

XenoQ™ Assays designed to detect human tumor xenografts in a mouse background and to identify gene expression changes that are crucial to malignant transformation, invasion and metastasis.

Bar Harbor BioTechnology, Inc. Trenton, ME, USA.

Experimental research in humans is limited for both ethical and practical reasons, and this can be extended to other non-human primate models as well. This limitation is partially overcome by the use of readily accessible rodent models, but it is increasingly realized that the translational potential of these models is limited¹. Models in which human tissue is transplanted and maintained in immunodeficient mice offers exciting opportunities to bridge this translational research gap². Recent advances in the development of these human/mouse (Hs/Mu) chimeras has made them highly attractive surrogates for understanding the properties of normal and transformed human cells, and for therapeutic development. Gene expression (GEx) and genomic copy number (GCN) profiling is widely recognized as a valuable tool for defining the molecular properties and state of normal and abnormal cells³. A current limitation is that gene profiling products cannot be used reliably to characterize human cells grown in chimeric mice because of the similarities between the human and mouse genes. Through exploitation of the evolutionary heterogeneity between mouse and human, it should be possible to independently analyze species specific molecular profiles derived from mixtures of human and mouse tissues. Bar Harbor BioTechnology's (BHB's) platform utilize the sensitivity of quantitative PCR and SYBR® Green I , XenoQ™ Assays, to detect gene expression in a minimal amount of human tumor cells that are circulating in the mouse bloodstream without interference from mouse cell gene expression. Previous studies involving the use of mouse tumor models have often raised the question of whether human tumor data obtained from a xenograft model is impacted by gene expression changes within the mouse. Those studies have also required that the mice be sacrificed in order to remove and analyze the response of the implanted human tumor to therapeutic interventions.

Introduction

Limitations to experimental testing in humans. The overall goal of much of society's investment in biomedical research is to elucidate human biology and to cure human diseases. However, experimentation in humans is limited by numerous ethical and technical constraints. The most proximal alternative to human subjects are non-human primate models, but are limited by both the practical issues of low availability and high cost, and by the ethics of experimentation on higher mammals. Human cell lines maintained *in vitro* provide some opportunities for biological analysis and therapeutic testing, but are too far removed from the living human to extrapolate cause and effect with accuracy. There is a critical need for

improved *in vivo* models that more accurately defines human biology and disease.

Need for better rodent models for translational research. Non-primate animal models continue to be pivotal for elucidating basic principles of mammalian biology and disease. The most heavily exploited of these are rodent models for a number of reasons, including their small size, ease of breeding, genetic integrity and the ability to modify their genomes. In particular, the laboratory mouse has emerged as the key experimental mammal, with an enormous variety of different mouse strains and genetic alterations. However, the evolutionary separation between mice and humans is significant and this separation is increasingly appreciated to result in biological differences that make it challenging to

extrapolate mouse genes, proteins, biological attributes and therapeutic responses to human. For instance, mouse cancers are stable in terms of karyotype and GCN variation while human tumors are much more variable. Furthermore, the anti-CD28 monoclonal antibody shown to effectively suppress autoimmune responses in mice was canceled from Phase I human trials when it led to a disastrous systemic inflammatory response in all cases⁴. Efforts to improve mouse models are underway by the introduction of human genes⁵ but these models currently remain a far cry from humans.

Hs/Mu chimera models offer a capable surrogate for the analysis of human biology and disease.

The value of immunodeficient mouse models in which both normal and transformed human cells can be transplanted and maintained is increasingly evident and has provided a critical foundation for preclinical anticancer drug screening *in vivo* since the mid 1980s. Hs/Mu chimeras are currently used for the assessment of many different therapeutic strategies that are aimed at restraining tumor growth, including humanized antibodies, DNA based vaccines, cell-based therapies and conventional small molecule inhibitors that target tumor growth or angiogenesis². These models are applicable towards the study of most major forms of human cancer, including those that produce both hematopoietic⁶ and solid neoplasms⁷. Recently Hs/Mu chimeras have been used to elucidate stem cells that contribute towards cancer progression. This includes the identification of the CD133+ tumor stem cell fraction which is capable initiating human brain⁸ and colon cancer^{9, 10} and the discovery of mesenchymal stem cells within tumor stroma that promote breast cancer metastasis¹¹. Hs/Mu chimeras have also aided in the elucidation of cancer causing pathways and genes, such as the paracrine requirement for hedgehog (Hh) ligand signaling associated with tumor formation of Hh-expressing cancers¹², the identification of a polymorphism in ARLTS1 that predisposes patients to familial cancers¹³ and the involvement of PAR1 in promoting growth and invasion of breast carcinoma cells¹⁴. The growing needs for Hs/Mu chimeric models include not only cancer, but autoimmune disease, toxicology, infectious disease, and tissue and organ regeneration by stem cells². Overall, Hs/Mu chimeras are providing promising *in vivo* models for the study of human biology, disease, and therapy.

Genetically engineered mouse models (GEMMs) versus Hs/Mu chimeras for cancer research.

GEMMs offer another solution for cancer research and anticancer drug development, and the use of these models over Hs/Mu chimeras is a current subject of controversy and debate^{15, 16}. Advantages of GEMMs include the ability to evaluate therapies that require an immune response or intact tumor/host environment and the capacity to monitor gene expression using standard molecular profiling techniques, such as traditional Real-time PCR and microarray technologies. Disadvantages include the limited variety of available tumor types that are representative of the human disease, the rapid growth of tumors compared to human tumors, different reactions of compounds against mouse versus human tumors and intellectual property issues for many of the GEMMs¹⁷. Many proponents of GEMMs hold the view that Hs/Mu chimeric models have been problematic and disappointing in terms of cancer drug discovery¹⁶. With this being said, thus far drug evaluation in GEMMs that truly represent the biological physiology of the human equivalent of the disease has been minimal¹⁶. Compared to GEMMs, Hs/Mu chimeras more accurately model the physiology of human tumors with an abbreviated lag phase (weeks versus months), and will likely remain the model of choice for preclinical discovery and testing in the foreseeable future. In addition, there is currently much work underway to improve these models (see below), which will keep them at the forefront of cancer drug discovery.

Hs/Mu chimera models are becoming increasingly sophisticated.

The first immunodeficient mouse model that supported the growth of a human tumor dates back to 1969¹⁸. These athymic, *nude* (hairless) mice lack development of T-cells due to a mutation in the FoxN1 gene which is selectively expressed in the skin and thymus¹⁹. While they retain high NK-cell activity, humoral immunity and are resistant to engraftment of human hematopoietic cells²⁰, many solid human tumors survive and grow in *nude* mice²¹, and there is considerable usage of this xenograft model for drug screening and other forms of lead therapies²². The more recent development of mice that fail to develop cells of the adaptive immune system because they carry a defective *Rag* or *Prcdc* (*scid*) gene^{23, 24}, has further propelled Hs/Mu chimeric

models into the limelight. These two models have limited engraftment of hematopoietic stem cells due to high levels of host NK cell activity, and leakiness in the CB17-*scid* model allows the development of B and T cells as mice age. However, it was transplantation in the *scid* mouse that led to the notion of cancer stem cells²⁵, where a rare subset of tumor initiating cells was found to give rise to acute myeloid leukemia, similar to that seen in the original patients from which the tumors were derived. Introduction of the *scid* mutation to the NOD mouse genetic background²⁶ contributed additional immunologic defects and allowed optimized growth of many more leukemias and lymphomas. Further additional improvements came with specific gene inactivation mutations in the common chain of the interleukin 2 receptor (*Il2g*)^{27, 28}, which in combination with either the *Rag-nulls* or *scid* mutations, further compromised the immune system due to the shared involvement of *Il2g* in signaling with multiple high affinity cytokine receptor complexes²⁹. This particular model has consolidated the utility of the Hs/Mu chimera by providing a higher level of engraftment of difficult human tumors, such as the slow growing multiple myeloma³⁰, and is useful for other long term studies of normal human tissue and stem cell xenografts. Indeed, other genetic and methodological improvements will further advance Hs/Mu models. For example, the discovery and transfer of new mouse alleles or transgenic insertion of human genes that interact more appropriately with the human cognate proteins of a transplanted tumor³¹ will act to simulate human cancers more accurately. The use of orthotopic transplantation methods that promote the metastatic spread of the resultant tumor³² will strengthen the ability to select the most appropriate molecules for recommended use in clinical studies³³. Other improvements for cancer related studies include the transplantation of the human microenvironment along with the tumor to help support it³⁴, and the introduction of human xenobiotic drug metabolizing genes that allow better determination of the drug toxicity associated with anticancer drugs⁵.

In addition to genetic and methodological improvements, emerging technologies will further increase the overall utility and quality of information gained from Hs/Mu chimeric models. A recent example is fluorescent imaging technologies that allow metastasis of a human tumor to be visualized in

immunodeficient mice³⁵. Future advances in molecular profiling technologies will improve target discovery, cancer staging and therapeutic monitoring. The technology we propose is poised to advance this endeavor.

Need for better molecular profiling techniques that enhance the translational research potential of rodent models. The identification of genes that are associated with oncogenesis is a key objective in cancer research³⁶, and methods that permit life scientists and clinicians to monitor the activity of these genes are vital to this goal. This process, referred to as 'molecular profiling', is widely regarded to be integral for advancing life science research and clinical diagnosis in the 21st century. It is increasingly recognized that subsets of genes (hundreds rather than thousands) provide valuable gene profile definitions of any specific biological process. This fact is driving a market opportunity for affordable, accurate and easy to use technologies that most accurately measure these "signature" genes.

Gene expression analysis has the capability to identify cancer targets, monitor toxicological responses to drugs and develop biomarker signatures needed for diagnosis, prognosis, staging and treatment of cancer. Current platforms used for measuring gene expression include DNA and protein based microarrays, quantitative PCR (Real-time PCR), serial analysis of gene expression (SAGE), and high throughput sequencing, with Real-time PCR and DNA microarrays currently being the most used technologies. Real-time PCR affords several advantages over the DNA microarray, which includes an inherently wider dynamic range, higher specificity of target detection, more accurate quantitative determination of target sequences and a much simplified data analysis of the results. It also provides the ability to implement the technology within individual labs at will, rather than standing in queue for institutional "core services" or an outside microarray service provider. Moreover, it is common practice in the scientific community to validate the important subsets of genes identified by microarrays using Real-time PCR, as complications from the microarray data analysis can lead to a high false discovery rate.

The major limitation to the application of advanced gene-based molecular profiling to Hs/Mu chimera models is the lack of products designed to discriminate human and mouse genes. With the exception of cells from blood cancers, which can be isolated by flow cytometry, this is partially due to the fact that most solid tumors are complex mixtures of neoplastic and non-neoplastic cells. It is well known that one of the greatest challenges of oncology is the isolation of pure populations of tumor cells from normal tissue³⁷. In order to take full advantage of any type of Hs/Mu chimera model, one would ideally want to monitor the biological status of the human tissue without being confounded by preparation complications that arise from the sample contaminating mouse tissue. In addition, the isolation procedures themselves compromise the biological status and quality of the samples. Accurate analysis of the molecular changes associated with tumors or the tissue microenvironment could be better facilitated using a platform capable of distinguishing the genetic material of the human tumor from that of the mouse. These tissues cannot be analyzed using robust GEx microarray technology because the probes were designed to interrogate transcripts from a single species and have the potential cross-hybridize. In one estimate, using microarray technology to monitor gene expression profiles from xenografts, it was determined that approximately 15% of the probes failed to specifically hybridize to human cDNA transcripts³⁸, however, this number may be an underestimate. In another study, microarray based probes were specifically designed for the purpose of species specific detection of human and mouse transcripts derived from xenografts³⁹. In this study, the overall cross reactivity between probes hybridized to the orthologous species was 29%. Even for probes that are suitable for distinguishing between the orthologous transcripts derived from a xenograft, it is most often the case that microarray results need to be validated using Real-time PCR. High throughput sequencing on the other hand could be used to distinguish mouse from human genes, however,

this method requires laborious cDNA preparations, labor intensive data analysis and currently is at a very high cost, which makes the method impractical for routine use. In addition, microarray technologies often require significant amounts of RNA that is difficult to obtain from microdissected tissue and RNA enrichment or amplification techniques prior to gene expression analysis must be used, which can also introduce error into the results. Instead, the Real-time PCR technology we propose has the specificity and sensitivity to distinguish between orthologous transcripts from a very limited amount of material, including rare transcripts, such as those derived from residual cancers after a drug treatment or rare stem cell marker genes.

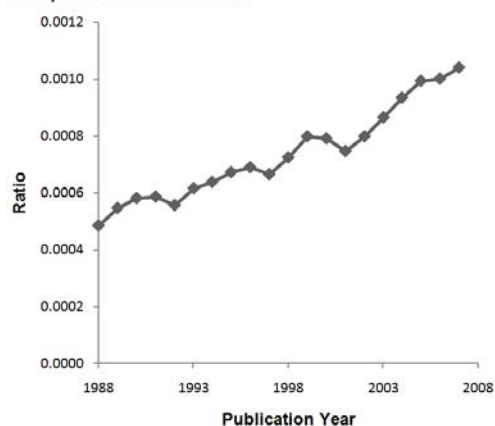
The ability to investigate the mouse host tumor microenvironment. The host participates in the induction, selection and expansion of neoplastic cells to a great extent and increased knowledge about the host environment has led to the development of several novel therapeutics⁴⁰. Few if any studies have been able to accurately address the gene expression patterns derived from the host tissues that surround a human tumor transplanted in Hs/Mu chimeras. This to a large extent is due to a lack of suitable molecular profiling tools that can accurately distinguish mouse from human genes. Real-time PCR assays that can selectively monitor human and mouse genes will allow for the analysis of the mouse host environment to a level that has never been possible before. Invasion of tumor cells into sites of metastasis is known to be dependent on the host environment, and studies in the *Ilr2g* deficient *scid* model have shown that sites of metastasis overlap with those of humans⁴¹. By providing an interspecies differential gene expression approach, our technology will greatly help in the analysis of the host environment at the sites of tumor transplantation and metastatic invasion, without the complications that arise from separation of human and mouse tissues. This may lead to the

development of new classes of therapeutics that are more able to specifically target stromal-tumor interactions that are critical for the progression of cancer.

Background and Significance

Potential of Real-time PCR assays for use in xenodetection. While molecular biology has advanced our collective understanding of cancer, there is still a lack of improved

Figure 1. Ratio of human-mouse xenograft publications to total publications in PubMed



treatments for patients. Despite many of their limitations, Hs/Mu tumor transplantation studies have helped to greatly speed the process of drug development for the purposes of fighting cancer. The great majority of successful cancer therapies developed in recent times have undergone xenograft testing¹⁶ resulting in over 1200 agents approved and used for treating cancer. In addition, almost half of all biotech drug development is focused on oncology and pharmaceutical companies currently have over 400 new agents in clinical trials⁴⁵. Research publications involving Hs/Mu chimeras has more than doubled in the past 20 years, and will most likely continue to grow. (Figure 1).

Drugs tend to fail late in clinical testing trials which is the most expensive part of the process, with investments of \$500 million or more accumulating towards the end stages²². Only about 11% of potential anticancer drugs that yield promising data in mice are approved for use in humans⁴⁶. This high rate of ineffective

compounds that enter into the clinical testing phase indicates a need for predicting efficacy prior to clinical testing in humans. Better animal models and methods for analyzing them have the potential to increase success at the early pre-clinical stage. Towards this endeavor, the molecular profiling of Hs/Mu chimeras has the potential to accelerate the discovery of cancer targets, biomarkers and therapeutics; however, the current limitation is the lack of products designed to adequately discriminate between human and mouse genes.

Scientific and technical basis of technology.

PCR is a revolutionary method capable of detecting rare and unique fragments of gDNA (genomic DNA) or cDNA (a DNA copy of RNA). Through the combination of oligonucleotide primers complementary to the target DNA molecule, free nucleotides and DNA polymerase, the PCR results in an approximate doubling of DNA after each cycle produced by a thermocycling device, which iterates the entire process through multiple cycles, resulting in the exponential amplification of the target sequences. Real-time PCR, also known as Real-Time PCR, has gained broad acceptance in virtually every area of life science through its ability to accurately estimate the relative copy number of gDNA/cDNA targets from tissue samples. As described in the Background and Significance, Real-time PCR affords several advantages over DNA microarrays. This includes an inherently wider dynamic range, better resolution and the ability to implement the technology within individual labs rather than as an institutional "core service". In lieu of the increased gene density afforded by the microarray, it is common practice in the scientific community to validate the important subsets of genes identified by this technology using Real-time PCR, as complications from the microarray data analysis can lead to a high false discovery rate. We have elevated the Real-time PCR technology to a new level, whereby the expression of hundreds of genes in single biological samples can be accurately analyzed. In doing so, BHB has been able to provide the life science market with cost effective, advanced solutions for molecular profiling through a product distribution partnership with Lonza.

Materials and Methods

Bar Harbor BioTechnology's targets the growing need to accurately analyze biologically focused sets of genes simultaneously by Real-time PCR. Each Real-time PCR assay is designed to be capable of detecting two parameters. The first is "conventional" gene expression profiling of cDNA transcripts derived from mRNA. The second is to measure gene copy number (GCN) variation present within the genome - a recently discovered phenomenon in biomedical research. GCN variations are more frequent in humans than previously expected⁴⁷ and play a major role for disease risk, onset, and progression. This is not only true for cancer, where it is well established that changes in the DNA itself are responsible for cancer type, stage, and prognosis, but for many other diseases^{48, 49}. Each current BHB Real-time PCR array is comprised of either 96 or 384 distinct primer sets for gene amplification, assembled into a molecular profiling array whereby the overall relationship between the gene content is geared toward a specific biological process. The oligonucleotide primers define the gene sets and are the core component of the Real-time PCR technology. The primers are designed using a proprietary computer algorithm to be gene-specific and are "in silico" and "wet lab" validated for optimal performance. The use of SYBR-Green by BHB as the means of detection requires an increased rigor of evaluation compared to probe-based detection schemes. The primers for the assays are deposited robotically into each well of the array and stabilized by proprietary methods to ensure long product shelf life. BHBs mid-density gene detection arrays overcome several impediments that complicate molecular profiling; namely, gene content assembly, primer design, experimental set-up, cost, data analysis, and fill a critical niche between monitoring expression levels of all genes by microarray approaches and conventional Real-time PCR applications.

BHB developed XenoQ™ Assays to offer several advantages over previous xenograft experimental methodologies. XenoQ™ Assays are available as fully customizable assays and can be arranged in 48, 96 and 384-well formats. Validated Primer sets have been designed with a special focus in cancer, toxicology and immunology. We will continue to expand our database and will design primer sets that

researchers would like to add to their XenoQ™ Assays. Because XenoQ™ Assays are effective at detecting minimal numbers of human cells found in the bloodstream of the mouse, a researcher has the option of taking serial blood draws as opposed to sacrificing the mouse in order to study tumor responses to therapy over time. This minimizes cost and variability by eliminating the need to sacrifice multiple mice. In addition, preclinical xenograft studies are all too often followed by failure of a particular drug/therapy in clinical trials as a result of analyzing and detecting both human and mouse genetic changes. XenoQ™ Assays will minimize these failures by allowing researchers to specifically focus on how the drugs/therapies are impacting the human tumors without cross-detection of genetic changes in the mouse models. Measuring the response of a human tumor to drugs/therapies will be done faster and more accurately using XenoQ™ Assays while significantly reducing the experimental costs of the preclinical trials.

XenoQ™ Assays have dual functionality; they can be used to quantitate gene copy number variation as well as gene expression. XenoQ™ Assays have been designed and tested using strict protocols. A method for primer design has been developed that results in the amplification of human targets and these primer sets have no specificity for mouse targets. Each primer set has been rigorously tested against both genomic DNA (gDNA) and complementary DNA (cDNA). When tested in a 384-well format, each primer set can successfully detect gene expression in as little as $\geq 60\text{ng}$ of human gDNA in a background of up to $\leq 1\mu\text{g}$ of mouse gDNA; equivalent to $\geq 0.14\text{ng}$ Hs gDNA in a background of $\leq 2.33\text{ng}$ Mm gDNA per $10\mu\text{L}$ reaction. Ct values for the gDNA-validated human primers fall in the range of 23-35. When tested against cDNA in a 384-well format, each primer set can detect human cDNA derived from an equivalent of $\geq 125\text{ ng}$ of RNA with a background of mouse cDNA derived from an equivalent of $\leq 2\mu\text{g}$ of RNA; equivalent to $\geq 0.3\text{ng}$ Hs cDNA with a background of $\leq 4.65\text{ng}$ Mm cDNA per $10\mu\text{L}$ reaction. Ct values for the cDNA-validated primers fall in the range of 22 - 36.9. cDNA was derived from Stratagene REAL-TIME PCR Human Reference Total RNA (CAT. #750500) and Stratagene REAL-TIME PCR Mouse Reference Total RNA (CAT. #750600).

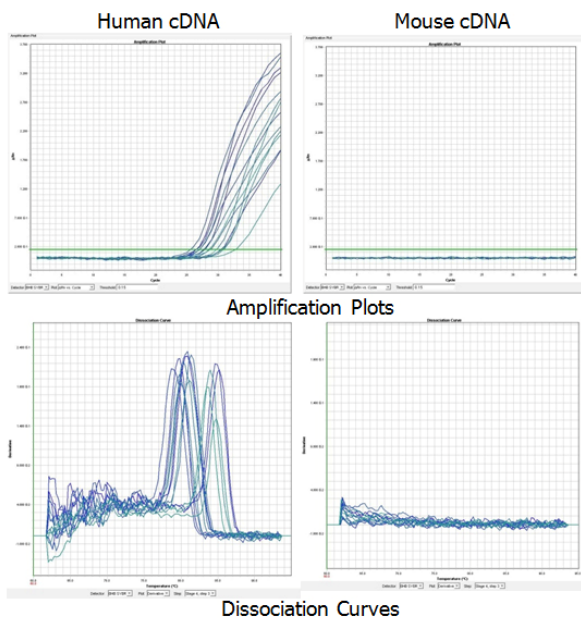
Analysis

A major complication of Real-time PCR measurements is the ability to statistically determine which genes show change. The traditional approach toward measuring gene expression changes from Real-time PCR data has been to normalize the results of a gene of interest with respect to a housekeeping gene (aka, a reference or normalizer gene). It is increasingly recognized that this conventional form of normalization is flawed because normalizer genes themselves change in expression, with the collateral effect of compromising complete Real-time PCR datasets. A major advantage of BHB's data analysis is its Global Pattern Recognition (GPR) algorithm⁽⁵⁰⁾, patent pending), which is optimally suited to identify significantly changed genes within a Real-time PCR dataset. This unique algorithm and accompanying software is a major asset that provides a true statistical analysis of results based on biological replicate consistency. Global Pattern Recognition™ Software reliably tabulates statistical significance (p-value) of gene expression changes on the fly allowing you to immediately focus on the real biology. Simply log into GPR™, select the XenoQ™ Assay that you ran on your real-time PCR instrument, upload your data and submit for analysis. An HTML or Excel® formatted file will be generated that gives a ranked list of genes by p-value, fold change value, and links to MGI and NCBI gene pages. GPR™ analyzes the data generated by the XenoQ™ Assays and determines what the real changes in gene expression are. GPR™ Software provides a true statistical analysis of results based on consistency in the data and accordingly it is optimally suited to detect small, but reproducible changes. Only after the genes are statistically ranked is the magnitude of the change calculated. A typical XenoQ™ Assay experiment would utilize 'biological replicates' (Bio-Reps). Bio-Reps are defined as samples collected from separate and closely matched biological samples. They are processed

individually under closely matched conditions. GPR™ Software processes the data derived from groups and reveals the 'constellation' of changing genes. Each constellation can be evaluated for the most likely biological context providing the researcher with a better understanding of the overall results. Each constellation may also provide the researcher with a step-wise treatment plan that can revert the tumor phenotype back to a more normal state. GPR™ Software globally positions the expression level of each gene with respect to all genes within an experiment. This can be done without prior assumption that a gene (normalizer) has an invariant expression level. GPR™ Software is unbiased in that it enables the experimental data to define the invariant normalizer genes, not the experimenter. The use of any gene as a potential normalizer also maximizes the use of the limited real-estate on XenoQ™ Assays by eliminating the loss of wells used to contain potentially erroneously predefined normalizers.

Results and Discussion

XenoQ™ Assays were validated using the following procedures. Human gDNA template was run against primer sets alone or with a background of mouse gDNA in quadruplicate reactions using a hot-start based SYBR® Green I detection system. The reactions are run using the following Applied Biosystems 7900HT Real-Time PCR System conditions: one (1) cycle of 50°C for 2 minutes, one (1) cycle of the recommended Master Mix Specific Hot Start Temperature and Time, forty-five (45) cycles of: 95°C for 10 seconds, 67°C for 20 seconds and 72°C for 40 seconds (data acquired at 72°C). The run is complete after the acquisition of a standard dissociation curve.



XenoQ™ Assay primers were QA/QC tested using both genomic and complementary DNA. The above figures depict amplification plots and dissociation curves derived from XenoQ™ Assays performed using human or mouse cDNA. XenoQ™ Assays guarantee no mouse cDNA cross-reactivity.

The first round of evaluation begins with validation of primer pairs in gDNA. Primer pairs that generate a multi-peak dissociation curve (DC test) when run against human gDNA are eliminated. Once primer pairs pass the DC test, they are then evaluated using the Ct test. If the mean Ct of the quadruplicates is ≥ 37 cycles, they are eliminated. In addition, primer pairs run against mouse gDNA whose mean Ct values are ≤ 37 cycles are also eliminated. Finally, human gDNA and mouse gDNA were added

simultaneously over a range of concentrations in order to determine threshold requirements for human gDNA input and threshold allowances for background mouse gDNA. It is recommended to use ≥ 60 ng human gDNA with a background of ≤ 1 μ g mouse gDNA.

The second round of evaluation involves testing the primer pairs in cDNA. Human and mouse RNA are DNase-treated followed by the validation of its integrity using an Agilent BioAnalyzer 2100. Samples with (a) genomic contamination or (b) RNA degradation are not used. It is critical that the RNA that is used for XenoQ™ Assays is of the highest quality. Following Agilent analysis of RNA, RNA is transcribed into cDNA using the following kit: MessageSensor RT Kit (Cat#1745, AB/Ambion). Primer pairs are subjected to the same Applied Biosystems 7900HT Real-Time PCR System conditions as above. Primer pairs are eliminated as a result of two occurrences: First, any primer pair with a Ct value of > 37 cycles when run against human cDNA is eliminated. Second, any primer pair with a Ct value of ≤ 37 cycles when run against mouse cDNA is eliminated. It is recommended to use human cDNA derived from an equivalent of ≥ 125 ng of RNA and mouse cDNA derived from an equivalent of ≤ 2 μ g of RNA.

References

1. Bernard, D., Peakman, M. & Hayday, A.C. Establishing humanized mice using stem cells: maximizing the potential. *Clin Exp Immunol* **152**, 406-414 (2008).
2. Shultz, L.D., Ishikawa, F. & Greiner, D.L. Humanized mice in translational biomedical research. *Nat Rev Immunol* **7**, 118-130 (2007).
3. Nevins, J.R. & Potti, A. Mining gene expression profiles: expression signatures as cancer phenotypes. *Nat Rev Genet* **8**, 601-609 (2007).
4. St Clair, E.W. The calm after the cytokine storm: lessons from the TGN1412 trial. *J Clin Invest* **118**, 1344-1347 (2008).
5. Gonzalez, F.J. & Yu, A.M. Cytochrome P450 and xenobiotic receptor humanized mice. *Annu Rev Pharmacol Toxicol* **46**, 41-64 (2006).
6. Dick, J.E. & Lapidot, T. Biology of normal and acute myeloid leukemia stem cells. *Int J Hematol* **82**, 389-396 (2005).
7. Villadsen, R. In search of a stem cell hierarchy in the human breast and its relevance to breast cancer evolution. *APMIS* **113**, 903-921 (2005).
8. Singh, S.K. et al. Identification of human brain tumour initiating cells. *Nature* **432**, 396-401 (2004).
9. O'Brien, C.A., Pollett, A., Gallinger, S. & Dick, J.E. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445**, 106-110 (2007).
10. Ricci-Vitiani, L. et al. Identification and expansion of human colon-cancer-initiating cells. *Nature* **445**, 111-115 (2007).
11. Karnoub, A.E. et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* **449**, 557-563 (2007).
12. Yauch, R.L. et al. A paracrine requirement for hedgehog signalling in cancer. *Nature* **455**, 406-410 (2008).
13. Calin, G.A. et al. Familial cancer associated with a polymorphism in ARLTS1. *N Engl J Med* **352**, 1667-1676 (2005).
14. Boire, A. et al. PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell* **120**, 303-313 (2005).
15. Becher, O.J. & Holland, E.C. Genetically engineered models have advantages over xenografts for preclinical studies. *Cancer Res* **66**, 3355-3358, discussion 3358-3359 (2006).
16. Sharpless, N.E. & Depinho, R.A. The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat Rev Drug Discov* **5**, 741-754 (2006).
17. Jaffe-S Ongoing battle over transgenic mice. *The Scientist* **18** (2004).
18. Rygaard, J. & Povlsen, C.O. Heterotransplantation of a human malignant tumour to "Nude" mice. *Acta Pathol Microbiol Scand* **77**, 758-760 (1969).
19. Nehls, M., Pfeifer, D., Schorpp, M., Hedrich, H. & Boehm, T. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* **372**, 103-107 (1994).

20. Ganick, D.J., Sarnwick, R.D., Shahidi, N.T. & Manning, D.D. Inability of intravenously injected monocellular suspensions of human bone marrow to establish in the nude mouse. *Int Arch Allergy Appl Immunol* **62**, 330-333 (1980).
21. Fogh, J., Fogh, J.M. & Orfeo, T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* **59**, 221-226 (1977).
22. Kelland, L.R. Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development. *Eur J Cancer* **40**, 827-836 (2004).
23. Bosma, G.C., Custer, R.P. & Bosma, M.J. A severe combined immunodeficiency mutation in the mouse. *Nature* **301**, 527-530 (1983).
24. Mombaerts, P. et al. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869-877 (1992).
25. Lapidot, T. et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**, 645-648 (1994).
26. Shultz, L.D. et al. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol* **154**, 180-191 (1995).
27. Shultz, L.D. et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* **174**, 6477-6489 (2005).
28. Ishikawa, F. et al. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. *Blood* **106**, 1565-1573 (2005).
29. Sugamura, K. et al. The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annu Rev Immunol* **14**, 179-205 (1996).
30. Miyakawa, Y. et al. Establishment of a new model of human multiple myeloma using NOD/SCID/gammac(null) (NOG) mice. *Biochem Biophys Res Commun* **313**, 258-262 (2004).
31. Takenaka, K. et al. Polymorphism in Sirpa modulates engraftment of human hematopoietic stem cells. *Nat Immunol* **8**, 1313-1323 (2007).
32. Troiani, T. et al. The use of xenograft models for the selection of cancer treatments with the EGFR as an example. *Crit Rev Oncol Hematol* **65**, 200-211 (2008).
33. Bibby, M.C. Orthotopic models of cancer for preclinical drug evaluation: advantages and disadvantages. *Eur J Cancer* **40**, 852-857 (2004).
34. Tassone, P. et al. Combination therapy with interleukin-6 receptor superantagonist Sant7 and dexamethasone induces antitumor effects in a novel SCID-hu In vivo model of human multiple myeloma. *Clin Cancer Res* **11**, 4251-4258 (2005).
35. Mitsiades, C.S. et al. Fluorescence imaging of multiple myeloma cells in a clinically relevant SCID/NOD in vivo model: biologic and clinical implications. *Cancer Res* **63**, 6689-6696 (2003).
36. Schutte, M. et al. Exon expression arrays as a tool to identify new cancer genes. *PLoS ONE* **3**, e3007 (2008).
37. Maitra, A., Wistuba, II & Gazdar, A.F. Microdissection and the study of cancer pathways. *Curr Mol Med* **1**, 153-162 (2001).
38. Bandapalli, O.R. et al. Global analysis of host tissue gene expression in the invasive front of colorectal liver metastases. *Int J Cancer* **118**, 74-89 (2006).

39. Schwartz, D.R. et al. Hu/Mu ProtIn oligonucleotide microarray: dual-species array for profiling protease and protease inhibitor gene expression in tumors and their microenvironment. *Mol Cancer Res* **5**, 443-454 (2007).
40. Liotta, L.A. & Kohn, E.C. The microenvironment of the tumour-host interface. *Nature* **411**, 375-379 (2001).
41. Nakamura, M. & Suemizu, H. Novel metastasis models of human cancer in NOG mice. *Curr Top Microbiol Immunol* **324**, 167-177 (2008).
42. Brinkman, B.M. Splice variants as cancer biomarkers. *Clin Biochem* **37**, 584-594 (2004).
43. Zhang, C. et al. Profiling alternatively spliced mRNA isoforms for prostate cancer classification. *BMC Bioinformatics* **7**, 202 (2006).
44. Cuperlovic-Culf, M., Belacel, N., Culf, A.S. & Ouellette, R.J. Data analysis of alternative splicing microarrays. *Drug Discov Today* **11**, 983-990 (2006).
45. Freedomia Group, Freedomia Focus on Biotechnology: Pharmaceuticals. 1-23 (2007).
46. Kola, I. & Landis, J. Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov* **3**, 711-715 (2004).
47. Redon, R. et al. Global variation in copy number in the human genome. *Nature* **444**, 444-454 (2006).
48. Hollox, E.J. et al. Psoriasis is associated with increased beta-defensin genomic copy number. *Nat Genet* **40**, 23-25 (2008).
49. Weiss, L.A. et al. Association between microdeletion and microduplication at 16p11.2 and autism. *N Engl J Med* **358**, 667-675 (2008).
50. Akilesh, S., Shaffer, D.J. & Roopenian, D. Customized molecular phenotyping by quantitative gene expression and pattern recognition analysis. *Genome Res* **13**, 1719-1727 (2003).
51. Stangegaard, M., Dufva, I.H. & Dufva, M. Reverse transcription using random pentadecamer primers increases yield and quality of resulting cDNA. *Biotechniques* **40**, 649-657 (2006).
52. Ross, D.M., Watkins, D.B., Hughes, T.P. & Branford, S. Reverse transcription with random pentadecamer primers improves the detection limit of a quantitative PCR assay for BCR-ABL transcripts in chronic myeloid leukemia: implications for defining sensitivity in minimal residual disease. *Clin Chem* **54**, 1568-1571 (2008).
53. Andersson, A., Bernander, R. & Nilsson, P. Dual-genome primer design for construction of DNA microarrays. *Bioinformatics* **21**, 325-332 (2005).
54. Liu, S., Tinker, N.A., Molnar, S.J. & Mather, D.E. EC_oligos: automated and whole-genome primer design for exons within one or between two genomes. *Bioinformatics* **20**, 3668-3669 (2004).

Contact Information

Bar Harbor BioTechnology, Inc

18 River Field Road

Trenton, ME 04605

Phone: 1.877.BHB.4441

E-mail: sales@barharborbio.com

Website: <http://www.bhbio.com/products/xenog>