

Addressing the Complexity of the HLA System with Luminex[®] Assays

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Professor of Medicine

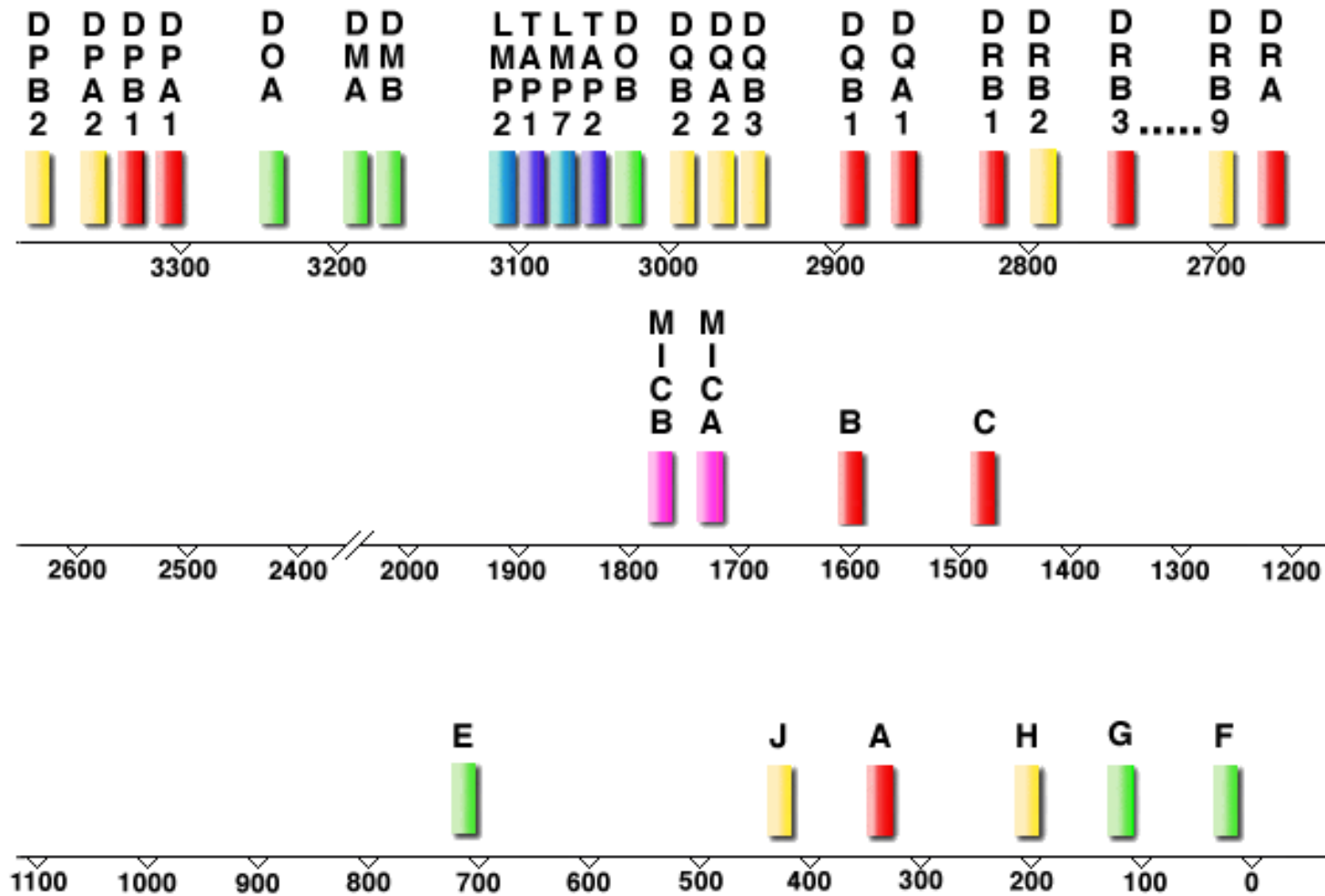
Director, JHU Immunogenetics



Histocompatibility and Transplantation

- Histocompatibility evaluations are performed for solid organ and hematopoietic stem cell transplantation (HSCT)
- Requirements:
 - HLA typing
 - Screening and identification of HLA specific antibodies
- Confounded by polymorphism of HLA System



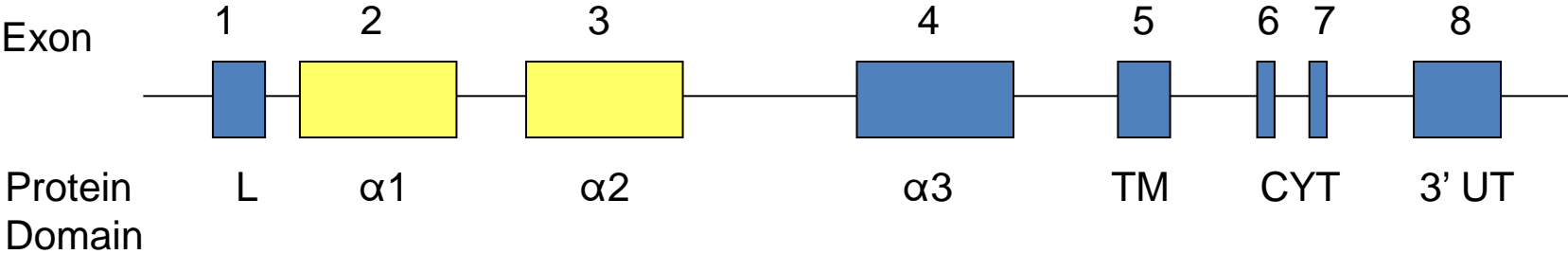
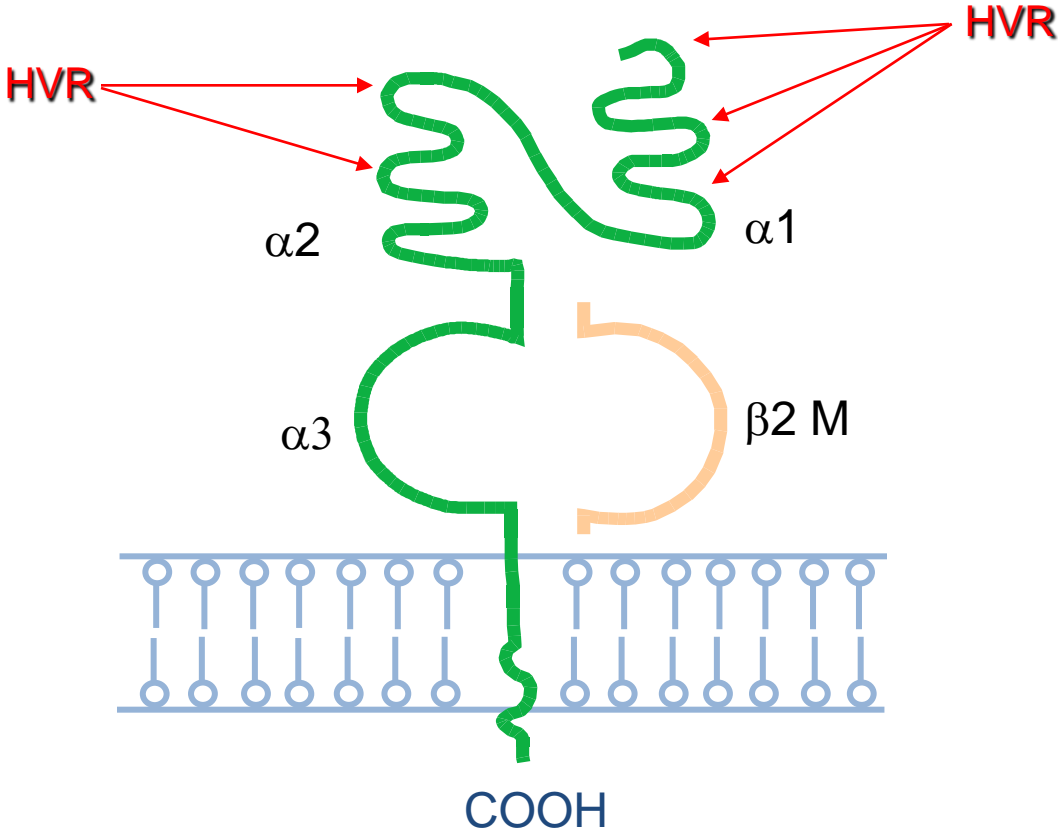


Human MHC - HLA System

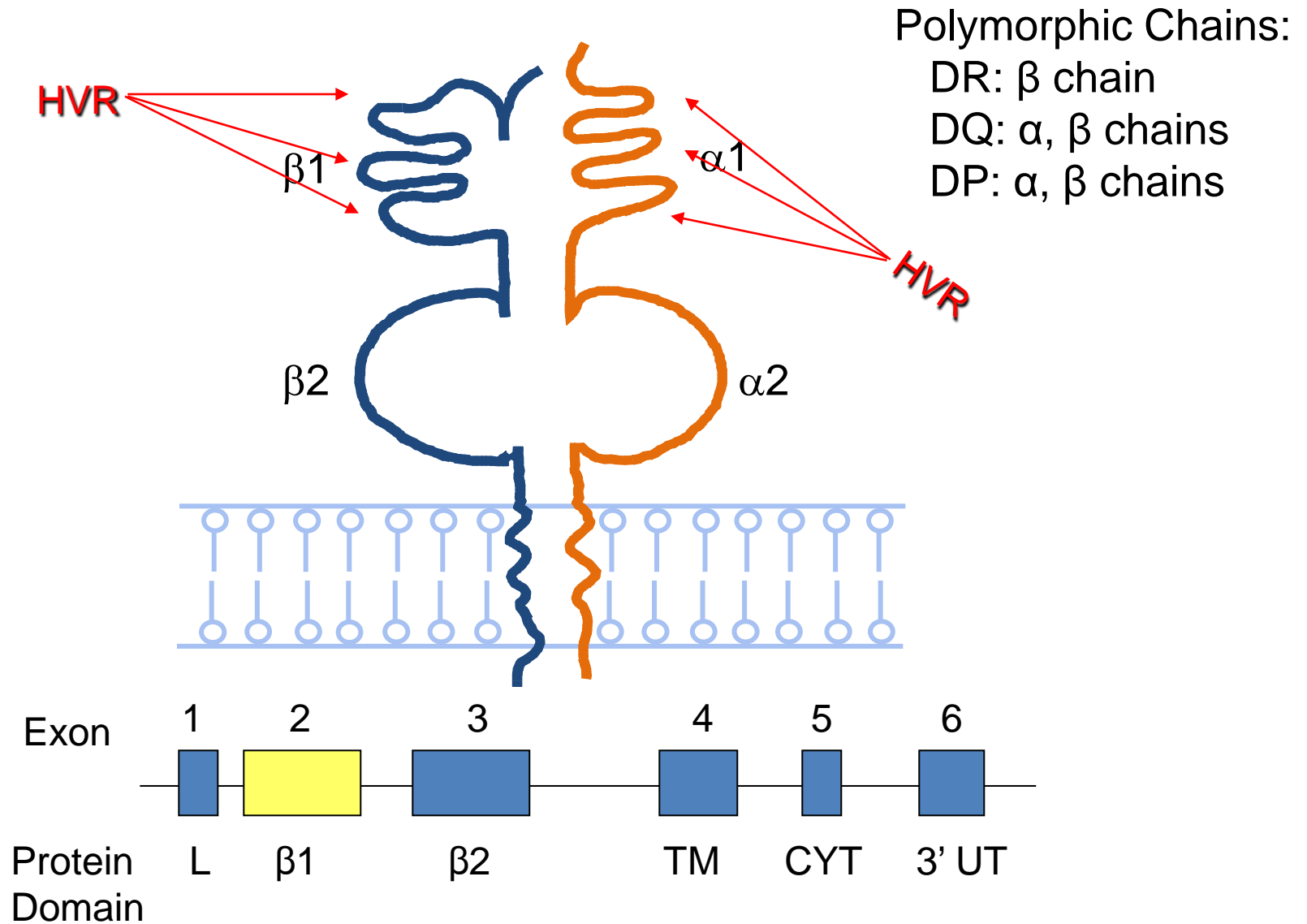
Class I loci: **HLA- A, B, C**

Class II Loci: **HLA-DR, DQ, DP**

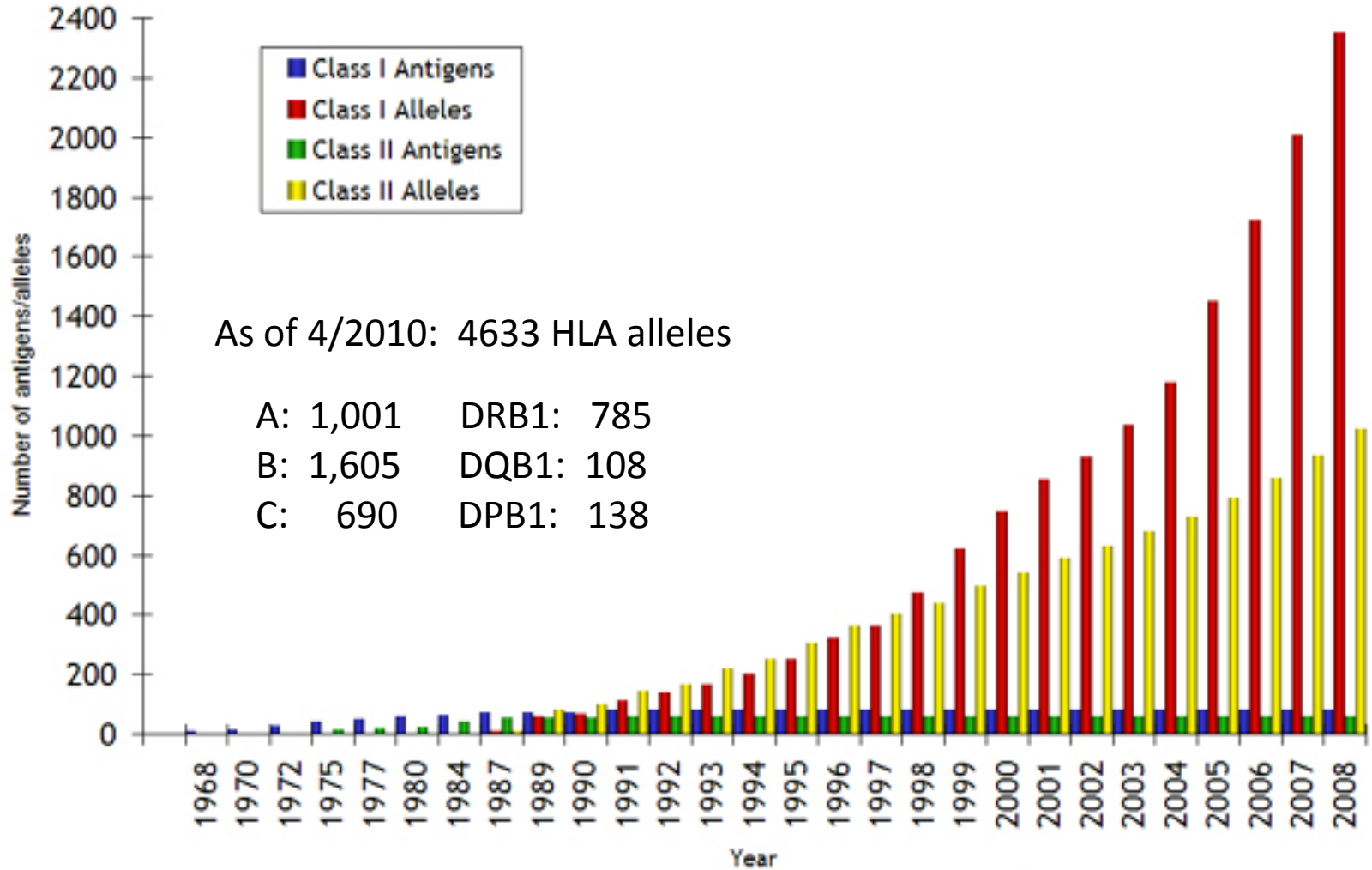
MHC class I



HLA class II



HLA Polymorphism



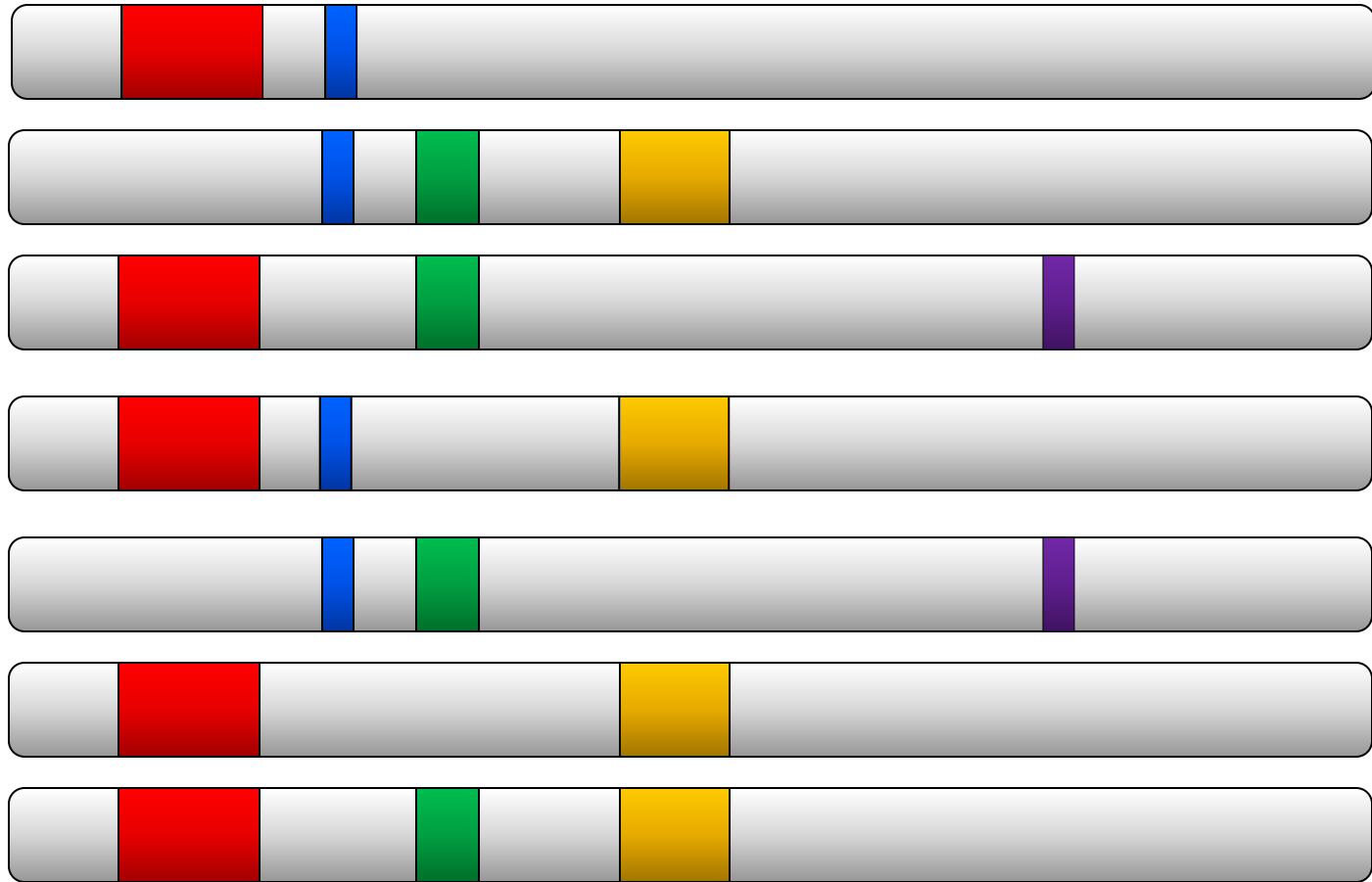
HLA Typing Requirements

- Solid Organ Transplantation
 - OPTN/UNOS requires antigen level typing for HLA-A, B, and DR antigens.
 - HLA-Cw, DQ, DP optional, but increasingly used.
- HSCT
 - NMDP requires typing of all common, well defined alleles^a
 - Must type for certain “null” alleles, eg., C*04:09N

^a Cano P, et al. Report of the ad-hoc committee of the ASHI. Human Immunol. 2007; 68:392



HLA allele variability occurs in patterns or motifs



Typing is confounded by extensive homology and shared sequences

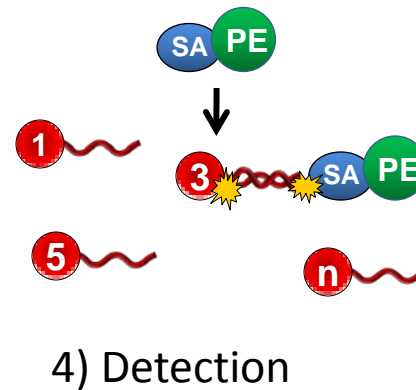
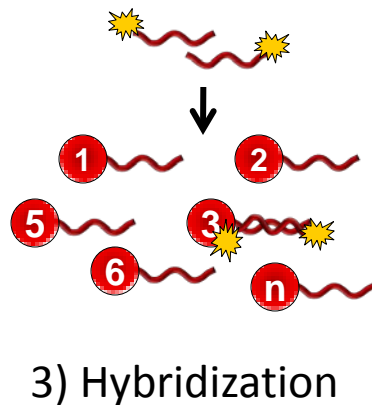
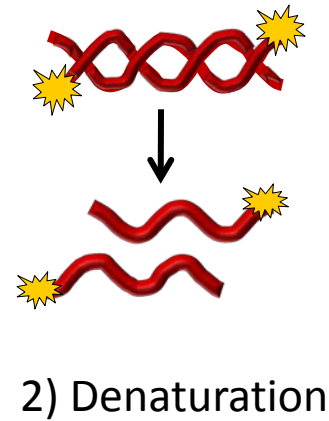
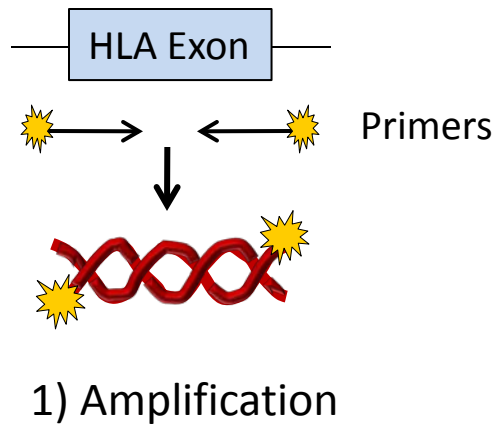
Desirable Features of Typing Assays

- Capability to use multiple probes and primers
- Rapid processing: 4-5 hours required for “on-call” deceased donor typing.
- Capability for full or partial automation
- Software to analyze probe hit patterns for allele assignments.



Reverse SSOP

(sequence specific oligonucleotide hybridization)



Limitations of Molecular Typing Assays

- Reverse SSOP assays use one set of hybridization conditions – designed to accommodate most probes
- Less than optimal temperature/stringency for some probes results in false + and – reactions.



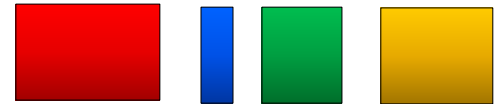
Limitations of Molecular Typing Assays

- Some false + and false – reactions.
- Current assays provide “intermediate” level of typing resolution – “allele groups”.
 - Expanded by supplemental, “high definition” kits
 - Potential with FLEXMAP technology
- Additional typing required for :
 - alleles that differ outside of exons 2 or 3 for class I; exon 2 for class II.
 - A*24:09N exon 4
 - B*51:01N exon 4
 - Cw*04:09N exon 7
 - resolution of ambiguous heterozygotes



Ambiguous Heterozygotes

Probe for sequence motif



Individual 1

allele 1



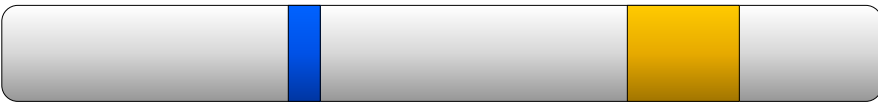
allele 2



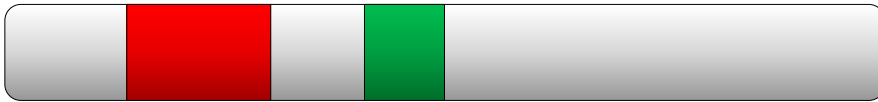
+ + + +

Individual 2

allele 3



allele 4



+ + + +

Ambiguous combination -- get same results for two individuals with different HLA types

Luminex[®] Assays for HLA Typing

- While current assays cannot resolve all HLA alleles – NO single technique can!
- Current Luminex[®] SSOP assays provide:
 - Rapid turn around
 - Antigen level typing sufficient for solid organ transplantation
 - Intermediate allele level typing that can permit genotyping and/or supplemental information for HSCT.



Detection of HLA Specific Antibodies

- Increasing importance –
 - Solid organ transplants
 - Bone marrow /HSCT



Sensitization to HLA: Immunologic Barrier to Transplantation

- Associated with reduced graft survival
- Increases waiting time
- Affects most, if not all transplanted organs
 - Greater impact on women and African-Americans
- Can result in hyperacute rejection
- Rates of sensitization:
 - >36% of national solid organ wait list
 - 28.7% of HSCT candidates (JHMI data)



HLA Specific Antibodies

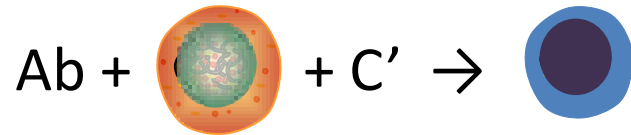
- Causes of sensitization:
 - Transfusion
 - Prior transplant
 - Pregnancy
 - Crossreactivity with some viruses and bacteria
- Numbers of defined HLA antigens:

Locus:	A	B	C	DRB1	DRB3-5	DQ	DP
# AGs	26	53	10	19	3	9	6



Prior to Luminex® -

For 50 years, detection of HLA specific antibodies used cell-based tests:



Complement dependent cytotoxicity
(CDC)

Limitations:

- CDC detected only C' fixing Abs
- Both assays detect non-HLA Abs
- Sensitivity

CDC <<<<< Luminex

FCXM << Luminex

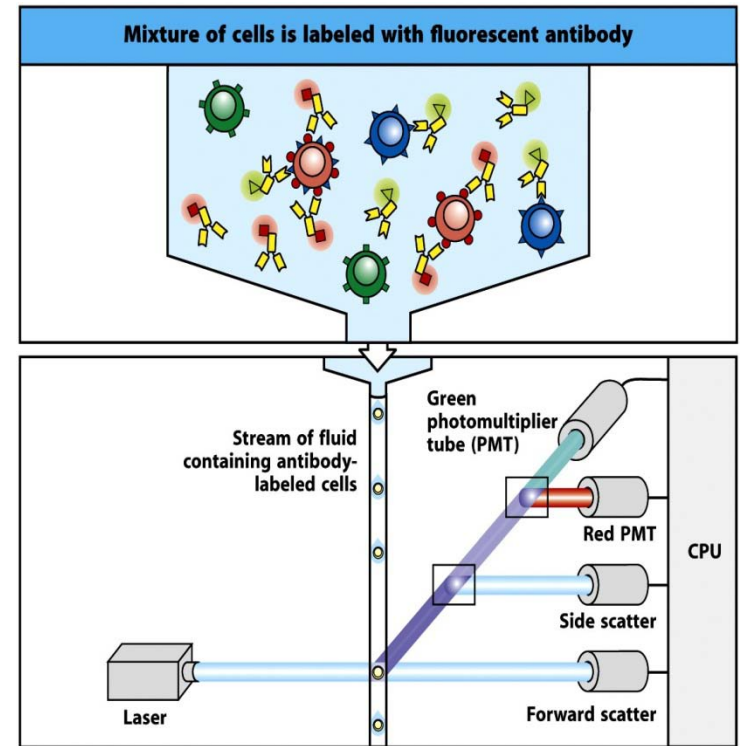


Figure A-26 part 1 of 2 Immunobiology, 7ed. (© Garland Science 2008)

Flow Cytometry (FCXM)

Solid Phase Immunoassays: Strengths

- Eliminates need for viable lymphocytes
- Very high sensitivity
- High throughput – 96 sera in 2-4 hours
- Greatly increased specificity
- Unaffected by most non-HLA-specific Abs
- Semi- to fully automated
- Small serum volume requirement
- Rx on continuous scale: more precise measurement



HLA Antigen Targets in Solid Phase Immunoassays

Target	Strengths	Weaknesses	Utility
Pooled antigens	Fast, least expensive	No specificity	Screening for presence of Ab
Phenotypes	Provides specificity and strength	Can't see all abs	Specificity and strength determination
Single antigens	Detects all Abs	May not measure strength well	Confirm presence/absence of particular Abs

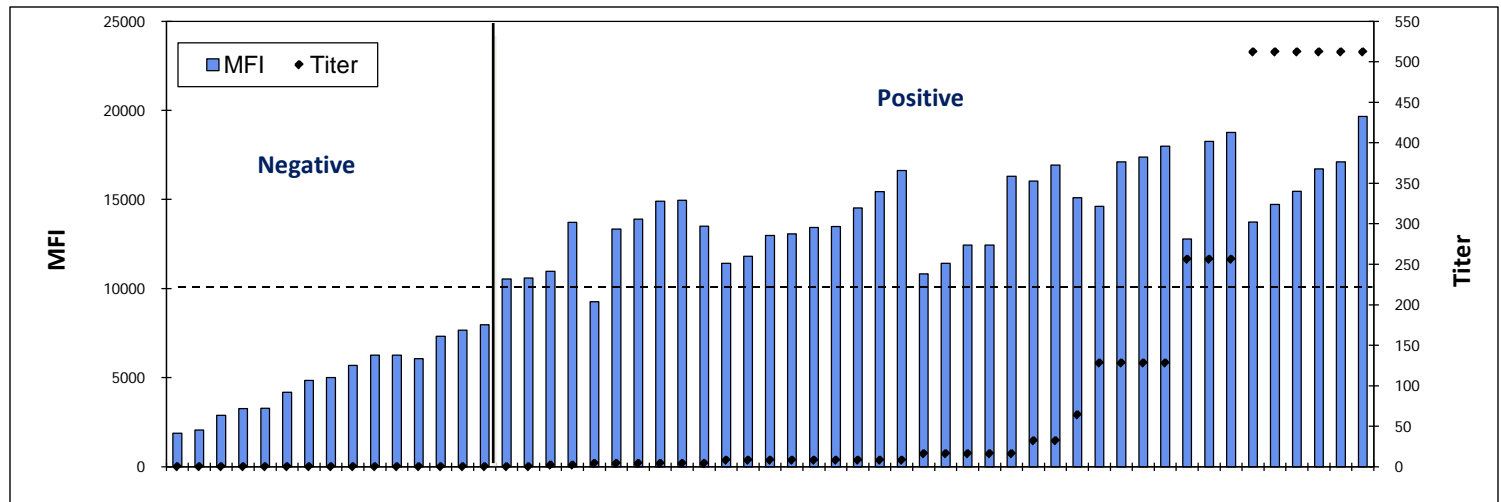
Information from Luminex[®] Ab tests

- Soluble HLA antigens as targets → Enhanced sensitivity and specificity
 - Has permitted of detection of Abs to specific alleles
- Evaluation of antibody strength
 - Permits “virtual crossmatches”
 - Facilitates monitoring for desensitization
 - Post-transplant monitoring for *de novo* Abs
- PRA: measurement of reactivity with panel
- cPRA (calculated PRA) - % incompatible donors

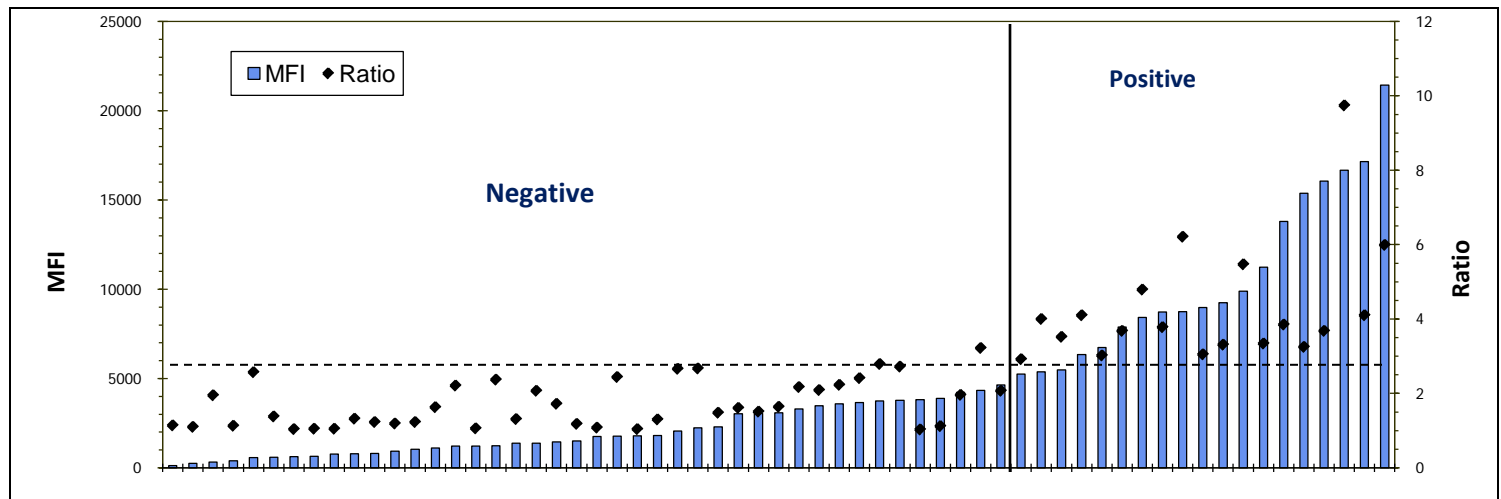


Correlations with Luminex® Phenotype Panels

CDC XM
 $r = 0.83$



FCXM
 $r = 0.85$



Crossmatch Prediction

XM Type	Correct Positive	Incorrect Positive	Incorrect Negative	Correct Negative	%Correct
CDC	34	6	4	92	92.6
Flow	73	5	4	45	92.9

Data Used: Luminex® phenotype panel results
Donor and recipient phenotypes
Previous mismatches
± Single antigen beads



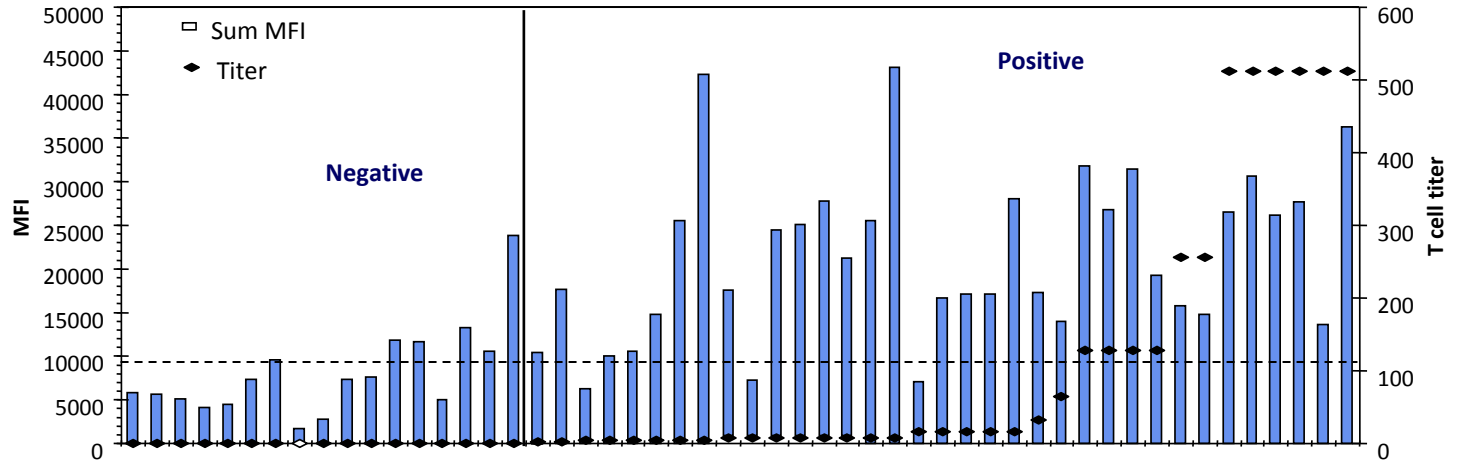
Issues with Antibody Detection

- Antigen variability
 - amount
 - condition

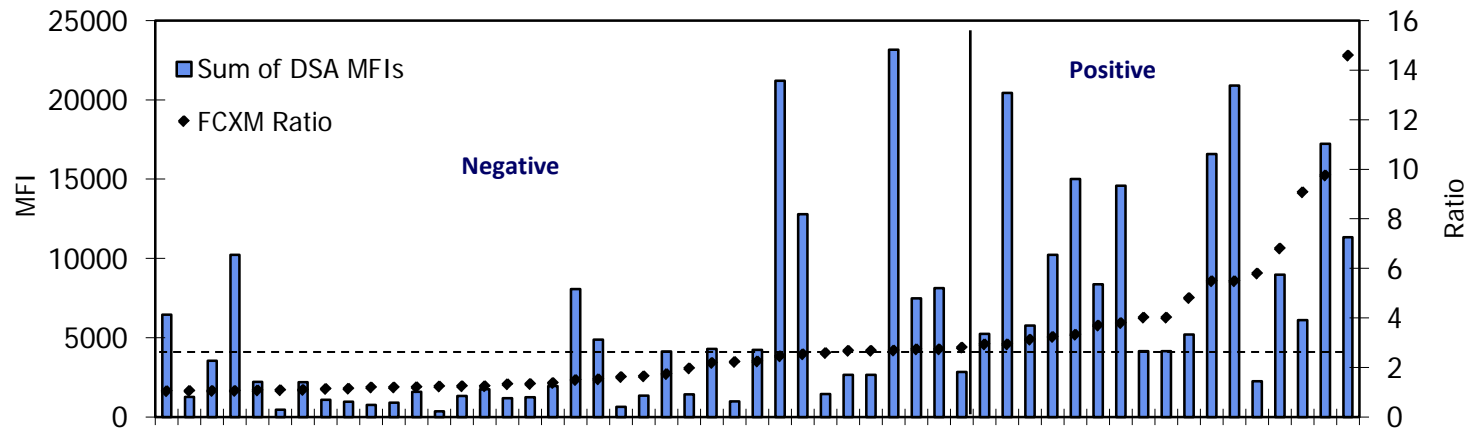


Correlations with Luminex® SAB

CDC XM
 $r = 0.60$



FCXM
 $r = -0.24$



Issues with Antibody Detection

- Antigen variability
 - amount
 - condition
- Interference by external factors
 - IVIg
 - thymoglobulin
- Interference by intrinsic factors
- Relevance of low antibody levels



Impact of IVIg on Negative Controls of Luminex[®] Phenotype Panels

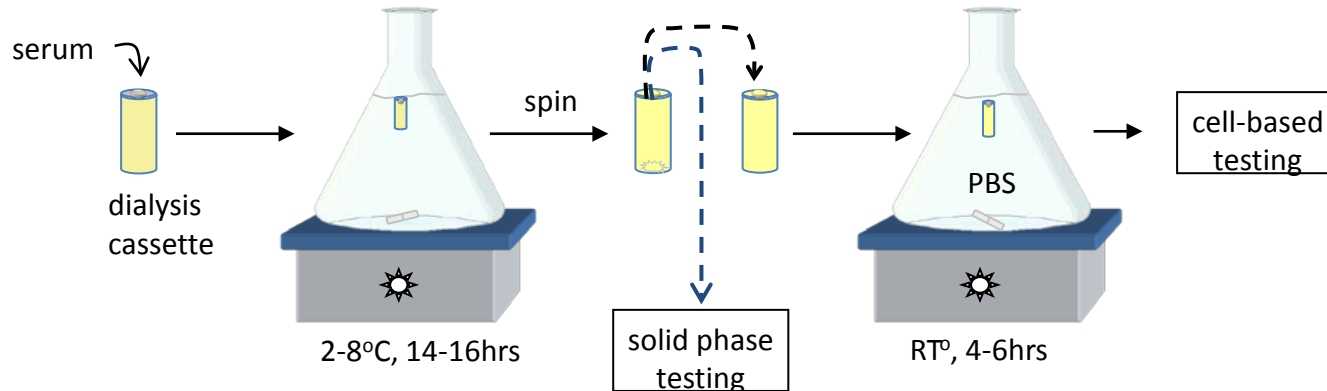
Time relative to IVIG	Neg Ctrl 1 ^a	Neg Ctrl 2	Neg Ctrl 3	Neg Ctrl 4	Post Ctrl
Pre-IVIg ^b	276	243	629	255	21,213
36 hrs post IVIg ^b	1311 (4.75x↑)	973 (4x↑)	3038 (4.8↑)	2401 (9.4↑)	23,399 (1.1↑)
6 days post IVIg ^b	367	261	666	546	20,320

^a Mean normalized MFI

^b IgM depleted sample



Interference Intrinsic to Serum Samples



- Increased background reactivity due to:
 - High serum levels of IgM
 - Immune complexes
- Removed by IgM depletion
 - Euglobulin ppt. by hypotonic dialysis superior to DTT inactivation

Antibody	IgM Depletion	DTT	% Difference
A*32:01	12,735	2765	78.3
A*30:01	9904	1463	85.2
Post Ctrl	15261	7052	53.8

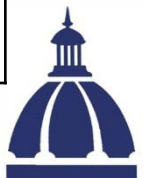
Ab Specificity and Strength After IgM Depletion

Before Treatment

DQA	DQB	MFI
04:01	02:01	8564
04:01	04:02	6073
03:03	03:01	3141
02:01	03:03	2649
05:01	02:01	1653
05:03	03:01	808
06:01	03:01	545

After Treatment

DQA	DQB	MFI
05:03	03:01	20,036
05:05	03:01	20,026
05:01	02:01	19,173
04:01	04:02	19,130
06:01	03:01	19,079
05:05	03:01	17,010
04:01	02:01	13,095



Reactivity with Self-Antigens

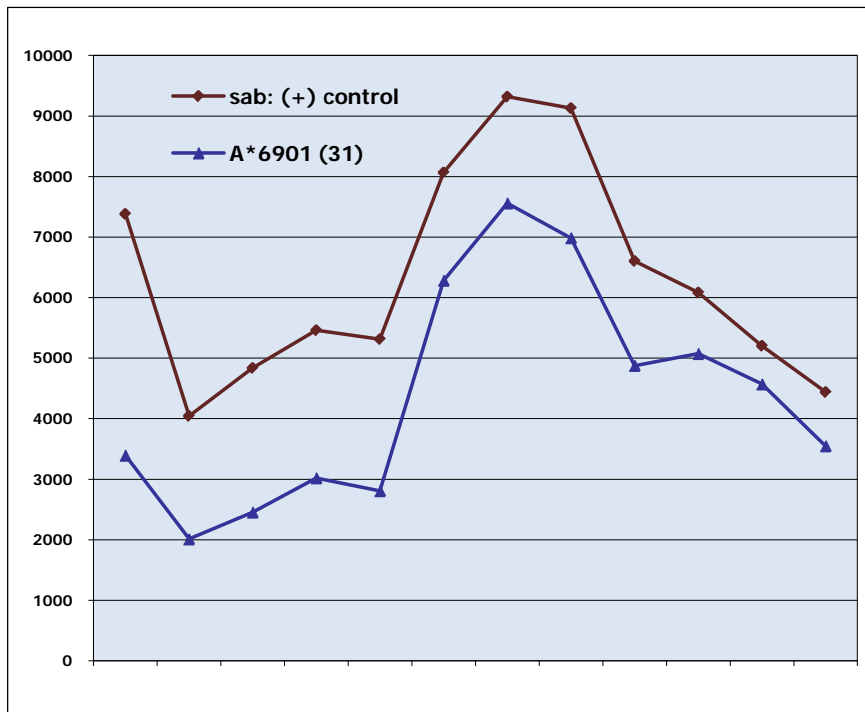
10/14/09 Sample – IgM Depleted	MFI	4/14/09 Sample – IgM Depleted	MFI
A*23:01	8184	B*15:01	18,619
A*24:01	7870	A*23:01	11,868
A*02:01	5005	A*02:01	3207
A*02:03	3576	A*02:03	2807
C*07:02	2092	C*07:02	1806
Pos Ctrl	11,455	Pos Ctrl	16,221
Neg Ctrl	768	Neg Ctrl	90

Reactivity was not observed with autologous antigens on phenotype assay.
Highest reactivity was with B62 and B63, MFI >16,000.

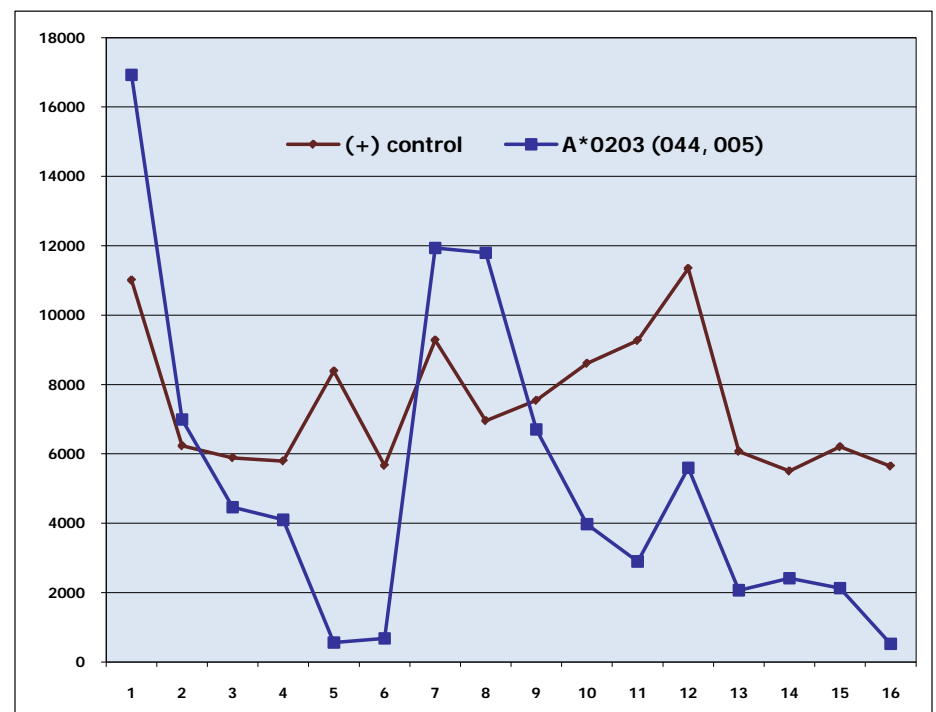


Variance in Control Reactivity

Single Ag Beads



Single Ag Beads



QC Considerations for HLA Luminex Assays

- HLA typing
 - Recognize potential false + and false – probe reactions
 - Consider possible allele dropout
 - Alternative methods for ambiguity resolution
- HLA specific antibody detection
 - Know your assays – determine “holes” and “hyper-reactive” antigens
 - Account for day-to-day and lot-to-lot variability
 - Re-correlate threshold values for virtual XMs when new assay lots change in sensitivity.



Luminex[®] Assays: Invaluable Tools in Histocompatibility and Immunogenetics

- Most widely used platform
- Rapid, multiplexed HLA typing by reverse SSOP
- Highly sensitive and specific detection of HLA specific antibodies
- Additional applications:
 - KIR genotyping
 - MICA typing and antibody detection
 - Cytokine gene polymorphism



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