



Microarray data generation – optimized reagents and protocols for optimal results

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Abstract

The increasingly utilized technology of DNA microarray analysis is a powerful tool, giving biologists the potential to monitor an entire transcriptome and assess the expression of thousands of genes simultaneously. To generate high quality, interpretable data, great care must be taken in several distinct but interrelated procedures. In the present study, we describe how the Pronto![™] Plus Systems provide versatile, end-to-end solutions for self-printing microarrays to achieve highly reproducible and robust array results. Our data were generated using microarrays printed onto UltraGAPS[™] slides to assess parameters of target labeling and microarray hybridization. For all analyses, labeled cDNA target was generated from 5 µg of total RNA template using the ChipShot[™] Labeling System by direct incorporation of Cy@ dye-labeled nucleotides. Deviation from the labeling protocol by varying the concentration of either unlabeled or Cy@-labeled deoxynucleotides dramatically affected both the total yield of labeled cDNA and the robustness of hybridization signal. We also titrated the molar amount of Cy@-labeled deoxynucleotides incorporated into the target cDNA that was hybridized to arrays. We determined that an optimal range exists where resource usage (sample RNA, Cy@-labeled nucleotides) is balanced with the extent (signal-to-noise and number of features detected above background) and reproducibility (as measured by %CV) of data generation.

Together, our observations demonstrate that the Pronto![™] Plus Systems provide a framework for achieving superior, reproducible signal detection for more reliable data interpretation during a microarray experiment.

5µg total RNA	ng yield	pmol	FOI	FOI:Yield ratio
Cy@3	1200-2400	100-170	20-35	1.25%-2.70%
Cy@5	900-2400	45-120	12-25	0.60%-1.50%
1.5µg mRNA	ng yield	pmol	FOI	FOI:Yield ratio
Cy@3	350-650	40-75	25-45	5.50%-8.50%
Cy@5	325-650	20-50	15-35	4.00%-6.00%

Table 1. Metrics for assessing cDNA labeling quality

Following a cDNA fluorescence incorporation reaction, absorbance readings should be taken using undiluted cDNA. As evidenced by the figures in this poster, best results from microarray hybridizations are obtained with cDNAs that fall into the above ranges.

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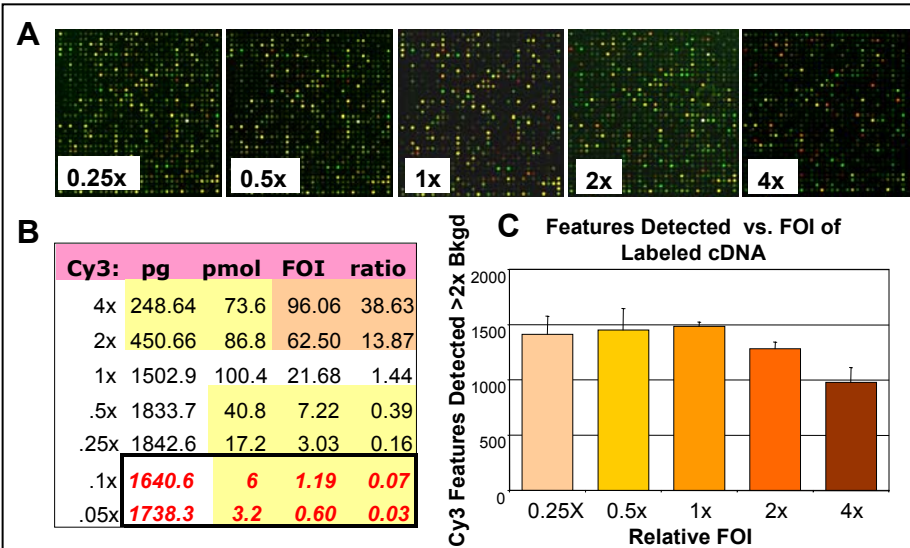


Figure 1. Titrating FOI of hybridized cDNA - More isn't Better

By altering the ChipShot™ Labeling System protocol, labeled cDNA was generated from total RNA that had varying frequencies of incorporation (FOIs) of Cy@3 dye.

A) Equivalent amounts of each labeled cDNA (40 pmol per full array) were hybridized to custom 4K human cDNA arrays. “1x” represents the FOI of 21.68 that falls within the recommended amount to be hybridized (see Table 1).

B) Concentrations of both unlabeled dNTPs and Cy@3-labeled dCTP were adjusted to increase or decrease FOIs outside the normal yields. Yellow and tan fields denote yields below and above, respectively, anticipated results. Italicized red numbers show cDNAs that were not hybridized due to the very low yield of incorporated Cy@3 dye.

C) Features detected >2x background from the 4K arrays were plotted. Low FOI cDNA shows a similar level of detection as the 1x amount, but much more volume is required for hybridization. High FOI cDNA shows a reduction in features detected, through either quenching or duplex destabilization.

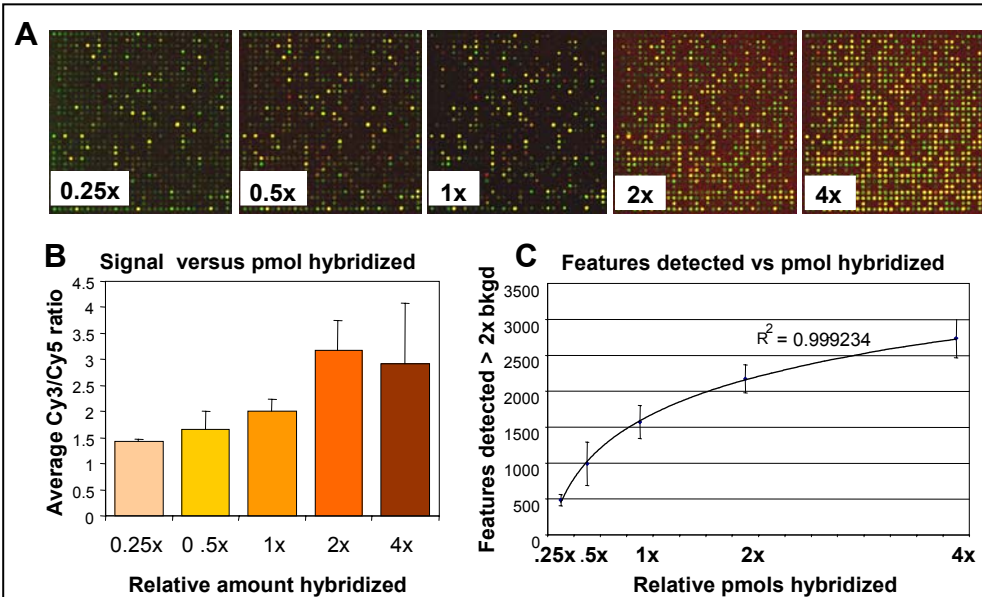


Figure 2. Titrating pmol of hybridized cDNA - More isn't Better II

For total RNA substrate, the Pronto!™ Plus protocol recommends using 40 pmol (“1X”) of incorporated Cy@dCTP for labeled cDNA hybridization onto one full array.

A) Using custom 4K human cDNA arrays, the amount of labeled cDNA hybridized (both Cy@3 and Cy@5) was titrated (**A**, identical exposures) and the overall Cy@3/Cy@5 ratio (**B**) and number of genes detected above 2x background (**C**) were plotted.

B) Data in **B** reveal that, at higher concentrations, ratios are biased toward the Cy@3 channel, thereby altering expression data interpretation. Moreover, error bars ($n=4$ slides) reveal higher variability with overloaded hybridizations.

C) The graph in **C** demonstrates that reducing the labeled cDNA hybridized to less than half of the recommended amount leads to a steep reduction in the number of genes detectable above 2x background. Since these custom arrays only have 3,300 DNA spots after the controls are removed, increasing labeled cDNA increases the risk of false-positive scoring of data.

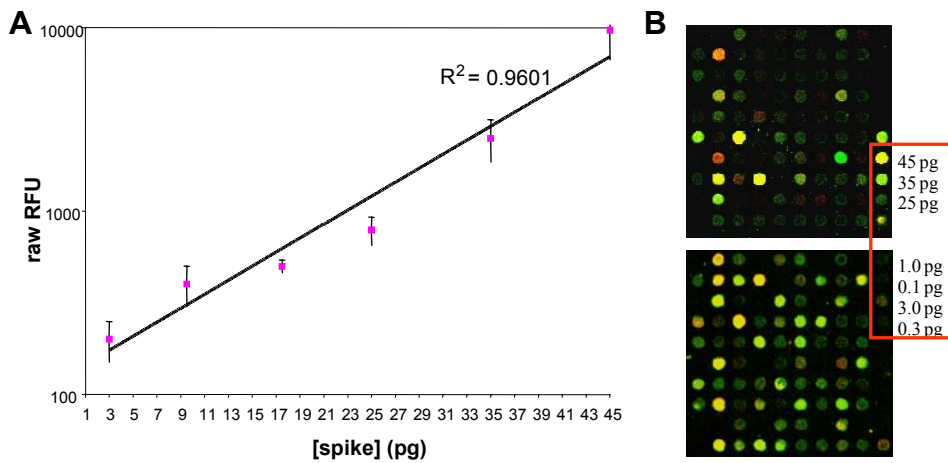


Fig 3. Sensitivity using Pronto!™ Plus Systems

Known pg quantities (indicated in the graph in **A**) of *in vitro*-transcribed, polyadenylated bacterial control RNAs were spiked into 5 μ g total RNA labeling reactions following the ChipShot™ labeling system protocol. Labeled cDNAs were purified and hybridized to custom 4K human cDNA arrays printed on UltraGAPST™ slides. Signal from 3 pg of spiked RNA in the 5 μ g labeling reaction was reproducibly detected above 2x background (see graph in **A** and representative array data in **B**). Depending on cell type and growth state, this level of sensitivity could correspond to a detection limit of ~2-10 copies per cell in 5×10^5 cells. Detection of fold changes is another measure of sensitivity important to microarrays. The graph in **A** demonstrates that, at a level of 25 pg or higher, 1.5-fold changes in expression are reproducibly detectable. At lower expression levels, RFU signals become too compressed for accurate differentiation.

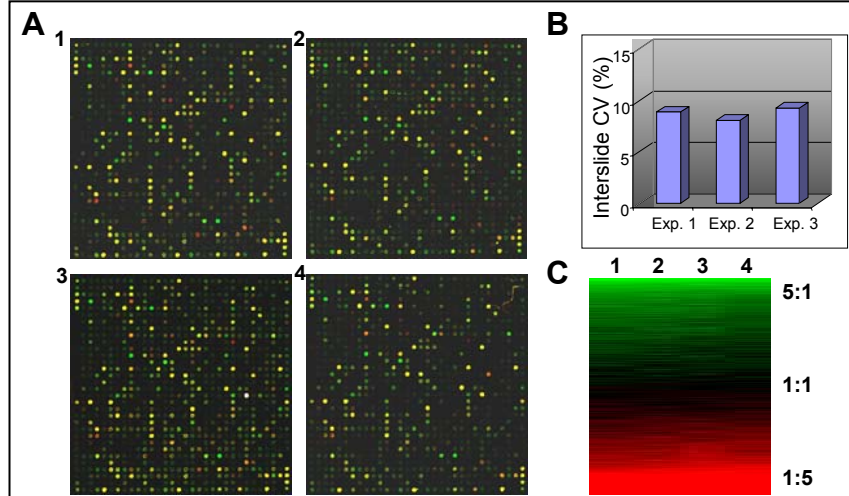


Fig 4. The goal: superior reproducibility

The combination of the Pronto!™ Plus reagents and protocols with UltraGAPST™ slides yields highly reproducible data both within and between experiments. **A** shows four representative arrays using cDNA that was labeled using the ChipShot portion of the Pronto!™ Plus System and total RNA generated from the SV Total RNA Isolation portion. Reproducibility was quantitated by measuring % CV (Std. Dev./Mean) for $n=3$ to 4 arrays, for three independent experiments (**B**). Moreover, differential gene expression patterns were found to be very consistent between arrays (**C**). For **C**, expression data (ratios of normalized Cy@3/Cy@5) were inputted into the Cluster and Treeview programs (Michael Eisen laboratory) for analysis.

Summary

- Protocols in the Pronto!™ Plus Systems have been optimized to generate the most meaningful and reproducible data possible.
- Pronto!™ Plus Systems, in conjunction with UltraGAPST™ slides, provide microarrays with end-to-end solutions to their experimental needs
 - Increased sensitivity
 - Reproducibility and consistency
 - Convenience
- Optimized protocols give users instant technical expertise