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A method for global protein expression and antibody screening on high-density filters of an arrayed cDNA library

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ABSTRACT

We have developed a technique to establish catalogues of protein products of arrayed cDNA clones identified by DNA hybridisation or sequencing. A human fetal brain cDNA library was directionally cloned in a bacterial vector that allows IPTG-inducible expression of His_6-tagged fusion proteins. Using robot technology, the library was arrayed in microtitre plates and gridded onto high-density *in situ* filters. A monoclonal antibody recognising the N-terminal $RGSH_6$ sequence of expressed proteins (RGS·His antibody, Qiagen) detected 20% of the library as putative expression clones. Two example genes, GAPDH and HSP90α, were identified on high-density filters using DNA probes and antibodies against their proteins.

For construction of the human expression library hEx1, cDNA was prepared from fetal brain poly(A)$^+$ RNA by oligo(dT)-priming (Superscript Plasmid System, Life Technologies). Products were size-fractionated by gel filtration and directionally (*SalI–NotI*) cloned into a modified pQE-30 (Qiagen) vector for IPTG-inducible expression of His_6-tagged fusion proteins (pQE-30NST, GenBank accession no. AF074376). *Escherichia coli* SCS1 cells (Stratagene) carrying the plasmid pSE111 with the *lacI*^{Q} repressor and the *argU* gene for a rare arginine tRNA (1) were transformed by electroporation. PCR analysis of 96 clones revealed an average insert size of ~1.5 kb (range 0.5–5.0 kb).

The library was plated onto 2×YT-AKG agar plates (230 mm × 230 mm Nunc Bio Assay Dishes containing 2×YT agar, 100 µg/ml ampicillin, 15 µg/ml kanamycin and 2% glucose) and grown at 37°C overnight. Using a picking/gridding robot (2), 193 536 colonies were picked into 384-well microtitre plates (Genetix) containing 2×YT-AKG medium supplemented with freezing mix (0.4 mM $MgSO_4$, 1.5 mM Na_3-citrate, 6.8 mM $(NH_4)_2SO_4$, 3.6% glycerol, 13 mM KH_2PO_4, 27 mM K_2HPO_4, pH 7.0). Bacteria were grown in microtitre wells at 37°C overnight, and 9216 or 27 648 clones were gridded onto 222 mm × 222 mm filter membranes in a duplicate pattern (Fig. 1). Nylon filters (Hybond-N$^+$, Amersham) were gridded for DNA hybridisations and processed as described (3). For protein analysis, polyvinylidene difluoride (PVDF) filters (Hybond-P, Amersham) were gridded, incubated on 2×YT-AKG agar plates at 30°C overnight and induced for protein expression for 3 h at 37°C on agar plates containing 1 mM IPTG. These protein filters were processed on pre-soaked blotting paper, i.e., denatured in 0.5 M NaOH, 1.5 M NaCl for 10 min, neutralised for 2× 5 min in 1 M Tris–HCl, pH 7.5, 1.5 M NaCl and incubated for 15 min in 2× SSC. Filters were air-dried and stored at room temperature.

For global protein expression, high-density filters were screened with the monoclonal antibody RGS·His (Qiagen). This antibody recognises the N-terminal sequence $RGSH_6$ of fusion proteins over-expressed from pQE-30 vectors and labelled ~20% of the hEx1 clones (Fig. 1A). Negative clones have inserts in incorrect reading frames with stop codons leading to short polypeptides that cannot fold into stable structures and are degraded within the host cell (4). Two example proteins, GAPDH (35.9 kDa, Swiss-Prot P04406) and HSP90α (84.5 kDa, Swiss-Prot P07900) were chosen for detailed analysis. A set of three DNA filters (80 640 clones) were screened with cDNA probes. Two hundred and six (0.26%) clones were positive with a human GAPDH probe (Fig. 1B), and 56 (0.07%) clones were identified with a human HSP90α probe. About 25% of these clones were positive with the RGS·His antibody. To confirm the expression of GAPDH or HSP90α proteins by these clones, protein filters were screened with antibodies against GAPDH (Fig. 1C) or HSP90α, respectively. Fifty-seven percent of the GAPDH and 72% of the HSP90α clones detected by the RGS·His antibody were also positive with the protein-specific antibodies. Sequence analysis showed that the remaining clones had inserts in an incorrect reading frame or expressed truncated GAPDH which reacted poorly with the GAPDH antibody.

In turn, 100% of the anti-GAPDH- but only 35% of the anti-HSP90α-positive clones were detected by the RGS·His antibody. All RGS·His-negative HSP90α clones had inserts in incorrect reading frames but nevertheless expressed proteins detected by the HSP90α antibody on western blots (data not shown). This indicates HSP90α molecules without a His_6 tag, suggesting translational start sites within cDNA inserts. Three anti-HSP90α positive clones contained inserts that were not recognised by the cDNA probe and turned out to be unrelated

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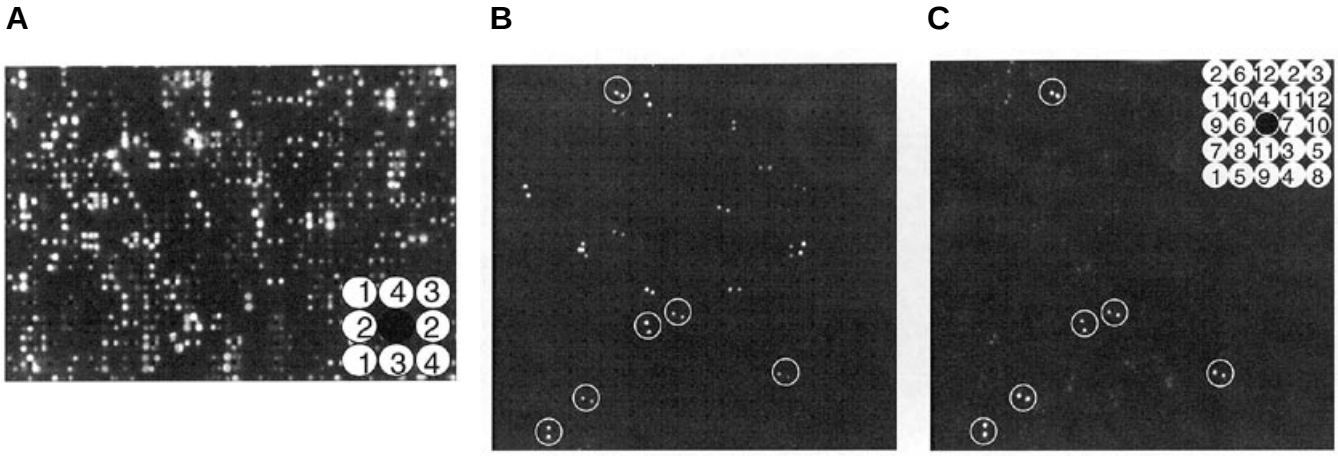

Figure 1. Identification of cDNA clones expressing recombinant fusion proteins on high-density filters. (**A**) RGS·His antibody detection (gridding pattern of 3×3 surrounding ink guide dots as shown in lower right corner). (**B**) DNA hybridisation with a GAPDH cDNA probe, as described (3). (**C**) Screening with a polyclonal anti-GAPDH antibody (corresponding sections of filters featuring 5×5 gridding patterns as shown in upper right corner; identical clones are circled). Before antibody screening, filters were soaked in ethanol, bacterial debris was wiped off in TBST-T (20 mM Tris–HCl pH 7.5, 0.5 M NaCl, 0.1% Tween 20, 0.5% Triton X-100), followed by washing 2× 10 min in TBST-T and 10 min in TBS. Filters were blocked in blocking buffer (3% non-fat, dry milk powder in TBS, 150 mM NaCl, 10 mM Tris–HCl, pH 7.5) for 1 h and incubated with antibody for 2 h (1:2000 diluted monoclonal RGS·His, Qiagen, or 50 ng/ml monoclonal anti-HSP90α, Transduction Laboratories, Lexington) or for 16 h (1:5000 diluted rabbit anti-GAPDH). After washing for 2× 10 min in TBST-T and 10 min in TBS, filters were incubated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit Ig (Pierce) for 1 h, washed 3× 10 min in TBST-T, 10 min in TBS and 10 min in AP buffer (1 mM $MgCl_2$, 0.1 M Tris–HCl, pH 9.5) and incubated in 0.25 mM Attophos (JBL Scientific, San Luis Obispo) in AP buffer for 5 min. Filters were illuminated with long-wave UV light and images were taken using a high resolution CCD detection system. Image analysis was done using Xdigitise software (written by Huw Griffith) which is available on request.

sequences. These sequences were analysed using BESTFIT (Wisconsin Package Version 9.1, Genetics Computer Group, Madison) but no common motifs of significant homology were found. This limited antibody specificity is not surprising as it reflects cross-reactivity which is not usually tested against a whole library of proteins as in our method.

The main advancement of our technique over existing technology (e.g. λgt11 libraries; 5) is its high-throughput link between DNA sequence information and protein expression as a resource for unlimited future use. Having screened a library for protein expression once, we can always go back and identify products of new genes as they are discovered, attributing first functional information to them. Based on screenings with the RGS·His antibody, 37 830 putative expression clones were re-arrayed into new microtitre plates, and high-density protein and DNA filters were prepared and are available from the Resource Centre of the German Human Genome Project (http://www.rzpd.de).

The main technical problems of our approach are inherent in cDNA library and filter hybridisation technology. Oligo(dT)-primed cDNA is biased towards 3′-ends of genes, and, subject to insert size, N-terminal parts of larger proteins are often missing. To include a maximum number of epitopes for antibody screening, complementary random-primed cDNA libraries should be used. Quantification of signal intensities on filters is largely based on arbitrary thresholds for manual or automated image analysis. Therefore, our approach is exclusively based on positive clones to be confirmed by sequencing and/or protein characterisation.

We envisage two main fields of application for our method. First, catalogues of protein products can be established for different tissues and developmental stages. As these proteins are expressed from arrayed cDNA clones, their identity can easily be checked by high-throughput gene identification techniques (e.g. oligonucleotide fingerprinting; 6). Therefore, gene expression patterns of normal and diseased tissues can be translated to the protein level, keeping a direct link to already existing DNA sequence data. Second, our method should also enable high-throughput analysis of antibody specificity and other protein–protein interaction or ligand-receptor systems (7), including non-protein molecules.

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