



nCounter® Gene Expression

Tech Note

Strategies for Successful Gene Expression Assays

Introduction

Biological samples exhibit a wide range of gene expression, and designing experiments that accurately measure both high- and low-expressing genes within a study can be challenging for even the most experienced scientist. In all multiplexed gene expression experiments, the very high expression of some genes can sometimes interfere with the ability to detect others. Diverse sample types complicate the effort to understand and apply strategies that work successfully for many assays and produce the best results.

The nCounter® platform is extremely robust and has been used successfully in many challenging scenarios. Results using purified total RNA, cell lysates, and fragmented RNA from formalin-fixed, paraffin-embedded (FFPE) tissue are highly correlated. NanoString provides several pre-made gene expression panels that examine up to 770 genes at once and custom CodeSets for up to 800 targets. This Tech Note provides opportunities to improve experimental design when studying large collections of genes or when working with certain sample types. Also provided is a simple attenuation technique that enables accurate counting of large sets of genes even when there are highly expressed targets within the sample. NanoString suggests considering these specific strategies outlined before starting your experiment.

Data Collection with the nCounter Analysis System

NanoString's nCounter technology delivers digital, multiplexed measurements of gene expression, providing simultaneous counts of the abundance of hundreds of mRNA transcripts following solution-based hybridization with target-specific probes (Geiss *et al.*, 2008). Two probes are used to detect each transcript: a reporter probe that carries the fluorescent barcode, and a biotinylated capture probe that immobilizes the complex for data collection. Up to 800 unique probe pairs, each pair specific to a particular nucleic acid target, are combined with internal controls to form a CodeSet.

After hybridization of the CodeSet with target nucleic acids, samples are transferred to the Prep Station, which contains a fluidic processing system that removes excess probes, unbound targets, and other extraneous material (FIGURE 1). Purified probe:target complexes are deposited onto a streptavidin-coated imaging surface and immobilized *via* the biotinylated capture probe. Immobilized reporters are then aligned, stretched, and immobilized again at the other end of each complex in order to create parallel fluorescent barcodes that can be imaged. An automated fluorescence microscope in the Digital Analyzer scans the cartridge, and the ordered fluorescent segments on the attached reporter probe identify each target molecule of interest.

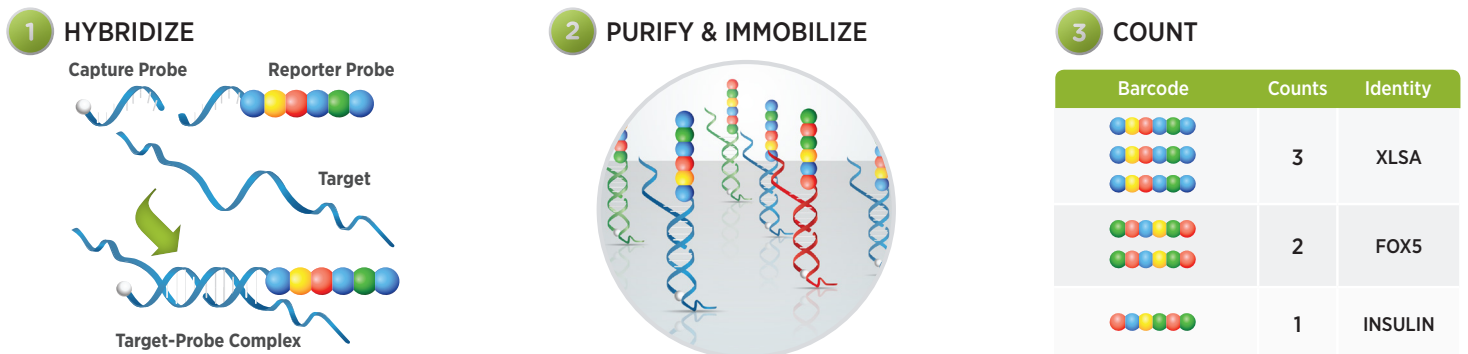


FIGURE 1: NanoString's nCounter platform. (1) Solution phase hybridization forms complexes between nucleic acids and target-specific capture and reporter probes. (2) Excess probes are removed using magnetic bead separation, then the remaining complexes are immobilized and aligned. (3) A fluorescent imaging system is used to scan the cartridge surface, providing highly accurate digital counts of nucleic acids corresponding to each colored barcode.

Molecules That Count®

Saturation of the imaging surface, in which case the image analysis system becomes unable to discriminate overlapping probe:target complexes, is rare but can become more likely when working with certain sample types or large CodeSets. Typical causes are excessive input material or highly expressed genes (an excess of 1 million total counts). As a consequence of saturation, data can be more difficult or impossible to analyze. This Tech Note is intended to provide greater understanding of how to identify and address such concerns in experimental designs more prone to saturation.

Sample Type Considerations

Sample input recommendations for nCounter assays were developed using purified total RNA from a variety of tissues, of which mRNA typically composes 5-10% (5-10 ng in a 100 ng sample no larger than 5 μ L). Many other sample types provide high-quality results with minor adjustments to sample volume or concentration as outlined below. Please consult with your Field Applications Scientist or contact NanoString Support at support@nanosttring.com if you have any questions about how to ensure the best results from your experiment.

Formalin-fixed, paraffin-embedded (FFPE)-derived samples have been shown to provide high-quality results due to NanoString's enzyme-free nCounter chemistry*, and mRNA degradation does not typically affect data quality since probes recognize a relatively short 100-base target region. NanoString recommends increasing the sample input by up to 300 ng in some cases to provide better results (see the *Analyzing FFPE Specimens with the nCounter CNV Assay* Tech Note for more details.) Carefully evaluate RNA quality using an Agilent Bioanalyzer® to measure nucleic acid fragmentation. NanoString recommends that at least 50% of the area under the trace be greater than 300 nucleotides in length for optimal performance (**FIGURE 2**).

Unpurified cell lysates can be used successfully with nCounter assays because the fluidic processing protocol that removes unbound probes also eliminates unwanted cellular debris. Cell lysates contain chaotropic salts that can affect the probes' melting temperature and reduce hybridization efficiency. To minimize this impact on hybridization efficiencies, NanoString recommends using no more than 4 μ L of cell lysate in each hybridization reaction. Ensure that the cell lysate used is sufficiently concentrated such that it contains the recommended minimum sample input of 100 ng, equivalent to 10,000 cells per hybridization reaction depending on cell size and type.

Blood samples can be assayed using purified total RNA, unpurified blood lysates, or specific blood fractions such as PBMCs isolated from whole blood. NanoString recommends the use of a commercially available kit to collect and purify RNA from blood just as you would for biological fluids such as sputum or urine. Although β -globin is highly expressed in blood, attenuation is not necessary unless your CodeSet has been designed to measure β -globin expression (see the next section). For unpurified RNA, NanoString recommends collecting blood lysate samples in specialized PAXGene tubes. Suggested protocols for preparing blood samples can be obtained by contacting NanoString Support (support@nanosttring.com).

Single cell gene expression assays also warrant special considerations but are not discussed here. Read the *Sorted Cells or Low Input RNA Gene Expression* protocol for more information.

* Single Cell assays require amplification prior to using the nCounter Analysis System.

Considerations for Highly Expressed Genes

Certain samples are especially prone to causing signal saturation when they include highly expressed genes. Individual reporter probes may not be easily distinguished in such circumstances, affecting the accuracy of results. One million or more total counts across all targets is a strong indication that saturation has occurred and may warrant modifications to the experimental design. (An individual highly expressed gene may have as few as 5,000 or as many as 200,000 counts, but the impact it has on saturation depends on the size of the CodeSet and the expression levels of other genes.) Even targets that would not be a concern in heterogeneous samples can become highly concentrated in samples containing a single cell type, such as β -globin in whole blood RNA purifications.

Saturation is of particular concern in larger CodeSets and panels because multiple genes expressed at moderately high levels—such as 20 genes with 50,000 counts per gene—can produce a cumulative effect comparable to a single, more highly expressed gene. Similar care should be used when measuring transgenes in genetically engineered systems. Assays using samples from cultured cells may require a lower input amount as these often contain a much higher proportion of mRNA.

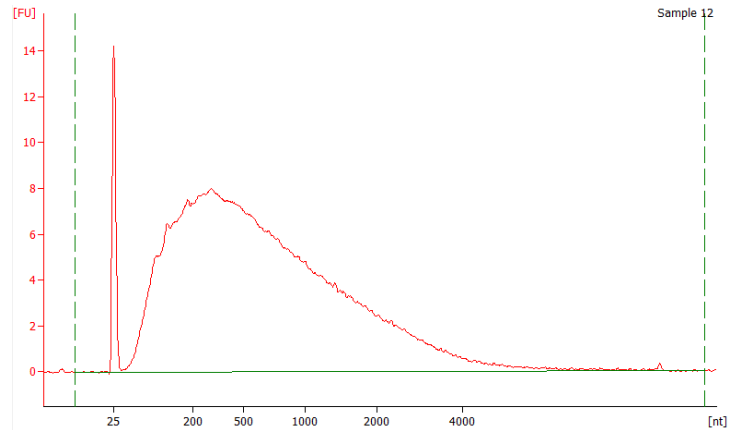


FIGURE 2: Ideal fragmentation profile for nucleic acids. This fragmentation profile was obtained using an Agilent 2100 Bioanalyzer® and an Agilent RNA 6000 Nano Kit to assay 300 ng of total RNA purified with a commercial FFPE extraction kit. The majority of the sample is greater than 300 nucleotides in length.

In most cases, exceeding the optimal reporter binding density—the average number of fluorescent spots per μm^2 —has a minimal impact on data because normalization to positive controls can correct for signal reductions. NanoString recommends a reporter binding density of 0.05-2.25 per μm^2 . Values between 2.25-3.00 do not necessarily indicate assay failure but are cause for concern and will be flagged by the nSolver Analysis Software. (Reporter density values can also be found in every RCC file.) Values above 3.00 should be reviewed carefully and may not produce reliable data.

Information about highly expressed genes for a particular biological system may be available from published data. To increase overall success, NanoString recommends researchers perform a pilot run before experiments involving a new CodeSet. Highly expressed genes can be identified early before they interfere with the results from many dozens or hundreds of samples in large experiments. Pilot experiments and consideration for highly expressed genes are of particular importance when using larger CodeSets and pre-designed panels that include between 200 and 800 targets because these potentially include a greater number of highly expressed genes.

Optional Instrument Protocols and Settings

Modifications to your typical nCounter assay protocol could further optimize an experiment. Titrating sample volumes across 12 assays during hybridization can determine if a lower input amount will sufficiently reduce very high counts while ensuring that counts for low-expressing genes remain well above background. The standard sample input amount is 100 ng, but in most cases this can be reduced to as little as 50 ng by using the High Sensitivity protocol on the Prep Station (see the *nCounter System Enhancement* Tech Note for more details). If financial constraints or limited sample amounts preclude a pilot run, NanoString recommends that researchers evaluate data after the first 12 assays to determine if the results match expectations before continuing with the experiment.

Users may also choose to image additional fields of view (FOV) with the Digital Analyzer. Target and background counts will both increase. However, it is expected that the increase in background counts will be proportionately lower when additional FOVs are sampled. nCounter cartridges can be rescanned multiple times at different settings with no additional material costs and with minimal loss of data quality. NanoString also recommends using one of the lanes for a blank sample (water only) to facilitate background subtraction and improve detection of low-expressing genes.

Strategy for Attenuating Highly Expressed Genes

Optimization of sample input amount and CodeSet design might be able to alleviate the impact of highly expressed genes in many cases. However in situations where overall reporter probe density continues to exceed optimal guidelines, some data loss may occur. The remainder of this Tech Note describes an attenuation strategy utilizing competitive inhibition to maintain high quality data even in the presence of highly expressed transcripts.

To develop this attenuation strategy, NanoString used the nCounter Human Reference Gene Expression Kit. This CodeSet detects 8 high-expressing and 10 medium- or low-expressing housekeeping genes present in most tissues. The CodeSet was hybridized with 100 ng each of human heart, liver, or colon total RNA purchased from Stratagene®. Unlabeled target-specific oligonucleotides, referred to as “inactive” probes, were used to attenuate the signal from each corresponding reporter probe. (Inactive probes may be purchased through any oligonucleotide supplier.) The indicated concentration of inactive probe was added to the hybridization mixture.

For the data that follow, all hybridizations were performed following the protocol in the *nCounter Gene Expression Assay Manual* using a 30 μL final reaction volume and 65°C incubation for a minimum of 16 hours. Samples were hybridized in triplicate and processed using the nCounter Prep Station and nCounter Digital Analyzer. To account for slight technical variability in hybridization and purification efficiencies, raw data were normalized to internal positive spike-in controls present in every reaction (Geiss *et al.*, 2008). Mathematical calculations were performed using Microsoft® Excel, though customers may also utilize NanoString's free nSolver™ Analysis Software.

Results

An attenuation strategy based on competitive inhibition was developed to reduce the overall image density without sacrificing the quantification of low-expressing genes. This involved adding excess inactive probes for the target(s) of interest to the hybridization reaction in proportion to the desired level of attenuation. For example, the standard hybridization reaction contains 25 pM of each reporter probe. To attenuate reporter counts for a particular gene by 90%—that is, to count only 10% of its endogenous expression level—it is necessary that 90% of the final reporter probe concentration (for that target) in the hybridization mixture be replaced with an inactive form. Therefore, we added 225 pM of inactive probe to the existing 25 pM of active probe, bringing the total concentration to 250 pM. Higher or lower attenuation levels can be achieved by making similar adjustments to the ratio of active to inactive probes.

Attenuation ratios are always approximate, and the effective level should be calculated by comparing the number of counts obtained with an unattenuated sample against that obtained after attenuation. This comparison produces an attenuation factor that can be used in future experiments to convert the number of attenuated counts in a sample to the equivalent unattenuated counts and makes possible direct comparisons of gene counts in a multiplexed sample.

It is important to use the same amount of RNA in both the attenuated and unattenuated assays to calculate the normalization factor accurately and to remain consistent in the amount of inactive probe used for future assays. Choose reference genes appropriate for the tissues or cell lines in your experiment since they may also be expressed at high levels in some cases. Furthermore, the count comparison must be performed under conditions where the amount of RNA used does not result in counts exceeding the optimal dynamic range.

The same attenuation strategy could be adapted for use with nCounter Elements General Purpose Reagents. These GPRs are reporter and capture tags that can be assigned to targets of interest using user-supplied oligonucleotide probes. Of these, probe A is used to link a reporter tag to a corresponding nucleic acid target (each tag/target pair has its own probe A). Rather than supplement the hybridization reaction with an inactive form of the reporter tag, the user can introduce an inactive form of probe A that binds to the nucleic acid target but not to the reporter tag.

Differential Tissue Gene Expression Profiles

Basal expression levels of the housekeeping genes were first examined in human total RNA from three different tissues: heart, liver, and colon. (All subsequent experiments were performed with colon RNA.) Genes were grouped based on their expression level as either high-expressing (FIGURE 3A) or medium-/low-expressing (FIGURE 3B) to typify the range of counts obtained using RNA from different tissues. As expected, expression levels varied significantly, not only between individual genes but also between tissue types for the same gene.

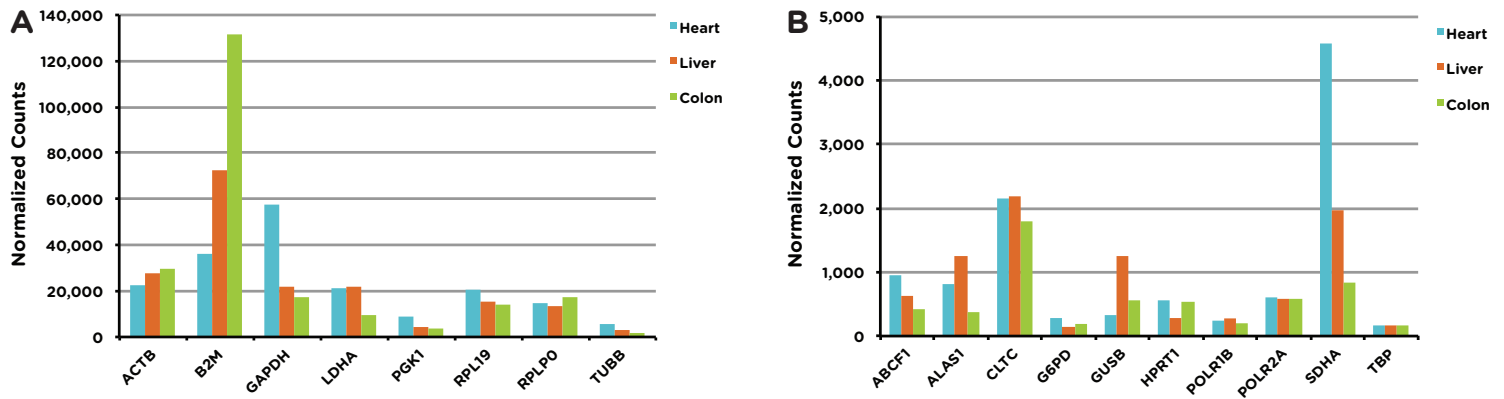


FIGURE 3: Expression profiles of differentially expressed genes in various tissue types. (A) Expression level profiles of 8 high-expressing housekeeping genes from heart, liver, and colon tissues. (B) Expression level profiles of 10 medium- and low-expressing genes from heart, liver, and colon tissue.

Attenuation Control Is Specific

To demonstrate the specificity of inactive probes used for attenuation, NanoString compared the counts obtained for all 18 genes in attenuated vs. unattenuated samples when only the 8 high-expressing genes were attenuated by 90% (FIGURE 4A). In the attenuated assays the counts for each of these genes were reduced by 7% to 17% compared to unattenuated counts. (The unattenuated *B2M* measurement was 131,862 normalized counts, greater than the scale of the y-axis.) Including the attenuating probes for high-expressing genes in the hybridization reaction had no effect on counts of the medium- and low-expressing genes (FIGURE 4B). Thus, this strategy affected only the attenuated genes and not counts for unrelated genes that were queried in the same multiplexed hybridization.

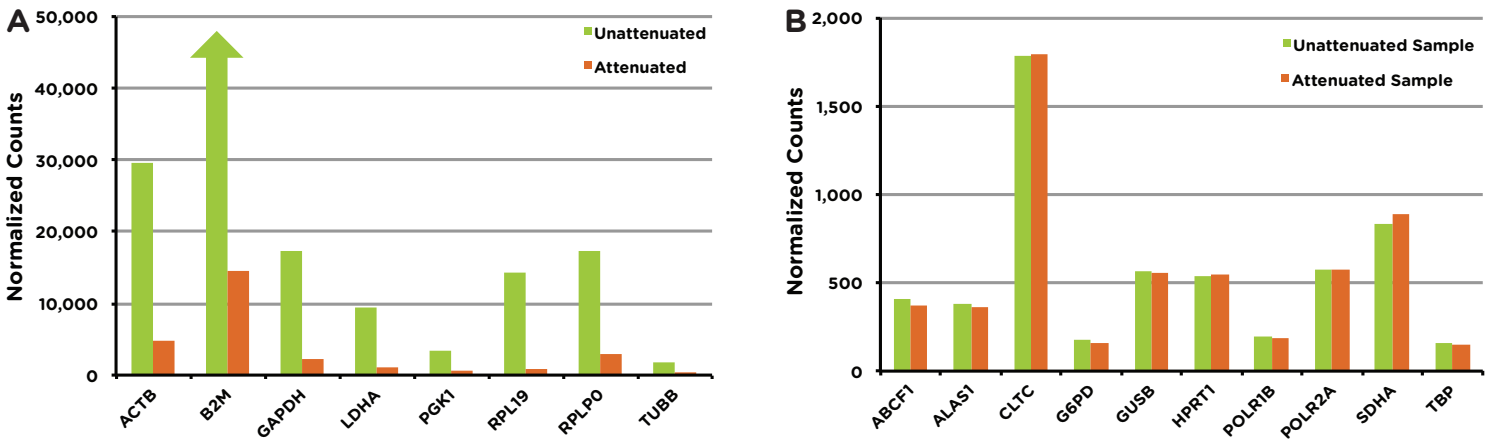


FIGURE 4: Gene counts attenuated by competitive inhibition. (A) 8 high-expressing housekeeping genes were equally attenuated to 10% of normal expression level (90% attenuated). (B) The 10 medium- and low-expressing genes in the same hybridization were unaffected by attenuation of the high-expressing genes.

Attenuation Control Can Be Fine-tuned

To demonstrate control over the degree of attenuation, NanoString attenuated the 8 high-expressing genes to varying degrees (FIGURE 5A). By adjusting the ratio of active to inactive probes, counts for each target were decreased to approximately 25, 50, and 75% of their 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 produces a slope of 0.230. The correlation coefficient between unattenuated and attenuated counts was greater than 97%. As shown previously, including attenuating probes for high-expressing genes had no effect on the counting of low- and medium-expressing genes (FIGURE 5B) since the slopes and correlations were unaffected.

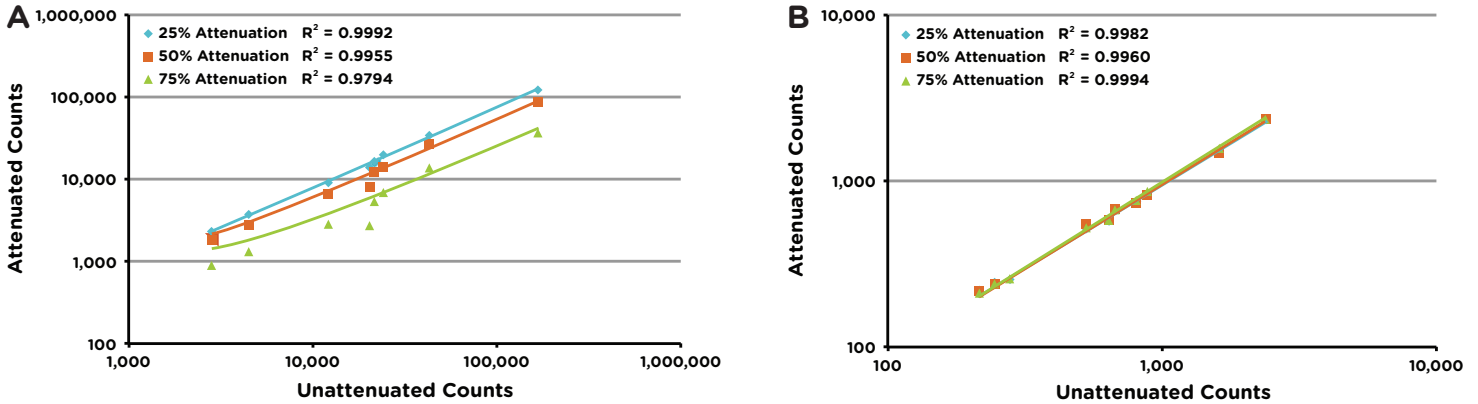


FIGURE 5: Fine-tuned attenuation retains target gene specificity. (A) Unattenuated counts of 8 high-expressing housekeeping genes were plotted against counts attenuated by increasing degrees (25%, 50%, and 75%). (B) Similar analysis of the medium- and low-expressing genes in the same hybridizations.

It was also possible to differentially attenuate two highly expressed genes in the same hybridization reaction. For example, *B2M* was attenuated to 1% of its original level (99% attenuation; 99.6% measured), while *GAPDH* was attenuated to 75% of its original level (25% attenuation; 33.4% measured) in a single tube (FIGURE 6). When these two genes were attenuated, there was no effect on the counts of other high-expressing but unattenuated genes. The counts for the ten medium- and low-expressing genes in the CodeSet were also unaffected (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 counts. For the previous example (FIGURE 6), the *B2M* gene was attenuated 99% (99.6% actually measured) and provided an attenuation factor of 260.4. The *GAPDH* gene was attenuated 25% (33.4% actually measured) and provided an attenuation factor of 1.5. Once this factor is calculated, it will remain constant in other experiments as long as the same amount of “inactive” probes are included in the hybridization. To calculate the corrected counts, the attenuation factor was multiplied by the attenuated counts; no adjustment is required for unattenuated counts.

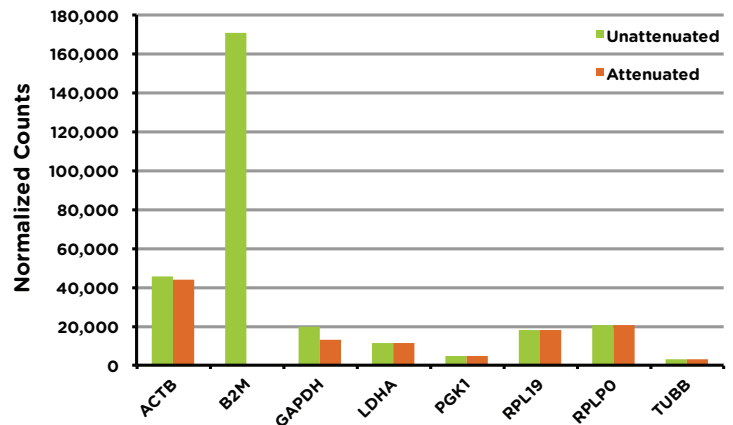


FIGURE 6: Attenuation can be differentially controlled in the same hybridization. The CodeSet of 18 housekeeping genes was hybridized to colon RNA. *B2M* and *GAPDH* were attenuated by 99% and 25%, respectively, in the same hybridization.

Conclusion

The nCounter platform provides accurate expression profiles of targets across a wide dynamic range in a single multiplexed reaction. When working with larger CodeSets and pre-made panels it is important to review considerations specific for the type of sample being used as well as the expected gene expression levels for both high and low expressing genes. It is necessary to take careful precautions when working with samples with challenging chemistry, or which contain a few genes expressed at very high levels that may interfere with image analysis. Either scenario can diminish data quality, especially for low-expressing genes. Simply increasing the sample amount to increase counts without a review of the various considerations is rarely a viable solution and may be counterproductive. Understanding the challenges posed by each sample type can help determine the appropriate course of action.

Modifications to the principle features of sample hybridization and processing—increasing or decreasing sample volume and concentration and using more sensitive instrument settings—will help optimize the detection of probe:target complexes. These changes are often minor and do not require additional handling steps. Rather, they reflect a necessary tradeoff to maintain the considerable flexibility offered by the nCounter platform: impurities in cell lysates must be below certain thresholds, fragmented nucleic acids must be above a certain size, and the instrument settings may need to be adjusted to offer greater sensitivity.

NanoString Technologies also developed a strategy of competitive inhibition to overcome concerns regarding extremely high-expressing genes when the aforementioned approaches are insufficient. Adding inactive probes that will compete with labeled reporter probes reduces the potential for saturation by highly expressed genes. Moreover, this Tech Note shows that the attenuation of a single target or group of targets does not interfere with results obtained for the remaining genes queried in the same CodeSet.

Attenuation is more likely to be required when previous knowledge suggests that some of the genes studied with a particular CodeSet are very highly expressed. It 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 adaptations described in this Tech Note, coupled with the intrinsic sensitivity and specificity of the nCounter platform, makes this system ideally suited to address the challenges of large multiplex expression profiling experiments regardless of the expression level of any particular gene of interest or any challenging sample type.

Considerations outlined in this Tech Note are valuable for all experiments. Please consult with your Field Applications Scientist or contact NanoString Support at support@nanosttring.com if you have any questions about how to ensure the best results from your experiment.

References

- Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T, James JJ, Maysuria M, Miton JD, Oliveri P, Osborn JL, Peng T, Ratcliffe AL, Webster PJ, Davidson EH, Hood L, Dimitrov K. (2008) Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol* 26(30):317-25.

TABLE 1: Counts can be corrected by using a calculated attenuation factor.

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

NanoString Technologies, Inc.

530 Fairview Ave N
Suite 2000
Seattle, Washington 98109

CONTACT US

info@nanosttring.com
Tel: (888) 358-6266
Fax: (206) 378-6288
www.nanosttring.com

SALES CONTACTS

United States: us.sales@nanosttring.com
Europe: europe.sales@nanosttring.com
Japan: japan.sales@nanosttring.com
Other Regions: info@nanosttring.com