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# AhR/Arnt:XRE interaction: Turning false negatives into true positives in the modified yeast one-hybrid assay

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ABSTRACT

Given the frequent occurrence of false negatives in yeast genetic assays, it is both interesting and practical to address the possible mechanisms of false negatives and, more important, to turn false negatives into true positives. We recently developed a modified yeast one-hybrid system (MY1H) useful for investigation of simultaneous protein–protein and protein:DNA interactions in vivo. We coexpressed the basic helix–loop–helix/Per-Arnt-Sim (bHLH/PAS) domains of aryl hydrocarbon receptor (AhR) and aryl hydrocarbon receptor nuclear translocator (Arnt)—namely NAhR and NArnt, respectively—which are known to form heterodimers and bind the cognate xenobiotic response element (XRE) sequence both in vitro and in vivo, as a positive control in the study of XRE-binding proteins in the MY1H system. However, we observed negative results, that is, no positive signal detected from binding of the NAhR/NArnt heterodimer and XRE site. We demonstrate that by increasing the copy number of XRE sites integrated into the yeast genome and using double GAL4 activation domains, the NAhR/NArnt heterodimer forms and specifically binds the cognate XRE sequence, an interaction that is now clearly detectable in the MY1H system. This methodology may be helpful in troubleshooting and correcting false negatives that arise from unproductive transcription in yeast genetic assays.



Since their introduction nearly two decades ago, the yeast two-hybrid (Y2H)[1] [1] and yeast one-hybrid (Y1H) [2] in vivo genetic assays have been widely used for identification and characterization of protein–protein and protein:DNA interactions. With the arrival of the postgenome era, these two systems, together with their mammalian and bacterial counterparts [3,4], are being applied extensively to search networks of interactions in pathways and genomes [5–7].

A general pitfall of genetic assays is the all-too-common observation of false positives and false negatives in yeast reporter assays [8]. Endogenous proteins can affect the protein–protein or protein:DNA interactions under investigation either positively (false positive) or negatively (false negative). In addition, some activation domain (AD) fusions autoactivate transcription without protein or DNA binding as a prerequisite, and some AD fusions can bind or regulate promoter sequences by themselves without interaction with the DNA-binding domain (DBD) fusion in the Y2H or designated DNA target element in the Y1H. In the case of the Y2H, the DBD fusion may also activate transcription independent of the AD fusion [9–11]. In addition, there exist a relatively small number of "sticky" or "promiscuous" proteins frequently detected using multiple baits. Moreover, some physically true, yet physiologically irrelevant, interactions may also be categorized as one class of false positives [12].

Compared with false positives, false negatives have received much less attention. However, as researchers shift their focus from detecting any protein that interacts with their protein or DNA targets of interest toward mapping all of the protein–protein and protein:DNA interactions on a genome- or pathway-wide scale, the issue of false negatives becomes more important [12]. False negatives stem from various factors, including weak interactions beyond the detection limitations of a yeast-based genetic system, proteins not stably expressed or folded improperly in the host cell, proteins not localizing to the nucleus, and posttranslational modifications not provided by the host cell's machinery. In addition, high expression levels of some hybrid proteins can be toxic to the host cell, and eukaryotic regulatory proteins may interfere with the function of their yeast homologs. Also, fused domains or epitope tags can have unintended effects by perturbing protein struc-

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[1] *Abbreviations used:* Y2H, yeast two-hybrid; Y1H, yeast one-hybrid; AD, activation domain; DBD, DNA-binding domain; AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; bHLH/PAS, basic helix–loop–helix/Per-Arnt-Sim; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCB, polychlorinated biphenyl; XRE, xenobiotic response element; DRE, dioxin response element; MY1H, modified Y1H system; 3-AT, 3-aminotriazole; SD, minimal synthetic dropout medium; TSS, transformation and storage solution; PCR, polymerase chain reaction; MCS, multiple cloning site; cDNA, complementary DNA; NAhR, $AhR_{6-436}$; NArnt, $Arnt_{82-464}$; ONPG, *ortho*-nitrophenyl-β-D-galactopyranoside; SEM, standard error measurement; S/N, signal-to-noise.

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ture or occluding the site of interaction. Furthermore, false negatives can be due simply to unproductive transcription of the reporter gene caused by an inappropriately positioned DNA target in the promoter and/or insufficient AD potency [6,9,12,13].

We examined the human aryl hydrocarbon receptor (AhR) and its partner aryl hydrocarbon receptor nuclear translocator (Arnt), which are basic helix–loop–helix/Per-Arnt-Sim (bHLH/PAS) proteins that regulate genes involved in the metabolism of carcinogens such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyls (PCBs). Because AhR alone is incapable of DNA binding, Arnt regulates the transcriptional activity of AhR by heterodimerization; thus, the AhR/Arnt heterodimer targets the cognate xenobiotic response element (XRE), 5′-TNGCGTG, also known as the dioxin response element (DRE) [14–19].

In this article, we demonstrate that a false negative interaction between the AhR/Arnt heterodimer and cognate XRE DNA site can be turned into a positive control in our modified Y1H system (MY1H) developed to investigate simultaneous protein–protein and protein:DNA interactions in vivo [20]. The methodology presented may assist in troubleshooting and correcting false negatives that arise from unproductive transcription in yeast genetic assays.

## Materials and methods

Reagents were purchased from BioShop Canada (Burlington, ON, Canada), enzymes were purchased from New England Biolabs (Pickering, ON, Canada), and oligonucleotides were synthesized by Operon Biotechnologies (Huntsville, AL, USA) unless otherwise stated.

### *Bacterial and yeast strains*

*Escherichia coli* SURE strain (Stop Unwanted Rearrangement Events, Stratagene, La Jolla, CA, USA) or *dam*$^-$/*dcm*$^-$ C2925 (New England Biolabs) was used for standard cloning and rescue of plasmids from yeast cells. SURE cells were used for routine cloning of DNA with secondary structures likely to be rearranged or deleted in conventional strains. C2925 is a methyltransferase-deficient *E. coli* strain used for growth and purification of plasmids free of *dam* and *dcm* methylation; this allows cloning to be performed with *dam*- or *dcm*-sensitive restriction sites. *Saccharomyces cerevisiae* YM4271 [*MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4-Δ512, gal80-Δ538, ade5::hisG*] was purchased from Clontech (Palo Alto, CA, USA) and used for plasmid construction via homologous recombination and reporter strain construction.

### *Construction of reporter strains*

Four yeast reporter strains were classified into two sets; three-copy strains and six-copy strains were created according to the Matchmaker One-Hybrid System User Manual (Clontech) for reporter assay analysis in the MY1H system. Three-copy strains YM4271[pHISi-1/XRE-3] and YM4271[pLacZi/XRE-3] contain three tandem copies of the consensus XRE (5′-TTGCGTG) [21] upstream of the *HIS3* and *lacZ* reporter genes, respectively. Similarly, the six-copy strains YM4271[pHISi-1/XRE-6] and YM4271[pLacZi/XRE-6] contain six tandem copies of the consensus XRE upstream of the *HIS3* and *lacZ* reporters, respectively. 3-Aminotriazole (3-AT) titration assay revealed that 30 mM 3-AT, necessary for inhibition of background *HIS3* expression, was sufficient to suppress background growth from both YM4271[pHISi-1/XRE-3] and YM4271[pHISi-1/XRE-6] on minimal synthetic dropout medium lacking histidine (SD/–H). Details of reporter strain construction are provided in the supplementary material.

### *Transformation, DNA preparation, and plasmid rescue*

Recombinant plasmids were transformed into *E. coli* by the standard transformation and storage solution (TSS) procedure [22]. Plasmids were isolated using the Wizard *Plus* SV Minipreps DNA Purification System (Promega, Madison, WI, USA). Yeast transformations were performed using either the standard lithium acetate method (Yeast Protocols Handbook, Clontech) or the transformation procedure developed by Dohmen and coworkers [23]. Transformants were selected by leucine prototrophy. Isolation of yeast plasmids was performed using the Zymoprep II Yeast Plasmid Miniprep Kit (Zymo Research, Orange, CA, USA). Polymerase chain reactions (PCRs) were performed with Phusion high-fidelity DNA polymerase (New England Biolabs). PCR products and DNA fragments for cloning were purified using a QIAquick Spin Kit, MinElute Kit, or QIAEX II Gel Extraction Kit (Qiagen, Mississauga, ON, Canada).

### *Plasmid construction*

All new constructs were confirmed by DNA sequencing on an Applied Biosystems (ABI) 3730XL 96 capillary sequencer at the DNA Sequencing Facility in the Centre for Applied Genomics, Hospital for Sick Children (Toronto, ON, Canada).

#### *pCETT2*

Plasmid pCETT2 (Fig. 1) was constructed by homologous recombination in YM4271 by insertion of a GAL4 AD gene upstream of multiple cloning site (MCS) II in pCETT [20]. This was achieved by use of *Sma*I-linearized pCETT and the T2AD fragment amplified by mutually primed synthesis [24] from pGAD424-MCS II [20] with oligonucleotides 5′-AAAGACGTCGCATGCAACTTCTTTTCTTT and 5′-ATTGACGTCAAGCTTGCATGCCGGTAGAGGT. The homology between the T2AD fragment and both ends of *Sma*I-linearized pCETT allows generation of pCETT2. In pCETT2, the gene encoding amino acids 768 to 881 of the GAL4 AD is inserted between $P_{ADH1(T)}$ and

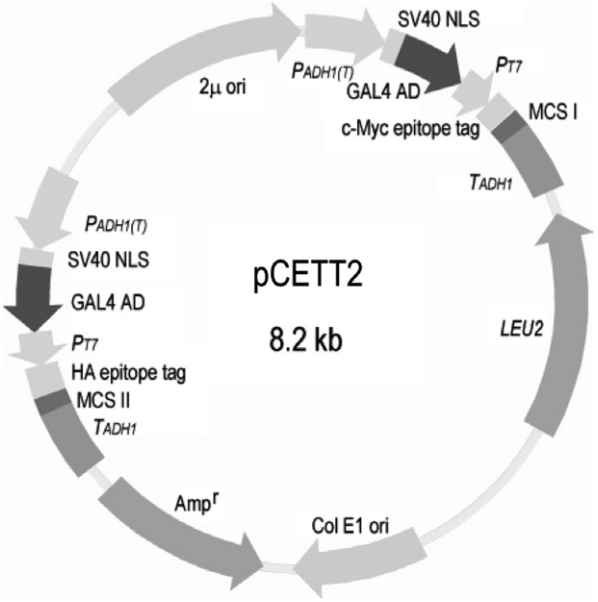


**Fig. 1.** Plasmid pCETT2 for coexpression of two AD fusion proteins in the MY1H system. The plasmid contains unique restriction sites located in the MCS I and MCS II regions. Both MCS I and MCS II are at the 3′ end of the open reading frame of the GAL4 AD sequence, allowing two fusion proteins, each of which combines the GAL4 AD and cloned protein of interest to be expressed at low levels from a truncated constitutive *ADH1* promoter.

$P_{T7}$ upstream of both MCS I and MCS II (in pCETT, this gene is inserted between $P_{ADH1(T)}$ and $P_{T7}$ upstream of MCS I only). Therefore, both monomers expressed from pCETT2 are expressed as GAL4 AD fusion proteins.

*pCETT/NAhR/NArnt and pCETT2/NAhR/NArnt*

Both human AhR (pRc-CMV/AhR) and human Arnt variant 3 (pRc-CMV/Arnt) complementary DNA (cDNA) [25] were generously provided by Patricia Harper and Allan Okey (Department of Pharmacology and Toxicology, University of Toronto). Details regarding the construction of the human $AhR_{6–436}$ fragment (NAhR) and the human $Arnt_{82–464}$ fragment (NArnt) are provided in the supplementary material. The NAhR and NArnt fragments contain cDNA fragments encoding the bHLH/PAS domains of each protein, respectively. The NAhR fragment was amplified and inserted into the *Sal*I and *Xba*I sites of MCS I in pCETT and pCETT2 to generate pCETT/NAhR and pCETT2/NAhR, respectively. To be consistent with a previously reported in vitro study [18], the NArnt fragment was based on the sequence of human Arnt variant 1, which we generated from the human Arnt variant 3 template provided in pRc-CMV/Arnt. The NArnt fragment was then inserted into the *Bam*HI and *Xho*I sites of MCS II in pCETT and pCETT2 to give pCETT//NArnt and pCETT2//NArnt, respectively.[2] Similarly, the NAhR fragment was inserted into the *Sal*I and *Xba*I sites of pCETT//NArnt and pCETT2//NArnt to generate pCETT/NAhR/NArnt and pCETT2/NAhR/NArnt, respectively.

*HIS3 reporter assay*

The integrated *HIS3* reporter strain was transformed with an AD fusion plasmid. The transformants were plated on SD/–H/–L plates. Interactions between the proteins under investigation and corresponding DNA target element were determined by activation of the *HIS3* reporter gene, which was measured by spotting 15 μl of diluted fresh cell resuspensions at $OD_{600} \sim 0.01$ on SD/–H/–L control plates and SD/–H/–L testing plates that contained 30 mM 3-AT. A positive interaction was indicated by growth of colonies on both types of plates.

*lacZ reporter assay*

Two commonly used β-galactosidase assays, qualitative X-gal colony lift filter assay and quantitative *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) liquid assay, were performed as described previously [20]. In the ONPG assay, results are presented as means ± standard error measurements (SEMs) from three or four independent experiments, each performed in triplicate.

## Results

*Initial trials with pCETT: Fusion of the GAL4 AD to NAhR only*

To construct a positive control in the study of XRE-binding proteins in our MY1H system, we first tried to coexpress both NAhR and NArnt from pCETT in yeast strain YM4271[pHISi-1/XRE-3], in which three copies of the XRE site were integrated into the yeast genome upstream of the *HIS3* reporter gene. Arnt can homodimerize, although it preferentially heterodimerizes [14]. To minimize the effect of Arnt homodimerization on reporter activation, NAhR is expressed as the fusion to the GAL4 AD (GAL4AD–NAhR, AD at N terminus) and NArnt is expressed as the independent protein with no AD fusion. The transformant expressing GAL4AD–NAhR and NArnt did not grow on SD/–H/–L plates containing 30 mM 3-AT, nor did the corresponding controls (Fig. 2A). Because increasing the copy number of XRE sites integrated upstream of the reporter gene may increase the probability of the NAhR/NArnt heterodimer binding at the promoter and/or optimize the distance between the promoter and site of transcription initiation, we constructed the six-copy strain YM4271[pHISi-1/XRE-6], wherein six copies of the XRE were integrated upstream of the reporter. However, coexpression of GAL4AD–NAhR and NArnt from pCETT in the YM4271[pHISi-1/XRE-6] strain did not show positive signal by *HIS3* assay as well (Fig. 2B).

The X-gal colony lift filter assay shows similar results to those of the *HIS3* assay. After 60 min incubation, both three- and six-copy transformants that coexpress GAL4AD–NAhR and NArnt proteins developed a faint blue color indistinguishable from their corresponding controls (Fig. 3A and B; see also Fig. S1A and B in the supplementary material). The quantitative ONPG assay provides more information than the *HIS3* and colony lift assays (Fig. 4A and B). When pCETT is used as the coexpression vector, the control transformant that contains vector only with no inserted genes gives background expression at 1.38 ± 0.06 in the three-copy strain, whereas it gives background expression at 4.83 ± 0.30 in the six-copy strain; therefore, increasing the copy number of DNA target elements increases background expression. When GAL4AD–NAhR and NArnt are coexpressed, the ONPG values are 3.16 ± 0.37 and 11.31 ± 0.81 in the three- and six-copy strains, respectively. Although the absolute value increases in the latter case, the actual signal-to-noise (S/N) ratios in both cases are not statistically different (2.29 vs. 2.34).

*Next generation trials with pCETT2: Fusion of the GAL4 AD to both NAhR and NArnt*

Plasmid pCETT2 is a double AD vector based on pCETT for coexpression of two AD fusion proteins in the MY1H system (Fig. 1). The difference between pCETT2 and pCETT lies in the expression cassette of the second protein; in pCETT, the GAL4 AD is fused to the protein expressed from only MCS I, whereas in pCETT2, the GAL4 AD is fused to proteins expressed from both MCS I and

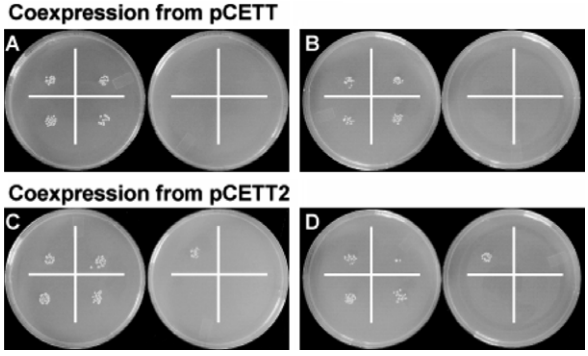


**Fig. 2.** *HIS3* reporter assay for detection of NAhR/NArnt:XRE interaction from protein expression vector pCETT or pCETT2 in YM4271[pHISi-1/XRE-3] (A and C) and YM4271[pHISi-1/XRE-6] (B and D) strains. The transformants that coexpress both GAL4AD–NAhR and NArnt (or GAL4AD–NAhR and GAL4AD–NArnt in the cases of panels B and D) as well as their corresponding controls were grown as patches on SD/–H/–L control plates (left in each subfigure) that select only for the presence of plasmid. These were replica-plated on SD/–H/–L testing plates containing 30 mM 3-AT (right in each subfigure) that select for *HIS3* reporter activity (30 °C, 5 days). The transformants on the same plate were identified according to the following order. (A and B) Upper left, pCETT/NAhR/NArnt; upper right, pCETT/NAhR; lower left, pCETT//NArnt; lower right, pCETT. (C and D) Upper left, pCETT2/NAhR/NArnt; upper right, pCETT2/NAhR; lower left, pCETT2//NArnt; lower right, pCETT2.

[2] Note that the recombinant plasmids pCETT and pCETT2 are named in the format "plasmid/MCS I/MCS II." When MCS I only is inserted with a gene, the format is "plasmid/MCS I"; when MCS II only is inserted with a gene, the format is "plasmid//MCS II."

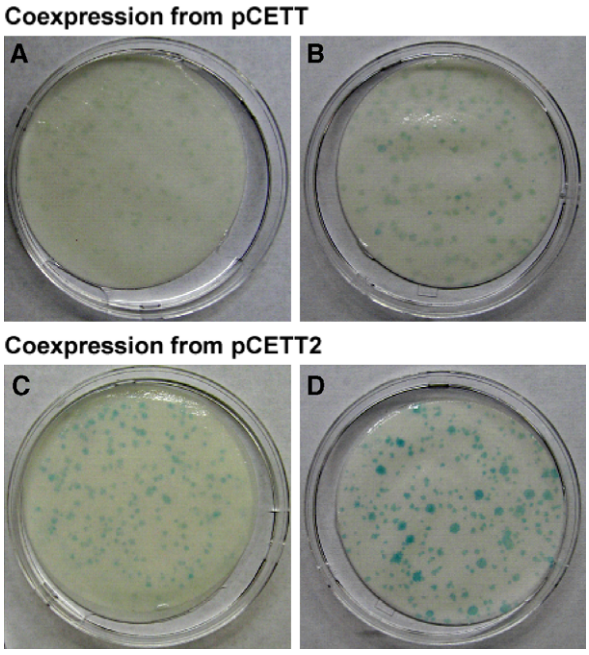


**Fig. 3.** Colony lift filter assay for detection of NAhR/NArnt:XRE interaction from protein expression vector pCETT or pCETT2 in YM4271[pHISi-1/XRE-3] (A and C) and YM4271[pHISi-1/XRE-6] (B and D) strains. The X-gal colony lift assay was performed after 4 days growth of transformants that were transformed with pCETT/NAhR/NArnt (A,B) or pCETT2/NAhR/NArnt (C and D) and plated on SD/–L/–U plates at 30 °C. Photos were taken after 60 min incubation at 30 °C. The colony lift filter assay results of their corresponding controls are shown in Fig. S1 in the supplementary material.

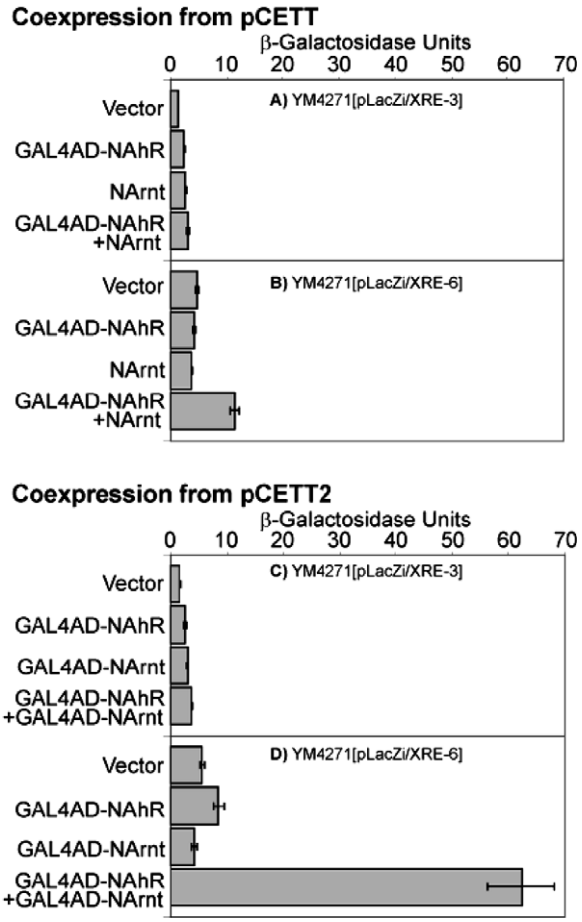


**Fig. 4.** ONPG assay for detection of NAhR/NArnt:XRE interaction from protein expression vector pCETT or pCETT2 in YM4271[pHISi-1/XRE-3] (A and C) and YM4271[pHISi-1/XRE-6] (B and D) strains. The horizontal axis indicates the mean values of β-galactosidase units. Error bars represent SEMs from at least three independent trials conducted in triplicate.

MCS II. In addition, the transcription of both genes is under the control of the same truncated *ADH1* promoter, leading to comparable low levels of protein expression [26].

From pCETT2, we coexpressed GAL4AD–NAhR and GAL4AD–NArnt in yeast strain YM4271[pHISi-1/XRE-3]. This time, transformants clearly grew normally on SD/–H/–L test plates containing 30 mM 3-AT, whereas the corresponding controls, which expressed either GAL4AD–NAhR or GAL4AD–NArnt or neither, were still clear on test plates (Fig. 2C). The same test was performed in YM4271[pHISi-1/XRE-6] with the same results (Fig. 2D). Because the *HIS3* reporter assay we performed is qualitative and shows only growth or death, this assay did not indicate a difference in signal from the three- or six-copy strain.

Results from the X-gal colony lift filter assay were consistent with the *HIS3* reporter assay. The cells transformed with pCETT2/NAhR/NArnt developed vivid blue color in the filter assay regardless of whether the plasmid was transformed into the three- or six-copy strain (Fig. 3C and D). The corresponding controls that express only one or neither of the two proteins showed no blue color development even after 60 min incubation (Fig. S1C and D in the supplementary material). Furthermore, when GAL4AD–NAhR and GAL4AD–NArnt were coexpressed in the six-copy strain, blue color began to show after 20 min, whereas in the three-copy strain, color started to develop after 40 min. After 1 h incubation, the former transformant showed blue color much more intense than the latter transformant.

The ONPG assay quantitatively confirmed the results obtained from both *HIS3* and colony lift assays. When GAL4AD–NAhR and GAL4AD–NArnt were coexpressed in YM4271[pLacZi/XRE-3], the ONPG value was 3.82 ± 0.13; its corresponding negative control transformed with pCETT2 gave an ONPG value of 1.59 ± 0.13 (Fig. 4C), corresponding to an S/N ratio of 2.40. In contrast, when GAL4AD–NAhR and GAL4AD–NArnt were coexpressed in YM4271[pLacZi/XRE-6], the ONPG value was 62.3 ± 5.9; its corresponding negative control transformed with pCETT2 gave an ONPG value of 5.67 ± 0.52 (Fig. 4D), corresponding to an S/N ratio of 10.98. In addition, ONPG values from these two negative controls in yeast strains with different copy numbers of XRE sites indicate again that increasing the copy number of DNA target elements also increases the background expression (1.59 ± 0.13 for three-copy strain vs. 5.67 ± 0.52 for six-copy strain).

As expected, the controls that express either GAL4AD–NAhR or GAL4AD–NArnt give only ONPG values close to their corresponding negative controls that express GAL4 AD only. Although Arnt can homodimerize and target the symmetric sequence 5′-CACGTG [27], we did not anticipate that the Arnt homodimer would target the asymmetric XRE sequence, 5′-TTGCGTG, and our data show that GAL4AD–NArnt displays activity indistinguishable from background.

## Discussion

### *Are false negatives due to unproductive transcription?*

Yeast model systems have already been used to study the protein–protein and protein:DNA interactions of AhR and Arnt. In 1995, successful isolation of a cDNA encoding Arnt was reported by using the recombinant AhR bHLH/PAS domain as a probe in cDNA library screening in the Y2H [15]. This work was followed

by two similar systems developed to investigate the AhR/Arnt heterodimer's (full-length or bHLH/PAS domain only) response to different AhR ligands in the Y1H system [17,19]. Therefore, we believe that the factors leading to the false negative observations in our initial experiments with pCETT may include unproductive transcription of the reporter gene from inappropriate positioning of XRE sites in the promoter region, masking effects of endogenous proteins that interact with DNA sites in the promoter region, steric hindrance from the NAhR/NArnt heterodimer that adversely affects transactivation of the GAL4 AD, and/or insufficient transactivation potency of a single AD on the heterodimer.

The above possibilities can be classified into two categories: target DNA element and AD. There are numerous factors that affect levels of gene transcription. From the standpoint of artificial transcription factor design, by using a DBD that binds its cognate response element with higher affinity or by using an AD that possesses stronger activation potency, levels of gene transcription can be increased [28]. Alternatively, increasing the potential for the DBD to find its specific target element by increasing the stoichiometry of cognate DNA sequences in the promoter [28,29] or multimerizing the AD on a single transcriptional factor [30] can also produce similar effects.

The NAhR/NArnt:XRE interaction in our MY1H initially showed a false negative. To turn this false negative into a true positive, the tactics mentioned above were combined to increase the levels of reporter gene transcription.

*Copy number of XRE target sites*

Transcription factor binding sites can function as regulated enhancer elements when multimerized and ligated to a promoter [28,31]. Therefore, increasing the copy number of XRE sites will likely increase the number of transcription factors bound in the promoter region, thereby synergistically increasing the activation potency of the reporter gene. As a matter of fact, it is not uncommon in nature for multiple DNA response elements to exist in a given gene's promoter region. For instance, yeast transcriptional activator GAL4 binds to multiple sites on DNA to activate transcription synergistically; the presence of two such sites can more than double the level of transcription from a single site [32]. In the case of XRE, at least six XRE sites have been identified in the upstream region of both rat and human *CYP1A1* genes [33], and eight XREs are located within 2.3 kb of the 5′-flanking region of the human *CYP1B1* gene [34].

Although the synergistic activation roles of these different XREs on gene regulation are unclear, in vitro experiments revealed that the AhR/Arnt heterodimer has different affinities for these XRE variants sharing the 5′-GCGTG core sequence [33,34]. These different XRE sites in the same promoter may be used to perform complicated regulation of downstream gene expression, as in the case of cI repressor and Cro proteins in phage λ or GAL4 in yeast [32,35]. Alternatively, it is also possible that these XRE sites are just evolutionary footprints from the different positions where XRE sites were placed to find a favorable spatial conformation for optimal synergistic gene regulation. These trials eventually led to a fraction of the total sites being physiologically relevant. Therefore, the presence of more XRE sites may simply enable transcription factors to perform their regulatory functions more advantageously.

However, the presence of more target elements in the promoter region also risks increased nonspecific interactions from endogenous biomolecules. Background expression will be more "leaky" as the number of DNA targets increases. Indeed, background expression increases by 3.6-fold in our studies when the number of XRE sites integrated into the yeast genome increases from three to six. The increase in background expression can become overwhelming when too many binding sites are present in the promoter. We constructed a yeast strain with 23 integrated copies of XRE sites in a pilot experiment and found that reporter gene activation from the negative control was so high that in the X-gal colony lift filter assay, the negative controls developed blue color within 5 min and intense blue color after 15 min of incubation.

When we increased the number of XRE sites integrated into the yeast genome, we still observed false negatives in our first trials with pCETT. Thus, we reevaluated the role of increased stoichiometry of target elements, which may be only one of many factors responsible for false negatives. Because insufficient transactivation potency from the single AD may also contribute to the false negative [36], the double AD expression vector pCETT2 was also employed to increase gene transcription levels.

*Double AD system*

In addition to increasing the number of transcription factors bound to DNA to trigger synergistic activation [32], oligomerization of the AD on a single transcriptional factor is another strategy for enhancement of gene transcription. For example, a transcription factor containing two copies of the VP16 AD upregulated transcription fivefold over the single copy AD activator [30].

Our strategy for AD oligomerization differs from that discussed above due to the heterodimeric nature of the NAhR/NArnt complex. By use of the double AD coexpression plasmid pCETT2, heterodimerization of GAL4AD–NAhR and GAL4AD–NArnt intrinsically leads to doubling the local concentration of ADs present at the promoter. This double AD heterodimer is somewhat different in structure from the multimerized AD on a single transcriptional factor, but we expected our strategy to yield a similar effect.

When both NAhR and NArnt proteins were coexpressed as GAL4 AD fusion proteins in strain YM4271[pLacZi/XRE-3], the sensitive *HIS3* reporter selection assay showed positive growth, demonstrating that doubling the AD concentration in the promoter region led to detectable transcriptional activation in the MY1H system. However, the ONPG assay showed the synergistic activation of this double AD in the three-copy strain to be insignificant (S/N = 2.40). Once the two fusion proteins were coexpressed in strain YM4271[pLacZi/XRE-6], the synergistic activation of this double AD was so considerable that the S/N ratio increased more than fourfold compared with the S/N ratio for the single AD heterodimer in the six-copy strain or the double AD heterodimer in the three-copy stain. This result proves that both increasing the number of DNA binding sites and increasing the number of activation domains contribute to the considerable augmentation of transcription potency of the reporter gene through synergistic activation and that implementation of both strategies was critical for avoiding the false negative result.

*Synergistic activation is due to both increase of XRE target sites and doubling of AD*

The data from trials with pCETT and pCETT2 reveal three important points. First, increase of the stoichiometry of XRE sites in the yeast genome from three to six raises background expression. Thus, it is feasible to "amplify" the signal from a specific protein:DNA interaction by increasing the copy number of DNA elements. However, the presence of too many DNA target elements can adversely affect the differentiation of true positive and negative signals. Second, only when both NAhR and NArnt are expressed as GAL4 AD fusion proteins can the reporter be activated in the MY1H system. This result indicates that the double AD system leads to more efficient reporter transcription. Third, and most significant, the considerable increase in S/N ratio requires increase of the copy number of XRE sites as well as attachment of ADs to both NAhR and NArnt. Overall, the data indicate that the combina-

tion of increasing target element stoichiometry and increasing the concentration of activation domains can make a false negative truly positive.

Another potential bonus of introducing synergistic activation into our MY1H system is that it increases the chances for detection of only weakly active proteins that would be considered inactive, and therefore missed, during testing of large libraries by genetic assays.

In summary, by increasing the copy number of XRE sites integrated into the yeast genome and doubling the GAL4 activation domains, we successfully turned a false negative NAhR/NArnt:XRE interaction into a true positive control that paves the way to further study of XRE-binding proteins in our MY1H system. Given that there is no general means for addressing all possible false positives and false negatives, the methodology we presented here provides an effective and productive way to pinpoint false negatives that arise from unproductive transcription.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2008.07.026.

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