INSTRUCTIONS

Immobilized *E. coli* Lysate Kit

PIERCE

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Screening a Library

Isolating a recombinant DNA clone which encodes a particular gene or mRNA sequence is accomplished by screening a recombinant DNA library. A recombinant DNA library consists of a large number of recombinant DNA clones. Only some of the clones in the library actually encode the desired sequence, so a procedure for determining the correct clones must be devised. The desired clone may confer a phenotype which will make it selective under certain conditions and it is possible to use these conditions for screening. For example, by selecting a restriction site which is in the middle of a gene such as ampicillin or tetracycline resistance genes in pBR322, sensitivity of the recombinant clone to the antibiotic results.

Once the recombinant DNA is synthesized, it is necessary to introduce the hybrid molecule into bacterial cells. *Escherichia coli (E. coli)* is the usual bacterial host. The vector may be either a plasmid or a phage. The library of clones produced can be screened either by hybridization (hybridizing individual clones with a DNA or RNA probe) or immunological screening (using antibodies that recognize the desired protein to identify the desired clones). If the immunological screening system is used, the DNA insert sequence is cloned in a expression vector and expressed as part of a fusion protein or the DNA insert is transcribed into mRNA and translated *in vitro.*[1] Antibodies can be used for the detection of fusion proteins and *in vitro* translated mRNA.

The screening procedure is normally performed on bacterial colonies containing plasmids or cosmids or on bacteriophage plaques. When using an antibody to screen a library, the antibody must recognize the fusion form of the desired protein. The fusion form is thought to resemble the denatured form of the protein, comparable to that encountered on a Western blot. Therefore, if an antibody is used successfully on a Western blot it is assumed to be useful in library screening.[1] However false positive plaques may be identified and it is best to perform a second test to ensure the validity of a positive clone. Even though a cDNA encodes an antigenic determinant, it does not necessarily encode the protein of interest. The detection, especially by a monoclonal, may be against only a single epitope within a DNA sequence. The detected determinant may not be encoded by the desired sequence but by a sequence related only at the level of protein product structure.[1] The screening procedure itself involves the immobilization of synthesized antigenic material to a solid support followed by a sensitive detection procedure.[1] Normally, nitrocellulose or other membrane is used for the immobilization of the plaque and detection is accomplished via an enzyme-labelled secondary antibody.

Background

High background (low signal-to-noise ratio) is often a problem when screening libraries. There are many sources of high background. The antibody used as a probe must be of pure quality. The screening techniques are sensitive to immunological impurities and there are problems with cross-reactivity with other antigens produced as fusion proteins.[1] If a poor quality nitrocellulose is used, a general purple background may be observed. Without proper blocking of any membrane a generalized background may result.[2] It may be necessary to increase the blocking time. Some blocking agents, such as nonfat dry milk, may contain alkaline phosphatase activity or IgG that binds conjugates. To determine if the blocking agent could be the cause of high background, perform a control by omitting the primary and secondary antibody from the procedure. This can be done together and sequentially. Also with a general background, it is possible that the color development was continued too long. The reaction should be watched closely and stopped when the development is at the correct intensity. If high background colors continue, the problem lies in the membrane or the blocking procedure. High backgrounds may result when the concentration of secondary antibody used is too high. It is necessary to optimize the concentration of secondary antibody.[2]

If the primary antibody is particularly sticky, it may bind even to a blocked membrane. A control can be executed using a blocked membrane without plaques to determine if this is the case. Some antibodies are so sticky that they may not be appropriate for immunoscreening procedures.[2]

It is also possible that the host strain, phage library or culture media is contaminated with organisms producing the enzyme alkaline phosphatase.[2] This possibility can be tested by omitting the primary and secondary antibody incubations from the procedure. If color development is significant, there is contamination. To inactivate alkaline phosphatase, heat the plaque lift at 80°C for 20 minutes or incubate in 0.1 M acetic acid for 20 minutes prior to the blocking step.[2] If the inactivation is successful, test the effect of the treatment on primary antibody binding. If it is not possible to eliminate alkaline phosphatase contamination, use horseradish peroxidase for screening.

If the background is localized and unexpected plaques appear to be positive, the primary antibody may contain components that react with *E. coli* proteins. Crude antisera and ascites fluid often contain IgG components that bind to *E. coli* proteins.[2] This could be especially problematic if the titer or binding affinity of the *E. coli* binding antibodies is higher than that of the antibody to the protein of interest making the background of the false positive plaques high. Optimizing the dilution of primary antibody for screening may eliminate some of the nonspecific background. By adsorbing the antisera or ascites fluid with an extract of the bacterial strain to inhibit or remove the anti-*E. coli* IgGs, the nonspecific binding may be decreased even further.

Another cause of localized background could be that the labelled secondary antibody cross-reacts with bacterial proteins. In this case, preadsorb the diluted secondary antibody conjugate with an *E. coli* extract or immobilized support.

Removal of *E. coli* Reacting Contaminating Antibodies

Removal of *E. coli* reacting contaminating antibodies can be accomplished via the solution-phase bacterial extract method. However, the simplest way of adsorbing antibodies would be with the use of an immobilized support. By immobilizing the bacterial proteins onto agarose, the primary antibody preparations can be applied directly to a column. The *E. coli* binding proteins will bind to the column and the purified antibody preparation will elute in the void volume. This functions as a "reverse" affinity column. If the antigen is properly immobilized to prevent leakage, the affinity column can be regenerated and reused many times. Pierce has designed a kit for immunoscreening of expression libraries from *E. coli*. The kit provides a simple and efficient method of removing *E. coli*-reactive IgG's from their primary antibody preparations. Bacterial proteins have been partially purified from a suspension of *E. coli* cells (strain BMH 71-18) and immobilized onto agarose by a proprietary method. This yields an affinity support which is essentially leak-free.

Product Description

NUMBERS	DESCRIPTION
44940	**Immobilized *E. coli* Lysate Kit** **Contents:** *1 x 2 ml* column of Immobilized *E. coli* Lysate bacterial lysate from *E. coli* BMH 71-18, immobilized on 6% crosslinked beaded agarose. This column should be stored at 4°C. **BupH™ Tris Buffered Saline,** *500 ml.* Store at room temperature. **Regeneration Buffer,** *250 ml.* 0.1 M Glycine, pH 2.8. Store at 4°C. **Column Extender** This kit contains sufficient buffers for 10 regenerations of the Immobilized *E. coli* Lysate column.

Instructions for Use

1. Dissolve BupH™ Tris Buffered Saline in 500 ml of distilled water to prepare the TBS buffer.
2. Bring the 2 ml Immobilized *E. coli* Lysate column to room temperature.
3. Remove top and bottom cap sequentially to prevent air bubbles from forming in the gel.

4. Pour off storage solution.
5. Equilibrate column with 10 ml of TBS buffer.*
6. Place either 12 microfuge or 12-100 mm test tubes in a rack and place column above the first microfuge tube or in the first test tube.
7. Add 1 ml of sample to top frit of column.
8. After all of the sample has entered the gel, add 100 µl of TBS buffer.
9. Move column to next tube.
10. Apply 1 ml fractions of TBS buffer and move column to the next tube with each additional fraction added. (1:100 dilutions of each fraction can be made in TBS buffer to monitor the A_{280} coming off of the column).
11. When the absorbance readings have returned to baseline, the fractions with the highest protein concentration may be pooled.
12. Following protein separation, wash the column with at least 10 ml of Regeneration Buffer.
13. After regeneration, immediately wash the column with at least 10 ml of TBS buffer containing 0.02% sodium azide (or other suitable storage solution.)
14. Store the column upright at 4°C.

*It is especially important to wash the column well, if a horseradish peroxidase conjugate will be used, since sodium azide inhibits this enzyme.

Results

Easy-Titer™ ELIFA System (Product No. 77000) was used to assay the effectiveness of the Immobilized *E. coli* lysate column for removing *E. coli* reactive antibodies from a polyclonal, anti-ß-galactosidase antiserum. ß-galactosidase, 71-18 lysate, and a lysate from *E. coli* strain Y1090 were coated onto the nitrocellulose membrane. The primary antibody, rabbit anti-ß-galactosidase, was divided into two aliquots. One aliquot was cross adsorbed according to the "Instructions for Use". The other aliquot was dialyzed against TBS buffer in a System 500 Microdialyzer (Product No. 66350). All protein solutions were assayed with BCA Protein Assay Reagent Kit (Product No. 23225) to determine total protein content and subsequently diluted with TBS buffer to 10 µg/ml (for antigens) and 100 µg/ml (for antisera). Each antigen was tested for reactivity to crude and cross adsorbed anti-ß-galactosidase antiserum. Alkaline phosphatase-labelled goat anti-rabbit IgG was used for detection with PNPP as the substrate.

Results: The assay showed strong specific binding of both crude and cross adsorbed anti-ß-galactosidase antisera to the ß-galactosidase antigen. The assay also indicated high cross-reactivity of the crude antiserum to both the 71-18 lysate and Y1090 lysate. The purified antiserum gave a significant reduction in cross-reactivity against both lysates indicating that the lysate of strain BMH 71-18 is able to remove cross-reactivity from other *E. coli* strains (Y1090). It will likely eliminate or decrease cross-reactivity contributed by other *E. coli* strains. (Figure 1) There is no apparent loss of anti-ß-galactosidase activity from the antiserum due to the purification.

Nonspecific Binding
BSA < 3%

Antibody Recovery from Regenerated Column
The column is reusable at least four times with no loss in cross adsorbing capacity.

References

1. Current Protocols in Molecular Biology, p 6.0.3.
2. Berger, S.L.and Kimmel, A.R. eds.(1987). Guide to Molecular Cloning Techniques. *Meth. Enzymol.* **152,** 467-469.
3. Sambrook, Fritsch and Maniatis, Molecular Cloning, A Laboratory Manual, 2nd edition, p. 12.2-12.3.

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Figure 1

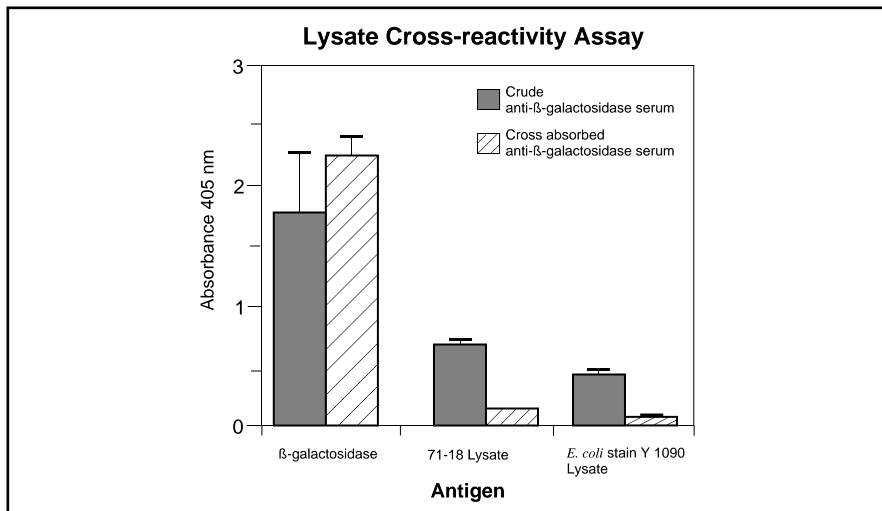

Figure 2