Improved procedure for screening expression libraries for novel autoantigens

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Abstract
The standard method for immunoscreening of a cDNA expression library is time-consuming because of the production of a large proportion of false positives during the first and second round of screening. This problem is more important when a sensitive chemiluminescence detection system is used. Due to the high sensitivity of the detection system, there is a need to avoid false positives which occur when the antibody reacts non-specifically. False positives are generally eliminated through absorption of the antibody with the host bacteria and by eliminating any clones, which react with antibodies present in normal sera. Here we present a method of obtaining almost identical bacteriophage plates by culturing phage in parallel, and show that this technique produces positive plaques in duplicate and eliminates false positives. Using this method, we successfully screened a human fetal spinal cord lambda gt11 cDNA library using purified immunoglobulin G (IgG) from patients with multiple sclerosis (MS) and Guillain – Barre syndrome (GBS).

Keywords: *Immunoscreening, Expression, cDNA Library, Autoantigen, Lambda*

INTRODUCTION

Probing an expression cDNA library with the purified antibody or whole sera can isolate antigen-encoding genes. Both radioactive (Sambrook *et al*. 1989) and non-radioactive (Higgs, 1994) methods are labor intensive because they produce large quantities of false positives during the first and second round of screening. The serial duplicate filter method (Snyder *et al*. 1987) is not always useful because the amount of absorbed expressed protein to the second filter is low. A previously recommended method (Tagu, 1993) for identifying false positives during the first round of cDNA library screening, using polymerase chain reaction (PCR) is useful only for the isolation of known genes. As an alternative, we describe here a method for isolating cDNA clones using parallel duplicate filters that produce true positives during different stages. This technique provides an advantage over screening in series, in which filters are overlaid onto the phage sequentially, as different phage and correspondingly different proteins are present at two different times on the filters. Culturing the phage in parallel eliminates this problem. Using purified IgG for screening the library is also important because it facilitates the pre-absorption step.

Pre-absorption of the serum against the bacterial host proteins is critical especially when low levels of antibody exist in the serum. Although removal of the anti–*E. coli* and anti-bacteriophage activity from serum is necessary to reduce the background, it will also eliminate antibodies, which can cross-react with both human and bacterial proteins. Another advantage of our method is that it uses an enhanced chemiluminescence detection system instead of radioactive which is more sensitive, convenient and safe.

There are so many reports about immunoscreening of different cDNA libraries prepared in lambda ZAP and lambda gt11 vectors (Hedstrand *et al*. 2001; Comtesse *et al*. 2000 and Moore *et al*. 1998). In this

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research we used lambda gt11 system. Of course, phage display is another strategy for making an expression cDNA library (Farrila *et al*. 2002; Hansen *et al*. 2001).

MATERIALS AND METHODS

Lauria–Bertani (LB) medium (Sambrook *et al*. 1989) was prepared using "Difco–certified yeast extract, tryptone and agar (Gibco, UK). Supported nitrocellulose filters (Hybond C-extra), secondary antibody (sheep anti-human IgG F (Ab') 2 HRP – linked) and the chemiluminescence detection reagents (ECL for Western blot, catalog number: RPN 2106) were obtained from (Amersham, Sydney, Australia). RX (blue–sensitive) autoradiograph film (Fuji) was from Hanimex, Perth. Clones used to validate the method presented here were obtained from screening a human fetal spinal cord cDNA library (HFSC) prepared by Dr. C. Campagnoni and colleagues, University of California, Los Angeles, USA. Fraction V BSA, and isopropyl thiogalactopyranoside (IPTG) were from Boehringer Mannehiem, Sydney. Tris – buffered saline (TBS): per liter, 30 ml 5 M NaCl, 20 ml 1M Tris – HCl pH 7.6 and TBS – T: TBS with 0.1% v/v Tween – 20 (Lab Supply, Perth).

a) Pre-absorption of IgG solution: Purified IgG from normal and MS sera (Richman *et al*. 1982) were pre-absorbed to remove the anti-*E. coli* and anti-bacteriophage activity before use in screening the λgt11 library. A non-recombinant phage was isolated from the HFSC cDNA library using white/blue color selection. Ten thousands phage from the library were plated on one 150 mm agar plate supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) 40 μg/ml final concentration. One of the blue plaques was picked up after an overnight incubation at 37°C. The plaque was added to 300 μl SM (Snyder *et al*. 1987) buffer containing one drop of chloroform, vortexed for 30 sec and incubated for at least 2 h at 4°C. The phage solution was replated three times in small plates until there were 100% blue plaques. One of non-recombinant blue plaques was added to 300 μl of SM buffer plus 1 drop of chloroform before incubation for 2 h at 4°C. The phage solution (top layer) was added to 100 ml of late phase culture of *E. coli* Y1090 and 900 ml of LB media (Sambrook *et al*. 1989) was added to the culture and incubated for 2 h at 37°C with 250 rpm shaking. The non-recombinant λgt11 phage was induced by rising the temperature to 45°C for 15 min. IPTG was added to 10 mM final concentration and incubation continued for a further 2 h at 37°C with 250 rpm shaking. The bacteria were harvested by centrifugation at 2000 g for 10 min at 4°C, resuspended in 50 ml of PBS and then lysed by three freeze-thaw cycles using liquid nitrogen and 37°C water bath respectively. The bacterial lysate solution was then sonicated 3×30 sec bursts at maximum power to reduce the viscosity and stored at -20°C until used.

A 137 mm nitrocellulose disc (Hybond-C extra) was soaked in the bacterial lysate solution for 30 min before incubation for 1 h in blocking solution (TBS-T containing 5% BSA). The filter was washed with TBS-T twice for 1 min, soaked in 50 ml IgG solution (diluted with PBS to 20 μg/ml total protein) and incubated overnight at 4°C with slow shaking. This procedure was repeated 3 or 4 times to make sure that all anti-bacteria and anti-β-galactosidase activity was absorbed. The IgG solution was tested with a dot blot procedure to make sure that there was no reactivity with the *E. coli* lysate proteins. In some cases when there was still some background activity, the IgG solution was mixed with the equal volume of bacterial lysate, incubated overnight at 4°C with rotation and then centrifuged at 100,000 rpm (rotor RP120AT-268 in a Sorvall RC M120 ultracentrifuge) for 15 min at 4°C. The supernatant was stored in 1 ml microcentrifuge tubes at -20°C until required.

b) Parallel lift method for immunoscreening: The bacterial parallel culturing method (Sambrook *et al*. 1989) was adapted for screening phage preparations, which produce duplicate filters carrying the expressed recombinant protein. An overnight culture of *E. coli* Y1090 was made in 10 ml LB (containing 0.2% maltose final concentration) and spinned at 2000g for 10 min at 4°C. The bacterial pellet was resuspended in minimal media. Bacteriophage and bacteria were mixed and incubated at 37°C for 20 min for uptake. The mixture was added to 3-5 ml of top layer agar, pre-warmed to 60°C, and poured onto a pre-warmed (37°C) LB plate. Both bottom and top

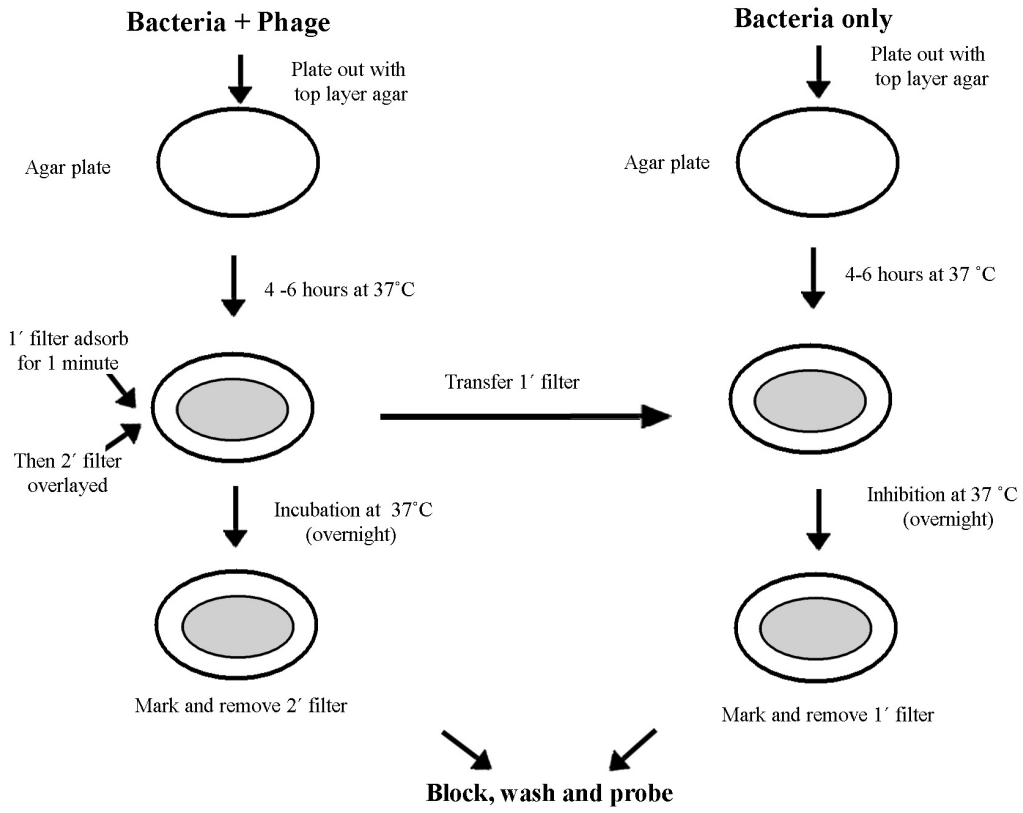

Figure 1. Parallel culturing of phage. Schematic illustration of the sequential steps for parallel phage culture.

layer agar as well as LB media were supplemented with $MgSO_4$ to 10 mM final concentration. Several combinations of phage were plated, at varying densities, to determine the optimum dilution. Lifts were taken from these phage plates using supported nitrocellulose filters pre-soaked in 10 mM IPTG, and air dried in a laminar flow on sterile 3 mm whatman paper.

Parallel lifts were obtained by incubating the phage plates at 37°C for 4-6 h until the plaques became visible. One filter was placed on the first plate, marked for later orientation and transferred to a second plate after 1 min. The second plate had only a bacterial lawn of *E.coli* Y1090, prepared at the same time as the phage was plated. The second duplicate filter was then placed on the first plate and marked. Both plates were incubated at 37°C for at least 4h to overnight (Figure 1). The treatment of both filters was the same from this stage onwards.

c) Enhance chemiluminescence (ECL) detection: Amersham's ECL Western blotting kit (Amersham Life Science, England), a sensitive non-radioactive method for detection of immobilized antigens (Higgs, 1994), was adapted for screening the expressed proteins from a cDNA library. The filters from above were washed with TBS-T and immersed in blocking solution (5% BSA in TBS-T) for 1h at room temperature with low speed shaking to block the non-specific binding sites. Washing the filters with TBS-T was similar for all stages of this procedure; two washes of 1 min, one of 15 min and a fur-

ther two of 5 min at room temperature with slow shaking, except for the last washing which had two extra 5 min washings. The filters were washed before and after incubation in IgG solution and the secondary antibody (anti-human antibody, horseradish peroxidase linked F [Ab'] 2, Amersham, England). Incubation with each antibody was for 1h at room temperature with low speed shaking. Finally, the filters were immersed in ECL detection reagents for 1 min and exposed to the radiography film for 15 min according to the manufacturer's procedure.

d) Obtaining disease specific clones: In order to obtain clones, which only react with antibodies, associated with autoimmune disease it is necessary to remove any clones, which also react with IgG present in normal sera. The positive clones at the first and second stages of screening were reacted with pooled normal human IgG, after stripping the nitrocellulose filters according to the recommended method by the manufacture of the ECL kit. The method for testing the striped filters against the pooled normal IgG was the same as above. Finally, the purified positive clones after tertiary screening were tested using the following procedure. 5 µl of each phage solution (containing approximately 100 recombinant phage) was added on top of an agar plate containing an Y1090 bacterial lawn in duplicate places in marked spots. Protein lifts were carried out after 2-3 h incubation at 37°C. The filter was then cut into two pieces, each carrying the same protein for incubation in MS and normal IgG, and processed for screening with the same method as above. With this method it is possible to check cross reactivity of more than 20 clones against two kind of IgG using just one 137 mm nitrocellulose filter.

RESULTS

The autoradiographs (shown in Figure 2) show that filters produced by growth in parallel, as described here, provide good positives in duplicate. By eliminating all clones, which also react with normal IgG, antigen-antibody reactions specific for autoimmune disease can be detected. Sequential screening can further enhance the specificity with the IgG prepared from individual patients and selecting for sequencing

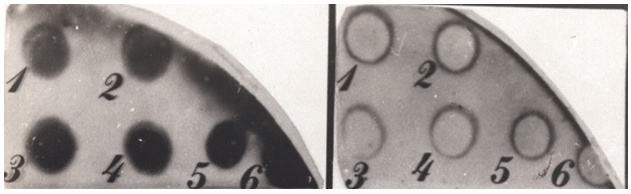

Figure 2. **Autoradiograph showing the cross–reactivity of the positive clones.** The left one shows the reaction of 6 positive clones after tertiary screening with pooled normal human IgG, the right one is the reactivity of the same clones with pooled MS IgG. Growing the phage in parallel and the production of identical duplicate filters.

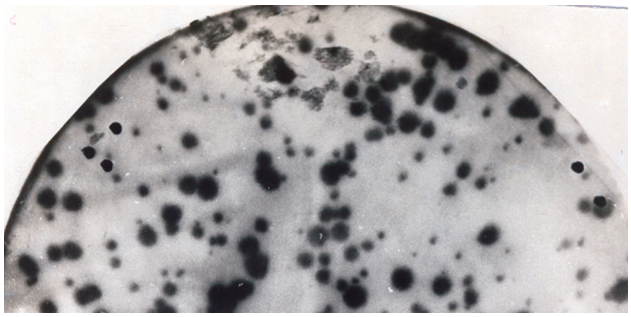

Figure 3. Two typical autoradiographs after tertiary screening. Two different positive clones, probed with pooled MS IgG as the primary antibody and anti-human IgG HRP–linked as the secondary antibody followed by 10 minutes exposure time.

only those clones, which react with IgG in a high percentage of sera from patients with the disease. Using the technique we have screened a HFSC cDNA expression library in lambda gt11 vector with IgG from patients with MS and GBS. Purified IgG from 5-pooled MS sera was used to identify the antigens expressed. Six clones were isolated from screening 2 million recombinant phage, which reacted with pooled MS IgG but not with the pooled IgG purified from 5 normal human sera (Figure 3). IgG from two patients with GBS resulted in the isolation of several clones. Analysis of the sequences of these clones is currently in progress.

DISCUSSION

The ability to obtain true duplicates of phage expression libraries by parallel filter lifts provides an obvious advantage over traditional serial lifts, eliminating the problems associated with false positives. Because

of presenting the expressed protein, by slower or rapidly growing phage, concurrently on both the original and duplicate filters, the same amount of protein is bound to both filters which results in a signal of equal intensity, and at the same position. Another advantage of this parallel screening protocol is that both DNA and protein lifts may be taken from the same original phage plate, if the protein lifts were both transferred to new plates; the phage plate could be allowed to grow to the desired density for a pair of DNA lifts. In addition, the immunological screening of true duplicates using a highly sensitive detection system such as ECL, enables successful isolation of recombinant protein antigens from cDNA expression libraries.

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