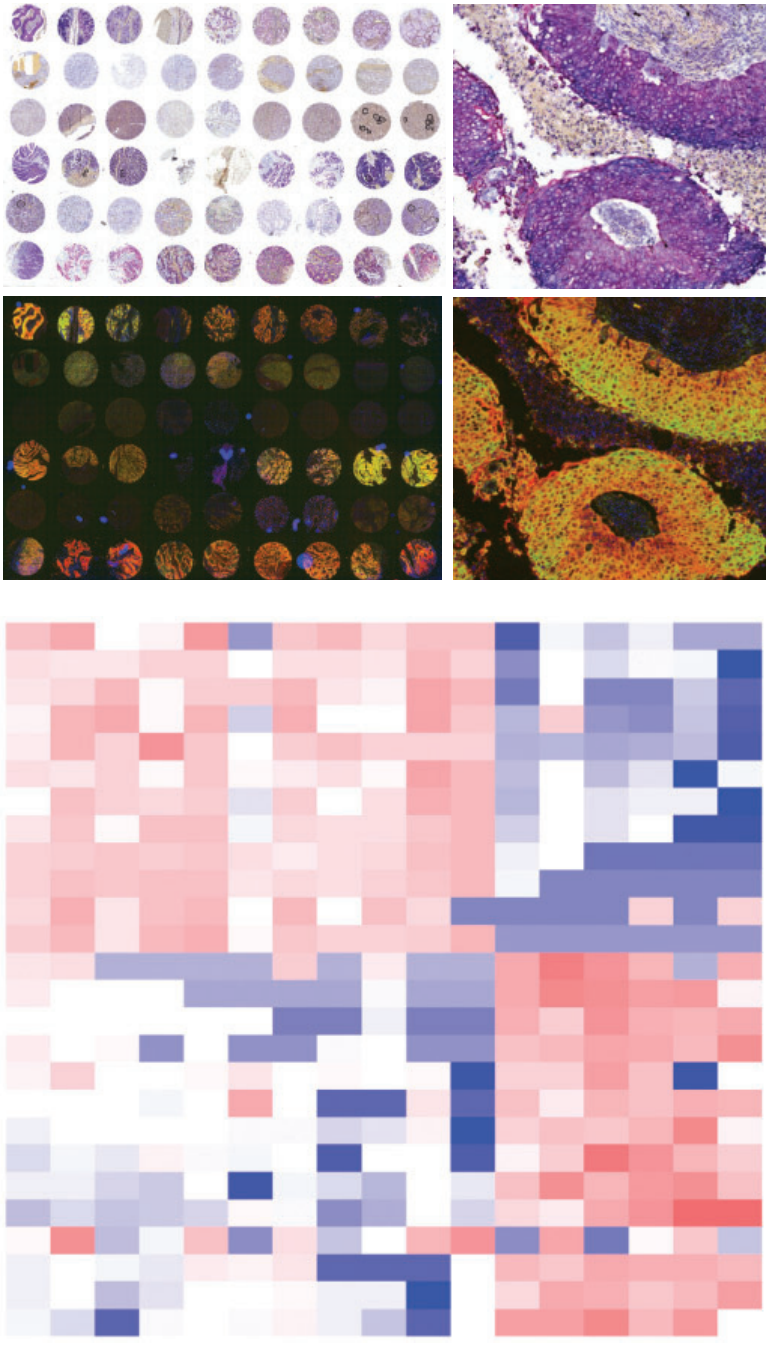
Affymetrix®
Revolutionize life

A Platform of Comprehensive Genomics Solutions for Analyzing FFPE Archival Tissues from Whole-Genome to Single Molecules



Introduction

Discovering biomarkers and developing clinical signatures from FFPE tissue

Cancer is a complex and heterogeneous disease and an increasingly leading cause of death worldwide. However, there remains much to be discovered about cancer biology, disease progression, and therapy. It is widely recognized that current treatment methods are largely ineffective for the majority of patients, and cancer researchers, pathologists, oncologists, and surgeons believe that a deeper understanding of cancer genetics will be imperative in identifying better treatment models. Meanwhile, fueled by next-generation sequencing and microarray technologies, discoveries of potentially causative mutations, structural and transcriptional variations, and related molecular events abound and will need to be replicated, validated, and ultimately translated into medical practice.

To tackle the challenge of validation, researchers believe that formalin-fixed paraffin-embedded (FFPE) cancerous tissues, especially those with good clinical annotation, will prove to be an important resource to accelerate this process.

Unfortunately, there are significant inherent issues associated with the use FFPE samples. Formalin is notoriously harsh on cellular and genetic material, and storage in paraffin causes degradation over time; thus, nucleic acids extracted from archived FFPE samples can be difficult to analyze due to nucleic acid fragmentation, nucleotide base modification, and cross-linking of nucleic acids to protein within the sample matrix. Additionally, PCR reactions of DNA and RNA derived from FFPE samples may suffer from inhibition and amplification bias, leading to poor sensitivity and accuracy. Tissue samples are typically heterogeneous combinations of cancer cells, some of which may be genetically variable as well as being contaminated with normal cells. Furthermore, tissues of interest are typically only available in small quantities, presenting challenges when multiple genes and replicate measurements are required. As such, researchers will benefit most from genetic analysis technologies that can perform well with small amounts of likely heterogeneous and often degraded nucleic acids.

Multiple platforms optimized for FFPE samples

Affymetrix has pioneered advances in new molecular techniques that enable effective genomic analysis of these precious FFPE samples.

The application of genomics technologies to FFPE sample analysis for the discovery and validation of clinical biomarkers typically falls into one of two strategic paradigms (Figure 1). In the first paradigm, microarrays could be applied to every stage of a development pipeline, from gene and transcript discovery to validation of microarray or *de novo* sequencing discoveries, to functional validation, and then ultimately to routine testing (Figure 1A).

In the second paradigm, microarrays and/or *de novo* sequencing are used for initial discovery to create a pool of candidate genes or transcripts. These pools are then progressively enriched to smaller and smaller subsets via a series of validation steps and then further enriched utilizing highly quantitative bead-based multiplex assays and single transcript *in situ* assays (Figure 1B).

Regardless of your strategy, Affymetrix can provide proven technologies and applications at every stage of FFPE analysis. Please see the following research product selection guide (Table 1) for a listing of products and services available from Affymetrix for your FFPE applications.

FFPE application and product overviews

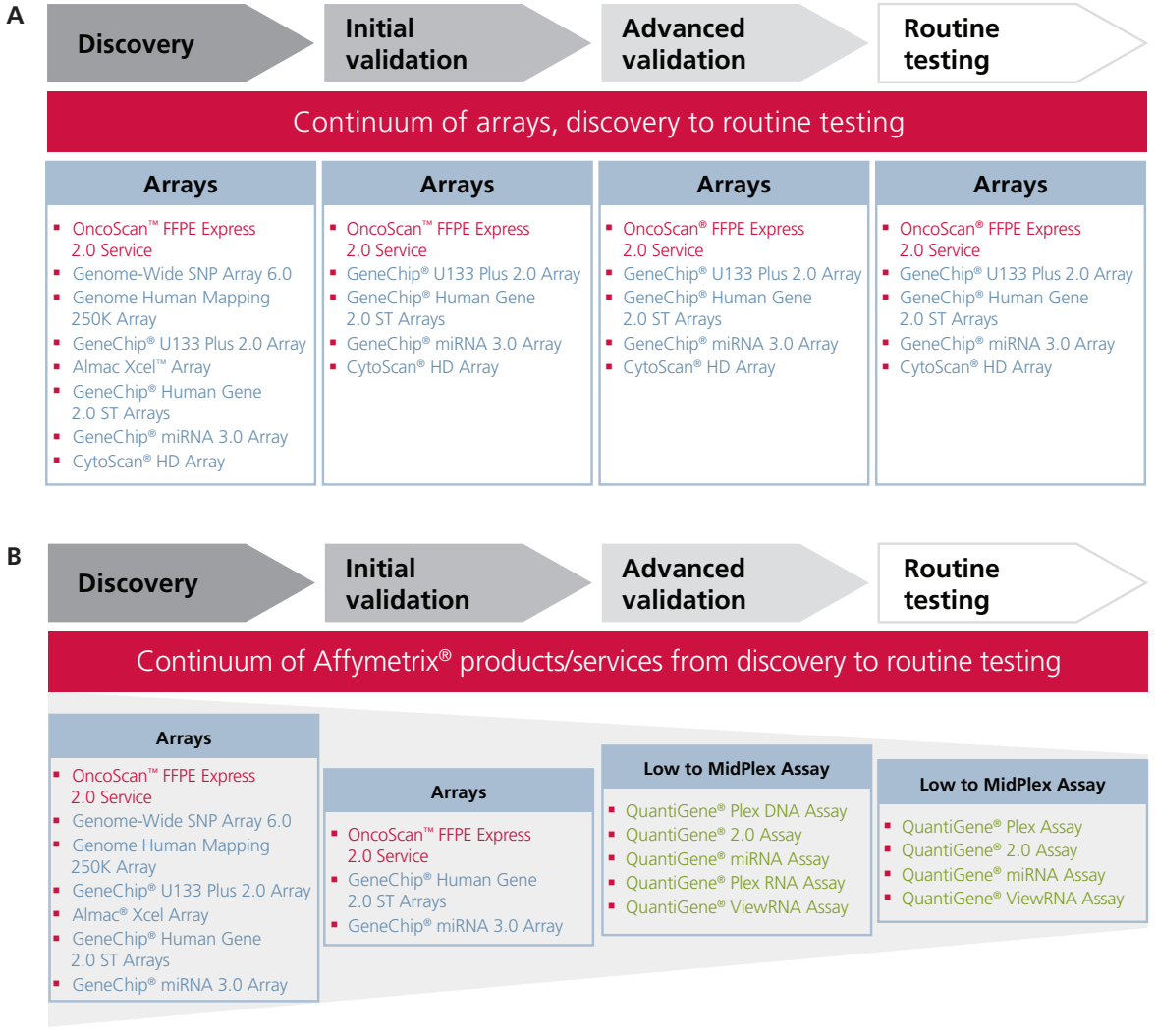


Figure 1: Applications of Affymetrix products in discovery, target enrichment, validation, and routine testing. Color coding – red: service; blue: arrays; green: assays. Source: Affymetrix, Inc.

DNA analysis

Whole-genome, high-resolution structural analysis of DNA copy number and LOH/UPD

OncoScan™ FFPE Express 2.0 Service from Affymetrix® Research Services Laboratory

The Affymetrix Research Services Laboratory offers a convenient and reliable solution for copy number analysis from problematic FFPE samples leveraging the OncoScan™ Assay.

OncoScan™ FFPE Express 2.0 Assay

OncoScan™ FFPE Express 2.0 Assay uses the unique Molecular Inversion Probe (MIP) technology, which enables exquisitely specific and sensitive detection of copy number changes from small amounts of input DNA in highly degraded FFPE samples. Each MIP probe requires only 40 bp of intact complementary DNA to bind.

Features and benefits

- Analysis of whole-genome copy number with high coverage of tumor suppressor genes and oncogenes
- Precise copy number dynamic range (0–30 copies and higher), allelic imbalance, and copy-neutral LOH data from a single sample
- Robust performance from just 75 ng of DNA from archived and highly fragmented FFPE samples

Bottom front cover image: Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: *British Journal of Cancer,* **104**:971-981, 2011.

Product selection guide

Product/service/assay	Molecule detected	Content description	Detection application
OncoScan™ FFPE Express 2.0 Service	DNA	334,000 markers	▪ Whole genome ▪ Copy number ▪ LOH
CytoScan® HD Array	DNA	2.6 million markers including 750,000 SNPs	▪ Whole genome ▪ Copy number ▪ LOH ▪ Mosaicism
Affymetrix® Genome-Wide Human SNP Array 6.0	DNA	1.8 million markers including copy number variants	▪ Whole genome ▪ SNPs ▪ Copy number ▪ LOH
GeneChip® Human Mapping 250K Array	DNA	250,000 SNPs	▪ Whole genome ▪ Copy number ▪ LOH ▪ SNPs
QuantiGene® Plex DNA Assay	DNA	33 targets/well	▪ Copy number
GeneChip® Human Genome U133 Plus 2.0 Array	RNA	~54,700 probe sets covering ~47,400 transcripts (~35,500 RefSeq) 3′ ends	▪ Biomarker discovery and clinical research ▪ Clinical test development ▪ Proven array for all disease types and human studies
Almac Xcel™ Array	RNA	~110,000 probe sets covering ~97,000 transcript 3′ ends and ~39,200 RefSeq transcripts	▪ Cancer biomarker discovery and validation of FFPE samples ▪ Premiere technology for clinical signature development
GeneChip® Human Gene 2.0 ST Arrays	RNA	~48,000 probe sets covering ~40,700 transcripts (~2 probes/exon) and ~11,000 lincRNA transcripts	▪ Whole-transcript gene expression profiling ▪ Transcript isoform detection ▪ lincRNA detection ▪ Biomarker discovery
GeneChip® miRNA 3.0 Array	RNA	miRNA 3.0 content: ▪ mature miRNAs: 1733 (human), 1,111 (mouse), 680 (rat) ▪ Pre-miRNAs (probes sets): 1658 (human), 855 (mouse), 486 (rat) ▪ Sno/scaRNAs: 2,216 (human)	▪ Global miRNA profiling ▪ miRNA biomarkers discovery
QuantiGene® 2.0 Assay	mRNA/DNA	▪ 1 mRNA or DNA/well	▪ Gene expression changes ▪ Fusion transcripts
QuantiGene® miRNA Assay	miRNA/ncRNA	▪ 1 miRNA/well	▪ Gene expression changes
QuantiGene® Plex Assay	miRNA/ncRNA	▪ 3–80 transcripts/well	▪ Gene expression changes
QuantiGene® ViewRNA Assay	mRNA/miRNA/ncRNA	▪ 1–4 RNAs/cell ▪ 1–2 RNAs/tissue	▪ Gene expression changes ▪ Single-transcript, single-cell detection

To learn more about Affymetrix, visit www.affymetrix.com.

Applications

Cancer researchers are using OncoScan™ technology to discover and validate novel clinical signatures using annotated banked and newly acquired FFPE samples. The goal is to translate these signatures into future assays for diagnosis, prognosis, and therapy response prediction. To facilitate clinical cancer research, Affymetrix has partnered with BioDiscovery, Inc., to provide the Nexus Copy Number™ Software (Figure 2) tool to enable the rapid analysis and correlation of copy number with clinical outcomes data. (PFS: Progression Free Survival, OS: Overall Survival, DOR: Drug of Response)

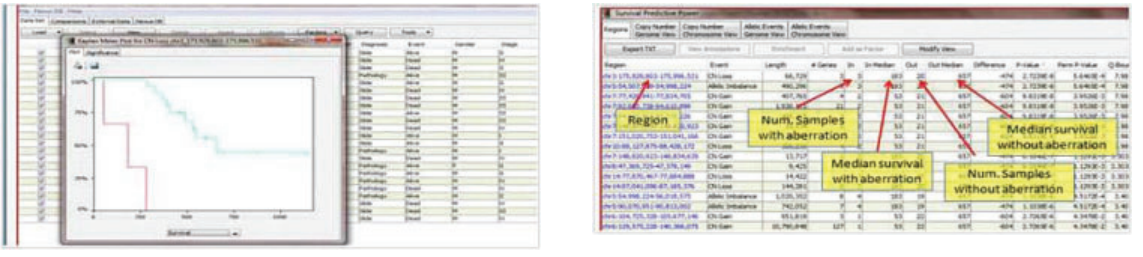


Figure 2: Data analysis and visualization by Nexus Copy Number Software. Source: BioDiscovery, El Segundo, CA.

FFPE Case 1: Analyzing copy number with somatic mutations in breast tumors using OncoScan technology

In a recent study, researchers determined copy number gains and losses using OncoScan technology in stage I/II breast tumors and identified 12 novel prognostic Copy Number Insertions (CNIs). A signature combining these novel CNIs with seven CNIs previously implicated as prognostic markers significantly improved prognostication separately for ER-, HER2+, luminal B, and triple negative tumors over clinical variables alone. In other words, the 19 CNIs signature discriminated risk of recurrence among early-stage breast tumors, independent of ER status (Figure 3).

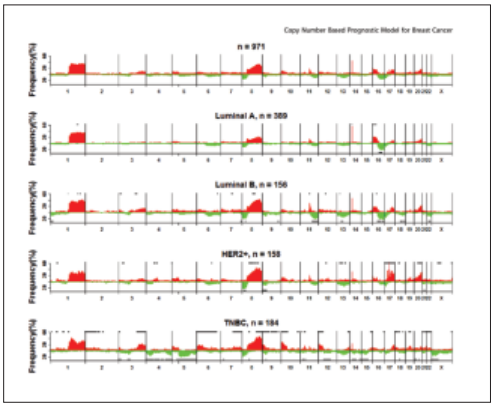


Figure 3: Pattern of CNIs for all 971 tumors and by subtype. The five panels show the percentage of samples showing gain (red) or loss (green) for all tumors (top) and individually for each clinical subtype identified using OncoScan technology. The horizontal black lines at the top (and bottom) of a panel associated with a particular clinical subtype indicate regions showing statistically significant increase in gain (and loss) frequencies (FDR <0.01) for this subtype compared with the other subtypes. Source: Thompson P. A., *et al. PLoS One* **6**(8):e23543 (2011).

Affymetrix® Genome-Wide Human SNP Array 6.0 and GeneChip® Human Mapping 250K Array

Affymetrix® Genome-Wide Human SNP Array 6.0 and GeneChip® Human Mapping 250K Array have been used frequently for cancer genome analysis of FFPE-derived DNA. Both products enable copy number detection, assessment of allelic imbalances such as LOH, as well as SNP analysis.

Loss of heterozygosity (LOH) is well documented as a common contributor to tumorigenesis leading to the loss of a wild-type allele and the unmasking of a recessive mutation. Scans of genomic copy number (CN) can reveal LOH due to hemizygous deletions, but LOH can also occur independently of a CN change when one chromosome or chromosomal region has been duplicated and its homolog has been deleted. When LOH occurs without CN change, it is commonly termed copy-neutral LOH. The ability of Affymetrix® SNP Arrays to combine CN and LOH detection for the identification of copy-neutral LOH has proven useful in many cancer research studies.

Features and benefits

- Whole-genome analysis of copy number changes, SNP genotypes, LOH, and copy-neutral LOH events
- Quality data from FFPE-derived DNA
- Proven performance—hundreds of publications, thousands of samples
- Complete reagent kit

FFPE Case 2: Analysis of somatic mutations and LOH in neuroblastoma

Affymetrix® SNP Arrays were used to compare primary neuroblastoma samples with paired blood from 22 children to detect somatic and LOH events across the genome. The majority of these LOH regions were associated with a reduction in CN, indicating that homozygosity arose due to a hemizygous deletion. Chromosome 11p also displayed frequent LOH, but in this region neither deletions nor gains were detected. Instead, for the four neuroblastoma samples exhibiting LOH on 11p, all remained diploid across the chromosome arm, indicating that frequent 11p LOH always occurred in the form of copy-neutral LOH for these samples (Figure 4).

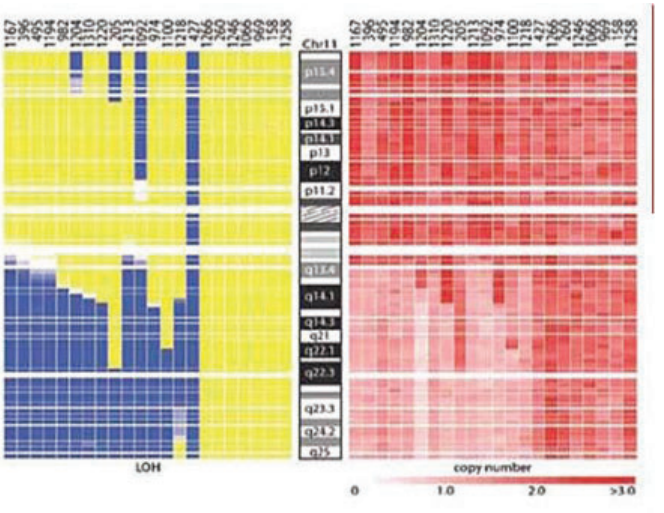


Figure 4: A total of 22 neuroblastoma tumors were characterized for both LOH (left) and CN (right) on a single SNP array. Blue = LOH; yellow = heterozygosity retained. On chromosome 11p, four samples displayed LOH without change in CN. In contrast, on chromosome 11q, 15 samples displayed LOH, 14 of which also showed an accompanying hemizygous deletion. Source: George R. E., *et al. PLoS ONE* **2**(2):e255 (2007).

QuantiGene® Plex DNA Assay

QuantiGene® Plex DNA Assay enables accurate quantitation of DNA copy number from cancer and normal FFPE tissue homogenates in a multiplex fashion without DNA extraction or PCR amplification, thus saving precious sample. In addition, QuantiGene Plex DNA Assay can be used to determine breakpoints of amplified DNA in cancer cells. With QuantiGene Plex DNA Assay, you can quantify single-copy differences in DNA copy number and multiplex up to 33 targets per well. This enables processing of more samples per plate with fewer reagents, less hands-on time, and increased accuracy.

FFPE Case 3: QuantiGene Plex multiplex DNA copy number variation analysis

Figure 5 shows the amplification of ERBB2/Her2 gene on chromosome 17 in breast cancer versus normal FFPE tissue; the multiplex panel includes other genes on chromosome 17 (PNMT, GRB7, SMARCE1, KAT7) and genes on chromosome 1 (MAPKAPK2) and chromosome 8 (FGFR1). The breakpoint on chromosome 17 lies between GRB7 (+9 kb from ERBB2) and TOP2A (+899 kb from ERBB2). As shown on the left, the ERBB2/Her2 DNA copy number correlates well with QuantiGene® ViewDNA *in situ* (FISH) determination of ERBB2/Her2 DNA copy number from the same breast cancer and normal breast FFPE tissue sections (ERBB2 [red dots]/CEP17 [green dots]).

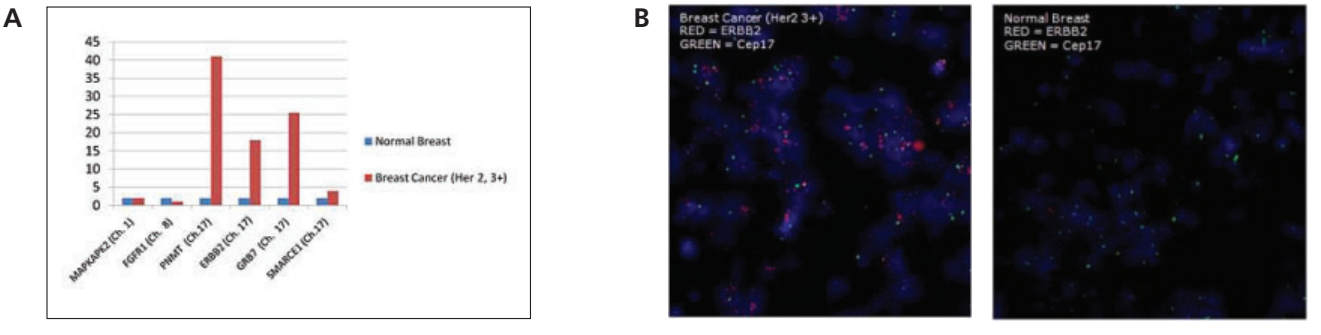


Figure 5: ERBB2/Her2 DNA copy number and chromosomal breakpoint determination in human breast cancer FFPE tissue homogenates. FFPE homogenates of normal breast tissue and breast cancer tissue (Her2, 3+) are prepared using a sample preparation kit (Affymetrix/Panomics). Homogenates are subjected to QuantiGene Plex DNA Assay using the standard protocol. Signals are normalized to MAPKAPK2. The gene copy numbers in the breast cancer tissue are determined by the ratio of signals between the two tissue types. A: QuantiGene® 8-plex DNA Copy Number Assay in breast cancer FFPE tissue and control normal breast FFPE tissue. Ratio of breast cancer/normal breast = ~7. B: DNA FISH Assay: Her2 amplified in breast cancer vs. normal breast. Ratio of breast cancer/normal breast = ~7. Source: Affymetrix, Inc.

RNA analysis

Whole-genome to single gene validation of RNA

3′ IVT arrays – GeneChip® Human Genome U133 Plus 2.0 Array

GeneChip® Human Genome U133 Plus 2.0 Array is the most widely used whole-genome expression array targeting well-characterized genes. A key advantage of the GeneChip® technology is that each array contains multiple unique probes targeting the 3′ end of the transcript, providing several independent measurements for sensitive and accurate gene expression detection. This array provides an unparalleled research publication legacy, allowing rapid data analysis and faster identification of cancer biomarkers.

Features and benefits

- Comprehensive content – complete coverage of the human genome for analysis of >35,000 transcripts
- Highest transcript coverage – 9 to11 unique probes per transcript for confident expression measurements
- High reproducibility – signal correlation >95%
- Complete workflow – flexible array formats, reagents, and instruments, enabling confident expression analysis
- Most peer-reviewed publications – thousands of published studies utilizing the GeneChip technology

FFPE case 4: The Pathwork® Tissue of Origin test

The Pathwork® Tissue of Origin test was developed using GeneChip Human Genome U133 Plus 2.0 Array. Through our Powered by Affymetrix™ Program, many companies are accelerating the development of clinical gene expression tests for routine use.

Pathwork Diagnostics has commercialized two FDA-cleared Tissue of Origin tests, one for use with FFPE and frozen tumor samples. The Pathwork Tissue of Origin test is the only 510(k) cleared molecular diagnostic test for identifying tumors of unknown origin. A study by Pillai R., *et al.* (*Journal of Molecular Diagnostics* **13**(1):48-56 (2011) showed that routine FFPE specimens can be reliably processed to yield high-quality gene expression microarray data. The Tissue of Origin test showed a high degree of agreement with the reference diagnosis for approximately nine of every 10 specimens. Tissues on the panel with SS results of ≤5 can be ruled out as the primary site with ≥99 percent likelihood. A multi-site inter-laboratory study has shown that the Tissue of Origin test results are highly reproducible, with 89.3 percent concordance between laboratories.

3′ IVT Arrays – Almac Xcel™ Array

Almac Xcel™ Array is a high-density, transcriptome-based microarray built on Affymetrix′ GeneChip® platform. Optimized for use with FFPE tissue, Xcel™ Array contains 97,000 transcripts, significantly more than is available on any other platform, and the technology is optimized for use with FFPE tissue. RNA extracted from FFPE samples tends to have a shorter median transcript length, and its analysis on generic arrays is highly unstable and non-reproducible. By designing probe sets specific to the 3′ extremities of transcripts, a much higher detection rate is possible. Almac Xcel Array is the optimal platform for biomarker discovery that enables the identification of biomarkers within multiple disease areas.

Features and benefits

- Ideal platform for biomarker discovery in multiple disease areas
- Contains more information than alternative arrays and a significant amount of proprietary information
- Optimized for use with FFPE and fresh frozen samples

To learn more about Affymetrix, visit www.affymetrix.com.

FFPE Case 5: Development and independent validation of a prognostic assay for stage II colon cancer

Almac is an expert in the optimization of high-density transcriptome-based microarrays based Affymetrix' gold standard GeneChip® platform to enable biomarker discovery and assay development. The two main product lines are the Xcel™ Array discovery platform for studies in multiple disease areas and Cancer DSA® Arrays for studies within specific disease research areas. All the arrays are optimized for use with FFPE-derived samples and have been designed using a combination of large-scale sequencing projects and carefully curated public databases.

Almac is also a Powered by Affymetrix™ partner which provides biomarker discovery and development solutions ranging from pre-clinical biomarker discovery, through full companion diagnostic development, biomarker clinical trial management, and clinical test delivery.

Almac developed a microarray-based gene signature for stage II colon cancer prognosis utilizing clinically relevant FFPE samples derived from a balanced set of 73 colon cancer patients with recurrent disease (high risk) and 142 patients with no recurrence (low risk) within five years of surgery. Source: Kennedy R. D., *et al. Journal of Clinical Oncology* **29**(35):4620-4626 (2011).

The 634-probe set signature identified high-risk patients that were confirmed in an independent validation set of 144 samples. The signature identified high-risk patients for recurrence and for cancer-related death. This gene signature represents a novel prognostic biomarker for patients with stage II colon cancer that can be applied to FFPE tumor samples using a high-density microarray.

GeneChip® Human Gene 2.0 ST Array

Affymetrix recently introduced GeneChip® Human Gene 2.0 ST Array, which offers an advanced and comprehensive gene expression profiling option for whole-transcriptome analysis—this includes more than 36,000 well-annotated mRNA and 11,086 long intergenic non-coding RNA (lincRNA) transcripts. Probes are distributed across the full length of the gene, providing a more complete and accurate picture of overall gene expression even in highly degraded RNA samples such as FFPE. Using GeneChip Human Gene 2.0 ST Array, you can analyze transcripts with alternative splicing, alternative polyadenylation, nonpolyadenylated messages, truncated transcripts, and profile degraded samples. GeneChip Human Gene 2.0 ST Array provides a confident solution for characterization of the molecular mechanisms of disease with a new level of resolution and accuracy.

Features and benefits

- Highest transcript coverage – get confident expression measurements of well-annotated content with up to 26 probes per transcript
- Whole-transcriptome analysis – capture the transcript isoforms you may miss with 3'-biased expression designs
- Simultaneous mRNA and lincRNA profiling – gain comprehensive understanding of the transcriptome
- High data correlation – achieve high inter- and intra-array strip signal correlation (R >0.99)

FFPE Case 6: Identification of expression signatures predictive of tumor sensitivity and resistance

As a potential target for novel cancer therapy, CD40 may mediate tumor regression through both an indirect immune activation and a direct cytotoxic effect on the tumor. Burington B., *et al.* (*Science Translational Medicine* **3**(74):74ra22 2011) used GeneChip® Human Gene 1.0 ST Array to analyze FFPE lymphoma samples to determine a 15-gene expression signature comprising CD40 target genes that are predictive of tumor sensitivity and resistance to CD40 stimulating therapy drugs. Identification of transcriptional variants in the CD40-activated pathway was only possible with GeneChip Human Gene 1.0 ST Arrays and would not have been possible on 3' array designs.

To learn more about Affymetrix, visit www.affymetrix.com.

GeneChip® Human Exon 1.0 ST Array

With almost 7 million probes covering 270,000 transcripts in the human genome, GeneChip® Human Exon 1.0 ST Array provides the highest resolution of exon-level and gene-level analysis available. Exon arrays provide the most comprehensive coverage of the genome, including empirically supported and predicted transcribed sequences, enabling the discovery of previously unidentified novel events.

Features and benefits

- Comprehensive coverage – detect transcriptional events that you would miss with other microarray platforms
- Two levels of analysis – measure expression of individual exons and entire genes, and detect alternative splicing information, all from a single experiment
- Superior exon-level analysis – get the most comprehensive coverage of the annotated genome, as well as predicted content for novel discovery, by interrogating more than 1 million exons

Discovery and validation of an adenocarcinoma vs. squamous cell carcinoma differentiation gene expression signature (Please refer to FFPE Case 10 of this brochure)

Using GeneChip® Human Exon 1.0 ST Arrays, Hall J. S., *et al.* (*British Journal of Cancer* **104**:971-981 2011) profiled 19 cervical squamous cell carcinoma (SCC) and nine adenocarcinoma (AC) FFPE samples from 10–16-year-old patients.

GeneChip® miRNA 3.0 Array

To advance gene expression research, Affymetrix introduced GeneChip® miRNA Array for global expression profiling of all miRNAs. This array is a powerful tool for studying the role of small non-coding RNA (microRNA, scaRNA, and snoRNA) and their importance in cancer and other diseases. MicroRNAs are small non-coding RNAs of approximately 18 to 25 nucleotides in length that are involved in gene expression regulation at the post-transcriptional level, leading to either translational repression or mRNA degradation. Additionally, microRNAs exhibit much higher stability in contrast to mRNAs, which allows expression profiling in routinely stored FFPE specimens, including samples that are more than 10 years old.

Features and benefits

- Comprehensive content – designed to independently interrogate pre-miRNA and mature miRNAs in the same experiment
- Unique content – includes snoRNAs and scaRNAs noted to be important in cancer research
- High sensitivity – detects 85% of miRNA transcripts at 1.0 amol
- High reproducibility – 0.95 reproducibility (inter- and intra-lot)
- Easy workflow – 45-minute assay without pre-amplification or purification steps
- Low sample input – requires as little as 100 ng total RNA

FFPE Case 7: Identification of liver transplant candidates

Barry C. T., *et al.* (*American Journal of Transplantation* **12**:428-437 2012) profiled miRNA from FFPE tissue samples to discover hepatocellular carcinoma (HCC) recurrence biomarkers that would allow the identification of patients who are good candidates for liver transplants, as they are at low risk of recurrence. Using a hierarchical clustering approach with a MIN-MAX refinement and a Cox regression analysis, 67 miRNA probes were found that could distinguish patients with and without HCC recurrence risk.

QuantiGene® Assays

QuantiGene® Assays are based on the clinically proven branched DNA (bDNA) signal amplification technology used in the 510(k)-cleared HIV and Hepatitis C viral load tests. QuantiGene Assays can be used to directly quantify mRNA, miRNA, ncRNA, fusion transcripts, and DNA copy number variations. QuantiGene Assays include single-plex and multiplex "grind and bind" assay formats for cell lysates and FFPE tissue homogenates as well as *in situ* hybridization assay formats for cell and FFPE tissue analysis. QuantiGene Assays work well on FFPE samples and do not suffer the issues common to PCR-based quantitation methods, including inhibition; bias; false negative and positive results; and poor sensitivity, precision, and accuracy, especially when analyzing low expressed genes.

QuantiGene® 2.0 Assay—Single-plex miRNA, RNA, or DNA analysis

QuantiGene® 2.0 Assay enables sensitive and direct quantitation of miRNA, RNA, or DNA copy number in any sample. This simple hybridization-based assay with an ELISA-like workflow results in a glow chemiluminescent signal that can be detected using a standard 96-well plate luminometer.

QuantiGene 2.0 Assays are widely used in quantitative gene expression of signal transduction pathways and for biomarker validation in retrospective and prospective FFPE samples. They are recommended for applications such as quantitation of miRNAs and fusion transcripts and testing large numbers of samples for small numbers of biomarkers (from one to less than 100).

Features and benefits

- Diversity – direct quantitation across diverse and difficult sample types, including FFPE and fresh frozen tissues, blood, urine, cells, miRNA, RNA, DNA, and more
- Streamlined – eliminates unnecessary steps and biases associated with miRNA, RNA, or DNA purification methods, cDNA synthesis, and PCR amplification
- Ignores degraded samples – insensitive to chemical modification of bases and RNA degradation associated with formalin fixation
- High specificity – uses multiple probes per target and is based on cooperative hybridization events to generate signals

FFPE Case 8: Detection of TMPRSS2-ERG fusion transcript isoforms in prostate cancer specimens

In an evaluation of clinical prostatectomy specimens, QuantiGene 2.0 Assay detected eight known TMPRSS2-ERG gene fusion subtypes from less than 200 pg of prostate cancer RNA. Fusion gene detection with one-step RT-PCR required more than 600 pg of RNA.

QuantiGene 2.0 Assay showed a concordant detectable fusion signal in all nine clinical samples that had fusion detected by nested RT-PCR or FISH. Moreover, bDNA detected gene fusion in two of 16 prostate cancer tissue specimens that were not detected by FISH or nested RT-PCR (Figure 6). These findings demonstrate a bDNA assay that is effective for detection of TMPRSS2-ERG gene fusion in prostate cancer clinical specimens, thus providing an alternative method to ascertain TMPRSS2-ERG gene fusion in human prostate cancer tissue. Source: Lu B., *et al. Urology* **74**(5):1156-1162 (2009).

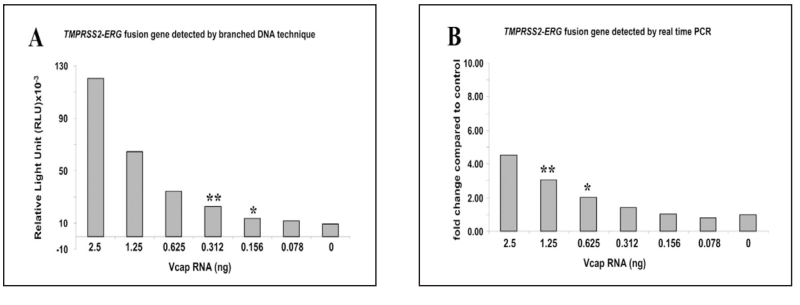


Figure 6: Detection of TMPRSS2-ERG fusion gene expression in total RNA from VCaP cells spiked with total RNA from normal human prostate tissue. A: Quantitation data for the TMPRSS2-ERG fusion gene from QuantiGene 2.0 Assay, which detected TMPRSS2-ERG gene fusion from less than 200 pg of prostate cancer RNA. B: Quantitation data for the TMPRSS2-ERG fusion gene from nested RT-PCR assay based on input of 600 pg of prostate cancer RNA. Reprinted from *Urology*, **74**, Lu, *et al.*, Detection of TMPRSS2-ERG Fusion Gene Expression in Prostate Cancer Specimens by a Novel Assay Using Branched DNA, 1156-1162, 2009, with permission from Elsevier.

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FFPE Case 9: Quantitative detection of fusion transcripts in H&E stained prostate tumors

QuantiGene® 2.0 Assay quantitatively detected Human TMPRSS2-ERG fusion mRNA in prostate tumors of patients treated with radical prostatectomy. The TMPRSS2-ERG fusion mRNA expression correlated well with ERG protein expression in randomly selected specimens. Direct detection of TMPRSS2-E RG transcripts from H&E section tissue homogenates enabled purification of tumor-specific RNA from benign contaminating tissue without extracting RNA. Prevalent gene fusions involving regulatory sequences of the androgen receptor-regulated prostate-associated gene TMPRSS2 and protein-coding sequences of nuclear transcription factor ERG result in frequent over-expression of ERG in prostate tumors. Emerging studies suggest oncogenic functions of ERG in prostate cancer.

H&E stained prostate cancer whole-mount FFPE section. Tumor area was macrodissected from benign tissue, homogenized in QuantiGene® 2.0 Tissue Solution Buffer, and TMPRSS2-ERG fusion mRNA was quantified and compared to ERG protein by IHC. Schematic representation of the expression of ERG oncoprotein (IHC) and TMPRSS2–ERG fusion mRNA was determined in prostate tumors of 35 CaP patients treated with radical prostatectomy by using bDNA assay. Consecutive tissue slides from whole-mounted FFPE prostate specimens were used for the two assays in a blinded fashion. Green triangles represent positive ERG oncoprotein staining; orange triangles represent the detection of TMPRSS2–ERG fusion mRNA (Figure 7). Hollow triangles indicate specimens with undetectable ERG oncoprotein or TMPRSS2–ERG fusion transcript.

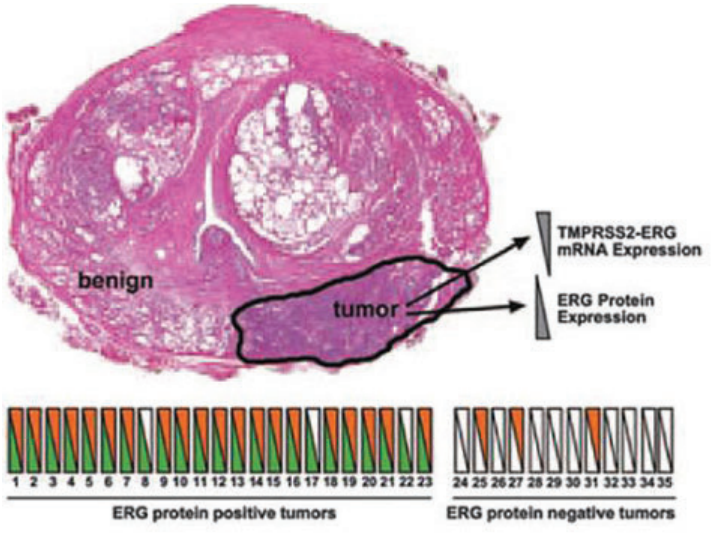

Figure 7: Detection of ERG oncoprotein by ERG–MAb in prostate cancer cells. Reprinted by permission from Macmillan Publishers Ltd: Furusato B., *et al. Prostate Cancer and Prostatic Diseases*, **13**:228-237, 2010.

QuantiGene® 2.0 miRNA Assay—Single-plex miRNA analysis

QuantiGene® 2.0 miRNA Assay provides direct measurement with excellent specificity of mature target miRNA. Based on proprietary chemistry for probe sets and branched DNA (bDNA) signal amplification, the assay provides accurate and precise miRNA quantitation.

Features and benefits

- Sensitive and specific – validated probe sets designed to target mature miRNA and cross-react with closely related family members
- Streamlined assay—direct analysis from lysates eliminates biases inherent to purification and amplification, providing accurate results with no additional steps (no miRNA isolation, cDNA synthesis, or PCR amplification)
- Variety – provides accurate results from a variety of samples, including purified miRNA or small RNA, exosomes, cultured cells, plant tissues, whole blood, and fresh frozen and FFPE tissues

QuantiGene® Plex Assay—Multiplex RNA and DNA analysis

QuantiGene® Plex Assays are a cost-effective way to generate high-confidence, high-quality, reproducible results for multiple targets across large numbers of samples. Identifying and assessing gene expression signatures allows researchers to identify targets and optimize lead compounds by tracking multiple genes associated with specificity, potency, and toxicity. QuantiGene Plex Assay is recommended for mRNA and DNA copy number applications where many biomarkers, from as few as three to up to 250, are to be quantified regardless of whether one or 10,000 samples are tested.

QuantiGene® Plex 2.0 RNA Assay and QuantiGene® Plex DNA Assay are widely used to profile and quantitate gene signatures for biomarker validation in retrospective and prospective studies using FFPE tissues and blood samples, validation of microarray and sequencing results, predictive toxicology, DNA copy number analysis, and more.

Features and benefits

- Efficient – save time and money with true same-well multiplexing of up to 80 RNA targets (RNA) or 34 DNA targets (DNA)
- No intermediary steps – eliminate steps for RNA and DNA purification, cDNA synthesis, and PCR amplification
- Robust – insensitive to chemical modification of bases and RNA degradation associated with formalin fixation
- Low input – typically requires less than 10 mL of blood per assay
- High specificity – uses multiple probes per target and based on cooperative hybridization events

FFPE Case 10: Distinguishing squamous cell carcinoma from adenocarcinoma in human FFPE cervical tumors: An example of microarray to mid-plex panel workflow continuum

Using GeneChip® Human Exon 1.0 ST Arrays, Hall, *et al.* profiled 19 cervical squamous cell carcinoma (SCC) and nine adenocarcinoma (AC) FFPE samples from 10–16-year-old patients. The gene signature derived was tested on a fresh-frozen non-small cell lung cancer (NSCLC) series with differential gene expression confirmed using QuantiGene Plex Assay. QuantiGene Plex 2.0 RNA Assay was used to validate a 26-gene signature from the exon arrays that distinguishes SCC from AC. Nineteen cervical (SCC) and nine cervical (AC) FFPE samples were used. Degradation and chemical modification of RNA in FFPE samples are known to hamper their use in expression profiling studies. In this study, 10–16-year-old FFPE samples were successfully used.

FFPE Case 11: Enrichment and validation of human melanoma biomarkers: An example of mid-plex panels to *in situ* RNA CISH workflow continuum

Validation of human melanoma biomarkers in FFPE samples is important in order to give prognostic and predicative value to these signatures. Sixty-two genes from the literature that were found to be of significance in melanoma were first validated using QuantiGene® Plex 2.0 Assay (Figure 8). Seven of the 62 genes that demonstrated the most significant difference in expression (p-values 1.28E-09–1.13E-06) between melanoma and matched normal skin were selected for testing using QuantiGene® ViewRNA ISH Tissue Assay. These seven candidate genes gave signal differences both in intensity and/or spatial recognition between melanoma and normal skin tissue (Figure 9).

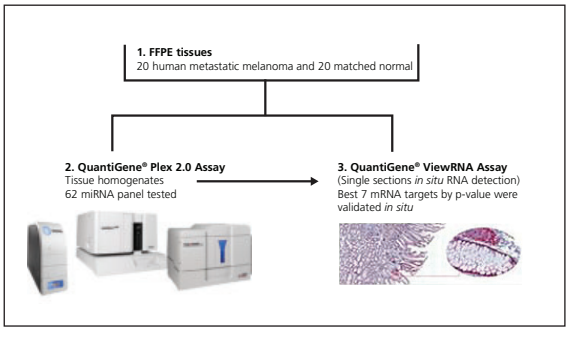

Figure 8: Schematic of gene expression workflow testing FFPE samples first using FFPE tissue homogenates. Sixty-two genes related to melanoma have been categorized from the literature using RNA expression to determine the prevalence of these genes in 20 frozen metastatic melanoma tissues as compared to their corresponding normal skin counterparts. (Note: Subsequent experiments were conducted using FFPE samples [data not shown].) RNA expression was quantified directly from melanoma and normal skin tissue homogenates by QuantiGene Plex 2.0 Assay based on the branched DNA technology. Genes demonstrating the most significant difference in expression between tissue types (p-values 1.28E-09–1.93E-06) were found to be significantly associated with melanoma as compared to normal skin. Source: Affymetrix, Inc.

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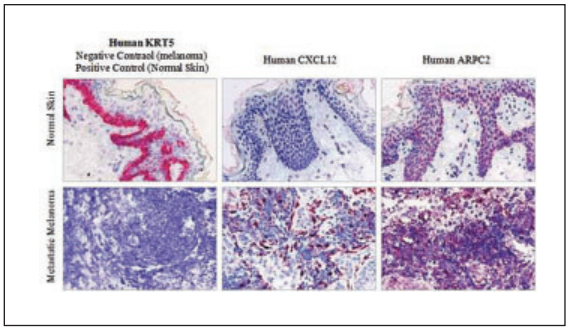

Figure 9: Differential expression of KRT5, CXCL12, and ARPC2 in metastatic melanoma and normal skin FFPE tissue sections. A. Bright-field images of KRT5, CXCL12, and ARPC2 from FFPE tissue sections of normal skin. B. Brightfield images of KRT5, CXCL12, and ARPC2 from FFPE tissue sections of matched metastatic melanoma skin. Nuclei are stained with hematoxylin (blue). Source: McMaster G. K. *et al.* Poster presented at the 4th International Symposium on Cancer Metastasis and the Lymphovascular System: Basis for Rational Therapy. New York City, NY, May 12-14, (2011).

QuantiGene® ViewRNA Assay—Single-plex and multiplex chromogenic *in situ* hybridization (CISH) assays

QuantiGene® ViewRNA Assays have the sensitivity and robustness to detect RNA molecules *in situ* at single-cell resolution and quantitate expression heterogeneity. This level of sensitivity and resolution is critical to understanding the important biological role played by lower expressed genes. As noted by Zhang L., *et al. Science* **276**(5316):1268-1272 (1997), 80 percent of mRNAs are present at fewer than five copies per cell.

QuantiGene ViewRNA Assays have broad applicability in cancer and disease research, stem cell biology and regenerative medicine, mRNA knockdown studies, neurobiology, biomarker validation, and more. These assays are a great alternative or companion to *in situ* immunohistochemistry and immunofluorescence assays when antibodies are not available or are inadequate.

Features and benefits

- Direct, spatial visualization – view RNA in complex tissue architectures while retaining tissue morphology
- Single-copy sensitivity at single-cell resolution
- Easy-to-use – simple hybridization assay to any RNA target, including ncRNAs
- Flexible probe design – target any gene or sequence within a gene with new assays developed in one week

FFPE Case 12: *In situ* detection of non-coding RNA biomarkers in human FFPE pancreatic tissue and fine needle aspirate biopsies

QuantiGene® ViewRNA ISH (*in situ* hybridization) Tissue Assay successfully analyzed non-coding RNA human satellite (HSATII) transcripts in pancreatic ductal tumor versus adjacent normal FFPE tissue and in fine needle aspirates (Figure 10). The HSATII positive epithelial cells were detected with excellent accuracy in both clinical sample types. Satellite repeats in heterochromatin are transcribed into non-coding RNAs that have been linked to gene silencing and maintenance of chromosomal integrity and are greatly over-expressed in human epithelial cancers. The over-expression of satellite transcripts in cancer may reflect global alterations in heterochromatin silencing and could potentially be useful as a biomarker for cancer detection.

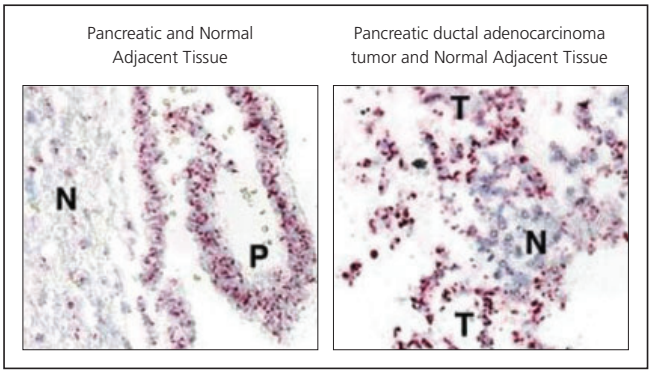

Figure 10: Over-expression of HSATII satellites in pancreatic cancers. A. Human pancreatic intraepithelial neoplasia (P) and normal adjacent tissue (N). B. Endoscopic ultrasound-guided fine-needle aspirate biopsy of pancreatic ductal adenocarcinoma confirmed tumor (T) and normal adjacent tissue (N). All images are at 200× magnification (scale bar, 100 mm). From Ting D. T. *et al. Science* **331**:593-596 (2011). Reprinted with permission from AAAS.

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FFPE Case 13: Tissue microarray (TMA) screening and validation of biomarkers

QuantiGene® ViewRNA Assay enables efficient mRNA screening of FFPE tissue samples by using tissue microarrays and *in situ* hybridization (ISH). An advantage of the two-color ISH assay is that housekeeping gene(s) can be included in the same section as a positive control for target gene(s) of interest while also serving as a control for the RNA integrity and for normalization. As a result, this saves precious clinical sample. Figure 11 shows an example using two probe sets, one a pan-housekeeping gene probe set as the positive control, and the other a pan-probe set for a number of keratins that identifies epithelial cells. In this example, a grade II non-small cell lung carcinoma is strongly stained for both pan-keratins and pan-housekeeping genes.

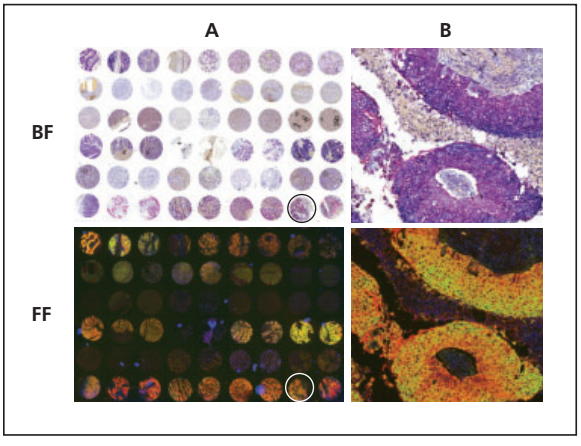

Figure 11: ISH of mRNA screening using tissue microarrays. A. Seventy 1-mm diameter cores per slide with cancer and normal tissues: esophagus, stomach, colon, liver, kidney, lung, and breast. The slide was probed using a pan-keratin probe set and a housekeeping gene pan-probe set. The pan-keratin probe set was labeled with the chromogenic dye Fast Red, and the pan-housekeeping probe set was labeled with chromogenic dye Fast Blue, which was imaged in brightfield (top left) and fluorescent (bottom left). Part of the circled non-small cell lung carcinoma in Panel A is shown in Panel B. B. Specific expression of keratin genes in the epithelial cells of a grade II non-small cell lung carcinoma are stained both red in bright-field (top right) and fluorescent (bottom right), whereas the housekeep-ing genes are blue in the brightfield (top right) and green in fluorescent (bottom right). Nuclei are stained with hemotoxylin (light blue, top right–brightfield) and DAPI (blue, bottom left). Source: Affymetrix, Inc.

Clinical Dx FFPE testing: Enabling the development of novel molecular diagnostic tests—Powered by Affymetrix™ Program

The Powered by Affymetrix™ Program provides a standardized platform for developing novel molecular diagnostic tests (Table 2). Affymetrix® platforms are fully scalable to support discovery of biomarkers and ultimately the development and validation of informative genetic signatures. Affymetrix' GeneChip® System 3000Dx v.2 has achieved CE marking and FDA clearance for *in vitro* diagnostic use. Affymetrix has a growing list of Powered by Affymetrix clinical diagnostic partners, including Pathwork Diagnostics, Almac, Signature Diagnostics, and GenomeDx with FFPE cancer tests.

1. Discovery	2. Array manufacturing	3. Instruments	4. Regulatory compliance	5. Market development
Developing your assays on one platform from start to finish can ensure efficiency, validation, and rapid time-to-market, while saving valuable time and resources.	Our industrial-scale manufacturing facilities offer you the flexibility to develop arrays to your exact specifications, such as the number of features or targets you require on each array.	Affymetrix has already developed the necessary instrumentation for microarray-based tests, so you can easily provide full solutions to your markets.	Having already met the requirements for European Union CE marking and United States FDA 510(k) clearance, the GeneChip® Scanner 3000Dx is positioned to assist in the development of diagnostic tests.	From expert technical support to the development of strategic marketing programs, Affymetrix can partner with you to ensure rapid technology adoption of your microarray-based assays.

Features and benefits

- Scalable platforms from many genes to single biomarkers
- Custom development
- Stable, proven platform
- cGMP manufacturing, IVD-cleared reagents and instrumentation
- Commercial clinical tests

Summary

Our comprehensive range of FFPE-oriented sample analysis tools makes for sensitive, accurate, and reproducible quantitation of RNA and DNA. Degraded genomic material, limited sample amounts, and PCR inhibitors are no longer barriers to discovery, validation, or clinical application. Offering a range of solutions—from single-cell, single-molecule resolution to whole-genome analysis—Affymetrix brings researchers closer to understanding the biology of cancer and increasing the power of their experiments. Talk to your Affymetrix representative to learn more about how you can use our tools for your own FFPE samples.

To learn more about Affymetrix, visit www.affymetrix.com.

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P/N EXP01599 Rev. 1