Methods 58 (2012) 376–384

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

# Quantitative real-time PCR as a sensitive protein–protein interaction quantification method and a partial solution for non-accessible autoactivator and false-negative molecule analysis in the yeast two-hybrid system

Richard H. Maier [1], Christina J. Maier [1], Helmut Hintner, Johann W. Bauer, Kamil Önder *

*Department of Molecular Dermatology, Paracelsus Medical University, Salzburg, Austria*

ARTICLE INFO

*Article history:*
Available online 13 September 2012
Communicated by Peter Uetz

*Keywords:*
Yeast two-hybrid
Protein–protein interaction
Relative quantification
Quantitative real-time PCR
False positives/negatives
Autoactivators

ABSTRACT

Many functional proteomic experiments make use of high-throughput technologies such as mass spectrometry combined with two-dimensional polyacrylamide gel electrophoresis and the yeast two-hybrid (Y2H) system. Currently there are even automated versions of the Y2H system available that can be used for proteome-wide research. The Y2H system has the capacity to deliver a profusion of Y2H positive colonies from a single library screen. However, subsequent analysis of these numerous primary candidates with complementary methods can be overwhelming. Therefore, a method to select the most promising candidates with strong interaction properties might be useful to reduce the number of candidates requiring further analysis. The method described here offers a new way of quantifying and rating the performance of positive Y2H candidates. The novelty lies in the detection and measurement of mRNA expression instead of proteins or conventional Y2H genetic reporters. This method correlates well with the direct genetic reporter readouts usually used in the Y2H system, and has greater sensitivity for detecting and quantifying protein–protein interactions (PPIs) than the conventional Y2H system, as demonstrated by detection of the Y2H false-negative PPI of RXR/PPARG. Approximately 20% of all proteins are not suitable for the Y2H system, the so-called autoactivators. A further advantage of this method is the possibility to evaluate molecules that usually cannot be analyzed in the Y2H system, exemplified by a VDR–LXXLL motif peptide interaction.



## 1. Introduction

Proteins are the principal agents of cellular processes. The function of most proteins is mediated through their interaction partners and often through large protein complexes. Therefore, investigating protein interactions is essential to understanding biological systems [1]. All proteins interact with other molecules, and the number of interactions per protein varies between 0.1 and 24 [2–5]. Proteins with a high diversity of interaction partners and multifunctional activities are called "high-connected nodes" or "hubs", and comprise an estimated 90% of the human interactome [6]. Elucidating the structures and functions of proteins is the main challenge of proteomics, one that is greatly facilitated by the study of protein–protein interactions (PPIs). The first high-throughput tool used in proteomics was two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). In more recent years this powerful method was augmented by mass spectrometry (MS) [7,8] and further enhanced by PPI techniques such as Tandem Affinity Purification in combination with MS for interaction partner analysis [9].

A second powerful method for the detection of PPIs is the Y2H system, invented in 1989 by Fields and Song [10]. This was further refined by many other investigators and used in proteome-wide studies to map the human interactome [11,12]. As with other PPI methods, especially in high-throughput applications, the major advantage of the Y2H system is its ability to screen vast numbers of candidates. Its main disadvantage is the high rate of false-positive and false-negative PPIs [5,13–18]. Perhaps the best strategy to reduce the rate of false positives/false negatives would be to combine automated Y2H analysis with 2D-PAGE/MS; however, this may not be technically feasible and the cost is likely to be prohibitive. Additionally, within the Y2H system is a major class of "false positives", the so-called autoactivators, which are proteins that can activate reporter genes without interacting with a second protein [19]. The mechanisms by which autoactivators function are not completely understood. This class of false-positives can increase the number of selected colonies per screen, which increases both the cost and workload of subsequent investigations to sort out the true interactions. A solution for false positives does not

* Corresponding author. Address: Department of Dermatology, Paracelsus Private Medical University Salzburg, Müllner Haupstraße 48, 5020 Salzburg, Austria. Fax: +43 662 44823044.
*E-mail address:* k.oender@salk.at (K. Önder).
[1] These authors contributed equally to this work.

1046-2023/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymeth.2012.09.001

yet exist, with the exception of additional complementary methods such as pull-down or immunoprecipitation analysis. Another problem for Y2H and other PPI technologies are the "false negatives". This term refers to proteins which should interact or are sometimes even reported to interact in the literature but are not identified by Y2H screening. In contrast to the well-known and described problem of autoactivators in the Y2H system, there seems to be no solution for the false-negative problem. Additionally, Y2H analysis requires that the bait protein be checked for autoactivity. This is cumbersome at best and it may not even be possible. We have developed a method which circumvents these problems.

Quantification of protein interactions can be indirect, such as the reporter activation measurement in the Y2H system. Many methodologies have been invented to signal interactions; the most common method for quantification is to detect β-galactosidase activity [20,21]. Other Y2H quantification methods utilize growth curve analysis [22], dual-luciferase assays [23], or fluorescence reporter protein detection [24–26]. In summary, all these techniques measure PPIs indirectly in the Y2H system. The readout of expressed reporter proteins as cell-growth or enzyme activity does not necessarily reflect the real amounts of mRNA expressed from the reporter genes due to variable mRNA/protein stability or translational activity.

Here we describe a new method for analysis and quantification of PPIs within the Y2H system by measuring the transcript level of reporter genes by quantitative real-time PCR (qRT-PCR). We designed DNA-probes for reporter gene amplification, optimized growth and analysis conditions, and constructed a set of known and affinity-measured PPIs to evaluate the sensitivity of this method. We demonstrated the functionality of the new method and the correlation with standard indirect genetic reporter measurements such as the growth of yeast cells or β-galactosidase quantification which are usually used in the Y2H system. The analyzed PPIs correlate well with direct protein-interaction measurements [e.g., surface plasmon resonance (SPR) measurements]. Additionally, we demonstrated greater sensitivity of this method compared to existing techniques. Due to the high sensitivity of qRT-PCR, it was possible to detect false-negative PPIs. Finally, we were also able to discriminate between autoactivators and authentic Y2H interactions, thus enabling screening using proteins which previously were not suitable for analysis in the Y2H system.

## 2. Materials and methods

### 2.1. Y2H plasmid construction

The amino acid sequences of the Test-Peptides P1, P1-a1, and P7-14 [27] were reverse translated into their nucleotide sequence. The obtained sequences were 5′-TATGGTTTGTGGATTTTGTGG TGTGATGAGGAGGGTTTGGATTTGGGT-3′ for P1, 5′-TATGGTAGGT GGATTTTGTGGTGTGATGAGGAGGGTTTGGATTTGGGT-3′ for P1-a1, and 5′-ACTACTTGGGAGGAGAGGTTGAGGTGTGAGGAGAATGGTTT GGGTGTT-3′ for P7-14. These and reverse complement oligonucleotides were designed with compatible ends for an EcoR1 site at the 5′ side and a BamH1 site at the 3′ side with an additional guanine nucleotide between the restriction site and the sequence to retain the reading frame. The pGADT7 vector (Clontech, Mountain View, CA, USA) was restriction digested with EcoR1 and BamH1 (both New England Biolabs, Ipswich, MA, USA). Respective oligonucleotides were annealed and ligated into the digested prey vector. As a bait protein, the nucleotide sequence coding for amino acids 300–928 of human retinoblastoma protein 1 (RB1; NCBI accession No. NM_000321) was cloned by recombination into the pBD-Gate2 vector [28]. The respective nucleotide sequence was PCR-amplified with gene-specific forward (5′-AAAAAGCAGGCTTGATGAATTCTCTT GGACTTGTAAC-3′) and reverse (5′-GTACAAGAAAGCTGGGTATT TCTCTTCCTTGTTTGAGGTAT-3′) primers as well as adapter primers as described by Brandner et al. [29]. A commercial human bone marrow cDNA library in the Gateway® compatible pCMV-SPORT6 vector (Invitrogen, Carlsbad, CA, USA) served as template. The resulting 1.9 kb PCR product was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the kit's instructions. This product was cloned through a BP recombination reaction into pDONR™/Zeo (Invitrogen) and subsequently recombined into the pBD-Gate2 vector according to the Gateway® cloning (Invitrogen) product manual.

Additional test plasmids were constructed by the same method using Gateway® BP and LR recombination. Human full-length peroxisome proliferator-activated receptor gamma (PPARG; NCBI accession No. NM_005037) was cloned into pAD-Gate2 [28] and human full-length retinoid x receptor alpha (RXR; NCBI accession No. NM_002957) was cloned into pBD-Gate2. For PPARG the following forward 5′-AAAAAGCAGGCTCCATGGTTGACACAGAGAT-3′ and reverse 5′-AGAAAGCTGGGTACTAGTACAAGTCCTTGTAGAT-3′ primers were used. The forward and reverse RXR primers were, respectively, 5′-AAAAAGCAGGCTTGATGGACACCAAACATTTCCTG-3′ and 5′-AGAAAGCTGGGTACTAAGTCATTTGGTGCGGC-3′. Each constructed plasmid was verified by standard dideoxy sequencing. Furthermore, in a previous Y2H screen [28] performed with the human vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR) as the bait molecule, the human Ski-interacting protein (SKIP; UniProt accession No. Q6P151) was identified (in pAD-Gate2) and used in the present study.

As a positive control for Y2H, the interaction between p53 and simian virus 40 (SV40) large T-antigen cloned in pBD-Gate2 and pAD-Gate2, respectively, [28] was used, and as negative control the two vectors pAD-Gate2 and pBD-Gate2 themselves were used.

### 2.2. Construction of an artificial Y2H LXXLL motif peptide library

An LXXLL motif-containing peptide library was generated using the oligonucleotide 5′-AAAAAGCAGGCTTG$(NNY)_7$CTG$(NNY)_2$($-$CTG$)_2(NNY)_7$TAATACCCAGCTTTCT-3′ (N is a mixture of all four deoxynucleotides; Y is a mixture of the deoxynucleotides C and T) as template for a PCR with the following forward 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTG-3′ and reverse 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTA-3′ primers. The resulting 120-bp PCR product was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega) according to the kit's instructions. The purified LXXLL PCR products were recombinated into the pDONR™/Zeo (Invitrogen) vector by BP reactions to create an entry library according to the Gateway® BP clonase II enzyme mix kit's instructions (Invitrogen). After transformation of the reaction mixture and propagation in *Escherichia coli*, plasmids were isolated and consecutively recombinated through an attL × attR recombination reaction [30] into the pBD-Gate2 vector. After bacterial transformation of the recombination products, the resulting *E. coli* colonies were subjected to plasmid isolation. Overall, $1 \times 10^6$ primary clones, representing at least the same number of different LXXLL-peptides, were obtained by this cloning reaction. Human VDR (NCBI accession No. NM_000376) was cloned into pAD-Gate2 as described [28]. To confirm the diversity of the created library, 20 randomly chosen clones were sequenced by standard dideoxy sequencing.

### 2.3. Standard Y2H analysis and quantification of protein–protein interactions

Bait and prey vectors were transformed together into the haploid yeast strain AH109 (MATα) from the Matchmaker™ Two-Hybrid System (Clontech) according to the kit manual, and one half of each transformation was plated onto medium lacking tryptophan