

internal positive spike-in controls present in every reaction (Geiss *et al*, 2008). Mathematical calculations were performed using Microsoft Excel.

Results

An attenuation strategy based on a competitive inhibition was developed to reduce the overall image density without sacrificing the quantification of low-expressing genes. This simple strategy involves including excess inactive probe for the target(s) of interest to the hybridization reaction. For example, the standard hybridization reaction contains 25 pM of each reporter probe. To attenuate the counting of a gene 90%; that is, to 10% of endogenous expression levels, it is necessary that 90% of the total probe in the hybridization mixture is inactive probe. Therefore, we add 225pM of inactive probe to the 25pM of “active” to bring the total concentration to 250pM. Higher or lower attenuation levels can be achieved by making similar adjustments to the ratios of “active” to “inactive” probes. The effective amount of attenuation is calculated by comparing the number of counts obtained with an unattenuated sample against that obtained in an attenuated sample. This comparison gives an “attenuation factor” that can be used to convert the number of counts in the attenuated sample to the equivalent unattenuated counts, making direct comparisons of gene counts in the multiplexed sample possible. It is important

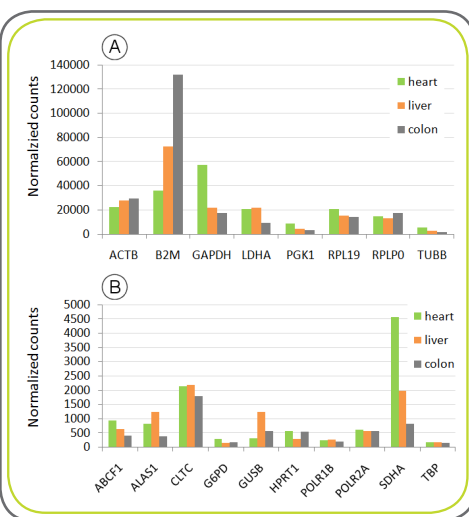


Figure 2. Expression profiles of differentially expressed genes in various tissue types. (A) Expression level profiles of eight highly expressed housekeeping genes from heart, liver, and colon tissues. (B): Expression level profiles of medium-low-expressed genes from heart, liver, and colon tissues.

to use the same amount of RNA in both the attenuated and unattenuated assay to calculate the normalization factor accurately. Furthermore, the count comparison must be performed under conditions where the amount of RNA used does not exceed the optimal counts.

Differential nCounter™ Gene Expression Tissue Profiles

The basal levels of expression of the housekeeping genes in various tissues as analyzed using the nCounter™ Analysis System are shown in Figure 2. Genes were grouped based on expression levels, into high or medium-low transcript levels, to illustrate the typical range of counts obtained using RNA from different tissues. As expected, expression levels vary significantly, not only between individual genes but also between tissue types for an individual gene.

Attenuation Control is Specific

To illustrate the effect of attenuation we compared the counts obtained for all 18 genes in attenuated versus unattenuated samples when only the 8 high-expressing genes were attenuated by 90% (Figure 3A). In the attenuated assays the counts for each of these genes were reduced 7 to 17% of unattenuated counts. The unattenuated B2M measurement was 131,862 normalized counts; this was greater than the Y-axis graph scale. Inclusion of attenuating probes for high-expressing genes in the hybridization had no effect on counts of the medium and low-expressing genes (Figure 3B). Thus, this strategy only affects the attenuated genes, and does not affect counts for unrelated genes that are queried in the same multiplexed hybridization.

Attenuation Control can be Fine-Tuned

To illustrate the ability to control the amount of attenuation, we attenuated the high-expressing housekeeping genes to varying degrees (Figure 4). By adjusting the “active” to “inactive” probe ratio, we were able to decrease the counts to approximately 25, 50 and 75% of unattenuated values. The slope of the linear regression on the graph reflects the degree of attenuation: 25% attenuation (75% normal expression) produces a slope of 0.758; 50% attenuation produces a slope of 0.534, and 75% attenuation (25% normal expression) produces a slope of 0.230. The correlation coefficient between unattenuated

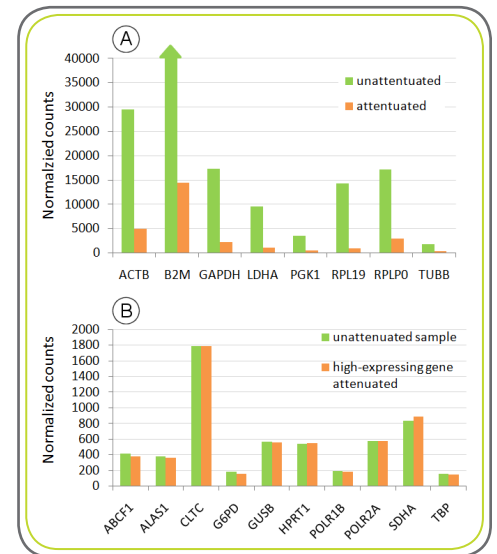


Figure 3. Gene counts attenuated by competitive inhibition. (A) The eight high expressing housekeeping genes described above were all equally attenuated to 10% normal expression level (90% attenuated). (B) The twelve medium-low expressing genes in the same hybridization were unaffected by attenuation of the high-expressing genes.

and attenuated counts was greater than 99%. As was seen above, including attenuating probes for high-expressing genes had no effect on the counting of low- and medium-expressing genes, since the slopes and correlations are unaffected.

Similarly, we were able to differentially attenuate two high expressing genes in the same hybridization. For example, the target B2M was attenuated to 1% of its original level (99% attenuation, actual measured was 99.6%), while the GAPDH gene was attenuated to 25% (33.4% actual measured) in a single tube (Figure 5). When these two genes are attenuated, there is no effect on the counts for the other, unattenuated genes. The counts for the ten medium-low expressing genes in the CodeSet were also unaffected by the attenuation (data not shown).

Once the degree of attenuation is known, a factor can be used to adjust the measured, attenuated counts to non-attenuated count equivalents (Table 1). The attenuation factor is calculated as the ratio of unattenuated to attenuated. For the example shown above (Figure 5), the B2M gene was attenuated 99% (actual 99.6%, giving a attenuation factor of 260.4) and the GAPDH gene was attenuated 25% (actual 33.4%; attenuation factor 1.5). To calculate the corrected counts in this experiment, the attenuated counts measured were multiplied by the attenuation factor. Once this factor is calculated, it

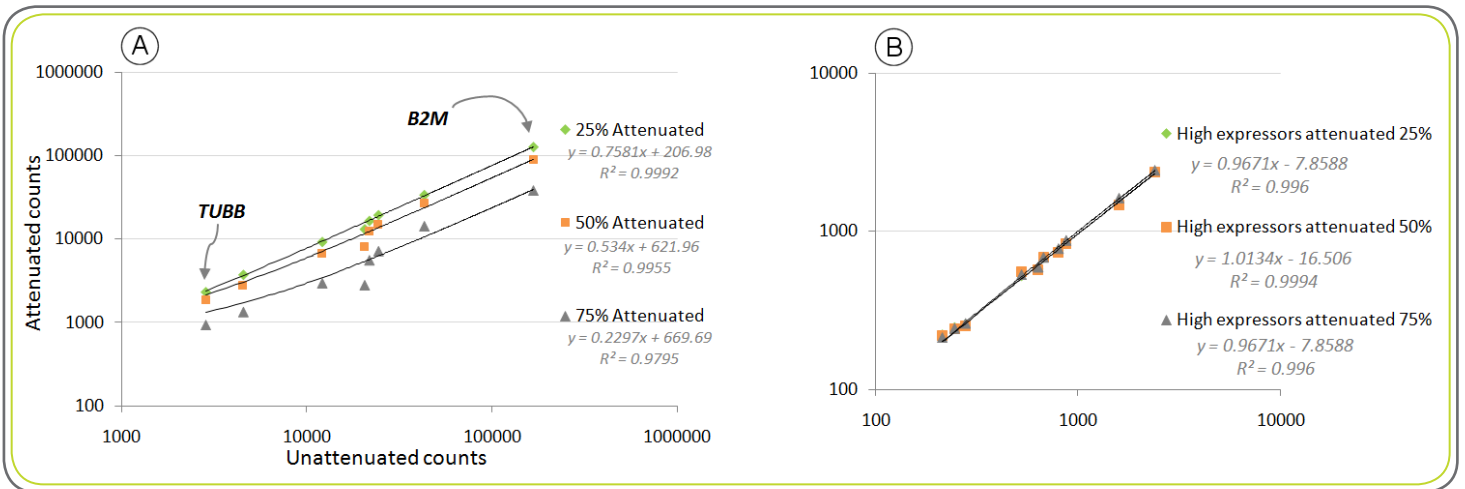


Figure 4. Fine-Tuned Attenuation Retains Target Gene Specificity. The eight high expressor housekeeping genes were attenuated by increasing degrees (25%, 50%, and 75%). (A) Unattenuated counts were plotted against the attenuated counts for the same genes. (B) Similar analysis of the medium-low expressing genes in the same hybridization as (A).

will remain constant in other experiments, as long as the same amount of “inactive” probes are included in the hybridization.

Discussion

The nCounter™ Analysis System provides accurate expression profiles of targets that are present across a wide range of levels in a single multiplexed reaction. It is occasionally necessary to analyze samples containing a few genes expressed at very high levels, or a large number of genes at moderately high levels. In extreme situations, the imaging surface can become saturated and the collection of data affected, especially for low-expressing genes.

Nanostring Technologies has developed a simple competitive inhibition strategy to overcome this issue. The approach is to add inactive probes that will compete out the labeled reporter probes that may saturate the flow cell imaging surface. Following a simple titration approach, it is possible to determine the level of attenuation

needed. Moreover, we have shown that the attenuation of single or group of genes does not interfere with the results obtained for the remainder of the genes interrogated by the particular CodeSet.

Attenuation is required when previous knowledge suggests that some of the genes studied with a particular CodeSet are very highly expressed. The attenuation procedure is also very useful in situations where final expression levels may be unknown or require further quantitative validation as is the case with whole genome microarrays and next generation sequencing (NGS) methods.

The attenuation technique coupled with the

intrinsic sensitivity and specificity of the platform makes the nCounter™ Analysis System the ideal choice for multiplex expression profiling experiments regardless of the levels at which your genes of interest are expressed.

How do I know if attenuation is required in my experiment?

In most cases the choice of genes to be included in your CodeSet will not be affected by their expression levels. However, it is important to alert NanoString during the design phase of your project if you suspect there might be genes expressed

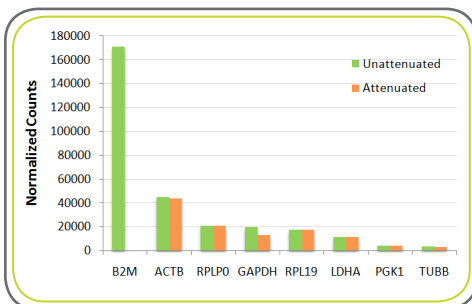


Figure 5. Attenuation can be differentially controlled in the same hybridization.

The CodeSet of eighteen housekeeping genes was hybridized to colon RNA, and B2M and GAPDH were attenuated 99% and 25%, respectively, in one hybridization.

Table 1: Counts can be corrected using an attenuation factor.

	Unattenuated Counts	Attenuated Counts	Attenuation Factor	Corrected Counts
B2M	170,809.2	656.0	260.3	170,809.1
ACTB	45,224.3	43,975.7		
RPLP0	20,919.9	20,741.7		
GAPDH	19,969.0	13,299.4	1.5	19,968.9
RPL19	17,688.7	17,803.0		
LDHA	11,699.5	11,232.3		
PGK1	4,354.6	4,343.5		
TUBB	3,387.4	3,224.7		
CLTC	2,320.1	2,261.5		
SDHA	1,730.8	1,660.4		
GUSB	867.3	785.6		
POLR2A	894.2	872.4		
HPRT1	658.5	631.8		
ABCF1	616.4	622.6		
EEF1A1	545.7	526.9		
ALAS1	579.5	585.0		
PPIA	327.6	328.0		
G6PD	323.3	320.9		
POLR1B	246.8	242.2		
TBP	212.9	207.4		

at extremely high levels in your samples. This will ensure that Nanostring is aware of the possible need to attenuate genes, and can supply the necessary advice or reagents with your CodeSet. If you have any questions or concerns, you can always contact your Field Application Scientist or Nanostring directly.

Purchasing Information

Product Description	Catalog No.
nCounter™ Analysis System Includes the Prep Station and the Digital Analyzer	NCT-SYST
nCounter™ GX CodeSet Gene Expression Custom CodeSet	GXA-P1CS
nCounter™ Human Reference GX CodeSet Gene Expression CodeSet profiling 18 endogenous reference genes	GXA-P1HR
nCounter™ Master Kit All reagents, sample cartridges, and consumables necessary for processing 48 Nucleic Acid Assays.	NAA-AKIT

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