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Notes & Tips

An optimized dexamethasone-methotrexate yeast 3-hybrid system for high-throughput screening of small molecule-protein interactions☆

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Yeast three-hybrid screening shows promise as a tool for engineering proteins with new functions, identifying drug targets, and cloning proteins based on function. We previously developed a dexamethasone-methotrexate (Dex-Mtx)[1] yeast three-hybrid system [1] based on the Brent "Interaction Trap" [2], which uses LexA as the DNA-binding domain (DBD) and B42 as the activation domain (AD). In our system, the DBD is expressed as a fusion protein with dihydrofolate reductase (DHFR) while the AD is expressed as a fusion protein with the glucocorticoid receptor (GR). Dex-Mtx, a chemically synthesized heterodimeric small molecule, induces dimerization of DBD-DHFR and AD-GR and thereby activates transcription of a downstream reporter gene (Fig. 1A). Here we describe the design of an optimized Dex-Mtx yeast three-hybrid system in which the genes encoding the DBD-DHFR fusion protein and the reporters have been integrated into the chromosome to stabilize the transcription readout.

While our Dex-Mtx yeast three-hybrid system generally worked quite well, the transcriptional readout was unstable. Variable levels of β-galactosidase activity, corresponding to reporter gene activation, were observed in individual colonies from identical strains. Furthermore, approximately 20% of colonies had no β-galactosidase activity at all. Instability of the transcriptional readout could be due to low-level expression of one of the gene products necessary for the assay or recombination of one of the essential plasmids to eliminate the gene of interest while retaining the selective marker. The system was originally developed with both of the yeast three-hybrid proteins and the *lacZ* reporter gene expressed from multicopy 2μ circle-based plasmids. This required transformation of three separate plasmids and constant selective pressure to maintain them in the yeast strain. The total copy number of plasmids with 2μ origins of replication is about 60 per cell; however, when multiple 2μ circle-based plasmids are present, the relative number of each plasmid likely varies. Clonal variation in relative plasmid copy number could affect the expression levels of the yeast three-hybrid proteins and the number of copies of the reporter gene resulting in variability in the transcriptional readout.

We envisioned that the Dex-Mtx yeast three-hybrid system would be used to screen for novel ligand-receptor interactions, simply by replacing the Dex and GR components with the ligand-receptor pair of interest. Thus, we wanted to stabilize the transcriptional readout, but do so in a manner that would make it straightforward to vary the AD fusion protein. Chromosomal integration provides a method for inserting the required genes into the yeast genome, such that the copy number is fixed and the risk of recombination or gene loss is minimized. The *DBD-DHFR* gene was integrated under the control of the inducible GAL1 promoter so that the level of transcription could be regulated. For the

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[1] *Abbrevations used:* Dex-Mtx, dexamethasone-methotrexate; DBD, DNA-binding domain; AD, activation domain; DHFR, dihydrofolate reductase; GR, glucocorticoid receptor; PCR, polymerase chain reaction.

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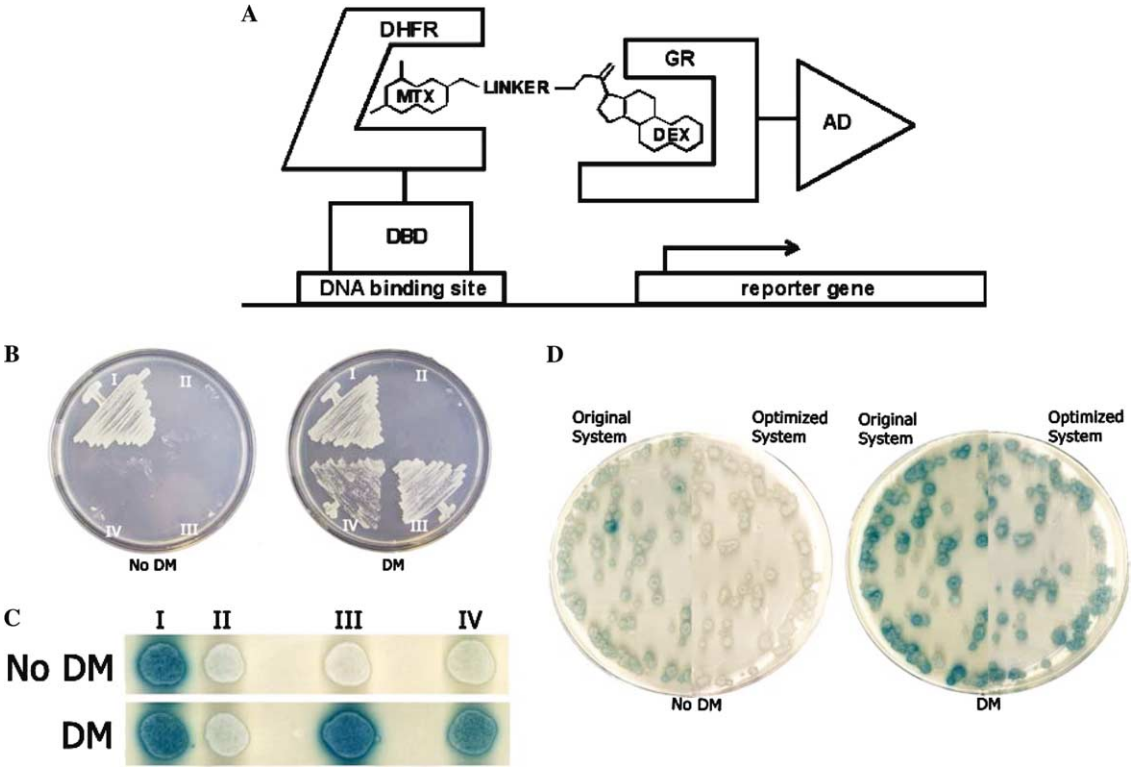

Fig. 1. An optimized dexamethasone-methotrexate yeast 3-hybrid system for high-throughput screening of small molecule-protein interactions. (A) Dexamethasone-methotrexate (Dex-Mtx) yeast three-hybrid system. A heterodimeric ligand (Dex-Mtx) bridges a DNA-binding domain-receptor fusion protein (DBD-DHFR) and a transcriptional activator domain-receptor fusion protein (AD-GR), effectively reconstituting a transcriptional activator and turning on transcription of a downstream reporter gene. (B and C) The optimized Dex-Mtx yeast three-hybrid system. Columns/segments I–IV on each plate correspond to yeast strains containing different DBD and/or AD chimeras, a *lacZ* reporter gene, and an integrated *LEU2* reporter gene: I, plasmid DBD-BAIT, plasmid AD-TARGET; plasmid *lacZ* reporter, integrated *LEU2* reporter. I is a direct protein-protein interaction used as a positive control; II, integrated DBD-DHFR, plasmid AD, plasmid *lacZ* reporter, integrated *LEU2* reporter. II has only the AD half of the AD-GR chimeric protein and is used as a negative control; III, integrated DBD-DHFR, plasmid AD-GR, integrated *lacZ* reporter, integrated *LEU2* reporter. III is the integrated Y3H system; IV, plasmid DBD-DHFR, plasmid AD-GR, plasmid *lacZ* reporter, integrated *LEU2* reporter. IV is the plasmid-based Y3H system. The plates were grown with or without 1 μM Dex-Mtx (DM) as indicated. (B) Dex-Mtx activates the *LEU2* reporter. Yeast strains were streaked onto synthetic complete media plates lacking histidine, uracil, tryptophan, and leucine and containing no glucose, 2% galactose, 2% raffinose, and either no Dex-Mtx (No DM) or 1 μM Dex-Mtx (DM) as indicated. Plates were incubated at 30 °C for 4 days. (C) X-gal plate assay of Dex-Mtx-induced *lacZ* transcription. X-gal plate assays were done as previously reported [4], except that plates were incubated for 3 days at 30 °C. (D) The integrated Dex-Mtx yeast three-hybrid system gives a more stable, consistent readout than the plasmid-based Dex-Mtx yeast three-hybrid system. The integrated yeast strain, V784Y, and the original strain, EGY48, containing the plasmid *lacZ* reporter pMW112 and the plasmid pKB521 encoding DBD-DHFR, were transformed side by side with the plasmid pBC398 encoding AD-GR and plated onto synthetic complete media lacking histidine, uracil, and tryptophan. The resulting colonies were replica plated onto X-gal plates containing either no Dex-Mtx (No DM) or 1 μM Dex-Mtx (DM) as indicated and incubated for 3 days at 30 °C.

integrated reporters, the widely used *LEU2* and *lacZ* genes were selected because of their complementary properties. The integrated *LEU2* reporter is very sensitive and allows for receptor/ligand pairs to be identified via a growth selection. These pairs can then be screened using the integrated *lacZ* reporter, which is less sensitive and can be used to rule out false positives.

Strain construction. The DBD-DHFR fusion protein was placed under control of the inducible GAL1 promoter by replacing the constitutive ADH promoter (P_{adh}) in plasmid pMW3eDHFR with the GAL1 promoter (P_{gal1}) from the plasmid pMW102. P_{adh} was cut out of pMW3eDHFR by restriction digestion with *Aat*II and partial restriction digestion with *Hin*dIII to obtain a 6953-bp fragment cut at the *Aat*II site upstream of P_{adh} and at the *Hin*dIII site just downstream of P_{adh}. P_{gal1} was obtained from pMW102 by restriction digestion with *Aat*II and *Hin*dIII to obtain a 3957-bp piece from the *Aat*II site upstream of P_{gal1} and the *Hin*dIII site just downstream of P_{gal1}. The DNA fragments were gel-purified, ligated together, and transformed into *Escherichia coli* TG-1 cells, and the resulting plasmid, pKB521, was purified using standard molecular biology techniques. Replacement of P_{adh} with P_{gal1} was verified by restriction analysis with the enzymes *Hin*dIII and *Aat*II. The integrating plasmid pKB523 encoding *lacZ* was prepared from pMW112, which contains the *lacZ* reporter downstream of 8 *lexA* operators. The 2μ origin of rep-

lication was cut out of pMW112 by restriction digestion at the two *Spe*I sites. The 9469-bp fragment was gel-purified, ligated, and transformed into *E. coli*. Correct removal of the 2μ origin of replication was verified by restriction analysis with *Spe*I.

The yeast strain EGY48 was chosen as the parent strain because it contains a *LEU2* reporter under the control of 6 *lexA* operators and is auxotrophic for histidine, uracil, and tryptophan. The gene expressing DBD-DHFR under control of P_{gal1} was integrated into the *ADE4* locus to give yeast strain V760Y. First, the P_{gal1}-*LexA-eDHFR-AdhT* and *HIS3* fragments were amplified from pKB521 to incorporate a 36-bp overlap of sequence identity between the two fragments. Fusion PCR was then performed to construct a P_{gal1}-*LexA-eDHFR-AdhT-HIS3* gene fragment suitable for integration. In order to integrate the PCR product into the *ADE4* gene locus, the 5′-sense PCR primer incorporated 60 bp of homology to the start of *ADE4*, and the 3′-antisense PCR primer incorporated 60 bp of homology to the end of *ADE4*. The PCR product was integrated into EGY48 using standard molecular biology techniques [3], and integrants were selected for on synthetic complete media lacking histidine. Integration of *DBD-DHFR* disrupted *ADE4*, resulting in adenine auxotrophy, which was verified by lack of growth on synthetic complete media lacking adenine. We next integrated V760Y with the *lacZ* reporter from pKB523. The integrating plasmid, pKB523, was linearized by digestion at the *Apa*I site in *URA3* and inserted into the *ura3* locus using standard molecular biology techniques [3] to make yeast strain V784Y. Integrants were selected for on synthetic complete media lacking uracil. Multiple copies of the *lacZ* reporter may have integrated into the chromosome, since the integration proceeds by insertion into, rather than replacement of, the *ura3* locus, and there is therefore nothing to prevent a second integration from occurring. Integration of both P_{gal1}-*LexA-eDHFR-AdhT-HIS3* and the *lacZ* reporter was verified by PCR analysis of chromosomal DNA. The final yeast strains used in the assays were prepared by transformation of EGY48, V784Y, and V760Y with the appropriate yeast-three-hybrid plasmids, listed in Table 1.

Results and discussion. While chromosomal integration of the genes necessary for the Dex-Mtx system should stabilize the transcriptional readout, the reduction in the number of copies of the genes could potentially cause an unwanted decrease in the sensitivity of the system. This proved not to be a problem, as the integrated system gave as strong, if not stronger readout as the plasmid-based system. In both systems, the *LEU2* reporter was activated in a Dex-Mtx-dependent manner, as the yeast strain was able to grow in the absence of leucine only when Dex-Mtx was added to the media (Fig. 1B, III and IV). Likewise, activation of the integrated *lacZ* reporter was Dex-Mtx dependent in both the integrated and the plasmid-based systems, as demonstrated by standard β-galactosidase assays on X-gal plates (Fig. 1C, III and IV) and in liquid assays (data not shown). The intensity of the blue color, corresponding to *lacZ* transcription, was not diminished in the integrated system, as might be expected due to lower levels of yeast three-hybrid proteins and the presence of fewer copies of the *lacZ* reporter gene. In liquid culture, the addition of 1 μM Dex-Mtx to the growth media resulted in a 15-fold activation in the integrated system, compared with a 4.5-fold activation in the plasmid-based system. Further, control assays in liquid culture showed that there were only background levels of β-galactosidase activity when Dex-Mtx was omitted or when only 1 μM Dex or 1 μM Mtx was added. A 10-fold excess of Mtx reduced Dex-Mtx-dependent activated

Table 1
Strains and plasmids used in this study

Name	Description	Source/reference
Strains	Genotype	
EGY48	*MATα trp1 his3 ura3 6lexAop-LEU2 GAL*$^+$	R. Brent/[5]
V784Y	*MATα trp1 6lexAop-LEU2 ade4::*P_{gal1}*-LexA-eDHFR*(*HIS3*) *ura3::x* (*8lexAop-lacZ*(*URA3*)) *GAL*$^+$	This study
V760Y	*MATα trp1 ura3 6lexAop-LEU2 ade4::*P_{gal1} *-LexA-eDHFR*(*HIS3*) *GAL*$^+$	This study
V1017Y	*EGY48* pBAIT pTARGET pMW112	This study
V780Y	*V760Y* pMW102 pMW112	This study
V781Y	*V784Y* pBC398	This study
V1018Y	*EGY48* pKB521 pBC398 pMW112	This study
Plasmids	Details	
pBAIT	P_{adh}*-LexA-BAIT* 2μ *HIS3* pBR ori amp^R	Origene
pTARGET	P_{gal1}*-B42-TARGET* 2μ *TRP1* pUC ori amp^R	Origene
pMW102	P_{gal1}/B42 2μ *TRP1* pUC ori kan^R	R. Brent/[6]
pMW112	*8lexAop-lacZ* 2μ *URA3* pBR ori kan^R	R. Brent/[6]
pMW3eDHFR	P_{adh}*-LexA-eDHFR* 2μ *HIS3* pBR ori kan^R	H. Lin/[1]
pBC398	P_{gal1}*-B42-*$(GSG)_2$*-rGR2* 2μ *TRP1* pUC ori kan^R	B. Carter/[4]
pKB521	P_{gal1}*-LexA-eDHFR* 2μ *HIS3* pBR ori kan^R	This study

lacZ transcription to near background levels, and a 10-fold excess of Dex did not effect Dex-Mtx activated *lacZ* transcription in the integrated strain as seen in previous results [1] with the plasmid-based system. However, we found that the integration of the *AD-GR* gene, when done in combination with both the *DBD-DHFR* and *lacZ* genes, diminished the cellular readout below detectable levels (data not shown). The relationship between the combination of integrated genes and the level of *lacZ* transcription is complex; however, preliminary data suggest that the *AD-GR* and *lacZ* genes, rather than the *DBD-DHFR* gene, are limiting.

Finally, we sought to determine whether integration of the yeast three-hybrid system increased the stability and consistency of the cellular readout. The plasmid expressing the AD-GR fusion protein was transformed into both the integrated strain, V784Y, and the original plasmid-based strain EGY48 containing the *lacZ* reporter on plasmid pMW112 and expressing the DBD-DHFR gene from plasmid pKB523. Positive transformants were replica-plated onto X-gal plates containing 1 μM Dex-Mtx (Fig. 1D). The percentage of white colonies, corresponding to false negatives, was dramatically reduced for the integrated system, from 20 to 3%, indicating that this system is much more stable than the plasmid-based system. Furthermore, unlike the plasmid-based strain, the integrated strain gave a highly consistent *lacZ* readout, with all the colonies showing a similar intensity of blue color on X-gal plates (Fig. 1D). This optimized Dex-Mtx yeast three-hybrid system should prove useful in the isolation of novel ligand-receptor pairs by enabling the successful screening of small molecule libraries, cDNA libraries, and protein libraries.

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