Goat anti-mouse immunoglobulin horseradish peroxidase (HRP) conjugate was diluted in PBS containing 10% BSA. This conjugate has been successfully used from various manufacturers; however, specific lots need to be titrated for optimal performance. One particular preparation of HRP-conjugated goat anti-mouse IgG, IgM and IgA (Catalog No. A 0412; Sigma Chemical, St. Louis, MO, USA) was diluted at 1/8000. One hundred microliters of the diluted conjugate were put into each well, and the binding was allowed to proceed for 45 min at room temperature on an orbital shaker. Plates were then washed 6 times with PBS/0.05% Tween-20.

3,3',5,5'-Tetramethylbenzidine (TMB) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was prepared according to the manufacturer's instructions, and 100 μ L were added to each well. The reaction was allowed to proceed about 30 min at room temperature, at which time the reaction was stopped by the addition of 100 μ L 0.18 M H₂SO₄. The plate was read at 450 nm with a microplate reader.

Sample antibody concentrations were estimated by comparing OD values to the OD values of the standard curve. An example of data from mice immunized with the SWM (110–121) peptide is shown in Figure 3. Inclusion of free unbiotinylated peptide has been shown to greatly diminish the signal generated in this assay (data not shown).

The assay described in this report should be helpful to most researchers wanting to prepare anti-peptide antisera or to monitor anti-peptide antibody responses for other experiments. We have used the above protocol, with appropriate modifications, in a number of systems with consistent success. In addition, this protocol could be adapted to other systems, besides monitoring anti-peptide antibodies, where a biotinylated substrate is obtainable.

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Increasing the Specificity of Colony Hybridization When Using Heterologous Probes

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There is considerable interest in studying the function of conserved genes in a variety of organisms, and this often requires identifying and cloning the homologous gene in the specific organism under investigation. This is generally feasible because the gene of interest has been cloned from another species, and its sequence is available. If a sequence from more than one organism is available and conserved regions have been identified, polymerase chain reaction (PCR) methods can be used with degenerate oligonucleotides to amplify a region of conserved sequences. Alternatively, screening of libraries with a heterologous gene can be used, especially if only one sequence is available. This approach is useful because a probe is already available and degenerate primers do not have to be designed and synthesized. In our studies of *Dictyostelium discoideum* genes that are homologous to yeast genes active in nucleotide excision repair and to the human genes responsible for xeroderma pigmentosum, we have developed a rapid strategy with improved probe preparation that has greatly facilitated the cloning of the homologous genes.

Screening libraries with heterologous probes require careful reduction of the hybridization stringency to enable the optimum detection of the specific signal against background hybridization. To clone the homolog of the yeast *RAD25* gene from *Dictyostelium*, we followed the conventional protocol of running a series of genomic Southern hybridizations at a constant temperature in decreasing concentrations of formamide (2) and chose the threshold condition of 42°C and 20% formamide, in which we detected a single strong signal with virtually no background. Once the conditions were established, we constructed a restriction map of the region surrounding the gene by analyzing multi-

ple restriction digests using the *RAD25* insert as a probe. We identified an appropriate fragment of genomic DNA containing the gene for cloning. Digested genomic DNA was then size-fractionated on a sucrose gradient, and the DNA from the fraction containing the gene was ligated into a vector with appropriate restriction sites. The DNA was electroporated into bacterial cells to generate a mini-library. In our hands, this step reduced the library complexity by at least 10-fold, which greatly facilitated screening. This method is particularly useful in organisms like yeast and *Dictyostelium*, which have few and relatively small introns, often allowing a good deal of the gene to be cloned as a single piece.

Even with this careful attention to hybridization conditions and library construction, the actual screening of the plasmid library proved to be problematic, resulting in many false positives. It is generally believed that false positives result from nonspecific hybridization of the probe to other cloned genes in the library. However, we have determined that these false positives are generally due to contamination of the heterologous probe with vector sequences even after substantial efforts to purify the insert before labeling.

In an initial attempt to screen a library containing the *Dictyostelium* homolog of the yeast *RAD25* gene, we used the pEP18 plasmid (1), from which we excised a 3.7-kb insert containing the 2.5-kb *RAD25* gene and flanking sequences. The fragment was

purified using the glass milk procedure (GENECLEAN®; Bio 101, La Jolla, CA, USA) and labeled by random priming. Using this probe, we identified approximately 35 putative positive colonies in a screen of over 75 000 total colonies, but none of them contained the *RAD25* homolog. Southern analysis of the plasmid preparations revealed that the purified yeast *RAD25* probe used in the screening was reactive with vector sequences (Figure 1). There was no hybridization to bands other than the vector, even though each preparation contained an insert of a different size.

Although it is clear that the false positives are the result of hybridization to vector sequences, we do not know why the probe hybridizes more strongly to some colonies than to the rest of the colonies in the library that also contain vector sequences. We suggest that this may be due to faster colony growth and/or a higher number of plasmid copies in these cells. Irrespective of the specific reason, the results in Figure 1 demonstrate that this reactivity is not due to hybridization with other cloned DNA fragments, but rather to hybridization with vector sequences.

We then used two gene-specific primers to amplify a segment of the yeast *RAD25* gene using the pEP18 plasmid as the PCR template and purified the PCR product on an agarose gel. As shown in Figure 2A (lanes 1 and 2), even this probe had a great deal of reactivity with vector sequences.

To eliminate this cross-hybridization with vector sequences, we added an

Figure 1. Southern hybridization demonstrating reactivity with vector sequences. 0.3 µg DNA of plasmid minipreps (prepared by the alkaline lysis method) was digested with *EcoRI-BamHI* to release the insert from the vector, and the digests were separated on a 0.8% agarose gel. The gel was blotted onto a nylon membrane and hybridized at 42°C in 20% formamide with the *RAD25* probe, radiolabeled as described in the text. The labeled band in lanes 1–19 corresponds to the expected size of the pUC18 vector *EcoRI-BamHI* fragment. pUC18 = 2.6 kb; pBluescript = 2.9 kb (lane 20).

Benchmarks

Figure 2. Southern hybridization with improved probe preparation. Panels A and B: lanes 1 and 2 contain an equal mixture of plasmid pUC18 (5 Prime → 3 Prime, Boulder, CO, USA) and pBluescript® (Stratagene, La Jolla, CA, USA), at 0.03 µg and 0.3 µg per lane, respectively. Lanes 3 and 4 contain 46 µg per lane of *Dictyostelium* genomic DNA, digested with *EcoRI* and *EcoRI-BamHI*, respectively. The probe was generated by amplifying a segment of yeast DNA from plasmid pEP18, using two sequence-specific oligonucleotides. The PCR was performed for 25 cycles as follows: denaturation for 1 min at 94°C, annealing for 2 min at 73°C and elongation for 3 min at 72°C. The PCR product was purified on agarose gels and eluted using GENECLEAN. Templates for PCR were (A) whole pEP18 plasmid and (B) the 3.7-kb *RAD25* fragment, excised from pEP18 and purified on a gel. Panel C: a restriction map of the genomic region of the *repB* gene. The location of the *repB* gene is indicated by the shaded region.

extra step of purification in which the 3.7-kb *RAD25* insert was purified from vector sequences on an agarose gel before being used as the template for PCR. The resulting PCR product was then repurified for use as the probe. As shown in Figure 2B, all hybridization with vector sequences was eliminated (lanes 1 and 2) while maintaining a strong signal with the *Dictyostelium RAD25* homolog (lanes 3 and 4). Upon screening the mini-library with this improved probe, we identified approximately 30 putative positive colonies from a screen of about 110 000 total colonies. In contrast to our initial experience, we obtained pure positive colonies in over 90% of the cases after only a single additional screening. Sequencing confirmed that all these clones were the *Dictyostelium RAD25* homolog, which was found to have 53% homology to the yeast gene.

The strategy outlined above is a dependable way of cloning homologous

genes using heterologous probes under conditions of low stringency. As we have shown, the probe preparation is particularly important. Even though the contaminating sequences constitute a small fraction of the labeled probe, they share 100% homology with the vector sequences and hybridize well at low stringency. This approach to probe preparation has dramatically increased the detection of homologous clones while reducing, by at least half, the number of cloning and hybridization steps needed for isolating a pure clone.

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