ARSHI

الجمعية العربية لتطابق الأنسجة
و الجينات المناعية

HLA typing technologies I
SSP,SSOP

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DCRC

Human Leukocyte Antigen (HLA)

The human leukocyte antigen system (HLA) is the name of the major Histocompatibility complex (MHC) in humans.

The super locus contains a large number of genes related to immune system function in humans.

This group of genes resides on chromosome 6, and encode cell-surface antigen-presenting proteins and many other genes.

The proteins encoded by certain genes are also known as *antigens*, as a result of their historic discovery.

The major HLA antigens are essential elements in immune function.

Why HLA typing ?

Transplanted organs are allografts, in which the donor organ and the recipient are genetically different

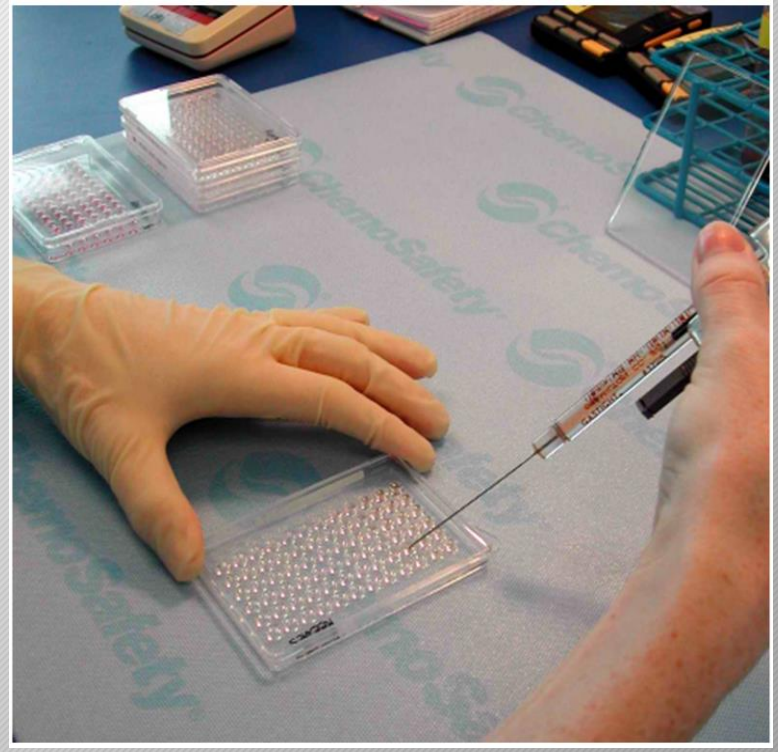
Compatibility (matching) of the HLA of the donor and the recipient increases the chance for a successful engraftment.

Help or confirm the HLA associated disease (Coeliac Disease , Ankylosing spondylitis).

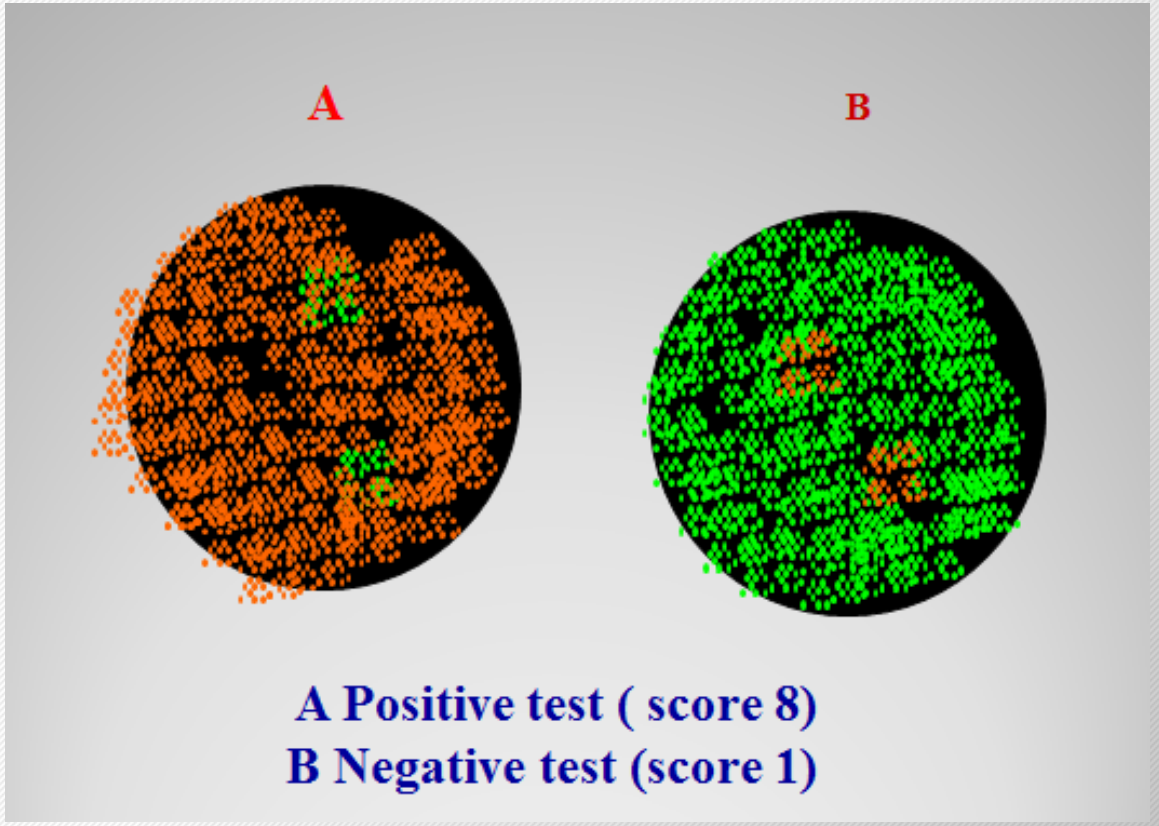
HLA typing Serology

- **Traditionally, HLA antigens have been defined using serological techniques. These techniques rely on obtaining viable lymphocyte preparations .**

- **serology performed adequately in typing family members, it proved unsatisfactory in typing unrelated donors for bone marrow transplantation.**



Appendix.1.HLA Serology



DNA –Based typing Methodology

During the last few years, DNA-based typing techniques have begun to replace the serological techniques in clinical applications. The DNA methods were initially applied to Class II typing, but more recently they have been used to determine Class I alleles. While Emergence of nucleotide sequence data for the alleles of HLA genes permitted the rapid development of many PCR-based techniques and reagents. Conversely, the PCR technique greatly reduced the effort required in subsequent sequencing of new alleles.

Polymerase Chin Reaction (PCR)

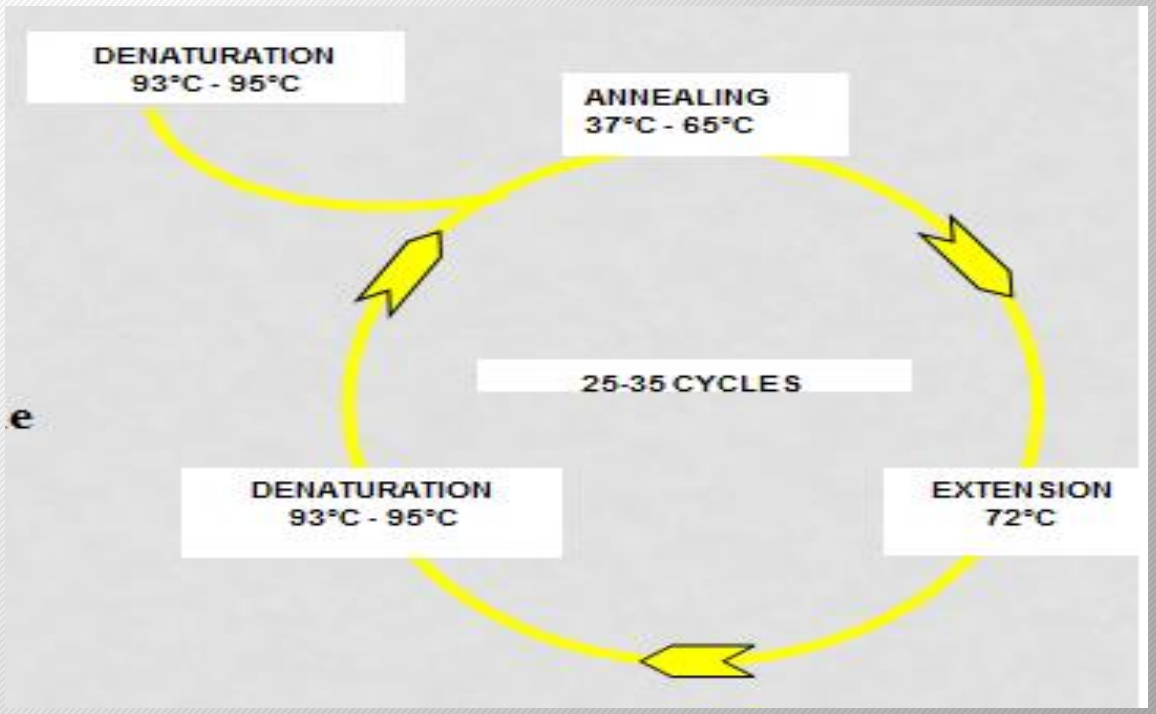
The PCR process is a simple and powerful method, which allows amplification of DNA segments in vitro through a succession of incubation steps at different temperatures. Typically, the double stranded DNA is heat denatured, the two primers complementary to the 3’ boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these consecutive steps is referred to as a cycle. The PCR process is based on the repetition of this and can amplify DNA segments .

Polymerase Chin Reaction (PCR)

Three distinct steps :

- ***Denaturation*: DNA melts and opens into single-stranded DNA**
- ***Annealing*: The primer binds to the target DNA**
- ***Extension*: The Taq polymerase extends the target DNA**

The above three steps are repeated in a cyclic manner in the same order to get an exponential amplification of the target DNA



Polymerase Chin Reaction (PCR)

PCR based methods may be broadly classified into three categories:

- Those which generate a product containing internally located polymorphisms which can be identified by a second technique (e.g. PCR-sequence specific oligonucleotide(SSO) probing, PCR-RFLP, PCR followed by sequencing).

- Those in which the polymorphism is identified directly as part of the PCR process, although there are post-amplification steps (e.g. PCR-sequence specific primer (SSP)).

Polymerase Chin Reaction (PCR)

- Conformational **Analysis** in which different mutations generate specific conformational changes in PCR products. e.g. heteroduplex analysis, single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE).

- The two methods most frequently adopted in clinical histocompatibility laboratories have been SSO and SSP

Polymerase Chin Reaction (PCR)

Essential components of successful PCR

- DNA: which serves as template
- DNA polymerase: an enzyme which catalyzes the replication of template DNA.
- Primer sequences: which provide specificity and allow initiation of replication.
- DNTPs: to incorporate into the newly synthesized DNA.
- Buffer : to ensure optimal pH for polymerase activity .
- MgCl: to provide divalent cations required by the polymerase.

Sequence Specific Primer technique (SSP)

- Successful HLA typing by SSP depends on the exquisite specificity of many pairs of primers to amplify only those sequences which anneal perfectly with a given primer pair;
- DNA is added to an array of microtiter tubes, each of which contains a different combination of primers.
- If DNA for the appropriate HLA allele (or group of alleles) is added to the SSP mix, the primers will amplify the sequence of interest, and the amplified product can be visualized by gel electrophoresis;

Sequence Specific Primer technique (SSP)

- In tubes where the appropriate DNA required by the primers is not present, no amplification will occur and no product will be visualized in the gel.

- SSP **discriminates** between the different alleles **during** the PCR process

- This shortens the post-amplification processing time to a simple gel electrophoresis detection step

Sequence Specific Primer technique (SSP)

Amplification during PCR may be affected by various factors:

A. Pipetting errors
B. Poor DNA quality
C. Presence of inhibitors….etc.

An internal control primer pair is included in every PCR reaction; The control primer pair amplifies a conserved region of the Human ß-globin gene which is present in all human DNA samples and its used to verify the integrity of the PCR reaction.

Sequence Specific Primer technique (SSP)

Interpretation SSP results is based on the **presence** or **absence** of a specific amplified DNA fragment.

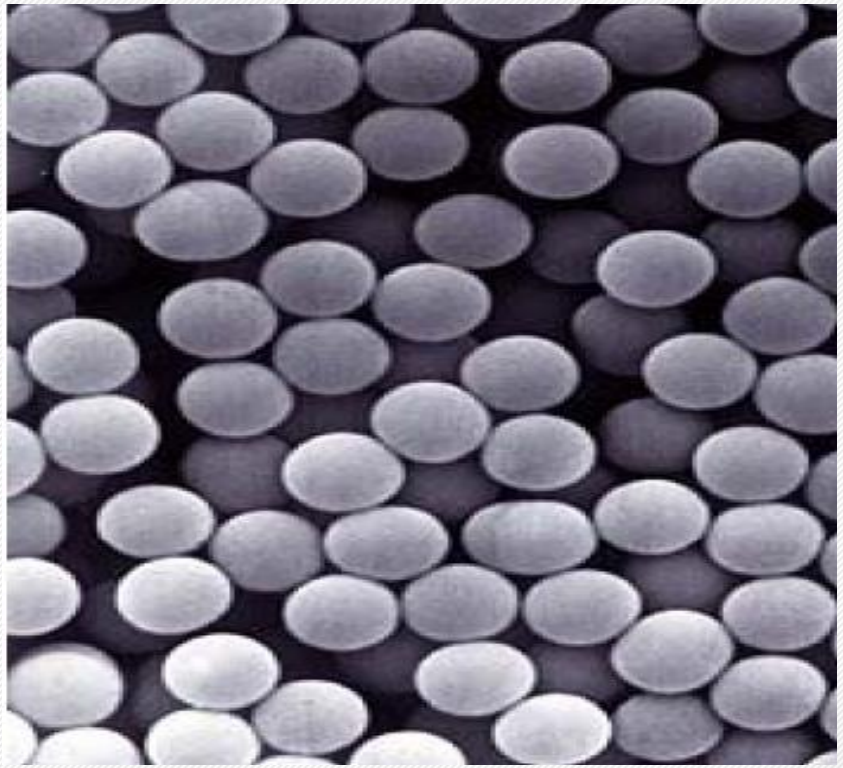
Sequence Specific Oligonucleotide technique (SSO)

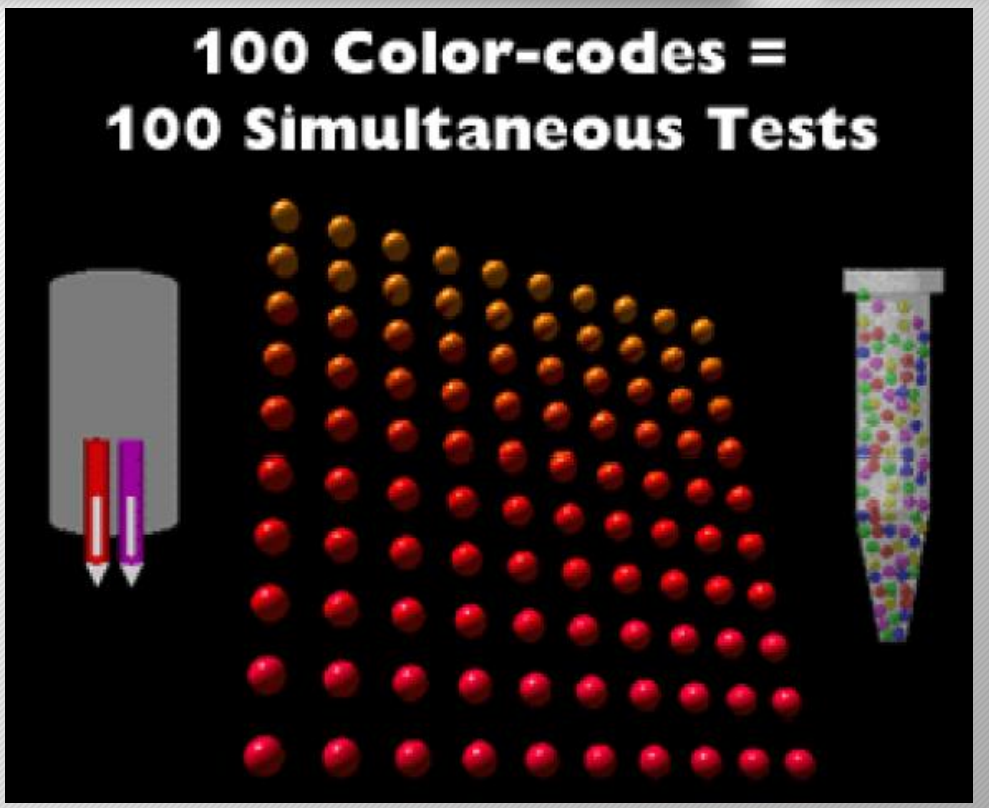
Hybridization of PCR-amplified DNA with sequence specific oligonucleotide (SSO) probes was the first molecular typing method used to detect HLA Class II alleles. The literature contains many alternative methods for SSO typing.

The main differences between these were:

- **The length of the DNA sequence of oligonucleotide probes**
- **Reporter molecule**
- **Detection method**

Sequence Specific Oligonucleotide technique (SSO)





Multiple Microparticles (Luminex)

Sequence Specific Oligonucleotide technique (SSO)

HLA typing using Luminex is a reverse polymerase chain reaction sequence specific oligonucleotide (PCR-SSO) system which involves PCR amplification of targeted regions within the MHC class I or II regions with group specific primers, followed by a process of probing the amplicon with Luminex beads, each coated with sequence specific oligonucleotide probes to identify the presence or absence of specific alleles. The assignment of HLA type is then based on the reaction pattern observed, compared to patterns associated with published sequences.

Sequence Specific Oligonucleotide technique (SSO)

- **Primers** used for the amplification are biotinylated. Amplification can then be either symmetrical, which therefore requires a denaturation step to create single strands or can be asymmetrical to generate an excess of a single strand. The single stranded product is then hybridised with a multiplex of up to 100 beads, all of which can be uniquely identified by their internal dyes and all of which are selectively coated with specific oligonucleotide sequences.

- The amplified DNA hybridise to complementary DNA probed on the beads. A washing stage may then be required depending on the Luminex typing kit used.

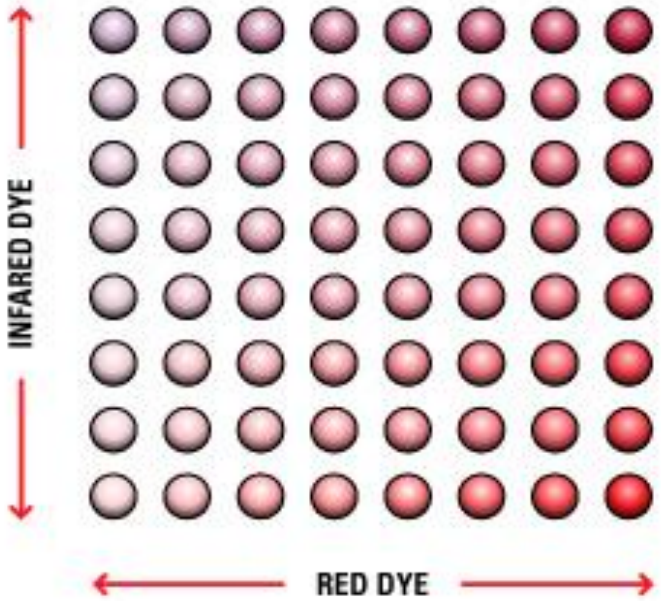
Sequence Specific Oligonucleotide technique (SSO)

- **Bound amplicon** is detected by labelling with a Streptavidin – Phycoerythrin (SAPE) conjugate, with Streptavidin binding to the biotin used to label the primers and phycoerythrin serving as the reporter dye for the presence of bound amplicon.

- Again a wash step may be required depending on the kit in use.

- **The Luminex platform** is used to identify any SAPE bound to the beads. The observed reaction patterns are used to assign HLA type. Positive and negative control beads are used to quality control the typing test.

Sequence Specific Oligonucleotide technique (SSO)



- Microspheres are dyed to create 100 distinct colors
- **Each microsphere has 'spectral address' based on red/infrared content**
- Microspheres are suspend able
- Microspheres are coated with capture reagent (oligo or antibody)
- Sample is added to microspheres
- Analyze is captured to microspheres
- Fluorescent reporter tag added

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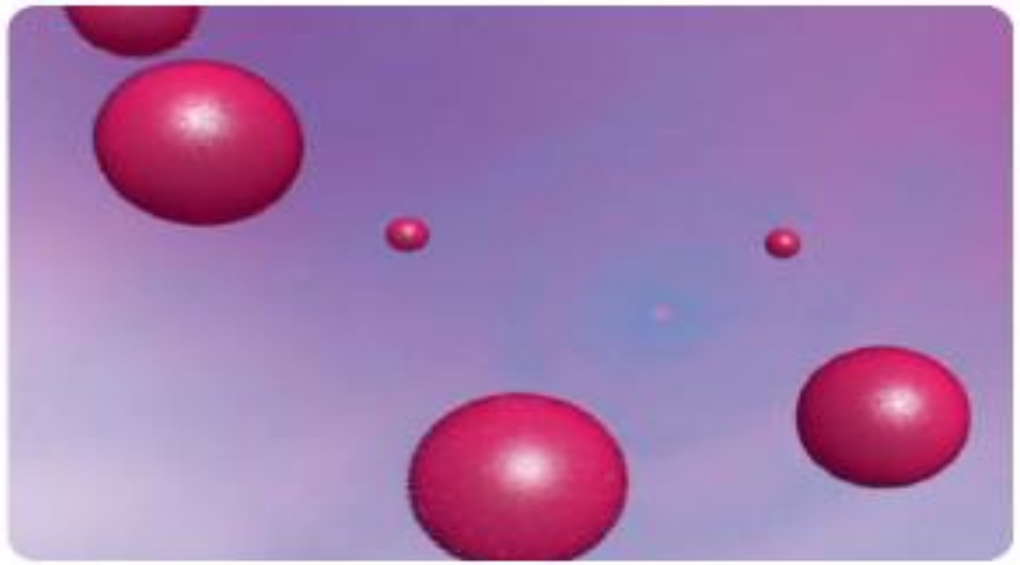

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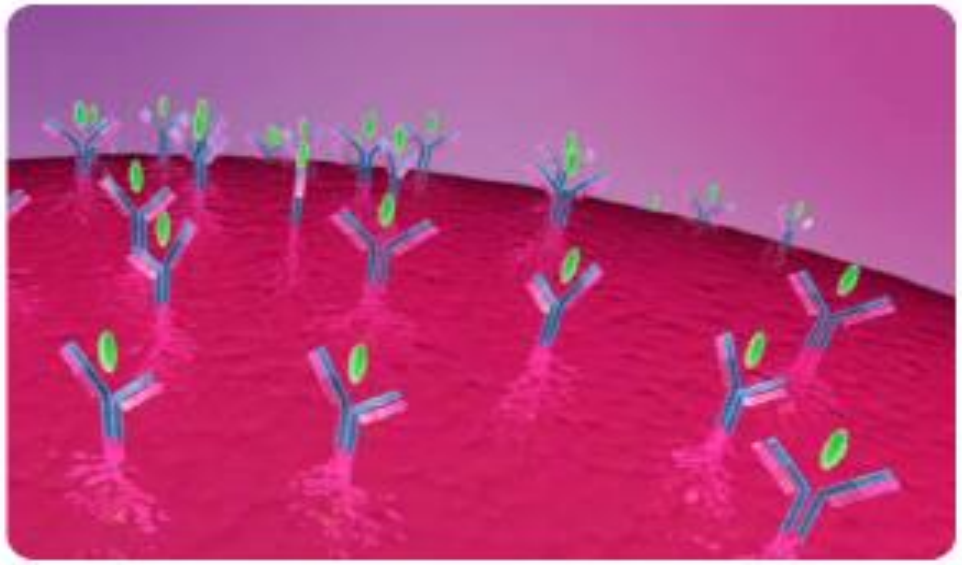

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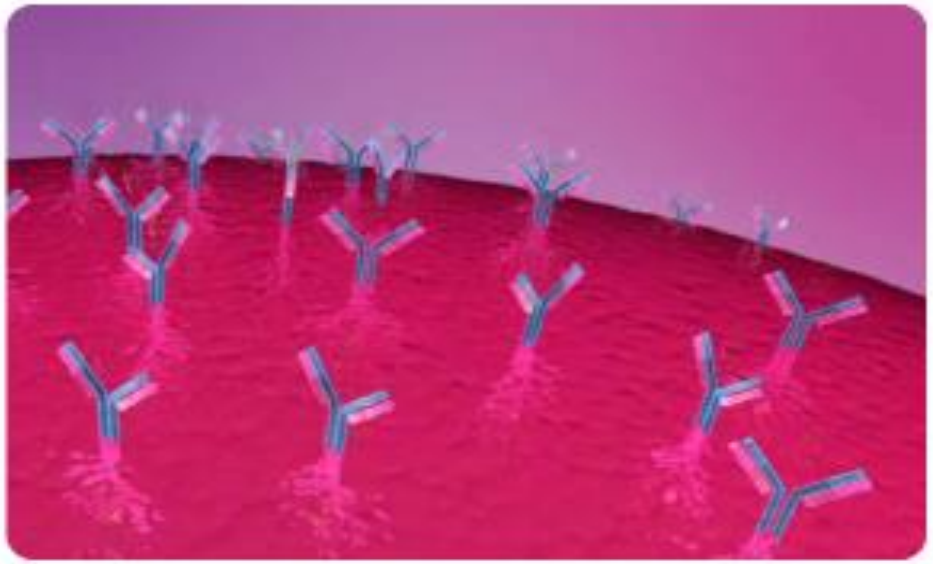

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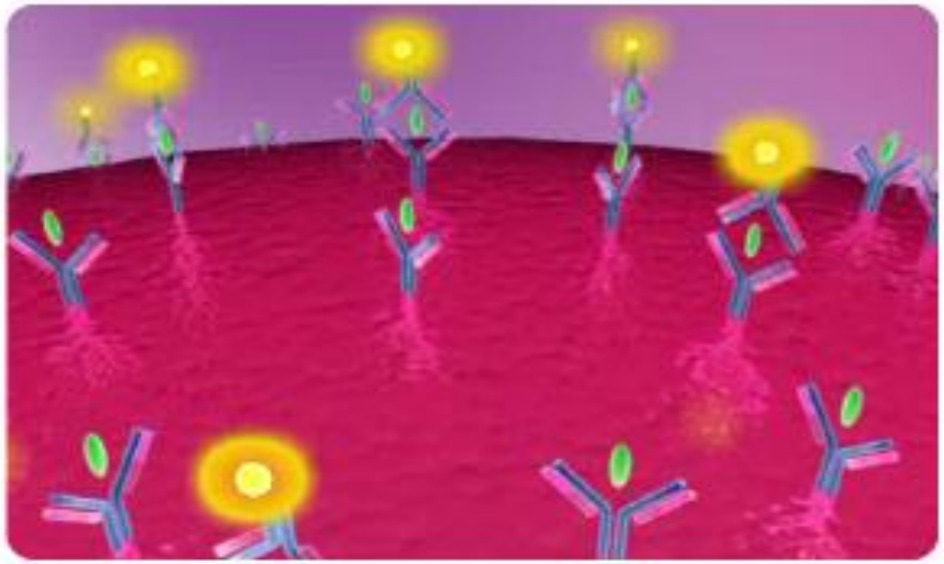

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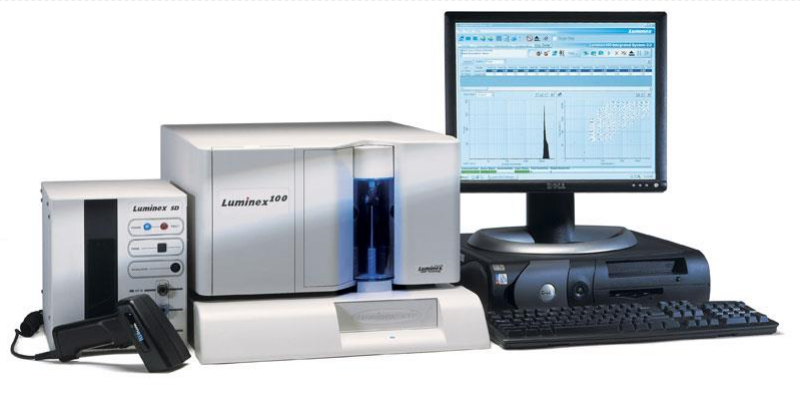

Assays are read using a compact microsphere analyzer

- Analyzer samples well
- Lasers excite fluorescent dyes - red laser for bead classification and green laser for assay result
- Multiple readings for each microsphere set
- Software reports results in real-time
- Up to 9600 results read in 1 hour

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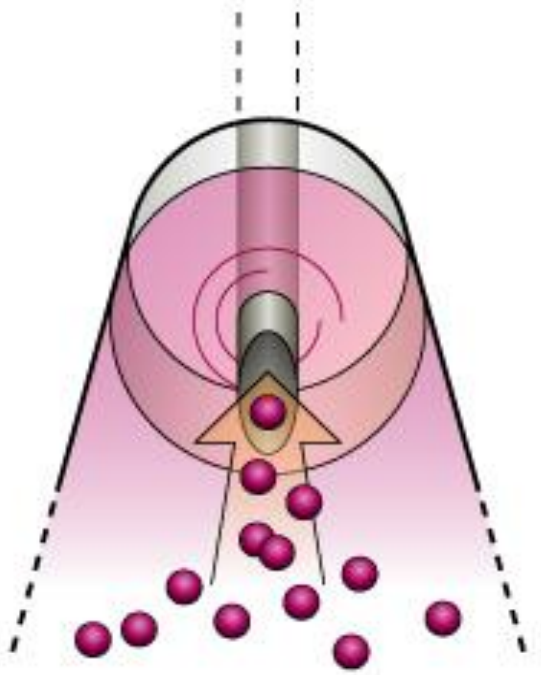

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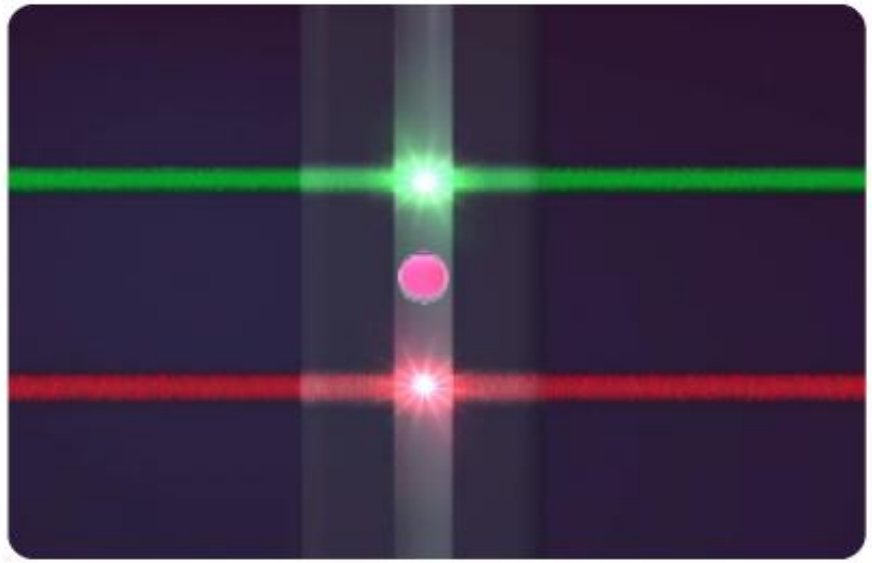

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Sequence Specific Oligonucleotide technique (SSO)

A. Pros

- Fairly rapid
- High volume
- Reasonable cost/test

B. Cons

- Single typing is more expensive
- Expensive equipment

HLA Typing by Molecular Methods

Advantages

- More accurate and precise than serology.
- Wider variety of samples can be used
- Less sample required, nonviable cells
- Becoming easier + automated
- Better matching

Taking home message

Many molecular methods currently in use in the histocompatibility laboratory have been described. The use of a specific technique will depend on the laboratory's requirements. The choice will be influenced by clinical urgency and requirement, sample numbers, availability of equipment, staff skills and budget.

Some laboratories, depending on their needs, may use a combination of methods. All techniques need to be continuously updated to allow for the detection of newly discovered alleles

Thank you

Any Questions ?