TITLE: HLA testing for Solid Organ Transplantation

PRESENTER: Idoia Gimferrer

Slide 1:

Hello, my name is **Idoia Gimferrer**. I am the **Co-Director of the Histocompatibility laboratory at Bloodworks NW (Seattle, WA).** Welcome to this Pearl of Laboratory Medicine on "**HLA testing for Solid Organ Transplantation.**"

Slide 2:

HLA stands for Human Leukocyte Antigen and it is a gene complex located in the short arm of chromosome 6.

There are 2 major classes, class I (A, B and C) and class II (DR, DQ and DP).

Both HLA classes present peptides to T cells, class I to CD8s and class II to CD4s.

Therefore, HLA is involved in keeping the homeostasis of the immune system when the peptide presented is derived from "self-proteins" and in immune responses when the T-cells recognize a non-self peptide (such those derived from viral proteins or cancer cells) .

Slide 3:

In this slide HLA class I is depicted on the left and HLA class II on the right.

HLA class I is composed by a monomorphic β2 microglobulin chain (in green) and a polymorphic α chain. The α chain is composed by 3 domains where domain 1 and 2 form the peptide-binding domain, which is considered the functional part of the protein.

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HLA class II is formed by two polymorphic chains, α and β. Both composed by 2 extracellular domains. The peptide binding domain of HLA class II is composed by the domains 1 of the α and the β chains.

Slide 4:

In solid organ transplantation a patient receives a graft that usually carries different HLA antigens than the patients. These allogeneic HLA will be recognized as "foreign" and trigger an allo-immune response, which could be humoral o cellular or a mix. Some patients have pre-formed HLA antibodies due to previous sensitization events and donors carrying these specificities should be avoided in order to avoid complications. To prevent graft rejection patient must be immunosuppress.

Slide 5:

In this cartoon you can see a heart transplant patient. Patient's immune system (cellular or antibodies) will attack and reject the allograft unless patient is immunosuppressed. In solid organ transplantation the goal is to find an organ that is immunologically compatible with the patient by determining donor and recipient HLA types and recipient's HLA Ab profile.

Slide 6:

The main role of the HLA lab is to assess and monitor the immunological risk of a donor-recipient pair. And since HLA is the most important player in the allo-response, we focus on HLA typing and HLA antibody testing.

HLA typing is done by molecular methods following ASHI and UNOS requirements. It is usually performed at low resolution, and for deceased donors a fast turnaround time (hours) is needed.

For HLA antibody testing we have two main methods: solid phase method, which usually is Luminex technology, and crossmatching.

Slide 7:

Molecular techniques for HLA typing start with a genomic DNA extraction from blood or lymphocyte followed by a Polymerase chain Reaction (PCR) amplification of the genes of interest. The different techniques have different abilities, such capability of processing

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several samples together or short time in getting results or levels of resolution for the HLA type. On the following slides, I will briefly explain the ones used by my laboratory. Importantly sequencing is mainly performed for high resolution HLA typing, which is not required for solid organ transplantation; therefore we do not used it in my lab, although some labs are opting to do it to increase accuracy in HLA type assignments.

Slide 8:

SSP stands for Sequence Specific Primer and is basically a PCR with primers designed to target specific polymorphic position that will allow identifying the HLA antigens or alleles of interest. Depending on the tray design can be used to get a full HLA type (A,B,C, DR, DQ, DP), or only one class (I or II) or even high resolution single locus typing. Also results could be obtained in few hours.

Real time PCR is the fastest method. It is a SSP-PCR that uses melting curve analysis to detect amplicons. The commercially available system is not a quantitative system and only allows for low resolution.

rSSO or reverse sequence specific oligonucleotides is based on Luminex technology and is very versatile. rSSO is good for batching several typings and can give 4 fields HLA resolution.

Slide 9:

My laboratory uses Real-time PCR as the main typing method for both recipients and donors. Real time allows for low resolution full typing in about 90 min, therefore it is especially advantageous for deceased donor HLA typing when on call.

Real time used SYBR green, which binds to double string DNA during the PCR extension step. After the PCR has finished a melting curve analysis is performed, that means that the double-stranded DNA-amplicon is heated and will open at a set temperature (depending on length and GC composition), then the SYBR green signal will be lost. This will be recorded by the instrument and graphed onto a dissociation curve. As an internal control, each well has a housekeeping gene amplicon that melts at much lower temperature than the potential HLA amplicon. The pattern of positive wells (wells with higher melting curve temperature amplicond) will be interpreted by the

software as a specific HLA type. In the case presented here sample was typed as HLA-A2 and A11.

Slide 10:

The other method that we use is rSSO that is a Luminex Technology. Luminex is based on a pool of "plastic" beads that are internally dyed with different combination of 2 colors. At the left is represented a set of beads ones with more "green" color and other with more "pink" colors. The Luminex instrument that it is a specialized flow instrument, reads the luminescent as the beads pass through it and the software interpret which bead is it. This Luminex technology can be used for both, HLA typing and HLA antibody testing. For HLA typing, each bead is covered by an oligonucleotide complementary to a specific HLA DNA sequence. Patient's locus specific amplicon is biotinylated during the PCR process and hybridized with the beads. Beads carrying the oligo complementary to the DNA sequence will bind. Streptavidin-PE is added to detect positive beads.

Slide 11:

The pattern of positive (in red) and negative (in blue) beads will be interpreted by the software to give an HLA type. In this case is B49 and B*52.

Slide 12:

The other important testing that the HLA laboratory does is HLA antibody testing. Per ASHI requirements, antibodies detection has to be performed by a solid phase method. One of the first one used was ELISA, but it has been substituted by Luminex technology due to its versatility and accuracy.

The other method is crossmatching which is used to confirm compatibility between a patient/donor pair. Depending on the organ transplanted it will be performed before the transplant or after.

Slide 13:

As shown on the left panel, Luminex beads for antibody testing carry HLA proteins and are incubated with patient serum. When serum has anti-HLA antibodies they will bind to the specific HLA-antigen and a secondary antibody (anti-human immunoglobulin with PE) is used to detect beads with bound patient's antibodies. The right panel shows the two main kinds of Luminex kits: - What is considered a "screen" assay, where each

beads carry multiple different HLA antigens (and here represented as a bead carry boxes of different colors). This is a "qualitative assay" and detects the presence or absence of HLA antibodies. – And the "Single Antigen Beads", where each bead carry one single HLA specificity. This test is used to "identify" the HLA target and is considered semi-quantitative assay. It is very sensitive and can detect very low levels of HLA antibodies. Finally there is a variation of the single antigen bead assay that uses complement (C') components (either C1q or C3d) to detect the capability of the HLA antibodies to bind C'. This is important because C 'binding donor specific HLA antibodies correlate with worse outcomes.

Slide 14:

This is an example of a single antigen run. Signal is measured in "Mean fluorescent intensity" units or MFI. Each lab used different cut off depending on the immunological risk accepted by its transplant center, but usually it is around 1,000 to 2,500 MFI. Specificities over the cut off are considered positive and donors with these HLA antigens may be avoided. These specificities are considered "unacceptable antigens". This test is also used in the post-transplant settings to detect and/or monitor the presence of donor HLA specific antibodies (or DSA).

Slide 15:

Unacceptable antigens are used to calculate the panel of reactive antibodies or CPRA that represents the probability for a patient to find a compatible donor. CPRA is calculated based on the frequency of these HLA antigens in the general population. This is an example of a patient that has a CPRA of 99%, meaning he or she will be compatible with only 1% of the potential donors.

Slide 16:

A crossmatch directly measure the presence of antibodies on patient's serum against donor cells. Donor lymphocytes are used as a target, because they are easy to isolate and because they express HLA, T cell express HLA class I only, but B cells express both, HLA class I and II.

There are two main techniques: -Complement dependent cytotoxicity or CDC, and – Flow cytometric XM. For CDC XM T and B lymphocytes are isolated and then separated

and plated on different trays. Cells are incubated with patient serum. Then, C' is added and if there are antibodies bound to the cells, the C' cascade is activated through the classical pathway and cells die. This is interpreted as positive XM. Cells are observed under a microscope to determine reactivity. XM by flow instrument is similar. Donor lymphocytes are also incubated with patient's serum. T and B cells specific markers (anti-CD3 and anti-CD19, respectively) are used to differentiate these cells and fluorochrome labeled anti-human immunoglobulin is added to detect patient Abs bound to the cells. Reactivity is read by the flow instrument. The presence of DSA against HLA-class I will cause T and B positive XMs and DSA against class II will XM positive only with B-cells.

Slide 17:

Lymphocytes are gated and fluorochrome makers allow differentiating T (CD3) from B (CD19) cells. Reactivity is measured in Mean Channel Shifts (MCS), and it is calculated as how many channels the patient's serum reactivity is shifted compare to the negative control serum. In this case it is also a positive crossmatch.

Slide 18:

Finally, a short comment on virtual Crossmatch. It is named "virtual" because no physical crossmatch is performed. It tries to predict the result of a XM based on donor's HLA and patient's HLA antibody profile. In the example presented here, patient has strong donor specific antibodies against HLA-A2 (also named DSA). And the prediction will be a positive XM.

Slide 19:

In summary, HLA laboratory testing for solid organ transplantation involves accurate typing for patients and donors and precise patients' HLA antibody profile determination. For some organs a physical XM pre-transplant is required (such kidney).

Post- transplant, monitoring of DSA against donor HLA is done by Luminex technology using single antigen kits.

The goal of the HLA testing is to provide an immunologic risk assessment for a given patient-donor pair based on donor and recipient HLA types and recipient's HLA antibody profile.

Slide 20: References

This is the reference slide

Slide 21: Disclosures

I don't have anything to Disclose.

Slide 22: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on "HLA testing for Solid Organ Transplantation."

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PEARLS OF LABORATORY MEDICINE

Pearl Title: HLA testing for Solid Organ Transplantation

Name of Presenter: Idoia Gimferrer

Affiliation: BloodworksNW

DOI:

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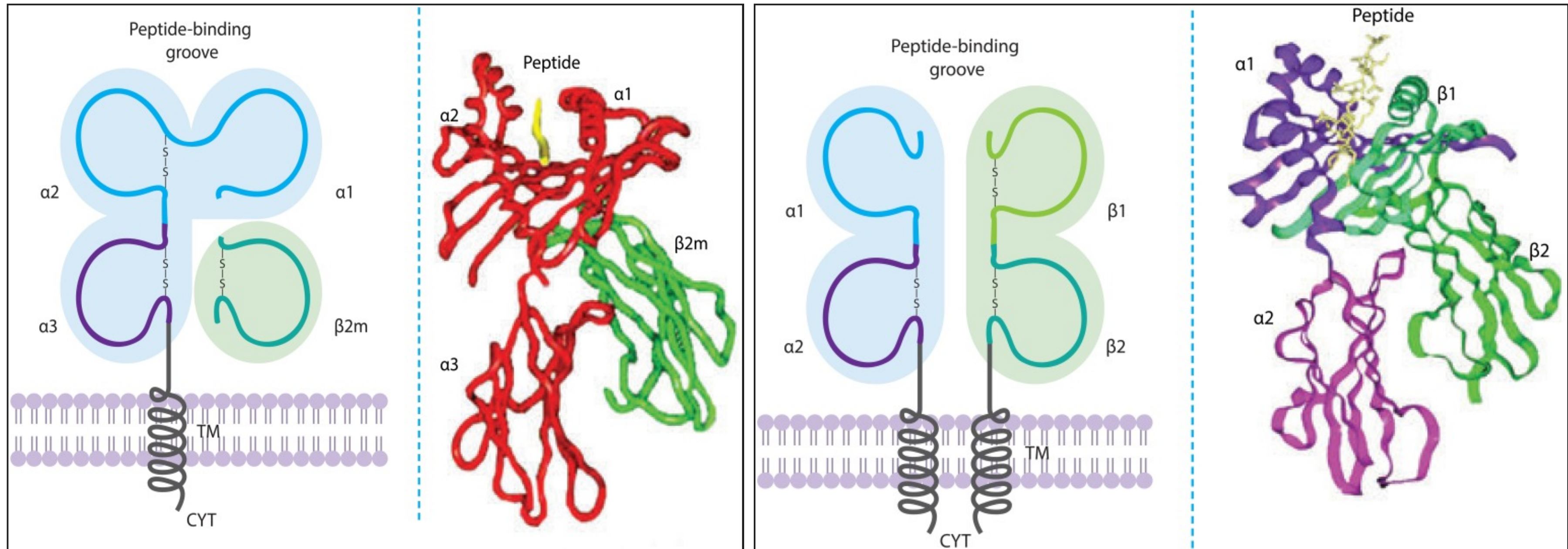
What is HLA?

- Human Leukocyte Antigen (HLA) is a gene complex located in chromosome 6 that codifies for a group of very polymorphic proteins.

- There are two major HLA classes:
  - Class I (A, B, C).
  - Class II (DR, DQ, DP).

- The role of both HLA classes is to present protein peptides to T cells. "Self-peptides" (derived from self-proteins) will maintain the homeostasis of the immune system. On the contrary, peptides derived from modified proteins (such cancer cells) or foreign organisms (such viruses) will activate the T cells and trigger immune responses.

HLA structure:



HLA-Class I **HLA-Class II**

Cruz-Tapias P, Castiblanco J, Anaya JM. Major Histocompatibility complex: Antigen processing and presentation. In: Anaya JM, Shoenfield Y, Rojas-Villarraga A, Levy RA, Cervera, editors. R. *Autoimmunity from Bench to bed side. 1st Ed. Bogota D.C. El Rosario University Press; 2013. p. 169-183* (Reproduced with permission).

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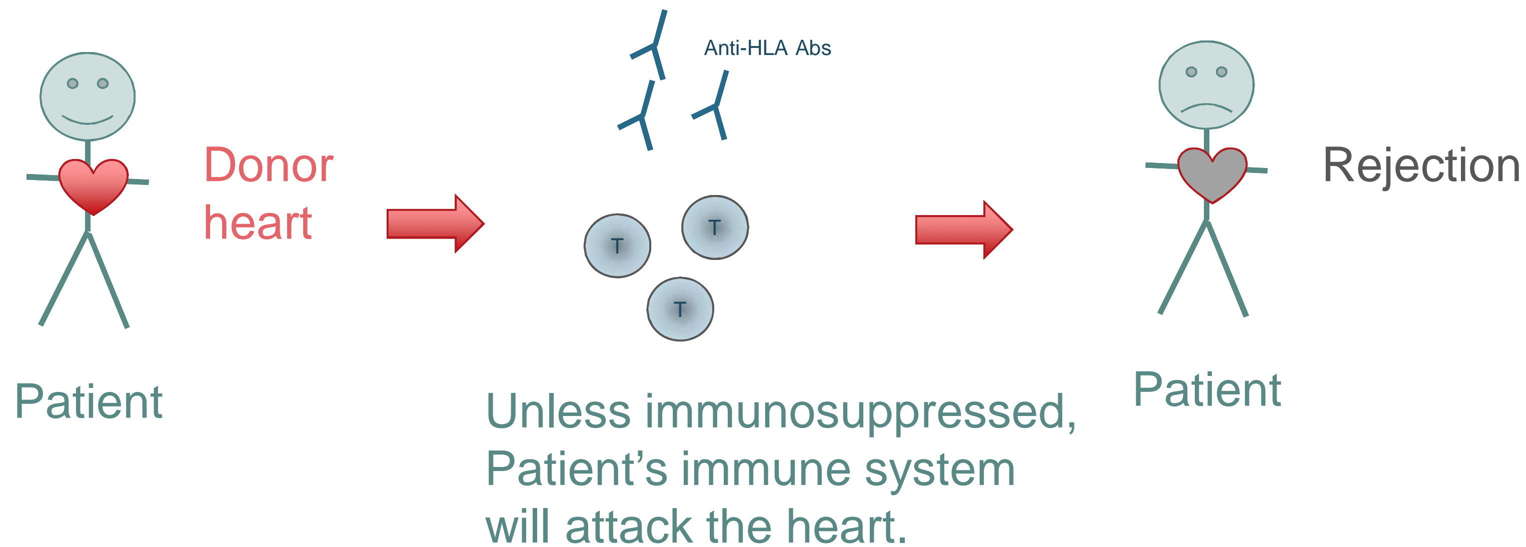
Solid Organ transplant

- In solid organ transplantation, a patient receives a graft that usually carries different HLA antigens.
- These allogeneic-HLA will be recognized by patient's T-cells as foreign and will trigger cellular and/or humoral allo-immune reactions.
- Some recipients have pre-formed antibodies against HLA antigens due to previous sensitizing events (pregnancy, transfusion or previous transplant). Donors carrying these HLA specificities should be avoided.
- Patients must be immunosuppressed to prevent graft rejection.

Solid Organ transplant



The goal is to find an immunologically compatible organ for a given patient, based on donor and recipient HLA types and recipient's HLA antibody profile.

HLA Testing in Solid Organ Transplantation

The main role of the HLA laboratory is to assess and monitor the immunological risk of a donor-recipient pair by performing:

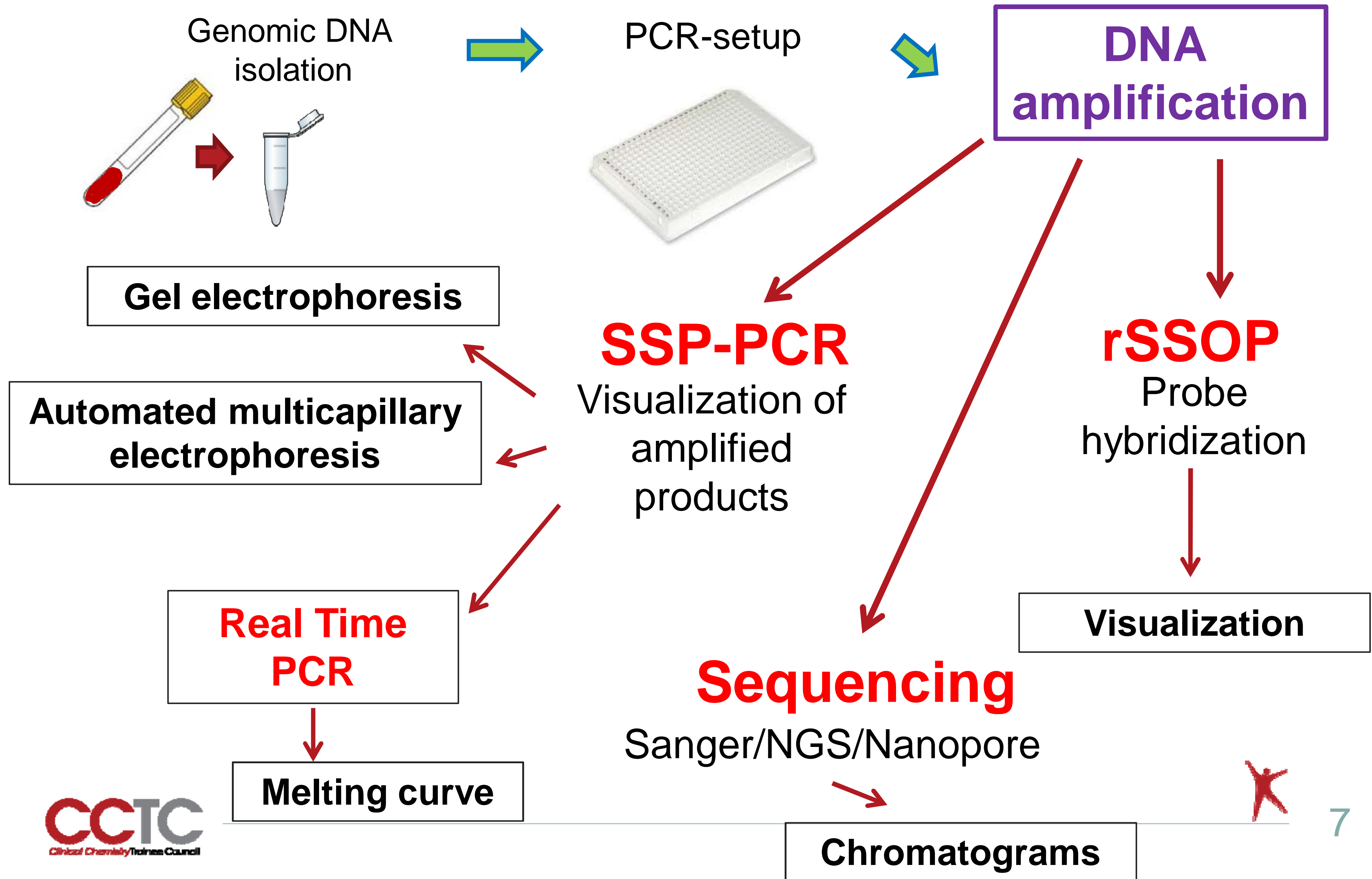
HLA typing:

- Molecular typing required by ASHI and UNOS
- Low resolution or antigen level
- Patients and donors
- Fast turnaround time (TAT) for deceased donors

HLA Antibody testing:

- Solid phase antibody (Ab) detection and identification
- Crossmatch (XM)

HLA Molecular Typing Techniques



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HLA Molecular Typing

Sequence Specific Primer -Polymerase Chain Reaction (SSP-PCR):

- Primers specific for polymorphic positions that allows to differentiate the HLA antigens or alleles of interest
- Fast (few hours)
- Depending on the tray design allows from full HLA low resolution typing or locus specific high resolution typing

Real time-PCR:

- PCR-SSP based
- Melting curve
- Fastest (less than 2 hours)
- Commercially available kits allow for low resolution only

Reverse Sequence Specific Oligonucleotide (rSSO):

- Initial PCR to amplify the HLA loci to be typed
- Luminex technology: Beads carry oligonucleotides complementary to the sequence of interest for hybridization
- Allows intermediate resolution HLA typing
- Efficient for high throughput

Dunckley H. HLA typing by SSO and SSP methods. Methods Mol Biol 2012;882:9-26.

Real Time-PCR

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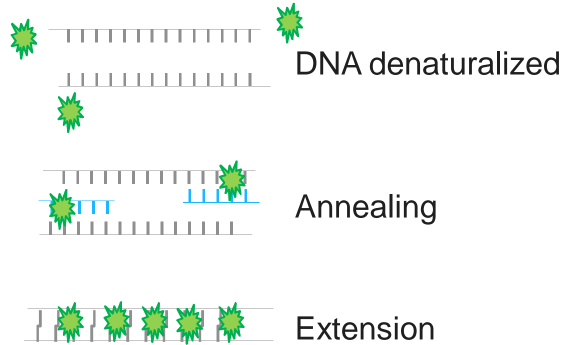
1- PCR Step



DNA denaturalized

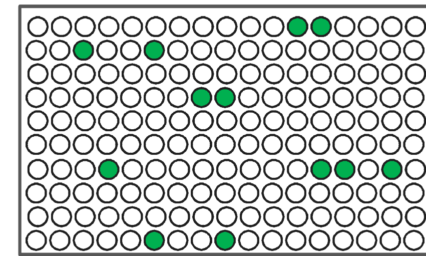
Annealing

Extension

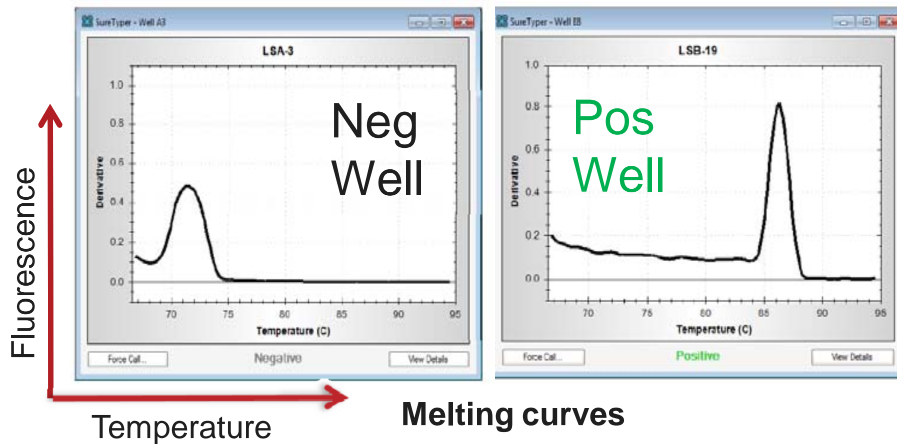
SYBR green binds to double string DNA

2- Melting curve

3- Interpretation



Well with amplicon (positive)



Melting curves

group	allele	antigen
A*02	**A*02: 01:01:01**, 01:01:02Le-01:03e, **01:04**, 01:05e-01:08e, 01:10e-01:13e, 01:15e, 01:18e-01:35e, 01:37e-01:51e, 01:53e-01:73e, 01:75e-01:115e, **02:01**, 02:02e, **04-05:01**, 05:02e, 05:04e, 05:06e, **06:01**, 06:02e-06:06e, 06:08e-06:11e, 06:13e-06:18e, **07:01**, 07:02e-07:07e, **08-09**, **11:01**, 11:03e-13d, **14**, 16d, **17:01**, 17:02e-18d, **20:01**, 20:02e-21d, **22:01**, 22:02e, **24:01**, 24:02e-25d, 29d, 31e, 34d, 41e-42e, 49d, 59e, 66e-68e, 70e-72e, 74d, 77e-79:01e, 89e-90e, 97e, 105e	A2
	A*02: 03:05e	*A203*
	A*02: 15Ne, 32Ne, 43Ne, **53N**, 82Ne-83Ne, 88Ne, 94Ne, 113Ne, 125Ne	*Null*
	A*02: 01:14Qe, 26e-28e, **30**, 33d, 36d-37e, 40:01e-40:02e, 44d-45d, 47e, 51e, 54e, 56:01e, 57e-58d, **60:01**, 60:02e-64d, 69e, 75e-76:01e, 80e, 84d-86d, 91e-92e, 96d, 99e-102e, 104e, 106e-109e, 111e, 115e-116e, 118e-121e, 123d, 126e-128e, 130e-134e, 137d-147e, 149e-151e, 153e-155e, 157e-168e, 170e, 172e-184e, 186e-194e, 196e-229e, 231e-236e, 238e-241e, 243:01e, 246e-252e, 254e-257e, 259e-263e, 265e-278e, 283e-299e, 301Ne-303e, 305Ne-308e, 310e-314Ne, 316e-333e, 335e-337e, 340e-344e, 346e-354e, 356Ne-369e, 371e-408e, 410e-411e, 413e-426e, 428e-430e, 432e-446e, 448e-452e, 455e-462e, 464e-465e, 467e-479e, 481e-503e, 506Ne-526e, 528e, 530e-543e, 545e-555e	-
A*11	**A*11: 01:01:01**, 01:01:02e-01:06e, 01:08e-01:27e, 01:29e-01:66e, **02:01**, 02:02e-03d, **04**, 05d, 07e, 09d, 12d-15:02e, 19d	A11
	A*11: 21Ne	*Null*
	A*11: 06e, 11e, 16e-18e, 20e, 22e-23e, 27e-30d, 32e, 33:02e-34e, 36e-43e, 45e-49e, 51e-93e, 95e-117e, 120e-129e, 131e-138e, 140e-157e, 159e-182Qe, 184e-190e, 192e-198e, 200e-208Ne, 210Ne	-

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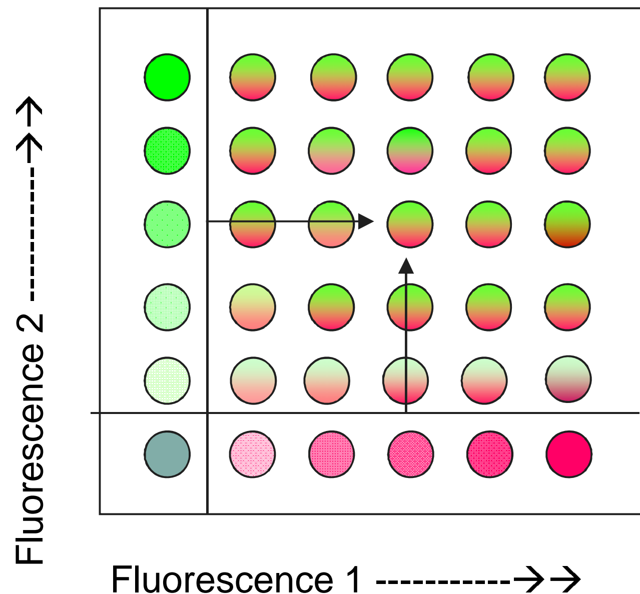
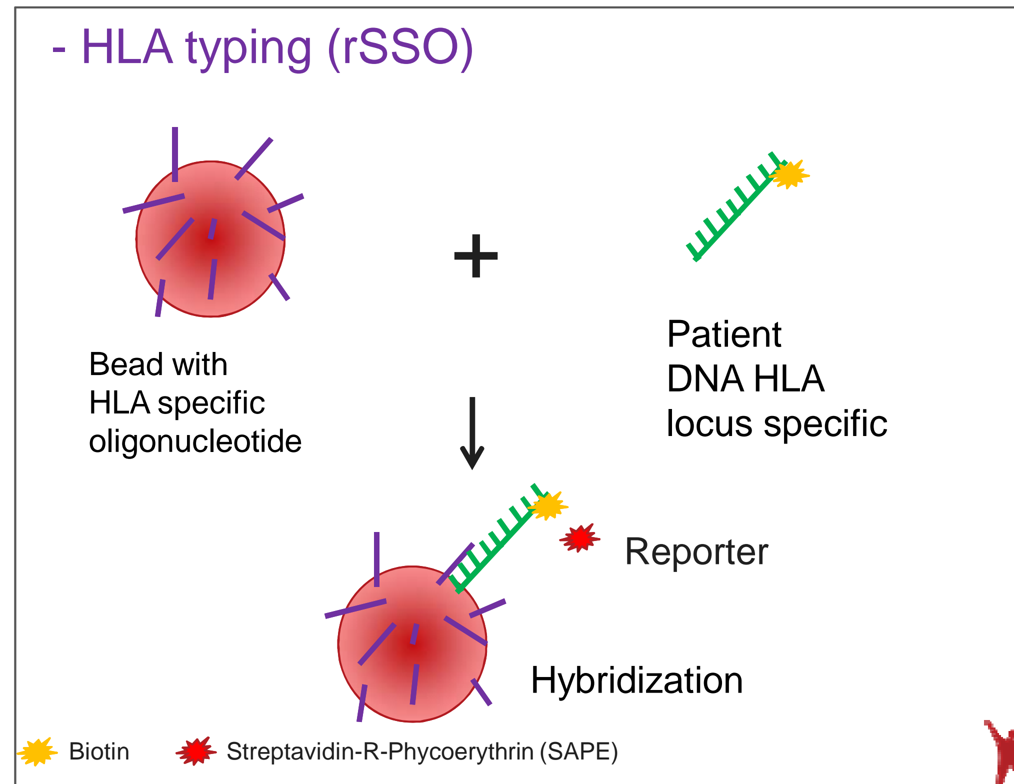
Ponchel F BMC Biotechnol 2003

Luminex Technology

The Luminex Technology is based on internally dyed beads. Different concentrations of red and infrared fluorophores were used to create distinct microsphere sets.



- Luminex system can be used for both:
  - HLA typing
  - HLA antibody testing



Reslova N. Front Microbiol 2017;8:1-17.

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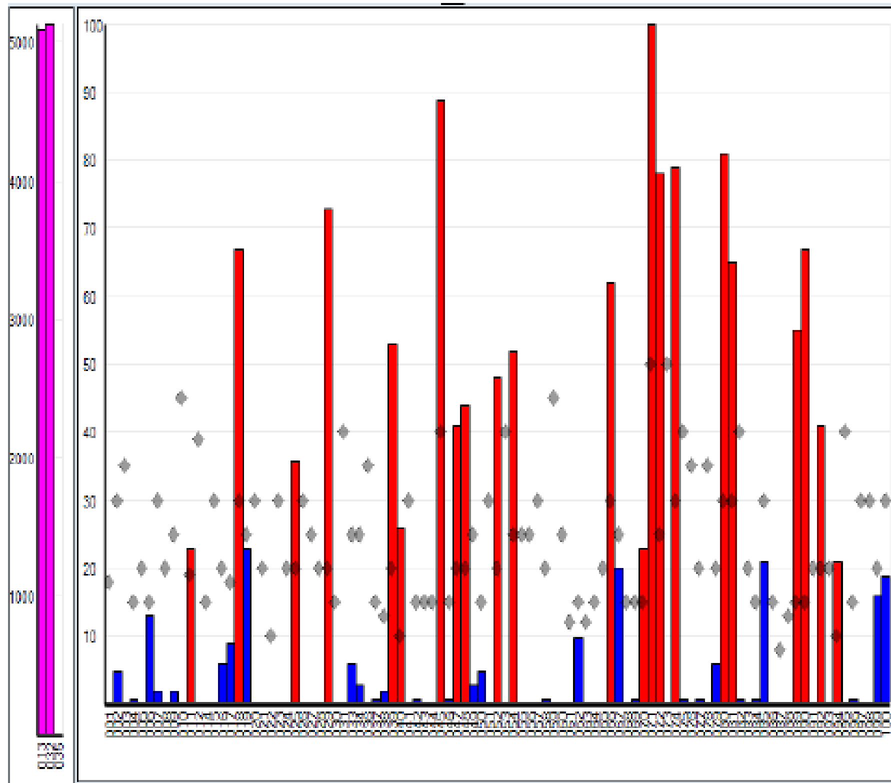
Real Time-PCR



Pairs | Force | Type/SubType | Match | Sero

B*49:01:01:01 B*52:01:01:01
B*49:01:01:01 B*52:01:01:02
B*49:01:01:01 B*52:01:01:03
B*49:01:01:01 B*52:01:01:04
B*49:01:01:01 B*52:01:01:05
B*49:01:01:01 B*52:01:06
B*49:01:01:01 B*52:01:07
B*49:01:01:01 B*52:01:08
B*49:01:01:01 B*52:01:10
B*49:01:01:01 B*52:01:11
B*49:01:01:01 B*52:01:12
B*49:01:01:01 B*52:01:13
B*49:01:01:01 B*52:01:14
B*49:01:01:01 B*52:01:17
B*49:01:01:01 B*52:01:18
B*49:01:01:01 B*52:01:19
B*49:01:01:01 B*52:01:20
B*49:01:01:01 B*52:01:21

B*49,52

HLA Antibody Testing

Solid Phase:

- ELISA. Few labs use it.
- Luminex Technology
  - Detection
  - Identification
  - Complement-binding

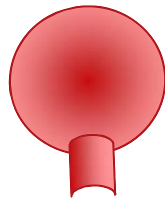
Crossmatch:

- CDC
- Flow Cytometry
- Virtual XM

Tait BD, Hudson F. Tissue Antigens 2010;76:87- 95.
Tinckam K. Transplant Rev 2009;23:80-93.

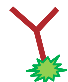
Luminex Technology

- HLA Antibody testing



Bead with HLA proteins

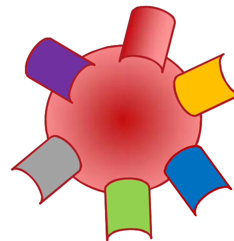
Patient serum with HLA Abs



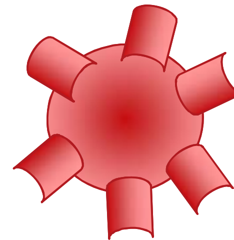
Anti-Human IgG PE

PE: R-phycoerythrin

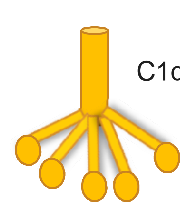
- Types of beads



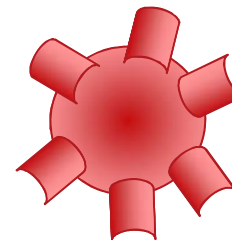
Multiple HLA antigens per bead
- Used for screening



Single HLA antigen per bead
- Used Ab identification



(or C3d) +



Identification of HLA Complement binding antibodies

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Luminex Technology

10000
8000
6000
4000
2000
0

X6 X4 X2

MFI Cut off. It is arbitrary.
Depends on immunological risk accepted

A
B
Bw
Cw

Positive

MFI: Mean fluorescence intensity. Semiquantitative measurement of the antibody bound to the beads.

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Calculated Panel of Reacting Antibodies (CPRA)

Unacceptable Antigens (CPRA:99)

One or more unacceptable antigens must be indicated in order to receive PRA Points. The unacceptable antigens should be able to support the PRA.

A:	1	9	23	24										
B:	12	44	45	57										
DR:	2	3	5	6	7	8	10	11	12	13	14	15	17	18
DR51\52 \53:	51	52	53											
DQ:	3	7	8	9										

List | Print | Status History | Edit | Remove | Change to KP | Backdate | Manage Attachments

- cPRA is calculated based on the "frequency" of these unacceptable HLA Ag in the population. Therefore, **cPRA relates to the frequency of the Ag in the donor population, does not relation to the antibody titer!**

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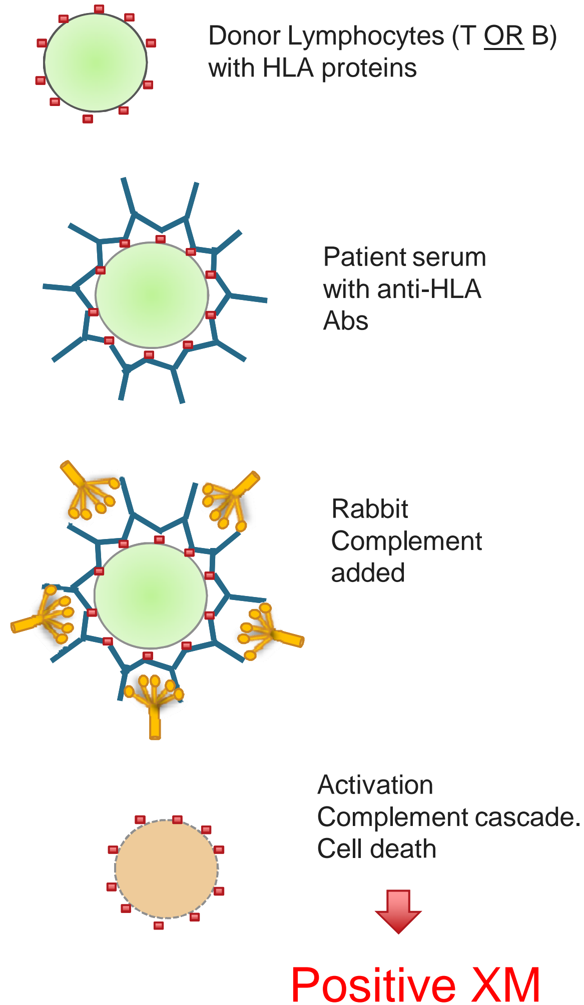
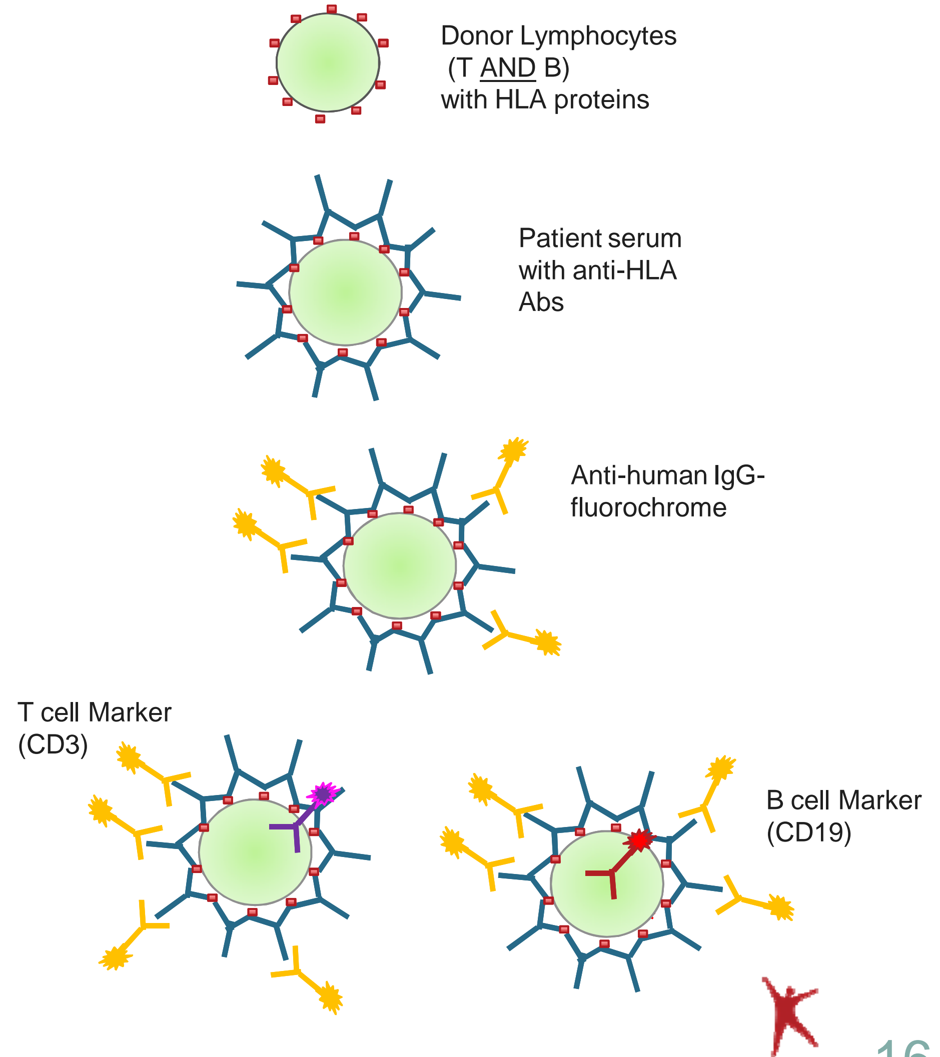
Crossmatches

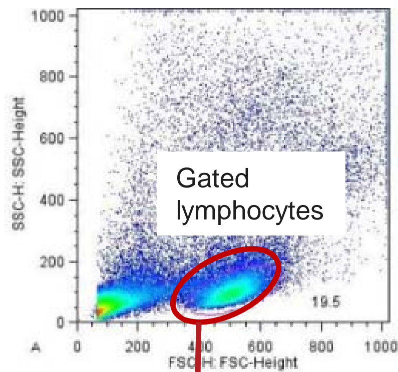
CDC



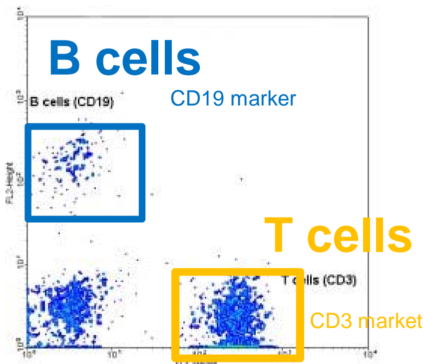
Flow Cytometry



Flow Cytometric XM



T and B lymphocyte are separated by cell surface specific markers labeled with fluorochrome.

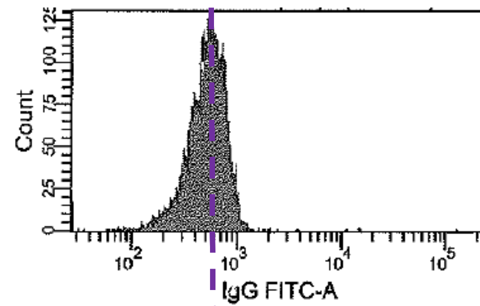


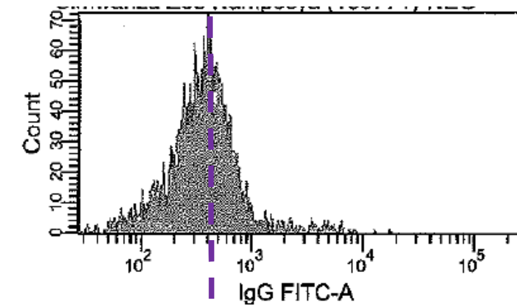
Patient's reactivity is measured in number of channels shifted over Negative control

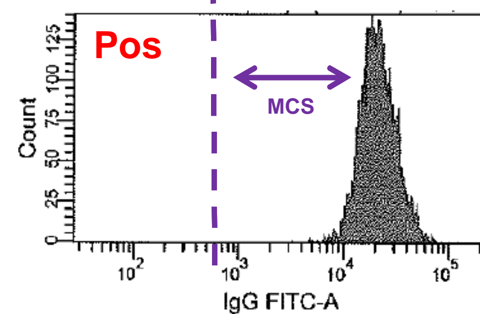
T cells

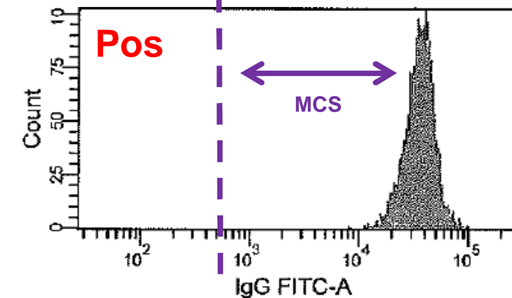
B cells

Neg C









MCS: Mean Chanel Shift

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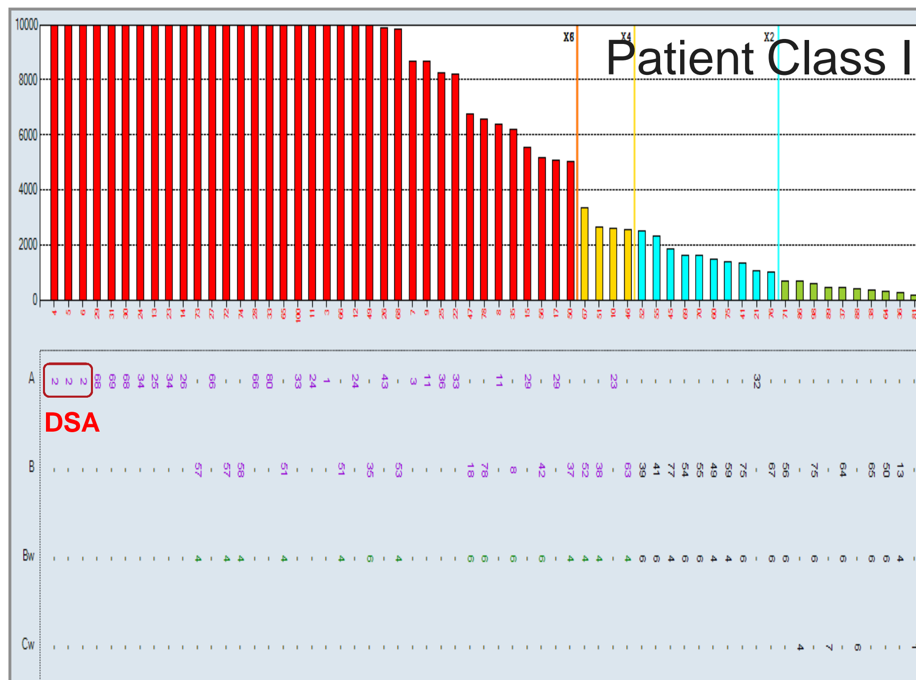
Virtual XM

- Prediction of the XM results based on donor HLA type and patient HLA ab profile



Donor: A2,3 B44,61 C9,16

- Patient has donor specific Abs (DSA) against HLA-A2.
- XM will be POSITIVE

Ellis TM. Hum Immunol 2010;73:706-710.

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Summary

- HLA laboratory testing for solid organ transplantation involves accurate typing for patients and donors and precise patient's HLA antibody profile determination.
- For some organs a physical XM pre-transplant is required (kidney).
- Post transplant, monitoring of DSA-HLA antibodies is done by Luminex technology using single antigen kits.
- The goal of the HLA testing is to provide an immunologic risk for a given patient-donor pair based on HLA typing and HLA Ab profile.

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AACC Better health through laboratory medicine.

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- **Employment or Leadership**:
- **Consultant or Advisory Role:**
- **Stock Ownership:**
- **Honoraria:**
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