**Lec 4 Immunological Technique**

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**Molecular Diagnostic Techniques( Polymorphisms)**

**Polymorphisms**

Structurally, mutations, variants, and polymorphisms are the same thing—

changes in the reference amino acid or nucleotide sequence. Alterations in

DNA or protein sequences shared by at least 2% of a natural population are

considered **polymorphisms.** The different versions of the affected sequences are referred to as *alleles.* Polymorphisms can involve a single base pair **(single-nucleotide polymorphisms or SNPs)** or millions of base pairs. Polymorphic changes may or may not have phenotypic effects.

Deleterious phenotypic changes are usually limited so that they do not reach

The required frequency in a population; however, some polymorphisms are

maintained because they are also associated with a beneficial phenotypic

effect. A well-known example of this is the A to T base substitution in the

beta-globin gene on chromosome 11 that causes sickle cell anemia. This

DNA substitution results in the replacement of glutamic acid (E) with

valine (V) at position 6 in the protein sequence (E6V). The mutation results

in abnormal red blood cells that do not circulate efficiently. The deleterious

effect has likely been maintained in the population because it is balanced by

a beneficial phenotype of resistance to *Plasmodium* species, which cause

malaria.

A highly polymorphic region in the human genome is the major

histocompatibility (MHC) locus on chromosome 6. The different nucleotide

sequences result in multiple versions or alleles of the human leukocyte

antigen (HLA) genes in the human population. These alleles differ by

nucleotide sequence at the DNA level (polymorphisms) and by amino acid

sequence. Each person will have a particular group of HLA alleles, which

are inherited from his or her parents. The HLA proteins coded for by these

alleles play important roles in the immune response and allow the immune

system to differentiate “self” from “non-self”. Other highly polymorphic areas of the genome include the genes coding for the antibody proteins and antigen receptor proteins in B cells and T cells, respectively. Polymorphisms are introduced in each cell through cell specific genetic events (gene rearrangements), followed by enzymatically catalyzed sequence changes (somatic hypermutation). These sequences differ from cell to cell, allowing for the generation of a large repertoire of antibodies and antigen receptors to better match any foreign antigen ,

Polymorphisms are found all over the human genome. Although there are

millions of SNPs, larger polymorphic differences occur less frequently.

Polymorphisms that create, destroy, or otherwise affect sequences in DNA

that are recognized by nuclease enzymes (restriction enzymes isolated from

bacteria) are detected as **restriction fragment length variations** or

**polymorphisms (RFLPs)** that differ among individuals. Repeat-sequence

polymorphisms, such as short tandem repeats (STRs) and variable-number

tandem repeats (VNTRs), are head-to-tail repeats of a single base pair to

more than 100 bp repeat units. STRs and VNTRs can be detected as RFLPs

or by using amplification procedures. STR testing has replaced RFLP

testing for human identification (DNA fingerprinting in forensics) and HLA

typing for parentage testing. STRs and VNTRs are the markers commonly

used to follow engraftment of donor cells into recipient blood and bone

marrow after allogeneic bone marrow transplantation.

In addition to the nuclear genome, mitochondria, located in the cytoplasm of eukaryotic cells, carry their own genome. The mitochondrial genome is circular, containing about 16,500 bp. Polymorphisms are also found in two regions of mitochondrial DNA sequences (hypervariable regions). These polymorphisms are not transcribed into RNA and do not affect protein structures. They are used for maternal lineage testing, because all maternal relatives share the same mitochondria and so have the same mitochondrial polymorphisms.

**Hybridization Methods**

**Specific Procedures**

Restriction enzyme cleavage methods are highly informative for investigating small genomes, such as those of microorganisms or plasmids.

For complex genomes, such as human DNA, such analyses are not practical, as the DNA is too large and complex to generate readable fragment patterns. How does one analyze specific DNA regions in a complex genome by RFLP without first cloning the region of interest? This question was addressed by Edwin Southern in the mid-1970s. The significance of his invention, the Southern blot, was that informative studies could be performed directly on large and complex genomes by cleaving the DNA into smaller fragments with restriction enzymes, separating the

fragments by gel electrophoresis, and identifying the region of interest through hybridization with labeled **probes** (short nucleic acids that bind to complementary sequences). **Hybridization** involves the binding of two complementary strands of nucleic acids, in this case, the template strand and a probe. A variation of the Southern blot, called the *northern blot,* was subsequently developed to analyze RNA structure and expression. Northern blots were mostly research tools and not used routinely for diagnostic purposes.

**Clinical Correlations**

**Western Blot**

The western blot was used for many years as a confirmatory test for the presence of

antibodies to HIV, the cause of AIDS, and to *Borrelia burgdorferi,* the cause of Lyme

disease. Although the western blot has been replaced by less labor-intensive methods in

the clinical laboratory, this highly specific method is still widely used in research

laboratories for protein analysis.

Detection of proteins and protein modifications can be done by a method

known as the **western blot.** In the western blot procedure, serum, cell

lysate, or extracted proteins are separated by gel electrophoresis and blotted

to a membrane. The probes for western blot are polyclonal or monoclonal

antibodies specific for the proteins of interest. Western blots may also be

probed with biological fluids such as serum to detect the presence of

antibodies produced in response to infection. Detection is performed with

secondary antibody–enzyme conjugates and color- or light-producing substrates.

**Array Methods**

Southern blotting and its variations allowed assessment of one or a few

molecular targets on as many samples as the gel system would allow. As

knowledge of genetic networks and pathways grew, it became apparent that

informative studies should include simultaneous analysis of many genes or

proteins to assess the true biological state of a cell or an organism. Thus

began the study of genomics. **Genomics** refers to the analysis of hundreds

to thousands of targets or whole genomes, rather than single genes.

The first methodology to perform these studies involved reverse-dot-blot

hybridization, that is, hybridization of a labeled sample to unlabeled

immobilized probes spotted or arrayed on a solid support. Modern arrays

can carry up to hundreds of thousands of probes. There are three basic types

of arrays: comparative genomic arrays, RNA expression arrays, and highdensity

oligonucleotide or SNP arrays. Comparative genomic hybridization

arrays are used to detect amplifications or deletions in DNA **(Fig. 1).**

Gene expression (mRNA synthesis) is measured using expression arrays,

where mRNA from the test material is converted into labeled cDNA, which

is hybridized to the probes. SNP arrays have single-nucleotide resolution

and can even be used to determine DNA nucleotide sequence. Generally,

thousands of targets with probes bound to a very small area, such as a

microscope slide, is referred to as a **microarray.**

Microarrays use highly specific unlabeled probes attached directly to a

solid support. The support can be glass slides or beads (bead arrays). The

test sample (nucleic acids or proteins isolated from cultures, cells, or body

fluids) is labeled and hybridized to the many immobilized probes.

Microarrays are used for a variety of applications, including detection of

chromosome microdeletions by virtual karyotyping and gene-expression

profiling. In the former method, genomic DNA is assessed for loss or gain

of genetic material at specific chromosomal locations compared with a

normal reference sample. In the latter method, the levels of mRNA

transcribed from thousands of genes are compared with normal reference

samples to look for up- or down-regulation of gene transcription.

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**Fig1**For array analysis, unlabeled probes are immobilized and hybridized to

labeled sample material (green). A reference material (red) is hybridized to the same array.

The results of the array are relative test:reference colors. In this example, a green color

indicates amplified gene regions, and the neutral yellow colors indicate no amplification or

deletion of those regions. A lack of red color, which would indicate deletion of a gene region,

is seen.

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Fig2: (A) Three of 100 to 400 bead colors, each with antibody to a different

analyte. The presence of multiple targets can be detected by the unique colors of the beads

that have associated fluorescence from the secondary antibody. Flow cytometry is used to

assay each bead separately for bound fluorescence. (B) For nucleic acid analysis, bead

array antibodies are replaced with single-stranded oligonucleotides complementary to the

test nucleic acid. If present, biotinylated sample DNA will hybridize to the sequences, and

the biotin-specific conjugate will generate a signal.

For array analysis, sample labeling is fluorescent, allowing dual detection

of the test sample and a reference sample that is hybridized to the array

along with it (see Fig. 1). This results in measurement of increased or

decreased amounts of test material relative to the normal reference. In bead

array systems, beads carry fluorescent labels specific to the probe they carry

so that bound sample, if present, can be detected in a flow cytometric

method.

Bead array assays are based on preparations of fluorescent beads of 100

to 400 different fluorescent “colors.” Each color bead is attached to an

antibody or a nucleic acid probe that will bind specifically to the target

protein or nucleic acid sequences. The target nucleic acid molecules may be

directly labeled, or for protein targets, a secondary antibody conjugated to a

fluorescent signal may be used to detect the presence of the target **(Fig. 2).** Because many beads are used, each bound to a different antibody or

probe, multiple targets can simultaneously be detected in a single assay run;

in other words, a multiplex assay is performed. Clinical applications include

HLA typing and respiratory virus panels.

**In Situ Hybridization**

**In situ hybridization (ISH)** refers to detection of targets in place as they

appear in tissues, cells, and subcellular structures. Labeled probes are used

to bind or hybridize to the targets. ISH is frequently used in pathology

studies of tissue and cell suspensions. **Immunohistochemistry** is a type of

ISH using labeled antibodies to detect the presence of clinically significant

protein targets, such as those expressed by tumor cells. Probes for these

tests are monoclonal antibodies linked to enzymes, such as horseradish peroxidase, that produce visible signals from chromogenic substrates.

Alternatively, enzyme-linked secondary antibodies recognizing the primary

antibody isotype may be used.Positive and negative controls must be included in ISH testing to ensure accuracy of the results. Normal tissue that expresses the protein target should serve as the positive control, whereas an adjacent section cut from the test tissue without the addition of the primary antibody and tumor tissues that do not express the antigen should serve as negative controls.

Ideally, the control tissues are processed with the test tissue. Control tissues

processed differently from the test tissue validate reagent performance but

do not verify the tissue preparation. If staining of positive control tissue is

not satisfactory, or if unwanted staining occurs in negative controls, all

results with the patient specimen should be considered invalid.

**Clinical Correlations**

**Programmed Cell Death Ligand (PD-L1)**

The programmed cell death ligand (PD-L1) is a transmembrane protein that suppresses the adaptive immune response when it is bound to a receptor on activated lymphocytes and dendritic cells called *programmed cell death protein 1* (PD-1). PD-L1 is found on some tumor cells and is thought to block recognition of the abnormal cells by lymphocytes that express PD-1. Detection of PD-L1 by immunohistochemistry guides the use of immunotherapy for those tumors that express the ligand.

**Fluorescence in situ hybridization (FISH)** methods use fluorescently

labeled probes and require specialized microscopes equipped to detect the

emitted fluorescent signals. FISH is commonly performed to detect specific

chromosome abnormalities, such as microdeletions or gene amplifications.

In these methods, probes ranging in size from a few thousand to hundreds

of thousands of bases long are covalently attached to the fluorescent dye.

FISH can be performed on non dividing (or interphase) cells or directly on

metaphase chromosomes from dividing cells. The DNA from the sample is

denatured into single strands. The probes are applied to prepared slides of

the cells or chromosomes, where they hybridize to their complementary

sequences. The resulting signals indicate if the targeted gene or region is

a bnormal. In addition to the test probes, reference probes that target the

centromeres of selected chromosomes are used to identify the chromosomes

of interest while assessing deletion or amplification **(Fig. 3).**

ISH methods are sensitive to the buffer and temperature conditions of

hybridization, a concept referred to as *stringency.* Protocols must be strictly

followed to avoid false-positive results caused by nonspecific binding of

probes or false negatives caused by failure of the probe to bind. Array

methods (comparative genome hybridization) complement FISH testing in

cases of multiple or complex genetic abnormalities as well as deletions and

amplification of genes.



Fig3: FISH analysis of the epidermal growth factor receptor gene. The gene probe

is labeled orange, whereas a probe complementary to the centromere of chromosome 7 is

labeled green. Normally, there are two chromosomes, each carrying one gene in each

nucleus (left). The image on the right shows gene amplification with multiple orange signals

associated with single green signals.