The University Health Network Microarray Centre

UHNMAC News

Quarterly newsletter Autumn 2009

Validating Array Data: Beyond quantitative PCR

Validation of microarray data is necessary to ensure that the data reflects the genuine biology and is not an artifact of the experimental technique. There are a number of validation methods that can be used and choosing the appropriate method depends on the number of genes of interest requiring validation, the amount and condition of experimental samples, budget, and access to specialised instruments.

Quantitative PCR (qPCR) is considered the gold standard of microarray data validation. However, this technique requires optimisation for each gene of interest, making it a tedious and costly validation method if you have more than 20 genes to validate or hundreds of samples to process. High throughput tools for quantitative expression profiling, such as Nanostring technology and the Bio-Plex system, can compliment qPCR experiments. In addition, the Ziplex array platform can also be used to validate array data from another platform. This report briefly describes several microarray data validation tools that are available through the UHNMAC Service Team.

Nanostrings (Nanostring Technologies) are the fluorescent barcodes that bind to target mRNA for gene expression analysis. The nCounter™ Analysis System measures RNA abundance by tagging with barcodes and counting individual mRNA molecules using a novel digital technology. This system is able to detect 1 copy of mRNA per cell, can evaluate up to 550 genes per reaction, and requires 10-100 ng of total RNA for analysis. The advantage of this system is that there is no enzymology (artifacts of reverse transcription) and no amplification. Although most CodeSets (collection of capture and reporter probes) are custom-made, off-the-shelf CodeSets are available for human kinase genes and human GPCR genes. A Virtual Cancer Reference Gene Set is also available, with more virtual gene sets coming soon. Nanostring technology is a cost-effective platform for the validation of 30 to 500 different genes or many samples.

How nanostrings work: Each gene sequence has a unique barcode (combination of fluorescent molecules) that is part of a target specific reporter probe. The reporter probe and target specific capture probe (linked with biotin) are hybridised to the sample to form a tripartite structure with target mRNA. The molecules are linked to a streptavidin-coated cartridge (via the biotin capture probe) and aligned by a magnetic field. The barcodes are then counted and tabulated.

The Bio-Plex system (Bio-Rad) is a multiplex analysis system that can simultaneously analyse up to 100 different biomolecules (proteins, peptides, or nucleic acids) in a single microwell. This suspension array system incorporates several technologies including fluorescently dyed bead (Luminex xMAP technology), a flow cytometer with two lasers and associated optics to measure the biochemical reactions that

occur on bead surfaces, and a digital signal processor to manage the fluorescent output. The Bio-Plex system is a cost-effective option for studies that require the validation of 20-80 different genes. For protein applications, various assays are available from a number of vendors.

How the Bio-Plex works: The system uses a liquid suspension array of 100 sets of 5.6 µm beads, each internally dyed with different ratios of two spectrally distinct fluorophores to assign it a unique spectral address. Each set of beads can be conjugated with a different capture molecule. The conjugated beads can then be mixed and incubated with sample in a microplate well to react with specific analytes. Capture molecules can include RNA, DNA, enzyme substrates, receptors, antigens, and antibodies. To detect and quantify each captured analyte, a fluorescently labelled reporter molecule that specifically binds the analyte is added. Following incubation, the beads are drawn through a flow cell where two lasers excite the beads individually. One laser excites the dyes in each bead, identifying its spectral address, and the other excites the reporter molecule associated with the bead, which allows the captured analyte to be quantified. Fluorescent signals are recorded, translating the signals into data for each bead-based assay.

The Ziplex System is a focused, low-density (400 features/probes per TipChip™ array) microarray platform that uses proprietary Flow-Thru Chip® technology. It is most often used to validate expression signatures following biomarker discovery studies using high-density microarrays. The molecular interactions between the arrayed probes and the target sample occur in 3-D microchannels within the TipChip™ array. The array consists of 220,000 microchannels and can accommodate up to 400 features (each feature has 70-100 microchannels). The Ziplex system uses chemiluminescence (rather than fluorescence) and a CCD camera to quantify hybridisation signal intensities. Xpress Chips (probes specific to genes involved with metabolism, inflammation, colon cancer and breast cancer) and custom Autograph Chips are available.

How the Ziplex works: Much like high-density arrays, the first and second strand synthesis of the RNA sample is performed. The second strand is then labelled with biotin by IVT. The concentration of labelled cRNA is measured and then hybridised to TipChip™ arrays. Hybridised targets are detected through sensitive chemiluminescent detection with a CCD camera.

Please contact us for more information about the Nanostring, Bio-Plex, or Ziplex service.

Feature Paper

Summary of: Khalil AM, et al. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. PNAS 2009, 106(28):11667

lincRNAs: Regulating the epigenome

Until recently, the idea that large intervening non-coding RNA (lincRNA) had biological significance was quite controversial. However, current studies (1-3) have provided evidence of evolutionary conservation of lincRNA, suggesting that at least some of these transcripts are functional. In mammals, several lincRNAs have been well characterised and have been found to play a role in *trans*-acting gene regulation (4), imprinting (5), regulation of nuclear import (6), and functioning in X-chromosome inactivation (7,8).

Earlier this year, the identification of 1600 lincRNAs based on a distinctive chromatin signature that marks actively transcribed genes was reported (2). The chromatin signature consisted of trimethylation of lysine 4 of histone H3 (at the promoter) and the trimethylation of lysine 36 of H3 (along the length of the transcribed region) (9). Chromatin-state maps were then generated using several cell types and "K4-K36" domains were located. Domains that corresponded to protein-coding genes were eliminated from the list, leaving previously uncharacterised K4-K36 domains; the majority of which encoded highly conserved lincRNA. This group had also previously reported that the *HOTAIR* lincRNA repressed gene expression by binding to the polycomb repressive complex (PRC)2, a methyltransferase that trimethylates H3K27 to repress the transcription of specific genes (4).

In this study, Khalil and his colleagues build on previous studies by analysing the chromatin-state maps of additional cell types and expand the catalog of human lincRNAs to about 3300 genes. An RNA immunoprecipitation followed by microarray hybridisation (RIP-Chip) assay was used to determine whether other lincRNAs are also associated with PRC2. Briefly, the RIP-Chip assay involved incubating the antibodies against two proteins of PRC2 with the nuclear extracts of three human cell types and hybridising co-precipitated RNAs on a custom exon-tiling array containing exons from 900 human lincRNA loci and 1000 human

protein-coding genes. The expression patterns of lincRNAs and protein-coding genes were also assayed on the exon-tiling array. The RIP-Chip and expression data suggested that a significant proportion of the newly discovered lincRNAs were physically associated with PRC2. This study also explored whether lincRNAs might be associated with other repressive chromatin-modifying complexes. In HeLa cells, 60% of the lincRNAs associated with CoREST (a corepressor of REST – RE1-silencing transcription factor) and not with PRC2, indicating that each complex has specific lincRNAs associated with it. The observation that 40% of the lincRNAs associated with both CoREST and PRC2 suggests that the two complexes share some regulatory targets.

To establish the functionality of lincRNAs, loss-of-function experiments were also performed. Six lincRNAs were individually knocked-down in siRNA assays. Interestingly, the sets of genes affected by each lincRNA did not show significant overlap, suggesting that each lincRNA has distinct target sets. The data also showed that lincRNA knock-down did not affect the expression level of nearby genes, suggesting that lincRNAs likely function by a *trans*-acting mechanism. This study also found that chromatin protein themselves (rather than chromatin-modifying proteins) were not associated with lincRNAs.

Although the experiments discussed in this paper focused on chromatin-modifying complexes that add repressive chromatin marks, it has been reported that other lincRNAs are associated with chromatin-modifying complexes that confer activating modifications (10). There may also be classes of lincRNAs that function in entirely different ways, such as the formation of paraspeckles (11). Further research will uncover the full range of biological diversity of lincRNAs.

References

1. Khalil AM, *et al*. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. PNAS 2009, 106(28):11667
2. Guttman M, *et al*. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 2009, 458:223
3. Ponjavic J, et al. Functionality or transcriptional noise? Evidence for selection within long noncoding RNAs. Genome Res 2007, 17:556
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11. Sunwoo H, *et al*. MEN varepsilon/beta nuclear-retained non-coding RNAs are up-regulated upon muscle differentiation and are essential components of paraspeckles. Genome Res 2009, 19:347

Recent Publications by UHNMAC Users

Hussein S, *et al*. Characterization of human septic sera induced gene expression modulation in human myocytes. Int J Clin Exp Med 2009, 2:131

Kovalenko A, *et al*. Caspase-8 deficiency in epidermal keratinocytes triggers an inflammatory skin disease. J Exp Med 2009, 206(10):2161

Veitch ZW, *et al*. Induction of 1C aldoketoreductases and other dose-dependent genes upon acquistion of anthracycline resistance. Pharmacogenet Genomics 2009, 19(6):477

Please let us know if you have published a study that involved one of our array products or services.

Announcements

Upcoming Meetings

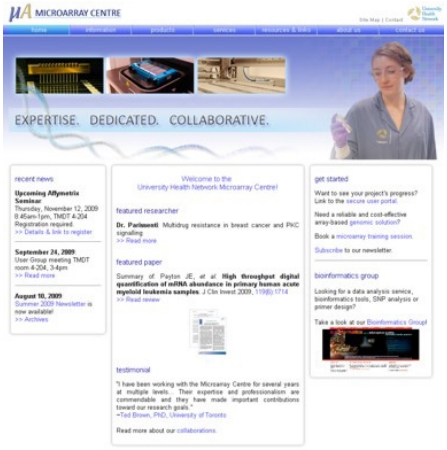
Affymetrix Medical Genomics Research Seminar

Thursday November 12, 2009
TMDT 4-204, 8:45am - 1pm
For more details, and to register, visit:
http://www.microarrays.ca/info/MedicalGenomicsSeminar_12Nov2009.pdf

Microarray User Group Meeting

Thursday November 26, 2009
TMDT room 4-204, 3-4pm
101 College Street, Toronto

The UHN Microarray Centre has launched its new website. Visit www.microarrays.ca to learn more!



Researcher Spotlight

Dr. W. Conrad Liles

Translational Research

As a clinical scientist, Dr. Liles' goal is to investigate disease pathogenesis in the laboratory and rapidly apply that knowledge to the treatment of patients. At the McLaughlin-Rotman Centre for Global Health, Dr. Liles' research focuses on the molecular immunopathogenesis of malaria. In 2007, Dr. Liles and his colleagues published a study that assessed brain transcriptional responses in cerebral malaria by comparing genetically resistant and susceptible inbred mouse strains that were infected with Plasmodium berghei strain ANKA. This study, which used the UHNMAC Affymetrix service, found that the genes that were differentially expressed in susceptible mice were associated with immune-related gene ontology categories and that both interferon-regulated processes and apoptosis played a central role in the pathogenesis of cerebral malaria. Most recently, Dr. Liles and his colleagues reported on a phase I/II clinical trial that used the peroxisome proliferator-activated receptor gamma agonist (rosiglitazone) for the treatment of *P. falciparum* malaria. This study found that patients who received rosiglitazone, in addition to standard antimalarial therapy, had better parasite clearance times and decreased levels of inflammatory biomarkers associated with adverse malaria outcomes.

Dr. Liles and his colleagues also study the regulation of myeloid development, the molecular basis of inherited neutrophil disorders, granulocyte transfusion therapy, mobilisation of CD34+ hematopoeitic stem cells, and the modulation of neutrophil function by colony-stimulating factors. More recent investigation by his team has concentrated on the role of the Fas (CD95)/Fas-ligand system in both spontaneous phagocyte apoptosis and phagocyte-mediated tissue injury and inflammation. His team is currently examining the role of this system in acute respiratory distress syndrome, atherosclerosis, sepsis, and multiple organ failure. His other clinical interests include primary (congenital) immunodeficiency disorders, fungal infections, zoonoses, and travel/tropical medicine.

Dr. Liles is a Professor and Vice-Chair of Medicine at the University of Toronto, the Director of the Division of Infectious Diseases at the University Health Network, a Senior Scientist at the Toronto General Research Institute, and an Affiliate Professor of Medicine at the University of Washington (Seattle). Dr. Liles holds a Canada Research Chair in Inflammation and Infectious Disease.

Dr. Roger Moorehead

Transgenic mouse models & the role of insulin-like growth factors in tumourigenesis

Dr. Moorehead's research examines the role of cell receptors in tumourigenesis. The receptors being investigated include the insulin-like growth factor receptor (IGF-1R), which is thought to play an important role in breast cancer development, and HER-2, another tumour-inducing receptor. Researchers are also studying the interaction between IGF-IR and HER-2 and their involvement in drug-resistant tumours. By focusing on type 1 insulin-like growth factor receptor (IGF-IR), a protein on the surface of the cell that signals the growth or death of the cell, Dr. Moorehead and his colleagues hope to determine the IGF-IR pathway in order to develop more effective cancer treatments. IGF-IR therapeutic treatments for breast cancer are currently in early-stage clinical trials.

Dr. Moorehead and his colleagues have also established transgenic mouse models, and primary cell lines derived from the tumours of these animals, to further their investigation in determining the role of IGF-IR. In 2008, Dr. Moorehead and his team characterised the first primary murine mammary tumour cell line with doxycycline-inducible IGF-IR expression, an important model to further examine the function of IGF-IR in mammary tumorigenesis. This study used the UHNMAC Microarray Service to identify genes regulated by IGF-IR. The results of this study suggested that at least some of the proliferative actions of IGF-IR are mediated through the proto-oncogene cyclin D1. In a more recent publication (July 2009), Dr. Moorehead and his colleagues revealed that two novel transgenic mouse models in which IGF-IR is overexpressed in either lung type II alveolar cells (surfactant protein C [SPC]-IGFIR) or Clara cells (CCSP-IGFIR) in a doxycycline-inducible manner had been produced. This study found that overexpression of IGF-IR in either cell type led to alveolar hyperplasia with papillary and solid adenomas.

Dr. Roger A. Moorehead is an Associate Professor in the Department of Biomedical Sciences at the University of Guelph. In May 2009, Dr. Moorehead received a special Lung Cancer Initiative grant from the Canadian Cancer Society, one of only five awarded across Canada.

Dr. Warren Chan

Nanoscience and biomedical engineering

At the Integrated Nanotechnology & Biomedical Sciences Laboratory (INBS) at the University of Toronto, Dr. Chan and his team (1) use nano- and microtechnology to study the genomic and proteomic changes associated with cancerous and virally-infected cells, (2) investigates nanoparticles interactions with biological systems to gain greater insight to the issue of nanotoxicity and to design nanoparticles for biomedical application, (3) and design quantum-barcode systems for high throughput multiplex detection of infectious disease markers.

Dr. Chan and his team have contributed to the advancement of nanotechnology-based drug delivery systems and cancer therapeutics. Unlike conventional drug therapies, in which the drugs are non-specifically distributed and quickly metabolised, nanotechnology enables drugs to be delivered specifically to the target cells and released intracellularly. Dr. Chan's group has also used UHNMAC Human cDNA microarrays to monitor the gene expression of cells following the uptake of gold nanorods. This study found that very few genes showed differential expression and concluded that gold nanorods could be used for therapeutic applications, such as thermal cancer therapy, due to their tunable cell uptake and low toxicity.

Dr. Chan and his colleagues have combined nano- and microtechnologies (quantum dots and microfluidics) to create a diagnostic system capable of multiplexed, high-throughput analysis of infectious agents in human serum samples. They have also published other studies involving quantum dots for the labelling of subcellular structures, quantification of protein expression, and surface marker labelling. Earlier this year, Dr. Chan's research on quantum dots was featured in the Globe & Mail.

Dr. Warren C. W. Chan is a Biomedical Engineer and Professor in the Faculty of Applied Science and Engineering, Institute of Biomaterials and Biomedical Engineering, at the University of Toronto. He also holds a Canada Research Chair in Bionanotechnology.