Antibody Screening & Identification

- Complement – Dependent Cytotoxicity (CDC)

  Terasaki & McClelland (1964) Nature 204, 998

- Enzyme-Linked ImmuneSorbent Assay (ELISA)

  Kerman et al (1996) Transplantation 62, 201

- Flow Cytometry

  Garavoy et al (1983) Transplant Proc 15, 1939 (crossmatching)

  Pei et al (1999) Human Immunol 60, 1293 (antibody screening)

NZBLOOD Te Ratonga Toto O Aotearoa

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Antibody Identification by Complement – Dependent Cytotoxicity

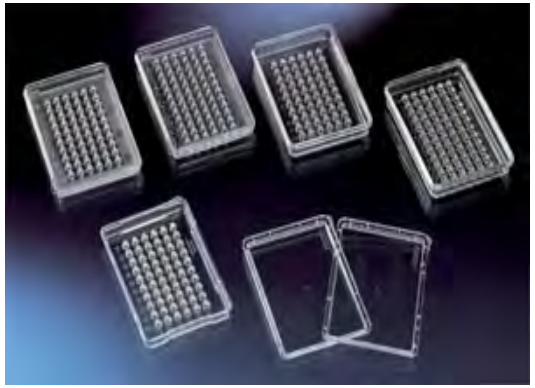
B cell Panel Reactive Antibody (BPRA) test –
patient serum + HLA typed random platelet donors

Antibody identification –
class I & II HLA typed cell panel. Frozen lymphocytes.
Good distribution of HLA types

Antibody Identification by Complement – Dependent Cytotoxicity (NIH extended assay)

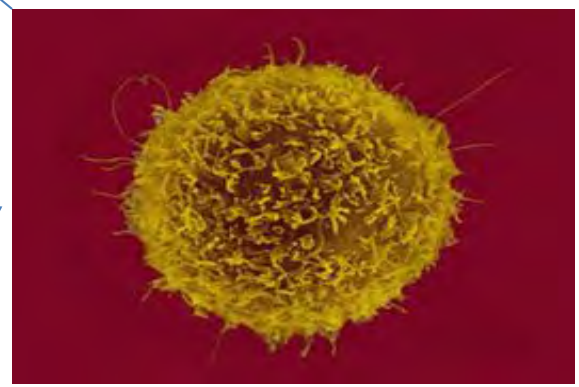
1. Monthly serum sample

+

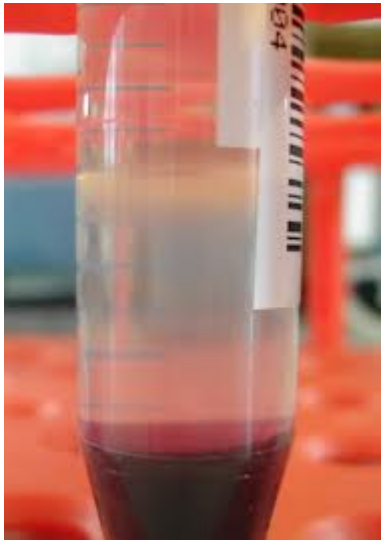


60-well ‘nunc’ trays.

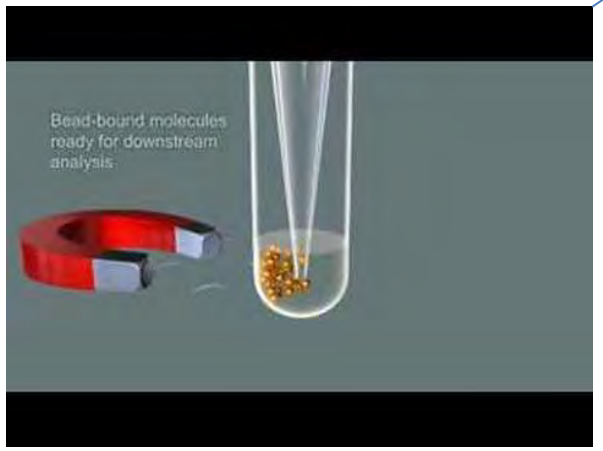
1. Serum ± DTT, 30’ RT
2. 1µl cells, 30’ RT
3. 5µl complement, 60’ RT
4. 5µl fluoroquench



B cell from random Platelet donors



2. Ficoll paque



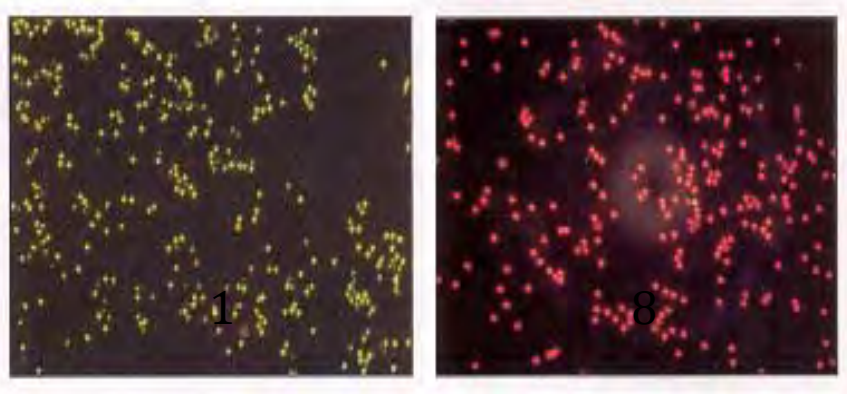
3. Lymphocytes + mAb CD19 Dynabeads

Control name	Description	Expected outcome / possible problem
ATSG	**Commercial IgG anti-T cell**	Should be negative with B cells. If positive may indicate wrong cell type used or poor cell viability
ABSG	**Commercial IgG anti-B cell**	Should be positive with B cells. If negative may indicate wrong cell type used or complement not added correctly. Reactivity may be acceptably reduced by DTT
ALSM	**Commercial IgM anti-lymphocyte**	Should be positive pre-DTT but negative post-DTT. If positive post-DTT, may indicate ineffective DTT treatment
PHS	**In house preparation of pooled IgG reactive patient serum**	Positive pre and post-DTT. If negative or only weakly positive may indicate complement not added correctly.
AB	**AB serum with no known CDC reactivity**	Total cytotoxicity should be less than 20%. If higher then cell viability may be unacceptably poor

Score	Interpretation	% cell death
0	Insufficient cells/Unreadable	
1	Negative	0 -10
2	Doubtful negative	11-20
4	Weak positive	21-50
6	Positive	51-80
8	Strong positive	80-100

Pre-DTT	**Post-DTT**	**Reactivity Specificity**
Positive	Positive (same value as pre-DTT)	IgG
Positive	Positive: pre - post DTT ratio >50%	IgG/IgM
Positive	Positive: pre - post-DTT ratio <50%	IgM/IgG
Negative	Positive	Negative/Prozone/Confounded
Positive (>3%)	Negative (<=3%)	IgM
Positive (<3%)	Negative (<=3%)	-
Negative	Negative	-

Score 1



Score 8

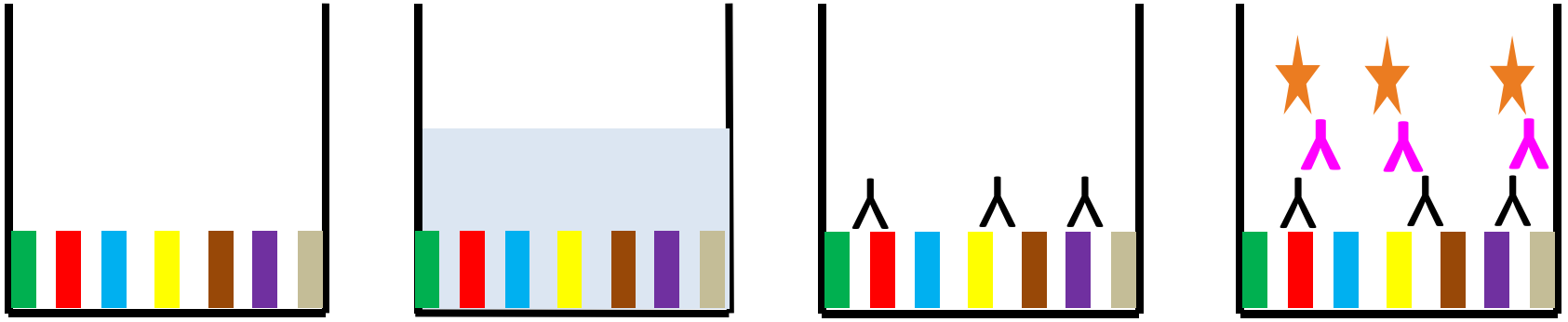
Lymphocyte Cell Panel for Antibody Identification

CELL 1		1025602	A2		B39	B44	DR4	DR15	DR51	DR53	DQ6	DQ7
CELL 2		1015262	A2	A32	B13	B63	DR7	DR15	DR51	DR53		
CELL 3		1317516	A2	-	B60	B58						
CELL 4		1022948	A26	A32	B38	B64	DR1	DR15	DR51		DQ5	DQ6
CELL 5		2882720	A11	A33	B13	B58	DR13	DR16	DR51	DR52		
CELL 6		2608913	A11	-	B35	B60	DR4	DR4	DR53			
CELL 7		1024438	A23	A24	B44	B57	DR7	-	DR53			
CELL 8		1012856	A1	A25	B18	B37	DR10	DR15	DR51		DQ6	
CELL 9		1020988	A2	A26	B35	B57						
CELL 10		2875950	A3	A11	B57	B60	DR4	DR7	DR53			
CELL 11		1026805	A2	A28	B35	B41						
CELL 12		1082391	A11	A33	B18	B55						
CELL 13		1001726	A3	A31	B47	B67	DR1	DR103			DQ1	DQ7
CELL 14		1090191	A2	A3	B38	B52						
CELL 15		1608854	A2	A68	B61	B65						
CELL 16		1065850	A2	A11	B48	B55	DR11	DR12	DR52		DQ7	
CELL 17		1025319	A31	A32	B51	B62	DR4	DR15		DR53	DQ1	DQ3
CELL 18		1056398	A2	A30	B18	B44	DR7	DR14	DR52	DR53	DQ1	DQ2
CELL 19		2898923	A31	A32	B35	B60	DR11	DR13	DR52	DR52		
CELL 20		1029743	A1	A2	B45	B50	DR7			DR53	DQ2	DQ7
CELL 21		1039409	A1	A32	B44	B65	DR7	DR13			DQ2	DQ6
CELL 22		2826864	A11	A24	B56	-	DR12	-	DR52			
CELL 23		2878430	A24	A33	B44	B54	DR4	DR7	DR53			
CELL 24		1906921	A30	A32	B13	B63	DR4	DR13	DR52	DR53		
CELL 25		2847444	A29	A68	B44	B65	DR4	DR7	DR53			
CELL 26		1029042	A3	A25	B18	B47	DR4	DR7		DR53	DQ2	DQ3
CELL 27		1031353	A2	A26	B41	B45						
CELL 28		1082035	A1	A29	B7	B65	DR1	DR15	DR51			
CELL 29		1111613	A2	A32	B38	B44	DR4	DR15			DQ3	DQ6
CELL 30		2538168	A24	A34	B13	B48						
CELL 31		1303347	A2	-	B62	B64	DR7	DR17	DR52	DR53		
CELL 32		1025306	A1	A2	B8	B49	DR4	DR17	DR52	DR53		
CELL 33		1028085	A1	A3	B37	B65	DR7	DR13	DR52	DR53	DQ7	
CELL 34		1016059	A2	A68	B62	B72						
CELL 35		2632284	A24	A30	B8	B13						
CELL 36		2864220	A23	A29	B44	-	DR7	-	DR53			
CELL 37		1028143	A1	A24	B8	B71	DR3	DR11	DR52			
CELL 38		1412450	A1	-	B7	-	DR15	DR17	DR51	DR52		
CELL 39		2752493	A32	A68	B27	-	DR11	DR13	DR52	DR52		
CELL 40		1028317	A2	A24	B7	B52						

Conclusion – CDC: a cellular method detecting antibodies against native protein on cell surface

ADVANTAGES	DISADVANTAGES
Easy to perform, detects C' fixing IgG and IgM antibodies. Can predict hypercute rejection.	Only detects complement fixing antibodies. Detects autoantibodies, irrelevant in transplantation
No special equipment required	Requires large well-maintained panel of HLA typed and viable cells. Cannot accurately determine individual specificities in highly sensitized patients
Long history of the assay	Not HLA specific. Antibody strength?
Directly comparable with the CDC cross match	Limited sensitivity. Subjective reading

ELISA - Enzyme Linked ImmunoSorbent Assay



1. Wells coated with HLA antigens, or coupled to mAb

2. Hydrate, wash

3. Add patient serum

4. Add secondary Ab (enzyme conjugated), Wash. Add substrate. Read.

<u>Required</u>:
ELISA kits - LATM, PRA-STAT, Quikscreen
Plate reader

<u>Secondary antibody</u>:
e.g. goat anti-human IgG
Linked to Horse Radish Peroxidase or Alkaline Phosphatase

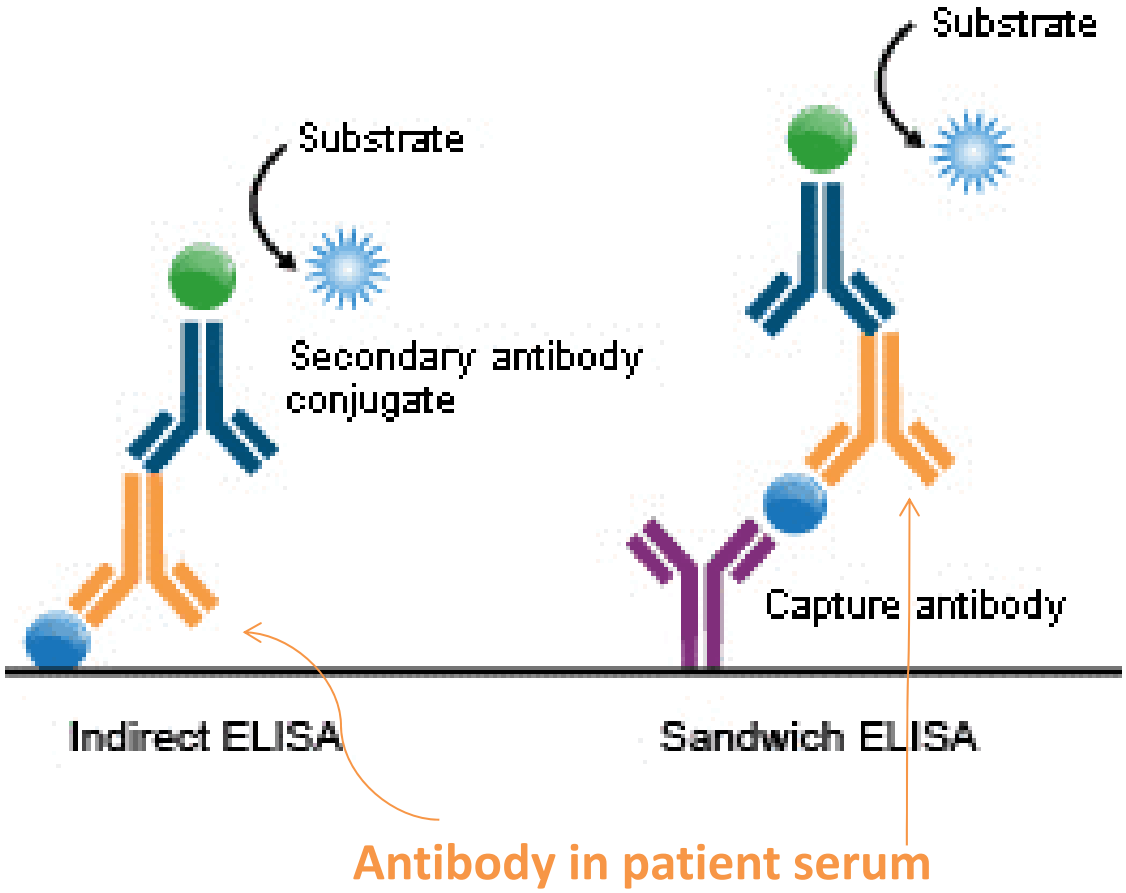
ELISA
enzyme linked immunosorbent assay

<u>Required:</u>
ELISA kits - LATM, PRA-STAT, Quikscreen
Plate reader

<u>Secondary antibody:</u>
e.g. goat anti-human IgG

<u>Enzyme</u>
Horse radish peroxidase
Alkaline phosphatase

<u>Substrates</u>
HRP: H_2O_2 + tetramethylbenzidine
AP: nitroblue tetrazolium +
bromochloroindolyl phosphate



Advantages of ELISA over CDC

- Sensitivity
- Panels of cells not required

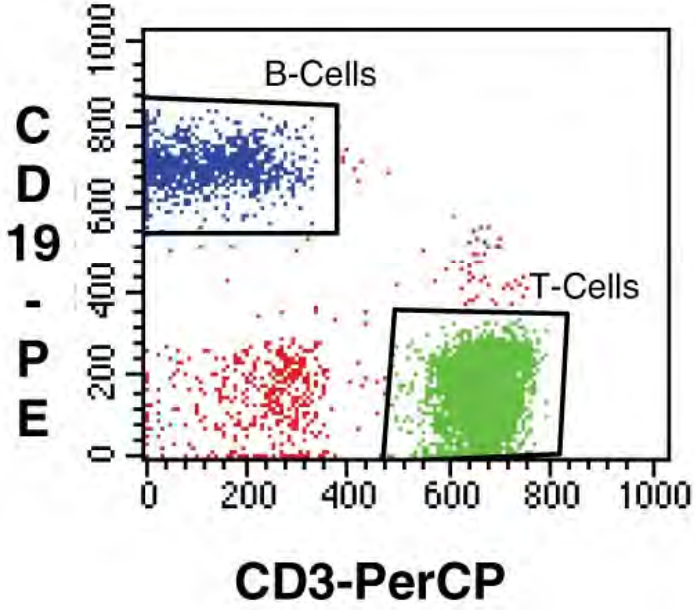
Drawbacks of ELISA

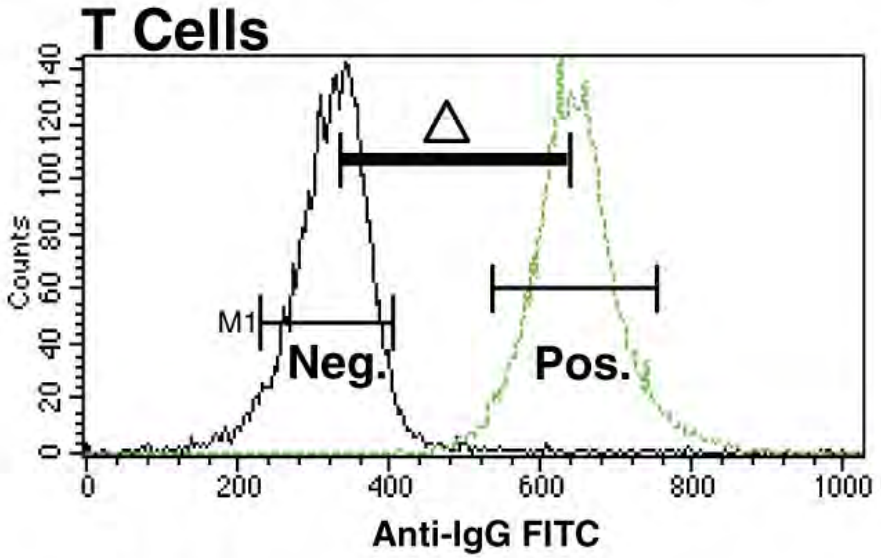
- weak positives and false negative reactions
- Weak HLA-Ab difficult to detect?
- Cutoff: positive/negative
- Limited antigen panels

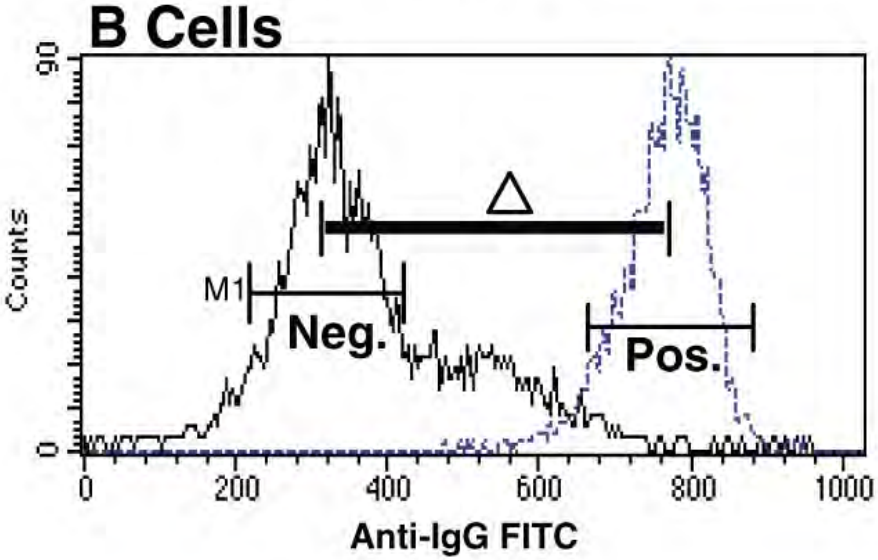
Technical Limitations

- Structural issues with isolated antigen?
- Specific antigen in too low concentration?
- Panel composition?
- Interference by immunoglubulins/therapeutic drugs
- Binding of non-HLA serum proteins e.g. after vaccination

3-Color Flow Cytometric Crossmatch







HLA Class I and II PRA Screening by FlowPRA Beads

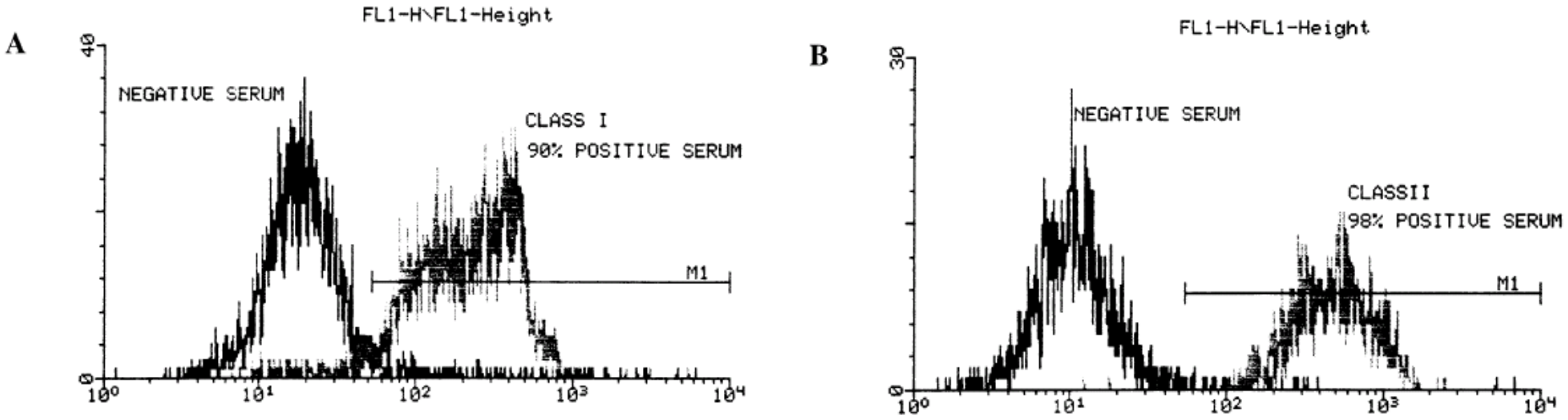
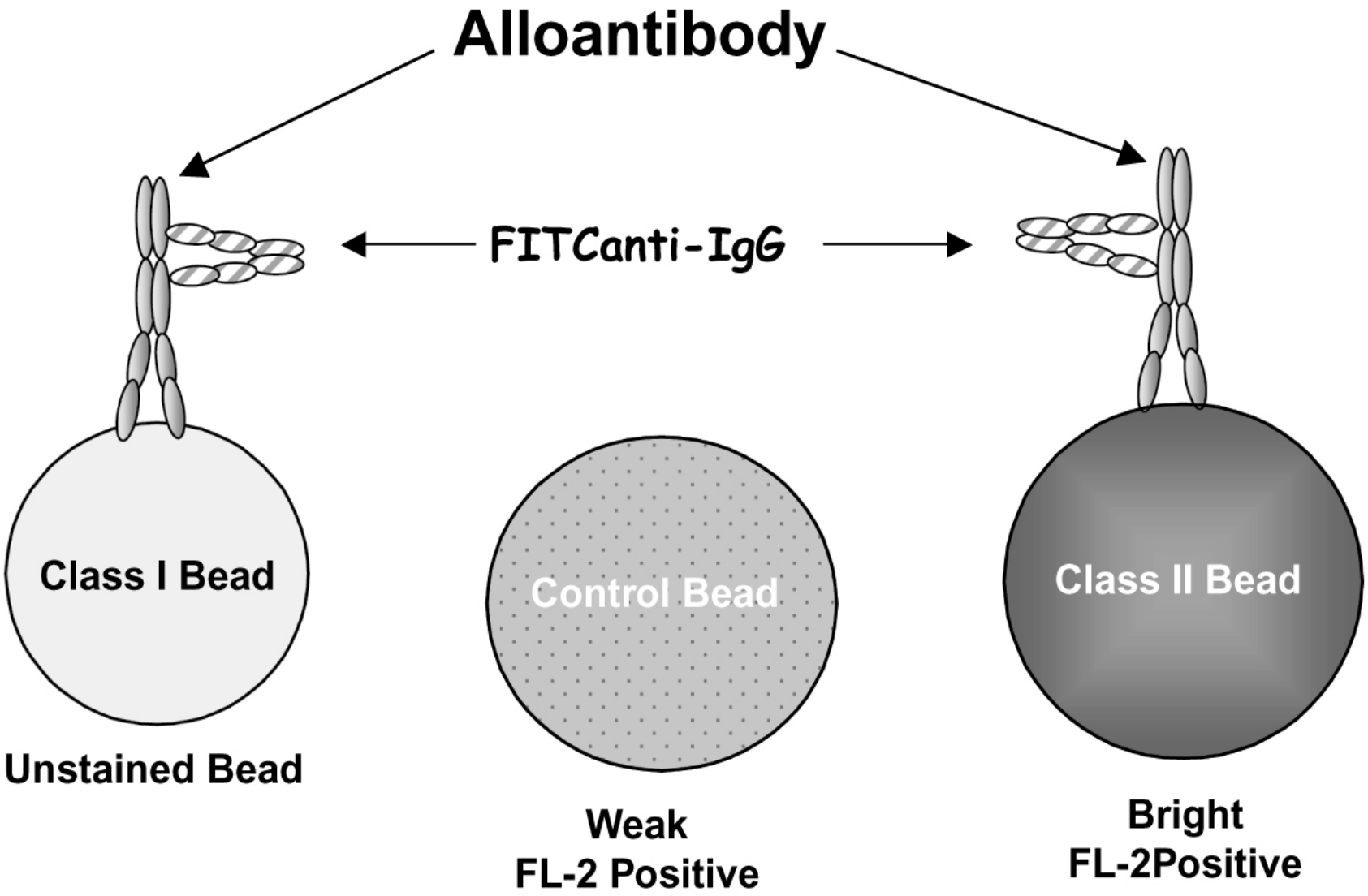


FIGURE 1 (A) Overlaid FL1 histograms of Class I microbead reactions with negative serum and with 90% positive serum. (B) Overlaid FL1 histograms of Class II microbead reactions with negative serum and with 98% positive serum.

Pei et al 1998 Human Immunol 59, 313

FlowPRA® ScreeningTest



Pei et al 1998; Bray et al (2011) Methods Cell Biol 103, 285

TABLE 1 Specificities of each FlowPRA bead

FlowPRA Class I Beads			FlowPRA Class II Beads			
Bead ID	Antigen Typing		Bead ID	Antigen Typing		
1	A11	B27,B48	31	DR15,DR9	DR53,DR51	DQ5,DQ9
2	A69,A29	B39,B55	32	DR4,DR15	DR53,DR51	DQ6,DQ7
3	A1,A29	B8,B45	33	DR16,DR4	DR53,DR51	DQ4,DQ5
4	A2,A24	B7,B55	34	DR8,DR14	DR52	DQ4,DQ5
5	A2,A25	B18,B65	35	DR4,DR7	DR53	DQ2,DQ8
6	A26,A24	B52,B62	36	DR15,DR18	DR51,DR52	DQ6,DQ4
7	A30,A68	B53	37	DR11,DR12	DR52	DQ5,DQ7
8	A2,A11	B13,B62	38	DR103,DR17	DR52	DQ5,DQ2
9	A23,A33	B45,B63	39	DR1,DR13	DR52	DQ5,DQ6
10	A23,A34	B44	40	DR9,DR10	DR53	DQ5,DQ9
11	A11,A23	B49,B52	41	DR15,DR12	DR51,DR52	DQ5,DQ7
12	A11,A24	B59,B60	42	DR16,DR14	DR51,DR52	DQ5
13	A24,A33	B44,B51	43	DR13,DR8	DR52	DQ5,DQ6
14	A23,A26	B41,B71	44	DR11,DR13	DR52	DQ5,DQ6
15	A3,A32	B50,B56	45	DR17,DR7	DR52,DR53	DQ2,DQ9
16	A2,A24	B54,B67	46	DR15,DR8	DR51	DQ6,DQ8
17	A2	B52,B73	47	DR15,DR4	DR51,DR53	DQ2,DQ6
18	A26,A34	B38,B75	48	DR15,DR17	DR51,DR52	DQ6,DQ2
19	A11,A33	B51,B54	49	DR15,DR7	DR51,DR53	DQ6,DQ2
20	A30,A31	B13,B71	50	DR1,DR7	DR53	DQ2,DQ5
21	A30,A36	B35,B71	51	DR15,DR11	DR52	DQ5,DQ6
22	A32,A68	B35,B7	52	DR7,DR13	DR52,DR53	DQ6,DQ9
23	A1,A32	B60,B64	53	DR15,DR13	DR51,DR52	DQ6,DQ2
24	A2,29	B7,B46	54	DR9,DR14	DR52,DR53	DQ5,DQ9
25	A30	B42	55	DR8,DR9	DR53	DQ2,DQ7
26	A2	B8,B58	56	DR17,DR14	DR52	DQ2,DQ5
27	A2,A3	B57,B65	57	DR1,DR11	DR52	DQ5,DQ6
28	A1,A30	B37,B57	58	DR17,DR4	DR52,DR53	DQ2
29	A3,A68	B7,B65	59	DR11,DR4	DR52,DR53	DQ7,DQ8
30	A32,A36	B53,B61	60	DR1,DR14	DR52	DQ5

TABLE 1. Recombinant human leukocyte antigen class I single antigen list

A	B	B	C
A*0101	B*07021	B*4701	Cw*0102
A*0201	B*0703	B*4801	Cw*0202
A*0203	B*0801	B*4901	Cw*0302
A*0204	B*1301	B*5001	Cw*03031
A*0205	B*1401(64)	B*51011	Cw*0304
A*0206	B*1402(65)	B*51022	Cw*0401
A*0210	B*1501101(62)	B*5103	Cw*0501
A*0301	B*1502(75)	B*52011	Cw*0602
A*1101	B*1503(72)	B*5301	Cw*0701
A*1102	B*1508	B*5401	Cw*0702
A*2301	B*1510(71)	B*5501	Cw*0801
A*2402	B*1511(75)	B*5502	Cw*0802
A*2403	B*1512(76)	B*5601	Cw*1202
A*2501	B*1513(77)	B*5701	Cw*1203
A*2503	B*1516(63)	B*5703	Cw*1402
A*2601	B*1801	B*5801	Cw*1502
A*2902	B*27052	B*5901	Cw*1601
A*3001	B*2708	B*67011	Cw*1701
A*31012	B*3501	B*7301	Cw*1802
A*3201	B*3701	B*7801	
A*3301	B*3801	B*8101	
A*3303	B*39014	B*8201	
A*3401	B*3902	B*8301	
A*3402	B*3905		
A*3601	B*4001(60)		
A*3603	B*4002(61)		
A*4301	B*4003(61)		
A*6601	B*4005		
A*6602	B*4101		
A*6801	B*4201		
A*6802	B*44021		
A*6901	B*44032		
A*7401	B*4501		
A*8001	B*4601		

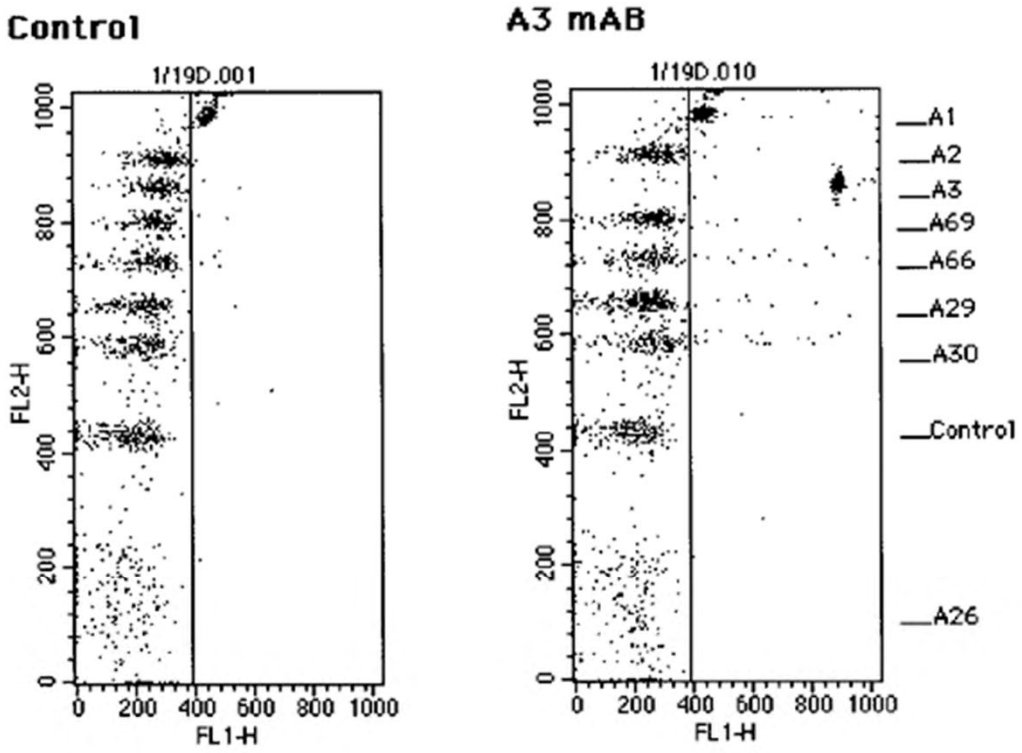


FIGURE 1. Example of a monoclonal antibody (mAb) (A3) reacting with a group of single antigen bead arrays. An eight-color bead array was shown in an FL1 versus FL2 dot plot. Each color of the beads was separated on the FL2 channel, and the typing of each antigen bead was marked on the right side of the graph. A shift of the FL1 channel indicated a positive reaction. The A3 mAb reacted specifically with the A3 antigen beads, compared with the control.

Pei et al (2003) Transplant 75, 43

Advantages

- Increased sensitivity was the ability to simultaneously assay for antibodies against HLA Class I and Class II.
- Reactivity is specific for HLA antigens (Class I and/or Class II).
- Lack of significant interference from non-HLA antibodies.
- Sensitivity comparable to the FCXM.
- Consistent source of antigen from stable cell lines.
- Not affected by subjective microscopic readings or cell viability.
- Semiquantitative assessment of antibody binding.

Disadvantges

- Cost – machine, kits
- Structural integrity of isolated proteins

NZBS - Platelet Immunofluorescence Test – to screen for platelet antibodies

Emergence of Luminex……