

# Whole-transcript Expression Analysis

## Gene Expression



Historically, microarrays have interrogated the few hundred bases proximal to the 3' end of each gene, and used expression at the 3' end to approximate expression of the entire gene. This approach is compatible with the 3' oligo(dT)-based priming and labeling assays and provides valuable insight into global gene expression. However, this approach assumes that the 3' end of each gene is clearly defined, that each transcript has an intact poly-A tail and that the entire length of the gene is expressed as a single unit.

These assumptions, however, do not apply to all genes or all samples. More than 60 percent of genes are known to be alternatively spliced<sup>1,2,3,4</sup>, yielding hundreds of thousands of transcript variants with potentially distinct functions. As many as 50 percent of disease-related point mutations may result in splice pattern changes<sup>5</sup>, and 20 percent of cancer-causing mutations can result in exon-skipping events.

Unfortunately, classical 3' expression microarrays do not discriminate between alternatively spliced transcripts that have identical 3' ends. Transcripts lacking a 3' exon because of alternative splicing, non-polyadenylation, genomic deletions or other non-canonical genomic events are not detected in 3' based expression experiments.

Genomic locus	Classical 3' Assay	WT Assay
Presumed standard transcript	●	●
Transcripts with undefined 3' end		●
Non-polyadenylated messages		●
Truncated transcripts		●
Alternative polyadenylation sites		●
Degraded samples		●
Genomic deletions		●
Alternative splicing		●
Alternative 5' start sites		●

Figure 1: Types of transcripts captured by a whole-transcript assay. Most of these cannot be detected with the classical 3' assay.

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### APPLICATION FOCUS

This Application Note describes how researchers are using Affymetrix' new whole-transcript expression profiling tools to characterize disease etiology and molecular mechanisms with a new level of resolution and accuracy. By utilizing the GeneChip® Whole Transcript (WT) Assay and Affymetrix' high-density microarrays to explore diseases like colon and brain cancer, these experiments have already revealed new mechanistic pathways, suggested potential treatment targets and identified new splicing regulatory mechanisms.

Affymetrix' whole-transcript analysis approach enables researchers to detect not only the level of expression, but also precisely what is being expressed, including alternative isoforms or genomic deletions. This has opened the door to new insights at a resolution not possible with the classical 3'-based microarrays.

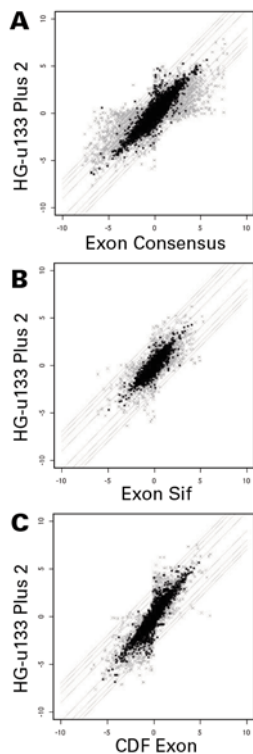


Figure 2: Scatterplots showing high correspondence between Affymetrix exon and standard expression arrays (Okoniewski M., *et al.*<sup>6</sup>).

“Since the classical microarrays have already been repeatedly validated experimentally, this provides strong evidence that exon arrays are also reliable, not only for probesets that can be successfully mapped to the existing arrays, but also for the many thousands of additional probesets that provide a more detailed coverage of the transcriptome.”

— Okoniewski M., *et al.*<sup>6</sup>

The combination of Affymetrix’ Whole Transcript (WT) Assay and high-density arrays, including GeneChip® Exon 1.0 ST Arrays and GeneChip® Gene 1.0 ST Arrays, provides a more complete and more accurate picture of overall gene expression, enabling researchers to detect transcript isoforms they didn’t know existed, and to discriminate between transcripts that previously appeared to look the same (Figure 1).

### Affymetrix’ Complete Product Solution for High-definition Expression Profiling

Affymetrix provides a complete product solution, standardized procedures, and world-class technical support to help researchers easily and quickly obtain biologically significant results in their research. The complete high-definition expression profiling system includes:

- High-density microarrays
- Conveniently packaged target amplification and labeling, array processing and various process control reagents
- Automated array processing with the Fluidics Station
- High-resolution scanner
- Basic analysis software
- Freely accessible online probe sequence and annotation resource
- Genome context design and array results viewer
- Complementary third-party statistical and pathway analysis tools

### Human, Mouse and Rat Exon 1.0 ST Arrays

With approximately four probes per exon and roughly 40 probes per gene, GeneChip® Exon 1.0 ST Arrays provide data for two complementary

levels of expression analysis in a single experiment—“exon-level” and “gene-level” analysis.

Exon-level analysis of multiple probes per exon provides the highest resolution microarray analysis, with the ability to analyze alternative splicing and differential expression of each exon within a gene. On a whole-genome level, this enables researchers to detect not only the level of expression, but also precisely what is being expressed. These differences may play a critical role in disease susceptibility and etiology.

In gene-level expression analysis, multiple probes on different exons are summarized into a single expression-level data point that represents all transcripts derived from each gene.

Exon arrays provide the most comprehensive coverage of all microarray designs, including empirically supported and predicted transcripts. The high level of coverage maximizes researchers’ ability to identify known and novel splicing events and make groundbreaking discoveries. Options are available to restrict analysis to subclasses of sequences, for a faster preview of the biology.

In addition, exon array probe design is based on genomic sequence, not UniGene clusters, so sequence annotations can be updated easily with each new genome build. With the annotations of each probe on the array anchored to the genome, the design makes it easy to integrate various types of genomic information—SNPs, mutations, chromosome deletions and amplifications—by referring to the same genomic structure. This enables rapid scientific advances from a systems biology perspective.

### GeneChip® Human Gene 1.0 ST Array

The Human Gene 1.0 ST Array, supported by the same whole-transcript assay as the exon array, is the latest addition to the family of Affymetrix' expression arrays offering whole-transcript coverage. Each of the well-annotated genes is represented by approximately 26 probes on the array spread across the full length of the gene, providing a more complete and more accurate picture of gene expression than 3'-based expression array designs. The Human Gene 1.0 ST Array utilizes a subset of the probes selected from the Exon 1.0 ST Array and focuses on well-annotated content at the gene level.

The Human Gene 1.0 ST Array—the most advanced and cost-effective gene expres-

sion profiling option for new microarray users—allows researchers to integrate microarray-based gene expression profiling more routinely in their research.

### Whole-transcript Array Results Validated by 3' Expression Microarrays

Recent studies demonstrate that results from high-resolution, whole-transcript Affymetrix arrays are consistent with those of Affymetrix' proven 3' gene expression microarrays. Okoniewski, *et al.*<sup>6</sup> recently compared the gene expression profiles of two established cell lines on the Exon 1.0 ST Array and classical 3'-based design, the GeneChip® Human Genome U133 Plus 2.0 Array. Using three mapping techniques, the two arrays showed a high degree of correspondence in terms of fold changes (Figure 2).

### EXON-LEVEL ANALYSIS IN PRACTICE

In addition to more accurate gene expression analysis, exon arrays provide the unique opportunity to unveil changes that occur along the entire length of the gene. Researchers are already detecting specific alterations in exon utilization that may play a critical role in disease mechanism and etiology.

#### Uncover Aberrant Splicing Events Linked to Disease State

Using Exon 1.0 ST Arrays, researchers at Millennium Pharmaceuticals have confirmed that specific "cassette" exons in the CD44 gene are highly expressed in primary tumor colon cancer cell lines, but not in metastatic colon cancer or HeLa cell lines. The team was able to monitor different exons independently because the array design covers the entire CD44 transcript, including the central part of the gene

where these variant cassette exons are located.

Their results suggest that CD44 splice variants might be used as a diagnostic or prognostic marker for colon cancer.

Figure 3 is a schematic representation of the Exon 1.0 ST Array's coverage of the CD44 locus, created with the Affymetrix Integrated Genome Browser (IGB). It shows different known RefSeq splice variants of CD44 in green. Exon 1.0 ST Array cov-

### PREVALENCE OF ALTERNATIVE SPLICING EVENTS

"A number of studies by different groups all reported finding alternative splice forms in a surprisingly large fraction of human genes, ranging from 40 percent to 60 percent."

— Lee C. and Roy M.<sup>1</sup>

"Adding to previous studies, the results indicate that at least 74 percent of human multi-exon genes are alternatively spliced." — Johnson, *et al.*<sup>2</sup>

### IMPORTANCE OF ALTERNATIVE SPLICING TO DISEASE RESEARCH

"Recent studies indicate that 70 to 88 percent of alternative splices change the protein product. The majority of these changes appear to be functionally interesting."

— Modrek B. and Lee C. J.<sup>4</sup>

erage of the CD44 gene is shown in blue and HG-U133 probe sets are shown in pink. The Exon 1.0 ST Array provides broad coverage of the entire CD44 transcript, including, but not restricted to the established RefSeq exons. This coverage enables researchers to monitor differential exon expression and uncover previously unidentified novel events.

Additionally, Gardina, *et al.*<sup>7</sup> demonstrated the ability of the Human Exon 1.0 ST Array to detect alternative splicing and differential gene expression in colon cancer samples compared with matched normal control tissues.

Their results suggest that aberrant splicing might be the mechanism of action for colon cancer, after finding several aberrant splicing events affecting the same functional pathways of cell architecture and the extracellular matrix. These results correlate extremely well with known cancer genes, pathways and many of the splicing events identified by the microarray data were subsequently verified by RT-PCR.

### Discover Causative Genes of a Disease by Identifying Unique Exon-skipping Events

Drs. Pim French and Justine Peeters of Erasmus Medical Center in the Netherlands discussed their work using the Human Exon 1.0 ST Array to classify glial tumors in the Summer 2006 issue of the *Affymetrix Microarray Bulletin*<sup>8</sup>.

French and Peeters used exon arrays to discover expression profiles with distinct splice variants that can be used to distinguish glioblastomas from oligodendrogliomas and help clinicians more accurately diagnose the multiple classes and variable prognoses of brain cancer.

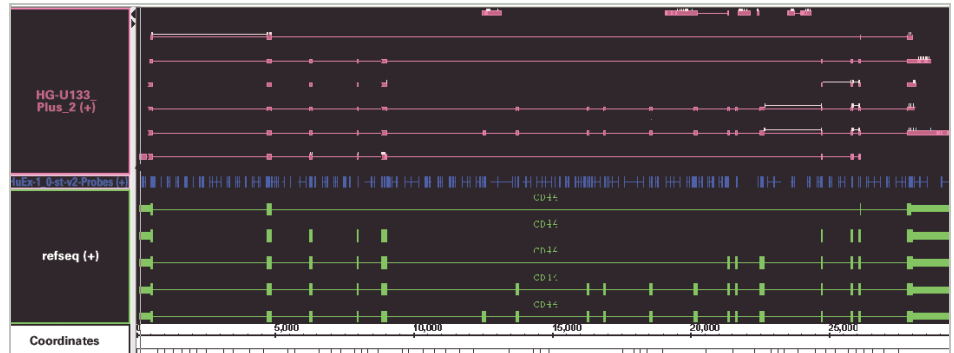


Figure 3: The Exon 1.0 ST Array provides coverage of the entire CD44 gene. Known RefSeq variants of CD44 are shown in green. Exon 1.0 ST Array coverage of the CD44 gene is shown in blue and HG-U133 probe sets are shown in pink.

The Erasmus team also used exon arrays to systematically survey the genome and compare different tumor samples to identify novel exon-skipping events and associated genes in individual patients. French and Peeters estimated that approximately 20 percent of mutations in glial tumors cause exon skipping; aberrant transcripts affected by exon skipping are potentially cancer-causing. They confirmed the findings from the exon arrays by sequencing regions surrounding the genes in which exon skipping occurred. The team identified mutations in those regions that could contribute to the exon-skipping phenotype, leading to discovery of novel potential targets for glioma cancer therapies.

### Uncover Splicing Regulatory Mechanisms

A recent proof-of-concept study by Bruno, *et al.*<sup>9</sup> at The University of Texas M. D. Anderson Cancer Center used exon arrays to examine exon skipping in the FGFR gene in glioma cell lines.

The team hypothesized that binding of the splicing inhibitory factor, PTB, to intronic splicing regulatory sequences within the FGFR gene, caused skipping of exon 3 in glioma cells. This hypothe-

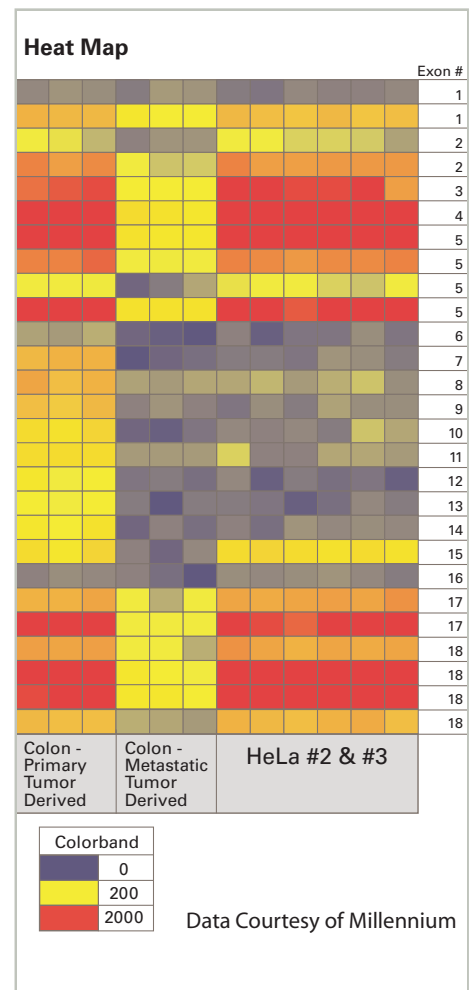


Figure 4: This heatmap image from Millennium scientists illustrating differential expression of CD44 exons in metastatic tumor versus primary tumor versus control HeLa cells.

sis was supported by evidence that PTB was over-expressed in glioma cells.

To directly test the hypothesis, the M. D. Anderson team treated human glioblastoma cells with antisense oligonucleotides targeting the intronic splicing regulatory sequences within the FGFR gene, to compete for binding with the PTB factor. If PTB contributes to the skipping event, then the antisense treatment should reverse the phenotype and trigger increased inclusion of exon 3. They evaluated whether exon arrays could be used to detect a change in exon 3 inclusion. Statistical analysis of the exon array data correctly identified the differential exon skipping of the FGFR gene with enhanced exon 3 inclusion as a result of antisense oligo treatment (Figure 5).

### Associate Splicing Patterns with Inherited SNPs

Dr. Jacek Majewski of McGill University and Genome Québec Innovation Centre discussed the detection of known and novel cases of alternative splicing using Affymetrix exon arrays during an October 24, 2006 *Affymetrix Microarray Bulletin* Symposia conference call<sup>10</sup>.

His group ran the CEPH samples from the International HapMap Project on the Affymetrix Exon 1.0 ST Array and demonstrated several differences in splicing patterns supported by linkage and/or association analyses, suggesting that they have underlying genetic causes.

Majewski's team also investigated how exon expression profiles segregated according to HapMap families. In several cases, they found SNP changes that resulted in exon skipping and alternative splicing.

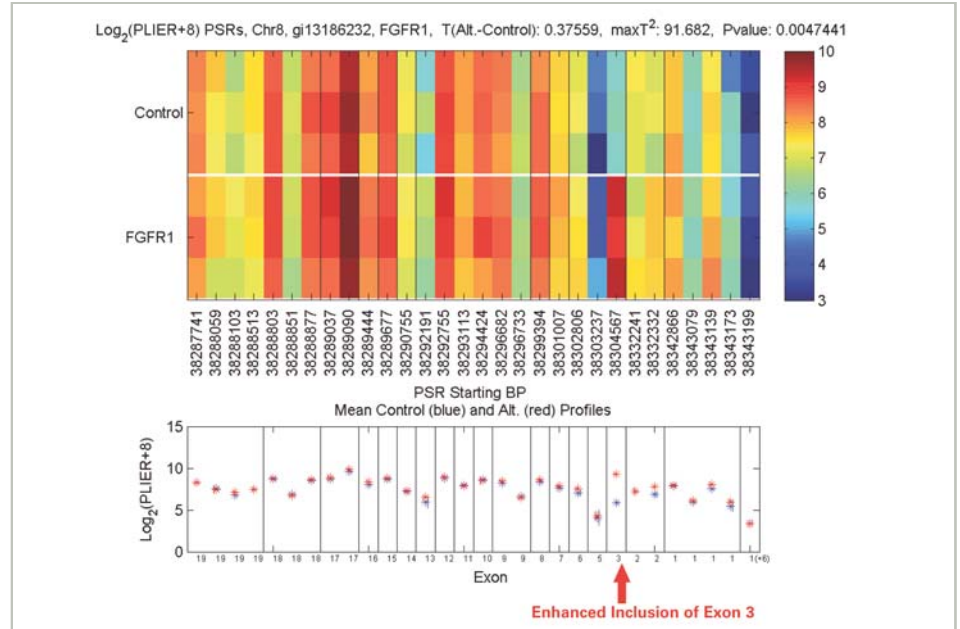


Figure 5: Heatmap image showing enhanced inclusion of exon 3 in FGFR alleles8.

### AFFYMETRIX HIGH-DEFINITION EXPRESSION PROFILING TOOLBOX

#### Assay and Reagent Solutions

Affymetrix provides a single source for highly reproducible, robust and consistent reagents. The complete system so-

lution for Exon 1.0 ST and Gene 1.0 ST Arrays includes WT assay reagent kits for sample amplification and labeling. Random-priming and linear amplification combined with a novel, robust fragmentation and labeling strategy enables researchers to generate targets along the entire length of the transcript.

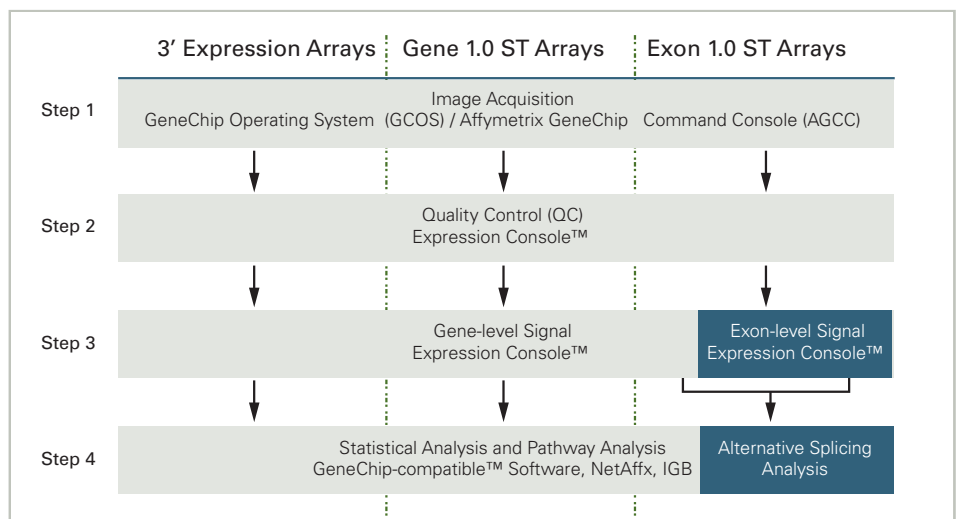


Figure 6: The data analysis workflow for Gene 1.0 ST and Exon 1.0 ST Arrays is similar to that of 3' expression arrays, utilizing GCOS or Affymetrix GeneChip Command Console (AGCC), Expression Console, GeneChip-compatible software, the NetAffx Analysis Center and IGB.

The 1 µg total RNA labeling protocol, which includes an initial rRNA removal procedure for optimal sensitivity, is required on exon arrays to obtain gene expression and alternative splicing information. The 100 ng total RNA labeling protocol—which omits the rRNA reduction step, offering the same high-level performance on the gene array with reduced hands-on time—is recommended for use with gene arrays.

Affymetrix WT Reagents include:

- PolyA Control Kit
- WT cDNA Synthesis and Amplification Kit
- WT Terminal Labeling Kit
- Sample Cleanup Module
- IVT cRNA Cleanup Kit
- Hybridization Controls
- Hybridization, Wash and Stain Kit

## DATA ANALYSIS SOLUTIONS

### Gene-level Analysis

Identifying and prioritizing gene-level expression changes using Exon 1.0 ST and Gene 1.0 ST Arrays is as simple as 3' expression analysis, and the workflows are similar. The GeneChip® Operating System (GCOS) or Affymetrix GeneChip® Command Console (AGCC) is used for initial image acquisition. Affymetrix Expression Console™ software then provides an easy-to-use analysis workflow for quality control (QC) and probe set summarization to attain gene-level signal data.

Affymetrix partners with a number of GeneChip®-compatible™ software providers, who provide statistical analysis solutions for generating lists of differentially regulated genes. Additionally, multiple software providers offer solutions for

pathway analysis—building and visualizing potential gene interactions by leveraging databases of published literature.

The NetAffx™ Analysis Center, freely available from Affymetrix, enables researchers to correlate these results with array design and annotation information. The Integrated Genome Browser (IGB) provides visualization tools to further explore genomes and corresponding annotations from multiple data sources.

### Exon-level Analysis

Expression Console also enables researchers to compute exon-level signal estimates for exon array data. To predict alternative transcript forms, GeneChip-compatible software that supports the analysis of Exon 1.0 ST Array data (Table 1) can be used to conduct statistical analysis of gene-level and exon-level sig-





	 <b>Biotique's Xray</b>	 <b>Genomatix' Chiplnspector</b>	 <b>JMP Genomics</b>	 <b>Partek® Genomics Suite™</b>
Supports analysis of GeneChip Human Gene 1.0 ST Array	Yes	Yes	Yes	Yes
Supports analysis of GeneChip Exon Arrays	Yes	Yes	Yes	Yes
Supports analysis for 3' expression arrays	No	Yes	Yes	Yes
Other GeneChip-compatible offerings	—	Regulation (ChIP-on-chip)	SNP Analysis	<ul style="list-style-type: none"> <li>• Tiling (Regulation, ChIP-on-chip)</li> <li>• SNP Analysis</li> <li>• Chromosomal Copy Number &amp; LOH Analysis</li> </ul>
Application Highlights	<ul style="list-style-type: none"> <li>• Fast MS Excel add-in offers familiar Excel interface</li> <li>• Rigorous normalization and analysis for unlimited numbers of CEL files on computers with 500 MB ram</li> <li>• Dynamically generated methods, results and array quality document suitable for publications</li> </ul>	<ul style="list-style-type: none"> <li>• Analysis based on single probes</li> <li>• Single probe curation based on latest genome annotation</li> <li>• Currently 37 arrays (12 genomes) supported, including promoter tiling arrays</li> <li>• Tight integration with other downstream Genomatix tools to derive greater biological insight</li> </ul>	<ul style="list-style-type: none"> <li>• Powerful SAS analytics enhanced by interactive JMP graphics</li> <li>• Sophisticated, automated experimental design tools</li> <li>• Quality control (QC) tools including batch effect removal</li> <li>• Extensive predictive modeling capabilities</li> </ul>	<ul style="list-style-type: none"> <li>• Fast and memory-efficient</li> <li>• Comprehensive statistics and interactive visualization</li> <li>• Removes batch effects</li> <li>• Easy-to-use workflows for exon, copy number and tiling arrays</li> <li>• Integration of results from multiple GeneChip technologies such as expression, regulation and copy number</li> <li>• Build and validate diagnostic and prognostic classifiers</li> </ul>
Website	<a href="http://www.orderXRAY.com">www.orderXRAY.com</a>	<a href="http://www.genomatix.de">www.genomatix.de</a>	<a href="http://www.jmp.com/genomics">www.jmp.com/genomics</a>	<a href="http://www.partek.com">www.partek.com</a>

Table 1: GeneChip-compatible products supporting both gene expression and alternative splicing analysis.

nal data. Pathway analysis, annotations from the NetAffx Analysis Center and IGB analysis of exon array data can all be conducted using workflows similar to 3' expression array data. For an introduction on managing data provided by exon arrays, see the Technical Note, *Identifying and Validating Alternative Splicing Events*, located at [www.affymetrix.com/support](http://www.affymetrix.com/support).

### Partners Providing GeneChip-compatible Products

Several Affymetrix partners provide GeneChip-compatible products for gene expression analysis, pathway analysis and alternative splicing analysis at the exon level.

### Additional Solutions and Resources

In addition to arrays, reagents and assays, Affymetrix offers instruments for streamlined array processing, including

the GeneChip® Scanner 3000 7G for array scanning and GeneChip Fluidics Station 450.

Affymetrix also provides a variety of supporting materials, which can be accessed via the Exon 1.0 ST and Gene 1.0 ST Array product pages at [www.affymetrix.com](http://www.affymetrix.com). This toolbox includes:

- Publications and references lists
- Webinars focused on alternative splicing and expression analysis using exon arrays
- GeneChip-compatible software demonstrations
- NetAffx Analysis Center, Expression Console, and IGB analysis demonstrations
- Technical Notes
- *Affymetrix Microarray Bulletin* interviews with researchers using exon arrays

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Gene-level Analysis Solutions	Pathway Analysis Solutions
Applied Maths' GeneMaths XT BioDiscovery's GeneSight Genedata's Expressionist® Genomatix' ChipInspector IMC's TeraGenomics SAS' JMP® Microarray Ocimum Biosolutions' Genowiz Partek® Genomics Suite Rosetta Biosoftware's Rosetta Resolver® System Spotfire's DecisionSite® for Functional Genomics Spotfire's DecisionSite® for Microarray Analysis Stratagene's ArrayAssist® VizX Labs' GeneSifter®	Ariadne Genomics' Pathway Studio® GeneGo's MetaCore Genomatix' BiblioSphere Pathway Edition Ingenuity Systems' Ingenuity Pathways Analysis (IPA) Stratagene's PathwayArchitect®

Table 2: GeneChip-compatible products supporting pathway analysis and gene-level statistical analysis.

## ORDERING INFORMATION

## Affymetrix WT Assay and Expression Arrays

Products	Species	Configuration	Content	Part Number	
GeneChip® Exon1.0 ST Arrays – high-definition single array for expression and alternative splicing	Human	Starter Pack	30 Arrays 30 reactions of reagents On-site training	900654	
		30-pack	30 arrays	900651	
		6-pack	6 arrays	900650	
		2-pack	2 arrays	900649	
		Mouse	Starter Pack	30 Arrays 30 reactions of reagents On-site training	900831
			30-pack	30 arrays	900819
	6-pack		6 arrays	900818	
	2-pack		2 arrays	900817	
	Rat		Starter Pack	30 Arrays 30 reactions of reagents On-site training	900848
			30-pack	30 arrays	900822
		6-pack	6 arrays	900821	
		2-pack	2 arrays	900820	
GeneChip® Human Gene 1.0 ST Arrays for gene expression analysis		Human	30-pack	30 arrays	901087
			6-pack	6 arrays	901086
	2-pack		2 arrays	901085	
	30-pack		30 arrays 30 reactions of reagents	901146	
	10-pack		10 arrays 10 reactions of reagents	901147	
	Reagents		WT Sense Target Labeling and Control Reagents	30 reactions	
	Hybridition, Wash and Stain Kit	30 reactions		90072	

	Kit		
Affymetrix Analysis Solutions	Expression Console™ NetAffx™ Analysis Center Integrated Genome Browser	Generate primary array analysis and QC Query functional annotations of array content and probe sequence Align array design and array data in the genomic context to integrate various genomic information	Freely available at <a href="http://www.affymetrix.com">www.affymetrix.com</a>

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