NHS Blood and Transplant

Laboratory techniques for detecting platelet and granulocyte antibodies

Matthew Hopkins

Clinical Scientist, H&I Filton

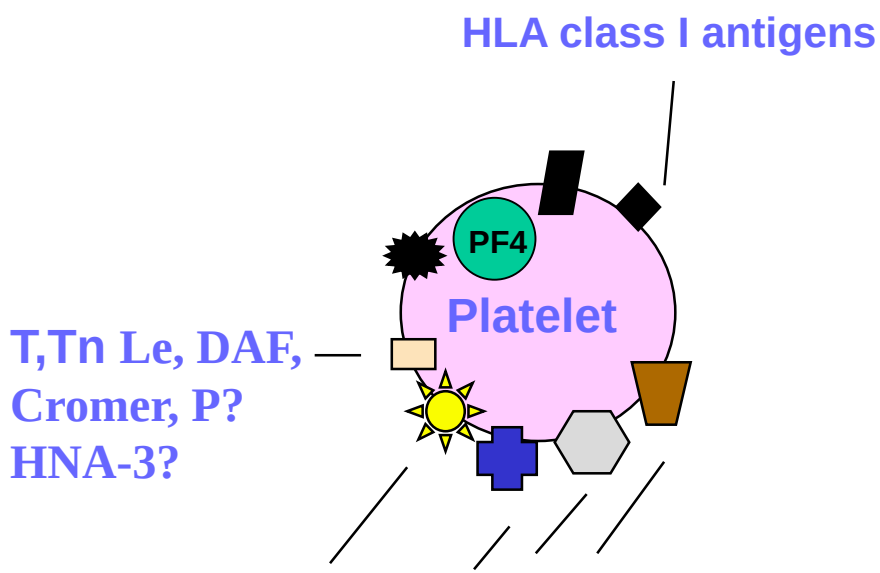
Presented at BSHI training day 01/11/2022

Outline

- Recap of human platelet antigens (HPA)
- Laboratory tests for platelet antibodies
- Heparin-induced thrombocytopenia (HIT)

Antigens on platelets

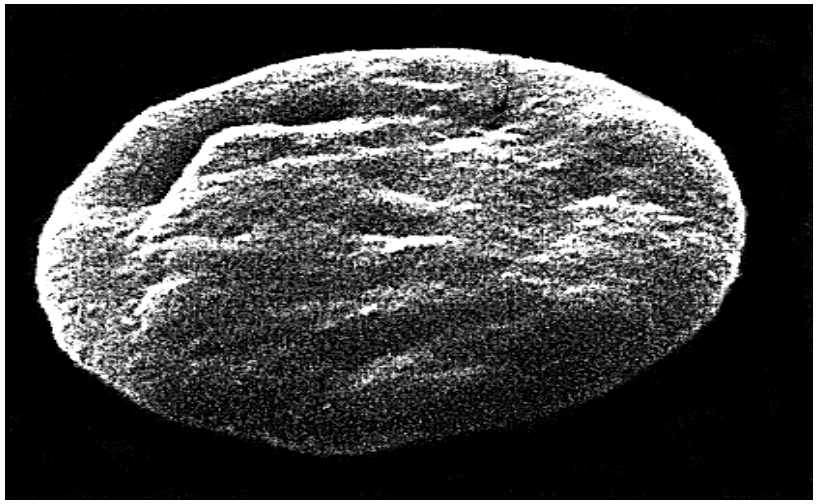
NHS Blood and Transplant

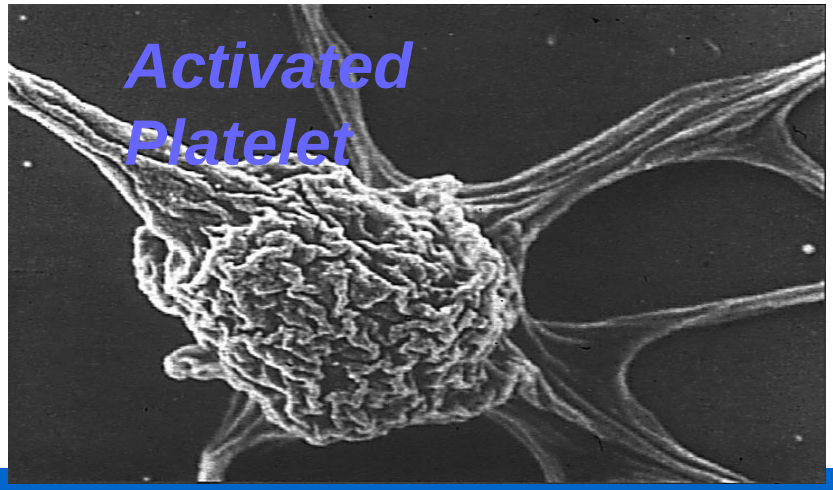


Platelet glycoproteins

GPIIb/IIIa
GPIa/IIa
GPIb/IX/V
CD109

} HPA-1 to 35

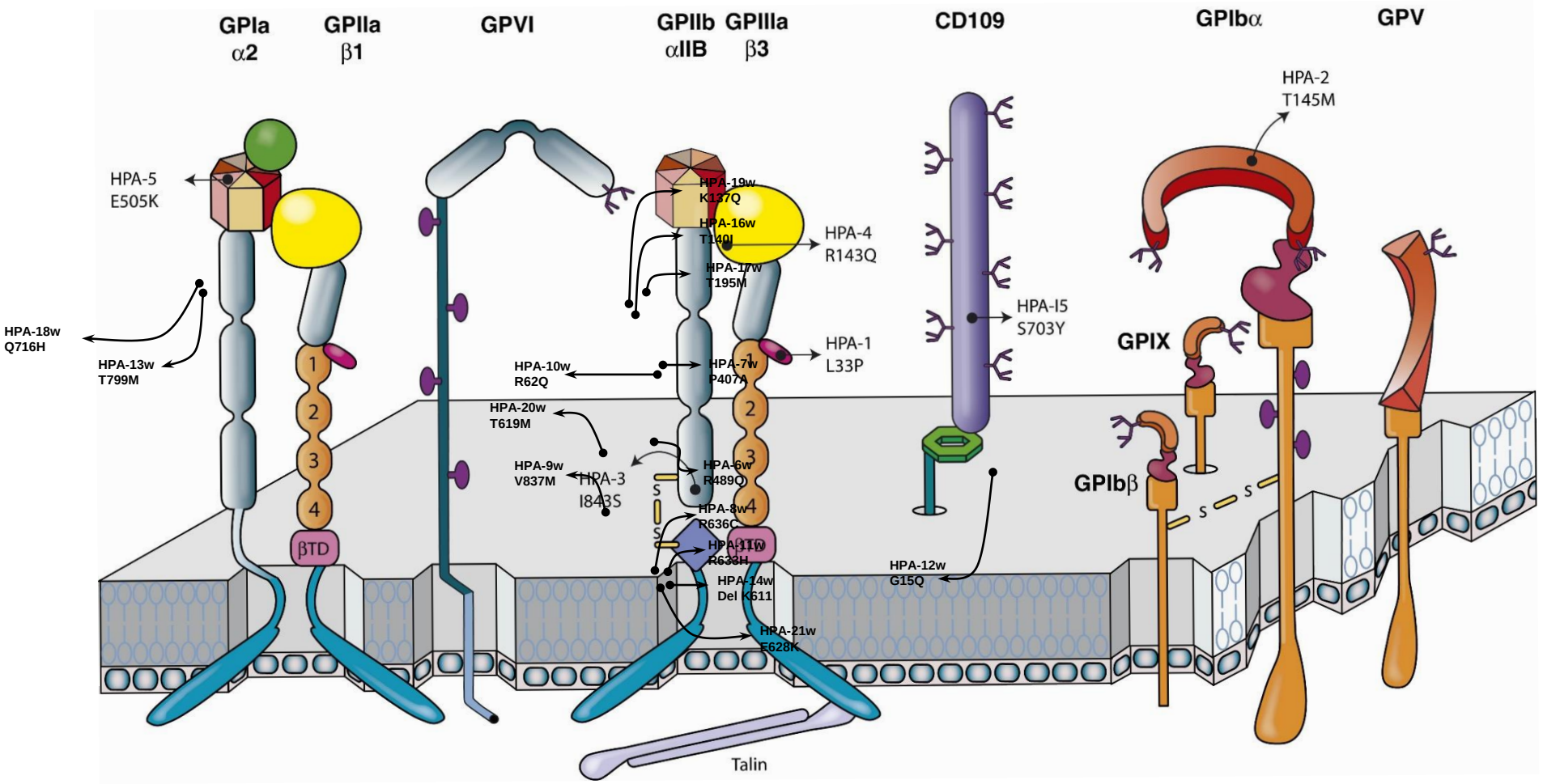




NHS Blood and Transplant

Overview of the human platelet antigens (HPA)

- The majority (28/35) are associated with the GPIIb/IIIa complex.
- The vast majority of HPA are bi-allelic systems, i.e. arise from a single amino acid substitution, except HPA-14bw, which results from an ‘in frame’ deletion of three nucleotides. (Recently, 3 HPA systems have been shown to be tri-allelic, i.e. there is a ‘c’ antigen; HPA-1, -5, -7 - these mutations are rare)
- The ‘a’ allele is always the high frequency form and ‘b’ the low frequency.
- The ‘w’ or workshop assignment is given to systems where antibodies to only one antigen has been described – this is the majority of recently identified HPA.
- The antigens may not be truly ‘platelet-specific’
  - GpIa/IIa (HPA-5) is also found on activated T-lymphocytes.
  - CD109 (HPA-15) is expressed on platelets, activated T-cells, endothelial cells and a subset of bone marrow progenitor cells


GPIa α2
GPIIa β1
GPVI
GPIIb αIIB
GPIIIa β3
CD109
GPIbα
GPV
HPA-5 E505K
HPA-18w Q716H
HPA-13w T799M
HPA-19w K137Q
HPA-16w T140I
HPA-17w T195M
HPA-4 R143Q
HPA-1 L33P
HPA-10w R62Q
HPA-7w P407A
HPA-20w T619M
HPA-9w V837M
HPA-3 I843S
HPA-6w R489Q
HPA-8w R636C
HPA-11w R633H
HPA-14w Del K611
HPA-21w E628K
HPA-12w G15Q
HPA-I5 S703Y
HPA-2 T145M
GPIX
GPIbβ
βTD
Talin

The most clinically significant platelet-specific alloantigens

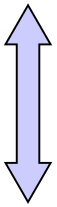
NHS Blood and Transplant

	Allele freq. (Cauc)	GP	Copies/cell	GP function
HPA-1a HPA-1b	84.5 % 15.5%	IIIa (CD61)	40K	Fg, vWF, Fn, Coll, Vn
HPA-2a HPA-2b	89.9% 10.1%	Ibα (CD42b)	20K	vWF
HPA-3a HPA-3b	60.3% 39.7%	IIb (CD41)	40K	
HPA-4a HPA-4b	100% 0.0%	IIIa (CD61)	40K	
HPA-5a HPA-5b	91.1% 8.9%	Ia (CD49b)	2-4K	Collagen
HPA-15a HPA-15b	50.0% 50.0%	CD109	0.5 -2K	Collagen

The clinical context of platelet-specific antibodies

NHS Blood and Transplant

- Neonatal alloimmune thrombocytopenia (NAIT) — *alloantibodies (HPA)*
- Refractoriness to platelet transfusions
- Bone marrow transplant recipients
- Post-transfusion purpura (PTP)



allo and/or auto antibodies

- Autoimmune thrombocytopenias — *autoantibodies (platelet GP specific but not HPA specific)*
- Drug induced immune thrombocytopenias, e.g. heparin, quinine and teichoplanin — *drug dependent antibodies*
  e.g. gold — *drug independent antibodies*
- Thrombasthenias, e.g. GT, BSS — *isoantibodies*

Types of tests for platelet antibodies

NHS Blood and Transplant

- Direct tests - membrane bound immunoglobulins on the patient's platelets
- Indirect tests - serum antibodies

- *Problems in platelet antibody detection:*

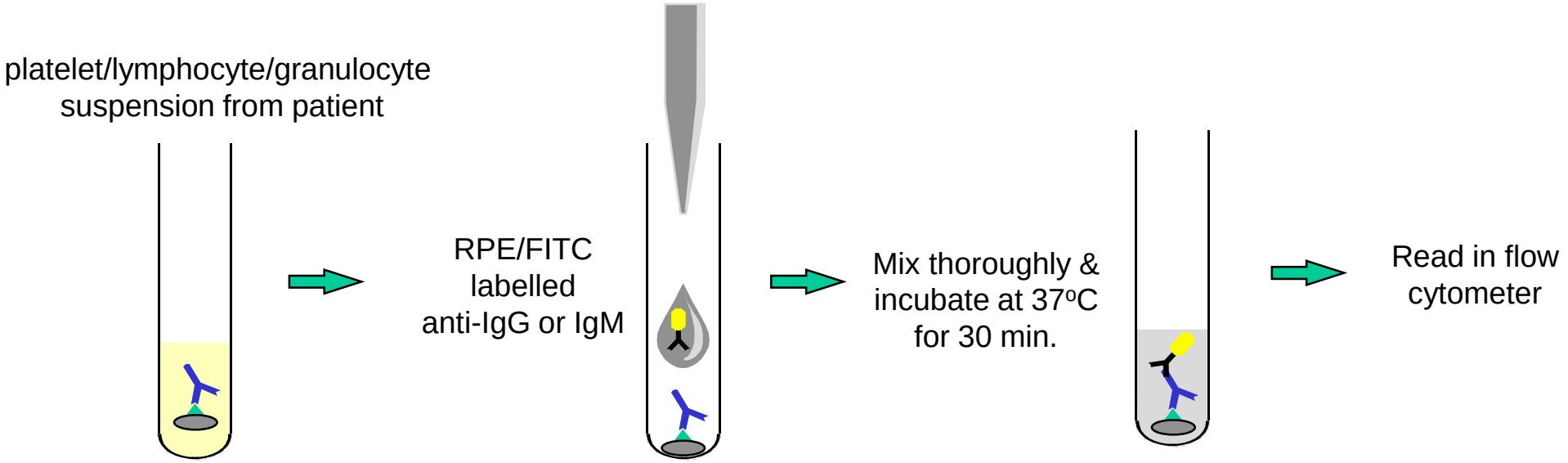
  - **Labile cells**, aggregate *in vitro*
  - **Cell activation** caused *in vitro* or during isolation may result in increased or decreased expression of membrane antigens
  - **Direct tests**: low numbers of platelets in circulation, platelet transfusions, IVIgG
  - **Ig binding** - $F(ab)_2$,
    - FcR
    - Non-specific
    - Internalized Ig
  - **Differentiation between platelet-specific and HLA antibodies**

NHS Blood and Transplant

Laboratory testing for platelet antibodies

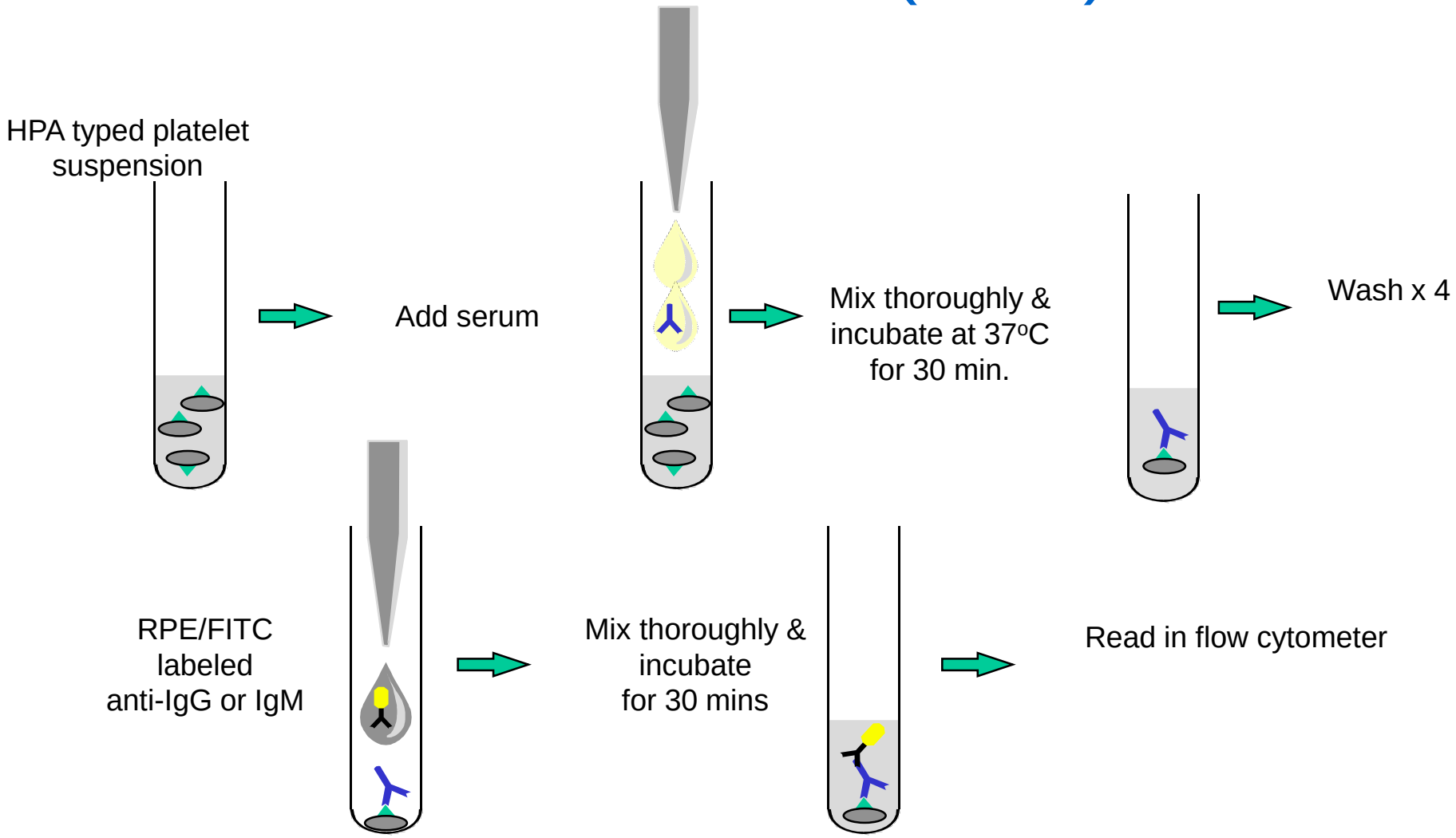
- Immunofluorescence tests
- MAIPA assay
- *Other ELISA assays (some commercially available)*
- Emerging technologies using rHPA, cell lines and Luminex

Direct Immunofluorescence tests

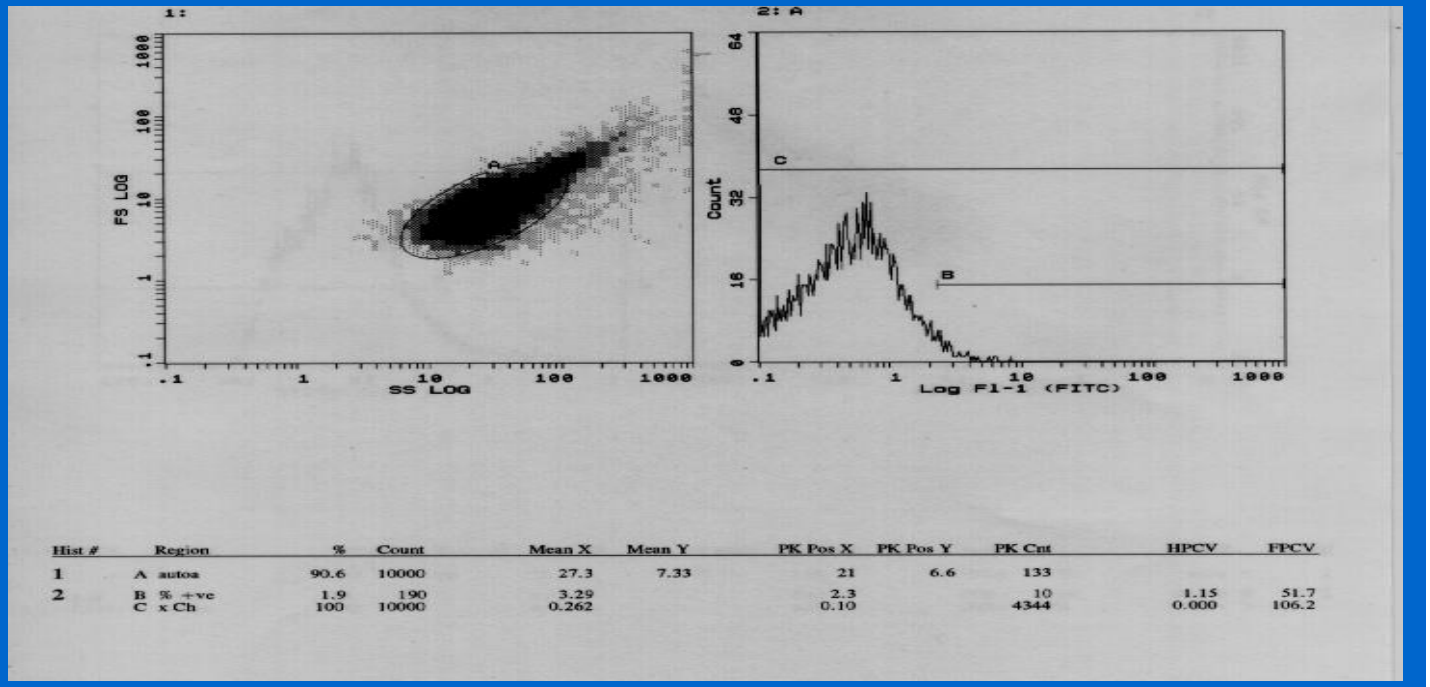


Indirect Platelet immunofluorescence test (PIFT)

NHS Blood and Transplant

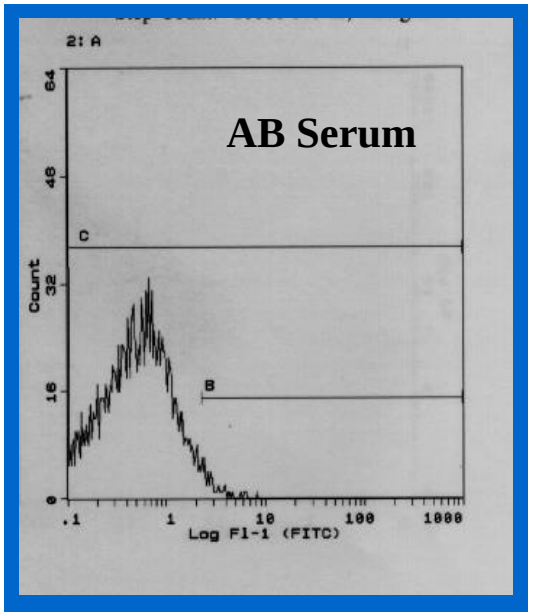


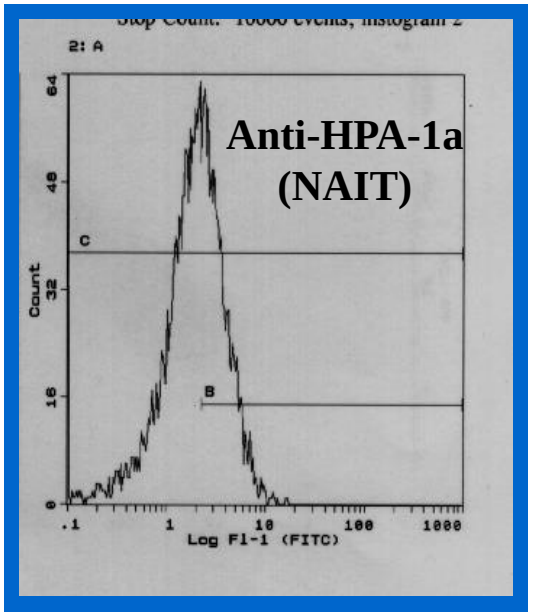
PIFT Results - Flowcytometry (1)

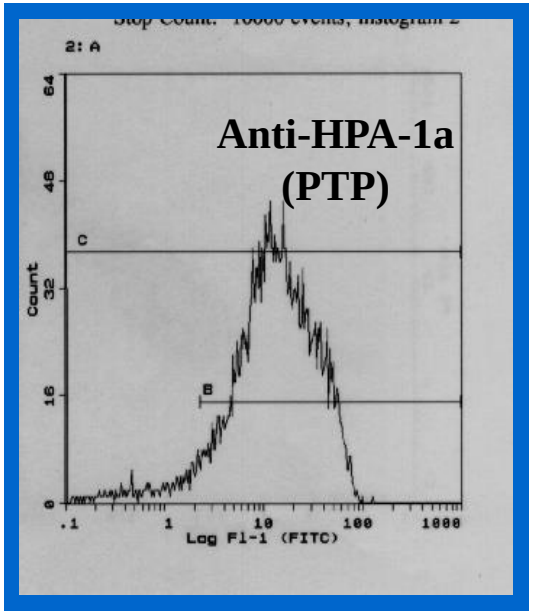


Hist #	Region	%	Count	Mean X	Mean Y	PK Pos X	PK Pos Y	PK Cnt	HPCV	FPCV
1	A autos	90.6	10000	27.3	7.33	21	6.6	133		
2	B % +ve	1.9	190	3.29		2.3		10	1.15	51.7
	C x Ch	100	10000	0.262		0.10		4344	0.000	106.2

PIFT Results - Flowcytometry (2)







	Negative	Weak Positive	Strong Positive
Mean Channel Fluorescence	0.262	1.81	10.4
% Positive Cells	1.9%	42.6%	90.3%

Advantages and disadvantages of indirect immunofluorescence tests

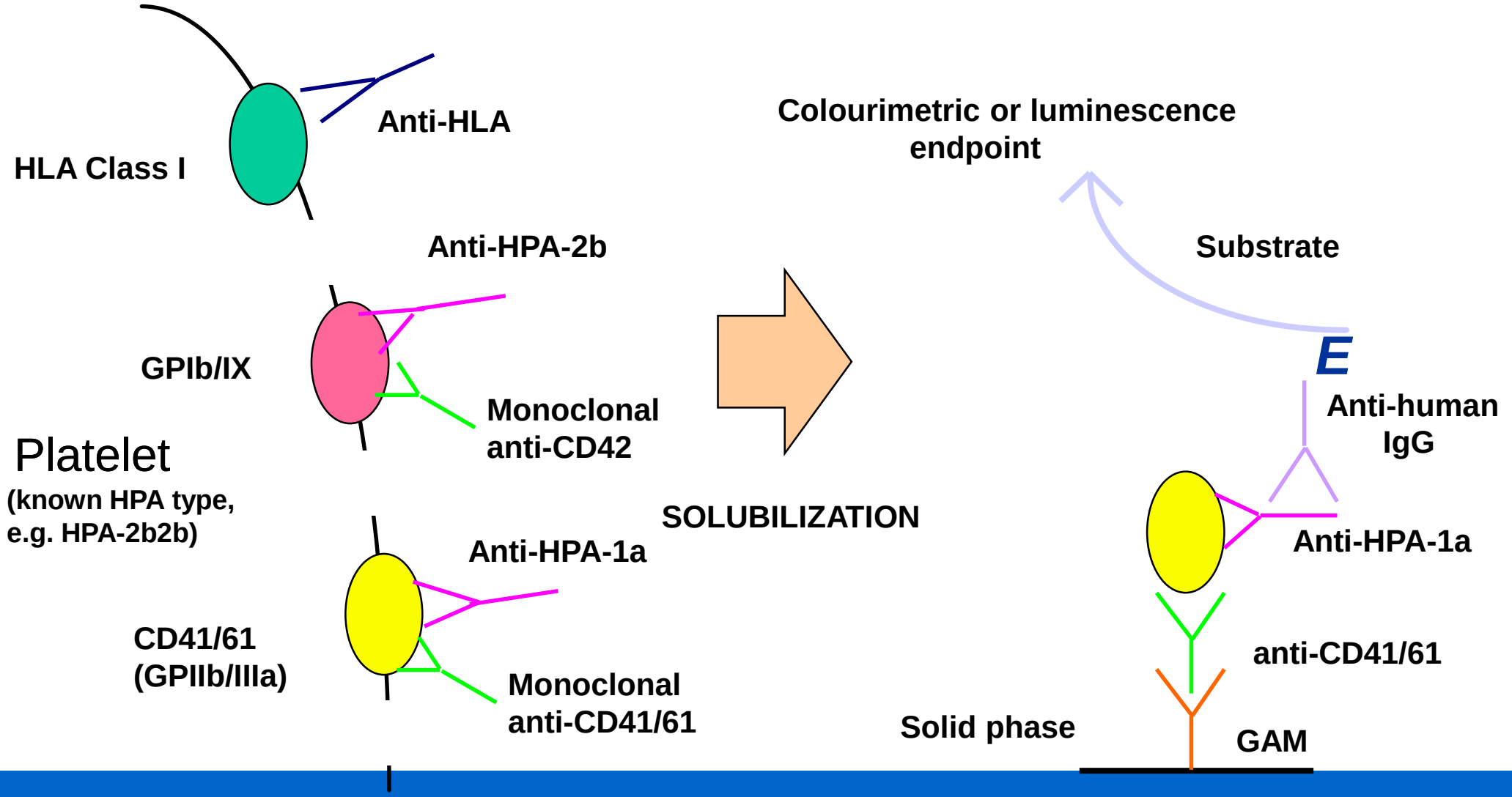
NHS Blood and Transplant

- *Advantages*
  - Sensitive, quick and cheap
  - Whole cell assays with potential to detect all antibodies to the membrane surface – important for some HPA, e.g. HPA-3a/b

- *Disadvantages*
  - May detect antibodies to HLA class I, ABH; IvIgG and immune complexes(?)

The monoclonal antibody immobilisation of platelet antigen (MAIPA) assay

NHS Blood and Transplant

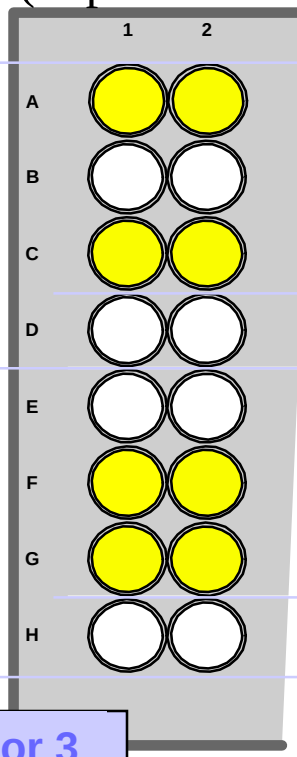


Results of MAIPA assay (patient serum contains anti-HPA-1a + HLA A2)

Blood and Transplant

(duplicate wells)

				Reaction patterns of:-
Mab anti GPIIb/IIIa	Tube 1	Donor 1	HPA-1a1a	**Anti-HPA-1a**
	Tube 2	Donor 2	HPA-1b1b	
	Tube 3	Donor 3	HPA-1a1a	
	Tube 4	Donor 1		**AB Serum**
Mab anti HLA	Tube 5	Donor 1	HLA-A1, A3	**Anti-HLA-A2**
	Tube 6	Donor 2	HLA-A1, A2	
	Tube 7	Donor 3	HLA-A2, A3	
	Tube 8	Donor 1		**AB Serum**



	Donor 1	Donor 2	Donor 3
	HPA-1a1a, HLA-A1,A3	HPA-1b1b HLA-A1,A2	HPA-1a1a HLA-A2,A3
PIFT results	+++	+++	+++

The advantages and disadvantages of the MAIPA assay

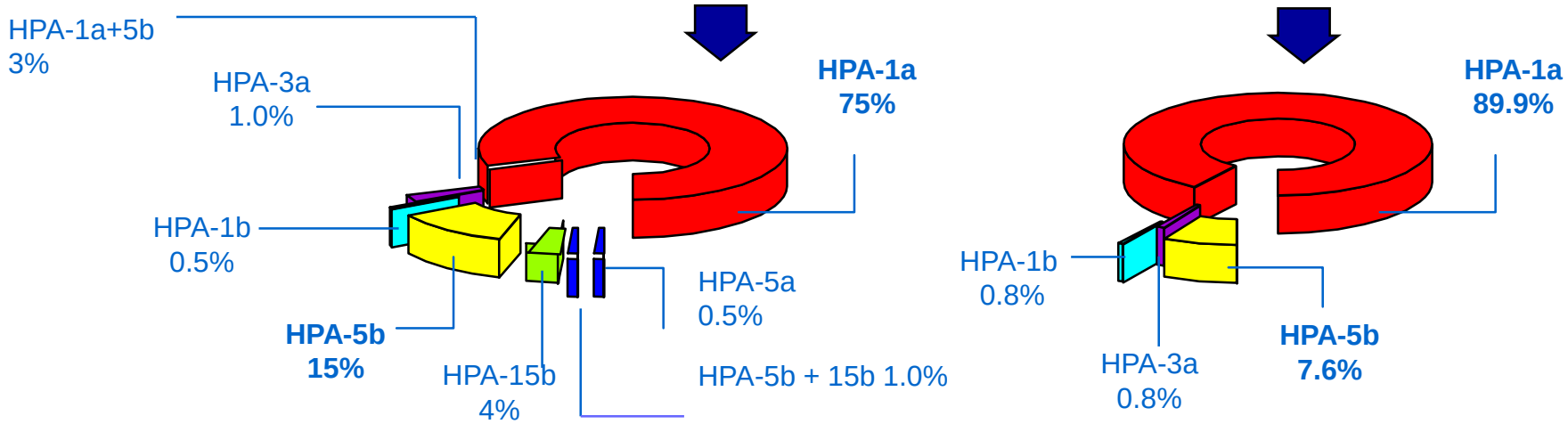
NHS Blood and Transplant

- *Advantages*
  - Specific and sensitive
  - Able to identify individual antibody specificities in complex antibody mixtures, differentiation from HLA class I antibodies
- *Disadvantages*
  - Need to know glycoprotein target antigen
  - Choice of monoclonal antibody can be critical
  - Solubilisation may modify the conformation of the native antigen e.g. HPA-3

Incidence of HPA antibodies in NAIT

NHS Blood and Transplant

	Ghevaert et al, 2007		Mueller-Eckhardt et al, 1989	
	Number	%	Number	%
Referrals	1148		348	
Patients with platelet-specific antibody	200	17.42	119	34.2



NHS Blood and Transplant

Do we miss antibodies?

Yes

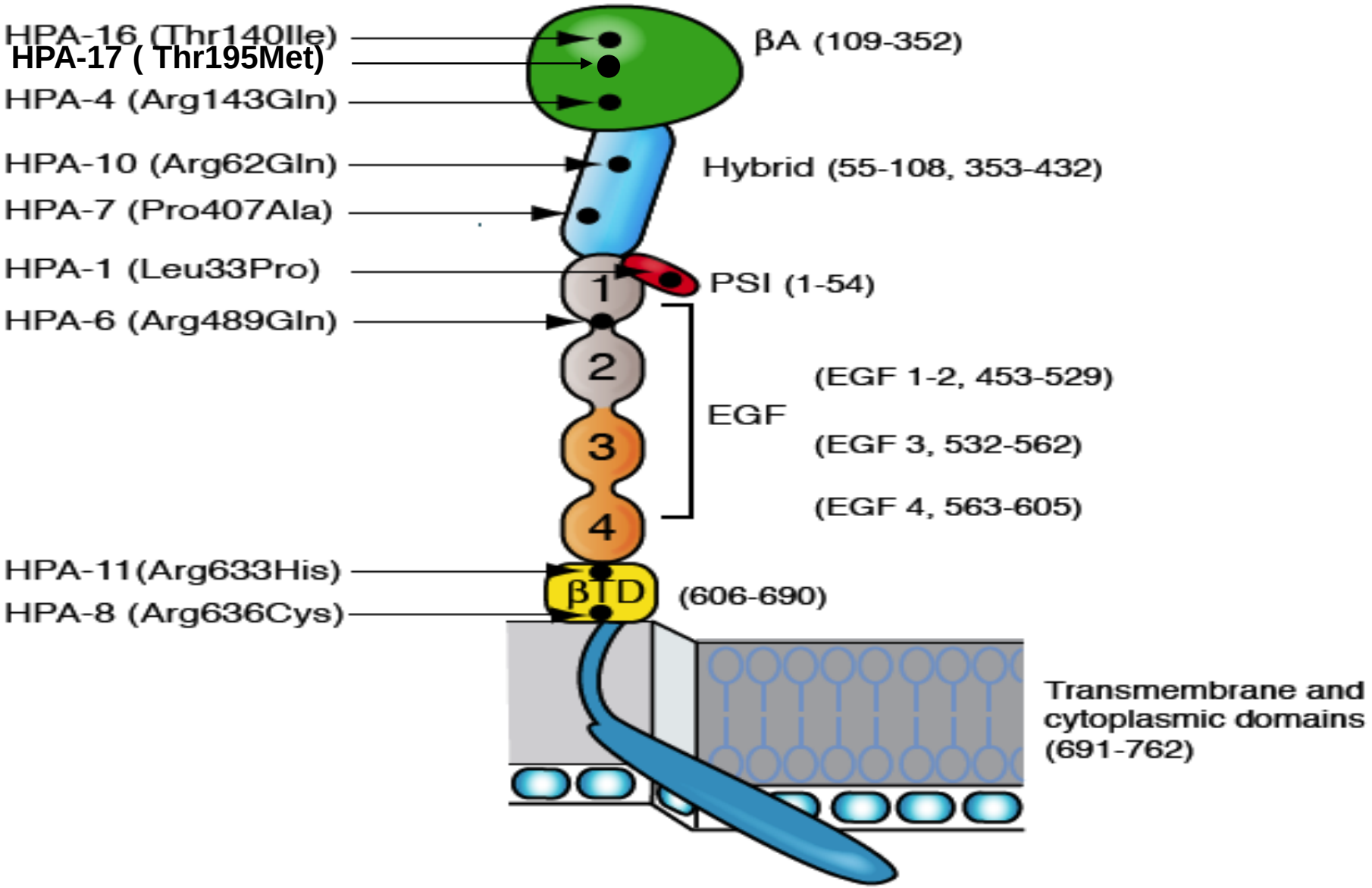
- The proportion of HPA-1b1b mothers in serologically negative NAIT cases is greater than expected

Why?

- Low affinity antibodies
- Isoforms of GPIIb/IIIa
- HPA-1a antibodies are polymorphic

HPA groups on the β3 integrin and domain localisation

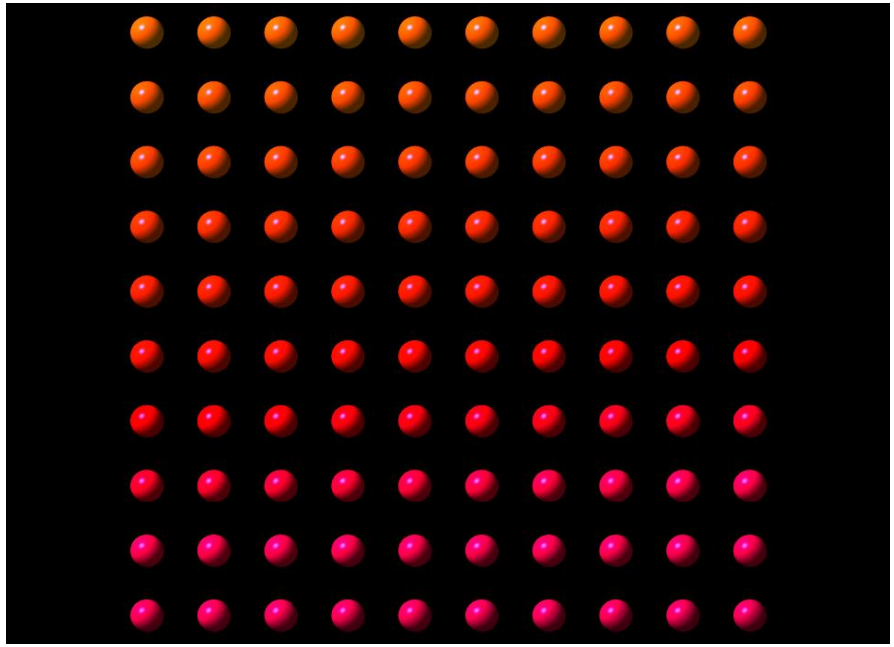
NHS Blood and Transplant

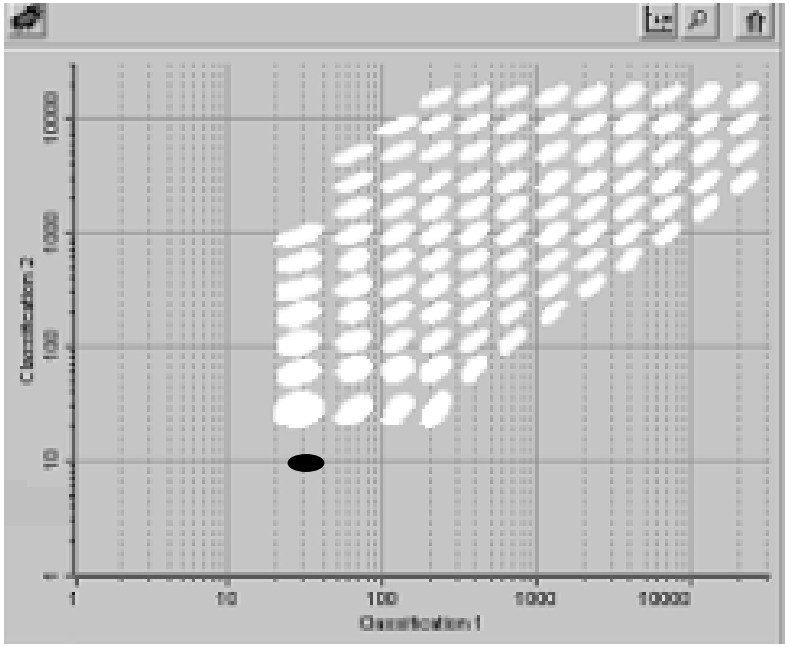


NHS Blood and Transplant

HPA antibody testing using fluorescent microbeads (Luminex technology)

Beads are of a uniform size: 5.6 µ diameter in 100 different colour intensities.





Data download from platelet antigens coupled to microbeads (PakLx)

NHS Blood and Transplant

SAMPLE ID:	9030/15
Minimum Cutoff (MC). If the MFI of the Con beads is < MC, the Adjusted Ratios are calculated using MC.	87

Antibody Target	GPIV	HLA	GPIIbIIIa (HPA-1,-3,-4)	GPIbIX (HPA-2)	GPIaIIa (HPA-5)
Result	Neg	Neg	Reactive	Neg	Neg

Bead Region	Glycoprotein Group	Antigen	MFI	Bead Reactivity	Adjusted Ratio 1	Adjusted Ratio 2	Adjusted Ratio 3
22	Con1	Con1	89				
26	Con2	Con2	90				
27	Con3	Con3	178				
11	POS	POS	17615				
29	GPIV	GPIV	176	Negative	-1.93	-2.52	-2.63
28	HLA Class I	HLA Class I	459	Negative	0.36	-0.22	-1.89
13	GPIIb-IIIa	HPA - 1a-3a-4a	19495	Positive	211.35	207.35	102.25
17	GPIIb-IIIa	HPA - 1a-3b-4a	18550	Positive	201.65	197.7	98.03
34	GPIIb-IIIa	HPA - 1b-3a-4a	503	Negative	-1.29	-3.07	-3.44
46	GPIIb-IIIa	HPA - 1b-3b-4a	387	Negative	-1.97	-3.82	-3.73
48	GPIIb-IIIa	HPA - 1ab-3ab-4a	17111	Positive	185.37	182.08	89.85
32	GPIIb-IIIa	HPA - 1a-3ab-4b	18676	Positive	201.87	198.33	97.75
33	GPIb/IX	HPA - 2a	190	Negative	-2.95	-4.01	-3.28

Commercial bead based assay for the detection of HPA antibodies

NHS Blood and Transplant

Detects antibodies against HPA-1, -2, -3, -4, -5, GPIV, HLA class I

Advantages

- Test results available after 2 hours
- 10uL of serum required
- Simple assay – beads + serum, wash, add conjugate, wash, test for bead associated fluorescence
- Sensitive for HPA-1a antibodies

Disadvantages

- Expensive
- Dependent on commercial supplier
- Limited range of beads with antigen combinations
- Unable to detect antibodies to HPA-15
- Validated for HPA antibodies but not for GP specific antibodies (AITP, thrombasthenias)
- Relatively insensitive to HPA-3a and HPA-5b antibodies compared to MAIPA
- Currently, cannot perform crossmatch or test for low frequency HPA

Porcelijn L *et al*. 54; 1486-92 (2014); Cooper N *et al*, Transfusion 56; 115-18 (2016)

Laboratory investigation in heparin induced thrombocytopenia (HIT)

NHS Blood and Transplant

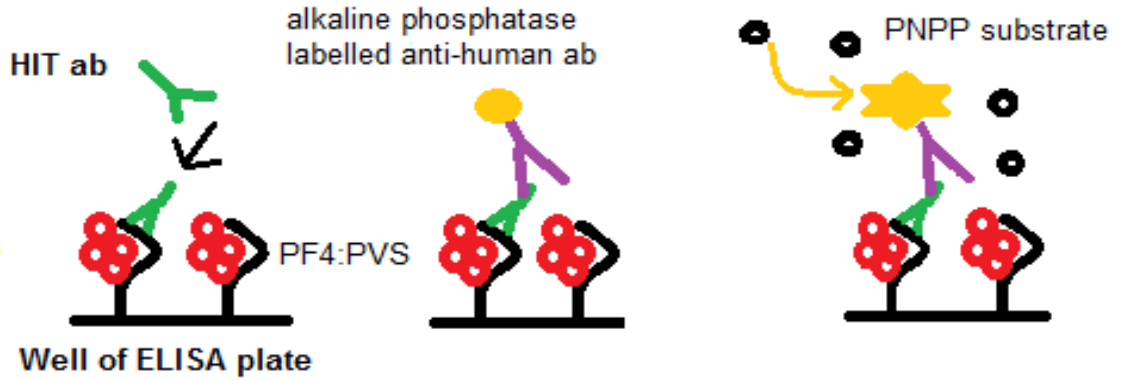
- Functional assays:
  - exploit the ability of HIT antibodies to activate platelets
  - the use of washed normal donor platelets provides high sensitivity in patients with clinical HIT
  - platelet aggregation can be used as an indicator of platelet activation
  - Should only be performed by specialist laboratories
- Immunochemical assays:
  - enzyme-linked immunosorbent assays (ELISA) for HIT antibodies, using the heparin-PF4 target antigen
  - ID-heparin/PF4 antibody test (gel card technology):
    - allows rapid detection, ? Sensitivity/reproducibility
  - ideally immunochemical tests should be followed up with a functional assay

Laboratory diagnosis of HIT using ELISAs

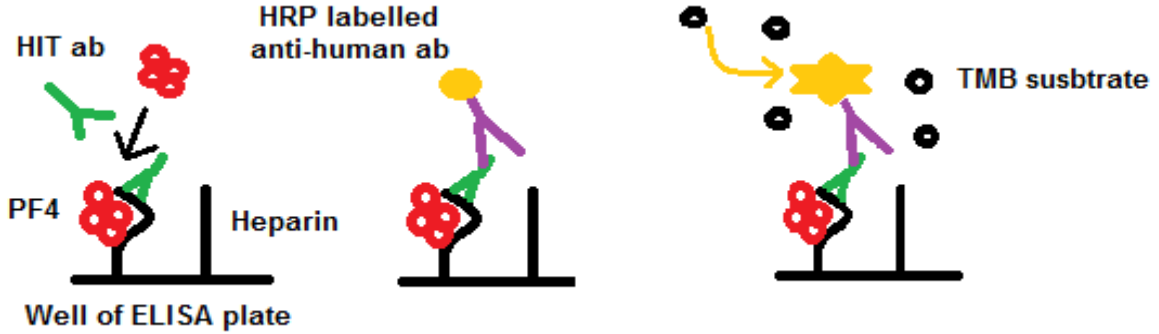
NHS Blood and Transplant

PF4 bound to heparin analogue

(Immucor kit)



PF4 added to bound heparin
(addition of platelet lysate as source of PF4, IL8, NAP2)
(Zymutest HIA)



How can we determine the clinical significance of HIT antibodies?

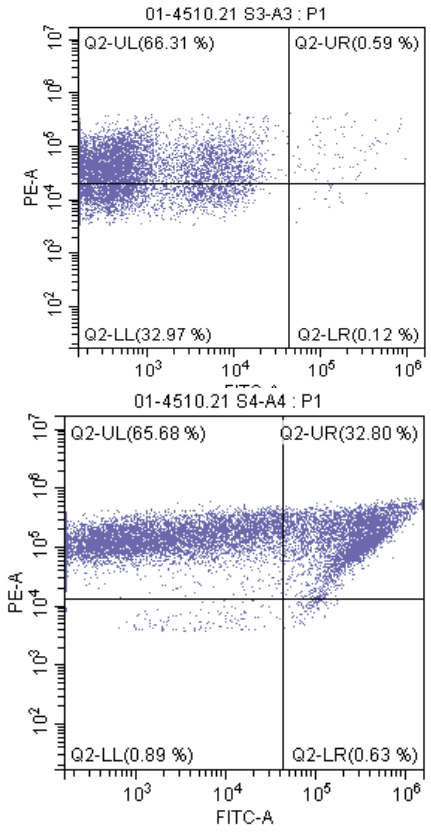
NHS Blood and Transplant

- Use an IgG conjugate rather than an IgGAM conjugate. IgG antibodies are generally regarded as being more pathogenic, although there are some documented exceptions.
- Addition of an excess of heparin inhibits antibody binding

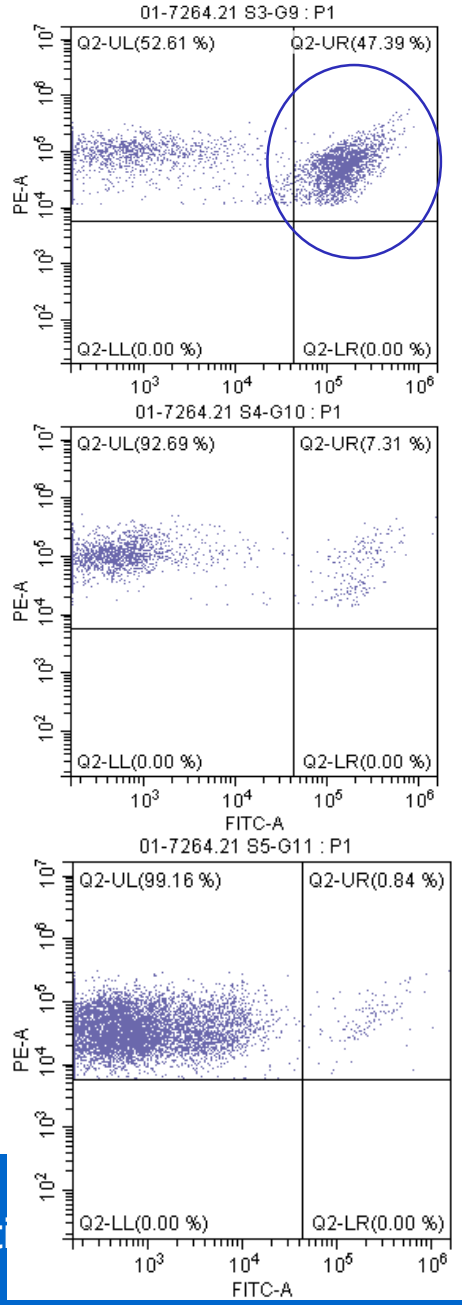
Results from a functional assay (Annexin V)	Heparin concentration (iu/mL)				
	0	0.1	0.3	0.6	100
Negative control	1.1	1.2	1.3	0.9	0.8
Positive control	0.6	21.5	50.9	47.2	0.8

- In ELISAs, OD>1.00 is associated with higher risk of HIT (BSH guideline - BJH, (2012), 159, 528-540

HIT Patient 1



VITT Patient 2



NHS Blood and Transplant

PATIENT SAMPLE + PLTS + buffer

3

PATIENT SAMPLE + PLTS + buffer + Heparin

4

PATIENT SAMPLE + PLTS + buffer + XS Heparin

5

Histoc ogenet

References

Ouwehand *et al*. Platelet immunology, present and future. *ISBT Science Series* 1, 96-102 (2006).

Allen D *et al*. 'Platelet and neutrophil antigens' *in* Practical Transfusion Medicine 5, 44-59 (2013).

Socher et al. Low avidity HPA-1a alloantibodies in severe neonatal alloimmune thrombocytopenia are detectable with surface plasmon resonance technology. Transfusion 49, 943-52 (2009)

Lucas G 'Human platelet antigens (HPA) and human neutrophil antigens (HNA) and their clinical significance' in *'Transfusion and Transplantation Science'.* Chapter 9, p187- 216 (2013)

Watson H, Davidson S, Keeling D. Guidelines on the diagnosis and management of heparin-induced thrombocytopenia: second edition. *British Journal of Haematology* 159, 528-540 (2012)

Porcelijn L *et al*. A new bead-based human platelet antigen antibodies detection assay versus the monoclonal antibody immobilization of platelet antigens assay. *Transfusion*, 54, 1486-1492 (2014)

Cooper N *et al*. A bead-based assay in the work-up of suspected platelet alloimmunisation. Transfusion, 56, 115-118 (2016)

Case study 1

– case referred to NHSBT consultant out of normal hours

- Presentation
  - 23 year old pregnant female
  - Previous miscarriage at 15 weeks, no live births
  - Maternal platelets 208 x 10^9/L (20/08)
  - IUGR at 20 weeks
  - Ultrasound at 36+3 showed ventriculomegaly with midline shift
  - Baby born at 37/40 (21/08), not breathing, meconium stained liquor, acidotic and covered in bruises, weight 3Kg, platelets were 12 x10^9/L and APPT was >100s
  - TORCHHS and CRP awaited.
  - Antibiotics started.
  - Ultrasound consistent with ICH

Case 1 - continued

Initial thoughts were possible IUGR detected at 20 weeks with bleeding detected post delivery.

1) Sepsis > acidosis > fetal distress > thrombocytopenia

OR

2) Thrombocytopenia > fetal distress > respiratory arrest > meconium stained liquor > acidosis

Advice given:

- Because of ICH, random stock platelets given immediately
- Order HPA-1a(-)5b(-) platelets
- Check platelet increments at 1 and 12 hours
- Keep platelets >100 x 10^9/L with HPA-1a(-)5b(-) platelets. Send samples for lab investigation for platelet antibodies

Case 1 - continued

NHS Blood and Transplant

Outcome and follow up (21/08)

- FFP and 20mL/Kg HPA-1a(-)5b(-) platelets transfused prior to transfer to regional centre.
- Platelets 136 x 10^9/L falling to 97 by night time
- *On 22/08*, platelets were 70 (AM) and 104 (PM)
- *On 23/08*, platelets were 138 (AM)
- No further platelets given but IvIgG (1g/Kg was infused).
- Baby having surgery for a stent
- Baby was in fact small for dates with weight being explained by large head size.

Case 1 - continued

NHS Blood and Transplant

- Laboratory results - HPA typing
  - Mother: HPA-1b1b,2a2a,3a3a,4a4a,5a5a,6a6a,9a9a,15a15b
  - Father: HPA-1a1a,2a2a,3a3b,4a4a,5a5b,6a6a,9a9a,15a15b
  - Infant: HPA-1a1b,2a2a,3a3b,4a4a,5a5a,6a6a,9a9a,15a15b
  - Comments?

Case 1 - continued

NHS Blood and Transplant

- Laboratory results – PIFT (IgG) results
  - Cell 1: 1a1a,2a2a,3a3a,5b5b – positive (MCF= 90.4)
  - Cell 2: 1b1b,2a2a,3a3b,5a5a – negative (MCF = within normal range)
  - Cell 3: 1a1b,2b2b,3a3b,5a5a – positive (MCF= 59.6)

  - Comments?

Case 2 - continued

NHS Blood and Transplant

- Laboratory results – MAIPA assay results
  - *GPIIb/IIIa*
  - Cell 1: 1a1a,3a3a – positive (OD = 3.277)
  - Cell 2: 1a1a,3b3b – positive (OD =3.225)
  - Cell 3: 1b1b,3a3a – negative (OD <0.15)
  - Cell 4: 1b1b,3b3b – negative (OD <0.15)

  - Negative cut off OD < 0.15

  - *GPIa/IIa* – negative for HPA-5a5a & 5b5b platelets
  - *GPIb/IX/V* – negative for HPA-2a2a & 2b2b platelets
  - *HLA class I* – negative

  - Comments?

Additional thoughts?

- Father homozygous for HPA-1a [heterozygous for HPA-5]

  -100% risk of next child being HPA-1a positive
  What about HPA-3b and -5b?

- antibody card and leaflet issued

- *Additional questions*
- When would you consider requesting a platelet crossmatch?
- What else would need to be considered in laboratory crossmatch results?
- When would you use HPA-1a(-)5b(-) platelets?
- What criteria are in place for donors of HPA-1a(-)5b(-) platelets for transfusions to fetuses and neonates?