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Enhanced yeast one-hybrid screens to identify transcription factor binding to human DNA sequences

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Dear Dr. Steidel,

Hereby we submit our revised manuscript entitled '***Enhanced yeast one-hybrid screens to identify transcription factor binding to human DNA sequences***' (JoVE59192) for publication at JoVE. We addressed all the editorial and reviewer's comments which we believe greatly improved the manuscript.

We deeply appreciate your consideration of our manuscript. Please do not hesitate to contact me if you have any queries.

Sincerely,

Juan I. Fuxman Bass

TITLE:

Enhanced Yeast One-Hybrid Screens to Identify Transcription Factor Binding to Human DNA Sequences

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SUMMARY:

Here, we present an enhanced yeast one-hybrid screening protocol to identify the transcription factors (TFs) that can bind to a human DNA region of interest. This method uses a high-throughput screening pipeline that can interrogate the binding of >1,000 TFs in a single experiment.

ABSTRACT:

Identifying the sets of transcription factors (TFs) that regulate each human gene is a daunting task that requires integrating numerous experimental and computational approaches. One such method is the yeast one-hybrid (Y1H) assay, in which interactions between TFs and DNA regions are tested in the milieu of the yeast nucleus using reporter genes. Y1H assays involve two components: a 'DNA-bait' (*e.g.,* promoters, enhancers, silencers, etc.) and a 'TF-prey,' which can be screened for reporter gene activation. Most published protocols for performing Y1H screens are based on transforming TF-prey libraries or arrays into DNA-bait yeast strains. Here, we describe a pipeline, called enhanced Y1H (eY1H) assays, where TF-DNA interactions are interrogated by mating DNA-bait strains with an arrayed collection of TF-prey strains using a high density array (HDA) robotic platform that allows screening in a 1,536 colony format. This allows for a dramatic increase in throughput (60 DNA-bait sequences against >1,000 TFs takes two weeks per researcher) and reproducibility. We illustrate the different types of expected results by testing human promoter sequences against an array of 1,086 human TFs, as well as examples of issues that can arise during screens and how to troubleshoot them.

INTRODUCTION:

A central problem in the biomedical field is determining the mechanisms by which each human gene is regulated. Transcription is the first step in controlling gene expression levels, and it is regulated by sets of transcription factors (TFs) that are unique to each gene. Given that humans encode for >1,500 TFs[1,2], identifying the complete set of TFs that control the expression of each gene remains an open challenge.

Two types of methods can be used to map TF-DNA interactions: TF-centered and DNA-centered methods[3] (**Figure 1A**). In TF-centered methods, a TF of interest is probed for binding to genomic DNA regions or to determine its DNA binding specificity. These methods include chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing, protein binding microarrays, and SELEX[4–6]. In DNA-centered methods, a DNA sequence of interest is probed to determine the set of TFs that bind to the DNA sequence. The most widely applied of such methods is yeast one-hybrid (Y1H) assays, in which interactions between TFs and DNA regions are tested in the milieu of the yeast nucleus using reporter genes[7–9].

Y1H assays involve two components: a 'DNA-bait' (*e.g.,* promoters, enhancers, silencers, etc.) and a 'TF-prey,' which can be screened for reporter gene activation[9,10] (**Figure 1B**). The DNA-bait is cloned upstream of two reporter genes (*LacZ* and *HIS3*) and both DNA-bait::reporter constructs are integrated into the yeast genome to generate chromatinized 'DNA-bait strains.' The TF-prey, encoded in a plasmid that expresses a TF fused to the activation domain (AD) of the yeast Gal4 TF, is introduced into the DNA-bait strain to fish for TF-DNA interactions. If the TF-prey binds to the DNA-bait sequence, then the AD present in the TF-prey will lead to the activation of both reporter genes. As a result, cells with a positive interaction can be selected for growth on plates lacking histidine, as well as overcoming a competitive inhibitor, 3-Amino-1,2,4-triazole (3-AT), and visualized as blue colonies in the presence of X-gal. Because the potent yeast Gal4 AD is used, Y1H assays can detect interactions involving transcriptional activators as well as repressors. In addition, given that TF-preys are expressed from a strong yeast promoter (ADH1), interactions can be detected even for TFs that have low endogenous expression levels, which are challenging to detect by ChIP[11,12].

Most published protocols for performing Y1H assays are based on introducing TF-preys into the yeast DNA-bait strains by transforming pooled TF-prey libraries followed by selection, colony picking, and sequencing to identify the interacting TF, or by transforming individual clones[8,9]. These are time-consuming protocols, limiting the number of DNA sequences that can be tested per researcher. A recent improvement of Y1H assays, called enhanced Y1H (eY1H), has dramatically increased the screening throughput by using a high density array (HDA) robotic platform to mate yeast DNA-bait strains with a collection of yeast strains each expressing a different TF-prey[10,13] (**Figure 1C**). These screens employ a 1,536 colony format allowing to test most human TFs in quadruplicate using only three plates. Further, given that TF-DNA interactions are tested in a pairwise manner, this approach allows for comparing interactions between DNA-baits (such as two noncoding single nucleotide variants) and between different TFs or TF variants[11,12,14].

Using eY1H assays, we have delineated the largest human and *Caenorhabditis elegans* DNA-centered TF-DNA interactions networks to-date. In particular, we have identified 2,230 interactions between 246 human developmental enhancers and 283 TFs[12]. Further, we have employed eY1H assays to uncover altered TF binding to 109 single nucleotide noncoding variants associated with genetic diseases such as developmental malformation, cancer, and neurological disorders. More recently, we used eY1H to delineate a network comprising 21,714 interactions between 2,576 *C. elegans* gene promoters and 366 TFs[11]. This network was instrumental to uncover the functional role of dozens of *C. elegans* TFs.

The protocols to generate DNA-bait stains and evaluate the levels of background reporter activity have been reported elsewhere[15–17]. Here, we describe an eY1H pipeline that can be used to screen any human genomic DNA region against an array of 1,086 human TFs. Once a yeast DNA-bait strain is generated and a TF-prey array is spotted onto the corresponding plates, the entire protocol can be performed in two weeks (**Table 1**). More importantly, the protocol can be parallelized so that a single researcher can screen 60 DNA-bait sequences simultaneously. To demonstrate the protocol, we screened the promoters of two cytokine genes CCL15 and IL17F. In addition, we show results from failed screens to illustrate the types of problems that may arise when performing eY1H assays and how to troubleshoot them.

PROTOCOL:

1. Preparations

1.1. Sc –U –H plates (150 mm Petri dishes)

NOTE: These plates will be used for growing the DNA-bait yeast strains.

1.1.1. Dissolve the drop-out mix, yeast nitrogen base (YNB), adenine hemisulfate, and ammonium sulfate in 920 mL of water, and pH to 5.9 with 5 M NaOH (approximately 1 mL per liter of media; see **Table 2** for composition). Pour into a 2 L flask and add a stir bar.

1.1.2. In a second 2 L flask, add the agar to 950 mL of water (do not add a stir bar as it will cause the agar to boil over in the autoclave).

1.1.3. Autoclave for 40 min at 15 psi on a liquid cycle.

1.1.4. Immediately pour the contents of the first flask, including the stir bar, into the agar containing flask. Add the glucose, mix well on a stir plate, and cool to 55 °C in a water bath.

1.1.5. Add the leucine and the tryptophan to the media. Mix well on a stir plate and pour into 150 mm sterile Petri dishes (~80 mL per dish). Dry for 3–5 days at room temperature, wrap in plastic bags, and store at room temperature for up to 6 months.

1.2. YAPD rectangular plates

NOTE: These plates will be used for growing the lawn for the DNA-bait strain and for mating with the TF array collection.

1.2.1. Dissolve powders (see **Table 3** for composition), except for agar, in 950 mL of water in a 2 L flask and add a stir bar.

1.2.2. In a second 2 L flask, add the agar to 950 mL of water (do not add a stir bar as it will cause the agar to boil over in the autoclave).

1.2.3. Autoclave for 40 min at 15 psi on a liquid cycle.

1.2.4. Immediately pour the contents of the first flask, including the stir bar, into the agar containing flask.

1.2.5. Add the glucose, mix well on a stir plate and cool to 55 °C. Pour media into the rectangular plates (see **Table of Materials**; ~70 mL per plate) using a peristaltic pump (5 mL/sec) and a 6 mm tubing. Dry for 1 day at room temperature, wrap in plastic bags, and store in the cold room for up to 6 months.

NOTE: Although the suggested media volume is 70 mL per plate, 50–80 mL per plate can be used. The three critical issues to consider when pouring plates are 1) that they are leveled so that the agar media has the same thickness throughout the plate (use a leveled table or surface for plate pouring and do not pour in stacks of more than seven plates), 2) to ensure the absence of bubbles in the agar media (bubbles should be popped using a sterile needle), and 3) drying the plates for only one day and wrapping the plates in plastic bags to avoid failures in pinning yeast.

1.3. Sc −Trp and Sc −U −Trp rectangular plates

NOTE: These plates will be used for growing the TF array collection (Sc −Trp) and to select diploid yeast after mating (Sc −U −Trp).

1.3.1. Dissolve the drop-out mix, YNB, adenine hemisulfate, and ammonium sulfate in 920 mL of water, and pH to 5.9 with NaOH 5M (approximately 1 mL per liter of media) (see **Table 4** for composition). Pour into a 2 L flask and add a stir bar.

1.3.2. In a second 2 L flask, add the agar to 950 mL of water (do not add a stir bar as it will cause the agar to boil over in the autoclave).

1.3.3. Autoclave for 40 min at 15 psi on a liquid cycle.

1.3.4. Immediately pour the contents of the first flask, including the stir bar, into the agar containing flask. Add the glucose, mix well on a stir plate, and cool to 55 °C.

1.3.5. Add the leucine, histidine, and uracil (omit the uracil for the Sc −U −Trp plates).

1.3.6. Mix well on a stir plate and pour into rectangular plates (~70 mL per plate) using a peristaltic pump (5 mL/sec) and 6 mm tubing. Dry for 1 day at room temperature, wrap the plates in plastic bags, and store in the cold room for up to 3 months.

1.4. Sc −U −H −Trp + 3AT + X-gal rectangular plates

NOTE: These plates will be used as readout plates for eY1H assays.

1.4.1. Dissolve the drop-out mix, YNB, adenine hemisulfate, and ammonium sulfate in 850 mL of water (see **Table 5** for composition). Do not pH. Pour into a 2 L flask and add a stir bar.

1.4.2. In a second 2 L flask, add the agar to 850 mL of water (do not add a stir bar as it will cause the agar to boil over in the autoclave).

1.4.3. Autoclave for 40 min at 15 psi on a liquid cycle.

1.4.4. Prepare 10x BU salts (1L) by combining 900 mL of water, 70 g of $Na_2HPO_4 \cdot 7H_2O$, and 34.5 g of $NaH_2PO_4 \cdot H_2O$. Mix using a stir bar to dissolve powders and adjust the pH to 7.0 using 5 M NaOH. Add water to bring to 1 L and autoclave.

1.4.5. Prepare the X-gal solution by adding 3.5 g of X-gal powder to a 50 mL plastic tube containing 42.5 mL of dimethyl formamide. Add X-gal powder to dimethyl formamide to dissolve more easily (this takes 30 min). Keep stock solution in the dark (either use opaque 50 ml tube or cover in foil). Store at -20 °C.

1.4.6. Immediately pour the contents of the first flask, including the stir bar, into the agar containing flask. Add the glucose and the 10x BU salts (see **Table 5** for composition), mix well on a stir plate, and cool to 55 °C.

1.4.7. Add the leucine, 3AT, and X-gal (see **Table 5** for composition).

1.4.8. Mix well on a stir plate and pour into the rectangular plates (~70 mL per plate) using a peristaltic pump (5 mL/sec) and a 6 mm tubing. Dry for 1 day at room temperature, wrap in plastic bags, and store in the cold room covered in aluminum foil (3AT and X-gal are light sensitive) for up to 1 month.

2. Spotting a TF array

2.1. Thawing the TF-prey array

2.1.1. Thaw the yeast glycerol stock plates with the TF-prey array on ice.

NOTE: TF-prey arrays can be generated as previously published[10,12,13,18].

2.1.2. Resuspend the yeast using a 12-channel pipette within 1–3 min before the next step.

2.2. **Spotting the yeast into Sc –Trp rectangular plates**

2.2.1. In the HDA robot (see **Table of Materials**), select multi-well 96 plates as source, 96 agar plates as target, and 96 long pin pads.

NOTE: Pin pads are not reusable and should be discarded.

2.2.2. Select the **Replicate Many** program to make two copies per 96-well plate. Do not use the recycle or revisit options to avoid back contamination of the frozen stocks.

2.2.3. Select the option to swirl up and down in the source to mix the yeast.

2.2.4. Bag the spotted array and incubate agar-side up at 30 °C for 2–3 days.

2.3. **Generating 384 colony arrays in Sc –Trp rectangular plates**

2.3.1. In the robot, select 96 agar plates as source, 384 agar plate as target, and 96 short pin pads.

2.3.2. Select the **1:4 Array** program. In this way, four 96 colony plates (each containing a different TF) will be consolidated into one 384 colony plate. Do not use the recycle or revisit options to avoid contamination between different plates.

2.3.3. Bag the plates and incubate the spotted 384-colony array agar-side up at 30 °C for 2 days.

2.4. Generating 1,536 colony arrays in Sc –Trp rectangular plates

NOTE: This will result in arrays containing four colonies for each TF-prey.

2.4.1. In the robot, select 384 agar plates as source, the 1,536 agar plates as target, and 384 short pin pads.

2.4.2. Select the 1:4 assay single source program. The goal is to copy each colony into four colonies to obtain quadruplicates. Use the recycle and revisit options as it involves copying four times each colony.

2.4.3. Bag the plates and incubate the spotted 1536-colony array agar-side up at 30 °C for 3 days.

2.5. **Amplifying the 1,536 colony array in Sc –Trp rectangular plates**

2.5.1. In the robot, select 1,536 agar plates as source, 1,536 agar plates as target, and 1,536 short

pin pads.

2.5.2. Select the **Replicate Many** program to replicate 3–4 copies. Use the recycle and revisit option, but throw out the pad when switching to a different plate of the array to avoid cross contamination.

2.5.3. Bag the plates and incubate the spotted 1536-colony array agar-side up at 30 °C for 3 days to use for mating steps (see below). After that, keep the plates at room temperature and copy again after 7 days for a new round of screening.

3. eY1H screen

3.1. Preparing DNA-bait strain lawns for mating

3.1.1. Spot the yeast DNA-bait strains on a Sc −U −H plate and grow for 3 days at 30 °C.

3.1.2. Streak the yeast into a 15 cm Sc −U −H plate using a sterile toothpick, so that each plate fits 12–16 different strains. Incubate one day at 30 °C.

3.1.3. Streak the yeast into a 15 cm Sc −U −H plate using a sterile toothpick, so that each plate fits 4 different strains. Incubate for one day at 30 °C.

3.1.4. Scrape the yeast using a sterile toothpick, making sure not to scrape any agar and add into a 1.5 tube with 500 µl of sterile water.

3.1.5. Add 10–15 sterile glass beads onto a YAPD rectangular plate. Add the yeast suspension onto the plate and shake thoroughly in all directions for 1 min to ensure the yeast is spread through all the plate.

3.1.6. Invert the plate immediately and tap so that the beads go to the lid. Remove and recycle the beads.

3.1.7. Bag the plates and incubate agar-side down for 1–2 days at 30 °C. Then proceed to the mating step.

3.2. Mating of yeast DNA-bait and TF array strains

3.2.1. Transfer the TF array to a YAPD rectangular plate with the robot. Select the 1,536 agar plate as source and target, and the 1,536 short pin pad. Select the **Replicate Many** program. Each TF array plate can be used to transfer to 3–4 YAPD plates (depending on the number of plates in the array). The TF array plates used for mating must be 2–3 days old but not more as mating may be inefficient.

3.2.2. Transfer the lawn of a DNA-bait strain to the YAPD plates already containing the TF array

with the robot. Select the 1,536 agar plate as source and target, and the 1,536 short pin pad. Select the **Replicate Many** program. Use a random offset in the source with a radius of ~0.6 mm to avoid taking yeast from the same spot, and mix on target to facilitate contact between yeast strains. Use the lawn containing the DNA-bait strains (section 3.1) as source, and the YAPD plates containing the TF array spotted in step 3.2.1 as target.

3.2.3. Bag the plates and incubate agar-side up at 30 °C for 1 day.

3.3. Selection of diploid yeast

3.3.1. Transfer the mated yeast from the YAPD plates to Sc −U −Trp plates with the robot. Select the 1,536 agar plate as source and target, and the 1,536 short pin pad. Select the **Replicate** program. Mix on source and on target.

3.3.2. Bag the plates and incubate agar-side up at 30 °C for 2–3 days (longer incubation leads to high background reporter activity).

3.4. Transfer to readout plates

3.4.1. Transfer the diploid yeast from the Sc −U −Trp plates to the readout rectangular plates Sc −U −H −Trp + 5mM 3AT + 0.4 mM X-gal using the robot. Select the 1,536 agar plates as source and target, and the 1,536 short pin pad. Select the **Replicate** program.

3.4.2. Bag the plates and incubate agar-side up at 30 °C for up to 7 days.

3.5. **Imaging of readout plates**

3.5.1. For DNA-bait strains with high background reporter activity, take pictures on days 2, 3, and 4. Otherwise, take pictures at days 4 and 7. Positive interactions are identified by growth and blue color of the yeast colonies and can be manually determined, or it can be determined using image analysis software.

REPRESENTATIVE RESULTS:

Three main factors should be considered when analyzing results from eY1H assays: the background reporter activity of the DNA-bait strain, the strength of the reporter activity corresponding to TF-DNA interactions, and the number of positive colonies. The background reporter activity (i.e., autoactivity) of the DNA-bait strain refers to the overall growth and color of the yeast colonies in the readout plate, even in the absence of a TF-prey. Ideally, non-autoactive strains show a background white or light brown color, with colonies for positive interactions being bigger and blue. Autoactive DNA-bait strains show yeast growth in media lacking histidine and a blue color in the presence of X-gal for all colonies in the plate, which is likely related to the binding of yeast transcriptional activators to the DNA-bait[8]. The strength of the reporter activity corresponding to the TF-DNA interactions detected (i.e., the size of the

colony and intensity of blue) depends on many parameters such as the affinity, the TF expression level in yeast, the number of binding sites and distance to the yeast minimal promoters located upstream of the reporter genes, and the background reporter activity of the DNA-bait strain. For example, a weak interaction may be easily detected in a low background bait but may be difficult to detect in an autoactive or uneven background bait. It is also important to note that reporter activity levels in yeast do not necessarily correlate with the regulatory activity in human cells, as the chromatin structure, nucleosome positioning, and distance effects are different between yeast and human. Further, interactions in human are likely to be influenced by the binding of other TFs and cofactors or may be masked by functionally redundant TFs[8]. Finally, interactions are considered positive in eY1H assays when at least two of the four colonies show reporter expression above background levels. However, we have observed that ~90% of interactions identified result from all four colonies corresponding to a TF being positive[10,12,19].

To illustrate the type of results that can be obtained using eY1H assays we screened the promoter regions (2 kb upstream of the transcription start sites) of the CCL15 and IL17F genes, against an array of 1,086 human TFs (**Figure 2**). The CCL15 promoter is an example of a non-autoactive DNA-bait where interactions, even weak ones, can be easily detected (**Figure 2A**). The IL17F promoter is an example of an autoactive DNA-bait with uneven background reporter activity, where some interactions can be detected while for several TFs it is uncertain whether the reporter activity is higher than background (**Figure 2B**).

Problems that can be encountered when performing eY1H assays

Although the screening eY1H protocol is straight-forward and robust, several problems can be encountered during the screen:

1) Colonies are too small and fail to transfer (**Figure 3A**): Although it is expected that some yeast expressing exogenous TFs may display slow growth given that yeast gene expression may be dysregulated, typically ~95% of TF-prey colonies display normal growth. If more than 10% of colonies fail to grow, the most frequent causes are problems with the media or with the yeast transfer. Suboptimal growth is frequently related to one of the media components losing activity (*e.g.*, uracil, histidine, or leucine), which can be solved by preparing fresh media with fresh stock solutions. Alternatively, this may also be related to a pinning offset that affects colony transfer. In this case, verify that the 1,536 pads pin the center of the yeast colonies in the source plates.

2) No yeast growth in a portion of the plate (**Figure 3B**): This issue is generally related with a failure in the mating step if the 1,536 pin pad fails to make contact with the yeast in the DNA-bait strain lawn, the TF array, or in the mating plate. In almost every case, this is due to uneven agar media level during plate pouring or due to excessive drying of the plate.

3) No interactions detected (**Figure 3C** and **D**): This issue is often related to either unintended inactivating mutations in the reporter genes, in particular LacZ (**Figure 3C**), or to high autoactivity that mask interactions (**Figure 3D**). To troubleshoot this problem, it is recommended to screen another independently obtained strain corresponding to the same DNA-bait.

4) The plate presents random blue spots (**Figure 3E**): This issue is often related to bacterial contamination. To solve this issue, streak the yeast to obtain individual colonies, and repeat the screen.

The above are the most frequent problems encountered when performing eY1H assays. Should other problems arise, preparing new media, confirming that appropriate settings for the HDA robot were used, and testing multiple strains per DNA-bait would likely solve most issues.

FIGURE AND TABLE LEGENDS:

Figure 1: Outline of eY1H assay screens. (**A**) Comparison between TF-centered and DNA-centered methods to identify protein-DNA interactions. (**B**) Schematics of eY1H assays. A DNA sequence of interest (promoter, enhancer, silencer, etc.) cloned upstream of the HIS3 and LacZ reporter genes is integrated into the yeast genome. The resulting DNA-bait strain is mated to a collection of yeast strains harboring TFs fused to the Gal4 activation domain (AD). Positive interactions are determined by the yeast's ability to grow without histidine and overcoming competitive inhibitor 3-AT, and turn blue in the presence of X-gal. (**C**) Pipeline for eY1H screens. A lawn of a yeast DNA-bait strain grown in a YAPD plate is mated in a YAPD plate to a 1,536 colony array expressing TFs fused to AD grown on a Sc −Trp plate. After one day, the yeast is transferred to a Sc −U −Trp to select for diploid yeast. After a 2–3 day incubation, the yeast is transferred to a Sc −U −H −Trp + 3AT + X-gal plate (readout plate) to identify protein-DNA interactions. Each interaction is tested in quadruplicate.

Figure 2: Examples of eY1H readout plates. (**A**) Interactions involving the promoter of CCL15, a non-autoactive bait. Background reporter activity for this bait is low (reduced growth in the absence of histidine and absence of blue color for non-interacting TFs). (**B**) Interactions involving the promoter of IL17F, an autoactive bait. Background reporter activity for this bait is high (growth in the absence of histidine and background blue color throughout the plate) and uneven, making it challenging to identify protein-DNA interactions. Strong, medium, and weak interactions are squared in red, orange, and yellow, respectively. The HGNC names of the interacting TFs are shown.

Figure 3: Problems in eY1H screens. (**A**) TF-prey array where multiple colonies failed to grow. (**B**) Readout plate where colonies in the lower left corner have failed to transfer. (**C**) Non-autoactive DNA-bait strain that does not display positive interactions. (**D**) Highly autoactive DNA-bait strain that does not display positive interactions. (**E**) Readout plate displaying multiple blue colonies due to contamination.

DISCUSSION:

The robotic eY1H mating screening approach described here greatly increases the throughput to identify the set of TFs that bind to a DNA region of interest, compared to previous library screening or arrayed screening approaches based on transformation. Further, the TF-DNA

interactions detected by eY1H assays are highly reproducible as 90% of interactions detected are positive for all four colonies tested per TF, and 90% of interactions retest in an independent screen of the same yeast DNA-bait strain[10,12,19]. More importantly, TF-DNA interactions detected by eY1H validate at a 40%–70% rate when tested in human reporter assays[12,20], in primary human cells (unpublished results), and in *C. elegans* knockout animals[11]. This is a similar validation rate to that observed for ChIP-seq data[21].

Although interactions identified by eY1H are highly reproducible when retesting the same yeast DNA-bait strain, testing different yeast strains for the same DNA-bait sometimes produces different, although overlapping, sets of TF-DNA interactions. This is usually due to differences in background reporter activity between strains. In addition, testing fragments of a DNA sequence results in the detection of more TF-DNA interactions than testing the full sequence, in particular when overlapping fragments are tested. This may be related with the assay being more efficient in identifying interactions that are close to the reporter minimal promoters, and because testing overlapping fragments reduces the chances that a binding site may be occluded by yeast nucleosomes. Thus, for small scale projects, it is recommended that overlapping 0.5–1 kb fragments of a regulatory region are tested and that two independent strains are screened for each DNA-bait sequence[8].

There are several critical steps in the eY1H screening protocol to avoid some of the issues presented in **Figure 3**. First, although most media ingredients are stable for several months (except for 3AT and X-gal), a lack of proper colony growth likely indicates that at least one of the ingredients may have lost activity and should be replaced. Second, it is important to prepare the rectangular plates so that the agar is leveled and so that they do not dry for more than one day to avoid failure in pinning when using the robotic platform. Finally, it is key to use the robotic platform programs as indicated in the protocol (revisit, recycle, mixing, etc.) for the yeast to be transferred effectively, for mating to be efficient, and to avoid cross contamination between yeast clones.

The examples we selected to illustrate the use of eY1H screens correspond to human gene promoters. However, other regulatory regions can also be tested including enhancers and silencers. For example, we have used eY1H assays to evaluate TF binding to human developmental enhancers and to *C. elegans* first introns[12,22]. In addition, given that interactions are tested in a pairwise manner, eY1H assays can be used to compare interactions between non-coding variants, and between TF coding sequence variants. For example, using eY1H assays we identified altered TF binding to 109 noncoding variants associated with different genetic diseases, and also differential interactions profiles for 58 TF missense mutations[12,14]. Although this protocol focuses on evaluating TF binding to human regulatory regions, DNA regions from other species can also be tested provided that a TF-prey is available or can be generated. Indeed, TF-prey arrays have been generated for *C. elegans*[10], *Drosophila melanogaster*[23], *Mus musculus*[24], *Arabidopsis thaliana*[25,26], and *Zea mays*[27]. Thus, with increasingly available resources, eY1H assays may be applied to additional systems.

Although eY1H assays have been instrumental to identify the repertoire of TFs that bind to

different regulatory regions in human and other species, they are not free of caveats[8,11,12,19,20,25]. One of the limitations is that interactions are tested in the milieu of the yeast nucleus and, although the DNA-baits are chromatinized, the chromatin structure in yeast may not reflect the chromatin structure in the species from where the DNA-bait originated and will not reflect cell type differences observed in vivo. Thus, interactions identified by eY1H assays must be validated in reporter or other functional assays. Of note, we and others have found TF-DNA interactions detected by eY1H validate at a 40%–70% rate in functional assays[11,12,20,23]. Another limitation of eY1H assays is that it cannot detect interactions involving TFs that require post-translational modifications absent in yeast to bind to DNA, TFs that are not properly folded in yeast when fused to the AD, and TFs that are missing from the array[8]. In addition, in the current format, eY1H assays do not detect interactions involving heterodimeric TFs, as each yeast colony in the TF array expresses a single TF-prey. Thus, further improvements in the assay will increase the breadth of TFs that can be tested and expand the capabilities of eY1H assays to identify novel TF-DNA interactions.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

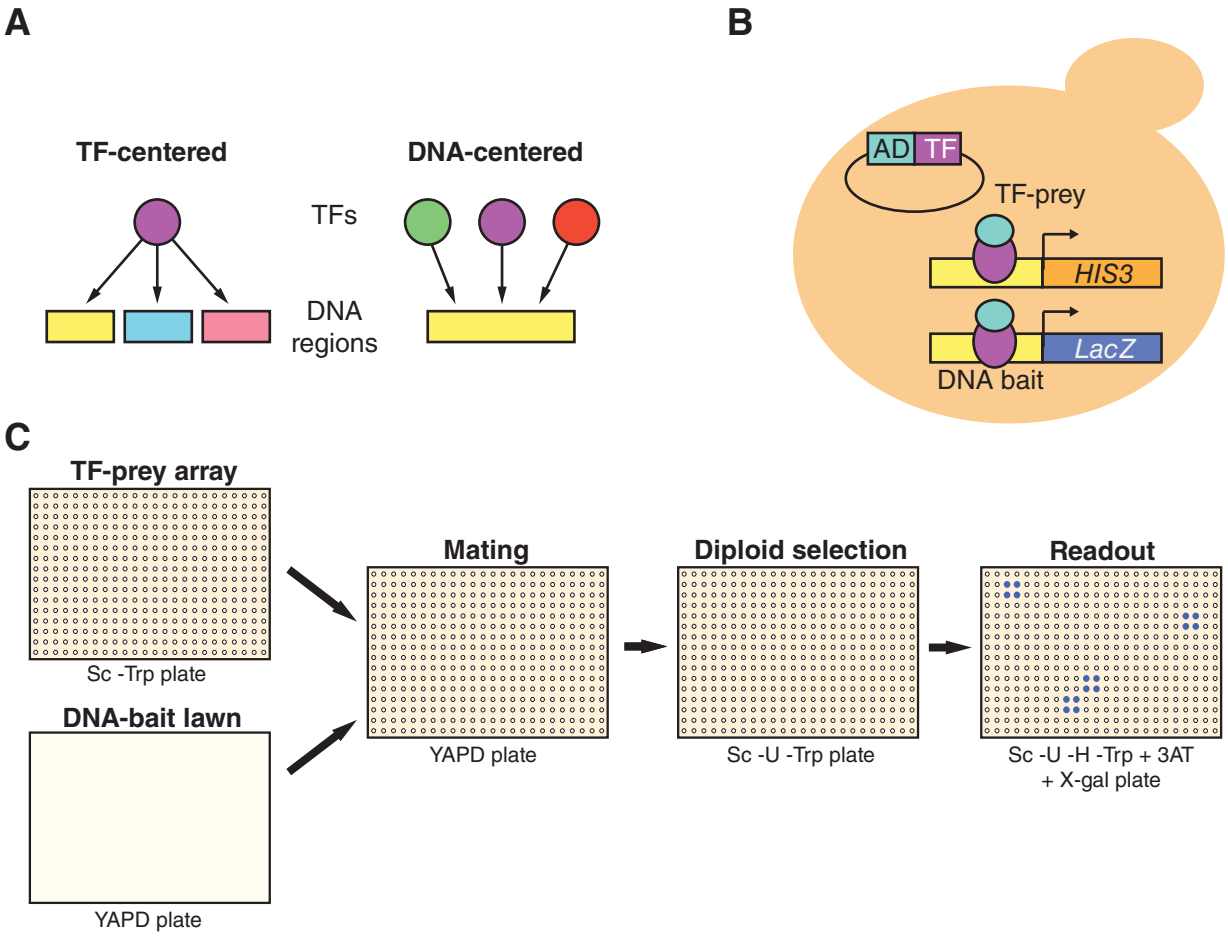


Figure 2

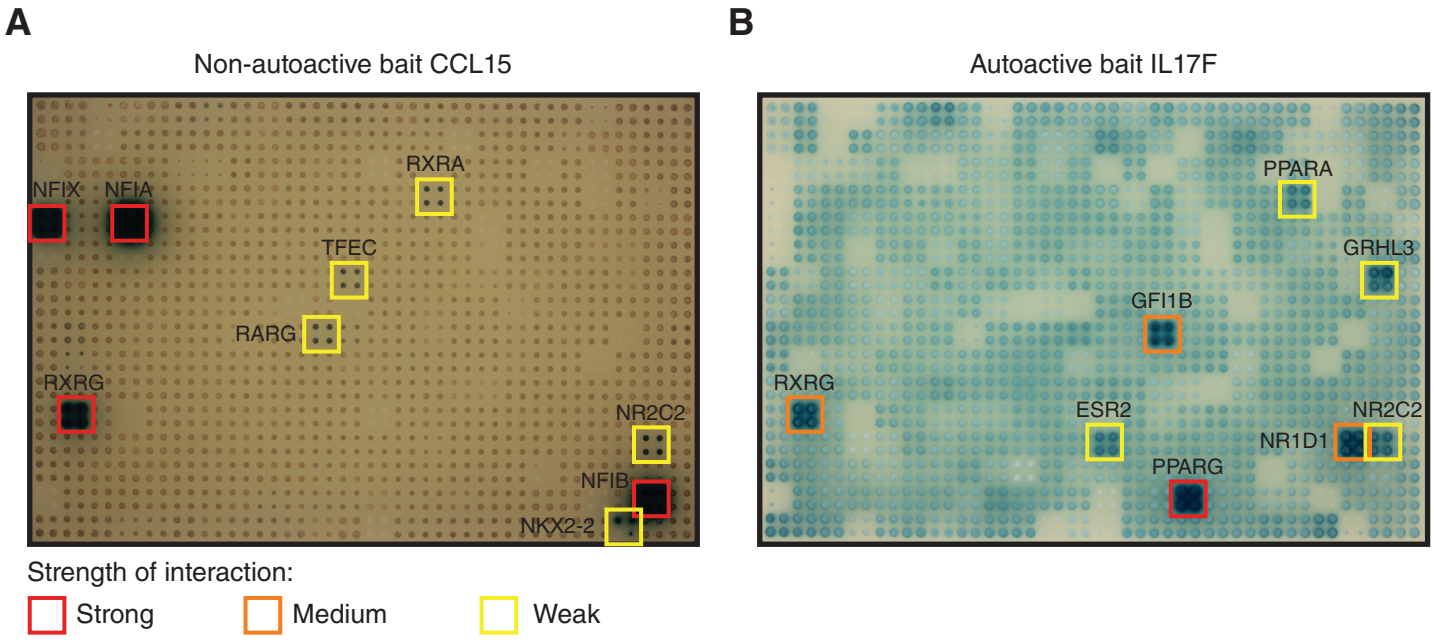
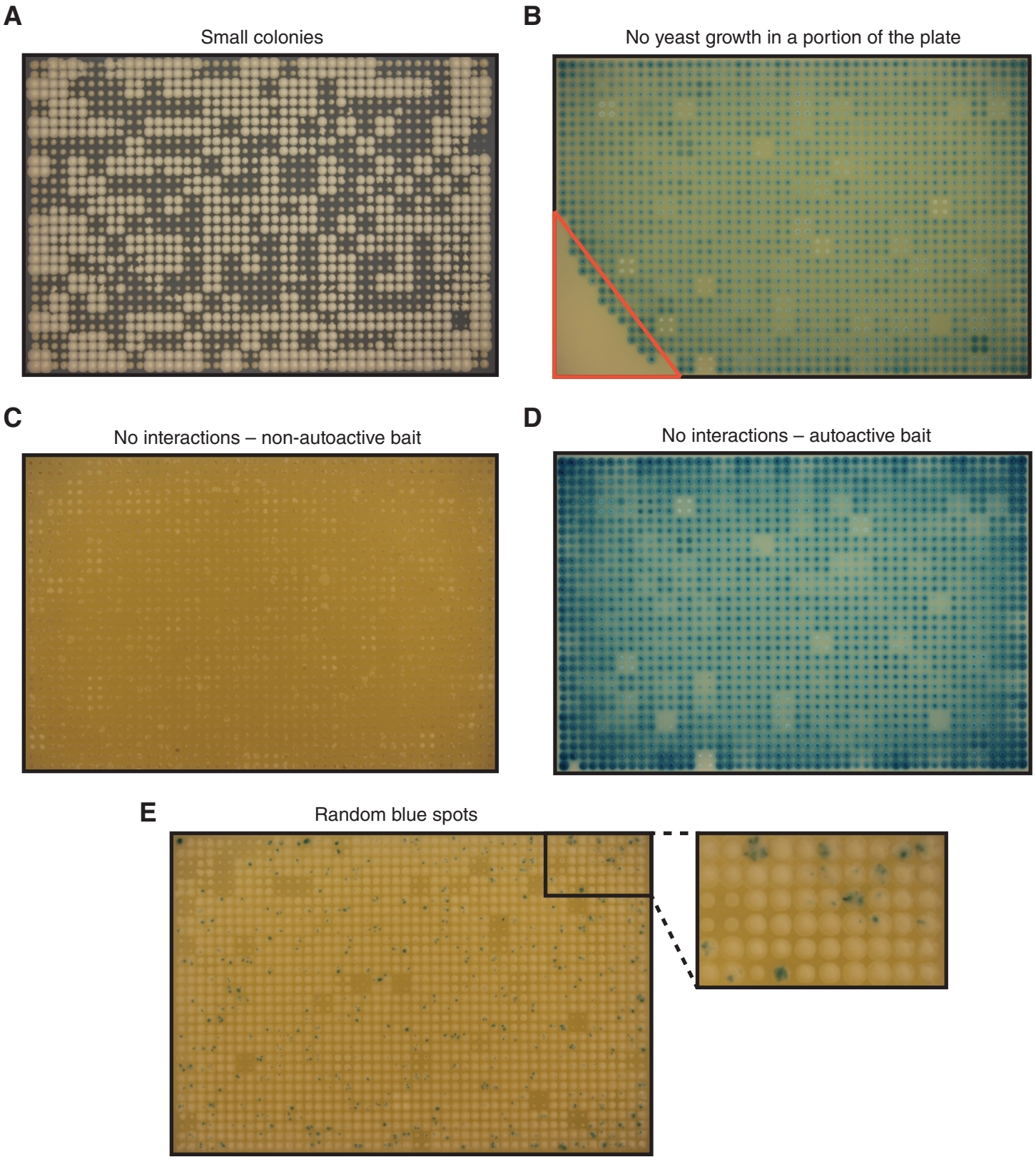


Figure 3



Spotting a TF array	
Day 1	Spot yeast into Sc –Trp plate in 96 colony format (**2.1** and **2.2**)
Day 4	Generate 384 colony TF array (**2.3**)
Day 6	Generate 1,536 colony TF array (**2.4**)
Day 9	Amplify 1,536 colony TF array (**2.5**)
Preparing DNA-bait strain lawns for mating	
Day 6	Spot the yeast DNA-bait strains (**3.1.1**)
Day 9	Streak yeast, 12-16 strains per plate (**3.1.2**)
Day 10	Streak yeast, 4 strains per plate (**3.1.3**)
Day 11	Prepare DNA-bait lawns (**3.1.4** and **3.1.5**)
Mating of yeast DNA-bait and TF array strains	
Day 12	Mating in YAPD plates (**3.2**)
Selection of diploid yeast	
Day 13	Selection of diploid yeast in Sc –U–Trp plates (**3.3**)
Transfer to readout plates	
Day 15	Transfer diploid yeast to readout plates (**3.4**)
Imaging of readout plates	
Days 17-22	Image acquisition of readout plates depending on background (**3.5**)

Table 2 Click here to access/download;Table;Table 2 110618.xlsx

REAGENT	QUANTITY (for 2 L)
Drop-out mix synthetic minus histidine, leucine, tryptophan and uracil, adenine, rich (2 g) w/o yeast nitrogen base	2.6 g
Yeast nitrogen base without amino acids and without ammonium sulfate (YNB)	3.4 g
Adenine hemisulfate dihydrate	160 mg
Ammonium sulfate	10 g
Agar	35 g
Glucose (40%, w/v) in water, sterile	100 mL
Leucine (100 mM), sterile filtered	16 mL
Tryptophan (40 mM), sterile filtered	16 mL

Table 3

REAGENT	QUANTITY (for 2 L)
Peptone	40 g
Yeast extract	20 g
Adenine hemisulfate dihydrate	0.32 g
Glucose (40%, w/v) in water, sterile	100 mL
Agar	35 g

Table 4

REAGENT	QUANTITY (for 2 L)
Drop-out mix synthetic minus histidine, leucine, tryptophan and uracil, adenine rich (2 g) w/o yeast nitrogen base	2.6 g
Yeast nitrogen base without amino acids and without ammonium sulfate (YNB)	3.4 g
Adenine hemisulfate dihydrate	160 mg
Ammonium sulfate	10 g
Agar	35 g
Glucose (40%, w/v) in water, sterile	100 mL
Leucine (100 mM), sterile filtered	16 mL
Histidine (100 mM), sterile filtered	16 mL
Uracil (20 mM), sterile filtered (omit for Sc –U –Trp plates)	16 mL

Table 5 Click here to access/download;Table;Table 5 110618.xlsx

REAGENT	QUANTITY (for 2 L)
Drop-out mix synthetic minus histidine, leucine, tryptophan and uracil, adenine rich (2 g) w/o yeast nitrogen base	2.6 g
Yeast nitrogen base without amino acids and without ammonium sulfate (YNB)	3.4 g
Adenine hemisulfate dihydrate	160 mg
Ammonium sulfate	10 g
Agar	35 g
Glucose (40%, w/v) in water, sterile	100 mL
10x BU salts	200 mL
Leucine (100 mM), sterile filtered	16 mL
3AT (2 M), sterile filtered	5 mL
X-gal (80 mg/mL) in DMF	4 mL

Name of Material/ Equipment	Company
3-Amino-1,2,4-triazole (3AT) ~95 % TLC	Sigma
Adenine sulfate (hemisulfate), dihydrate	US Biologicals
Agar High Gel Strength - Bacteriological grade	American International Chemical
Ammonium Sulfate	US Biologicals
D+ Glucose Anhydrous	US Biologicals
Drop-Out Mix minus His, Leu, Tryp and Uracil, adenine rich w/o yeast nitrogen base	US Biologicals
edge Multiparameter pH Meter	Hanna Instruments
Flat Toothpicks 750ct	Diamond
Glass Beads	Walter Stern
Glycerol ≥99%	Millipore Sigma
L-Histidine	US Biologicals
L-Leucine	US Biologicals
L-Tryptophan	Sigma
N,N-Dimethylformamide	Sigma
Omnipense Elite	Wheaton
Peptone, Bacteriological	American International Chemical
Petri Dish, 150x15 mm	VWR
PlusPlates	Singer Instruments
Precision Low Temperature BOD Refrigerated Incubator	ThermoFisher Scientific
RePads 1,536 short	Singer Instruments
RePads 384 short	Singer Instruments
RePads 96 long	Singer Instruments
RePads 96 short	Singer Instruments
Singer HDA RoToR robot	Singer Instruments
Sodium Hydroxide (Pellets/Certified ACS)	Fisher
Sodium Phosphate dibasic heptahydrate	Santa Cruz Biotechnology
Sodium Phosphate monobasic monohydrate	Santa Cruz Biotechnology
Uracil	Sigma
X-gal (5-Bromo-4-chloro-3-indoxyl-beta-Dgalactopyranoside)	Gold Biotechnology
Yeast Extract	US Biologicals
Yeast Nitrogen Base (powder) w/o AA, carbohydrate and w/o AS	US Biologicals

Catalog Number	Comments/Description
A8056-100G	Competitive inhibitor for products of HIS3 gene
A0865	Required for proper yeast growth
AGHGUP	Nutritive media for yeast growth
A1450	Nitrogen source in synthetic yeast media
G3050	Required for yeast growth
D9540-02	Synthetic complete media required for yeast growth
HI2020-01	To measure pH of selective media
	To streak yeasts on petridishes
100C	To spreak yeast when making lawns
G9012-1L	Required to make frozen yeast stocks
H5100	For yeast growth selection in selective media
L2020-05	For yeast growth selection in selective media
T-0254	For yeast growth selection in selective media
319937-1L	To make X-gal solution
W375030-A	For dispensing accurate volumes of media into Singer plates
PEBAUP	Protein source required for yeast growth
10753-950	For growing yeast baits for screening
PLU-003	To make rectangular agar plates to use with Singer Robot
PR205745R	To incubate yeast plates at constant temperature
REP-005	To transfer the TF-prey array, mate yeast, and transfer yeast to diploid selection and readout plates
REP-004	To transfer TF-prey array from 384 to 1,536 colony format
REP-001	To transfer TF-prey array from glycerol stock to agar plate
REP-002	To transfer TF-prey array from 96 to 384 colony format
	For transfering yeast in high-throughput manner
S318-1	For adjusting pH of selective media
sc-203402C	Required for LacZ reporter activity on X-gal
sc-202342B	Required for LacZ reporter activity on X-gal
U0750-100G	For yeast growth selection in selective media
X4281C100	β-galactosidase turns colorless X-gal blue to detect protein-DNA interaction
Y2010	Nutritious medium for growth and propagation of yeast
Y2030	Required for vigorous yeast growth

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Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise lines 65-68, 123-132, 142-148, 163-169 and 420-421 to avoid previously published text.

These lines were original, but given that they refer to a protocol, inevitably the text will be somewhat similar. We made additional changes we hope will satisfy the editors.

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References to companies and trademark/registered symbols were removed. We did keep references to HDA Rotor robot and PlusPlates, without indicating the company, as these resources are critical for the protocol and confusion with other resources and plates would affect the success of the protocol.

4. Lines 110-111, 130-131, 148-149, 170-171: Please remove the embedded tables from the manuscript and upload each Table individually to your Editorial Manager account as an .xls or .xlsx file. Then reference the table in the manuscript.

Tables were removed from the manuscript and are submitted as separate files. Reference to the tables was added in the manuscript.

5. 1.1.1: Please reference the table for composition.

The Table now is referenced.

6. 1.4.6: Please specify the flow rate.

The flow rate (5 mL per sec) and the tubing size (6 mm) are indicated in the revised manuscript.

7. Lines 190-196, 198-201: Please make these a numbered step and write the text in the imperative tense in complete sentences.

This section has been amended to include the recipes as numbered steps.

8. 3.1.7: Please specify the incubation temperature.

Incubation temperature was added.

9. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

The following paragraphs were added to the discussion:

"There are several critical steps in the eY1H screening protocol to avoid some of the issues presented in Figure 3. First, although most media ingredients are stable for several months (except for 3AT and X-gal) a lack of proper colony growth likely indicates that at least one of the ingredients may have lost activity and should be replaced. Second, it is important to prepare the rectangular plates so that the agar is leveled and so that they do not dry for more than one day to avoid failure in pinning when using the robotic platform. Finally, it is key to use the robotic platform programs as indicated in the protocol (revisit, recycle, mixing, etc.) for the yeast to be transferred effectively, for mating to be efficient, and to avoid cross contamination between yeast clones."

"Although eY1H assays have been instrumental to identify the repertoire of TFs that bind to different regulatory regions in human and other species, they are not free of caveats 8,11,12,19,20,25. One of the limitations is that interactions are tested in the milieu of the yeast nucleus and, although the DNA-baits are chromatinized, the chromatin structure in yeast may not reflect the chromatin structure in the species from where the DNA-bait originated and will not reflect cell type differences observed in vivo. Thus, interactions identified by eY1H assays must be validated in reporter or other functional assays. Of note, we and others have found TF-DNA interactions detected by eY1H validate at a 40-70% rate in functional assays11,12,20,23. Another limitation of eY1H assays is that it cannot detect interactions involving TFs that require post-translational modifications absent in yeast to bind to DNA, TFs that are not properly folded in yeast when fused to the AD, and TFs that are missing from the array8. In addition, in the current format eY1H assays do not detect interactions involving heterodimeric TFs as each yeast colony in the TF array expresses a single TF-prey. Thus, further improvements in the assay will increase the breadth of TFs that can be tested and expand the capabilities of eY1H assays to identify novel TF-DNA interactions."

10. References: Please do not abbreviate journal titles.

We are using the JoVE Endnote style downloaded from the JoVE webpage. Our reference format also matches other JoVE articles. Is there an updated format? Please advise.

11. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the Name of Material/ Equipment.

Table of equipment and materials has been amended.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In the manuscript "Enhanced yeast one-hybrid screen to identify transcription factor binding to human DNA sequences", the authors developed an efficient method to detect interactions between transcription factors and DNA regions. As demonstrated by the authors using promoters of CCL15 and IL17F, the enhanced yeast one-hybrid assay will take a researcher two weeks to carry out a high-throughput screen. This protocol is definitely of great efficiency by using a robotic platform. In addition, the authors summarized failed results and showed how to troubleshoot them. This manuscript does provide sufficient information to ensure efficacy and reproducibility, but some problems still need to be concerned. Overall, I recommend this work to be published in Journal of Visualized Experiments.

Major Concerns:
1.In the Protocol Section 1.4, Line 169, the concentration of X-gal in DMF is 160mg/ml; however, for X-gal solution in Line 198, the authors dissolve 3.25g X-gal powder in 42.5ml DMF; thus, final concentration will be 76.5mg/ml. Please check the agent amount and concentration.

We thank the reviewer for noticing the incongruence and we apologize for the confusion. The concentration is 80 mg/mL and it should be prepared dissolving 3.5g X-gal powder in 42.5ml DMF (adding the X-gal powder to the DMF increases the volume to ~43.75 mL bringing the concentration to 80 mg/mL). This has been amended in Table 5 and in the text.

Minor Concerns:
1.In the Abstract, Line 34 and the Introduction, Line 61, the authors say" 'TF-prey', which can

be screened for activation of the DNA-bait". It may be more accurate to say: 'TF-prey', which can be screened for activation of the reporter.

Lines 34 and 61 were changed to: "'TF-prey', which can be screened for reporter gene activation"

2. Section 2 in the Protocol may puzzle the readers, because the font for 2.1, 2.2, and 2.3 etc. are the same as their subtitles (i.e. 2.2.1, 2.2.2 etc). To distinguish from experimental procedures, main titles can be displayed in bold as Section 3.

Titles in Section 2 were added and displayed in bold as in Section 3. Text was amended to fit the same format.

Reviewer #2:
In this article Shrestha et al. illustrate how to perform targeted yeast one-hybrid screens using a method coined enhanced Y1H.
The article is well written and provides a detailed step-by-step guide to understand and reproduce the method. Given that eY1H could be used to identify TF-DNA interactions in any organism (provided that a TF prey array is available), the article should be of interest to a broad audience.
Before publication a few points should be considered:
1) Abstract and intro should mention that the method requires a robot (HAD singer robot). This is necessary not only to perform the assay in a 1536 colony format but also to achieve the mentioned throughput (lines39-40 and 97-98 in abstract and intro respectively).

Lines 37-38 of the abstract was changed to "*using a high density array (HDA) robotic platform that allows screening in a 1,536 colony format*" and lines 79-80 of the introduction were changed to "*by using a high density array (HDA) robotic platform to mate yeast DNA-bait strains*" to acknowledge the used of the platform.

2) Preparing even agar plates (in Singer plates) is a critical reagent for this method. It would be useful to mention any tip to get a perfectly leveled agar medium surface in each plate.

The following notes were added in step **1.2.5** to comment on the critical steps in plate pouring:

"Notes: Although the suggested media volume is 70 mL per plate, 50-80 mL per plate can be used. The three critical issues to consider when pouring PlusPlates are:

1) that the plates are leveled so that the agar media has the same thickness throughout the plate (use a leveled table or surface for plate pouring and do not pour in stacks of more than seven plates).

2) to ensure the absence of bubbles in the agar media (bubble should be popped using a sterile needle)

3) drying the plates for only one day and wrapping the plates in plastic bags to avoid failures in pinning yeast."

3) Singer plates are filled with 70 mL of medium. How critical is it to have the same volume in each plate (or how flexible is the robot to deal with differences in medium surface height across multiple plates)?

The following note was added in step **1.2.5:** *"Although the suggested media volume is 70 mL per plate, 50-80 mL per plate can be used."*

4) Are pin pads used in the robot disposable? If not, how are pins washed in between plates (this is a key step that is missing in the procedure)?

We added the following note to step 2.2.1. *"Note: pin pads are not reusable and should be discarded."*

5) Line 199. Replace "Put" by "Add or Pour".

6) Line 199. Is it necessary to mention that x-gal is from goldbio?
Recipe in line 199 was changed to the following: *"**1.4.5.** Prepare the X-gal solution by adding 3.5g X-gal powder to a 50 mL plastic tube containing 42.5 mL dimethyl formamide. Add X-gal powder to dimethyl formamide to dissolve more easily (this takes 30 min). Keep stock solution in the dark (either use opaque 50 ml tube or cover in foil). Store at -20C."*

7) Line 206. A reference for the TF-prey yeast stock plate preparation should be provided.

Reference for TF-prey array generation were added: *"TF-prey arrays can be generated as previously published [10,12,13,18]"*

8) Line 335. "The strength of TF-DNA interactions.." should be "The strength of reporter activity.." (as it is mentioned in the next line there are several parameters that affect reporter expression, thus Y1H does not provide direct measurement of interaction strength).

This was changed to "*the strength of the reporter activity corresponding to TF-DNA interactions*".

9) Line 336. Among the parameters it could be mentioned that each TF might reach different protein levels.

The text was amended the following way: "*depends on many parameters such as the affinity, the TF expression level in yeast, the number of binding sites and distance to the yeast minimal promoters located upstream of the reporter genes, and the background reporter activity of the DNA-bait strain*".

10) Line 455. Ref 24 reported a partial Arabidpsis TF prey-array. An additional reference for a more comprehensive TF prey-array could be added (Pruneda-Paz et al, Cell Reports 2014).

We thank the reviewer for pointing this out. The reference was added to the revised manuscript.

11) Fig 1C. Given that DNA-bait strains are in the background of the mating plate, are colonies visible in the Mating YAPD plate? (if not, mating plate drawing should be revised).

The DNA bait lawns look like white/cream color throughout the plate but no individual colonies can be observed. The figure was amended to represent that and avoid confusion with the background color of the agar plate.