Deposited research article

The influence of RNA integrity, purity and cDNA labelling on glass slide cDNA microarray image quality

Dachuan Guo, Belinda Cutri and Daniel R Catchpoole

Addresses: The Tumour Bank, The Oncology Research Unit, The Children's Hospital at Westmead, Westmead, NSW 2145, Australia.

Correspondence: Daniel R Catchpoole. E-mail: danielc@chw.edu.au

Posted: 16 July 2004

Genome ***Biology*** 2004, **5:**P13

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2004/5/8/P13

Received: 14 July 2004

This is the first version of this article to be made available publicly.

The influence of RNA integrity, purity and cDNA labelling on glass slide cDNA microarray image quality.

Dachuan Guo[1], Belinda Cutri[2] and Daniel R. Catchpoole[3]*

The Tumour Bank, The Oncology Research Unit, The Children's Hospital at Westmead, Westmead, NSW, 2145, Australia.

Running Title: Improving cDNA microarray quality assurance.

Submitted to *Genome Biology*

Date: 13/07/04

* Corresponding Author

The Tumour Bank,
The Children's Hospital at Westmead
Locked Bag 4001,
Westmead, NSW, 2145
Australia
Phone: 61-2-98451205
Fax: 61-2-98453078
Email: Danielc@chw.edu.au

Authors Email.

[1] dachuang@chw.edu.au
[2] b.cutri@hri.org.au
[3] danielc@chw.edu.au

Abstract

Background: The accuracy of gene expression measurements generated using cDNA microarrays is dependent on the quality of the image generated following hybridization of fluorescently labelled cDNA. It is not known how this image is influenced by sample preparation factors which such as RNA quality, cDNA synthesis and labelling efficiency.

Results: In this study we used a simple metric based on the ratio of the total feature (F) and background (B) fluorescence, which correlates with the visual assessment of 60 microarray images, to determine the influence of sample preparation on image quality. Results indicate that RNA purity (A_{260}/A_{280}) and integrity (18S:28S ratio) do not strongly influence microarray image quality. cDNA having an nucleotide to dye ratio greater than 100 produced poor microarray images, however, cDNA labelled more efficiently was not a guarantee of a better image. The data also indicate that the array image quality is not improved by loading more cDNA into the hybridization mixture however poor image quality did result from a disproportionate amounts of Cy5 and Cy3 labelled cDNA.

Conclusion: This study provides insight into the source of variation in microarray image analysis introduced during sample preparation and will assist in the standardisation of cDNA glass slide microarray protocols.

Introduction

The ability to extract meaningful gene expression data from microarray experiments is dependent upon the precision, sensitivity and reproducibility of the measured values for each gene on the array. In the case of cDNA microarray spotted onto glass slides, gene expression data is determined by the ratio of the relative signal of two fluorescent Cy-dye labelled cDNA preparations that have been hybridized to the microarray [1]. Following laser scanning of the hybridized array, a two colour image is generated which consists of signals from the two laser-excited fluors. Image analysis software digitally renders this image into pixels which are identified as either corresponding to spot features, or as coming from the background [2,3]. The intensity of the local background pixels is subtracted from the feature pixels for each gene transcript to give the corrected intensity for each fluorescence dye [4,5]. The ratio of the two fluorophore signals is consequently used as a relative measure of the gene expression.

As gene expression data is generated from a multi-channel image the quality of that image will greatly influence the accuracy of the gene expression values used for biological inference. High quality images are a prerequisite for obtaining high precision gene expression data [6,7]. Complex computational image metrics which draw upon different segmentation algorithms including fixed circle, adaptive shape and histograms [7,8], and background corrections involving Bayesian priors, non-linear filter called ‘morphological opening’ and best-fit methods [9] are currently being advanced. Such approaches seek to determine errors and variation in gene expression read-outs from microarray images introduced by fluorescence intensity fluctuations within the array, background from non-specific binding of labelled DNA, electronic noise and photon counting errors [9]. Despite these analytical

developments, data output differences between scanners, software availability, and a lack of appreciation of the statistical complexity of these approaches has restricted the adoption of a common image analysis approach within the microarray research community.

It has also been recognised that microarray generated gene expression data is also influenced by experimental factors that can be introduced during the fabrication of the cDNA microarrays themselves as well as the target preparation, labelling reactions and hybridization processes [10]. The spotting and deposition of the cDNA templates on the array slides is a recognised source of systematic bias in gene expression data [11]. Considerable effort is being placed on optimizing spotting conditions [12] as well as the surface chemistry [13] of the glass slides. The accuracy of gene expression evaluation is recognised to be influenced by the quality of the starting total RNA with this usually assessed by determining its purity using the spectrophotometry and calculating the $A_{260/280}$ ratio [14]. The integrity of the RNA is evaluated using the relative intensity of the 28S and 18S ribosomal bands following gel electrophoresis [6,14]. Preparation of the cDNA synthesis and fluorescent labelling reaction has also been examined. Various studies have compared cDNA labelling methods and have now shown that indirect labelling protocols, which incorporates amino-allyl modified dUTP (aa-dUTP) and subsequent coupling of Cy3 and Cy5 dyes is the preferred to direct labelling procedures as they result in higher labelling efficiency [15,16].

Despite these improvements, the direct relationship between the quality of starting RNA, or the degree of fluorescent dye labelling of the cDNA and the clarity of the resulting image has not been reported. In order to examine this relationship simply we have undertaken a systematic visual assessment of the microarray images

derived from 60 microarray experiments and compared the data which were generated according to a standard indirect labelling protocol [17] and qualitatively verified that this was an appropriate factor for distinguishing the visual quality of the cDNA microarray image. To assist with this ‘back to basics’ approach we used the ratio of the total combined feature fluorescence intensity (F) to total background intensity (B) (F/B ratio) per array. Using this graded visual assessment and the F/B ratio we examined the relationship between the microarray image clarity and several sample preparation quality assurance indicators. Specifically, the RNA purity, RNA integrity and cDNA labelling efficiency and the amount of cDNA present during hybridization.

Material and Methods

RNA Isolation and cDNA Synthesis – RNA Purity and Integrity

The data used in our analysis was collected from a variety of microarray studies ongoing within our laboratory with the RNA having been isolated from either frozen bone marrow sample or CCRF-CEM (CEM) cell lines. Fourteen bone marrow samples were obtained from the Children's Hospital at Westmead Tumour Bank, and were used with the approval of the hospital Human Research Ethics Committee as well as the Tumour Bank Committee.

RNA was isolated from frozen bone marrow samples using Trizol LS (Gibco BRL) according to a protocol optimized in our laboratory [18] whilst CEM cells (>1 x 10^7 cells) were lysed in 1ml Trizol LS and the RNA isolated according to the manufacturers protocol. RNA purity was assessed by determining the $A_{260/280}$ ratio by spectrophotometry. RNA (3μg) was loaded onto a 1% denaturing agarose gel, subject to electrophoresis and the RNA visualised under UV transillumination following staining with ethidium bromide. Gel images were taken and the intensity of the 28S and 18S ribosomal RNA bands determined by pixel density. The 28S:18S ratio was subsequently calculated and used as a measure of RNA integrity.

cDNA Microarray Hybridization – Labelling Efficiency

The technique used for the microarray cDNA preparation, indirect fluorescence labelling and hybridization is per the techniques used at The Institute for Genome Research (TIGR, USA) [17].

We combined Cy5-labelled cDNA derived from 'test' preparation with the Cy3-labelled cDNA from controls and mixed with 5xSSC buffer which contained 25% formamide, 0.1% SDS, 10μg human Cot1 DNA, 20μg heat-denatured ssDNA, 6μg

polyA, and 12μg yeast tRNA. This cocktail was denatured for 5 minutes at 95°C prior to being hybridized to cDNA microarrays over night at 42°C. The cDNA microarrays consist of either 6000 or 10500 sequence verified known human genes spotted onto Telechem® slides. cDNA microarrays were obtained from the Ramaciotti Centre for Genome Function at the University of New South Wales and the Peter McCallum Cancer Centre. Following hybridization, the microarray was wash in a pre-warmed (~50°C) 1xSSC solution containing 0.03%SDS for 5 minutes followed by successive 5 minute washes in 0.2xSSC and 0.05xSSC at room temperature. The microarrays were scanned on an Axon II Scanner with a multi-channel image generated which was subsequently analysed with Genepix software (Axon, USA).

Data Analysis - Calculations

For each array we determined the feature to background (F/B ratio) which is a simple quality metric for the entire microarray image based on 'signal to noise' principle (4,6,19) but calculated to represent the whole array as opposed to individual spots. To calculate the F/B ratio we selected the intensity values from the Genepix data file for each spot or 'Feature' (F) and corresponding local background

$$F/B = \frac{\sum_i (F_{532} + F_{635})}{\sum_i (B_{532} + B_{635})}$$

(B) for each of the Cy5 (635nm, red) and Cy3 (532nm, green) fluorescence channels detected. The F/B ratio for the entire array was calculated using the following formula….

…where i is each individual feature on the array.

The individual features on each array was also assessed for its signal intensity according to the following formula….

$$\text{Feature Intensity} = (F_{532} - B_{532}) + (F_{635} - B_{635})$$

The proportion of nucleotide coupled to each fluorescent dye moiety (N/D ratio) is established as a measure of cDNA labelling efficiency and was calculated as described in the TIGR protocol [17]. Similarly, the amount of cDNA and dye present in our final hybridization mixture was also calculated as previously described [17].

Results and Discussion

cDNA Microarray Image Quality Corresponds to F/B Ratio.

A practice undertaken by all who use cDNA microarray is the preliminary visual examination of the array image upon which broad assessment of the quality of the success of the hybridization and validity of gene expression data will be made. In this present study, we wished to evaluate the visual appearance of the entire microarray image and determine whether this was influence by downstream sample preparation quality assurance measures. To achieve this we used the F/B ratio, a broad scan of feature and background fluorescence intensity across the whole array, as a simple quantitative metric which is representative of the visual microarray image quality. It was chosen as it is computationally simple and accessible for all two-colour spotted cDNA microarray analysis software tools. We determined the F/B ratio for each of the 60 cDNA microarrays which was compared to the quality of the image generated for each of these experiments. Qualitative visual assessment of the microarray images was performed in a blinded fashion with the array images categorized into 4 group according to the criteria described in Table 1. The F/B ratio for each array was clearly correlated with the qualitative assessment of microarray image and is shown to steadily decrease with each category with the best microarray images having F/B ratios greater than 2.0 (Figure 1A).

The quality of an array experiment has also been determined by the number of features on the microarray which fluoresce at a level greater than specified lowest level cut-off which is often an arbitrary figure [6]. We determined the proportion of features in each microarray for which the signal intensity was negative once the intensity had been corrected for local background signal for which they would be

eliminated from further analysis [9]. Figure 1B demonstrates that our visual assessment of the microarray image quality corresponds to a greater number of features having measurable gene expression values that fluoresced at levels above background. The percentage of features was moderately inversely correlated to F/B ratio according to Pearson correlation analysis ($r = -0.503$, $p<0.01$) and thus the variability in the loss of readable spots on the array can be accounted for by the F/B ratio. This correlation was not affected by the removal of outlier data points. Our data demonstrates that there is a clear and logical relationship between the F/B ratio and microarray image quality. It is likely that the various image segmentation and fluorescence acquisition algorithms indicated above may create different scales of F/B ratios [8]. However, it is uncertain that these improved image analytical approaches will make the image look better upon visual assessment. The F/B ratio is not intended as a spatial, feature-by-feature or even pixel-by pixel image evaluation will only be useful when comparing image data generated using the same analysis tool.

cDNA Microarray Image Quality and RNA Purity and Integrity.

It is generally presumed that accurate gene expression data is dependent on the quality of the RNA isolated from the samples being examined as well as the efficiency of the cDNA synthesis, labelling and hybridization reactions. Despite this valid assumption, there has been scant experimental data to verify this assertion. Spectrophotometric analysis of RNA ($A_{260/280}$ ratio) in particular has been widely accepted as an important quality assurance measures for microarray experimentation [14] although, how the evaluation the purity of the RNA and the presence of contaminating genomic DNA and protein influences cDNA microarray gene expression profiles is not established. However, a high $A_{260/280}$ ratio (greater

than 1.8 to 2.0), whilst an indicator of sample purity, is not a guarantee for the intact nature of the RNA as determined by gel electrophoresis and the 28S:18S ratio [22].

In total, our data was generated from replicate microarrays of 29 cell line and bone marrow RNA preparations. To determine how microarray images are affected by the quality of the starting RNA, we binned our data into groups based on the nearest $A_{260/280}$ ratio for each of the samples and compared the F/B ratios of all microarray analyses with these samples. Figure 2A illustrates that RNA of lesser purity ($A_{260/280}$ ratio = 1.5-1.6) did yield quality images and that Group D microarrays, having a F/B ratio of ~1.0, actually came from experiments using more pure RNA ($A_{260/280}$ ratio = 1.8-2.0). Similarly, RNA integrity, as determined by 28S to 18S ratio, did not appear to influence F/B ratio (r = -0.160) nor associate with image quality groupings (Figure 2B). Our data demonstrates that the RNA purity and the 28S:18S ratio did not influence the final array image quality (Figure 2A,B). Whilst the quality of the feature and background signal on a cDNA microarray image is not influenced by starting RNA purity and integrity, this parameters should not however be ignored. Degrading RNA will influence the length of the cDNA transcript generated during reverse transcription [23], which may lead to ineffective hybridization, poor signal and ultimately, spurious gene expression values.

cDNA Microarray Image Quality and Labelling Efficiency.

How bright the fluorophore-labelled cDNA is prior to hybridization is also a recognised factor in cDNA microarray image quality assurance. The TIGR protocol used [17] states that for optimal hybridization 150 pmol of dye needs to be incorporated per sample at a ratio of less that 50 nucleotide/dye molecule, although as before, there is no definition as to how this value was experimentally deemed to be ‘optimal’. The instruction manuals from commercially available indirect labelling

kits state that when determining the amount of dye coupled to the cDNA set the criteria as being >40pmol of dye to >700pmol cDNA (Invitrogen)[24] or, at least 50 nucleotide per dye molecule in the case of the Stratagene Fairplay system [25]. In all these instances there is a shortage of detail in how these parameters have been interrogated with regards to determining the most favourable gene expression profile. The proportion of spots that were brighter than the background, or hybridization intensity, is one such measure of labelling efficiency [20,26].

As we have demonstrated that the proportion of measurable features is closely correlated to the F/B ratio (Figure 1) we compared the F/B ratio to the cDNA labelling efficiency calculated by the N/D ratio. There is a clear relationship between F/B ratio and a nucleotide to dye ratio (N/D) of less than 100 (Figure 3A). N/D ratio greater than 100 uniformly gave low F/B ratio values. This was confirmed when we compared data based on quality assessment of the images (Figure 3B) with the poorest images (Group D) having an average combined N/D ratio of 100. When the N/D ratio for each Cy dye fluorophore were examined separately we discovered that the Cy5 dye was most prone to inefficient indirect labelling and that this was associated with poor image quality (Figure 3B, group D). It is pertinent to note however, that well labelled cDNA probes having an N/D ratio less than 50, and even as low as 20 nucleotides per dye molecule, also resulted in F/B ratios of less than 1.5 which is the average F/B ratio for group C results. The lack of linear relationship between cDNA labelling efficiency and visual image quality was confirmed with there being no strong correlation between N/D ratio and F/B ratio ($r = -0.263$). Our finding that a low N/D ratio (<50) was not always associated with microarray images with high F/B ratio (Figure 3A) suggests that other factors apart from labelling efficiency must be considered in cDNA microarray quality assurance measures.

It has been considered [10] that adding more cDNA to the hybridization mixture will produce greater consistency to microarray data. It is intriguing that commercial kits do not specify an upper limit for the optimal amount of cDNA to be added during hybridization. Hence, we next examined the total amount (pmol) of cDNA and Cy dye which were added to each hybridization mixture. Figure 4A illustrates that adding more cDNA to the microarray slide during hybridization did not yield better microarray images. Rather, the poorest quality images (group D) were associated with increased levels of cDNA (data not shown).

This raises the prospect that cDNA hybridization preparations can be overloaded and that this can be a source of confounding. As the Cy5 dye is known to quench preferentially to the Cy3 dye, there is a temptation to balance the hybridization mixture based such that the amount of fluorescence from the two fluors is equal. However, a more pronounced affect on image quality was observed when we examined the ratio of the total Cy5 to Cy3 labelled cDNA in the hybridization mixture with F/B ratio. The poorest group of microarray images (Group D) had on average twice as much Cy5 labelled cDNA than Cy3, whilst the other quality assessment group each averaged 1.0 (Figure 4B) and despite the level of amount (pmol) for each of the fluorescent dyes being balanced in all cases (Figure 4C).

Conclusion

The methodological goal of all cDNA spotted glass slide microarray experiments is to generate a high specificity of spot hybridization with a low or minimal background. The fluorescent signal from the cDNA hybridized to the microarray generates a 16 bit multichannel TIFF image that is initially examined visually for the quality of the feature signals, the level of background fluorescence and presence of artefacts. The image is analysed computationally by specialist software that incorporates

segmentation algorithms [2,3,7,19] and background correction strategies [5,7] which determine the intensity of the emission peaks for the two fluorophores which are being generated by each individual pixel of the image, with each pixel being identified as part of a gene feature or as local background. An active area of biostatistics research is the development of image analysis approaches which improve the precision of the gene expression data extracted from cDNA microarray experiments through improved gridding of array images [7], intensity-based or spatial signal-background image segmentation [2,19], Bayesian approaches [21], pixel-by-pixel feature assessment [9] and use of 'signal to noise' measurement as quantitative control assessment of spot quality [4,6,19,20]. Whilst the value of such development can not be underestimated, the absence of consensus as to the 'best' approach, as well as the complexity of the statistics involved, means that the application of these image analysis applications are not common place. However, despite this, this study provides insight into the source of variation in microarray image analysis introduced during sample preparation and will assist in the standardisation of cDNA glass slide microarray protocols.

Acknowledgements

This research has been financially supported by funds raised through the philanthropic work of Kayaking for Kemo Kids for which we owe our gratitude. This research was completed by DRC in his capacity as Research Fellow of the University of Sydney, NSW, Australia.

References

[1] Duggan DJ, Bittner M, Chen Y, Meltzer P, Trent JM: **Expression profiling using cDNA microarrays.** *Nature Genetics* 1999, **supp 21**:10-14

[2] Nagarajan R: **Intensity-based segmentation of microarray images.** *IEEE Transactions on Medical Imaging* 2003, **22**:882-889

[3] Chen Y, Dougherty ER, Bittner ML: **Ratio-based decisions and the quantitative analysis of cDNA microarray images.** *J Biomedical Optics* 1997, **2**:364-374

[4] Chen Y, Kamat V, Dougherty ER, Bittner ML, Meltzer PS, Trent JM: **Ratio statistics of gene expression levels and applications to microarray data analysis.** *Bioinformatics* 2002, **18**:1207-1215

[5] Kim JH, Shin DM, Lee YS: **Effect of local background intensities in the normalization of cDNA microarray data with a skewed expression profile.** *Experimental and Molecular Medicine* 2002, **34**:224-232

[6] Yu J, Othman MI, Farjo R, Zareparsi S, MacNee SP, Yoshida S, Swaroop A: **Evaluation and optimization of procedures for target labelling and hybridization of cDNA microarrays.** *Molecular Vision* 2002, **8**:130-137

[7] Yang YM, Buckley MJ, Speed TP: **Analysis of cDNA microarray images.** *Briefing in Bioinformatics* 2001, **2**:341-349

[8] Ahmed AA, Vias M, Iyer NG, Caldas C, Brenton JD: **Microarray segmentation methods significantly influence data precision.** *Nucleic Acid Research* 2004, **32**:e50

[9] Brown CS, Goodwin PC, Sorger PK: **Image metrics in the statistical analysis of DNA microarray data.** *Proceeding of the National Acadamy of Science* 2001, 98:8944-8949

[10] Franssen-van Hal NLW, Vorst O, Kramer E, Hall RD, Keijer J: **Factors influencing cDNA microarray hybridization on silylated glass slides.** *Analytical Biochemistry* 2002, **308**:5-17

[11] Diehl F, Grahlmann S, Beier M, Hoheisel JD: **Manufacturing DNA microarrays of high spot homogeneity and reduced background signal.** *Nucleic Acid Research* 2001, **29**:e38

[12] Rickman DS, Herbert CJ , Aggerbeck LP: **Optimizing spotting solutions for increased reproducibility of cDNA microarrays.** *Nucleic Acid Research* 2003, **18**:e109

[13] Dolan PL, Wu Y, Ista LK, Metzenberg RL, Nelson MA, Lopez GP: **Robust and efficient synthetic method for forming DNA microarrays.** *Nucleic Acid Research* 2001, **29**:e107

[14] Baelde HJ, Cleton-Jansen A-M, van Beerendock H, Namba M, *et al*: **High quality RNA isolation from tumours with low cellularity and high extracellular matrix component for cDNA microarrays: Application to chondrosarcoma.** *Journal of Clinical Pathology* 2001, **54**:778-782

[15] Richter A, Schwagger C, Hentze S, Ansorge W, Hentze MW, Muckenthaler M: **Comparison of fluorescent tag DNA labelling methods used for expression analysis by cDNA microarrays.** *Biotechniques* 2002, **33**:620-628

[16] Xiang CC, Kozhich OA, Chen M, Inman JM, Phan QN, Chen Y, Brownstein MJ: **Amine-modified random primers to label probes for DNA microarrays.** *Nature Biotechnology* 2002, **20**:738-742

[17] Hegde P, Qi R, Abernathy K, Gay C, Dharap S, Gaspard R, Earle-Hughes J, Snesrud E, Lee N, Quackenbush J: **A concise guide to cDNA microarray analysis.** *Biotechniques* 2000, **29**:548-562

[18] Barbaric D, Dalla-Pozza L, Byrne JA: **A reliable method for total RNA extraction from frozen human bone marrow samples taken at diagnosis of acute leukaemia.** *J Clinical Pathology* 2002, **55**:65-867

[19] Wang X, Ghosh S, Guo S-W: **Quantitative quality control in microarray image processing and data acquisition.** *Nucleic Acid Research* 2001, **29**:e75

[20] Manduchi E, Scearce LM, Brestelli JE, Grant GR, Kaestner KH, Stoeckert CJ: **Comparison of different labelling methods for two-channel high-density microarray experiments.** *Physiological Genomics* 2002, **10**:169-179

[21] Kooperberg C, Fazzio TG, Delrow JJ, Tsukiyama T: **Improved background correction for spotted DNA microarrays.** *J Computational Biology* 2002, **9**:55-66

[22] Guo D, Catchpoole D: **Isolation of intact RNA following cryosection of archived frozen tissue.** *Biotechniques* 2003, **34**:48-50

[23] Wildsmith SE, Elcock FJ: **Microarrays under the microscope.** *Journal of Clinical Pathology: Molecular Pathology* 2001, **54**:8-16

[24] Invitrogen Superscript Indirect Labelling System Catalog Nos L1014-01 and L1014-02 Version B, May 2003

[25] **Fair Play® Microarray Labelling Kit Instruction Manual,** [www.stratagene.com]

[26] 't Hoen PAC, de Kort F, van Ommen GJ, den Dunnen JT: **Fluorescent labelling of cRNA for microarray applications.** *Nucleic Acid Research* 2003, **31**:e20

Table 1 Criteria for the qualitative assessment of cDNA microarray images.

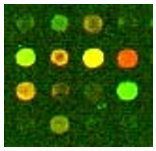
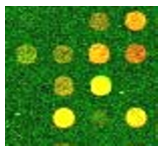
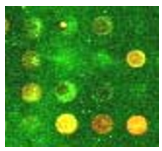
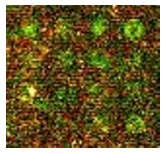
Category	Characteristic	Quality Assessment	Number	Example
A	bright spot with minimal background	complete reliable data	22	
B	bright spot and limited background	reliable data	20	
C	faint spot, high background or presence of minor artefacts	partial reliable data	13	
D	failed hybridization, excessive artefact	no reliable data	5	

Figure Legends

Figure 1 – F/B ratio corresponds to cDNA microarray image quality. *A*. Comparison of the average F/B ratio (*y-axis*) for each image quality category, described in Table 1. *B*. The average proportion expressed as a % (*y-axis*), of microarray features with in each array which were deemed undetectable due to having a negative signal intensity compared to local background intensity for each image quality category. The standard error is indicated by the error bars for each column.

Figure 2 – Evaluation of the influence of RNA quality on cDNA microarray images. *A*. Spectrophotometric assessment ($A_{260/280}$) of RNA purity compared to the F/B ratio. The mean F/B ratio for each bin is represented by the bars. *B*. RNA integrity as determined by the density of the 18S and 28S ribosomal bands is compared to the image quality category. The standard error is indicated by the error bars for each column.

Figure 3 – The effect of cDNA labelling efficiency on cDNA microarray image attributes. *A*. cDNA labelling efficiency, as determined by the average N/D ratio, for both Cy5 and Cy3 labelled cDNA preparations for each microarray experiment is directly compared to the F/B ratio. *B*. The labelling efficiency for both the Cy5 and Cy3 labelled cDNA preparations as well as the average (*combined*) N/D ratio is shown for each quality assessment category. The standard error is indicated by the error bars for each column.

Figure 4 – Analysis of the absolute amount of cDNA and fluorescent dye in the microarray hybridization mixture and its effect on image quality. *A*. Total cDNA (pmol) from the combined Cy5 and Cy3 labelled cDNA preparations for each microarray experiment is directly compared to the F/B ratio. *B*. The ratio of total cDNA (pmol) from the Cy5 and Cy3 labelled cDNA preparations is shown for each quality assessment category. *C*. The average amount of Cy5 and Cy3 fluorescent dye (pmol) in each hybridization in each quality assessment category. The standard error is indicated by the error bars for each column.

Figure 1

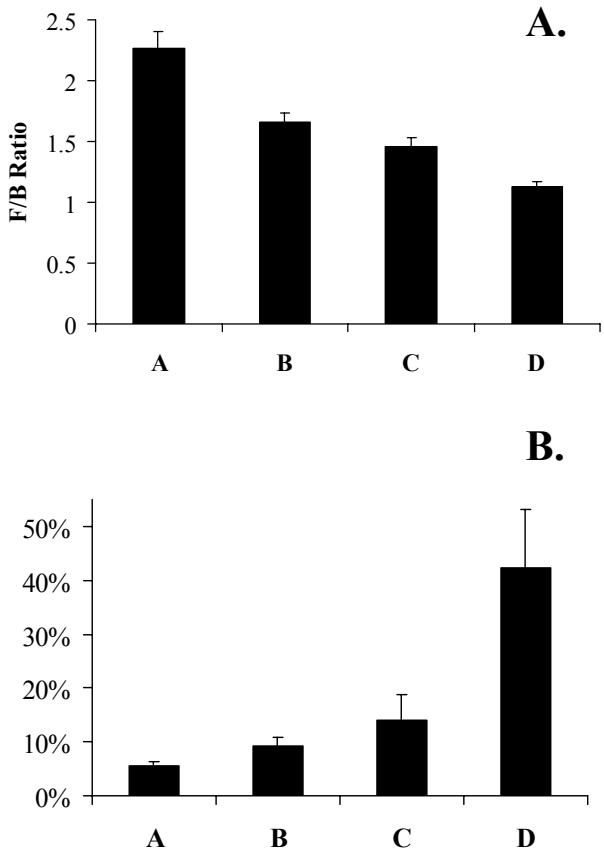
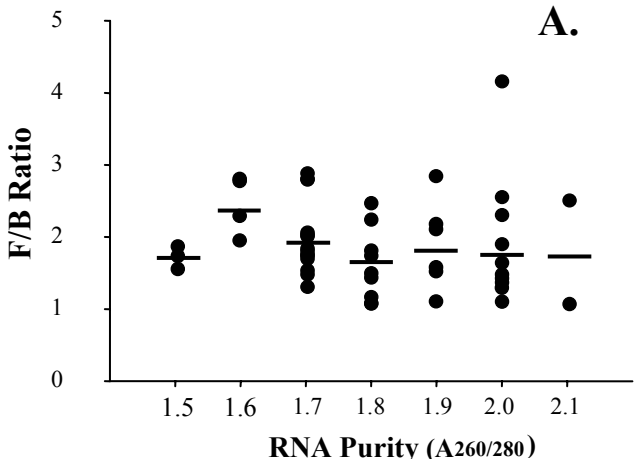


Figure 2


A.
F/B Ratio
5
4
3
2
1
0
1.5
1.6
1.7
1.8
1.9
2.0
2.1
RNA Purity (A260/280)

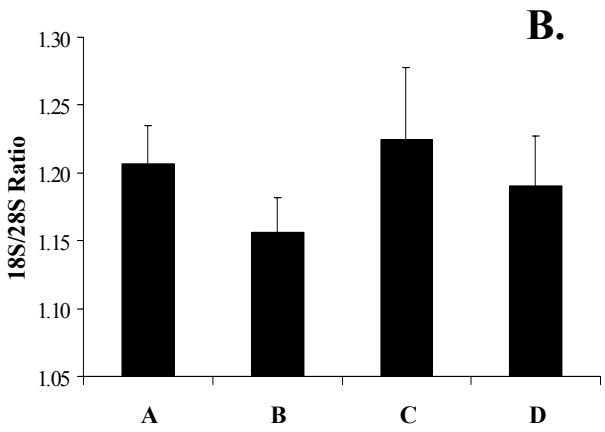
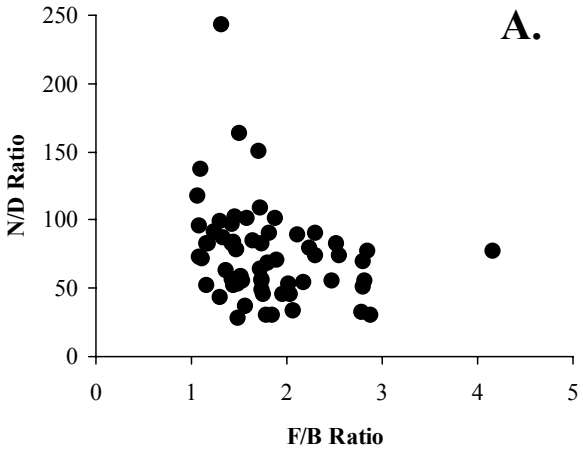

B.
18S/28S Ratio
1.30
1.25
1.20
1.15
1.10
1.05
A
B
C
D

Figure 3


A.
250
200
150
100
50
0
N/D Ratio
0
1
2
3
4
5
F/B Ratio

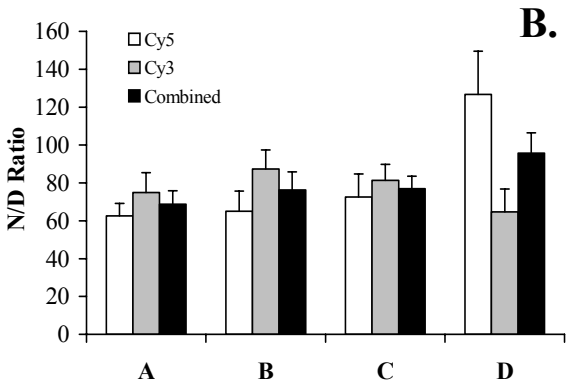
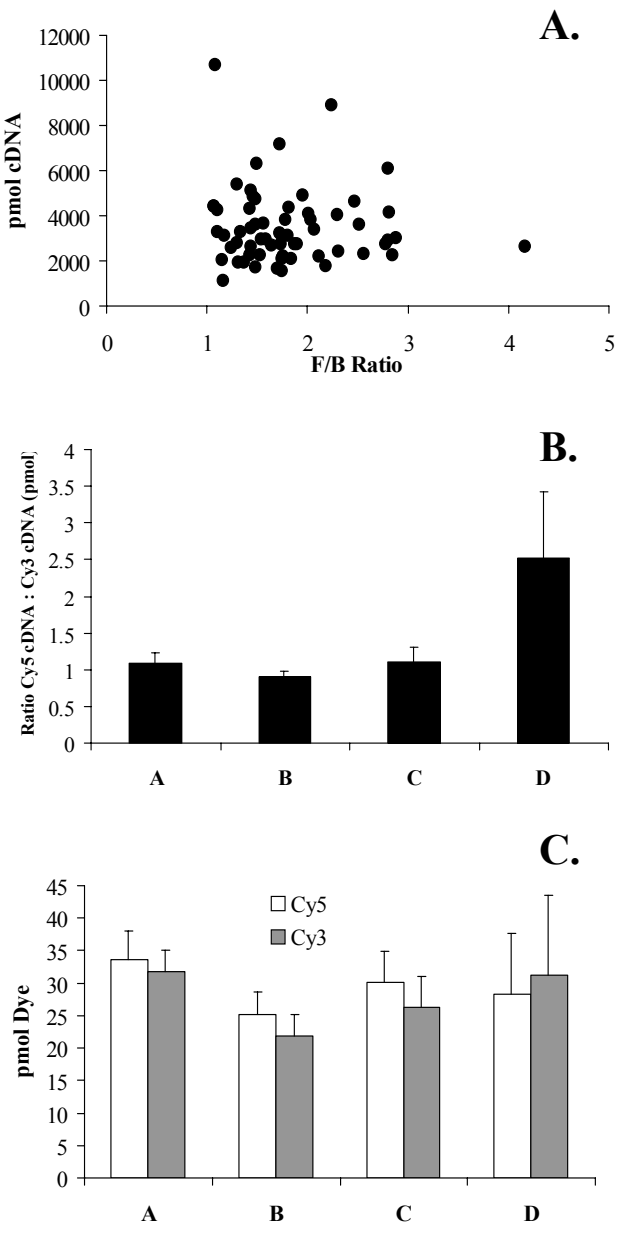

B.
160
140
120
100
80
60
40
20
0
N/D Ratio
Cy5
Cy3
Combined
A
B
C
D

Figure 4


A.
pmol cDNA
12000
10000
8000
6000
4000
2000
0
0
1
2
3
4
5
F/B Ratio
B.
Ratio Cy5 cDNA : Cy3 cDNA (pmol)
4
3.5
3
2.5
2
1.5
1
0.5
0
A
B
C
D
C.
pmol Dye
45
40
35
30
25
20
15
10
5
0
Cy5
Cy3
A
B
C
D