Chapter 8

Options and Considerations When Using a Yeast One-Hybrid System

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Abstract

Comprehensive mapping of protein–DNA interactions is essential to uncover the mechanisms involved in gene regulation. However, the data generated has been sparse given the number of regulatory elements and transcription factors (TFs) encoded in the genomes of metazoan organisms. Yeast one-hybrid (Y1H) assays provide a powerful "DNA-centered" method, complementary to "TF-centered" methods such as chromatin immunoprecipitation, to identify the TFs that can bind a DNA sequence of interest. Here, we present different technical variations that should be considered when using a Y1H system, including the type of DNA sequence to test, source of TF clones, as well as types of vectors and screening format. Finally, we discuss limitations of the assay and future challenges.

Key words Yeast one-hybrid, Protein–DNA interactions, DNA-centered, Reporters, Screening

1 Introduction

The study of gene regulation requires a comprehensive identification of all possible interactions between regulatory regions and the transcription factors (TFs) that bind to them. However, this has proven to be a daunting task given that the genomes of multicellular organisms harbor hundreds of thousands of regulatory regions and can encode for >1000 TFs [1–4]. To date, no single experimental approach has been able to interrogate the entire range of possible protein-DNA interactions (PDIs) demonstrating the need for complementary TF-DNA mapping methods. These methods can be classified as TF-centered, if they test the set of targets of a particular TF; or DNA-centered, if they interrogate the repertoire of TFs that bind to a DNA region of interest (Fig. 1a). The most widely used TF-centered methods are chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) that detects in vivo binding, and protein binding microarrays (PBMs), SELEX and bacterial one-hybrid assays that identify TF binding motifs [5, 6].

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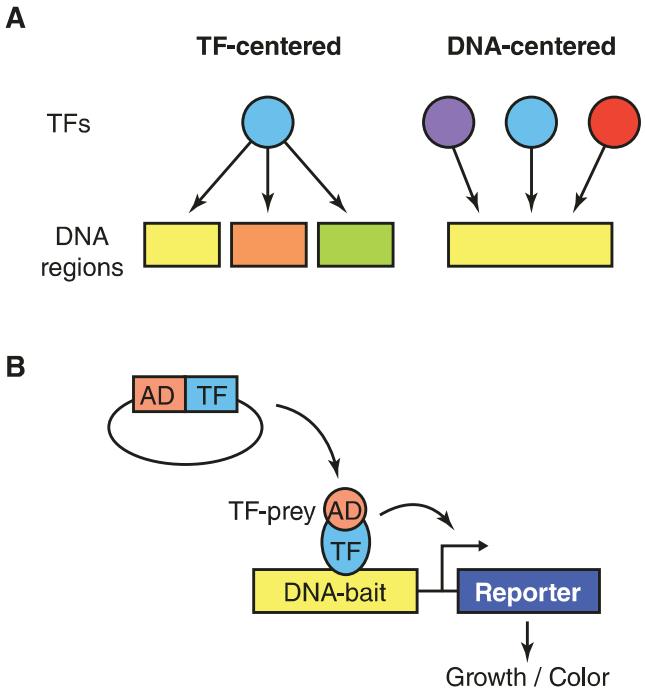

Fig. 1 Yeast one-hybrid (Y1H) assays. (**a**) Protein–DNA interaction mapping methods can be classified as TF-centered, if they interrogate the targets of a particular TF, or as DNA-centered, if they interrogate the repertoire of TFs that bind to a DNA region of interest. (**b**) Schematics of Y1H assays. A DNA sequence of interest (DNA bait) is cloned upstream a reporter gene and is used to "fish" for interacting TF preys (i.e., TFs fused to the yeast Gal4 activation domain, AD). A positive interaction is detected by growth (auxotrophic markers) or color (enzymes such as β-galactosidase)

Yeast one-hybrid (Y1H) assays are the most commonly used DNA-centered methods to map PDIs [7] (Fig. 1b). This method, which detects interactions in the milieu of the yeast nucleus, involves two main components: (1) a reporter construct comprised of a DNA sequence of interest (the DNA bait) cloned upstream of a reporter gene; and (2) a plasmid encoding for a protein prey (frequently a TF) fused to the activation domain (AD) of the yeast TF Gal4. When both components are introduced into a yeast cell, the bait can be used to "fish" for interacting preys. If the TF prey binds to the DNA sequence of interest, the AD will induce the expression of the reporter gene. Given that transcription is induced by the yeast AD, interactions involving both activators and repressors can be detected.

Y1H assays have been successfully used to identify PDIs between TFs and multiple types of DNA sequences including promoters, enhancers, and silencers [8–11], noncoding disease variants [10], and short DNA sequences containing TF binding motifs [12, 13]. In addition, different TF resources can be used in the screens such as a collection of TFs present in a particular organism, TF missense vari-

ants associated with disease, and TF isoforms [10, 14]. Y1H assays can circumvent many limitations faced by TF-centered methods. For instance, Y1H assays can evaluate the binding of hundreds of TFs to a given DNA sequence in a single experiment. Further, given that PDIs are tested within the yeast nucleus, this assay is less biased than ChIP-seq towards highly and broadly expressed TFs [9, 10]. Nevertheless, Y1H assays are not free of caveats as they fail to detect interactions involving heterodimer TFs, and interactions identified in yeast may not be relevant in the endogenous context.

Different formats and pipelines have been developed to perform Y1H assays. Therefore, multiple technical variations need to be considered when selecting the most appropriate system, including the type of DNA bait, reporter gene, prey source, cloning approach, yeast strains and screening format (Fig. 2). Throughout this chapter, we discuss different technical alternatives to consider as well as their advantages and limitations.

2 Y1H Workflow

When designing Y1H experiments, it is important to consider the different alternatives in the workflow (Fig. 2). Multiple types of DNA sequences can be selected as DNA baits which are cloned upstream of one or more reporter genes, and then introduced into yeast to produce DNA-bait strains [15]. Libraries or collections of TF preys are generated by cloning cDNA or individual TFs into low or high copy number vectors. Once both components of the Y1H system are generated, the TF preys are introduced into the DNA-bait strains by transformation or by mating, and screened for reporter gene activity to determine positive PDIs. These assays can be performed as library or arrayed screens.

3 DNA Baits

3.1 Types of DNA Sequences

Y1H assays can be used to detect TF interactions with short DNA sequences such as TF binding motifs or noncoding allele variants [10, 12, 13] as well as with complex DNA elements such as regulatory regions [8–11] (Fig. 2). Promoters [7–9, 11, 16], enhancers [10, 17, 18], and silencers [19] up to ~2 kb can be selected as DNA baits. However, PDIs occurring in the proximal 0.5–1 kb are more likely to be detected given that most regulatory interactions in yeast are short-range [20]. These complex regions are regularly obtained by PCR from genomic DNA using high-fidelity enzymes.

Short DNA sequences containing noncoding allele variants can also be tested by Y1H assays to determine altered TF binding [10]. These variants can be introduced into the primers during the initial

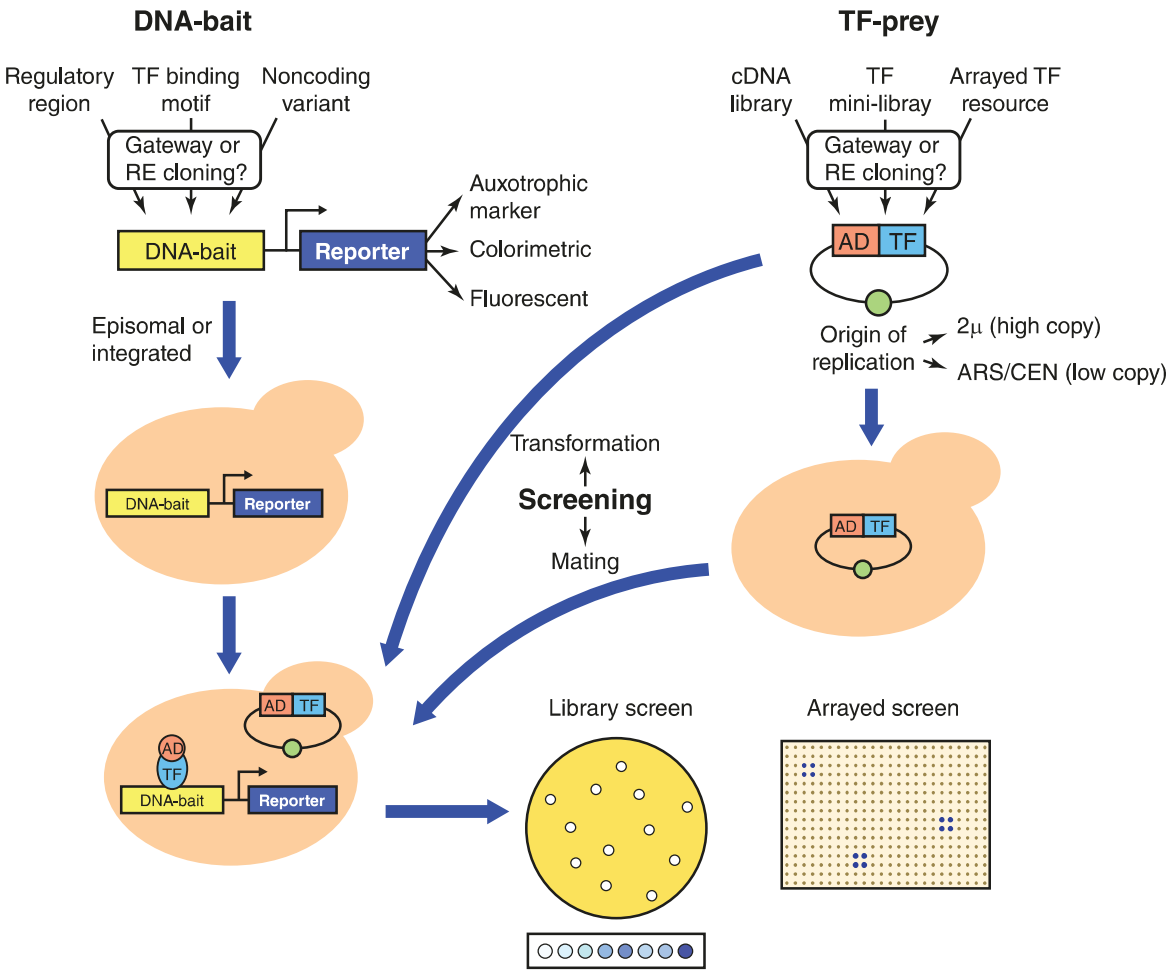

Fig. 2 Outline of variations and considerations in Y1H assays. DNA sequences corresponding to complex regulatory regions, TF binding motifs or noncoding variants can be cloned upstream of reporter genes using restriction enzymes (RE) or Gateway technology. These constructs are most frequently integrated into the yeast genome to generate DNA-bait strains. DNA-bait strains are then transformed or mated with a collection of prey clones (ORFs fused to the Gal4 activation domain, AD) originated from a cDNA library, a TF mini-library or an arrayed TF resource. Vectors encoding the preys can be low (ARS/CEN) or high (2μ) copy number. Screening can be performed as a library screen where positive colonies must be sequenced and retested or in an arrayed format where the location of the positive colonies indicates the identity of the interacting TF

cloning steps involved in generating the DNA baits, thereby circumventing the need for patient samples. Alternatively, noncoding variant sequences can be generated by annealing oligonucleotides. Short DNA fragments containing TF binding sites can also be tested in Y1H assays, as single copy or multiple tandem copies [12]. When screening tandem copies of motifs it is important to consider that TF binding can occur at the junctions of two consecutive motifs or between the motif and the vector [13]. Therefore, mutated motifs and sequences with spacers between motifs should be tested as controls.

3.2 Reporter Genes

Multiple reporter genes have been used in Y1H assays (Fig. 2). These include auxotrophic genes such as HIS3, URA3, LEU2, and TRP1 that allow the yeast to grow in the absence of histidine, uracil, leucine,

and tryptophan, respectively; and genes that can be measured in colorimetric assays, such as *LacZ* that encodes for bacterial β-galactosidase which converts the colorless X-gal into a blue compound.

Auxotrophic genes are useful for library screens as they allow to select for the few positive PDIs among the thousands of bait–prey pairs tested. Most Y1H systems use HIS3 as a reporter gene, as the His3p enzyme encoded by HIS3 can be inhibited by the competitive inhibitor 3-amino-1,2,4-triazole (3AT), allowing for the titration for strong and weak interactions. Given that the screening with auxotrophic genes is based on selection, sporadic mutations that allow the yeast to grow could be selected for and mistaken for true PDIs. Therefore, PDIs detected should be retested using gap repair or independent TF-prey transformation as previously described [21].

To increase the confidence in the PDIs detected, Y1H systems regularly use two reporter genes such as HIS3 and LacZ. These reporter constructs can either be present in the same yeast strain or in different strains [10, 19, 20, 22, 23]. In either case, only PDIs detected using both reporters are considered positive which results in fewer technical false positive interactions than observed using a single reporter [20].

3.3 Episomal or Integrated Reporter Constructs

DNA-bait reporter constructs can be introduced into yeast by transformation using episomal plasmids or by integrating the reporter into fixed locations in the yeast genome (Fig. 2). Using episomal constructs is simpler and faster than using integrated constructs, as multiple steps are required to generate and confirm integration. However, copy number variation between cells can often lead to variable reporter activity even in the absence of TF preys as some cells may have higher number of plasmids, resulting in many false positive PDIs. Integrated reporter constructs, overcome this problem as each construct is present in the same number in every yeast cell. In addition, integrated DNA baits are chromatinized, which may reflect a more endogenous setting for PDIs to occur compared to the open DNA that episomal reporters present.

3.4 Cloning Method

Restriction enzyme based cloning has traditionally been used to generate DNA baits and TF preys. However, cloning of each DNA sequence requires an individual design and set of enzymes, which limits the application to large-scale projects. Gateway technology has been successfully used to generate DNA baits (and TF preys), which has enabled Y1H assays to become a high-throughput approach to study PDIs. Gateway cloning is advantageous in that hundreds of cloning reactions can be performed in parallel allowing constructs to be generated much faster over traditional cloning methods. In addition, many clone sources (promoters, ORFs, TFs) are now publicly available. In the case of DNA baits, Gateway cloning allows DNA sequences to be shuttled to the HIS3 and LacZ reporter vectors, as well as to additional vectors to determine expression in vivo using GFP or luciferase reporters [8, 10].

4 TF Preys

4.1 Clone Source

Prey clones can be obtained from different sources such as cDNA libraries, TF mini-libraries (i.e., a pooled collection of TF clones) and TF arrayed collections (Fig. 2). Using a cDNA library, rather than a TF mini-library or collection, allows the identification of uncharacterized TFs. However, cDNA libraries present several limitations: (1) in a given species TFs correspond to only 5–10% of protein-coding genes, (2) TFs are expressed at lower levels compared to other genes, (3) many TFs may not be expressed in the source tissue for the cDNA, and (4) clones can be truncated or be in any of the six frames. Therefore, only a low fraction of clones present in the cDNA library will correspond to TFs. This implies that, unless millions of transformants are screened, many PDIs will be missed.

TF mini-libraries overcome some of the limitations of cDNA libraries. These resources comprise individually cloned TFs into Gateway compatible vectors that are pooled together and used to transform DNA-bait strains. The advantage compared to cDNA libraries is that all clones are in frame, correspond to TFs, and have similar representation. The disadvantages are that potential PDIs with uncharacterized TFs will be missed, and that generating individual clones may be time-consuming and expensive. However, several clone resources are now available for multiple species including human [10], mouse [24], *C. elegans* [20], *D. melanogaster* [18], and *A. thaliana* [25], all of which can be used for pooled and arrayed screens.

Regardless of whether a cDNA or a TF mini-library is used, these screens require extensive sequencing of yeast colonies to identify the interacting prey. This is alleviated by performing screens with TF arrays. In this type of resource, TF clones are organized so that the identity of the TF in each position is known. This allows for pairwise testing of PDIs either by transformation or by mating, without the need for sequencing to identify the interacting pair.

4.2 Plasmid Copy Number

Plasmid copy number determines the expression level and abundance of the TF prey (Fig. 2). Low-copy plasmids commonly feature an ARS/CEN origin of replication and are present at 1–2 copies per cell [26]. When performing Y1H assays, low copy plasmids encoding the TF-prey protein can either be transformed into haploid yeast containing the DNA bait or mated with a DNA-bait strain to create diploids. However, one study found that many PDIs detected by transforming low copy plasmids could not be detected when tested by mating [20]. Alternatively, high copy plasmids that typically contain a 2μ origin of replication can be used, which results in higher expression of the TF-prey

protein [20]. This leads to more PDIs being detected, both by transformation and by mating. However, some interactions detected by transformation of 2μ vectors cannot by reproduced by other Y1H pipelines [20].

4.3 Cloning Method

Similar to the cloning of DNA baits, individual cloning of TFs into AD containing vectors using restriction enzymes can be time consuming if the goal is to generate a large TF-prey resource. For example, each TF contains unique combinations of restriction enzyme cut sites within its respective coding region and, thus will require different enzymes to avoid cut sites potentially present. Gateway cloning can be used to generate hundreds of TF-prey fusion proteins simultaneously making this a high-throughput method for generating TF libraries and arrays. TF preys can be designed so that ORF sequences are in frame with the AD with the simple addition of gateway attB sites to each of the cloning primers [27]. Gateway compatible resources, including a large selection of Gateway compatible destination vectors, allows these TF clones to be tagged with fluorescent proteins or epitope tags (FLAG, HA, T7, etc.) for subsequent validation and follow-up studies.

5 Screening Approach

5.1 Library Versus Arrayed Screens

Y1H assays can be performed by library or arrayed screens (Fig. 2). Library screens involve the transformation of DNA bait strains using cDNA libraries or TF mini-libraries, followed by selection using an auxotrophic reporter (most frequently HIS3) and sequencing of positive interactions. Given that different TFs may have different representation in the libraries, particularly in those generated from cDNA, these studies require screening of thousands of transformants. Therefore, these screens are time-consuming and can only be used if few DNA baits need to be assayed. Further, some interactions may be missed if the clones are not present in the library, if the screening depth is insufficient, or in the case of weak interactions that lead to slow colony growth.

Arrayed screens involve ordered collections of individually cloned TF preys that are transformed or mated in/with DNA baits, allowing the identification of PDIs based on the location of the yeast colonies that express the reporter genes. This alleviates many of the issues presented by library screens as each TF prey present in the array will be screened with each DNA bait, and sequencing of positive interactions is not required as the identity of the interacting TF prey can be determined by the position in the array. This approach allows for more PDIs to be detected, including weak interactions. Moreover, arrayed screens take considerably less time to perform and allow for the comparison of PDIs between different DNA baits such as noncoding variants (e.g., risk versus nonrisk

alleles) [10, 20]. However, the major disadvantage of arrayed screens is that they require the generation of a comprehensive collection of individually cloned TFs.

5.2 Screening by Mating Versus Transformation

In order to detect PDIs in Y1H assays, TF-prey constructs must be introduced into the DNA-bait yeast strains. This can be accomplished by (1) transforming the TF-prey constructs into the DNA-bait strain, or (2) mating haploid yeast strains, one expressing the TF preys and the other one containing the DNA bait, to generate a diploid yeast strain where both components are present (Fig. 2). While transformation-based screens usually detect more PDIs than mating-based screens, PDIs captured by this method are less reproducible [20, 28]. Additionally, transformation-based screens are more time-consuming as each TF prey must be transformed into every DNA-bait strain and are less amenable for automatization using robotic platforms.

5.3 Yeast Strains

Host DNA-bait and TF-prey strains (in case the screen is performed by mating) need to carry the appropriate auxotrophic markers to select for transformants, integrants, and diploid cells, and to evaluate reporter activity when testing for PDIs. Studies involving both Y1H and Y2H assays have found that using different combinations of bait and prey strains yield overlapping, yet different sets of PDIs [20, 29]. When Y1H screens are performed by mating, the optimal combinations of yeast strains among the ones tested is Yα1867 (*MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6 ura3-52 ade2-101 trp1-901 his3-Δ200*) as the host prey strain and Y1H-aS2 (a modified YM4271 strain to increase integration efficiency into the HIS3 locus) as the host bait strain [20].

5.4 Enhanced Yeast One-Hybrid (eY1H) Assays

eY1H assays alleviate many of the issues described in this chapter by pairwise testing hundreds of interactions using a robotic platform [20]. In this pipeline, interactions are tested by mating in a 1536 array colony format, allowing the interrogation of up to 380 TF preys per plate evaluated in quadruplicate (plus negative controls and empty wells for plate orientation). This pipeline also incorporates other improvements such as simultaneously evaluating two integrated reporter genes (HIS3 and LacZ), using 2μ vectors for the TF preys, and using the Mybrid software for automatic quantification of reporter activity [16, 20]. Overall, this reduces the number of false positive and false negative PDIs by considering interactions that occur two or more times with both reporters simultaneously. Current TF-prey resources are composed of 837 *C. elegans* TFs (~90% of total) and 1086 human TFs (~75% of total), and can therefore be screened using only three plates per DNA bait [10, 20]. This greatly increases the throughput allowing a single researcher to screen ~60 DNA baits per week. The main

disadvantage of this approach is the upfront cost, as it requires a specialized robot to perform the assay and is only justified if hundreds/thousands of DNA-bait strains are being tested.

6 Limitations

As with any experimental system, Y1H assays may lead to the detection of false positive interactions or result in true interactions being missed (false negatives). Missed PDIs can arise from different sources: (1) the TF is absent from the clone source, (2) the TF was not tested due to some clones failing to be transformed into yeast (library screens), (3) the TF-prey protein is not properly expressed or folded correctly in yeast, (4) the TF binds to DNA only as a heterodimer (current Y1H systems can only test monomers or homodimers as TFs are expressed one at a time), (5) the TF requires posttranslational modification(s) to bind DNA, and/or (6) the DNA sequence bound by a TF is occluded by yeast nucleosomes or it is located far away from the yeast minimal promoter.

There are two different types of false positives to be considered when performing Y1H assays. Technical false positives are interactions that are not reproducible when repeating the assay, and can be identified by retesting detected PDIs one or multiple times. Biological false positives, instead, are PDIs that can be consistently detected in yeast when repeating the assay but never occur in vivo. Determining whether an interaction detected by Y1H assays is a biological false positive is challenging because methods used to perform in vivo validations, such as ChIP or reporter assays, can also miss true biological interactions [30]. For example, an interaction may be missed by ChIP if it is not assayed in the appropriate cells or conditions. In addition, interactions tested in TF knockout backgrounds may be masked by functionally redundant interactions with other TFs. Ultimately, a comprehensive identification and functional validation of PDIs requires multiple complementary approaches, both DNA- and TF-centered.

7 Future Directions

Y1H assays represent a powerful tool to study PDIs. While many modifications and improvements have been made to this technique throughout the years, technical limitations still exist. One feature of Y1H approaches that can be improved is the ability to detect heterodimeric interactions. In its current setup, the Y1H method is designed to interrogate the binding of TF monomers or homodimers and, thus are likely missing many

heterodimer -dependent PDIs. A low-throughput study has successfully utilized Y1H assay to test a PDI involving the BBX32-COL3 heterodimer, but this adaptation has yet to be scaled up to use in high-throughput setups [31].

Another limitation of Y1H assays is the throughput by which PDIs can be interrogated. eY1H techniques have made considerable advances by increasing the number of baits and preys that can be tested, but are still limited by the number of pairwise matings that can fit in a single plate and by the low throughput of DNA-bait generation. Incorporation of next generation sequencing techniques represents the next evolution in interrogating high-throughput PDIs as both TF preys and DNA baits can be barcoded with unique identifiers. A similar approach has been used to test binary high-throughput protein–protein interactions using a Cre recombination-based system [32]. As Y1H clone resources become more readily available, and improvements to increase the throughput and the breadth of TFs amenable to the assay are made, the Y1H system will continue to expand its current capabilities for interrogating PDIs.

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