Applications

Note 221 • March 2010

# Photometric quantification of the β-galactosidase activity for the analysis of the relative interaction strengths between signal transduction proteins from *Schistosoma mansoni* in the Eppendorf BioPhotometer plus

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## Abstract

**The Src tyrosine-kinase SmTK3 is involved in processes which control mitotic activity and differentiation in *Schistosoma mansoni*. Using the yeast two-hybrid system it was possible to identify several interaction partners of SmTK3, and their relative interaction strength were determined via a β-galactosidase liquid assay. The indicator reaction of this assay was the conversion of the substrate ONPG (ortho-nitrophenyl-β-D-galactopyranoside) to ONP (ortho-nitrophenol) and D-galactose. The increase in ONP was measured in the Eppendorf BioPhotometer plus *via* absorbance measurements at 405 nm. Using this reaction, the relative interaction strength of potential SmTK3 binding partners could be determined. Thus, a subunit of a multi enzyme complex, which is involved in chromatin-reorganization processes, could be identified as the strongest partner.**

## Introduction

The trematode *Schistosoma mansoni* is a pathogenic parasite of humans and animals, and it is the causative agent of the tropical schistosomiasis (bilharzia), one of the most prevalent parasitic diseases worldwide, second only to malarial in the tropics [1]. Schistosomes are dioecious, and the adult stages parasitize the venous system of the final host in a state of permanent pairing contact. One unique feature of the biology of schistosomes is that mitotic and differentiation processes leading to the development of the female reproductive organs (ovary and vitellarium) are triggered by this pairing contact with the male [2,3]. Paired females produce approximately 300 eggs per day, which are responsible for the pathogenicity of *Schistosoma* [4]. Hence, the elucidation of the pairing-induced development of the female gonads may be crucial for the development of novel strategies to combat this parasite. One molecule, whose involvement in mitosis, and differentiation processes was demonstrated in previous studies, is the Src tyrosine-kinase SmTK3 [5,6]. This signal transduction molecule consists of different functional domains, such as an SH4 domain, which enables an association of the molecule to the plasma membrane following myristylization. This domain is connected at its C-terminus to an SH3 domain *via* the “unique site”. This SH3 domain is required for binding to the proline-rich sequences of interaction partners, which are located downstream of Src kinases in a signaling hierarchy. Next to the SH3 domain is a SH2 domain, which binds to phosphorylated tyrosine residues of interaction partners acting upstream in a signaling cascade. At the C-terminus the catalytic tyrosine kinase domain is located, which is required for autophosphorylation as well as substrate phosphorylation [7]. Due to the central role of the SmTK3 in gonad development in *S. mansoni*, it was of interest to identify binding partners of this kinase, whose identity could unravel the signal transduction pathways in which SmTK3 is involved.

The yeast two hybrid system (Y2H) is a molecular tool, which is frequently used to identify protein-protein interactions [8]. Its function is based on transcription factor (TF) -mediated, inducible reporter-gene activity. For this purpose among others the yeast TF Gal4 is used. Gal4 consists of an transcription activation domain (AD) and a DNA binding domain (BD) [9]. Binding of this TF to the DNA occurs *via* the upstream-activating sequence of a minimal promoter, which controls transcription of the reporter genes. The AD is responsible for transcription initiation. Only if both domains are present and in contact to each other, the Gal4 TF is able to bind the upstream-activating sequence.
For Y2H analyses, both domains of the Gal4 TF have to be cloned separately into different vectors. The vector encoding the Gal4 BD additionally contains the sequence of a known gene (bait), for which these interaction studies were performed. Furthermore, this vector contains a growth selection marker for the amino acid tryptophan. The second vector contains the Gal4 AD, as well as the sequence of a potential interaction partner (prey). This prey may be either a known protein, or an unknown protein derived from a cDNA library. With the help of such a Y2H library it is possible to  to search for potential interaction partners by performing a Y2H screening with a bait construct. The vector that is used for the prey construct contains a growth selection marker for the amino acid leucin. Interaction studies are performed using yeast strains which are auxotrophic for the above mentioned amino acids. Thus, it is possible to select for yeast containing both vectors following transformation. Finally, reporter gene activities (further amino acid selection markers and/or enzymes) indicate the interaction of proteins.

In order to be able to quantify protein-protein interactions, the reporter gene *lacZ* is used. *LacZ* is integrated into the genome of appropriate yeast strains, and its expression is controlled by a minimal promoter, which is activated by the Gal4. The *lacZ* gene encodes the β-galactosidase (β-Gal), which is able to cleave the substrate ONPG (ortho-nitrophenyl-β-D-galactopyranoside) to ONP (ortho-nitrophenol) and D-galactose, thus generating a yellow color. The intensity of color formation depends on the activity of the enzyme and can be measured photometrically. Thus relative interaction strengths of two proteins can be determined since the reporter gene *lacZ* is only expressed under the control of a reconstituted Gal4 TF. Substrate conversion correlates with the amount of β-Gal, whose expression level depends on the strength of interaction. Conversion of ONPG to ortho-nitrophenol was measured in the Eppendorf BioPhotometer plus at 405 nm.

The Y2H screening of a *Schistosoma* cDNA library led to the identification of interaction partners of the src tyrosine kinase SmTK3 [10], whose relative interaction strengths were determined in this study using a β-Gal liquid assay.

## Materials und Methods

### Cloning of the *S. mansoni* Y2H-cDNA library

The Matchmaker™ III system [11] was used as the basis for cloning of a Y2H cDNA library based on Gal4. The mRNA from adult Schistosomes was reverse transcribed into cDNA using oligo dT primer. Subsequently, the cDNA was cloned into the *SmaI*-site in frame with the Gal4 AD into the prey vector pGADT7-Rec [11], which contained the growth selection marker Leu-2 [10].

### Cloning of the bait sequence of SmTK3

In order to identify putative downstream interaction partners of the Src kinase SmTK3, a bait construct was cloned into the pGBKT9 vector consisting of the “unique site“ and the SH3-domain (US-SH3-bait) [10,11].

### Co-transformation of the yeast

Co-transformation of yeast cells (AH109, Clontech) with bait and prey vectors was performed using the lithium acetate method [12]. To this end, 0.1 µg prey and bait plasmid DNA were mixed with carrier DNA, followed by the addition of 100 µL competent cells. To increase uptake of plasmid DNA, a 40 % PEG/LiAc solution (polyethylene glycol/lithium acetate solution) was used. The cell-DNA mix was incubated for 30 minutes at 30 °C and 200 rpm. Following the addition of 70 µL dimethyl sulfoxide (DMSO), the cells were heated to 42 °C for 15 minutes and subsequently cooled on ice for 2 minutes. Following brief centrifugation, the cells were resuspended in 200 µL 1x TE buffer. The cell suspension was streaked on the appropriate selection plates and incubated at 30 °C for 2-4 days.

### β-galactosidase-liquid assay

Yeast cells transformed with bait and prey plasmids were grown over night (o/n) in 5 mL selection medium and incubated at 30 °C and 200 rpm. Of this culture 2 mL were transferred to complete medium (1xYPD) and incubated until an $OD_{600}$ of 0.5 to 0.8 was reached.

The cells were then pelleted by centrifugation, resuspended in 1.5 mL Z-buffer and centrifuged again. The pellets were resuspended in 300 µL Z-buffer, thus resulting in a volume reduction from 1.5 mL to 300 µL and a concentration factor of 5 (1.5 / 0.3 = 5). Two aliquots of 100 µL each were transferred to fresh reaction tubes, six aliquots of Z-buffer (100 µL each) were treated the same way. Subsequently, in order to measure β-Gal enzyme activity, the yeast cells were lysed via freeze-thaw cycles. The enzymatic color reaction was initiated by the addition of 700 µL Z-buffer with 1.9 µL β-mercapto ethanol as well as 160 µL ONPG solution (solubilized in Z-buffer) to the samples. The samples were incubated at 30 °C until considerable coloring of the solution could be observed. The reaction was stopped by the addition of 400 µL 1 M $Na_2CO_3$. After centrifugation, the absorption values of the samples, including one reference reaction, were measured in the Eppendorf BioPhotometer plus at 405 nm instead of at 420 nm, the wavelength recommended by Clontech. Previous measurements at 420 nm (photometer by a different manufacturer) and 405 nm (BioPhotometer plus) had shown that the small difference in wavelength did not have an impact on the final results.

Calculation of the β-Gal units was conducted using the following formula:

$$\beta\text{-galactosidase activity} = \frac{1000 \times OD_{405}}{t \times V \times OD_{600}}$$

The calculated β-Gal units are equivalent to the amount of enzyme which cleaves 1 µmol ONPG per minute and yeast cell [13]. The $OD_{405}$ results from the measured absorption of the ortho-nitrophenol; t is the incubation time in minutes; V equals the volume of 0.1 mL with a concentration factor of 5; and the $OD_{600}$ value is equal to the measured absorption of the o/n liquid culture at 600 nm. With this method a statement of the relative binding strengths for protein-protein interactions can be made.

**Settings on the BioPhotometer plus:**
In order to determine the optical density of the yeast culture, the method $OD_{600}$ was chosen on the BioPhotometer plus. Culture medium without cells was used as a blank value. An example of the results is shown in fig. 1.


**Fig. 1: Determination of the optical density of yeast cultures at 600 nm.**
**A** Method "$OD_{600}$" was chosen
**B** Determination of the blank value by measuring culture medium without cells
**C** Determination of the optical density (example 1)
**D** Determination of the optical density (example 2)

The absorption of ONP for the determination of β-galactosidase activity was performed at 405 nm in the Eppendorf BioPhotometer plus. To this end, for "absorbance", the wavelength 405 nm was chosen, and the absorption values were measured for each sample following determination of the blank value (an example is shown in fig. 2). The detections were carried out in Eppendorf UVettes®.

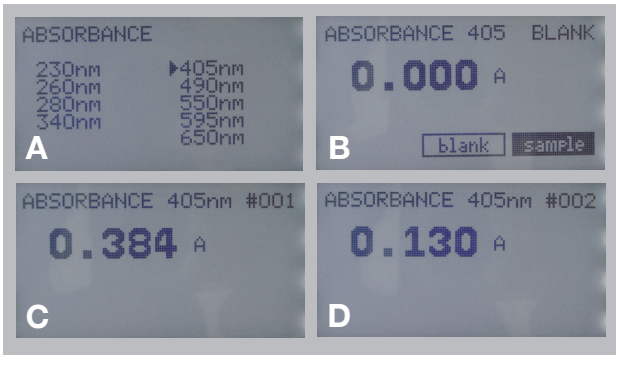


**Fig. 2: Determination of the absorption at 405 nm.**
**A** Choice of wavelength via the key "Absorbance"
**B** Determination of the blank value
**C** Absorption of the sample at 405 nm (example 1)
**D** Absorption of the sample at 405 nm (example 2)

## Results and Discussion

To investigate signaling pathways in which the Src tyrosine-kinase SmTK3 is involved, a Y2H screening was performed. For the identification of interaction partners which act downstream of this tyrosine kinase, the SH3 domain with the N-terminal "unique site" (US-SH3) was used as bait. This led to the identification of 39 prey clones, which were sequenced. According to subsequent data bank analyses, these clones were allocated to 9 homology groups. These included diaphanous proteins, the eukaryotic translation initiation factor eIF4$\gamma$2, the BAF60 subunit of the SWI/SNF complex, a YME1-like metalloprotease and a mRNA (guanin-7) methyltransferase. Further clones displayed homology to a quinolinate phosphoribosyl transferase, an SH3-binding protein from the brown rat (*Rattus norvegicus*) and a Smad protein (Hrsmad 2/3) from the sea squirt *Halocynthia roretzi*.
For further 18 clones, only a weak homology to human pericentrin B could be determined *in silico* [11].

For confirmation of the discovered interactions, and to analyze the contribution of the "unique site" and the SH3 domain, respectively, to these interactions, one representative clone from each homology group, was re-transformed into the yeast strain AH109 together with either a SH3- or an SH3-US-bait construct. Subsequent growth was performed under selective conditions. Cells of the AH109 strain transformed with the US-SH3 bait construct alone were used as a negative control. In contrast to the negative control, only yeast which were co-transformed with both bait and prey constructs were able to grow under conditions selecting for reporter gene activity. The relative activities of $\beta$-Gal, which was induced and expressed as a result of the interactions between bait and prey proteins, were determined via substrate conversion of ONPG at an absorption wavelength of 405 nm. For these measurements, the Eppendorf BioPhotometer plus was used.

The results determined for the relative interaction strengths of both SmTK3 constructs with their potential binding partners are summarized in fig. 3. For all clones, interactions with both constructs could be detected. As expected, no evidence for interaction was found for the negative control. The SH3-bait construct as well as the US-SH3-bait construct showed the strongest interactions with the BAF60-subunit of the SWI/SNF complex (SWI/SNF-BAF60). This protein is a subunit of a multi-enzyme complex, which is involved in reorganization processes of chromatin [14].

Latest studies have revealed that tyrosine phosphorylation by Src kinases may also occur in the nucleus, where they play a role in structuring processes of chromatin, triggered by growth factors [15]. These results suggest that the Src kinase SmTK3 could phosphorylate the BAF60 subunit of *S. mansoni* inside the nucleus, and thus influence processes involved in chromatin reorganization. The results of this $\beta$-Gal liquid assay further demonstrate that the "unique site" of the SmTK3 contributes strongly to the binding efficiency of this kinase with its partners. For almost all interaction partners of the SmTK3, an increase in the relative strength of interaction is observed in the presence of the "unique site".

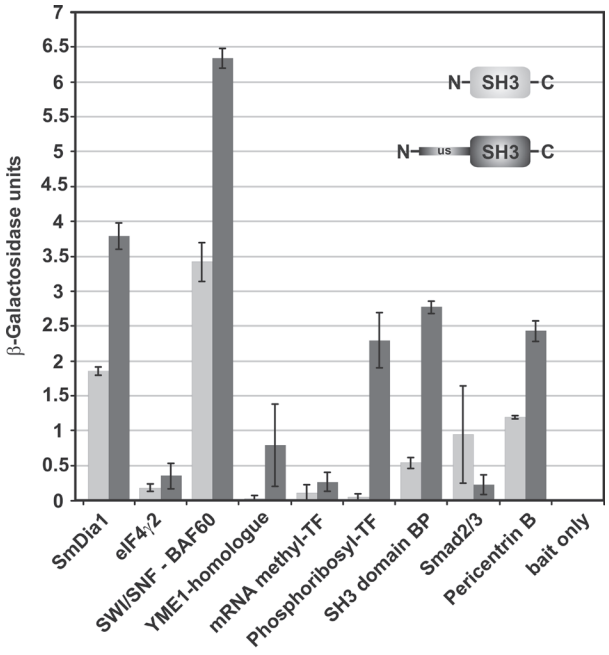


**Fig. 3: Results of the $\beta$-Gal liquid assay for quantification of the relative interaction strengths between SmTK3 and interaction partners identified in the Y2H screen.**

The figure shows the calculated units of $\beta$-Gal activity of the prey clones from the Y2H screening with the SmTK3 SH3 domain (light gray bars) and the SmTK3 SH3 domain in combination with the "unique site" (dark gray bars), respectively. Representative prey clones were first co-transformed with the bait constructs into AH109 yeast cells and subsequently used for this assay (n=6). The used prey clones were Diaphanous (SmDia1), the eukaryotic translation initiation factor (eIF4$\gamma$2), the BAF60 subunit of the SWI/SNF complex (SWI/SNF-BAF60), the YME1-like metalloprotease (YME1-homologue), the mRNA (Guanin-7) methyltransferase (RNA methyl-TF), the quinolinate-phosphoribosyl transferase (Phosphoribosyl-TF), the SH3 binding protein (SH3 domain BP), the Smad protein (Smad2/3), and pericentrin B. As a negative control, AH109 cells were transformed with the bait plasmid US-SH3 alone (bait only).

## Conclusions

Y2H screenings are suitable to identify protein-protein interactions and to detect potential binding partners for proteins of interest. By measuring the cleavage product ortho-nitrophenol (ONP) at 405 nm in the photometer, the relative interaction strengths can be determined *via* indirect measurement of β-Gal activity

The Eppendorf BioPhotometer plus convinces through its clear data display and user friendliness throughout the process. The measurement of ONP at 420 nm, as recommended by the manufacturer (Clontech), may be performed at 405 nm without significant impairment of the results.

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