for large-scale protein quantification

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The tandem affinity purification (TAP)-tag has rapidly gained a wide popularity, mostly in studies on protein interactions, but lately also in large-scale protein quantification studies. We have developed an immuno-quantitative real-time PCR (qPCR) method to achieve rapid, sensitive and accurate quantification of TAP-tagged (and protein A-tagged) proteins in yeast with a detection range between 10^7 and 10^{10} molecules. The immuno-qPCR protein quantification showed an excellent correlation to the published *in vivo* fluorescent protein (GFP)-based large-scale protein quantifications, but allowed for a much higher sensitivity. The correlation with published data from the large-scale Western blotting-based quantification of the TAP-tag was lower, but the sensitivity of detection was on roughly the same level. The practical use of the immuno-qPCR approach was demonstrated by analysis of osmo-regulated proteins, where the 2000-fold increase in expression of Catalase (Ctt1p), from an extremely low basal expression, could be accurately quantified. All steps of the method, from cell growth, to protein extraction and determination and the immuno-qPCR reaction itself are potentially amenable to automatization. Therefore, since the TAP-tag and protein A are useful in most model organisms, the immuno-qPCR method is both generic and suitable for large-scale studies.

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1 Introduction

All cells have the ability to adapt to changes in the environment by altering their macromolecular and metabolite compositions. Understanding how these changes are brought about and which signalling pathways are involved is one of the fundamental questions of biology. A key to enable these studies is the ability to accurately measure the amounts of all classes of molecules.

Recent years has seen a revolution in the way biological studies are conducted, which has been brought about by the systematic sequencing of a large number of genomes from all major groups of organisms. The availability of the complete DNA sequences has in turn spawned the development of methods to study cellular changes in macromolecular levels on a truly global scale. The study of RNA on a large scale is termed transcriptomics and is usually performed by various developments of the microarray technology. The study of proteins on a global scale is much more difficult, due to their extremely heterogeneous nature, mediated by variations in amino acid sequence and a plethora of modifications. The first technique for global analysis of proteins was 2-D PAGE [1], which became truly powerful with the

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Abbreviations: Ct, threshold cycle; **FACS,** flow cytometry; **GFP,** green fluorescent protein; **TAP,** tandem affinity purification; **qPCR,** quantitative real-time PCR

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development of mass-spectrometric identification of trypsin digested proteins (reviewed in Aebersold and Goodlett [2]). However, 2-D PAGE suffers from several drawbacks such as the inability to separate very hydrophobic proteins (prominently *e.g.* integral membrane proteins), the inability to separate proteins with p*I*s and molecular weights outside of the gel and finally also the poor ability to analyse proteins with a low expression.

Developments in MS has led to methods addressing several of the problems with 2-D PAGE, thus the use of isotope-coded affinity tags in combination with multidimensional LC-MS/MS can quantify proteins of a wider range in expression directly from protein mixtures [3] and the analysis of membrane proteins can be achieved by digesting the membrane proteins soluble domains prior to analysis [4]. However, despite the promising nature of the LC-MS/MS-based methods, they still suffer from the drawback of being highly time consuming and requiring access to expensive equipment and a high level of technical expertise.

Especially in *Saccharomyces cerevisiae*, a range of genome wide collections has been constructed which have direct applications for proteomics. In one of these collections, green fluorescent protein (GFP) has been inserted after the ORF of each yeast gene [5]. Another collection has the tandem affinity purification (TAP)-tag, consisting of calmodulin-binding peptide followed by a TEV-cleavage site and two copies of the IgG binding domain of protein A [6], inserted after essentially all yeast protein coding genes [7]. It is noteworthy that both these tags have been developed for other applications, the TAP-tag was originally developed for purification of protein complexes [6] and the GFP-tag has mainly been used to localize proteins, *e.g.* on a global scale in yeast [5]. The TAP-tag collection has been used to quantify most yeast proteins by Western blotting using an antibody raised against the TAP-tag [7]. Western blotting is rather time consuming and involves several steps which are difficult to perform in an automated fashion. Furthermore, quantification of Western blots is subject to the same hurdles as all gel images (*e.g.* how to subtract background, gel distortions, variations in blotting). However, one advantage of the Western blotting approach is the ability to detect post-transcriptional modifications (*e.g.* signal peptide removal, alternative splicing forms, major covalent modifications). The GFP-collection has recently been used in combination with flow cytometry (FACS) for global quantification of proteins in yeast [8]. This set-up is currently considerably less sensitive than the TAP-tag/Western approach, but has the advantage of being able to supply information on biological noise [8, 9]. It should also be emphasized that 2-D PAGE and the MS-based approaches do not require genomic tagging of individual genes, with the potential problems this might cause.

We have developed an immuno-quantitative real-time PCR (qPCR)-based method to quantify the TAP-tag. Immuno-qPCR is a highly sensitive technique which combines the specificity of antibodies with the amplifying power of PCR to which it is linked [10]. It is an improvement of immuno-PCR [11] that used agarose gel electrophoresis for detection. By using real-time PCR the assay gets more quantitative and a sensitive and precise assay is achieved without the need of any post-PCR handling. Immuno-qPCR has previously been used for detection and quantification of a wide variety of proteins, *e.g.* vascular endothelial growth factor [10], mumps antibodies [12], prostate-specific antigen [13], rotavirus [14] and prion proteins [15] and have in most of the cases been 10–1000 times more sensitive than the corresponding ELISA. However, each of those assay systems is of use for only one protein. By developing an assay against a commonly available tag, such as the TAP-tag or GFP, the assay gets vastly more useful, in principle allowing global protein quantification of all organisms in which the construction of genomically tagged collections is feasible. The immuno-qPCR is also highly reproducible, it can be performed in the 96-well format and we believe that the combination of sensitivity and ease of use makes the method useful for large-scale analysis. In our hands, the immuno-qPCR method yields superior results to the Western-based analysis of the identical extracts. In addition, the low detection threshold allows accurate quantification of proteins such as Ctt1p with an extremely dynamic expression profile.

2 Materials and methods

2.1 Strains and growth conditions

Strains were wild-type BY4741 or derivatives thereof containing TAP-tagged genes as indicated [7]. The strains were both precultured and grown in YPD medium with 0 or 1 M NaCl as indicated. Cells used in Fig. 8 were grown in E-flasks on a rotary shaker to OD $\approx$ 0.6. All other cells were grown in 50 mL falcon tubes with 5 mL media, the OD at harvest was about 0.6 except for the protein extraction comparison (see below).

2.2 Preparation of protein extract

Two extraction procedures were compared. Five yeast strains with different proteins TAP-tagged were grown in triplicates in 5 mL YPD in 50 mL falcon tubes. Two samples corresponding to 1.7 OD units were taken from each culture in mid-log phase (OD $\approx$ 1) and in postdiauxic shift cells (3 day cultures, OD $\approx$ 10). The cells were harvested by centrifugation at $14\,000 \times g$. One tube was extracted by vortexing together with glass beads and 100 μL Milli-Q water using a FastPrep (FP120, Savant) four times 20 s at speed 6 at 4°C, followed by the addition of 100 μL of $2 \times$ SDS-mix (0.1 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 4% β-mercapto-ethanol) and 5 min incubation at 100°C. The extract was centrifuged for 5 min at $14\,000 \times g$ and the supernatant was transferred to a new tube. From the other tube the proteins were extracted following the procedure described in Ghaemmaghami *et al.* [7]. Briefly, the proteins were extracted from

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the cell pellets by addition of a boiling SDS buffer followed by centrifugation at 14 000 × *g* for 5 min. The supernatant was transferred to new tubes. For both methods, the total protein concentration was determined using BioRad Dc Protein Assay (BioRad Laboratories, Sweden), preceded by a TCA precipitation step, but otherwise according to the manufacturer's instructions. The same amount of total protein was added in the immuno-qPCR reactions for all samples.

2.3 Antibodies and DNA conjugation

A polyclonal anti-protein A affinity purified antibody derived in chicken (CPA-65A, Immunology Consultants Laboratory, USA) was used both in Western blot analysis and in immuno-qPCR as capture antibody and detection antibody. For detection in immuno-qPCR, the antibody was conjugated covalently to a DNA-label as previously described [13]. The sequence of the 66 bases long DNA label was TCTCCTCATCTCACCTACCAAGCCTGCGCCTTGCTCCT TTGGTTCGAGTTGGTGATTGGTGAGGAG. Primers used for PCR amplification and quantification of the DNA label were CTCCTCATCTCACCTACCAA and CTCCTCACCAAT CACCAACT.

2.4 Immuno-qPCR

The immuno-qPCR was partly optimized using pure protein A (Sigma–Aldrich, P6031) as analyte. After thorough optimization this was the protocol that was used. Robostrips (AJ Roboscreen, Germany) were coated with capture antibody by incubation of 25 μL of anti-protein A antibody (1 μg/mL in carbonate buffer, pH 9.6) over night at 4°C. The wells were washed three times with wash buffer (5 mM Tris-HCl, pH 7.75, 0.154 M NaCl, 0.005% Tween 20) before being blocked with incubation buffer (PBS containing 5% milk, 0.05% Tween 20, 4 μg/mL IgY and 0,1 mg/mL Herring sperm DNA) for 1 h at 37°C. After washing three times with wash buffer, 25 μL of protein extract diluted at least 50 times in incubation buffer was added and incubated for 1 h at room temperature. The wells were washed six times with wash buffer and incubated with the detection antibody/DNA conjugate, diluted 10 000 times in incubation buffer, for 1 h at room temperature. After washing six times with wash buffer and ten times with Milli-Q water real-time PCR was run. Real-time PCR was performed on a BioRad iQ5 real-time PCR instrument using BioRad SYBR Green Supermix with a final primer concentration of 0.3 μM and total volume of 25 μL. 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. After the run, a melt curve was performed.

2.5 Calculation of LOD

To get a number of the LOD of the developed immunoassay, a standard curve of known concentration of protein A was made and six samples without protein was analysed. The apparent protein concentration of the negative samples was calculated from the standard curve and the LOD was set as the mean plus three times the SD. The limit was then transformed to number of TAP-tags by multiplying with two. Protein A has four IgG binding domains (Sigma data sheet) whereas the TAP-tag contains two IgG binding domains. Therefore, a factor of two should be used in converting the protein A standard curve values into the corresponding TAP-tag amount, *i.e.* 1×10^7 protein A molecules corresponds to 2×10^7 molecules of TAP-tag.

2.6 Investigation of effect of SDS-buffer concentration and unspecific binding in immuno-qPCR

To investigate the effect of the SDS containing extraction buffer on the immuno-qPCR, a serial dilution of the extraction buffer with 4 ng/mL protein A was made in incubation buffer containing the same concentration of protein A. The protein samples were analysed with immuno-qPCR. To investigate the unspecific binding of the detection antibody/DNA-conjugate to other components in the protein extracts than the TAP-tagged proteins a serial dilution of wild-type yeast protein extract was made. The protein samples were analysed with immuno-qPCR.

2.7 Western blot analysis

Extract of TAP-tagged Hxk2p was serially diluted in extract from a nontagged strain. They were analysed in single samples. In all cases, protein samples with the same total protein amount *per* well were run on a 10% polyacrylamide gel and blotted onto a NC membrane in a semi dry blot equipment for 1 h at 10 V and 70 mA/gel. The membrane was blocked with 5% milk in TBST (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl and 0.05% Tween 20) for 1 h at room temperature and thereafter washed 5 min with TBST. The anti-protein A antibody (1 mg/mL) was diluted 2000 times in TBST with 5% milk and incubated with the membrane over night at 4°C. The membrane was then washed 3 × 5 min with TBST. HRP-conjugated anti-chicken antibody (Upstate, USA) diluted 5000 × in TBST was added to the membrane and incubated for 1 h at room temperature. The membrane was washed three times 5 min with TBST. The signal was developed using the ECL plus Western Blotting Detection System (GE Healthcare, Sweden) according to the manufacturer's instructions. Western blot data were analysed using the ImageJ software [16].

3 Results

3.1 Optimization of the immuno-qPCR assay

Immuno-qPCR is a development of the ELISA technique for extremely sensitive detection of specific proteins (summarized in Fig. 1). Briefly, a capture antibody is used in combi-

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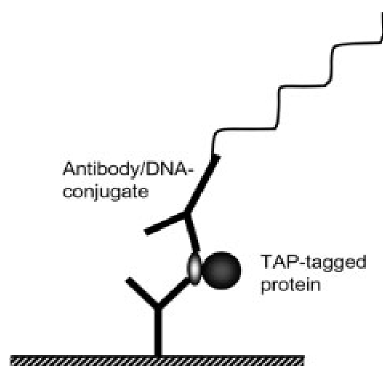

Figure 1. Schematic representation of the immuno-qPCR assay.

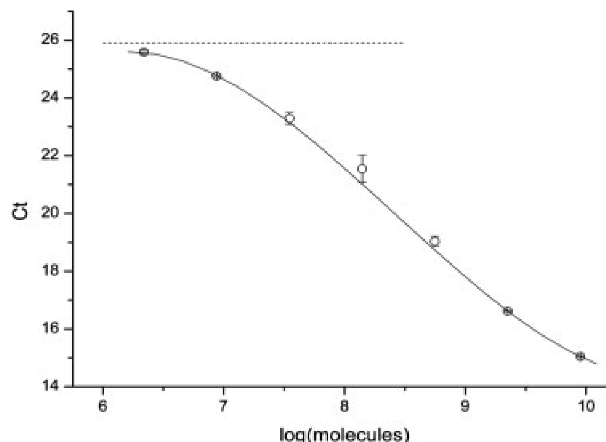

Figure 2. A representative standard curve of protein A. Samples were run in duplicates with SD as error bars. The dotted line shows the mean Ct value of two control samples without protein A.

nation with a DNA-conjugated detection antibody which allows for subsequent quantification by real-time PCR. We set out to develop the immuno-qPCR for the detection of the TAP-tag [6]. To this end, we started out with a variety of antibodies directed against either the Calmodulin binding peptide or the protein A domain. After trial of different combinations of capture and detection (DNA-conjugate) antibodies, we have achieved the best results with anti-protein A chicken IgY for both capture and detection. An advantage of this choice of antibodies is that it permits detection not only of the TAP-tag, but also of the widely used protein A tag. Another characteristic with IgY antibodies is that it does not bind to protein A by its constant region, as many IgG antibodies, but only by the variable region. In addition to choice of antibodies, we have also optimized choice of reagent concentrations, buffer composition and PCR-tube design. For example, we found milk powder in high concentration to be superior to block unspecific binding in the yeast whole cell extracts compared to BSA. We also found it very important to use a PCR tube that fits the real-time PCR cycler well. The most commonly used tube for immuno-qPCR, the TopYield strips (Nunc, Denmark), does not fit in the iCycler iQ5 PCR block and can therefore not be used in this instrument. The Robostrips (Roboscreen), however, have the same shape as regular PCR tubes and fit well giving a good heat transfer. The total assay time, excluding the over night incubation of capture antibody was about 5.5 h out of which 2 h were the real-time PCR. The incubation times might be possible to reduce and also more rapid PCR could be considered.

To allow for direct linkage between the qPCR signal and protein amount, we have used pure protein A to create a dose-response type standard curve in which the threshold cycle (Ct) is plotted as a function of protein A amount (Fig. 2). The Ct value is a measure of the cycle number at which the PCR amplification curve intersects with the set threshold value (which is an arbitrary fluorescence value in the exponential phase of the PCR). The LOD was calculated to be about 5×10^6 (as described in the Section 2) and the upper limit was roughly 10^{10} molecules of protein A (Fig. 2) after which the signal levels off (data not shown). The SD showed no bias towards either end of the standard curve. The RSD of technical triplicates was calculated to be about 8% on an average, which compares favourably to the 20–28% RSD reported for (intralaboratory) protein quantification by 2-D PAGE [17].

For extraction of proteins from yeast, we commonly use vortexing with glass beads followed by boiling in an extraction buffer composed of Tris, SDS and β-mercaptoethanol. Since the presence of SDS and β-mercaptoethanol might be suspected to influence the binding step of the immuno-qPCR or the PCR itself, we decided to investigate the extent to which it would be necessary to dilute samples. Undiluted extraction buffer had a strongly negative influence on the detection of constant amount of protein A as seen from the increased Ct values, while higher dilutions of the extraction buffer than 50 times had a negligible effect (Fig. 3A). It is also conceivable that components of the whole cell protein extract could have a strong influence on the assay, *e.g.* as found previously for the chloramphenicol-acetyltransferase (CAT)-reporter assay [18] or as a result of increased unspecific binding. We therefore tested whether a protein extract devoid of the TAP-tag would give signal above the background Ct, and indeed, a strong unspecific signal was detected with protein extracts diluted less than approximately 50 times (corresponding to a maximum total protein amount of 0.1 μg/μL) (Fig. 3B). In summary, the combination of the extraction buffer effect and the unspecific binding indicates that protein extracts have to be diluted at least 50 times (and contain less than 0.1 μg/μL total protein) to avoid errors in the quantification. However, this parameter most likely needs to be optimized for the specific system under study.

3.2 Comparison of quantification with immuno-qPCR and Western analysis

The TAP-tag has been used for large scale determination of protein abundance in yeast, with quantification performed using Western analysis [7]. However, Western blotting is a

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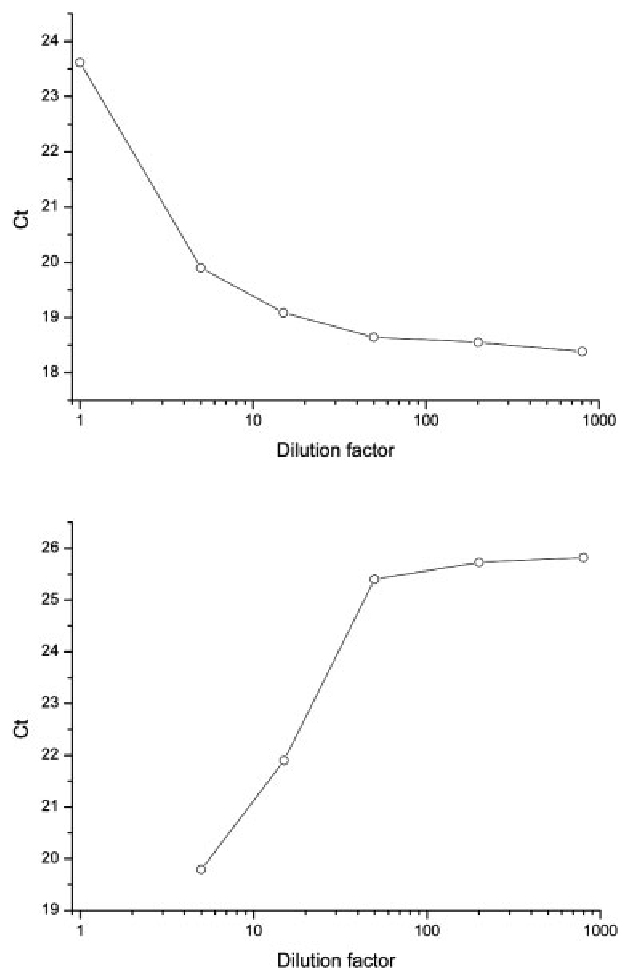

Figure 3. Ct values *versus* dilution factor. (A) Samples with 4 ng/mL of protein A in SDS-buffer was diluted to maintain the same concentration of protein A while the SDS-buffer concentration was decreasing. (B) A serial dilution of protein extracted from a wild-type strain not containing the TAP-tag.

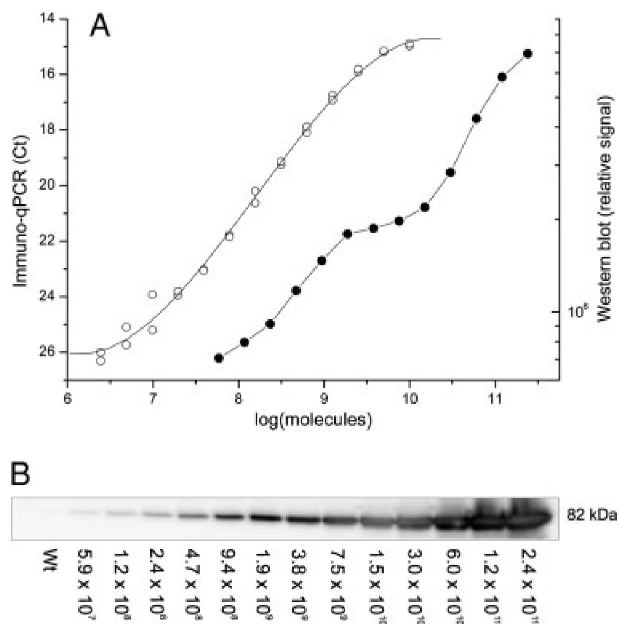

Figure 4. (A) Comparison of dilution series of TAP-tagged Hxk2p analysed by immuno-qPCR (open circle) and Western blot (filled circle). (B) Image of the Western blot membrane showing the band used for quantification. Number of molecules in each lane is indicated below.

tedious, labour-intensive method when applied to large collections of strains. We therefore decided to perform a comparative study of our immuno-qPCR method *versus* Western analysis of identical samples. Two-fold dilution series of protein extract with TAP-tagged Hxk2p was analysed both with immuno-qPCR and Western blot. The immuno-qPCR was made in duplicates while the Western blot was made with single samples. The immuno-qPCR results were plotted as Ct *versus* log(molecules) and the Western blot results as signal intensity in log scale *versus* log(molecules). The signal intensity of the Western blot was plotted from data not subjected to background subtraction (Fig. 4A). The immuno-qPCR curve has a smooth sigmoidal shape while the shape of the curve from the Western blot is irregular. The Western blot signal reaches the background at a protein copy number of about 5×10^7 (Fig. 4A), also seen in an image of the Western blot (Fig. 4B). It is evident that the Western blot signal was only useful for a span of approximately 1.5 log units, as compared to the response of the immuno-qPCR which had a useful range of approximately 3 log units. This means that the Western analysis would be hampered by the need for running more dilutions/sample, further contributing to the time needed for large-scale analysis. The qPCR method, in contrast is conceivably easy to adapt for automatization.

3.3 Comparison of immuno-qPCR quantification and the published TAP-tag and GFP-tag quantifications

We wished to compare quantification with immuno-qPCR with the published TAP-tag quantification method [7]. To this end, we first evaluated our glass bead-based extraction method (method I) in comparison to the method used in the previously published paper (method II). The two methods differed strongly in the amount of protein which was extracted from two identical cell pellets. For log-phase cells, both methods yielded approximately 2 μg/μL. However, considering that the total volume of protein extract was about 200 μL with method I and only 50 μL with method II, the first method yielded about four times the amount of protein. The superiority of method I was even more evident when using cells from postdiauxic shift phase of growth, where method I yielded approximately 3 μg/μL and method II yielded approximately 1 μg/μL which gives a huge (>10 times) difference of protein amount extracted. Surprisingly, the relative abundance of the five tested proteins, expressed in fmol/mg protein, was roughly similar irrespective of the method used

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and independent of the growth state of the cells (Fig. 5). Thus, despite the differences in the amount of protein extracted, the composition of the protein mixture seemed relatively similar, indicating that the two methods differ mainly in the percentage of cells being disrupted. Especially for postdiauxic shift cells, the lower protein concentration achieved with method II means that less total protein can be used in the immuno-qPCR analysis, thereby raising the limit threshold for protein detection (*i.e.* proteins will need a higher abundance to be detectable with method II compared to method I). In view of these results, we have chosen to use the glass-bead based method I for all subsequent experiments.

The expression of 24 proteins, chosen to represent proteins of various levels of expression (with emphasis on low expression), biophysical properties (*e.g.* membrane *versus* soluble proteins) and different subcellular localization, was then analysed with immuno-qPCR using a standard curve of known concentration of protein A that was recalculated to the corresponding number of TAP-tags (Table 1). The amount expressed as fmol/mg of total protein in the immuno-qPCR assay was plotted against the published amount of copies/cell from Ghaemmaghami *et al.* [7] (Fig. 6). The immuno-qPCR was run on three independent biological replicates and the average RSD of the measurement of the samples was 16%. Only few of the Ghaemmaghami results were made in duplicate and the SD is shown with error bar. Only for one of the proteins, Cdc27p, we did not obtain a signal above background with the immuno-qPCR method

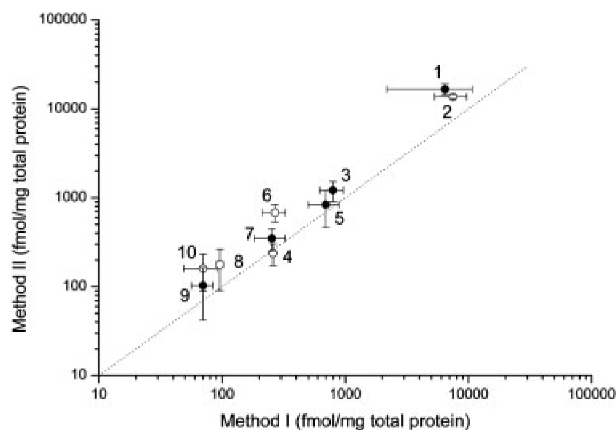

Figure 5. Correlation of protein expression between samples extracted by vortexing with glass beads and then boiling with an SDS-buffer (method I) and samples extracted with the method described in Ghaemmaghami *et al.* [7] (method II). Proteins were extracted from cells in mid-log phase (open circle) and from postdiauxic shift cells (filled circles). 1 and 2 are Hxk2p, 3 and 4 Snf1p, 5 and 6 Gpd1p, 7 and 8 Puf3p and 9 and 10 Fps1p. Samples were run in biological triplicates and the SDs are shown with error bars.

Table 1. TAP-tagged genes from Fig. 6. Quantity from Ghaemmaghami *et al.* [7] (Western blot) *versus* immuno-qPCR derived values (this study) and the protein abundance from Newman *et al.* [8] (GFP-FACS). Also given is the localization of the proteins taken from Huh *et al.* [5]

Gene	Ghaemmaghami (copies/cell)	i-qPCR (fmol/mg)	Newman (abundance)	Localization
ERG8	80	804	225	Everywhere
CDC23	80	75	n.d	Nuclear
RRN3	138	230	n.d	Nucleolus
PRI1	197	92	n.d	Everywhere
CDC13	319	111	n.d	Everywhere
SEN2	319	41	n.d	Mitochondria
CTT1	319	30	n.d	Everywhere
RRN11	476	42	n.d	Microtubule/nucleolus
SNF1	589	837	273	Everywhere
CDC27	593	n.d	50	Everywhere
PUF3	846	658	204	Everywhere
FPS1	907	384	172	Membrane
MCD1	1 041	286	138	Everywhere
TPO1	1 470	343	414	Membrane
ARH1	1 600	301	n.d	Mitochondrial IM
CLF1	2 136	218	n.d	Nuclear
NDC1	3 029	837	565	Nuclear pore
RPA43	4 072	746	358	Nucleolar
GPP2	5 000	952	238	Everywhere
HXT7	7 350	817	n.d	Membrane
HXT1	23 300	1 760	n.d	Membrane
DAK1	23 600	1 360	511	Everywhere
HXK2	114 000	5 600	10 709	Everywhere
PMA1	1 260 000	5 560	6439	Membrane

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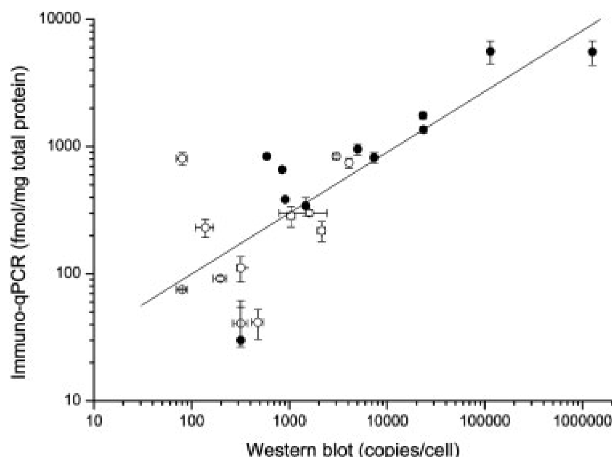

Figure 6. Protein expression of 23 different proteins measured with immuno-qPCR (in triplicate) and compared to published data from Western blot [7]. Western blot data are mainly derived from single sample (closed circle), however, some samples were performed in duplicate (open circle). The SD is shown with error bars where possible and the linear regression is drawn as a solid line ($R = 0.78$).

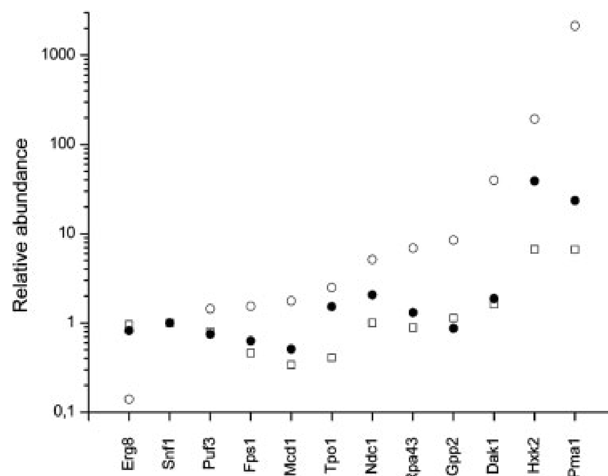

Figure 7. Proteins quantified in the present study by immuno-qPCR (open square), and also quantified in both datasets from Western blot [7] (open circle) and from GFP-FACS [8] (closed circle). Proteins plotted in order of increased abundance according to the quantification in [7]. The protein abundance in all cases expressed as fold change relative to Snf1p.

and it was therefore omitted from the graph. The correlation between the two methods was reasonable, but not exceptional, with an *R*-value of 0.78. However, the deviation between the methods is clearly higher for proteins of low abundance. There was no visible difference on extraction or SD of proteins from different localization of the cell. For 12 out of the 24 proteins, with a clear bias towards those with a high expression, there was also data from GFP-FACS measurements of Newman *et al.* [8]. This data were compared with the immuno-qPCR quantification and the Western blot quantification from Ghaemmaghami *et al* [7] in a plot where the fold change relative to Snf1p was plotted for each data set (Fig. 7). It was evident that the immuno-qPCR method gave results more consistent with the Newman GFP-FACS derived values (Pearson correlation = 0.95) than with the Ghaemmaghami TAP-Western data (Pearson correlation = 0.72).

3.4 Practical uses of immuno-qPCR

Four proteins that previously had been found to display an expression dependent on the salt concentration of the growth medium [19, 20] were chosen to exemplify the possibility of measuring protein expression fold changes with immuno-qPCR. The proteins were Hxk2p, Dak1p, Ctt1p and Gpp2p and the respective TAP-tag strains were used. Cells were grown to mid-log phase in YPD or YPD + 1 M NaCl at 30°C. The quantification was made as above using a standard curve of known concentration of protein A. Samples were quantified in triplicates and the results are plotted in Fig. 8 normalized to the amount of total protein in the samples. Duplicate samples were also analysed on Western blot and

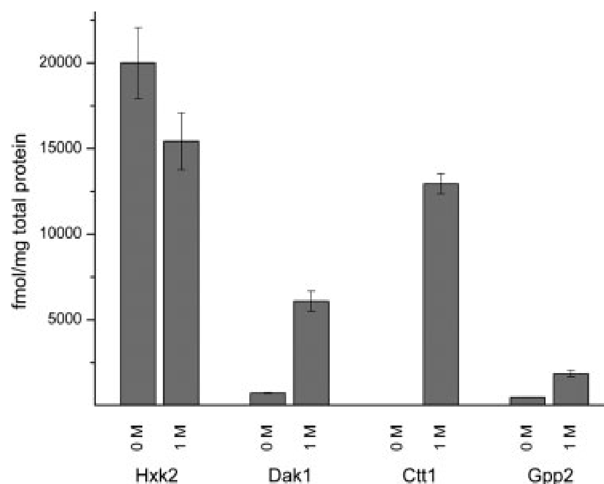

Figure 8. Protein expression of four different proteins grown on YPD or YPD + 1 M NaCl. The samples were analysed in triplicates and SD is shown with error bars. The expression of Ctt1p in regular YPD was very low, 6.7 ± 0.8 fmol/mg total protein.

relative quantification was made using the signal intensity, the results are shown together with immuno-qPCR results in Table 2. The expression of Hxk2p did not change to any large extent in our present analysis and was therefore used for normalization of the Western blot data. As expected, the expression levels of Gpp2p, Ctt1p and Dak1p were all increased in the cultures with 1 M NaCl, both as measured with immuno-qPCR and with Western blot. The expression of Ctt1p was affected the most with an increase of almost 2000 times as calculated from the immuno-qPCR data. This nicely complements previous experiments [21], in which

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Table 2. Protein expression fold change as a response to addition of 1 M NaCl in growth media

Protein	Immuno-qPCR	Western blot
Hxk2p	0.8 ± 0.1[a)]	1 ± 0.2
Dak1p	8.4 ± 0.9	2.9 ± 0.8
Ctt1p	1900 ± 200	[b)]
Gpp2p	3.9 ± 0.4	2.6 ± 0.3

a) Protein expression fold change is shown plus or minus one SD.
b) The expression of Ctt1p in YPD without 1 M NaCl was not detected above the background in Western blot.

catalase activity, *CTT1* mRNA and LacZ reporter gene activity only gave a basal, nonquantifiable level in the absence of osmotic stress. The Ctt1-TAP grown in YPD without NaCl had too low expression to give signal in the Western blot above the background and therefore no fold change could be calculated for this protein. The calculated fold change of Gpp2p and Dak1p were all lower with Western blot data than with immuno-qPCR data.

4 Discussion

A sensitive assay for quantification of TAP-tagged proteins has been developed. The detection limit was calculated to be about 5×10^6 molecules of protein A, corresponding to 1×10^7 molecules of TAP. This is slightly higher than our previously published immuno-qPCR for quantification of prostate specific antigen [13], the difference is probably mainly due to the different antibodies. The sensitivity of an assay is highly dependent on the affinity and specificity of the antibodies to the target [22] and may therefore differ between assays. The whole cell extracts needed to be diluted at least 50 times before immuno-qPCR analysis, causing a reduced sensitivity. However, compared to Western blotting the sensitivity is still roughly ten times higher as shown in Fig. 4. The 50 times dilution is needed due to effects of SDS in the extraction buffer and unspecific binding of components, *e.g.* proteins, in the extract. The SDS effect is probably partly a negative effect on the capture antibody and antigen binding, but a negative effect was also seen on the PCR itself. The amplification curves from samples with undiluted SDS buffer showed flatter slopes than the diluted samples (data not shown), indicating inhibited PCR. Interestingly, this effect was seen even though the PCR was run after a total number of 22 repeats of washing after the addition of the SDS containing sample. Most likely, the SDS is bound to the well and released into the reaction at the denaturation step of the PCR.

The major advantages of the immuno-qPCR method are the smooth shape of the standard curve and the ease with which the background value can be calculated. In contrast, the Western blot assay suffers from problems with signal saturation and background subtraction. Furthermore, because the sample number is strongly limited on Western blot gels, it is almost impossible to obtain a representative standard curve and at the same time analyse a reasonable number of samples. Using immuno-qPCR and the 96-well format, a standard curve can be run at the same time as many unknown samples are analysed which will vastly improve the quality of the data. The other problem with Western blot, background subtraction, arises from the difficulty to define a correct background and correct area to use for quantification, since background signal can be unevenly distributed over the membrane and contribute differently to different wells. In the immuno-qPCR, the background value is easily defined by including a negative control sample.

The comparison of our immuno-qPCR data on protein expression to the published TAP-tag-Western derived data [7], showed that the correlation was reasonable. However, the correlation was lower for the proteins of low abundance. Recently, the collection of GFP-tagged strains was used for quantification of proteins using FACS-analysis [8]. The GFP-FACS data are obtained from measurements on living cells and are therefore not subject to technical problems deriving from extraction of proteins and subsequent quantification. Twelve of the proteins in our analysis were also quantified in the FACS-study and it was clear that these quantities correlated much better with our immuno-qPCR data than with the dataset from TAP-Westerns. Since we have demonstrated that the extraction method difference does not give a major difference in relative protein abundance, we are left with suggesting that the deviant protein quantifications derive from the Western blot analysis. This in turn leads us to suggest also that the low abundance proteins are more accurately quantified by immuno-qPCR than by TAP-Western analysis. We conclude that the *in vitro* immuno-qPCR method gives protein quantification results in good agreement with the *in vivo* GFP-based method. The immuno-qPCR method is therefore especially suitable in complementing the GFP-method for quantification of proteins with a low abundance.

The protein expression analysis of the different yeast strains grown on YPD with and without 1 M NaCl clearly showed the potential of the immuno-qPCR technique. Differences in expression were compared using quantification made from a standard curve. The upregulation of Dak1p, Ctt1p and Gpp2p as a response to salt stress was consistent with earlier results [19–21] and was also shown on Western blotting. Ctt1p was expressed to a very low level without salt induction and was on the border to be detected with the immuno-qPCR and could not be detected above background with the Western blot. The expression was calculated to be upregulated almost 2000 times with the data from the immuno-qPCR. The already published 2-D data show lower upregulation but most likely overestimates the expression of proteins close to background and therefore gives a lower

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value of upregulation. Generally the Western blot data also showed lower upregulation than the immuno-qPCR. But the data from Western blot were analysed just by relative comparison of the signal, without any standard curve included, and the calculations are therefore likely to be incorrect. As shown above, the dose-response curve of Western blots is irregular and the results will often be underestimations of protein quantities.

The method described in this paper is rapid, sensitive and accurate, which also leads us to suggest using TAP in combination with immuno-qPCR as a reporter gene with the same potential uses as the commonly used LacZ/β-galactosidase and CAT. Using immuno-qPCR for detection also avoids the negative effects of protein extract amount on reporter enzyme activity [18]. We have performed a trial in which *COX17* promoter drives expression of the TAP-tag followed by a 3′untranslated region (3′UTR) from *COX17*. Expression of this construct in a wild-type strain and in a *puf3*Δ::KanMX strain gave a 2.9-fold increase in TAP expression, which compares well to the data from a similar LacZ-expressing plasmid (data not shown) and which also fits well with the published *COX17*-specific effect of a *puf3*-deletion [23].

By using the TAP-tag for detection, the same antibody and immuno-qPCR assay can be used for investigation of almost any protein in organisms amenable to genetic alterations (*e.g. S. cerevisiae*). This is a major advantage compared to having to obtain specific antibodies to each individual protein and conjugating DNA-labels to them. One challenge with immuno-qPCR is otherwise the conjugation of a DNA-label to the detection antibody. Therefore, to have a universal immuno-qPCR assay for all proteins that can be tagged with TAP is highly advantageous. Because the TAP-tag is not restricted to yeast, proteins from other species ranging from *Escherichia coli* to humans [24], can be analysed with this immunoassay. For the future, it would be extremely useful to develop the immuno-qPCR assay for all commonly used protein-tags, *e.g.* GFP and antibody epitopes.

In conclusion, we have developed an immuno-qPCR assay that is sensitive, precise and easy to use. Furthermore, all steps of the method, from cell growth to protein extraction and determination and the immuno-qPCR reaction itself are amenable to automatization. We have shown that it is useful for protein expression quantification, fold change calculations and also for quantification of the TAP-tag as a reporter gene. Because the TAP-tag is not species specific this immunoassay could also be used in other organisms than *S. cerevisiae.*

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