

Molecular Pathology for Toxicologic Pathologists: Techniques and Applications

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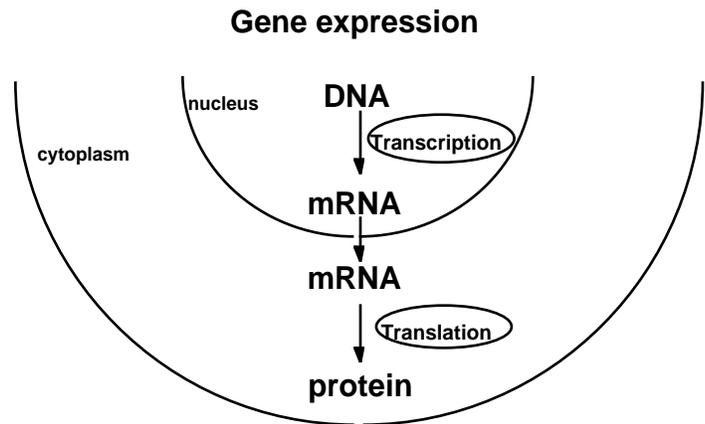
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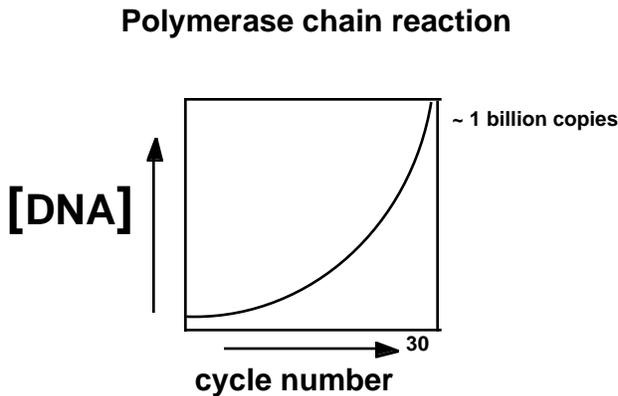
I. INTRODUCTION

Gene expression is the process by which deoxyribonucleic acids (DNA) code, via the translation of copies of mRNA, for the production of specific proteins. mRNA copies are made from DNA in the nucleus by the process of transcription and then modified and transported to the cytoplasm where they usually become associated with ribosomes to produce protein. Gene expression can be assessed by measuring either cellular mRNA or protein. The presence of mRNA strongly suggests active gene expression, however, not all mRNA is necessarily translated into protein.

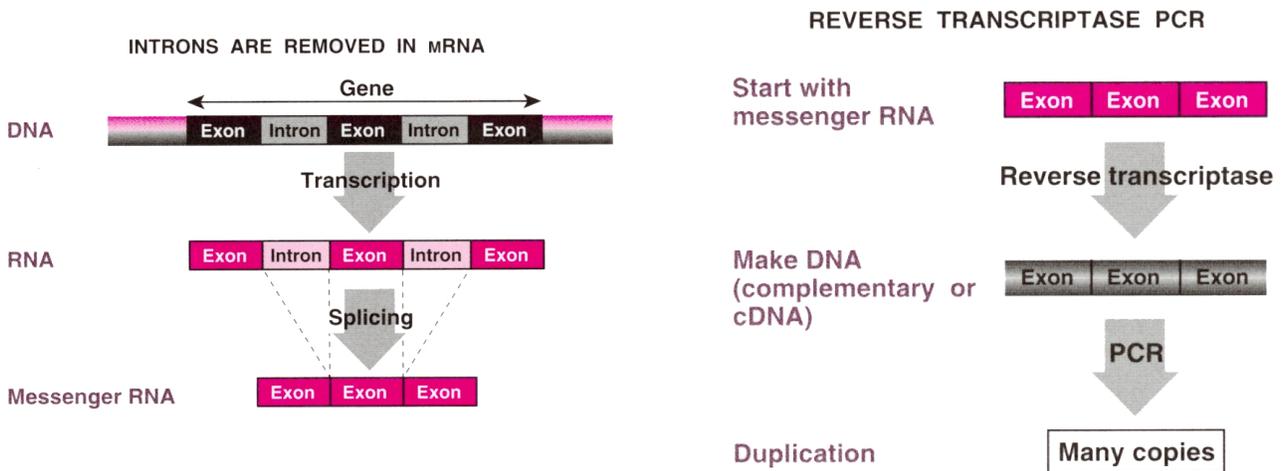
The polymerase chain reaction (PCR) and *in situ* hybridization (ISH) have revolutionized the study of genes, gene expression, and microbial infections and many of these molecular biology advances will greatly impact diagnostic pathology and toxicology research. Analysis of altered genes and gene expression can potentially provide mechanistic information about toxicity and carcinogenicity. Slide-based techniques have been developed to study mutations, gene expression, clonality, tumor cell origin, and the sequence of molecular events in cancer development in specific cell populations. This review presents a basic overview of the concepts, methodology, and applications of established and emerging molecular biology techniques in the study of DNA, RNA, and proteins.



PCR, first described about 13 years ago, is fast becoming an essential molecular biological tool in toxicologic pathology. It is a relatively simple technique to amplify specific DNA sequences to detectable and analyzable levels, and it allows the efficient generation of large amounts of DNA from minimal amounts of even crude starting material. Billions of copies of a specific molecule of DNA can be generated by reactions in less than 2 hours. PCR can amplify DNA



derived from freshly frozen tissue or partially degraded tissues (such as those found in paraffin-embedded, fixed tissues) or from mRNA. For mRNA a complementary DNA (cDNA) copy is made using reverse transcriptase (RT) prior to the PCR reaction (called RT-PCR). Additional refinements of the RT-PCR technique have made it possible to make quantitative measurements of mRNA transcripts.



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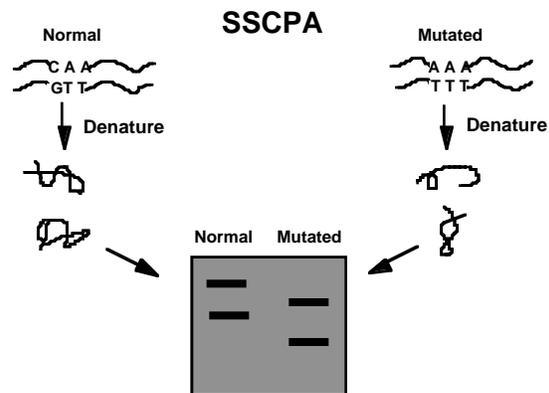
II. DNA

1. mutation analysis

PCR is commonly employed for detection of point mutations in oncogenes and tumor suppressor genes in the analysis of germline mutations and somatic mutations in tumor cells. Techniques that have commonly been used following PCR for the detection of single base changes include single-strand conformation polymorphism analysis (SSCPA), restriction endonuclease digestion analysis, allele-specific oligonucleotide hybridization (ASO), and direct sequencing.

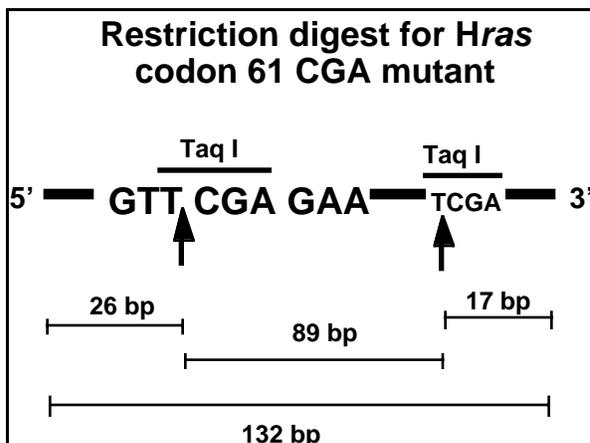
a. PCR/SSCPA

SSCPA, a quick and effective technique for the detection of single nucleotide base substitutions and the most widely used of the scanning technologies, is based on the principle that single-stranded DNA takes on sequence-based secondary structural conformation under non-denaturing conditions. Single-stranded molecules which vary by as little as a single base substitution may form a different three-dimensional configuration and migrate at an altered speed in a non-denaturing polyacrylamide gel. PCR is carried out by using oligonucleotide primers flanking the target sequence of a gene, and either the primers are labeled or, most often, a labeled deoxynucleoside triphosphate is incorporated in the reaction. Both radioactive and non-radioactive methods have been described. PCR products with altered band migration are identified on the polyacrylamide gel and further analyzed by DNA sequencing or restriction enzyme digestion to define or confirm mutations.

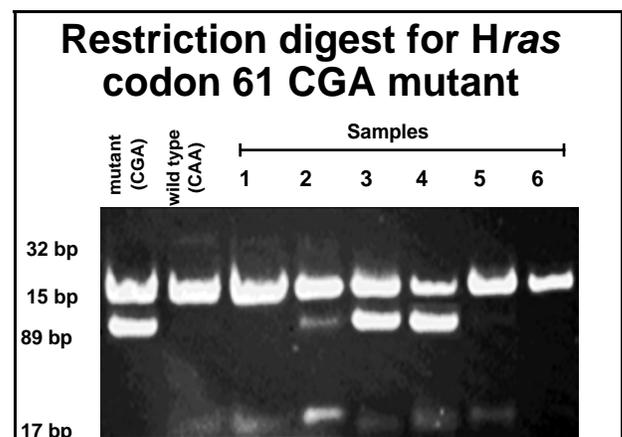


b. PCR/RFLP

Mutations can be identified when they occur in restriction enzyme recognition sequences and render the mutated site either sensitive or resistant to cleavage. Restriction enzymes cut DNA in a sequence specific manner. For some techniques the restriction digest is used to remove the wild-type (normal) sequences, leaving only the mutated sequences intact for further analysis. Some techniques use specific primers that either recognize specific mutations or introduce a restriction site into the PCR product.



Lee and Drinkwater, 1995

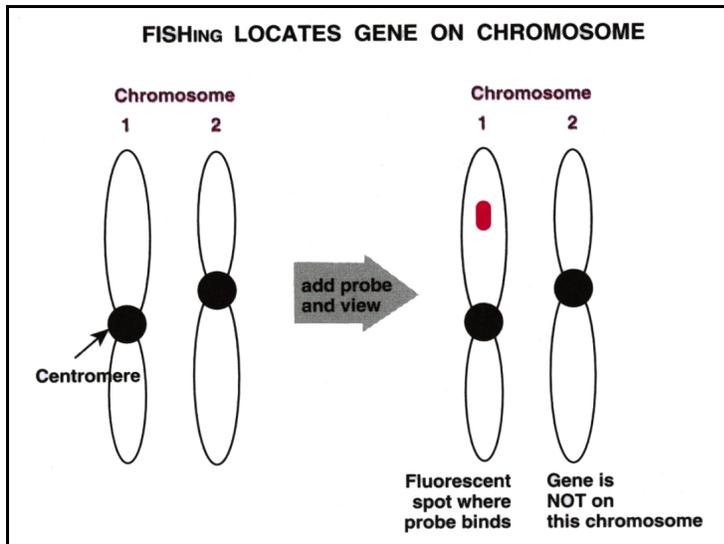


Malarkey, unpublished data

within a normal metaphase chromosomal spread. Regions of gain of DNA sequences are seen as an increased, and losses as decreased, color ratios of the two fluorochromes used to detect the labeled DNAs. Evidence indicates that many more genes than the currently known oncogenes may undergo amplification in human tumors. This technique offers promise for finding such genetic alterations in animals

e. FISH

Flourescence in situ hybridization (FISH) is useful for detecting aneuploidies, quantifying large scale chromosomal damage following exposure to clastogens, and mapping genes to specific chromosomes.



It is a sensitive non-radioactive technique commonly used in human cytogenetics with great potential for application in rodents. However, there are obstacles for the use of this technique in the mouse. Success has been limited because the 40 acrocentric mouse chromosomes are of similar size and difficult to separate by flow cytometry; nonetheless, probes have been developed for the mouse.

Figure from: D.P. Clark and L.D. Russell (1997) *Molecular Biology Made Fun and Simple* with permission from Cache River Press, Vienna, Illinois, USA.

f. loss of heterozygosity (LOH)

Individuals inherit one chromosomal allele from each parent, and LOH refers to the loss, by deletion or a recombinational event, of one of those alleles. Due to the recessive nature of tumor suppressor genes, mutations in these genes are frequently accompanied by an LOH in the chromosome region containing the normal allele. Allelotype analysis that reveals frequent losses of specific chromosomal regions in neoplasms provides presumptive evidence for the existence of tumor suppressor gene involvement in tumor development. It is assumed that the lost or deleted chromosomal regions harbor a gene with tumor suppressor-like activity. The demonstration of LOH has been instrumental in the discovery and characterization of novel tumor suppressor genes. So far, more than a dozen tumor suppressor genes have been identified in human tumors, and many more are believed involved in both human and animal tumorigenesis.

Other than p53, little is known about the involvement of tumor suppressor genes in experimental carcinogenesis. Allelotypic analysis for LOH has recently been applied in the study of chemically induced mouse lung, skin, and liver tumors. Studies utilize F1 hybrid mice and involve PCR amplification for the analysis of

previously defined chromosomal markers, such as microsatellites. Frequent allelic losses were demonstrated in both the lung and skin tumors providing probable evidence for the involvement of tumor suppressor genes in the tumor development. Consistent LOH appears uncommon in murine hepatocarcinogenesis. As more studies are performed, the significance of these chromosomal losses and the role of tumor suppressor genes in experimental carcinogenesis may become evident.

g. DNA chip technology (see also microarray of gene expression

below)

This technology has been developed to aid in the screening of polymorphisms and mutations, as well as mapping of genomic DNA clones. DNA chips will revolutionize genetic screening much the way silicon chips have transformed electronics.

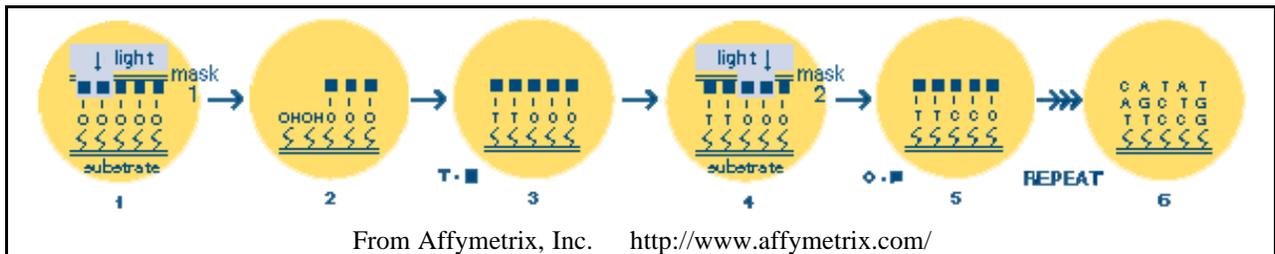


Photo: Bob Sacha

The computer chip makers and DNA chip makers both use a technique called “photolithography” to make microscopic “etches”, however with modifications scientists at Affymetrix Inc. in Santa Clara, CA have used the technology to synthesize short chains of DNA on chip surfaces. A gene chip, which is made of glass, can hold up to 409,000 distinct testing spots each with a unique known sequence. In other words, thousands of Southern hybridizations (DNA-DNA) of an individual's DNA sample can be performed simultaneously on a glass slide about 1/2 the size of a postage stamp. Since hybridizations can theoretically be specific to find point mutations, a patient's

DNA can be screened for possible mutations in cancer genes or other genes related to disease. Soon it should be possible to put a few of your cells into a gene-chip scanner and quickly analyze your genetic risks for scores of diseases. Also, tumor prognosis or specific therapeutic approaches for cancer may be dictated by the types of mutations occurring in, for example, the *K-ras*, *p53*, or *BRCA1* genes.

A gene chip begins as a glass slide about 1/2 the size of a postage stamp that contains hundreds of thousands distinct microscopic squares. Upon each square specific DNA molecules, hundreds of sequences long, can amazingly be synthesized. The highly specialized technique for DNA synthesis utilizes protecting groups on nucleic acids as “masks” to block the addition of nucleotides. Light is used to “unmask” a specific square when the appropriate nucleotide base is to be added to the molecule. DNA from the individual (can be from a tumor or perhaps peripheral



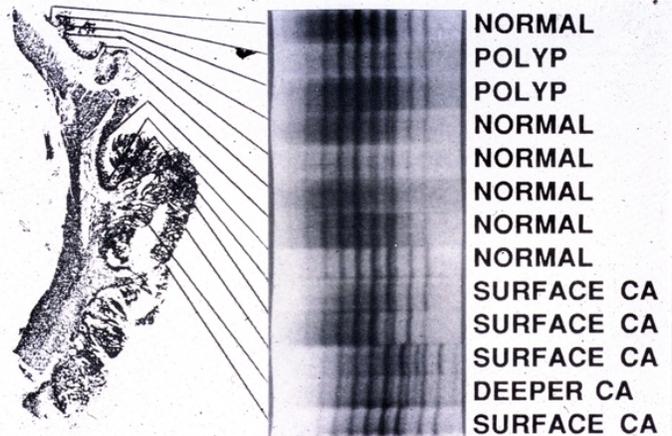
blood leukocytes) is fragmented by restriction endonucleases and then labelled with fluorescent probes prior to hybridization. The chip is then put into an optical scanner which identifies the hybridized probes.

This same technology can be used to study gene expression (mRNA) by making cDNA copies of mRNA as described below under **microarray-based analysis of gene expression**.

h. microsatellite instability

The mammalian genome contains numerous tracts in non-coding regions of short di, tri, and tetranucleotide repetitive sequences referred to as microsatellites. For example, there are more than 100,000 CA/GT repeats that have a chain length greater than 24 bases. Investigations have reported variations in the number of repetitive unit sequences and changes in the fingerprint patterns for microsatellites in human tumors of the colon, stomach, pancreas, and endometrium. These changes were not found in adjacent normal tissue from the same individual. This and other evidence indicates that, for some cancers, a continual accumulation of mutations occurs within individual tumor cells. The evidence has led to the emerging concept that genomes of cancer cells are unstable and that microsatellites might provide a

sensitive indicator for genetic instability and hypermutability in tumors. This hypothesis is based on the notion that the mutation frequency within these cells is high, and microsatellite alterations reflect the numerous mutations that have occurred throughout the genome. Loeb (1994) proposes that mutations in stability genes, for example those involved in DNA replication, DNA repair, or chromosomal segregation, may be an early event in carcinogenesis and contribute to the increase in the overall level of mutations throughout the genome.



Depiction of microsatellite instability in human colorectal tumor (Shibata, et al. 1994, Nature Genetics 6:273-281.)

Alterations in microsatellites can be used as a measure of genomic instability, and recent demonstration of microsatellite instability in different human and animal tumors has provided good evidence for the so-called "mutator phenotype". Using SSCPA, PCR, and/or direct sequencing, mutations in microsatellites have been found in rat colon tumors induced by heterocyclic amines (Canzian, et al. 1994) but not in mouse liver tumors (Fox, et al. 1997). Some of the rat colonic tumors were also found to have mutations in the *APC* gene, a gene believed involved with the development of colorectal polyps and carcinomas in humans and described as familial adenomatous polyposis.

2. Diagnostics

a. Helicobacter infection (an example case).

Infection of the mouse liver by *Helicobacter hepaticus* has recently become important for the interpretation of carcinogenicity bioassays and other research utilizing rodents. Many species of the genus *Helicobacter* have been identified in mammals, and their pathogenicity varies, with some species inducing significant

disease while others appear to merely colonize the gastrointestinal tract. *Helicobacter* species identified in mice include *H. muridarum*, *H. rappini*, *H. hepaticus*, and *H. bilus*. Although colonizers of the mouse gastrointestinal tract, *H. muridarum* and *H. rappini* are not generally considered to be pathogenic. In susceptible strains of mice, *H. hepaticus* causes acute focal, nonsuppurative, necrotizing, hepatitis which progresses to chronic, active hepatitis characterized by minimal necrosis, hepatocytomegaly, oval cell hyperplasia, cholangitis along with the presence of relatively few infectious organisms not usually apparent on routine histologic examination. A/JCr, B6C3F1, BALB/cAnCr, C3H/HeNCr,

SJL/NCr, and SCID/NCr strains of mice are variably sensitive to the development of hepatitis due to *H. hepaticus* infection, and the A/JCr and male B6C3F1 mice have an increased incidence of hepatocellular neoplasms associated with infection. *H. bilus* can be found in the bile, liver, and intestines of mice and may be associated with chronic hepatitis, although its pathogenicity is, as yet, undetermined, and it is not known whether *H. bilus* causes liver tumors in mice.

There are multiple approaches to identifying a study (or group of mice) as being infected. Definitive diagnosis of *H. hepaticus*-associated liver disease in mice is based on the presence of histologic alterations along with demonstration of the infectious agent. While culturing organisms from fresh or frozen tissue or feces is desirable, retrospective

analysis will depend upon alternative methods, including PCR-based assays, that can be applied to archival tissues. *H. hepaticus* organisms have been detected in lesioned liver by methods such as Steiner or Warthin-Starry histochemical stains, immunofluorescence, *in situ* hybridization, immunohistochemistry, and ultrastructural analysis. A serum ELISA test for circulating IgG antibodies against *H. hepaticus* has been used to provide information for determining *in vivo* infection status in pathogenesis studies. PCR-based diagnostic assays for *Helicobacter hepaticus* potentially offer greater specificity, increased sensitivity, and decreased expense and time expenditure compared to culture, histochemical stains, or ultrastructural analysis. Recently a number of PCR-based assays have been described for the identification of murine helicobacters in rodent tissues or feces; and

Murine Helicobacters		
Agent	species	disease
<i>H. hepaticus</i>	mouse	hepatitis, liver tumors, IBD*
<i>H. bilus</i>	mouse / rat	hepatitis / IBD*
<i>H. muridarum</i>	mouse	gastritis
<i>H. trogonatum</i>	rat	-
<i>H. rodentium</i>	mouse	-
" <i>F. rappini</i> "	mouse	-

*IBD=inflammatory bowel disease / typhlocolitis

PCR detection of <i>H. hepaticus</i>			
Chemical	Helicobacter hepatitis	Steiner stain	PCR/RFLP
Triethanolamine (5)	80%	80%	100%
AZT (20)	35%	10%	55%
Scopolamine (20)	5%	5%	60%

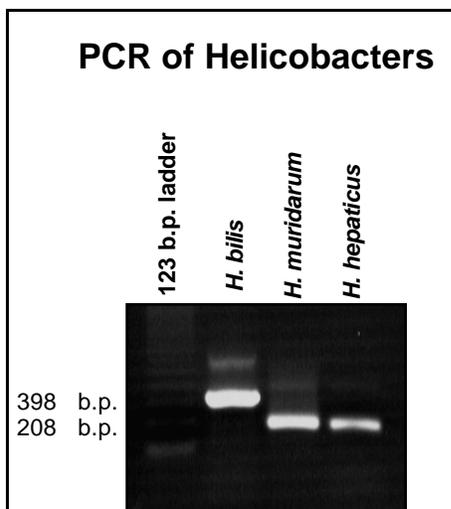
Malarkey, et al. 1997. Tox Path 25 (6):606-612

some have reported success at detecting *H. hepaticus* in formalin-fixed, paraffin-embedded tissues.

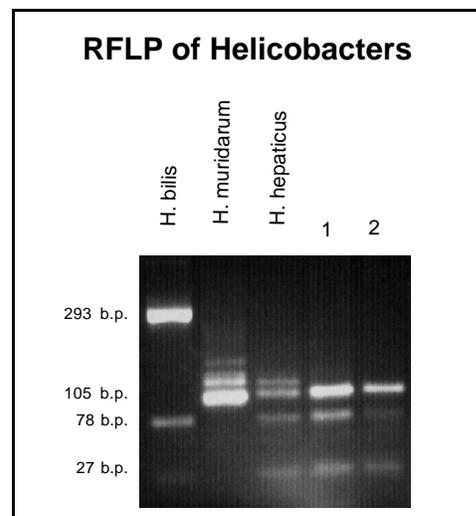
Presently, the specificity of many PCR-based assays for the identification of the Campylobacteria family, which includes *Helicobacter sp.*, should be regarded with caution. Specificity of PCR-based molecular tests are dependent on the degree of variability in the primary nucleotide sequences of the 16S ribosomal RNA (rRNA) gene. These sequences are nearly identical in phylogenetically closely-related bacteria. For example, the region of the 16S rRNA gene amplified in the assay described by Malarkey, et al. (1997) for *H.*

hepaticus has similarities with other *Helicobacter* species not reported in the mouse, such as *H. canis*, *H. trongontum*, *H. mustelae*, as well *H. rappini*, found rarely in the mouse and not considered a murine hepatic pathogen, making them indistinguishable by the PCR-RFLP. It is possible that as newly discovered species of *Helicobacter* are characterized, some may have spans of nucleotide sequences indistinguishable by PCR from that of *H. hepaticus*. Confidence in the diagnosis of *H. hepaticus* hepatitis can be gained by using multiple PCR-based assays at different regions of the 16S rRNA gene to distinguish *Helicobacter* organisms based on sequence polymorphisms.

Diagnostic PCR amplification of genes for the identification of presumptive



Malarkey et al 1997



Malarkey et al 1997

low level bacterial infection from formalin-fixed paraffin embedded archival tissues has proven problematic presumably because fixation is inherently detrimental to nucleic acid molecules and the PCR. The determinants of sensitivity of the PCR assay appear related to the bacterial load and distribution in the liver, amount of tissue analyzed, and the detrimental effects of formalin fixation and paraffin embedding procedures on the bacteria in the tissue and the PCR reaction.

Shortening the duration of fixation, increasing the initial sample size, amplifying relatively small fragments (less than 200 base pairs), using a nested primer amplification method, and/or increasing the number of amplifying cycles have all been shown to be helpful strategies for achieving successful PCR results from fixed paraffin-embedded tissues. In the studies *H. hepaticus* infection in mice by Malarkey, et al. (1997),

PCR detection of <i>H. hepaticus</i>			
Chemical	Helicobacter hepatitis	PCR-RFLP results	
		Frozen liver	fixed liver
Triethanolamine (5)	80%	100%	80%
Cobalt Sulfate (20)	55%	NA	0%
AZT (20)	35%	55%	10%
Scopolamine (20)	5%	45%	40%

Malarkey, et al. 1997. *Tox Path* 25 (6):606-612

results in liver tissues appeared primarily dependent on the duration of fixation and bacterial load, and not necessarily on the presence or absence of hepatitis, origin of the sample from neoplastic or non-neoplastic liver, or sex of the mouse. In some cases PCR methods were more sensitive than histologic evaluation and thus may provide the first evidence of *H. hepaticus* infection. The finding of PCR positivity in the absence of histologic alteration may be due to identification of an early infection or of a strain of *H. hepaticus* that is of low or minimal pathogenicity.

In summary, the impact of infection of the mouse liver by *Helicobacter hepaticus* on research and the bioassay is not completely understood and our understanding of the pathogenesis depends on achieving a reliable diagnosis.

III. mRNA

a. northern blotting

Northern blotting is the RNA counterpart to Southern blotting and is used to detect a particular RNA in a mixture of RNAs. The differences between the two procedures stem from the fact that RNA is single stranded and must be denatured, ensuring that all RNA molecules have an unfolded, linear conformation, for effective electrophoresis. The individual RNAs are separated according to size by gel electrophoresis and transferred to a nitrocellulose filter. The filter then is exposed to a labeled DNA probe and subjected to autoradiography. The assay will assess the steady state level of specific mRNAs at the time the RNA was extracted from cells or tissue.

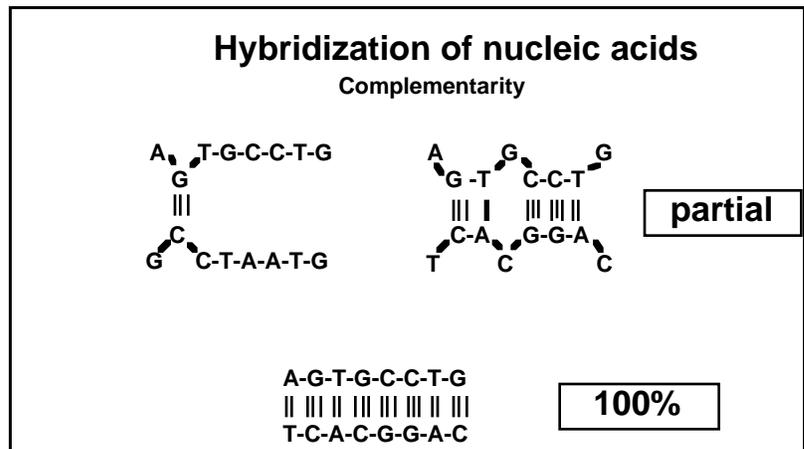
b. Rnase (nuclease) protection assay

A method for detecting and quantitating specific RNA molecules based on the principle that endonucleases digest single-stranded but not double-stranded nucleic acids. In the technique, a radiolabeled ssDNA probe (added in excess) specifically hybridizes with complementary target RNA in a mixed population of cellular RNAs and is then subjected to digestion with an endonuclease. The endonuclease degrades all the unprotected ssRNA and DNA molecules leaving the double-stranded RNA-DNA complex intact. The protected RNA-DNA hybrid is detected by

autoradiography after gel electrophoresis and the amount of intensity of the resulting band is proportional to the original quantity of RNA in the sample.

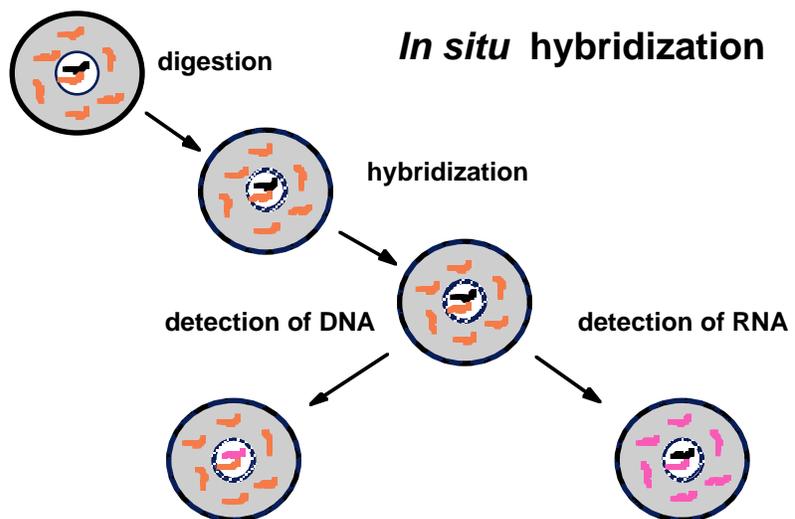
c. ISH

ISH, described over 25 years ago and only recently widely utilized, can localize specific sequences of DNA and RNA within cells and thus provide data on individual cells rather than an average of total cellular populations. ISH is based on the principle that cell and tissue-bound RNA and DNA sequences will hybridize with labeled probes of complementary sequence. Sites of hybridization can then be visualized microscopically. ISH also allows the maximal use of tissues, through serial sections, that may be of limited supply. The technique can be extremely sensitive and detect the relative amounts of mRNA contained in a single cell. Over the last decade there have been numerous refinements of ISH technology that have allowed for some new applications in basic cancer research.



ISH has been used for the detection and localization of gene expression, microbial infections, single-copy genes to individual chromosomes, translocations, deletions, amplifications, and other chromosomal abnormalities. ISH has proven a useful tool in the study of *c-H-ras* and *c-myc* expression in chemically induced liver tumors in B6C3F1 mice. In conjunction with immunohistochemistry and PCR analysis, ISH can add to our understanding of the role of oncogenes in tumorigenesis, as described in studies of *myc* in human malignancies.

Methodology. The success of this technique depends on many factors including the number of copies of DNA or RNA present, extent of preservation or fixation, type and size of probe, stringency conditions for hybridization, ionic strength of reaction, proportion of the nucleotide bases G and C, proportion of mismatch bases, method of probe labeling, and techniques of signal



detection. Some protocols are claimed to have a 90% success rate on the first hybridization attempt despite the source or type of tissue.

Of importance is the inclusion of proper controls including cells known to express the target gene, non-expressing cells, use of prehybridized probes, hybridization with non-specific vector sequences, and use of corresponding sense probes that should not hybridize to the specific mRNA.

Fixation. The evaluation of many fixatives for ISH has revealed that 4% paraformaldehyde seems to achieve the best overall results. Excellent results have been attained, however, with many of the cross-linking aldehyde fixatives, including formalin-fixed, paraffin-embedded tissues. Immediate quick fixation is recommended to inactivate RNA degradation by endogenous ribonucleases. Fixatives such as Bouin's, Zenker's, and Carnoy's should be avoided because they generally provide less preservation and retention of mRNA. When mRNA is abundant the length and time of fixation is less critical. Fresh frozen sections have also been used successfully with quick fixation from a few minutes to a few hours. The use of frozen sections for ISH, despite imparting a loss in morphologic detail, obviates the need for deparaffinization and protease digestion -- steps that might contribute to false negative results.

Pretreatment of slides. Pretreatment of slides with aminopropyltriethoxysilane, poly-l-lysine, or organosilane increases adherence of tissue sections. Following deparaffinization, protease digestion (proteolysis) is performed with using enzymes such as proteinase K, pepsin, or pronase to unmask the target nucleic acids and allow penetration of the probe. The degree of protease digestion is related to the length of fixation and tissue type; generally the longer the fixation, the greater the amount of digestion required. For some protocols treatment with acid solutions and/or excessive heat are required to achieve optimal results. A balance between adequate digestion and retention of morphology must be determined for each tissue type, probe, and reaction. DNase or RNase digestion may be performed to remove nontarget nucleic acid sequences.

Probe selection. Probes can be single-stranded (ss) or double-stranded (ds) DNA or RNA and ideally should be less than 400 bases long to permeate the cell matrix. ssRNAs are preferred for most experiments because RNA:RNA hybrids are more stable, and digestion with RNase removes unhybridized and partially hybridized ssRNA, thus decreasing non-specific, false positivity. dsDNA probes are most commonly used for the detection of DNA. Oligonucleotide probes (oligoprobes) of 20-40 bases of ssDNA are usually made by chemical synthesis, while longer ssDNA probes are made from clones or unidirectional PCR. The production and use of oligonucleotide probes does not require advanced expertise with basic molecular biology technologies because cloning technology is not necessary, labeling can be carried out in a single step, and oligoprobes can be constructed based on published cDNA maps and are increasingly available from commercial sources. Oligoprobes are somewhat less sensitive than ss or dsDNA probes with longer sequences because relatively few labeled nucleotides can be incorporated per molecule of probe; however, sensitivity is increased by using cocktails of up to 20 different specific oligoprobes simultaneously. The attainment of high specificity under stringent conditions is possible with oligoprobes.

There are multiple ways to label the probes using either radioactive (^{35}S , ^{32}P , or ^3H) or non-radioactive (biotin, alkaline phosphatase, digoxigenin, fluorescein) labels. In general the radioisotopic labels are best for quantitative analysis and have increased sensitivity and decreased resolution compared to non-isotopic methods. However, in the last ten years dramatic advances in non-isotopic labeling and detection systems have greatly enhanced their sensitivity. These systems are yielding a high resolution and decreased non-specific background allowing better localization of the target. Non-isotopic methods are relatively simple and nonhazardous, have a shortened procedure and turn-around time, and occasionally have sensitivity equivalent to that of radioisotopic methods.

Applications of ISH

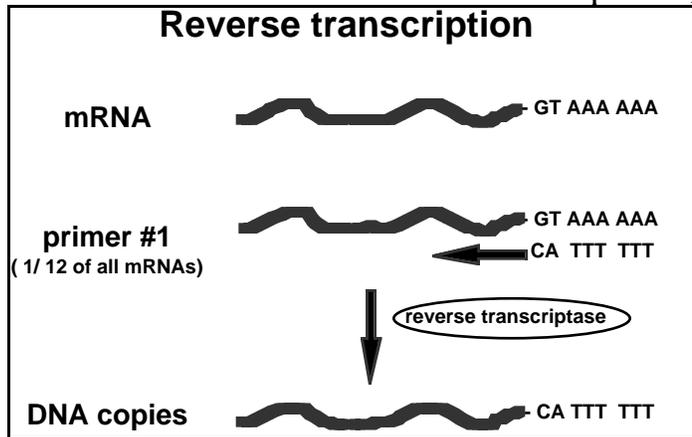
Oncogene/tumor suppressor gene overexpression. ISH has proven a useful tool in the study of *Brcal*, *c-H-ras* and *c-myc* expression in chemically induced liver tumors in B6C3F1 mice (Malarkey et al., unpublished results; Nelson, et al. 1990). In conjunction with immunohistochemistry and PCR analysis, ISH can add to our understanding of the role of oncogenes in tumorigenesis, as described in studies of *myc* in human malignancies (Tervahauta, et al. 1992).

Measurement of cell proliferation. The expression of histone genes is known to be tightly coupled to DNA synthesis, and the presence of histone mRNA appears to be a reliable marker of cells in the S-phase fraction. Detection of histone mRNA is proposed to be more accurate than proliferating cell nuclear antigen (PCNA) immunohistochemistry as an endogenous marker for S-phase cells (Alison, et al. 1994), and examination of archival tissue is possible. Because the histone genes are conserved between species, the same probes can be applied to human and animal tissue. Determination of histone mRNA has been used for measuring cell proliferation in normal and malignant human tissues (Chou, et al., 1990) and in regenerating rat liver after partial hepatectomy (Alison, et al. 1994).

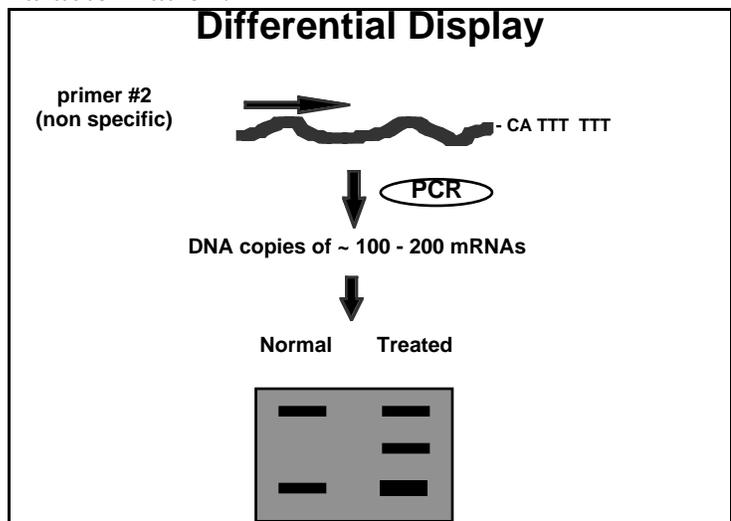
d. differential display of mRNAs

Comparisons of gene expression in different cell types can provide underlying information for understanding biologic processes that dictate development and disease. The analysis of changes in gene expression (either induction, enhancement, or suppression) in cells which have been exposed to certain chemicals or undergone neoplastic transformation is of prime interest in toxicological pathology. The technique called differential display of mRNA allows identification of new genes or the characterization of differentially expressed genes by comparing expression between two samples. Differential display offers a marked savings of time over cloning-based or subtractive hybridization techniques in searching for new genes, allows simultaneous detection of differentially expressed genes between different cell populations, is a simplistic method initially requiring only PCR apparatus and a sequencing gel, and requires only a small amount of RNA.

The aim of differential display is to amplify partial cDNA sequences from subsets of the approximately 15,000 mRNAs in any given cell using RT-PCR. Primer pairs are designed to amplify cDNA copies of 50-100 mRNAs at a time. The first primer anneals at the junction of the polyadenine tail which is naturally added on to the 3' untranslated sequence of almost all mRNAs. The second primer, an arbitrary primer of about 10 nucleotides of defined sequence, is added to the reverse transcriptase mixture along with polymerase and a radioactive nucleotide prior to PCR amplification. Amplified fragments are separated on a denaturing gel, and fragments present in one sample and absent from the next are determined by inspection of a suitably exposed autoradiograph. Once identified, DNA is cut directly from the gel and available for reamplification, Northern blotting, cloning, sequencing, and further characterization.



Novel mammalian genes have been identified and characterized utilizing differential display. The technique has been used successfully to find a novel gene induced by dioxin in rat liver. Further characterization of that gene has led to the discovery that it has homology with an, as yet, undefined human gene. Furthermore, investigators demonstrated a dose-related increase gene expression in rat liver and



constitutive expression in other organs including brain, lung, and kidney. Others researchers using the differential display technique have identified genes expressed specifically in human brain tumors or rat brain after treatment with cocaine or amphetamine. As the technique is refined further and more widely used, information will be gained regarding genes involved with chemical toxicity and carcinogenicity.

Differential display of mRNAs

•Aftermath

1 Characterize the gene

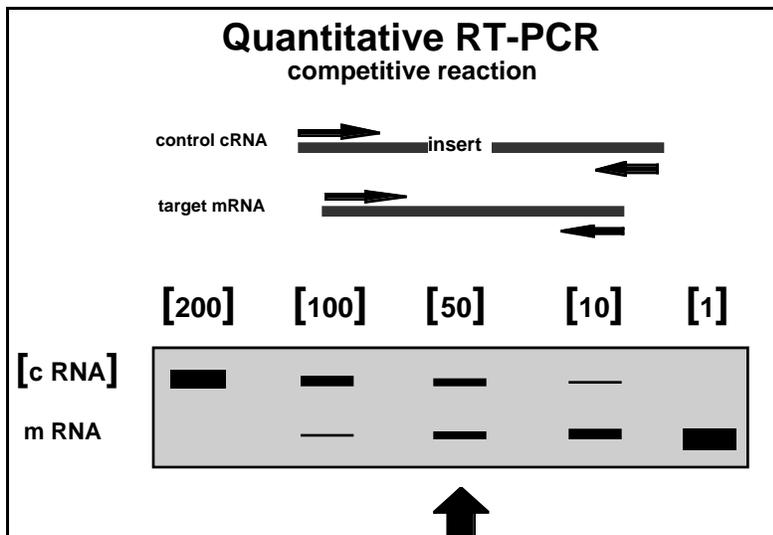
- k DNA sequencing
- k check cross-species homology
- k analyze expression in other organs and effects of treatment

e. quantitative reverse transcriptase PCR (QRT-PCR)

If RNA instead of DNA is used as the initial sample, producing a DNA copy of the RNA using reverse transcriptase before starting the PCR becomes necessary. Reverse transcriptase will generate cDNA from mRNA. Some of the commercially available polymerases function both as a reverse transcriptase and a DNA polymerase allowing the production of the first strand cDNA and cDNA amplification in the same tube. Assessment of gene expression by PCR is proving to be an extremely useful technique, especially in situations in which only a limited number of cells is available. Additional refinements of the RT-PCR technique have made it possible to make quantitative measurements of mRNA transcripts. QRT-PCR is full of both great potentials as well as problems. The results are dependent on the efficiency of the amplification and variability can be very large, since PCR can also easily amplify errors, and thus preclude accuracy and reliable quantification. Furthermore, none of these approaches control for the variability present in the RT reaction. The techniques must be carefully designed and executed for each experiment and lab in order to achieve reliable results.

i. competitive and non-competitive QRT-PCR

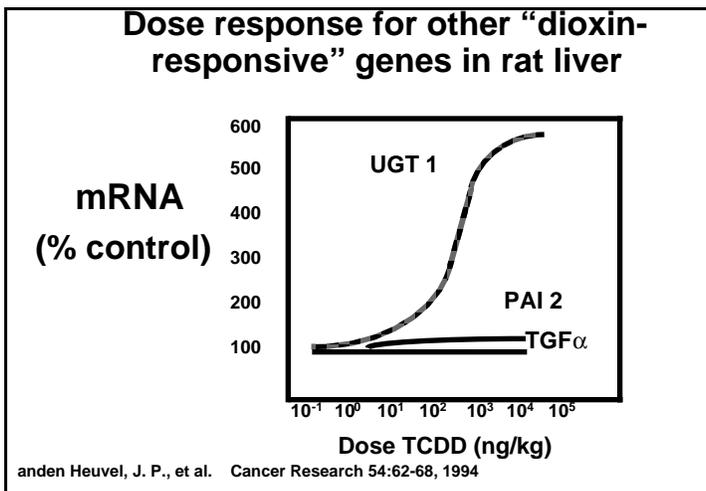
Although PCR has had a profound impact in many areas of research, its application to the quantitation of nucleic acids has proven problematic. This difficulty is due primarily to the exponential nature of PCR, as small variations in the amplification efficiency dramatically affect the yield of amplification product. Despite this limitation, absolute quantitation of RNA can be readily achieved using a



technique called competitive RT-PCR, which involves the spiking of individual reactions with known amounts of control RNA template that is coamplified with the target of interest. The control RNA (cRNA) is made by in vitro transcription and is identical to the target RNA except for a small deletion, insertion, or restriction site introduced by mutagenesis. The control

template is amplified by the same primers as the endogenous target to minimize differences in amplification efficiency. After a standard concentration curve is generated by amplifying serial dilutions of spiked control RNA, direct quantitative comparisons between RNA levels can be made. The point at which the concentration of target and control are equimolar (arrowhead) can be used to calculate the original quantity of target RNA. Endogenously expressed RNA can serve as an internal control.

This methodology has been used to study dose-response relationships of dioxin-responsive genes in the rat liver (Vanden Heuvel, et al. 1994). The investigators found dose-related increases in the expression of the enzymes cytochrome p450-1A1 and UDP-glucuronosyl-transferase-1 (UGT 1) but no increases in transforming growth factor-a (TGFa) or plasminogen activator inhibitor-2 (PAI2) at four days following a single exposure to dioxin. This type of data



adds to our understanding of the pertinence of the dynamic response of gene expression for certain environmental chemicals that pose potential human health hazards. The amount of gene expression may also be used as a biomarker of human exposures to environmental chemicals (Vanden Heuvel, et al. 1993). Some advantages of this technique include the benefit of examining many genes concurrently and the ability to quantify absolute levels of a given mRNA and attain a high sensitivity of detection.

Noncompetitive RT-PCR differs only in that the quantification is estimated based on conditions in which there is no competition for the components of the PCR. The standard RNA is added in a linear fashion within one order of magnitude of the target. The increasing series of standard amounts is co-amplified with equal amounts of total experimental RNA which generates a linear scaled graph. The intercept point (between native and standard) is then used to estimate quantity of the target mRNA.

ii. real-time (kinetic) QRT-PCR

Real-time QRT-PCR is a relatively new approach that is based on the measurement of the target DNA produced during each cycle of an amplification reaction. The traditional amplification product measurements have been the “end point” analysis which are the determinations made after it is completed. Real-time product monitoring offers the greatest potential for improved QRT-PCR.

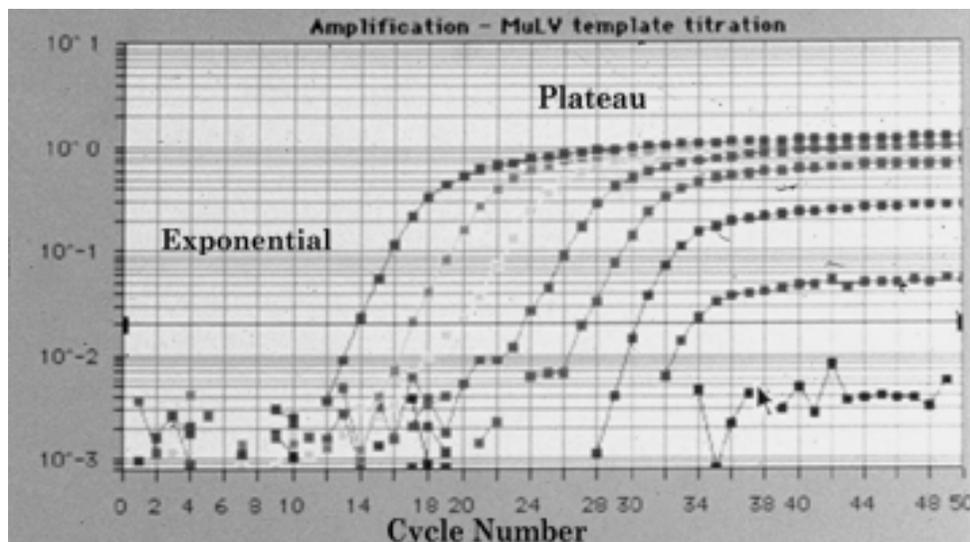
Two methods have been designed to provide real-time detection of amplified products, the ABI PRISM 7700 and 5700 Sequence Detection Systems (Perkin Elmer Applied Biosystems) and the LightCycler™ (Idaho Technologies). The quantitative ability of this detection method comes from being able to monitor the accumulation of amplification products using either fluorogenic probes or intercalating dyes. The methods are based on the kinetics of the PCR reaction and the fact that, under optimal conditions, the cycle number is proportional to the amount of starting material. The PCR occurs in two phases, the exponential phase and the plateau phase. The exponential phase occurs during the early and middle cycles and the amount of cycles before it enters this phase is dependent on the amount of starting cDNA. Relative differences in the number of cycles required to reach the midpoint of the exponential phase can be used to mathematically calculate (based on controls of known amounts of cDNA) the starting concentrations of cDNA. During the plateau phase the components of the reaction mixture (i.e. supply of nucleotides, polymerase activity, primer concentration) become limiting. Furthermore the numerous single stranded products may re-anneal with each other rather than with the primers.

The PRISM 7700 system uses probes (TaqMan; Perkin Elmer) to specifically detect the target sequence in the presence of nonspecific amplification products. A specific fluorogenic probe, that hybridizes internally to the amplified product, is added to the reaction. The 5' → 3' exonuclease activity of the *Taq* polymerase hydrolyzes the probe which abolishes the suppression of the reporter fluorescent dye. The fluorescence emission is measured during each cycle through a fiber optic lines positioned above an optically non-distorting tube caps. The amount of fluorescence detected is proportional to the amount of accumulated PCR product.



f. microarray-based analysis of gene expression

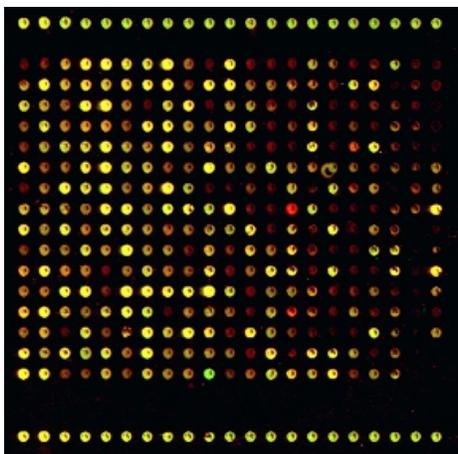
Microarray technology is one of several developing approaches to comparatively analyze genome-wide patterns of mRNA expression. Simply stated, the recently described cDNA microarray or DNA chip technology allows for the simultaneous



monitoring of the relative expression levels for hundreds to thousands of genes between two populations of cells (ie between tissue from treated and control animals or between an individual's diseased and normal tissues).

The technology uses specific complementary cDNA sequences or cDNA inserts of a library for PCR amplification that are arrayed as “microdots” on a glass slide. Each “microdot” represents a pure population of a specific cDNA and with high speed robotics as many as 1000 cDNA sequences are arrayed per cm². The microarrays serve as gene targets for hybridization to cDNA probes made from RNA samples of cells or tissues. A two-color fluorescence labeling technique is used to label the probes and during simultaneous hybridization fluorometric signals reflect the relative abundance of specific gene expression. The ultimate goal is to develop arrays which contain every gene in a genome against which mRNA expression levels can be quantitatively assessed.

In the laboratory of Dr. Cynthia Afshari at the NIEHS, RTP, NC (<http://www.niehs.nih.gov/envgenom/abstract/z01smart.htm>), microarray technology is being



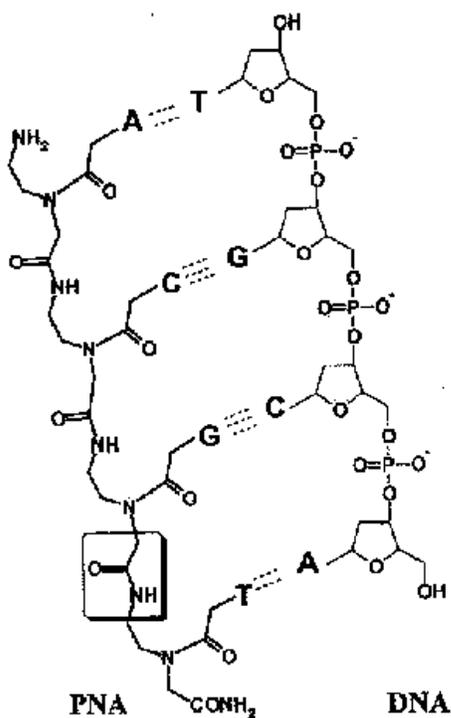
developed to identify toxicants and carcinogens in humans and rodents. The patterns of gene expression may reveal data that lead to assessing exposures as well as elucidating the mechanisms of action for environmental agents. For example, treatment of model systems (such as dioxin, peroxisome proliferators, or estrogen) may provide a “molecular signature” that can be used to gain insight into affects of unknown agents or determine crosstalk between combinations of agents.

IV. PNA (Peptide Nucleic Acids)

PNA, as first described by Nielsen, et al in 1991, was originally designed as a reagent to specifically bind double stranded DNA in order to control gene expression. The intent was to develop targeted antisense and antigene therapeutic drugs. PNA is an analog of DNA in which the entire negatively-charged sugar phosphate backbone is replaced with a peptide-like backbone. The peptide backbone consists of repeated units of N-(2-aminoethyl)glycine linked by amide bonds and this backbone supports the four natural nucleotide bases of adenine, cytosine, guanine, or thymine which are at spacing equal to that of the DNA bases.

There are a number of unique structural and hybridization properties of PNA that offer many potential biological and diagnostic applications than that of traditional oligonucleotides. For example, PNA is not prone to degradation by nucleases or proteases, thus offering high biological stability. PNA, having a neutral backbone and proper interbase spacing, binds to its complementary nucleic acid sequence (DNA or RNA) according to Watson-Crick base-pairing rules with higher specificity and affinity. Furthermore, the stability of the PNA/DNA duplex is essentially independent of salt concentration in the hybridization solution. There is also a higher thermal stability of the PNA/DNA duplexes, and this stability is strongly affected by imperfect matches. PNA/DNA duplexes with a mismatch is more destabilizing than a mismatch in DNA/DNA duplex. This property of discrimination makes PNA attractive for use in assays to detect specific mutations in DNA molecules.

PNA technology exploits the unique properties of PNA and is emerging as a breakthrough new technology which appears destined to revolutionize genetic diagnostics, therapeutics, and the study of gene function. Techniques are currently being developed to utilize PNA in PCR, arrays, mutation detection, in-situ hybridization (ISH), fluorescence in-situ hybridization (FISH), selective suppression of wild-type genes in the detection of low levels of oncogenes, biosensors in conjunction with mass spectrophotometry, gene therapy, and diagnostics.



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Nielsen, P.E. 1999. *Curr Opin Biotech* 10:71-75

V. Analysis of proteins

Western blotting

Method for the detection of a particular protein in a complex mixture that utilizes a 3 step procedure of gel electrophoresis, the specificity of antibodies, and the sensitivity of enzyme assays. The protein mixture is first separated on an SDS-polyacrylamide gel. Then a thin nitrocellulose membrane is applied to the face of the gel to bind and transfer the proteins. An electric field drives the proteins out of the gel and into the membrane (a.k.a.= blotting). The membrane is soaked in a solution containing the specific antibody to the protein of interest and the band is visualized after treatment and development of an enzyme-linked secondary antibody.

immunohistochemistry

The immunohistochemical detection of specific proteins on tissue sections is the pathologists' forte (as well as the bane of our existence). Don't forget to use proper controls and test the antibody's specificity!!!!!!

IV. slide-based techniques

Slide-based PCR techniques have been recently described and are being developed to allow the analysis of DNA or RNA from small subpopulations of cells in a tissues section. Heterogeneity of mutations among neoplastic cells has been observed in both human and animal tumors. Some studies describe techniques with careful microdissection of small areas of tumors from histologic sections followed by a PCR-based mutation detection assay.

a. ISH (see above)

b. Microdissection of tissue

New slide-based microdissection techniques have recently been described and applied in the analysis of DNA, RNA, and/or protein from small subpopulations of cells in tissue sections, and in some investigations have helped delineate the sequence of molecular events of human tumor development and progression. The precision of microdissection varies among the techniques, and, until laser capture microdissection became available in 1997, methods have been capable of analyzing no fewer than about 50 cells from a histologic section. The results will aid in our understanding of the sequence of mutational events and gene expression in tumor development and progression, and furthermore may aid in studying clonality, tumor diagnosis, and prognosis of neoplasms.

i manual scraping

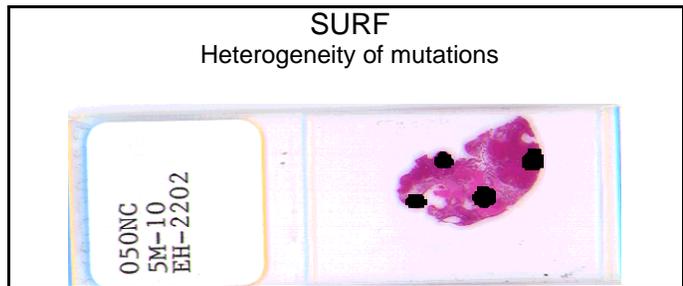
Be careful not to contaminate. Need we say more?

ii. glue

Recently a technique using a starch-based adhesive has been suggested as an alternative for the selection and study of small subpopulations of cells (Turbett, et al. 1996). Briefly, a starch adhesive glue was placed over the areas of interest as small as 1 mm² and the dried spots of glue were removed along with the tissue and placed into a tube for subsequent DNA isolation. Similar techniques are currently being applied by some investigators for the study of mRNAs.

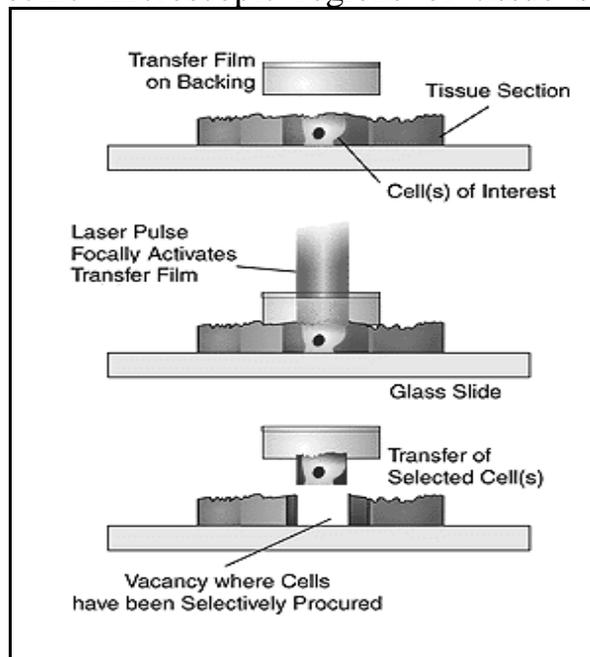
iii. SURF

A specialized technique called selective ultraviolet light radiation fractionation (SURF) allows for the selection of a subpopulation of as few as 30 cells from surrounding tissue in a histologic section (Shibata, 1992). Ink dots are applied over the tissue to protect subpopulations of tumor cells from short-wave ultraviolet light exposure. The unprotected cells are damaged so that DNA targets are not amenable to PCR amplification. After the appropriate exposure to ultraviolet light the protected cells are then cut away from surrounding tissue, which is mounted on plastic slides, and used as the DNA source for mutational analysis.



iv. Laser capture microdissection

Laser capture microdissection is a promising new method for procuring as few as 2-3 pure cells from specific microscopic regions of tissue sections with precision and without contamination (Emmert-Buck, et al 1996). The microdissection is accomplished when a laser beam focally activates a special transfer film which bonds specifically to the targeted cells identified by microscopy within the tissue section. The transfer film with the bonded cells is then lifted off the thin tissue section, leaving all unwanted cells behind. Multiple homogeneous samples can then be analyzed by PCR.



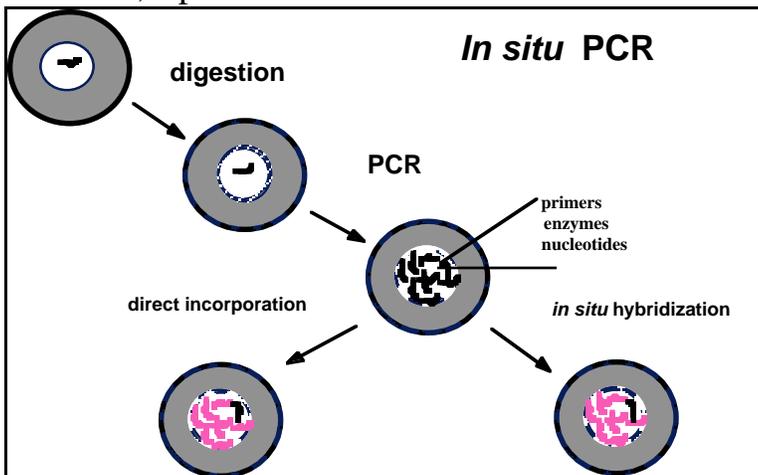
website: <http://dir.nichd.nih.gov/lcm/lcm.htm>

c. IN SITU PCR (IS-PCR)

By combining the localizing ability of ISH with the amplifying capability of PCR, the technique of IS-PCR has allowed the detection of low copy numbers of specific DNA and mRNA sequences in tissue sections and cell preparations. IS-PCR has been successful for the detection of low copy number viral DNAs, single-copy genes, immunoglobulin gene rearrangements in cell suspensions and cytopins, and for the analysis of low-level gene expression. The greatest success has been gained in the study of infectious diseases and, in particular, in the diagnosis of viral latency. The advantages of IS-PCR include elimination of concern over contamination-based false positives, ability to assess the histologic and subcellular distribution of the target, and ability to detect as few as 1 -10 copies in a cell. IS-PCR also has been extended to studies of RNA after the *in situ* synthesis of the corresponding cDNA through the use of reverse transcriptase. This approach has been used successfully for the detection of RNA viruses and for mRNAs.

Detection threshold of major hybridization assays	
	<u>detection threshold</u>
<i>In situ</i> PCR	1- 10 copies / cell
<i>In situ</i> hybridization	> 10 copies / cell
Southern blot	1 copy / 100 cells
Slot blot	1 copy / 200 cells

The procedure seems straightforward; however, many scientists have been unable to achieve reproducible results. Results from work with archival formalin-fixed, paraffin-embedded materials have been limited and have required



experimental designs of moderately high complexity. A number of variables can influence the success of this technique including factors related to fixation, tissue pretreatment, efficiency of the PCR reaction, retention of the amplified product *in situ*, and sensitivity for the detection of the amplified product. IS-PCR should be used only when alternative methods such as IHC, SURF, ISH, or filter hybridization

techniques fail to answer the specific scientific or diagnostic question. The development of IS-PCR is time-consuming and technically demanding but it offers great potential for improving our understanding of biological systems.

The enticing promise of IS-PCR is that by amplifying DNA or RNA within cells, the sensitivity of ISH will be elevated to permit the detection of single copy DNA sequences or low copy mRNAs in individual cell preparations or tissue sections. From the time that PCR was first conceived, researchers have recognized

the tremendous potential of being able to combine this technique with ISH. The advantages include elimination of concern over contamination-based false positives, ability to assess the histologic and subcellular distribution of the target, and ability to detect as few as 1 -10 copies in a cell. IS-PCR has been successfully performed using fresh, frozen, cell suspensions, or paraffin-embedded archival fixed tissues.

Methodology

Fixation and pretreatment. Major obstacles have been overcome in the development of IS-PCR. Parameters were established to expose adequately the target nucleic acid sequences in the cells or tissue to the PCR reaction mixture while still maintaining tissue integrity and cellular morphology. Solutions to some of the problems lay in the fixation and appropriate protease treatment of the cells or tissue and the development of equipment designed specifically for IS-PCR.

The fixative of choice is NBF for at least 8 hours (Nuovo, 1994). Choosing a crosslinking fixative is essential because it appears necessary for retention of the amplified products in the tissue. Non-crosslinking acetone or ethanol fixation alone can have deleterious effects on the reaction, and PCR product can easily be washed from the tissue. In general, Carnoy's, Bouin's, and Zenker's solutions should be avoided since they may not achieve sufficient crosslinking, and components of the fixatives may interfere with the PCR (Nuovo, 1994).

Fixation and protease treatment are critical to the success of IS-PCR. The protease treatment must be titrated so that it digests the tissue sufficiently to permit exposure of the target DNA to the reaction mixture, but not so much that the amplified DNA would be washed away. The concentration of protease and the length of time of protease digestion differ according to the specific enzyme used, the fixative, the length of fixation, and the tissue or cell type containing the target.

PCR on slides. There are several other issues that make IS-PCR more difficult than routine solution PCR. To prevent mispriming, the reaction mixture must be added to the tissue at temperatures greater than 70°C, a procedure introduced by Nuovo (1994) called "hot starting". This modification has allowed the detection of as few as one human papilloma virus copy per cell in formalin-fixed, paraffin-embedded samples. Also, the reaction mixture must be sealed over the tissue to prevent evaporation and drying of the tissue during the temperature cycling. Sealing the reaction mixture has been accomplished in a number of ways, including immersing the slide in mineral oil or securing a coverslip over the tissue using nail polish. Some researchers have even suggested mounting tissues onto glass slides cut into pieces small enough to be placed into 0.5 ml PCR tubes containing the reaction mixture.

To achieve adequate thermocycling, initial protocols involved placing the slides on top of thermocycler block designed for solution PCR or in hot air ovens. Although successful results have been achieved, the techniques utilizing investigator-modified solution-PCR equipment may have contributed to inconsistent and irreproducible results. Today, a number of biotechnology companies offer specialized equipment dedicated to IS-PCR that will help optimize thermocycling temperatures while efficiently maintaining reaction mixtures on the slide. This equipment may provide optimal conditions to improve the consistency, reproducibility, and reliability of the technique.

Signal detection. Labeled nucleotide can be incorporated directly into in situ amplifiants (direct IS-PCR) or, alternatively, ISH can be performed after in situ amplification using labeled oligonucleotide probes (indirect IS-PCR). For the direct method non-isotopic labels, such as digoxigenin, have been most commonly used. A reportedly high occurrence of false-positive results by the direct method is supposedly due to misprimed DNA products or DNA repair mediated by the *Taq* polymerase. Nuovo (1994) appears to have eliminated some false-positive reactions by pretreating the tissues with a solution of dideoxynucleotides to block DNA repair. Detection by the indirect method is recommended for most experiments and has been performed using either isotopically or non-isotopically labeled probes.

The efficiency of the PCR amplification on the slide is not well defined and has led to some controversy. Some results have suggested that the PCR on the slide is highly inefficient and that perhaps only a 50-fold increase of the original target is produced after 30 cycles (Nuovo, 1991). Despite the inefficient amplification, however, the amplifiants are enough to be easily detected by ISH or possibly by a suitably labelled product of direct IS-PCR. Further scientific analysis of the PCR efficiency and specificity may clarify these issues.

In situ RT-PCR. IS-PCR also has been extended to studies of RNA after the in situ synthesis of the corresponding cDNA through the use of reverse transcriptase. This approach has been used successfully for the detection of RNA viruses and for mRNAs. Usually the tissue is first treated with DNase overnight to remove native DNA. Alternatively, it may be possible to design primers that span an intron so that the amplification is specific for the spliced RNA target making DNase treatment unnecessary.

Controls. To confirm the sensitivity and specificity, stringent controls are essential and should include known positive and negative cells or tissues, use of non-complementary primers or oligonucleotides in place of the original primers, amplification mixtures lacking *Taq* or primers, and detection of a known endogenous single-copy gene.

Applications of IS-PCR

IS-PCR has been successful for the detection of low copy number viral DNAs, single-copy genes, immunoglobulin gene rearrangements in cell suspensions and cytopins, and for the analysis of low-level gene expression. The greatest success has been gained in the study of infectious diseases and, in particular, in the diagnosis of viral latency. Applications of IS-PCR for the study of RNA associated with toxicity, carcinogenesis, and cancer progression are being developed.

Potentially, researchers may be able to visualize single cells bearing premalignant mutations or karyotypic alterations. In our laboratory we have tried, unsuccessfully, to identify individual neoplastic and non-neoplastic mouse pulmonary epithelial cells which contain a mutation in the *K-ras* oncogene. Our hope was to elucidate events of tumor initiation and progression using a direct method of IS-PCR on histologic sections of mouse lung. To date, we do not know of any publications in peer-reviewed journals applying IS-PCR for point-mutation analysis. Further refinements of IS-PCR may enhance the utilization of this technique for mutational analysis or perhaps a technique such as laser capture microdissection will suffice.

V. FIXATION

Techniques have been successfully performed using fresh, frozen, fixed, or paraffin-embedded fixed tissues or cell suspensions. Fixation can be detrimental to nucleic acid molecules and thus negatively affect the success of the techniques, however fixation is essential for some of the slide-based techniques (ISH and IS-PCR) and, of course, for the preservation of archival tissues. Fortunately, most of the PCR-based techniques can be successfully applied to paraffin embedded tissues fixed in neutral buffered formalin. There are many optimizing strategies reported to help overcome some of the detrimental effects of fixation on PCR. Shortening the duration of fixation, increasing the initial sample size, amplifying products less than 200 bp, using a nested primer method, and increasing the number of amplifying cycles have all been shown to help achieve positive results for PCR.

For ISH, short fixation of frozen tissue sections with 4% paraformaldehyde seems to achieve the best overall results. Furthermore, when analyzing for RNA, immediate fixation is strongly recommended to inactivate RNA degradation by endogenous ribonucleases. The use of frozen sections for ISH, despite imparting a loss in morphologic detail, obviates the need for deparaffinization and protease digestion -- steps that might contribute to false negative results. For IS-PCR, the fixative of choice is neutral buffered formalin for at least 8 hours. Choosing a crosslinking fixative is essential because it appears necessary for retention of the amplified products in the tissue. Non-crosslinking acetone or ethanol fixation alone can have deleterious effects on the reaction, and PCR product can easily be washed from the tissue. In general, Carnoy's, Bouin's, and Zenker's solutions should be avoided for ISH and IS-PCR since they provide less preservation, they may not achieve sufficient crosslinking, and/or components of the fixatives may interfere with the PCR.

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