Two-hybrid reporter vectors for gap repair cloning

Jennifer I. Semple¹, Graham Prime², Lise J. Wallis², Christopher M. Sanderson¹, and David Markie²

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Yeast two-hybrid analysis is a valuable approach to the discovery and characterization of protein interactions. We have developed vectors that can indicate the presence of an insert when used in two-hybrid bait and prey construction by gap repair cloning. The strategy uses a recombination cloning site flanked by sequences encoding the GAL4 activation and binding domains. After gap repair cloning in standard hosts carrying an ADE2 reporter gene, disruption of GAL4 by an insert can be identified by the development of red colony color, while empty vector plasmids produce white colonies. Function in yeast two-hybrid applications was initially validated using known interacting proteins in pair-wise analyses, and subsequently, the bait vectors were used in library screens with the mouse Mad2l2 and human Mcd1 proteins, identifying a number of putative new interactions for these proteins. These vectors should facilitate high-throughput yeast two-hybrid screens in which large numbers of bait and prey constructs may be required.

INTRODUCTION

Since its description in 1989 (1), yeast two-hybrid analysis has become a widely used and invaluable tool for the identification of new protein interactions. Increasingly, two-hybrid analysis is being used in high-throughput approaches to address protein interactions on a global scale, and attempts at interaction analysis of entire viral (2), bacterial (3), yeast (4–6), and fruitfly proteomes (7) have been undertaken, with similar approaches in progress in the nematode *Caenorhabditis elegans* (8). To accommodate the scale of these analyses, significant methodological improvements have been required, including the development of hosts with multiple reporter genes to decrease false positives (9), mating-based approaches for screening libraries (4,6,10–12), and the use of gap repair approaches to directly clone DNA fragments in yeast. This last approach, based on the ability of yeast to recombine homologous sequences in vivo (13), can be used to construct plasmids from DNA fragments with overlapping terminal sequences (14).

Sequences as short as 25–30 bp can direct homologous recombination in yeast, enabling the addition of tags by PCR for cloning DNA fragments into linearized vectors (15). Gap repair cloning directly in a yeast host has clear advantages for large-scale yeast two-hybrid screens, where many bait and prey constructions may be required. However, an issue with using this methodology is the background of clones without inserts resulting from residual nonlinearized vector molecules. To expedite bait and prey cloning, we have developed vectors that can indicate the presence of an insert in standard yeast two-hybrid host strains. The strategy for insert indication is based on a disruption of function approach. Bait vectors were constructed by placing the *GAL4* activation domain sequence downstream of a binding domain sequence, and prey vectors were constructed by placing the sequences in the opposite orientation. Both of these arrangements reconstitute *GAL4* function from the empty vector that is capable of activating standard two-hybrid reporter genes. Insertion of appropriate DNA fragments at the

cloning site disrupts *GAL4* function, but maintains expression of fusion proteins similar to conventional yeast two-hybrid bait and prey vectors, respectively. The *ADE2* reporter gene, present in commonly used PJ69-4A and AH109 host strains, is most convenient to detect loss of *GAL4* function as it can be scored directly by colony color.

MATERIALS AND METHODS

Yeast Strains, Media, and Manipulation

The two-hybrid yeast strain PJ69-4A (9) was used as a host, and strain PJ69-4 α was developed to provide a suitable mating partner with identical genotype. To achieve this, the mating type of PJ69-4A was switched by transient expression of the *HO*-endonuclease from plasmid pGAL-HO (16). As expression of the *HO*-endonuclease from this plasmid is *GAL4*-dependent and the PJ69-4A host is null for this gene, the entire *GAL4* gene was first cloned at the *SalI* site of pGAL-HO. Mating type switching was then carried

¹MRC Rosalind Franklin Centre for Genomics Research, Cambridge, UK and ²University of Otago, Dunedin, New Zealand

out as described previously (16), and the resulting strain was subsequently cured of the plasmid by growth in nonselective conditions to produce PJ69-4 α .

Yeast were routinely grown in YPAD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L D-glucose, 50 mg/L adenine hemisulfate, 15 g/L agar when required). For selection, SD medium was used (6.7 g/L yeast nitrogen base without amino acids, 20 g/L D-glucose) with appropriate supplements for the PJ69-4A host to produce selective dropout media (20 μ g/mL adenine hemisulfate, 20 μ g/mL uracil, 20 μ g/mL L-histidine, 20 μ g/mL L-tryptophan, 20 μ g/mL L-methionine, 100 μ g/mL L-leucine). For optimal growth and the development of red color, bait strains were grown on SD medium supplemented with 14 g/L acid hydrolyzed casein (SD+C medium) and 20 μ g/mL each of adenine hemisulfate and uracil. The lithium acetate method (17) was used, with minor modifications, for all yeast transformations.

Construction of Vectors

To produce the bait vector pGBAD-B, a DNA fragment that contained the *GAL4* activation domain sequence was placed in-frame with the *GAL4* DNA binding domain sequence in plasmid pGBD-C1 (9), and the multiple cloning site was replaced with a synthetic DNA fragment containing the *attB1* and *attB2* sequences flanking a *Bam*HI restriction site (Figure 1). This vector was designed for cloning DNA fragments that incorporate a termination codon at the end of an open reading frame. A matched non-indicator bait plasmid (pGBD-B) was constructed by directly replacing the cloning site of pGBD-C1 with the same synthetic DNA fragment.

A second indicator bait plasmid was constructed that relies on frame-shifting rather than a stop codon to report the presence of an insert. This was achieved by replacing the cloning site of pGBAD-B with an alternative sequence that adds a single nucleotide (cytosine) between the recombination sites and a TA dinucleotide downstream that creates a stop codon in the appropriate reading frame (Figure 1). This plasmid was designated pGBAE-B.

In a similar approach for constructing insertional indicator vectors for prey cloning, the *GAL4*-binding domain sequence was placed downstream of the activation domain sequence in pACT2 (BD Biosciences Clontech, Palo Alto, CA, USA), and the original cloning site was replaced as before to produce pACTBD-B. This vector relies on a termination codon at the end of the incorporated open reading frame to indicate insertion. To provide a matched non-indicator prey vector, the cloning site of pACT2 was

also directly replaced with the new cloning site, producing pACT2-B.

A second indicator prey plasmid, pACTBE-B, which relies on a frameshift rather than a stop codon, was constructed in the same way as pGBAE-B, except that a TG rather than a TA dinucleotide was inserted to create the required stop codon (Figure 1).

To provide a plasmid capable of expressing an ochre termination suppressor in yeast, a 914-bp *Sal*II-*Hind*III fragment, containing the *SUP4-o* allele from pYAC4, was ligated into

Figure 1. Two-hybrid vectors. (A) Vector structure. The two indicator bait vectors pGBAD-B and pGBAE-B are based on the pGBD-C1 vector and are identical in overall structure to one another (left). The non-indicator bait vector pGBD-B (not shown) is similar in structure but lacks the *GAL4* activation domain. The two indicator prey vectors pACTBD-B and pACTBE-B (right) are based on pACT2. The non-indicator prey vector pACT2-B is similar but lacks the *GAL4*-binding domain sequence. (B) Vector cloning sites. The *attB1* and *attB2* sequences are incorporated in the cloning sites of all vectors and consequently are included as part of the A1 and A2 tags added to PCR fragments for cloning. In the indicator vectors, the cloning site maintains an open reading frame between the two *GAL4* domain sequences, as indicated by the triplet spacing. For indicator function in the pGBAD-B and pACTBD-B vectors, an in-frame stop codon (TAA) is incorporated at the end of the insert fragment to terminate translation of the fusion protein, thereby extinguishing *GAL4* function. Indicator function for pGBAE-B and pACTBE-B is based on frameshifting. In these vectors, the addition of one nucleotide between the recombination sequences and two nucleotides downstream (shown in boxes) maintains the reading frame prior to cloning, but incorporation of an open reading frame by gap repair cloning (shown as NNN) shifts the reading frame with subsequent translation termination directly after the A2 recombination site.

Sall-*Hind*III-digested YCplac111, a yeast-*Escherichia coli* shuttle vector that carries the yeast *LEU2* gene (18). After construction in *E. coli*, this plasmid (pSUP4) was transferred to the two-hybrid host strains for use as a mating partner to test for intact open reading frames from inserts cloned in pGBAD-B.

Cloning of Open Reading Frames as Baits and Preys

Gene fragments were obtained by PCR using Expand™ High Fidelity DNA Polymerase (Roche Diagnostics, Indianapolis, IN, USA) from mouse or human first-strand cDNA with gene-specific primers that add short sequence tags. The A1 tag (5'-GAATTCACAAGTTTGTG-CAAAAAGCAGGCTGG-3') was added in frame to sense primers, and the A2 tag (5'-GTCGACCACTTTGTACAAGAAAGCTGGGTG-3') was added in frame to antisense primers. An in-frame ochre stop codon was added to the antisense tag (5'-GTCGACCACTTTGTACAAGAAAGCTGGGTGTTA-3') when required for cloning in the pGBAD-B and pACTBD-B vectors. The resulting DNA fragments were cotransformed into yeast hosts with *Bam*HI-linearized vector DNA and plated with selection to enrich for functional plasmid reconstruction by gap repair cloning. For bait cloning, the selective medium was SD+C supplemented with uracil (20 µg/mL) and limiting adenine (20 µg/mL), which allows selection for the *TRP1* gene on bait vectors but provides better color development in these host strains than standard -trp dropout medium. Standard -leu dropout medium with limiting adenine was used for prey cloning. Individual colonies were tested by PCR for the presence of an insert of the correct size, but were not further characterized by DNA sequencing.

Pair-Wise Mating for Interaction Testing

For interaction testing, single colonies from bait and prey constructions were suspended in sterile water, mixed in appropriate combinations, and 5-µL aliquots were arrayed onto the

surface of a YPAD plate for overnight growth at 30°C. Following replica plating to -trp-leu dropout medium and growth to establish diploids, testing for reporter gene activation was achieved by further replica plating to -trp-leu-his and to -trp-leu-ade dropout medium.

Two-Hybrid Library Screening

Plasmid cDNA libraries in the vector pACT2 were obtained from BD Biosciences Clontech and transformed into yeast host strains. The resulting colonies were harvested, pooled, made to 15% glycerol, and frozen at -70°C in aliquots of at least 10⁸ viable cells. For Mad212 screens, a single aliquot of frozen library was thawed, added to 100–200 mL YPAD broth, and grown overnight at 30°C with shaking. For Mccd1 screens, frozen aliquots (5 × 10⁸ viable cells) were used directly for mating without prior overnight growth in YPAD.

A pool of 6–12 individual colonies from bait constructions was made for library screens and grown overnight in SD+C broth supplemented with 100 µg/mL adenine hemisulfate and 20 µg/mL uracil. For each screen, 5 × 10⁸ freshly grown bait cells were mixed with a similar number of cells from the library culture and spread over a 140-mm diameter plate of YPAD agar. After 4–6 h incubation at 30°C, the cells were harvested and spread over selective plates of -leu-trp-his dropout medium with a total surface area of around 2000 cm². Colonies were picked after 3 days growth and checked for activation of second and third reporter genes (9). Confirmation of interactions was undertaken in a similar manner to that described by Petermann et al. (19). In brief, prey inserts were amplified using vector-specific primers with attached A1 and A2 tags, and “rescued” by gap repair cloning with *Bam*HI-digested pACT2-B (a non-indicator vector) into a “naïve” host of appropriate mating type. No attempt was made to differentiate clones with inserts, and all colonies were mated to bait strains using a replica plating approach. Confirmation of a specific interaction in the yeast two-hybrid system was assumed if multiple rescued prey colonies from a primary

clone demonstrated growth on -ade and -his dropout medium after mating with the original bait strain, but not with an unrelated bait strain. For clones that demonstrated a reproducible and specific yeast two-hybrid interaction, interaction sequence tags (ISTs) were then obtained by sequencing the PCR fragments from prey inserts, and the corresponding genes were identified by searching nucleic acid databases.

RESULTS

Strategy for Vector Design

In addition to the reconstruction of *GAL4* function to allow a simple assay for disruption by insertion, some consideration was given to the sequences used for recombination cloning. A cloning site common to all vectors, which included the *attB1* and *attB2* sequences (Figure 1B), was used so that appropriately tagged DNA fragments could be readily cloned as both baits and preys in yeast, as well as providing compatibility with the Gateway™ system (Invitrogen, Carlsbad, CA, USA) for cloning the same fragments into *E. coli*.

Two alternative approaches were used to produce termination after translation of the insert fragment and prior to the downstream *GAL4* domain in the vectors. In the first, a stop codon was incorporated at the end of open reading frames during PCR, and the resulting DNA fragments were cloned into the pGBAD-B and pACTBD-B indicator vectors or into the pGBD-B and pACT2-B non-indicator vectors. This approach has the advantage that the C terminus of the fusion protein is completely defined and contains no extra peptide tag that may interfere with interactions. In addition, subsequent mating to a yeast strain expressing an ochre termination suppressor allele from the pSUP4 plasmid (see the Materials and Methods section) can be used to confirm that the cloned insert encodes an open reading frame through restoration of *GAL4* function, although this was not utilized for the routine cloning of baits and preys described in this study. However, one disadvantage of incorporating a stop codon

in the insert is that it limits utility when cloning the same fragment in Gateway destination vectors designed to express C-terminal fusion proteins. For this reason, a second approach was developed that relies on insert-dependent frameshifting in the vectors for reporting insertion, but maintains the reading frame between the insert and the downstream *attB2* sequence (Figure 1B) for Gateway cloning.

Performance in Gap Repair Cloning

As expected, the use of indicator vectors for gap repair cloning of PCR fragments as baits and preys produced a mixture of red and white colonies on appropriate medium containing limiting concentrations of adenine (Figure 2). The development of red color was improved by including acid-hydrolyzed casein in the medium (SD+C) when cloning baits, presumably because this supplement provides amino acids that are limiting, but not essential, for growth of the host strains. Similar supplementation was precluded for prey cloning, as selection for the *LEU2* gene can not be applied, but color development was adequate on standard dropout medium to allow differentiation of colonies.

To determine the efficacy of red colony color as an indication of insertion, individual clones were amplified using the A1 and

Figure 2. Colony color as an indicator of insert status in the bait vector pGBAD-B. Disrupted *GAL4* function fails to activate the *ADE2* reporter gene resulting in the production of red colony pigmentation in clones that contain inserts. White colonies represent clones with intact *GAL4* function owing to the absence of inserts.

A2 sequences as PCR primers and analyzed on agarose gels. In one such analysis, a total of 96 colonies from six different open reading frames cloned in the pGBAD-B plasmid were examined (see Figure S1 in the Supplementary material that can be found online at www.BioTechniques.com). Ninety-four colonies produced a fragment of the expected size, one colony produced no fragment from PCR, and one colony produced two fragments, suggesting a greater than 95% cloning efficiency when red colonies were selected. This result was representative of most gap repair cloning experiments, although

when secondary bands were visible in the original PCR sample used for cloning, an increase in the number of inserts of incorrect size was found.

In gap repair cloning experiments carried out in parallel, it was also observed that the total number of background white colonies was relatively constant for each preparation of *Bam*HI-digested vector. However, the number of white colonies was varied between different preparations of linear vector. This suggests that white colonies are largely the product of residual nonlinearized vectors following restriction enzyme digestion,

which may vary between preparations. An exception occurred when attempts were made to clone as bait the mouse β -catenin gene, which encodes a protein with a transcriptional activation domain. Very few red colonies were observed, and all contained inserts of aberrant size. Similar analysis of the abundant white colonies demonstrated that most contained the correct insert, suggesting autoactivation of the *ADE2* reporter gene by the fusion protein, thereby making insertion undetectable by colony color. Such auto-activating baits are unsuitable for library screening and can be excluded at this early stage of bait construction.

It was also observed from a series of gap repair cloning experiments that although the number of background white colonies was usually consistent, the number of red colonies varied but correlated with the amount of insert DNA, as determined by band intensity of the PCR product. In general, the use of crude

Figure 3. Replication of known protein interactions. (A) Mouse *Bub3* and *Mad211* genes were cloned as baits into pGBD-B and pGBAD-B. Fragments of mouse *Bub1* and *Mad1* genes were cloned as preys in pACT2-B and pACTBD-B. After pair-wise mating of the bait and prey strains and establishment of the diploid strains, interaction testing was undertaken by replica plating to selective media. Clear growth is seen for all vector combinations with the Bub1-Bub3 interaction, but differential growth is seen for the Mad1-Mad211 interaction. (B) Testing of the pGBAE-B and pACTBE-B vectors was undertaken by cloning four human genes in both plasmids and carrying out pair-wise matings. The protein interactions Csnk2b-Csnk2b, Csnk2b-Csnk2a2, and Atp6v1g1-Atp6v1e1 were reproduced as expected, although once again, variations in growth were noted. In this case, the apparent interaction strength was dependent upon whether Csnk2b was used as bait or prey.

Table 1. Results of Parallel Yeast Two-Hybrid Screens Using Mouse Mad2l2 Protein as Bait

Vector	Accession No. Matching IST	Corresponding Locus Name	Prey Description/Domains/Function	ISTs
pGBD-B	NM_023429	Ociad1	Expressed in ES cells and developing vasculature	18
	NM_023056	1810009M01Rik	Lr8 protein	5
	NM_025931	Rabl4	Putative ras-related GTP-binding protein, Rabl4	5
	XM_358819	9530039J15Rik	Noncoding 3' UTR only	2
	NM_178647	Cggbp1	Includes 300-bp 5' UTR	2
	NM_183426	BC019206	Strawberry notch homologue, DExH box helicase	1
	NM_011591	Timm17b	Protein translocation across inner mitochondrial membrane	1
	NM_026307	0610039D01Rik	Similar to proteins involved in divalent cation tolerance	1
	NM_020493	Srf	Noncoding 3' UTR only	1
	XM_128800	Map4k3	Noncoding 3' UTR only	1
	BC002262	—	Single EST and genomic sequence match	1
pGBAD-B	NM_023429	Ociad1	Expressed in ES cells and developing vasculature	12
	NM_183426	BC019206	Strawberry notch homologue, DExH box helicase	4
	NM_025931	Rabl4	Putative ras-related GTP-binding protein, Rabl4	3
	NM_023056	1810009M01Rik	Lr8 protein	2
	NM_145625	Eif4b	Includes 700-bp 5' UTR	1
	AK010603	Atp6v1e1	Noncoding 3' UTR only	1
	BC057660	Clta	Clathrin subunit involved in receptor-mediated endocytosis	1
	BG296586	Mm.328595	Two EST and genomic sequence match	1
	NM_011619	Tnnt2	Troponin T2, cardiac	1

Locus name entries shown in bold were identified in library screens with both vectors. The number of times a gene was identified in each screen is shown under the interaction sequence tags (ISTs) column. ES, embryonic stem; UTR, untranslated region; EST, expressed sequence tag.

PCR preparations directly in gap repair cloning produced more red colonies than when attempts were made to purify the DNA fragment, indicating that loss of DNA yield during purification more than outweighed any deleterious effect the components of the PCR may have on yeast transformation efficiency. Following this observation, gel purification of PCR products prior to cloning was undertaken only when significant secondary bands were observed. However, in such situations, the indicator vectors offer the most value in gap repair cloning, allowing easy identification of infrequent clones obtained from limiting amounts of DNA.

Replication of Known Interactions

The function of the insert indicator vectors pGBAD-B and pACTBD-B was initially tested. The coding regions of mouse *Bub3* and *Mad2l1* genes were cloned as baits in pGBAD-B and pGBD-B, and appropriate fragments of mouse *Bub1* (codons 1–505) and *Mad1* (codons 536–717) were cloned as preys

in pACTBD-B and pACT2-B. This arrangement provides the ability to test for two known protein interactions from the spindle assembly checkpoint, Bub1-Bub3 (20) and Mad1-Mad2l1 (21), as well as providing negative interaction controls (Bub3-Mad1 and Mad2l1-Bub1). The expected interactions were confirmed, with no growth seen on the negative controls (Figure 3). The Mad1-Mad2l1 combinations resulted in less growth than the Bub1-Bub3 combinations, suggesting a difference in interaction affinity, and the indicator vectors appeared slightly more sensitive at detecting the Mad1-Mad2l1 interaction than non-indicator vectors. This effect was not so marked when the interactions were tested using the less stringent *HIS3* reporter gene.

The function of the pGBAE-B and pACTBE-B vectors was subsequently tested with two further well-characterized protein interactions—the first between the α' and β subunits of the human CK2 enzyme (encoded by *CSNK2A2* and *CSNK2B*, respectively) and the second between two subunits of the human vacuolar ATPase

proton pump (encoded by *ATP6V1G1* and *ATP6V1E1*). The CK2 enzyme is composed of four subunits: two regulatory β subunits that homodimerize and two peripheral α or α' catalytic subunits that interact with the β subunit dimer (22). As expected, the Csnk2b protein interacted specifically with itself and with the Csnk2a2 protein in both orientations of bait and prey, although once again variations in growth were noted. In this case, the interaction strength was dependent upon whether Csnk2b was used as a bait or prey. In comparison, the protein subunits of the vacuolar ATPase proton pump, Atp6v1g1 and Atp6v1e1, demonstrated similar interaction in both orientations and did not interact with any of the other baits or preys tested (Figure 3).

Function of Bait Vectors in Library Screens

Although the new vectors were successful in replicating a small number of known interactions, the most demanding yeast two-hybrid application

Table 2. Results of Parallel Yeast Two-Hybrid Screens Using Human Mccd1 Protein as Bait

Vector	Accession No. Matching IST	Corresponding Locus Name	Prey Description/Domains/Function	ISTs (No.)
pGBD-B	NM_005051	QARS	Glutaminyl-tRNA synthetase	10
	NM_025072	PTGES2	Prostaglandin E synthase 2	9
	NM_003316	TTC3	Tetratricopeptide repeat domain 3, E3 ubiquitin ligase	4
	NM_004270	CRSP9	Co-factor required for Sp1 transcriptional activation	4
	XM_291018	USP34	Ubiquitin specific protease 34	2
	NM_016223	PACSN3	Protein kinase C and casein kinase substrate in neurons 3	2
	NM_001831	CLU	Complement lysis inhibitor	1
	NM_022909	CENPH	Centromere protein H	1
pGBAD-B	NM_025072	PTGES2	Prostaglandin E synthase 2	10
	NM_005051	QARS	Glutaminyl-tRNA synthetase	3
	NM_014608	CYFIP1	Cytoplasmic FMR1 interacting protein 1	2
	NM_016223	PACSN3	Protein kinase C and casein kinase substrate in neurons 3	2
	NM_001831	CLU	Complement lysis inhibitor	1
	NM_003316	TTC3	Tetratricopeptide repeat domain 3, E3 ubiquitin ligase	1
	NM_004270	CRSP9	Co-factor required for Sp1 transcriptional activation	1
	NM_022909	CENPH	Centromere protein H	1
pGBAE-B	NM_025072	PTGES2	Prostaglandin E synthase 2	7
	NM_003316	TTC3	Tetratricopeptide repeat domain 3, E3 ubiquitin ligase	3
	NM_004663	RAB11A	Member RAS oncogene family of small GTPases	2
	NM_004270	CRSP9	Co-factor required for Sp1 transcriptional activation	1
	NM_005051	QARS	Glutaminyl-tRNA synthetase	1
	NM_014608	CYFIP1	Cytoplasmic FMR1 interacting protein 1	1
	XM_291018	USP34	Ubiquitin specific protease 34	1

Locus name entries shown in bold were identified in library screens with all three vectors. The number of times a gene was identified in each screen is shown under the interaction sequence tags (ISTs) column.

is library screening. It is possible that the presence of both the *GAL4* activation and DNA binding domain sequences in the indicator vectors may predispose to false positives or that the decapeptide HPAFLYKVVD added to the C terminus of fusion proteins by pGBAE-B may increase the false negative rate. In an initial experiment, the full coding region of the mouse *Mad2l2* gene was cloned into the two bait vectors pGBAD-B and pGBD-B, and parallel screens were undertaken using a mouse day 7 embryo library (Matchmaker™; BD Biosciences Clontech). The analyses produced very similar results, with four genes identified in common between the two screens that together accounted for 50 out of a total of 64 clones analyzed (Table 1).

Finally, we carried out parallel screens with the recently described human Mccd1 protein (23) using bait vectors pGBD-B, pGBAD-B, and pGBAE-B across a human erythroleukemia library (Matchmaker). The bait was constructed by amplifying the *MCCD1* sequence from codon 25 to 119, excluding the N-terminal hydrophobic mitochondrial localization sequence (23), because it causes promiscuous interaction in a yeast two-hybrid screen (24). Again, vectors performed consistently with each screen, identifying seven to eight interaction partners and a core of four preys in common between the three screens (Table 2). An additional five preys were identified in various combinations in at least two of the three screens, and one prey (Rab11a) was identified twice but in only one screen.

DISCUSSION

Comparison of Indicator and Non-Indicator Vectors

The indicator vectors clearly performed comparably to non-indicator vectors in the four previously reported interactions chosen for pair-wise testing, although some observations from this experiment are worth noting. The first of these is the apparent poor detection of the Mad1-Mad2l1 interaction for both classes of vector, suggesting a lower interaction affinity for this protein pair. This could reflect either a weaker physiological interaction for these two proteins, or it may be an artifact arising from the use of isolated protein fragments in a foreign environment. However, it did allow the direct comparison of vectors for their relative performance in detecting weak

interactions, and both the pGBAD-B and pACTBD-B indicator vectors were slightly more sensitive than their non-indicator counterparts. As the constructs are identical apart from the presence of a second *GAL4* segment, it is not clear why this should be the case, although it is possible that the longer 3' untranslated region of fusion transcripts from these vectors may result in increased protein expression.

A second observation of interest is the difference in reporter gene activation seen in the Csnk2a2-Csnk2b interaction, depending on which protein was expressed as a binding or activation domain fusion. Although this interaction was tested only with the pGBAE-B and pACTBE-B indicator vectors, we have observed more extreme examples of this phenomenon with other protein interactions and vectors (data not shown). Differential interference with interaction function produced by alternate domain fusions is likely to be protein-specific and may be one cause of false negative results in yeast two-hybrid screens.

Vector Performance in Library Screens

One of the potential problems with the new vectors in library screening is the possibility of reconstitution of *GAL4* activity from bait constructs through spontaneous recombination, deletion, or base substitution. Such events may enhance the false positive rate among primary clones to an extent that the screens are overwhelmed, making the new vectors unusable. We routinely incorporate in our library screens a procedure of prey rescue and interaction reconfirmation, aimed at exclusion of a class of false positives that are not dependent upon interaction. Clones arising from reconstitution of *GAL4* function in bait constructs should be excluded by this procedure, and significantly, baits constructed from the new vectors did not give rise to obviously higher numbers of excluded clones. This suggests that even if these events occur with the new bait vectors, the frequency is low enough to be accommodated in the standard screening procedure without a noticeable increase in effort.

Among the clones that remain after the reconfirmation procedure, it is not possible to formally exclude, without extensive further studies, false positives that could arise from nonphysiological interaction of fusion proteins in yeast. However, preys that were replicated between screens accounted for the majority of clones, demonstrating a similar overall performance of indicator and non-indicator vectors. The significance of preys that were restricted to just one of the vector screens is more difficult to evaluate with respect to vector function, but these were limited to clones identified only once or twice in any screen, suggesting that the depth of library screening is limiting rather than a systematic issue with false positive production or an increased false negative rate from the new vectors.

New Interactors for the Mouse Mad212 and Human Mccd1 Proteins

In addition to providing support for the performance of the new indicator vectors, the library screens undertaken have provided putative new interactions for the human Mccd1 protein and the mouse Mad212 protein that may assist functional characterization. Although further studies are required to validate these results, it seems that Mad212 is capable of interacting with a number of proteins and may be implicated in a wider range of cellular functions than previously suspected. The three screens undertaken with Mccd1 also produced generally consistent results that may assist with establishing a functional role for this protein.

Overall, the new indicator vectors performed comparably to standard vectors in common yeast two-hybrid applications. In addition, they can expedite the construction of baits and preys by significantly decreasing the effort involved in characterizing the products of gap repair cloning and should prove useful in high-throughput yeast two-hybrid projects requiring construction of large numbers of baits and preys.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address for correspondence:

David Markie
 Pathology Department
 Dunedin School of Medicine
 University of Otago
 P.O. Box 913
 Dunedin, New Zealand
 e-mail: david.markie@stonebow.otago.ac.nz