Construction of a Reporter Plasmid that Allows Expression Libraries to be Exploited for the One-Hybrid System

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The two-hybrid system, now firmly established as a routine method of molecular biology, determines protein/protein interactions involved in many cellular processes (7,8). Some components of cellular processes exert their influence by protein/DNA interactions. A modification of the two-hybrid system, coined appropriately the one-hybrid system (10), has been developed and allows screening of expression gene libraries for proteins that directly interact with DNA target sequences. Wang and Reed (13) used two plasmids: The first one, the reporter plasmid, contains the DNA binding site for the transcription factor Olf-1, cloned in three copies upstream of the GAL1 promoter in front of the HIS3 gene. The second plasmid contains the GAL4 activator domain driven by the ADC1 promoter upstream of inserts from a CDNA library. An earlier example of the one-hybrid system identified the DNA binding site for NGF1-B by genetic selection in yeast (14). Li and Herskowitz (10) isolated ORC6, a component of the yeast origin recognition complex, by the one-hybrid system. The reporter plasmid used in their system carried the lacZ gene, which allows blue/white selection for the recognition of positive protein/DNA interactions. Since the reporter plasmids employed in (10) and (13) with the DNA binding sites carry the LEU2 marker, it is not possible to use them with MATCHMAKEr™ libraries (Reference 4; CLONTECH Laboratories, Inc., 1020 East Meadow Circle, Palo Alto, CA 94303-4230, USA. Internet: yluo@clontech.com).

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Figure 1. The structure of the plasmid pRW95-1. The 2-kb Apal/SacI fragment from pRS315HIS (13), indicated by the double-ended arrow, was cloned into pPC86 (5), resulting in pRW95-1. The polylinker is located upstream of the GAL1 promoter in front of the HIS3 gene. The plasmid contains the yeast-selectable marker TRP1 and the terminator T-ADC. In addition, the plasmid carries a yeast centromere (CEN6), a yeast replication origin (ARS4), a bacterial replication origin (ori) and the ampicillin-resistance gene (Amp).
For construction of the new reporter plasmid, pRW95-1, a SacI/ApaI fragment of pRS315HIS (13), carrying the HIS3 reporter gene preceded by the GAL1 promoter, was inserted into the larger SacI/ApaI fragment of pPC86 (5). The resulting plasmid, pRW95-1 (Figure 1), consists of the HIS3 reporter gene downstream of the GAL1 promoter. The DNA binding sites under investigation can be cloned upstream of the GAL1 promoter by using the multiple cloning site. In many features, this plasmid is similar to pBM2389 and pHR307a as described by Liu et al. (11). pRW95-1 has, however, the advantage of a multiple cloning site to facilitate the insertion of the cis-DNA elements for which the cognate binding proteins are being sought, and its total length is 7 kb, as opposed to the 9.5 kb of pBM2389.

To test the system, three copies of the olfactory cyclic nucleotide channel, the Olf-1 DNA binding site (13), was inserted into a partially BamHI-digested pRW95-1, resulting in plasmid pRW95-2. The plasmid pGAD424-Olf-1 was constructed by cloning the Olf-1 cDNA insert, flanked by the GAL4 transactivation domain and the T-ADC from Y11 (13), into the SphI sites of pGAD424 (1). The plasmid Y11, encoding the Olf-1 protein, and the plasmid 59.7, with the Olf-1 DNA binding sites, were described previously (13). For sequencing the DNA binding site in pRW95-2, the primer T-ADC (5'-TTGATTGGAGACTTGACC-3') can be used. The yeast strain yWAM2 (MATα Δgal4 Δgal80 URA3::GAL1-lacZ lys2801amberhis3-D200 trp1-Δ63 leu2 ade2-101 ochre CYH2) was transformed according to the PLATE method (6).

After cotransformation of the yeast strain with the plasmids pRW95-2 and pGAD424-Olf-1, the transformants were plated on selective media with and without histidine (Figure 2). Yeast transformed with the plasmids Y11 and 59.7, as well as with the plasmids pGAD424-Olf-1 and pRW95-2, can grow in the presence of 5 mM 3-amino-triazole on media lacking histidine (1). No growth on plates lacking histidine was observed when transforming...
This means that transactivation only binds to the Olf-1 DNA binding site, pGAD424-01f-
yWAM2 occurs when the fusion protein of Table 1. Measurement of
Figure 3. The structure of the plasmid pRW95-3. The 4.9-kb SauI/ScaI fragment from pLacZ (3) was cloned into pcBl66 (S), resulting in pRW95-3. The polylinker is located upstream of the CYC1 promoter in front of the lacZ gene. The plasmid contains TRP1, T-ADC, CEN6, ARS4, ori and Ampas in pRW95-1.

yWAM2 with pRW95-1, and either pGAD424-01f-1 or pGAD424 and with pRW95-2 and pGAD424 (Figure 2). This means that transactivation only occurs when the fusion protein of Olf-1, with the GAL4 activation domain, binds to the Olf-1 DNA binding site, thus switching on the GAL1 promoter, which in turn activates the HIS3 gene.

In contrast to the low-copy-number CEN plasmid pRW95-1, the plasmid pGAD424-01f-1 is a high-copy-number plasmid. Therefore, the selection for the library plasmid is easier because, when plasmid rescue was performed, 90% of the plasmids obtained were found to be the library plasmid (data not shown).

In a series of cloning steps (K. Roder, unpublished results), we have constructed a plasmid, pRW95-3 (Figure 3), that contains the lacZ gene as the reporter. This permits quantitative measurements for protein/DNA interactions. Three copies of the Olf-1 DNA binding site (13) were inserted in the appropriate location of pRW95-3 to give rise to pRW95-4. We have tested this plasmid in the presence and absence of the expression plasmid pGAD424-Olf-1. The results shown in Table 1 confirm the interactions of the Olf-1 binding protein with its target sequence. A preliminary qualitative test can be made by subjecting the transformants to an in situ plate assay for β-galactosidase (9). This assay is sufficiently sensitive to differentiate between real and false positives.

In summary, we have constructed two reporter plasmids, pRW95-1 (HIS3) and pRW95-3 (lacZ), that allow commercially available expression libraries to be used in the one-hybrid system.

REFERENCES


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Device to Facilitate Colony Hybridization


Identification of specific genomic and cDNA clones propagated in bacteria is generally accomplished by transferring the bacterial colonies onto nylon or nitrocellulose filters, followed by hybridizing the filters with labeled probes (3,5). A critical step in this procedure is lysis of the bacterial cells and denaturation of the DNA on the filters. Commonly, this is performed by wetting the filters in a denaturing solution consisting of 0.5 N NaOH/1.5 M NaCl, followed by neutralization with a solution consisting of 1 M Tris-HCl, pH 7.4/1.5 M NaCl (5). During these steps, adequate care has to be taken to ensure that the duration of denaturation and neutralization of each of the filters is kept uniform. In addition, since each filter is transferred between solutions, care should be taken to prevent cross contamination of the colonies by the solutions getting on the top surface of the filters. This procedure, although effective, can be cumbersome and time-consuming, particularly when dealing with a large number of filters. Identification of false positives due to cross contamination can be a problem, particularly when using high-density colony filters. Alternative methods using a microwave oven to lyse cells and denature the DNA on filters have also been used—however, only with nylon filters (1). In addition, the duration of the treatment and the limited number of filters that can be processed at a time does not significantly decrease the time expended.

We describe the use of a simple and inexpensive device to facilitate colony lysis. This device, shown in Figure 1, consists of a support constructed from closely meshed fiberglass netting (window screen available at any hardware supply store) held in place by a frame made of Delrin® plastic (Delrin 500 series thermoplastic; Du Pont, Wilmington, DE, USA). Filters (Hybond™-N nylon filters; Amersham, Arlington

Figure 1. Schematic of the support device depicting the fiberglass netting in a plastic frame held together using plastic screws.