Angiogenesis **6**: 93–104, 2003.

Review

Generation and use of a tailored gene array to investigate vascular biology

Amanda L. Evans[1,2], Andrew S. Sharkey[1,2,*], Samir A. Saidi[1,3,*], Cristin G. Print[1,2,*], Roberto D. Catalano[1,2], Stephen K. Smith[1,3] & D. Stephen Charnock-Jones[1,3]

[1]*Reproductive Molecular Research Group,* [2]*Department of Pathology, University of Cambridge, Tennis Court Rd, Cambridge, UK;* [3]*Department of Obstetrics and Gynaecology, The Rosie Hospital, Robinson Way, Cambridge, UK*

Received 2 April 2003; accepted in revised form 4 June 2003

Key words: angiogenesis, array, HUVEC, normalisation, transcript

Abstract

Vasculogenesis, angiogenesis and vascular remodelling are complex processes where the fate of several cell types is determined by different signalling networks. Many of these networks ultimately function by changing the abundance of RNA transcripts within the cells which constitute blood vessel walls. Researchers can now map these transcript abundance changes using gene array technology. In this review, we describe the design, production and use of a gene array specifically tailored to investigate vascular biology. We describe the advantages of tailored gene arrays, and give detailed protocols based on our experience to allow the reader to use such gene arrays to generate meaningful data. We list the issues to consider when choosing and verifying the genes and splice variants included in an array, and describe our use of *Arabidopsis* sp. RNA spikes for quality control. We present data that illustrates the absolute necessity for both technical and biological replicates to be incorporated in the design of gene array experiments using primary cells such as HUVECS. Finally, we describe methods for the normalisation and interpretation of the data that gene arrays produce. The approach to gene array technology described here is easily within reach of the budget and expertise of most academic research groups.

Abbreviations: QC – quality control; HUVEC – human umbilical vein endothelial cells; PCR – polymerase chain reaction

Introduction

The recent development of DNA array technology has substantially altered the conduct of angiogenesis research. This technology allows the abundance of a large number of transcripts within complex RNA populations to be determined simultaneously. For the first time this offers researchers the prospect of understanding the subtle interactions between multiple genes that may underlie complex cellular behaviours. For example, vasculogenesis, angiogenesis and vascular remodelling are complex processes involving interaction between several cell types within and outside the vessel wall [1–3]. We are using gene arrays to determine the transcript abundance regulation that underlies these processes.

Gene arrays come in two main types: large generic arrays and small tailored arrays. Large generic gene arrays, are available commercially or by collaboration with dedicated gene array laboratories. One well established large generic technology is the Affymetrix Genechip system (Affymetrix Inc. Santa Clara, California, USA, http://www.affymetrix.com) [4] in which two-dimensional arrays of synthetic oligonucleotides are synthesised using a combination of photolithography and solid phase DNA synthesis [5, 6]. Affymetrix gene chips allow thousands of transcripts to be analysed in a single hybridisation, and are ideal for experiments in which researchers wish to search for unexpectedly regulated genes. While we, and others have shown that this approach can be extremely informative for angiogenesis research [7], it is inappropriate for many experimental designs. Commercial generic arrays are expensive and academically produced generic arrays of limited availability. Therefore, generic gene arrays may be impractical when a large number of experimental replicates are required. In addition, the genes included in most generic arrays are inflexible, and frequently omit genes and splice variants of interest. An alternative approach is for individual research laboratories to construct relatively small tailored gene arrays focussed on their specific research interests. These have the advantage of low cost, which allows researchers to

*Authors contributed equally to the work described in this publication.

Correspondence to: Dr Stephen Charnock-Jones, Reproductive Molecular Research Group, Department of Obstetrics and Gynaecology, The Rosie Hospital, Cambridge, CB2 2SW, UK. Tel: +44-1223-336875; Fax: +44-1223-215327; E-mail: dscj1@cam.ac.uk

perform sufficient replicates to maximise experimental power. In addition, tailored gene arrays encourage researchers to perform well planned, hypothesis driven experiments investigating a focused set of transcripts. New genes and splice variants can be added whenever required, and detailed quality control (QC) performed.

Small tailored gene arrays also offer the option to use radioactively labelled complex cDNA probes. These have been used successfully since 1983 [8], and require as little as 3–5 μg of total RNA. In contrast most fluorescent labelling techniques require 20–50 μg of total RNA [9]. Although cDNA amplification methods such as the SMART™ system (BD Biosciences, Clontech, Oxford, UK) allow fluorescent arrays to be used with small amounts of starting material, they are yet to gain wide acceptance due to concerns about linearity and representation. The ability to use small amounts of starting material is essential for many studies involving angiogenesis. In the female reproductive tract for example, only small samples of tissue or cells are available from biopsies.

To perform studies requiring multiple replicates with limited amounts of RNA, at reasonable cost, we have designed and produced a tailored gene array focused on the processes involved in vascular biology (endothelial and smooth muscle cell proliferation, apoptosis, migration, differentiation, activation and morphogenesis). This tailored array currently comprises 988 unique cDNAs (1171 total cDNAs including controls and multiple sequences from some genes) generated by PCR and spotted in duplicate onto a nylon membrane. These membranes are hybridised with ^{33}P-labelled complex cDNA probes derived from 5 μg of total cellular RNA. We have validated the production, hybridisation and analysis steps required to utilise this tool (Figures 1a, b). This approach brings the ability to monitor the abundance of hundreds of transcripts within reach of any academic research group. The aim of this review is to promote the use of gene arrays by vascular biologists who have no previous genomics experience. Therefore, we will describe the gene array methods developed in our laboratory in simple terms, but we will provide sufficient detail to allow our methods to be easily replicated.

Clone selection and verification

The array method described here is based on spotting cDNA fragments generated by PCR onto nylon membranes. The selection of the cDNA fragment to be amplified is a crucial step, since fragments must show little or no homology to other genes. For closely related gene families (such as the VEGF family), we have generated specific fragments which can distinguish family members [10, 11], but for most genes we have obtained appropriate fragments from the IMAGE consortium (http://www.hgmp.mrc.ac.uk). Ideally, cDNA fragments should be relatively short (0.6–2 kb). The inclusion of 3′ non-coding regions often allows discrimination between related transcripts that share close homology within their coding regions. Careful attention was also given to selection of cDNAs that would hybridise either to every splice variant of a transcript, or to individual splice variants of interest. We have found that web-based software provided by Compugen (http://www-labonweb.com/) is helpful to predict splice variants. The 988 cDNAs chosen for the array were selected to include the molecular pathways believed to be important in vascular biology. These include cell adhesion, apoptosis, signalling, cell cycle regulation, extracellular matrix remodelling and angiogenesis. The entire list can be seen at http://www.obgyn.cam.ac.uk/genearray. A significant proportion of the clones held by the IMAGE consortium have been miss-assigned therefore, all the clones included on the array have been validated by re-sequencing.

Generation of PCR products

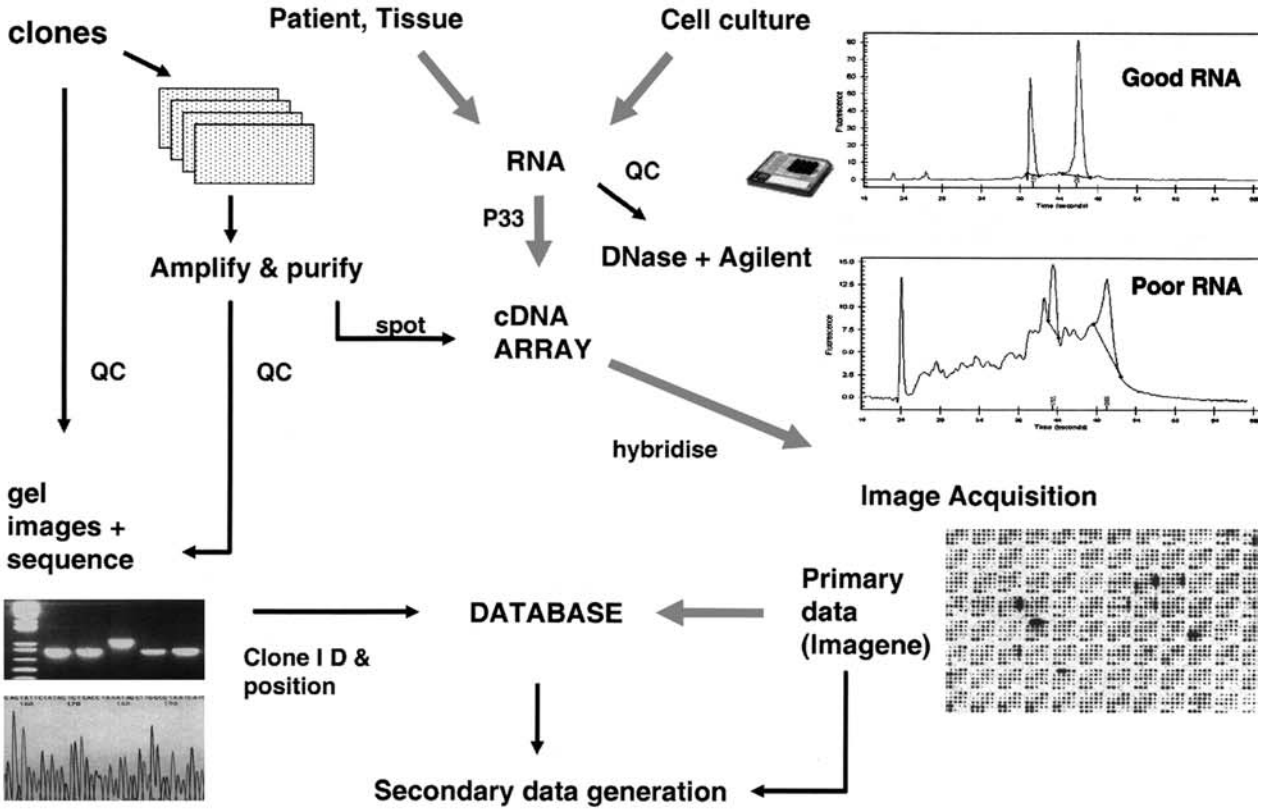
Amplification of cDNA clones requires a robust protocol to amplify hundreds of different inserts of varying sizes using a universal primer set (see Protocol 1). A variety of PCR conditions and enzymes were tested to optimise the yield of PCR product in a 96 well format (Figure 2). Biotaq DNA polymerase (Bioline, London, UK) consistently generated the most PCR product at the lowest cost. Randomly chosen wells from each plate were checked on 1.2% agarose gels (Figure 1a, Protocol 2); the results forming part of the QC information for each batch of arrays. In order to produce enough purified cDNA to print a large batch of nylon membranes (approx. 600), with sufficient cDNA to achieve satisfactory sensitivity, approximately 40–50 μg of each purified PCR product was prepared in 100 μl of water. Eight PCR reactions each of 50 μl were sufficient to provide this amount from each clone. The PCR products were pooled, purified and concentrated using the Millipore Multiscreen 96 system (Millipore, Watford, UK) with a vacuum manifold as described in Protocol 2.

Quantification of PCR products prior to spotting

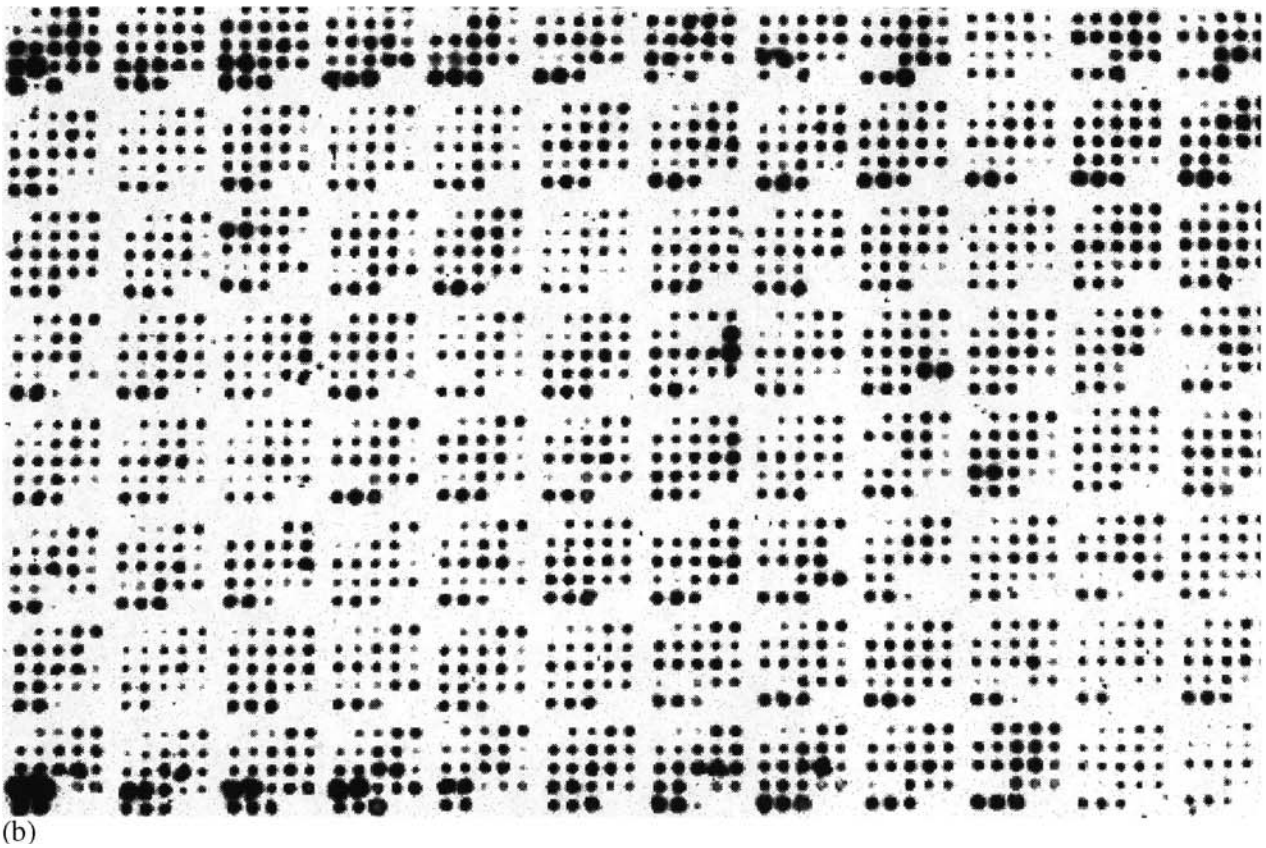
PCR products were quantified with SYBR® Green I (Sigma, Poole, UK), an ultrasensitive stain for double stranded DNA. This method uses Blue Fluorescence Imaging on a Molecular Dynamics (Buckinghamshire, UK) Storm 860® Phosphoimager and required only 2 μl of each product [see protocol 2]. This method was rapid and contributed to the archive of QC data relating to each batch of nylon membranes. The concentration of each cDNA product was adjusted to approximately 400 μg/ml to ensure even spotting.

Printing of nylon membranes

Contact printing was carried out using a BioRobotics MicroGrid robot (BioRobotics Inc., a subsidiary of



(a)



(b)

Figure 1. (a) Production and use of the angiogenesis array, (b) array image produced by probing with radiolabelled cDNA produced from 5 μg total human secretory phase endometrium RNA.

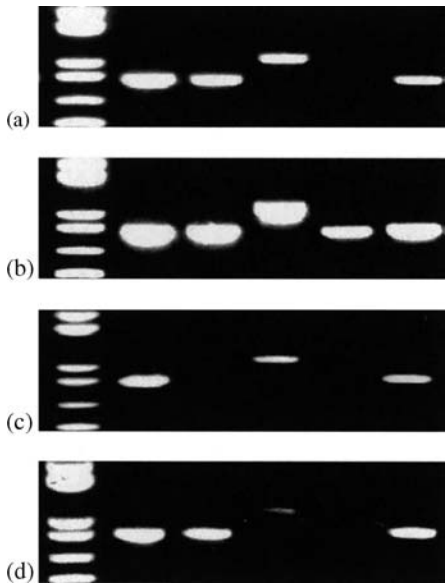


Figure 2. Testing DNA polymerases for efficiency of amplification. PCR amplification of five IMAGE clones carried out under identical conditions, using four different polymerises using the protocol described in protocol 1. Bioline taq DNA polymerase gave consistently the best yield. (a) YieldAce® Stratagene 2.5 U/reaction, (b) Biotaq® Bioline 1.25 U/reaction, (c) ABgene 2 × Master Mix 1.25 U/reaction, (d) Promega 2 × Master Mix 1.25 U/reaction.

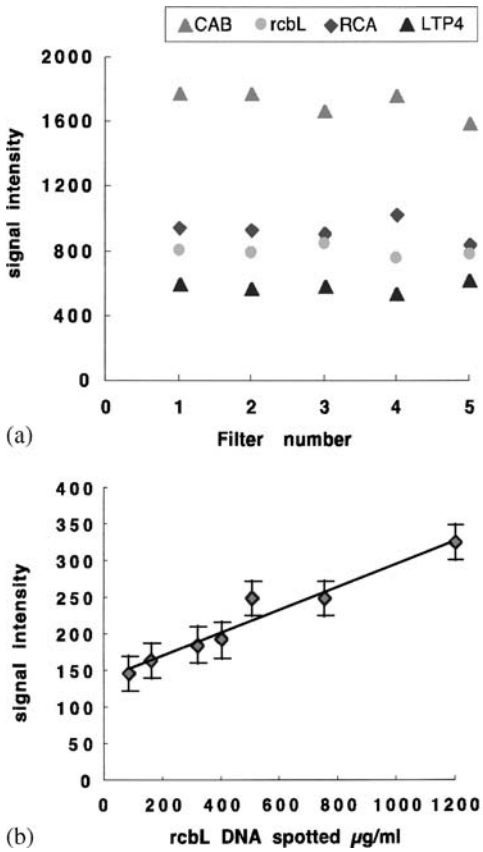


Figure 3. (a) Reliable detection of *Arabidopsis* sp. RNAs. Radiolabelled cDNA was produced from five separate aliquots of 5 μg human placental RNA. Prior to labelling each was spiked with *Arabidopsis* sp., RNA (500 pg to 1 pg as protocol 3). Following hybridisation, image analysis and normalisation the means of each spike duplicate across the five membranes was plotted. The low coefficients of variance for each spike RNA indicates close agreement between the five hybridisations.

	coefficient of variance
Spike 1 CAB Chlorophyll a/b binding protein	0.056
Spike 2 RCA RUBISCO activase	0.078
Spike 3 rbcL Ribulose-1 -5-biphosphate carboxylase/ oxygenase	0.050
Spike 4 LTP4 lipid transfer protein	0.057
Spike 5 LTP6 lipid transfer protein (not shown)	0.046.

(b) Linearity of signal intensity to the amount of DNA spotted is maintained after reverse transcription. rbcL ribulose-1-5-biphosphate carboxylase/oxygenase dilution series of DNA spotted in addition to the set of *Arabidopsis* sp. in 3a, each point is the mean signal intensity of 10 spots. Five membranes were hybridised to a radiolabelled mix of 50 pg rbcL RNA spiked into 5 μg human placental RNA. Errors ± 1 SE.

Apogent Discoveries, Hudson, New Hampshire, USA) and a 96 pin tool to create a 12 × 8 metagrid, 5 × 5 subgrid matrix yielding a theoretical maximum of 25 × 96 (2400) individual spots (Figure 1b). The purified PCR products were spotted in water in duplicate onto pre-cut (12 × 8 cm) Hybond N^+ (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK). Each spot was printed with two strikes of a 0.4 mm diameter solid pin. and contained approximately 14 ng of DNA product. After printing, the membranes were air dried, soaked in a denaturing solution (0.5 M NaOH, 1.5 M NaCl for 10 min at room temp.), neutralised (0.5 M Tris pH 7.4, 1.5 M NaCl for 4 min at room temp.), air dried, cross linked (70,000 $\mu J/cm^{-2}$) using a Stratalinker® UV Crosslinker model 1800 (Stratagene, La Jolla, California, USA) and stored dry at room temperature. This protocol is superior to either baking or air drying [12].

Validation and sensitivity

To enable QC and validation of the hybridisation we have incorporated several controls into the design of the array. These comprised:

(i) negative controls, (i.e. target DNAs that should not hybridise to complex cDNA generated from total RNA) such as salmon sperm DNA. Poly dA(40–60), and human Cot-1 DNA (enriched with Alu and Kpn repeats, [13]) were also spotted to confirm that hybridisation to these sequences was efficiently blocked by the prehybridisation step.

(ii) targets which may be used as normalisation controls (β-actin, cyclophilin, 18S ribosomal RNA, hypoxanthine ribosyl transferase, glyceraldehyde-3-phosphate dehydrogenase, ubiquitin and histidyl tRNA synthetase). This enable a suitable endogenous control to be chosen for subsequent real-time PCR confirmation of transcript abundance changes.

(iii) Exogenous cDNA clones from Stratagene's Spot Report® Exogenous Array Validation System (Stratagene, California, USA). These cDNAs from *Arabidopsis*

thaliana show no cross-hybridisation to human sequences. They provide a convenient method to monitor interarray variability and the sensitivity of the arrays. For example known amounts of cRNA corresponding to each of the spot report cDNAs were 'spiked' into five separate 5 μg aliquots of the same placental total RNA. The amounts of each spike RNA used are shown in Protocol 3. The spiked total RNA samples were then labelled and hybridised to five separate arrays. Following image analysis and normalisation, the mean intensity of the cDNA spots corresponding to each of the spikes was determined (Figure 3). As expected the CAB RNA, which was added at 500 pg, gave the strongest signal. The results for each spike exhibited low coefficients of variance (Figure 3) indicating reliable detection of these RNAs in the placental RNA sample. We were also able to determine the sensitivity of the arrays using this data. By depositing 14 ng cDNA per spot on the membrane we were able to detect 10 pg of mRNA (LTP4) at a signal intensity five times above background. Thus we are able to detect transcripts at an abundance of approximately 0.01% with good consistency.

RNA preparation and QC

High quality RNA is essential for efficient labelling and optimal hybridisation. Several issues need to be considered: labelling of degraded RNA or RNA contaminated with protein or carbohydrate will be variable, producing misleading results. In addition, contaminating genomic DNA may increase the background signal and therefore should be eliminated. Finally, when limited amounts of tissue or cells are available, the method chosen should produce a high RNA yield [14]. Of a wide variety of methods tested, Trizol® (Gibco, Life Technologies, Paisley, UK) produced the highest yield with acceptable purity. After isolation, all RNAs are treated with DNAse (using DNase I at 8 units/100 μl RNA for 30 min at 37 °C, Ambion, Inc., Texas, USA), followed by a further Trizol® extraction. Samples were checked for genomic DNA contamination by PCR using primers for the Histydyl tRNA Synthase gene (forward 5′ CCGCAGGTCGAGACAGC 3′, reverse 5′ TCATCAGGACCCAGCTGTGC 3′; 94 °C 4 min (1 cycle); 94 °C 30 s, 65 °C 30 s, 72 °C 30 s (30 cycles); 72 °C 3 min (1 cycle)). PCR products of 186 and 270 bp are produced from cDNA and genomic DNA respectively. The integrity and purity of the RNA are determined using an Agilent Technologies 2100 Bioanalyser, (Agilent technologies UK Limited, Cheshire, UK). This instrument gives detailed information about RNA quality and quantity and requires only a 50–500 ng of sample. Inspection of the electrophoretograms produced reveals any RNA degradation or genomic DNA contamination present in the samples and ensures that only high quality RNAs are labelled (Figure 1a).

Production of labelled cDNA from total RNA samples

Reverse transcription is used to produce cDNA from total cellular RNA (5 μg) labelled to high specific activity with ^{33}P-dCTP (Amersham PLC, Amersham, UK). To minimise non-specific priming the reaction is performed at 48 °C with an anchored Oligo d(T) primer using the EndoFree Reverse Transcriptase (RT)™ system (Ambion®, Texas, USA). EndoFree RT produces greater signal intensities and better sensitivity compared to Superscript™ II RT (Gibco, Life Technologies, Paisley, UK) [15]. Residual RNA is removed and the probes purified using NICK columns (Amersham Pharmacia Biotech) [Protocol 3]. Incorporation is checked using a scintillation counter (Tri-Carb1600Tr Liquid Scintillation Analyser Packard Instrument Company, Connecticut, USA).

Hybridisation

We have tested a number of different buffers and hybridisation conditions. The best results were obtained using ExpressHyb© Hybridisation Solution (BD Biosciences Clontech, USA) (Figure 1b). Membranes are hybridised in 15 cm × 4 cm roller bottles in a Hybridisation Oven (Hybaid Limited, Middlesex, UK) enabling continual mixing in a small volume. Both prehybridisation and hybridisation buffer include non-specific blocking agents [Protocol 4]. Stripping and re-use of membranes has been suggested as a way to reduce costs [16]; however, this can lead to loss of signal. We have not tested the performance of stripped membranes. We recommend drying the membranes by baking at 60 °C for 1 h prior to exposure to phosphor screens [Protocol 4].

Image processing and primary data acquisition

Membranes are exposed to Low Energy Storage Phosphor Screens (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK) optimised for use with ^{33}P, and scanned at high resolution (50 μm) using a Molecular Dynamics Storm® 860 Phosphoimager (Molecular Dynamics Inc, California, USA). Images are then transferred directly to IMAGENE 5® (BioDiscovery, California, USA) software, which provides sophisticated tools for spot finding, quantitation and data export.

Linearity

Image generation and data acquisition are complex repetitive tasks and therefore software automation to assist in this progress is advantageous. However, this leads to many of the underlying processes and data manipulations being hidden from the user. Along with others [17], we have found that scanner and phosphorimager software compress the data using non-linear

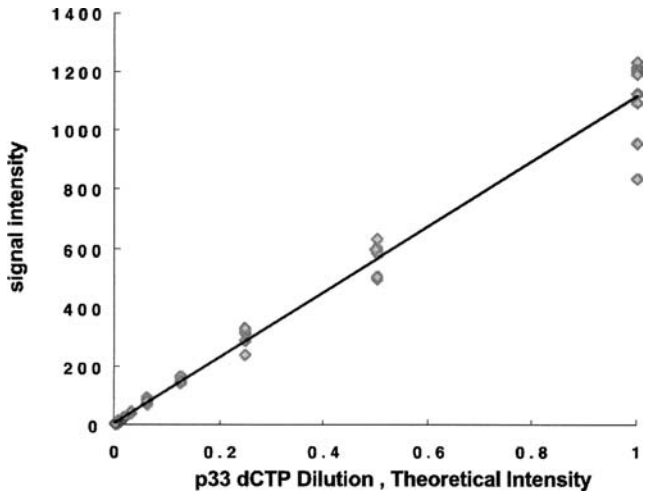
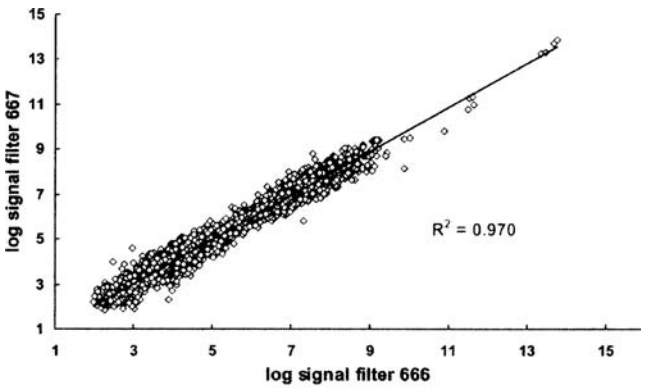


Figure 4. Validation of phosphorimager and image analysis software compatability. A 2 fold dilution series of ^{33}P-dCTP from 1×10^{-3} μCi/μl to 4×10^{-6} μCi/μl was produced. Eight spots of 1 μl of each dilution were manually deposited onto a nylon membrane, which was exposed to a phosphor screen for 48 h and the mean signal intensity for each spot determined. These values were plotted against the theoretical ^{33}P-dCTP dilution. The combination of Imagequant 4 and Imagene 5.1 produced the expected linear response indicating correct image processing.



Filter	663	664	665	666	667
663	1.000	0.979	0.970	0.978	0.983
664	0.979	1.000	0.979	0.982	0.980
665	0.970	0.979	1.000	0.980	0.980
666	0.978	0.982	0.980	1.000	0.985
667	0.983	0.980	0.980	0.985	1.000

Figure 5. Determination of Inter-array variability. Five aliquots of the same human placental RNA were labelled and hybridised to five membranes. Inter-array variability was assessed using scatter plots of mean signal intensity for each membrane against any other, as presented above for filters 666 and 667, for all filter combinations. There is a high degree of correlation.

transformations (e.g. square-root) and that some image processing software packages do not correct for this. This can introduce catastrophic artefacts into the data and so is a serious concern. We strongly recommend that the performance of the phosphorimager and image analysis software are validated. To do this we produced an artificial 'array' by manually spotting 2-fold serial dilutions of ^{33}P-dCTP onto a nylon membrane. Each dilution was spotted eight times. The mean signal intensity for each set of eight spots was determined and showed a direct relationship with the dilution factor.

The combination of Molecular Dynamics Storm® 860 Phosphorimager and ImageQuant 4 or Imagene 5.1 faithfully replicated the serial dilution curves of the input ^{33}P-dCTP (Figure 4). We found other image analysis software packages for which this was not the case.

Reproducibility and technical replication

Gene arrays are of little use if the data they produce is so noisy that only very large changes in transcript abundance can be detected above the noise. To reduce the effects of noise, each cDNA represented on the array is spotted in duplicate. In addition, for a small subset of genes, multiple cDNA sequences from distinct regions of the gene are spotted. When five aliquots of the same placental RNA were labelled and hybridised to five different arrays we found that less than 4% of the duplicates on each array differed by >50% (Table 1, Intra-array variability).

To determine inter-array variability, each membrane was compared to the other four. When the mean log signal intensity for each spot in an array is plotted against the results of another array as shown in Figure 5, regression coefficients were close to 1, indicating a close positive correlation. Figure 6 shows that the percentage of array elements in each pairwise comparison of these membranes (technical replicates) which appear to be regulated up or down by more than 2-fold. These 'apparent' changes (given that the same RNA was used for all experiments) represent false positive rates due to technical 'noise' and ranged between 0.5% and 5.0%. Combined with the data presented in Figure 5, this demonstrates that our tailored array is reproducible. However, averaging the results of two or more arrays (technical replication) reduces the impact of chance or technical differences between arrays.

Table 1. Intra – array variability within each of the arrays in Figure 5 less than 4% of the duplicated spots differed by greater than 50%.

Filter	Percentage of duplicates with errors >50%
663	2.9
664	3.3
665	2.4
666	1.8
667	3.1

Biological replicates

There is considerable variation in the behaviour of individual isolates of human umbilical vein endothelial cells (HUVEC) ([18] and our unpublished Affymetrix data). This is also likely to be true of other primary cells.

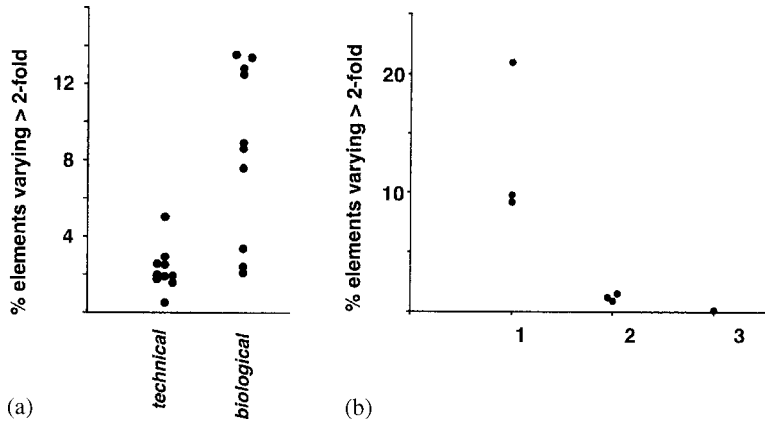


Figure 6. (a) Comparing of the degree of technical and biological variation evident when using our tailored gene arrays, 'technical' designates technical variation, where complex probes were prepared from a single RNA and hybridised to five separate filters. For each possible combination of any two filters, the % of array elements which varied >2-fold (up or down) is shown, *'biological'* designates biological variation, where RNA was isolated from five separate primary HUVEC cultures each collected from a different individual. Radiolabelled cDNAs prepared from each separate RNA were hybridised to five separate filters. For each possible combination of any two filters, the percentage of array elements which varied >2-fold (up or down) is shown. (b) Biological replicates are essential to reduce the incidence of false-positive results. HUVEC from 3 separate individuals were cultured for 24 h in the presence or absence of a low concentration of growth factor, which had no statistically significant effect on transcript abundance in these cells. RNAs were prepared from these cultures and used to generate radiolabelled cDNAs that were hybridised to filters. Averaging the results of any two or all three biological replicates reduced the % of false positives' (array elements that appeared to be regulated >2-fold (up or down) due to the idiosyncratic properties of a single HUVEC culture or due to chance).

Therefore, experiments using primary cells from a single individual donor may reveal idiosyncratic results that cannot be generalised. To illustrate this problem, we prepared RNA from HUVEC obtained from five different individuals. Labelled cDNA was generated from these RNAs and hybridised to our arrays. Pairwise comparisons between the different patients were performed. The percentage of array elements that appear to be regulated by more than 2-fold (up or down) between any two of these 'biological replicates' ranges between 2% and 13.5% (Figure 6a). Since no treatment has been applied to the HUVEC cultures used in this experiment, these differences represent genetic differences, as well as technical differences between cultures or hybridisations. There is approximately five times more variation between biological replicates (where labelled cDNAs are generated from cells from different individuals) than there is between technical replicates (where separate labelled cDNAs are generated from a single RNA).

To demonstrate the importance of biological replicates in a real experiment, HUVEC isolated from three individuals (three biological replicates) were treated with a low dose of a growth factor, and RNA collected after 24 h. Hybridisation of these RNAs to our tailored arrays showed that none of the 998 genes present on our array were regulated significantly in the cells derived from all three individuals. This was confrimed using Affymetrix genechips. However, if the experiment had been conducted only once using HUVECs donated by any one of the three individuals, we would have been mislead into concluding that a significant percentage of the transcripts interrogated by the arrays were regulated in response to the growth factor (Figure 6b, x-axis = 1). When the results for any two biological replicate experiments were averaged, the number of apparently regulated transcripts is substantially reduced (Figure 6b, x-axis = 2). Averaging all three biological replicate experiments further reduced the number of 'false positives' to zero (Figure 6b, x-axis = 3). Therefore, we believe that two, and preferably three or more biological replicates is essential for any gene array experiment. This is particularly true of experiments in which primary cell cultures or tissues from different individuals are used.

When planning complex experiments, a critical consideration is the number of replicates required, to ensure sufficient statistical power to distinguish between genuinely regulated genes and false positive results, due to biological variation [19]. Additionally, randomising the order of tissues (or operators) during the hybridisation process is fundamental to minimising bias [20].

Data normalisation and analysis

One of the biggest problems in extracting biologically relevant data from gene array experiments is making valid comparisons between arrays. Numerous factors influence the signal generated for any particular spot. Some of these relate to the specimen quality (protein contamination, RNA degradation), others to array quality and image processing (spotting efficiency signal quantification, and 'background' correction). Finally there is variation due to reverse transcription efficiency, hybridisation specificity and most importantly the specific activity of labelled RNA.

To obtain meaningful comparisons between data sets it is necessary to make some mathematical adjustment to the data. Although this is commonly termed normalisation, scaling is more often applied in practice. There are several normalisation methods currently in use for

microarray data. Internal reference methods utilise a set of predetermined genes expected to give consistent signal intensities across different experiments [21]. Internal globalisation methods allow normalisation to be dictated by the observed data rather than by the expression level of genes determined *a priori* to be expressed at consistent levels. There are a number of ways this can be done. Global scaling applies a constant scaling factor based on first-order statistics (e.g. the mean or median) to define a scaling quotient. Applying a constant factor, however, is not always appropriate when the error is non-linear. Rank transformation of data can deal with differing data distributions but is insensitive and may lead to data loss.

In most gene array experiments, only a few transcripts are regulated by the treatment or experimental condition under investigation, and the abundance of the majority of transcripts on the array is unchanged. In a graph comparing the abundance of transcripts in the two RNAs we would therefore expect the majority of transcripts (those which are not regulated) to lie on a diagonal line (e.g. Figure 5). However, this is frequently not the case following global scaling. Small differences in probe labelling, hybridisation and scanning may cause intensity-dependant errors, where all transcripts of a particular intensity are shifted off the diagonal (Figure 7a). If not corrected, this can lead to the mistaken conclusion that these transcripts are more abundant in one RNA population than another. An approach for dealing with this type of distribution discrepancy, as well as global scaling errors, is to use a smoothing function aiming to return the position of all unregulated transcripts to the diagonal. This is known as intensity-dependant normalisation. The effect is seen easily in a Bland–Altmann plot [22, 23], shown in Figure 7b, where the difference between the log signal intensities of transcripts within two RNA populations is plotted against the average of the log signal intensities. The majority of transcripts, which are not differentially expressed, should lie along the *x*-axis. Transcripts more abundant in one RNA, than the other, will lie above or below the *x*-axis. Any intensity-dependent deviation

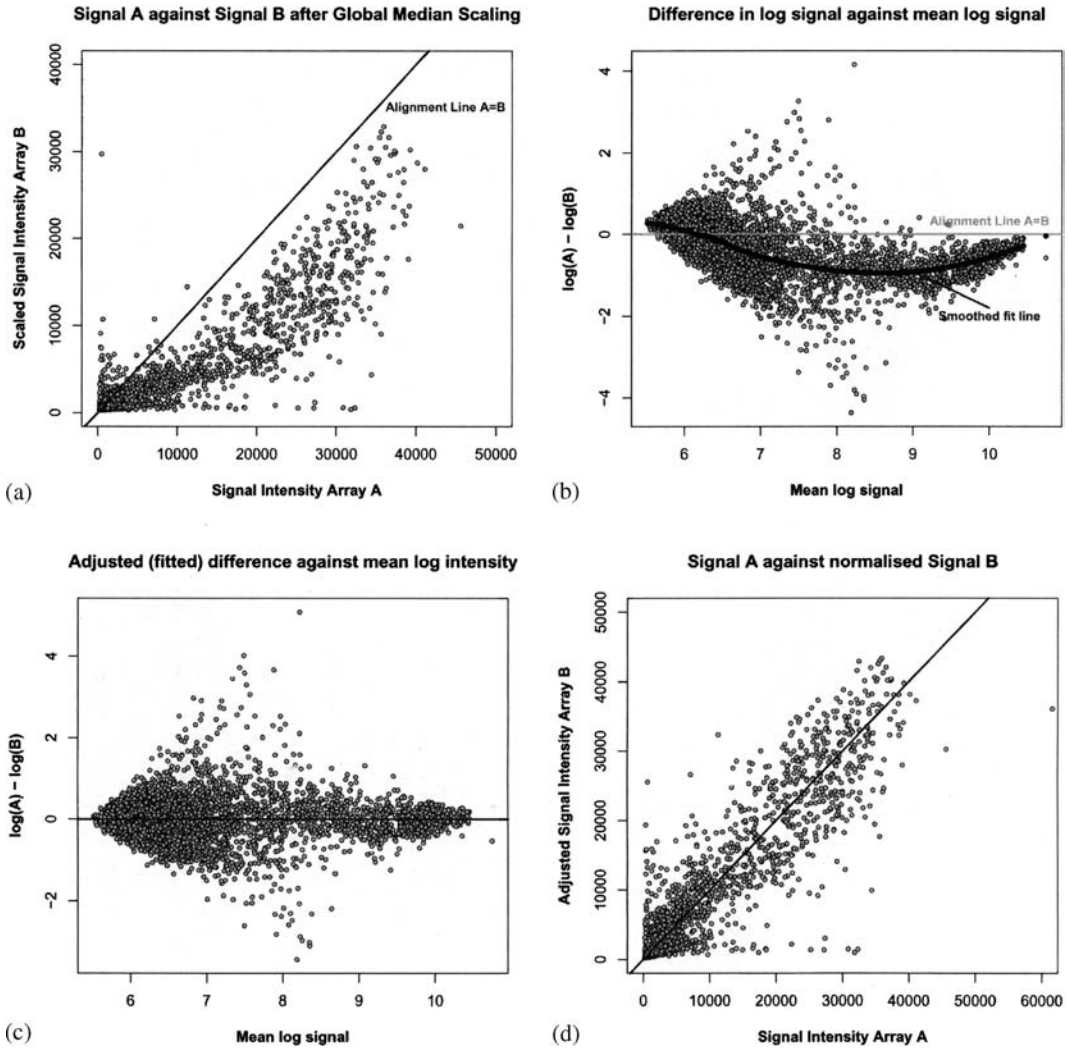


Figure 7. Loess normalisation to correct intensity dependent errors. Two closely related samples were labelled and hybridised. (a) Global scaling fails to correct intensity-dependent scaling errors, (b) rotation and log transformation. Difference in log signal intensity is plotted agains mean log signal intensity, (a Bland–Altman plot), demonstrates intensity-dependent deviation from the x-axis. A Loess smoothing regression is applied (red line) to correct the intensity-dependent deviation from the *x*-axis. (c) Corrected data and (d) transformed back to the original scale.

from this axis can be seen by applying a smoothing fit line as shown in Figure 7b. This allows the data points to be adjusted back to the *x*-axis to correct for the intensity-dependant error (Figure 7c). In most cases the smoothing function applied is derived from the 'Lowess' function [24] of the S statistical package (or its R equivalent, 'Loess'). The disadvantage of this method is that there is a tendency to over-fit data where there are few data points, typically at the upper range of expression values. This may have the effect of reducing fold-change values, even when these represent true biological differences. It is important, therefore to appreciate the effect of such normalisations on reducing the observed fold change in gene expression.

The aim of all normalisation methods is essentially to reduce inter-array variance due to noise, whilst maintaining the ability to detect significant biological differences in gene expression. More sophisticated methods have recently been developed to enhance sensitivity to identify significant change, despite normalisation. The methods of Yang [25], and Huber [26] attempt to improve the sensitivity of detection of differentially expressed genes over Loess-based normalisation alone. However, these methods are complex. We have therefore chosen to adopt the Loess approach to normalisation described above.

There is a further issue arising from the use of Loess for unpaired data sets, the concept of a reference array. Loess normalisation must be applied sequentially between arrays, typically against a standard reference array. For paired experiments the control array in each pair is a natural reference but there is no such natural reference in unpaired data sets (e.g. tumour and normal tissue from different patients). One approach [27] is to use an 'average array', comprising the geometric mean of the data (by genes), as a reference array, to which to normalise all the array data. An alternative approach is to select, as the reference array, the array which is closest (in terms of Euclidean distance) to this average array. A full description of this approach with an example of its application is available at http://www.obgyn.cam.ac.uk/genearray.

Following normalisation, advanced statistical methods are typically required to generate a list of candidate genes for validation by quantitative PCR. Student's *t*-test is often inappropriate in the context of gene array data, due to the large number of genes tested simultaneously. This leads to the problem of multiple comparisons [28] and susceptibility to a high false positive rate. Cyber-T [29] employs a Bayesian modification of the *t*-test, and SAM [30] uses a permutation-based approach to identify the false discovery rate for individual genes. Packages such as Bioconductor (http://www.bioconductor.org) or Genespring (Silicon Genetics, California, USA) can be used for data visualisation and clustering [31]. Reducing the data to a minimal number of components by the use of principal components analysis (PCA) or, more recently, independent component analysis (ICA) [32, 33] may lead to the identification of gene signatures involved in vascular remodelling.

Clearly the production of nylon filters requires multiple steps each of which incurs consumable costs. We have determined these and made allowances for possible failures of some of the steps and therefore the cost per filter is the maximum foreseeable. Thus to produce a batch of 400 filters the cost works out at £19.35 per filter. This compares very favourably with similar commercial filters which cost approximately £1200 for four (however, this does include the cost of some of the labelling reagents as well). We have also costed our optimised labelling and hybridisation protocols and the cost of the isotope, labelling and hybridisation reagents works out at £40 per hybridisation. Since a major portion of the labelling and hybridisation cost is attributable to the purchase of ^{33}P-dCTP, volume discounts may reduce this. The personnel cost associated with the generation of the filters has not been included in the figure above however once the protocols have been developed and optimised it is possible to produce the PCR products necessary and to generate the filters in approximately 6–8 weeks. In fact the most time consuming step is the assembly and verification of the cDNA clone collection. All in all using our optimised protocols we believe it is feasible for modest size laboratories to produce large number of filters which allow many biological replicates to be analysed.

Summary

Over 2000 microarray papers have been published in the last few years. The majority are now fluorescence based studies using commercially produced arrays of tens of thousands of features, but the range also includes a 59 gene rat array [34].

We believe small targeted gene arrays such as our angiogenesis array has an important place in this range, especially in situations where limited RNA is available (such as investigations into reproductive angiogenesis using small tissue samples) or when the use of primary cells and tissues necessitates multiple biological replicates. The array methods presented here are targeted, sensitive, flexible and relatively inexpensive. This type of array is well within the funding and expertise of many laboratories, but generation of meaningful results depends on careful validation of every step in the process. If this is done, tailored gene arrays provide a reliable tool to address the complex regulation of RNA transcript abundance that underlies much of vascular biology.

Acknowledgements

Amanda L. Evans, and Roberto Catalano were supported by WHO Rockfeller Foundation grant number RF99021/115. Andrew S. Sharkey was supported by a Meres Studentship from St. John's College, Cambridge. Sam Saidi was supported by MRC programme grant

Protocol 1. Amplification of cDNA inserts for spotting.

ANGIOGENESIS GENE ARRAY

- 11 × Master Plates glycerols −70 °C
- Grow fresh cultures 96 well plates 37 °C/48 h in LB/Amp 50 μg/ml
- Heat 25 μl culture + 75 μl dH_20 95 °C/10 min
- Lysates stored −20 °C

Spin 5 min 1000 rpm to pellet debris prior to use
Use supernatent as PCR template

96 well PCR

PCR mix	1 × 50 μl	100 × 50 μl
10 × Bioline buffer	5	500
2 mM dNTPs	5	500
M13F 10 μM	2.5	250
M13R 10 μM	2.5	250
50 mM $MgCl_2$	1.5	150
dH_20	31.25	3125
Bioloine Taq5 u/μl	0.25	25

Template (boiled lysate) 2

Parameters for PCR
(94 °C 1 min) × 1,
(94 °C 30 s, 55 °C 30 s, 72 °C 1 min) × 30,
(72 °C 7 min) × 1
Hold 4 °C

M13F (-20) GTA AAA CGA CGG CCA GTG
M13R (-48) AGC GGA TAA CAA TTT CAC AC
Random empty wells serve as negative controls

Protocol 3. Labelling of cDNA using EndoFree RT.

Reverse Transcription (EndoFree RT)

RNA 3–5 μg (cone., ≥0.7μg/μl)
1 μl 10 μM Anchored Oligo(dT) Primer
1 μl RNA Spike mix (below)
μl RNase-free H_2O upto to 8 μl total volume

70 °C, 5 min
48–50 °C, 5 min

2 μl 10X RT Buffer
4 μl dNTPs (2.5 mM dATP, dTTP, dGTP, 10 μM dCTP)
1 μl RNase Inhibitor
3 μl [α-^{33}P] dCTP 10 μCi/μl, 2500 Ci/mmol

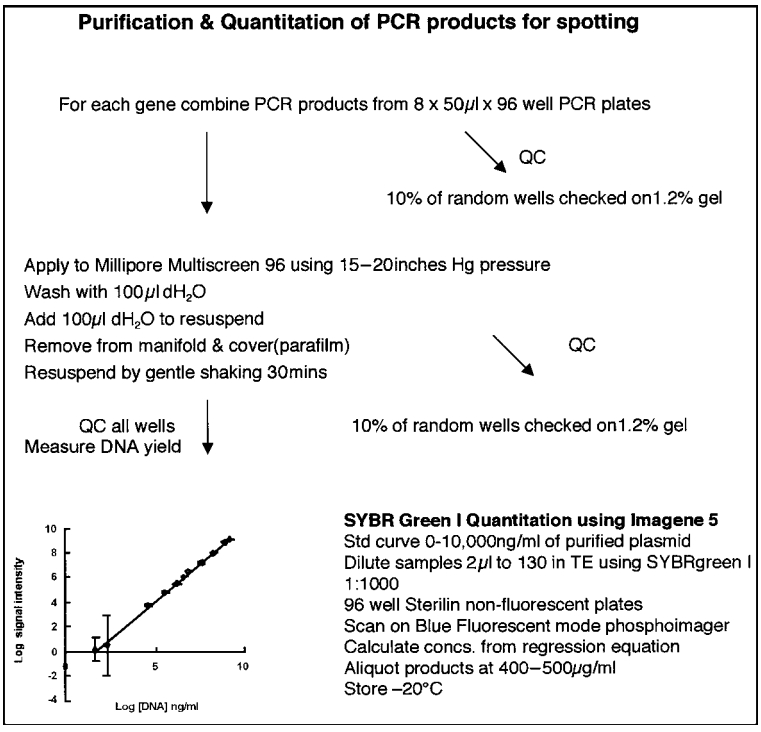
48–50 °C for 5 min.
1 μl of Reverse Transcriptase
Incubate at 48–50 °C for 2 h.

RNA SPIKE MIX

1 μl spike mix contains	Spike pg	CAB	RCA	rbcL	LTP4	LTP6
		500	100	50	10	1

Remove RNA
Add
2 μl 10% SDS
1 μl 0.5 M EDTA pH 8.0
3 μl 3M NaOH (fresh)
68 °C 20 min
RT 10 min
Add
10 μl 1 M Tris pH 7.5
3 μl 3 M HCI
Remove unincorporated dNTPS using Nick column (Amersham)

Protocol 2. Purification of amplified cDNA inserts for spotting.



number G9623012 awarded to SK. Smith and DS Charnock-Jones of The Molecular Reproductive Medicine Group. Cristin G. Print was supported by The Wellcome Trust fellowship number 59522.

Protocol 4. Hybridisation and washing nylon membrane.

Pre-Hybridisation of nylon membranes

Pre wet membranes in dH_2O
Pre-heat Express Hyb© buffer at 68 °C
Heat – denature at 95 °C, 5 min the following
10 µl human Cot-1 DNA (1 mg/ml)
10 µl polyA (1 mg/ml)
200 µl salmon sperm DNA (5 mg/ml)

Add to 10 mls Express Hyb.© pre-warmed
Pre-Hybridise at 65 °C 3 h at 5–7 rpm in a roller bottle

Hybridisation

Heat denature at 95 °C, 5 min the following
400 µl purified radiolabelled cDNA
10 µl MEDTA
5 µl human Cot-1 DNA (1 mg/ml)
5 µl poly A (1 mg/ml)
100 µl salmon sperm DNA (5 mg/ml)
Add to 4.5 ml pre-warmed Express Hyb©
Replace the.Pre-Hyb. Buffer with the hyb. Mix.
Hybridise 65 °C 16 h 5–7 rpm

Washing

Pour off the hyb. buffer		
2 × SSC/0.5% SDS	30 min 60 °C	Twice
0.1 × SSC/0.1% SDS	30 min 60 °C	Twice
Rinse dH_20 for 5 sec only		
Bake 60 °C for 1 h		
Expose to Molecular Dynamics LE screen 48 h		
Scan at 50 µm resolution		

We thank Khashayar Lessan, supported by Wellbeing grant number PG/282, for his contribution to the apoptosis gene set, and Nicola Johnson for use of the HUVEC data. We also thank Kate Day for excellent technical support.

Appendix A

Agilent	http://www.agilent.com/chem/labonachip
Apogent	http://www.apogentdiscoveries.com
Amersham	http://amershambiosciences.com
Bd Biosciences	http://bdbiosciences.com
Caliper	http://www.calipertech.com
Genespring	http://www.silicongenetics.com
Imagequant	http://www.mdyn.com/products/lmageQuant/default.htm
Imagene	http://www.biodiscovery.com/products/lmagene/imagene.html
SpotReport	http://www.stratagene.com

A comprehensive list of array web sites is available from Molecular Cloning, A Laboratory Manual, Volume 3, Sambrook and Russel. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

References

1. Folkman J, D'Amore PA. Blood vessel formation: What is its molecular basis? Cell 1996; 87: 1153–55.
2. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogeneic switch during tumorigenesis. Cell 1996; 86: 353–64.
3. Risau W. Mechanisms of angiogenesis. Nature 1997; 386: 671–4.
4. Lipshutz R, Fodor S, Gingeras TR, Lockhart DJ. High density synthetic oligonucleotide arrays. Nat Genet Sup 1999; 21: 20–4.
5. Lockhart DJ, Dong H, Byrne MC et al. Expression monitoring by hybridisation to high density oligonucleotide arrays. Nat Biotechnol 1996; 14(1112): 1675–80.
6. Adessi C, Matton G, Ayala G et al. Solid phase DNA amplification: Characterisation of primer attachment and amplification mechanisms. Nucleic Acids Res 2000; 28(20e87): 1–8.
7. Borthwick JM, Charnock-Jones DS, Tom BD et al. Determination of the transcript profile of human endometrium. Mol Hum Reprod 2003; 9(1): 19–33.
8. Sargent TD, David IB. Differential gene expression in the gastula of Xenopus Laevis. Science 1983; 222(4620): 135–9.
9. Duggan D, Bittner M, Chen Y et al. Expression profiling using cDNA microarrays. Nat Genet Sup 1999; 21: 10–4.
10. Clark DE, Smith SK, He Y et al. A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. Biol Reprod 1998; 59: 1540–48.
11. Sowter HM, Corps AN, Evans AL et al. Expression and localization of the vascular endothelial growth factor family in ovarian epithelial tumors. Lab Invest 1997; 77(6): 607–14.
12. English D, Parker B. Methods for Preparing PCR Based Arrays. Scleicher & Schuell Applications Notes for Biomolecular Screening, 1999.
13. Britten RJ, Graham DE, Neufeld BR. Meth Enzymol 1974; 29: 363–418.
14. Khodarov NN, Yu J, Nodzenski JS et al. Method of RNA purification from endothelial cells for DNA Array Experiments. Biotechniques 2002; 52: 72–4.
15. Hegde P, Qi R, Abernathy C et al. A concise guide to cDNA microarray analysis. Biotechniques 2000; 29: 548–62.
16. Bao Z, Wenli M, Rong S et al. Re-use of a stripped cDNA microarray. Br J Biomed Sci 2002; 59(2): 118–9.
17. Ramdas L, Coombes KR, Baggerly K et al. Sources of nonlinearity in cDNA microarray expression measurements. Genome Biol 2001; 2(11): 0047.1–7.
18. Novak JP. Characterisation of variability in large-scale gene expression data: Implications for study design. Genomics 2002; 79: 104–13.
19. Kerry S, Bland JM. Sample size in cluster randomisation. BMJ 1998; 316(7130): 549.
20. Draghici S, Kuklin A, Hoff B, Shams S. Experimental design, analysis of variance and slide quality assessment in gene expression arrays. Curr Opin Drug Discov Dev 2001; 4(3): 332–7.
21. Kroll T, Wolfl S. Ranking: A closer look on globalisation methods for normalisation of gene expression arrays. Nucleic Acids Res 2002; 30(11): e50.
22. Bland JM, Altman DG. Measuring agreement in method comparism studies. Stat Meth Med Res 1999; 8: 135–40.
23. Dunn G. Statistical methods in laboratory medicine. Stat Meth Med Res 1999; 8(2): 91.
24. Cleveland W. Robust Locally Weighted regression and Smoothing Scatterplots. J Am Stat Assoc 1979; 74: 829–36.
25. Yang Y, Dudoit S, Luu P et al. Normalization for cDNA microarray data: A robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 2002; 30(4): e15.
26. Huber W, Van Heydebreck A, Sültmann H et al. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 2002; Suppl 1: S96–104.
27. Workman C, Jensen L, Jarmer H et al. A new non-linear normalization method for reducing variability in DNA microarray experiments. Genome Biol 2002; 3(9): research0048.
28. Bland JM, Altman DG. Multiple significance tests: The Bonferroni method. BMJ 1995; 310(6973): 170.
29. Baldi P, Long A. A Bayesian framework for the analysis of microarray expression data: Regularized *t*-test and statistical inferences of gene changes. Bioinformatics 2001; 17(6): 509–19.

30. Tusher V, Tibshirani R, Chu G. Significance of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 2001; 98(18): 10515.
31. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998; 95(25): 14863–8.
32. Liebermeister W. Linear modes of gene expression determined by independent component analysis. Bioinformatics 2002; 18(1): 51–60.
33. Martoglio A, Miskin J, Smith SK, Mackey D. A decomposition model to track gene expression signatures: Preview on observer-independent classification of ovarian cancer. Bioinformatics 2002; 18(12): 1617–24.
34. De longueville F, Surry D, Meneses-Lorente G et al. Gene expression profiling of drug metabolism and toxicology markers using a low-density DNA microarray. Biochem Pharmacol 2002; 64(1): 137–49.