Diagnostic molecular pathology: current techniques and clinical applications, part I

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Recent revolutionary progress in human genomics is reshaping our approach to therapy and diagnosis. Nucleic acid–based testing is becoming a crucial diagnostic tool not only in the setting of inherited genetic disease (e.g., cystic fibrosis and hemochromatosis) but in a wide variety of neoplastic and infectious processes. Following diagnosis, molecular testing can help guide appropriate therapy by identifying specific therapeutic targets of several newly tailored drugs, thus playing an integral role in the application of pharmacogenomics *(Figure 1)*. Molecular diagnostics provides the necessary underpinnings for any successful application of gene therapy or biologic response modifiers. It offers a great tool for assessing disease prognosis and therapy response and detecting minimal residual disease. It is estimated that by the year 2005, more than 5% of all laboratory testing will be based on DNA or RNA analysis (1).

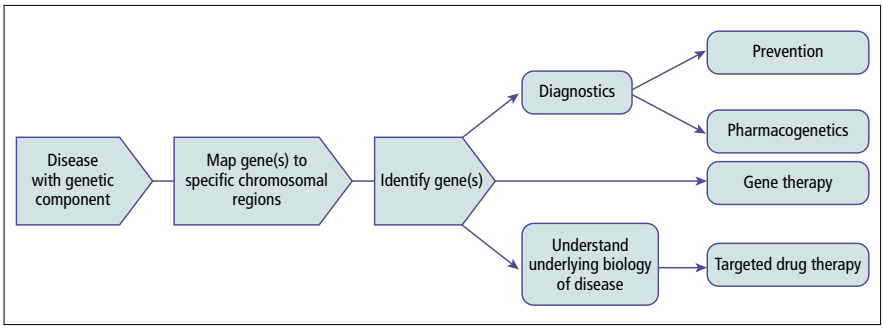

Figure 1. Steps involved in a genetic approach to the diagnosis and treatment of disease. Reprinted with permission from reference 1.

The newly established molecular pathology laboratory at Baylor University Medical Center positions our institution to provide state-of-the-art molecular testing as an integrated consultative element of our advanced patient care.

This, the first of a 2-part article, provides a general review of some principles and applications of molecular diagnostic techniques such as polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH), spectral karyotype imaging (SKI), and DNA chip technology.

MOLECULAR DIAGNOSTIC TECHNIQUES

Polymerase chain reaction and real-time PCR

As an inherent part of oncogenesis, genetic rearrangements provide a great target for many molecular diagnostic tests in oncology. Rearrangements juxtapose otherwise distant segments of our genomic DNA. By bringing nucleic acid sequences closer together, new fusion (chimeric) genes are formed through chromosomal translocations or deletions of intervening DNA sequences.

PCR is the most frequently used molecular technique in a molecular pathology laboratory. Using a pair of priming complementary sequences (oligonucleotide primers) flanking a location of interest, together with unique heat-resistant polymerases (DNA-copying enzymes), multiple copies of a targeted chimeric gene can be obtained *(Figure 2)*. Each PCR cycle involves 3 basic steps: denaturing, annealing, and polymerization. During denaturing, the 2 strands of the helix of the target genetic material are unwound and separated by heating at 90° to 95°C. During annealing, or hybridization, oligonucleotide primers bind to their complementary bases on the single-stranded DNA. This step requires a much cooler temperature, 55°C. Finally, during polymerization (at 75°C), the polymerase reads the template strand and quickly matches it with the appropriate nucleotides, resulting in 2 new helixes consisting of part of the original strand and the complementary strand that

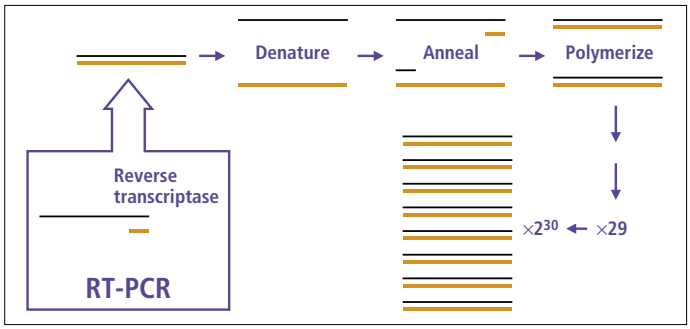

Figure 2. Basic steps of a PCR/reverse transcriptase PCR reaction. Each cycle involves 3 steps: denaturing, annealing, and polymerization. During denaturing, the 2 strands of the helix of the target genetic material are unwound and separated by heating. During annealing, or hybridization, oligonucleotide primers bind to their complementary bases on the single-stranded DNA. Finally, during polymerization, the polymerase enzyme reads the template strand and matches it with the appropriate nucleotides, resulting in 2 new identical helixes. After 30 to 40 cycles, millions of identical copies of the original DNA sequence are generated.

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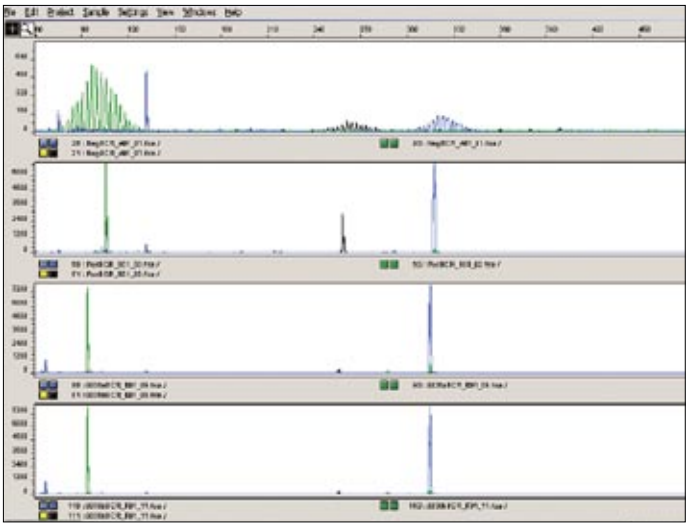

Figure 3. Capillary electrophoresis analysis of a PCR product using a sequencer for fragment analysis (ABI3100). A distinct peak indicates a positive amplification reaction.

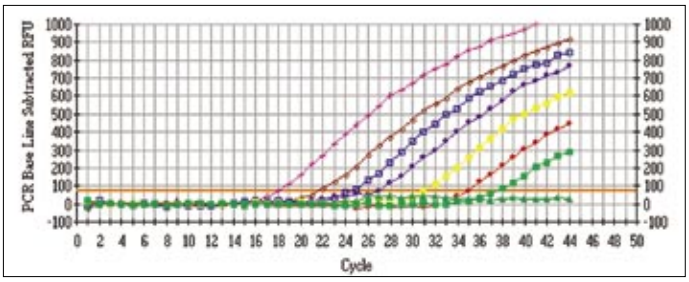

Figure 4. Amplification curve generated by the iCycler real-time PCR instrument. Fluorescence intensity is plotted against cycle number, showing the cycle numbers (threshold) at which the exponential increase in fluorescence occurs in each sample. A sample with a larger number of copies of the original target DNA sequence (top line) will reach the exponential increase at an earlier cycle (cycle 16).

was just assembled. The process is repeated 30 to 40 times, each cycle doubling the amount of the targeted genetic material. At the end of the PCR procedure, millions of identical copies of the original specific DNA sequence have been generated. Since these copies are identical in electrical charge as well as molecular weight, they are expected to migrate simultaneously, forming a single band, when applied to an electrophoretic gel.

If oligonucleotide primers used during the PCR cycles are labeled with fluorescent dye, the PCR product can then be analyzed in a capillary electrophoresis instrument, which tracks the fluorescence of the identical PCR sequences as they migrate. The computerized instrument then generates a graph depicting a peak of fluorescence at the migration location of the PCR product *(Figure 3)*.

PCR can also be used to amplify an RNA target sequence. This procedure is termed reverse transcriptase PCR (RT-PCR). The RNA sequence is first converted to a double-stranded nucleic acid sequence (cDNA) by using a reverse transcriptase enzyme borrowed from a retrovirus. The cDNA sequence can then be amplified by using the same PCR cycles already described. RT-PCR is used for detection of RNA viruses, such as HIV and hepatitis C, and of messenger RNA (mRNA) of originally large DNA translocations (>300–500 bp, such as *BCR-ABL*). Since RNA is not as stable as DNA, fresh samples are generally required for

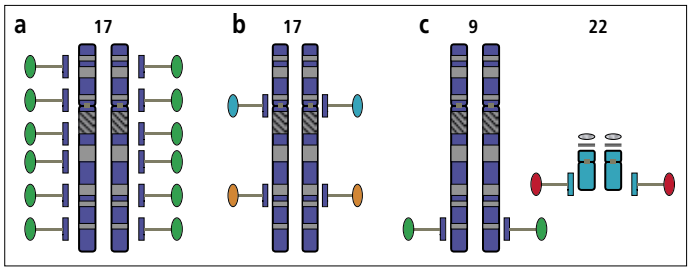

Figure 5. Fluorescent in situ hybridization probes: **(a)** chromosome 17 painting probe; **(b)** chromosome 17 centromeric probe (blue) and *HER2/neu* allele–specific probe; **(c)** chromosome 9 and chromosome 22 *BCR-ABL* allele-specific probes.

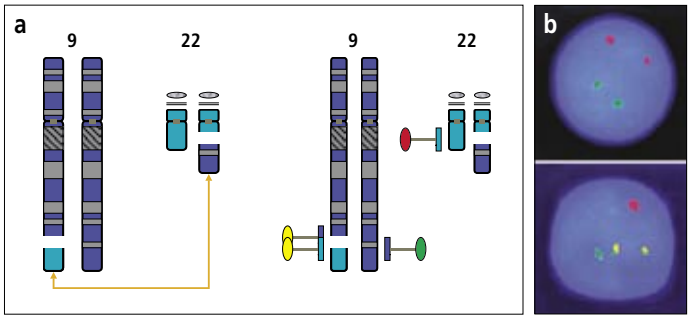

Figure 6. (a) Fluorescent in situ hybridization analysis for chromosomal translocation (9;22)(q34;q11.2), *BCR-ABL*. **(b)** A photomicrograph taken through a fluorescence microscope depicting (top) a normal nucleus with 2 green and 2 red signals and (bottom) a nucleus from a chronic myelogenous leukemia cell with 2 novel yellow signals, indicating a translocation. Figure 6b reprinted from reference 11. Copyright American Society of Hematology; used with permission.

RNA, whereas archival paraffin-embedded samples can be used for DNA.

The recent development of "real-time" PCR (Q-PCR) added great advantages to traditional PCR. As the name indicates, this technique allows for the real-time quantitation of PCR product following each of the 40 amplification cycles. The computerized Q-PCR instrument measures after each cycle the amount of fluorescence emitted from a dye intercalated in the double-helix DNA product; the amount of fluorescence is proportional to the number of copies of the amplification target. When a certain critical copy number is reached, the amount of fluorescence increases by an exponential amount. As depicted in the fluorescence vs cycle number plot in *Figure 4*, the cycle in which the critical copy number is reached (16th or 36th) is directly dependent on how many target DNA copies were present in the original sample before any amplification (100 copies vs 1). Q-PCR therefore offers a great rapid quantitative advantage. It is, moreover, less prone to contamination since the entire process of amplification and quantitation of the original target DNA for each sample is done in a single sealed tube. Q-PCR is of great utility in the assessment of minimal residual disease following novel targeted therapy against specific molecular defects as well as bone marrow transplantation for myelogenous leukemia. Not only can the presence or absence of leukemic cells carrying the target translocation (t15;17; inv 16; or *BCR-ABL*) now be evaluated, but a series of blood samples (or bone marrow aspirates) after transplantation can be compared to determine whether the number of *BCR-ABL*–positive cells in these samples is stable or is increasing. Results can be obtained in 2 hours and, depending on the instrument used, as many as 96 samples can be tested in a single run (2–6).

Fluorescent in situ hybridization

FISH is based on the use of fluorescence-labeled oligonucleotide probes that specifically attach to their complementary DNA sequence target on the genome and label that region with fluorescence color (e.g., Texas red, FITCI green, acridine orange). The labeled region can then be easily visualized under a fluorescence microscope. Currently, 3 types of probes are in wide use:

- Painting probes that identify an entire chromosome by attaching to overlapping sequences on its target (e.g., chromosome 17) and thus "painting" that chromosome with the chosen fluorescence color *(Figure 5a)*
- Centromeric probes that identify the centromeric region of a specific chromosome and thus help in enumerating the number of copies of that chromosome even in a nondividing cell (interphase state) *(Figure 5b)*
- Allele-specific probes that adhere to a specific target allele sequence such as the *p53* tumor suppressor gene or the *HER2/neu* oncogene (Figure 5b)

FISH offers great advantages over conventional cytogenetics in the study of chromosomal deletions and translocations and gene amplifications (7, 8). Conventional cytogenetics requires a time-consuming cell culture step and can be performed only with fresh tissue samples. FISH, on the other hand, can be performed on cells in dividing (metaphase) as well as resting (interphase) stages. It can be performed on fresh frozen as well as archival cytologic smears or paraffin-embedded tissue sections. This great versatility, in addition to the topographic advantage of fluorescent microscopic examination, which allows distinction between signals from tumorous and nontumorous cells, has fueled the field of "interphase cytogenetics" in both tumor and prenatal settings (9).

Currently, FISH is often used in evaluation of *HER2/neu* oncogene amplification in breast carcinoma and for detection of different translocations in chronic myelogenous leukemia and acute myelogenous leukemia (10).

A normal cell should have 2 copies of the *HER2/neu* oncogene, 1 on each of its 2 copies of chromosome 17. Using a centromeric chromosome 17 probe (green signal) in combination with an allele-specific probe for the *HER2/neu* oncogene (orange signal), visualization of 2 green and 2 orange signals in each cell is expected (a ratio of 1, Figure 5b). In a breast cancer cell undergoing *HER2/neu* oncogene amplification, 4 or more orange signals are visualized in nuclei showing only 2 green signals (a ratio of 2 or more).

Similarly, a combination of 2 allele-specific probes to the breakpoint regions on the long arms of chromosome 9 (green signal) and 22 (red signal) are used to detect t(9;22), the Philadelphia chromosome *(Figure 5c)*. In a nonleukemic cell, 2 green and 2 red signals are visualized. A t(9;22) will juxtapose 1 green and 1 red signal, leading to the appearance of a new yellow signal (color overlap) in addition to the remaining 1 green and 1 red signal that did not participate in the translocation *(Figure 6)*.

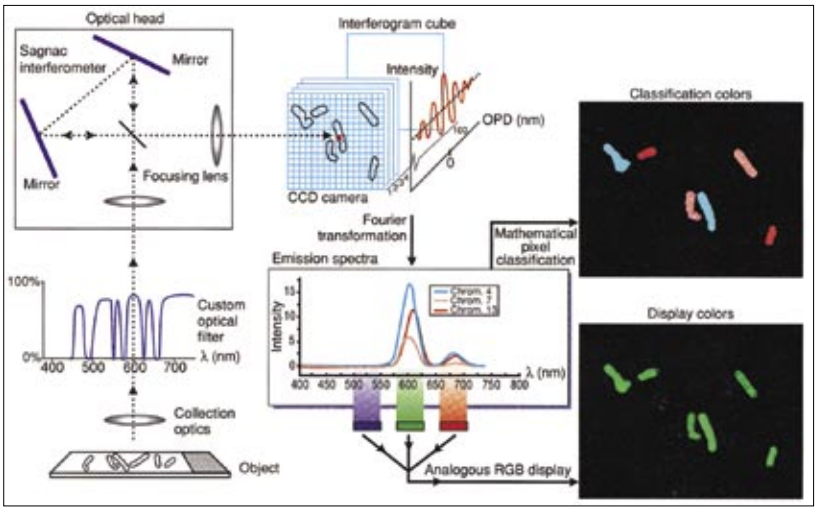

Figure 7. Spectral karyotype imaging. Diagrammatic illustration of computerized signal manipulation used to visualize slight variations in light spectra among different chromosomes. The 6 chromosomes depicted in the insets would have been indistinguishable by the naked eye. With the use of interferometer technology, they are identified as 3 different chromosomal pairs; each pair is assigned a different classification color and is identified accordingly. Reprinted with permission from reference 12. © 1996 AAAS (http://www.sciencemag.org). Permission from AAAS is required for all other uses.

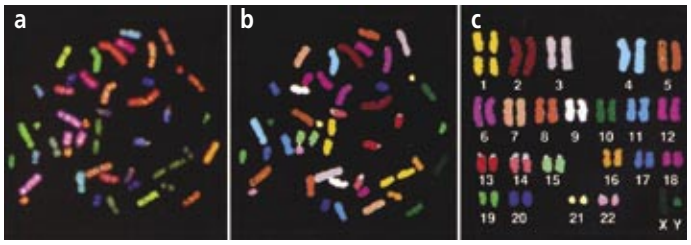

Figure 8. Spectral karyotype imaging: **(a)** display colors, **(b)** classification colors, and **(c)** final karyotype with all 24 chromosomes pair-matched and arranged in numerical order. Reprinted with permission from reference 12. © 1996 AAAS (http://www.sciencemag.org). Permission from AAAS is required for all other uses.

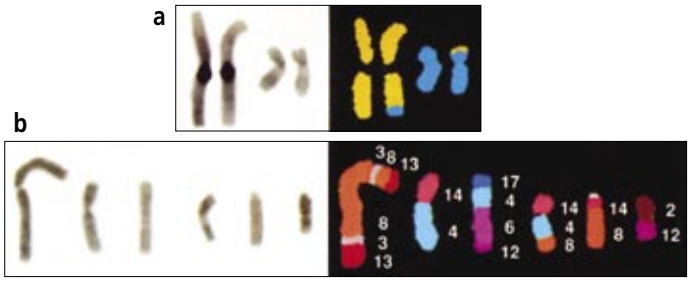

Figure 9. Spectral karyotype imaging: **(a)** Translocation (1;11) can easily be detected with the transposition of colored segments among chromosomes 1 (yellow) and 8 (blue). **(b)** Complex marker chromosomes occurring in breast carcinoma. Reprinted with permission from reference 12. © 1996 AAAS (http://www.sciencemag.org). Permission from AAAS is required for all other uses.

Spectral karyotype imaging

Introduced by Schrock et al (12), SKI is based on the use of 23 sets of chromosome-specific "painting" probes. Each probe is labeled with varying proportions of 3 fluorescent dyes, which allows each chromosome pair to be labeled by a light of unique spectral emission. A crucial component of this technology is the use of an "interferometer" similar to ones used by astronomers for differentiating light spectra emitted by different stars. Slight variations in color, undetectable by the human eye, are detected by this computerized device, which then reassigns an

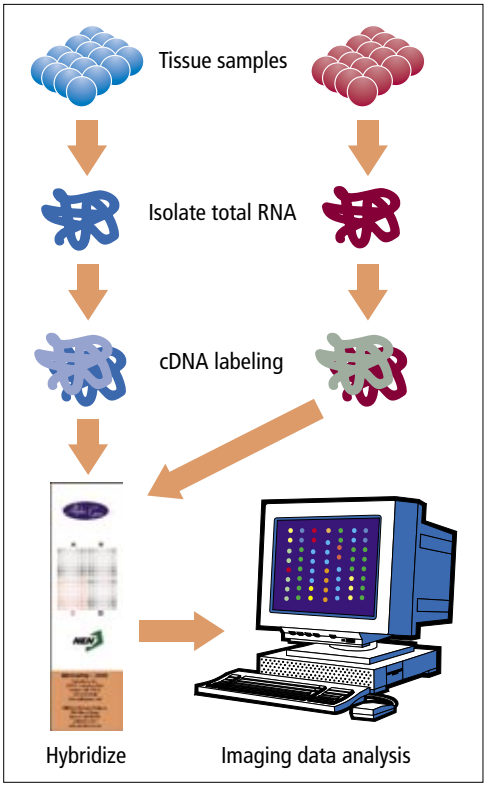

Figure 10. Steps involved in DNA microarray analysis. Each square on the glass slide contains 1200 cDNA loci for a total of 4800 in this example. Adapted with permission from Perkin-Elmer Life and Analytical Sciences.

easy-to-distinguish visual color (classification color) to each pair of chromosomes *(Figure 7)*. Currently complementing conventional cytogenetics, SKI is used on dividing cells in the metaphase stage. The cell karyotype is depicted on the digital screen, aligned according to chromosome pair color and numeric order *(Figure 8)*. The pathologist can then readily identify any numerical chromosomal abnormalities (aneusomy) or any shifting in colored chromosomal portions (translocations) *(Figure 9a)*. Previously indecipherable complex translocations occurring in tumor cells (e.g., breast cancer cells) can now be resolved by SKI, leading to analysis of "marker chromosomes" composed of an amalgam of fragments from different chromosomes *(Figure 9b)*.

DNA microarrays

Gene expression profiling using DNA microarrays holds great promise for the future of molecular diagnostics. This technology allows, in one assay, for simultaneous assessment of the expression rate of thousands of genes in a particular sample. The 2 types of DNA microarrays that are widely used are cDNA microarrays and oligonucleotide/DNA chips.

In cDNA microarrays, DNA sequences complementary to a library of mRNA from thousands of genes are mechanically placed on a single glass slide. The immobilized cDNA sequences serve as anchoring probes to which mRNA extracted from the tested sample will specifically attach during hybridization. If the tested mRNA is first tagged with a fluorescent dye, the intensity of fluorescence at each anchoring probe location would be proportional to the amount of mRNA (degree of expression) of the gene at that location. A microarray reader displays the intensity

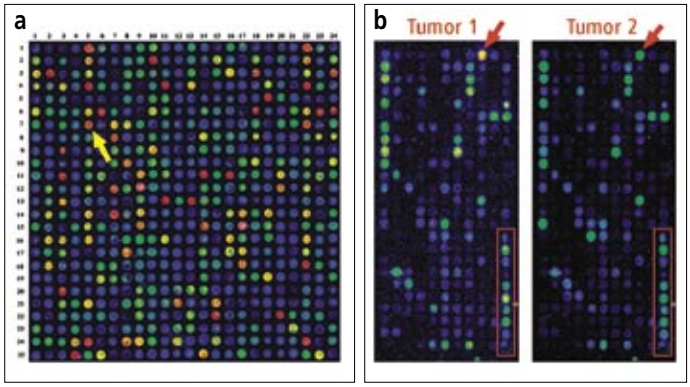

Figure 11. (a) A computerized DNA microarray reader displays fluorescence intensities at the different cDNA intercept locations. In this example, the gene indicated by the yellow arrow is markedly overexpressed in comparison to the genes in blue. **(b)** Variations in gene expression help identify patterns that may predict prognosis and treatment outcome. The red box indicates a group of genes related to a specific functional pathway. Reprinted with permission from Perkin-Elmer Life and Analytical Sciences.

of fluorescence at each cDNA location as a colored dot per gene location on a grid *(Figures 10* and *11)*. In the example shown, blue designates a normal gene expression rate, while green, yellow, orange, and red represent increasing degrees of gene expression. The computerized reader is linked to a database indicating the gene at each intercept location; a query can be made and the status of expression of a particular gene or group of functionally related genes can be recognized. For example, if the gene in column 5, row 7, of Figure 11a is *HER2/neu*, the red signal in that location indicates strong overexpression of *HER2/neu* in the tumor under analysis.

Oligonucleotide/DNA chips are silicon chips on which the "anchoring" oligonucleotide sequences are directly synthesized and serve as the immobilized probes to which the complementary specific mRNA will hybridize. DNA chips can be made with an astonishing density of gene arrays encompassing up to 12,000 or more genes on a single chip.

The applications of these technologies are limitless. By analyzing and comparing hundreds of tumor samples, databases of gene expression "fingerprints" are being built and specific patterns of expression linked to both prognosis and outcome of therapy (13–18). Patterns of gene expression in tumors are also linked to the primary site of origin. A lung primary tumor, for example, has a different fingerprint than an ovarian or colonic primary. This feature can be exploited in the setting of tumors of unknown primary, in which DNA microarrays have been shown to predict the correct primary site with an amazing accuracy rate of 99% (18). New molecular techniques for classifying neoplastic diseases are expected to soon complement the currently familiar histology-based classification systems (13).

The bioinformatics/statistical analysis workload generated by high-density microarrays is very taxing. To be of practical utility in the working molecular diagnostic laboratory, custom-tailored DNA microarrays are used, which have a small number of the most informative prognostic markers for a particular type of tumor. This brings down the cost and labor involved in the analysis. For example, a breast cancer prognostic chip would include a small array of cDNA targeting the currently recognized markers, such as *HER2/neu*, estrogen receptor, and progesterone receptor.

For tumors of unknown primary origin, chips that look at only 10 to 12 of the most informative genes are 80% to 88% accurate in identifying the primary tumor (18). Such chips are evidently much easier to manage in a diagnostic setting.

CONCLUSION

As many molecular techniques have made their expected transitions into the clinical arena, molecular diagnostics is becoming an integral part of our clinical practice at Baylor University Medical Center. Part II of this article will discuss the salient clinical applications of these techniques.

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