NHS Blood and Transplant

Measurement of anti-D in Pregnancy

Use of Column Agglutination Titration Scores and Flow Cytometry

Joint Meeting of UK NEQAS (BTLP) and the BBTS Blood Bank Technology SIG
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Royal College of General Practitioners, London

Fran Green and David Bruce: RCI NHS Blood and Transplant

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Introduction

- Anti-D can cause severe HDFN
- In the UK the concentration of the maternal anti-D is determined by quantification using a continuous flow analyser (CFA)
- The antibody is monitored throughout pregnancy to identify fetuses/infants at risk from HDFN

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BCSH guidelines (2007) suggest the following levels of anti-D should be used to guide management of pregnancies:

Low Risk: < 4 IU/mL: HDFN unlikely continue to monitor.

Moderate Risk: 4 – 15 IU/mL: Refer to specialist unit.

High Risk: >15 IU/mL: Refer to specialist unit.

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April 2014 RCI project team began testing maternal anti-D samples with an aim to:

"To determine if flow cytometry and titre scores established by column agglutination technology (CAT) could provide data that is at least of equivalent quality to that produced by the CFA"

PROJECT GROUP

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Elinor Curnow:	Statistics and clinical audit
Fiona Regan:	Clinical Director
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Nay Winn:	Clinical Director
Wendy Etheridge:	RSM
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Rationale for the study:

- Continuous flow analysers are supplied and maintained by only one company within the UK
- The quantification service (provided by RCI) is solely dependant on the future sustainability of this one company
- We need a contingency in case the company terminates its supply and maintenance of the CFA

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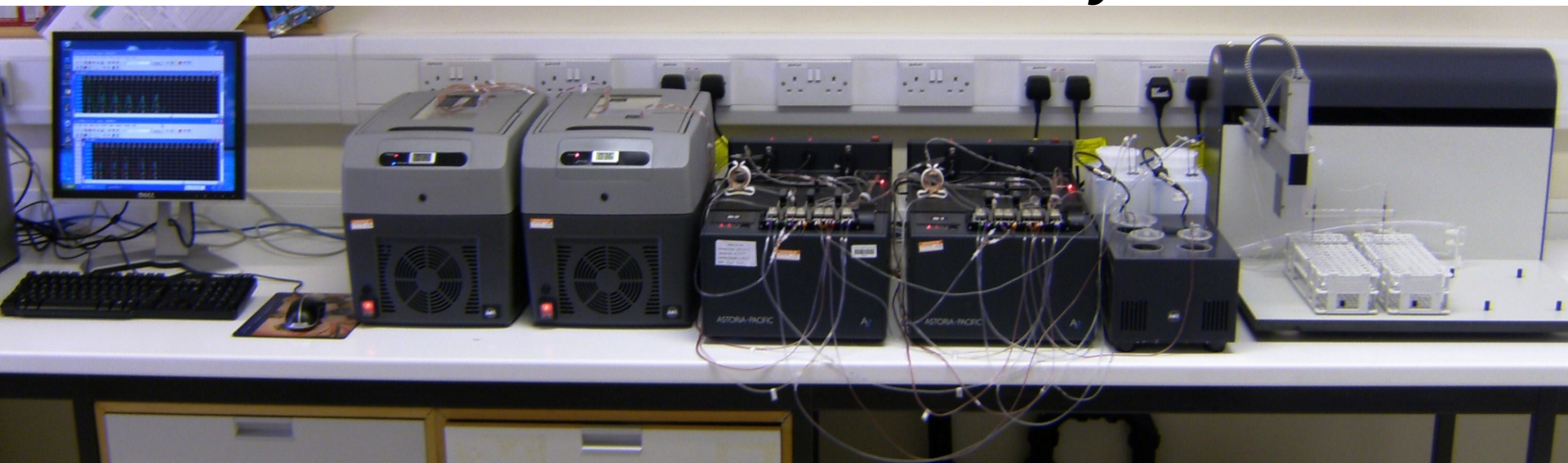
Rationale for the study:

- The current method in use in RCI is essentially the same as that described in 1968 by Marsh, Nicholls and Jenkins
- With advancements in blood transfusion science and serological testing it is time to reconsider alternative methods
- Advantageous to use methods which are in mainstream use for other laboratory purposes

The Astoria 2 AutoAnalyser



Study design

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1. Large prospective comparison of CFA with FC and CAT Titre Scores

2. Study to run for 12 months (April 2014 to April 2015)

3. Samples referred to RCI Filton and Newcastle for antibody quantification tested by all three methods

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Column Agglutination Titration Scores for the measurement of anti-D in pregnancy

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CFA was adopted in the UK in the 1970s because of its superiority to manual antibody titration by tube technique

CFA	Titration
– Process large numbers – Minimal cost – Accurate – Reproducible	– Poor reproducibility – Inherent subjectivity of the titre endpoint – Misleading without additional evaluation of the strength of reaction

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Disadvantages of using the AutoAnalyser

1. Intra-laboratory reproducibility CV ~10%

2. Inter-laboratory reproducibility CV ~20% (Fleetwood and McNeill 1990)

3. Difficult to standardise between laboratories with a multitude of variables

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With improvements in serological testing it is now reasonable to reconsider titration

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Improvements in serology methods

- Column Agglutination Technology
- Automated reading equipment
- Automated pipettes
- Standardised reagents
- (Titre scores)

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Determination of a titre score

- Doubling dilutions of plasma are prepared
- The reaction grade of each dilution is converted to a score
- The score of each dilution is summed to give the titration score

Grade	Score
4	12
3	10
2	8
1	5
+/-	3
0	0

Adapted from AABB technical manual 14th Edition

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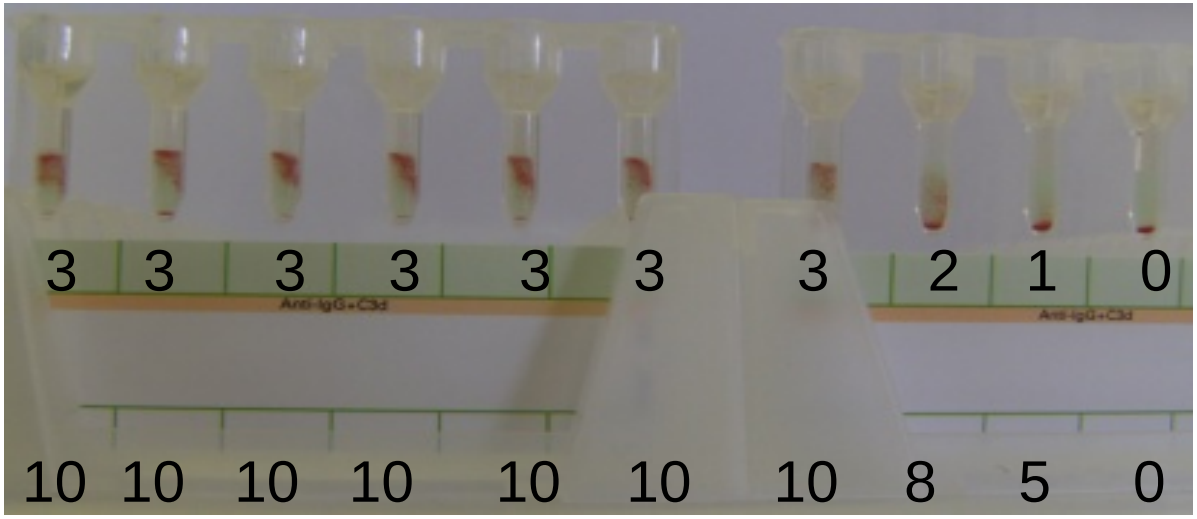
Example of a Titre Score

Dilution:	Neat	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Grade	3	3	3	3	3	3	3	2	1	0
Score	10	10	10	10	10	10	10	8	5	0



TOTAL TITRE SCORE: 83

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Sample		Plasma Dilution										Titre	Titre Score
		1	2	4	8	16	32	64	128	256	512		
1	Strength	3	3	3	2	2	2	1	1	+/-	0	256	67
	Score	10	10	10	8	8	8	5	5	3	0		
2	Strength	4	4	4	3	3	2	2	1	0	0	128	77
	Score	12	12	12	10	10	8	8	5	0	0		
3	Strength	2	2	1	1	1	1	1	1	0	0	128	46
	Score	8	8	5	5	5	5	5	5	0	0		

Method

- **Antenatal samples with anti-D were quantified against NIBSC standards on a CFA (Astoria 2 Flow Analyser).**
- **Serial dilutions of these samples were titrated using Bio-Rad IAT cards**
  - The reaction strength was determined using a Bio-Rad Banjo ID Reader and Maestro Master Software with the result expressed as a titre score.

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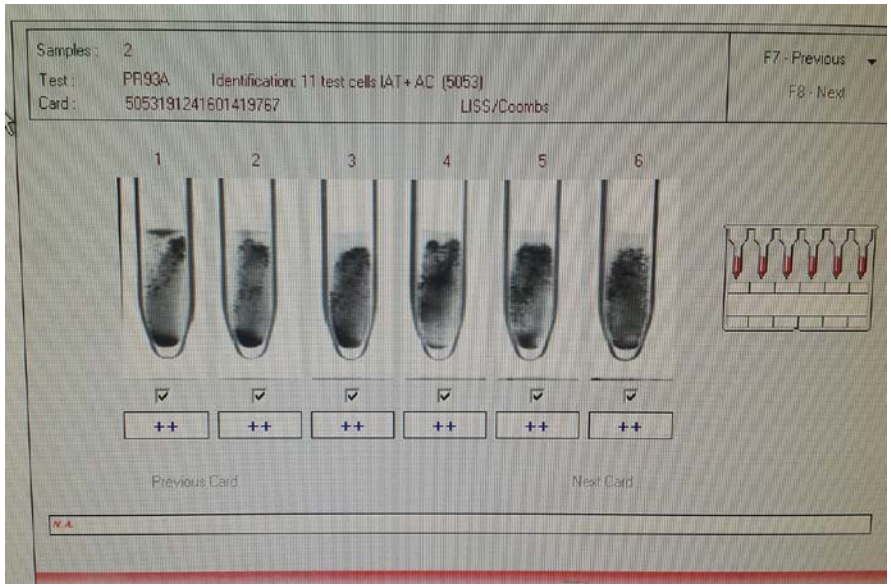
Bio-Rad Banjo ID Reader and Maestro Master Software



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Preliminary Data Analysis

Data from 108 anti-D samples tested between April 2014 and July 2014 was used:

- **to determine the titre score which best marks the threshold of clinical significance (i.e. a CFA results of 4IU/mL)**
- **to determine the titre score which best marks the threshold for a high risk of HDFN (i.e. a CFA results of >15IU/mL)**

Anti-D antibody CFA quantification result versus titre score, by risk group

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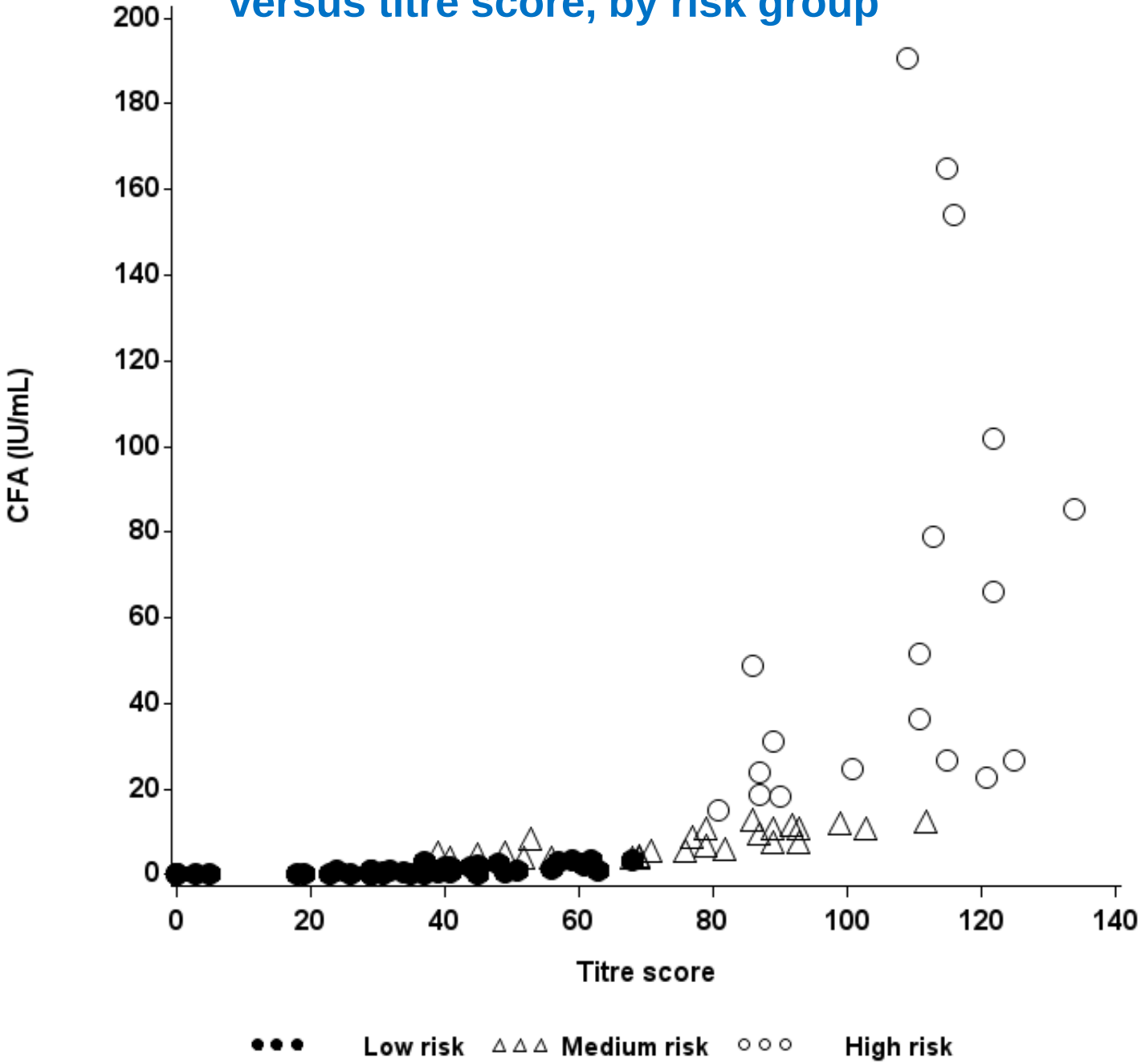

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Analysis suggests that a medium/high boundary of 80 or 85 and a low/medium boundary of 60 or 65 best describe the relationship between CFA and TS

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Results obtained up to the end of October 2014

n=126	Quant <4	Quant >=4
TS < 60	58	7
TS >=60	4	57

A titre score of less than 60 identified 58/62 samples with anti-D quantification <4IU/mL BUT

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But must consider the following:

- Nicolaides, K.H.& Rodeck, C.H. (1992) Maternal serum anti-D antibody concentration and assessment of rhesus isoimmunisation. *BMJ*, 304, 1155 – 1156
  - "In all pregnancies (n=49) with a maternal anti-D concentration ≤ 15 IU/mL the fetuses were at most mildly anaemic."

- The cut off chosen for all pregnancies deemed not at risk of HDN (4 IU/mL or equivalent parameter) ensures that there is NO Hb deficit on delivery

And must also consider:

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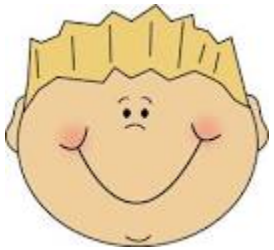
- For the 7 samples (out of a total of 65) with a TS < 60 and a CFA ≥ 4 IU/mL the values were as follows:

Sample	Patient	Titre Score	CFA IU/mL
1	a	39	5.2
2		49	5.4
3	b	41	4.0
4		45	5.0
5		58	5.0
6	c	49	5.4
7	d	56	4.0



- Interlaboratory reproducibility: CFA has a CV of about 20% (Fleetwood and McNeill 1990).

- Walsh CA, Doyle B, Quigley J, McAuliffe FM, Fitzgerald J, Mahony R, Higgins S, Carroll S, McParland P. (2014) Reassessing the critical maternal antibody threshold in Rh(D) alloimmunisation: a 16-year retrospective cohort study. *Ultrasound in Obstetrics and Gynaecology*. Apr 4. doi: 10.1002/uog.13383. [Epub ahead of print]

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Results obtained up to the end of October 2014 revisited

n=125	Quant <15	Quant >=15
TS < 80	85	1
TS >=80	12	27

A titre score of greater than 80 identified 27/28 samples with anti-D quantification >15IU/mL

Conclusion

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- CAT titre scores provide a simple method to monitor anti-D levels
- The method is sensitive to a wide range of anti-D concentrations as determined by the CFA
- The technique has the potential to replace the CFA by identifying those cases that require closer monitoring for risk of HDFN

Anti-D quantification by flow cytometry

AIM

- **The NHSBT Diagnostics strategy group identified a need to pursue an alternative methodology to Continuous flow analysis (CFA) for quantification of patient anti-D and anti-c levels**

Why?

- **Current method – old technology, not widely available**
- **Reliance on one company – lack of CE marking – maintenance difficulties**

- **The antibody levels should be reported in IU/mL and should be in the same range as those obtained by CFA so that clinical interpretation not affected by change in technology**

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Method Development

Method Development
Literature Review

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Austin, EB & McIntosh, Y. Anti-D quantification by flow cytometry: a comparison of five methods. Transfusion 2000;40:77-83

	1	2	3	4	5
Cell phenotype	R_1R_1	R_1R_1	R_1R_1	R_2R_2	R_2r
Cell diluent	LISS/0.5%BSA	LISS/0.5%BSA	LISS/0.5%BSA	PBS	PBS
Serum diluent	LISS/0.5%BSA	LISS/0.5%BSA	NISS/0.5%BSA	PBS	PBS/ 2%HSA
Volume of antisera	50µL	50µL	50µL	50µL	100µL
Volume of cells	50µL	50µL	50µL	50µL	10µL
Final cell concentration	0.5%	0.5%	0.5%	2.5%	9%
Serum:packed cell ratio	50:1	50:1	50:1	20:1	10:1
Cell-serum mixture incubation time, temperature	30 min, 37ºC	20 min, 37ºC	20 min, 37ºC	45 min, 37ºC	30 min, 37ºC
Wash reagent	PBS	LISS	LISS	PBS	PBS
Anti-human IgG dilution diluent	1/500 in LISS/BSA	1/500 in LISS/BSA	1/500 in LISS/BSA	1/40 in PBS	1/20 IN PBS/HSA
Cell-anti-human IgG mixture incubation time, temperature	30 min, 4ºC	30 min, 4ºC	30 min, 4ºC	30 min, 22ºC	15 min, 22ºC
Wash reagent	PBS	LISS	LISS	PBS	PBS
Final diluent	PBS	LISS	LISS	PBS	PBS/HSA
Standard range (IU/mL)	1.28-0.005	1.28-0.005	1.28-0.005	2.5-0.8	0.05-0.01

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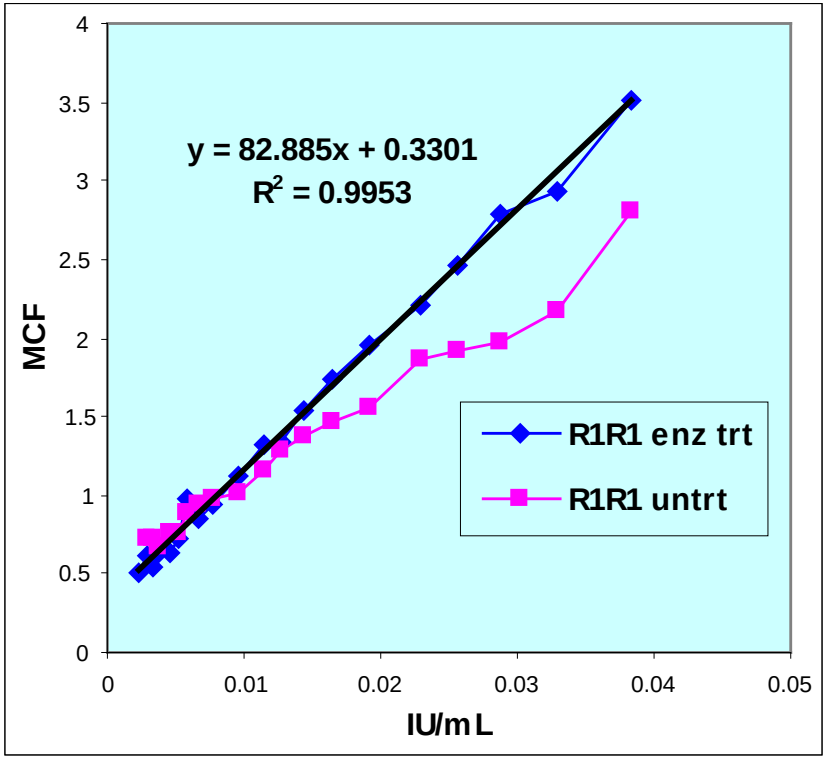
How does the chosen FC method differ from previously reported flow methods?

Simply by using enzyme-treated cells

Untreated vs Bromelain-treated cells

Pooled red cells
O R1R1 K-

Standard:
NIBSC: 73/515
(0.23 IU/mL)
Range:
0.0023-0.03833
IU/mL



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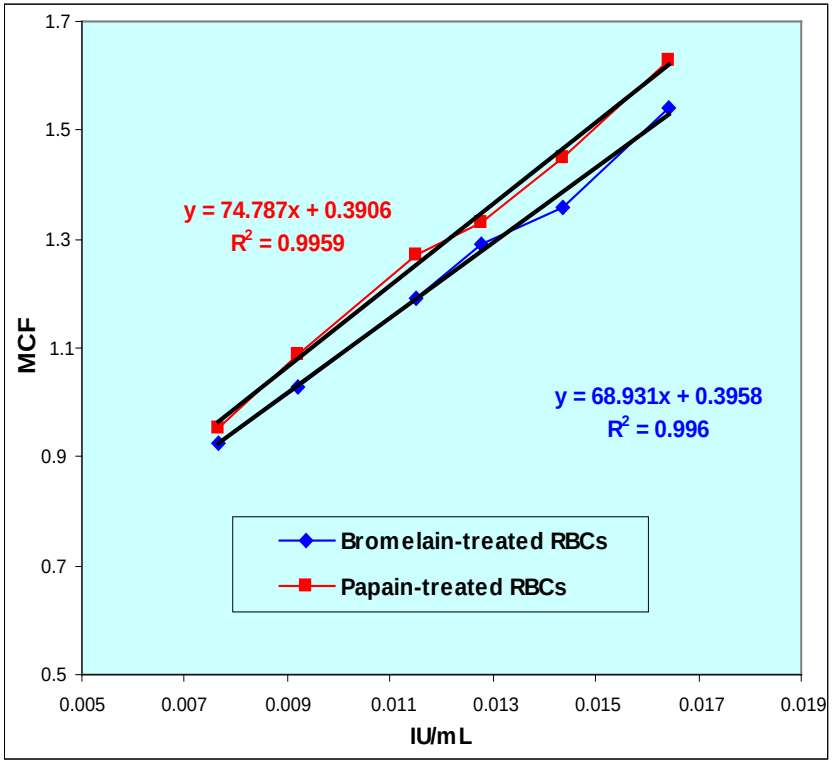
Bromelain vs Papain

Enzyme-treated pooled red cells
O R1R1 K-

Standard:
NIBSC: 73/515 (0.23 IU/mL)
Range: 0.007667-0.01643 IU/mL

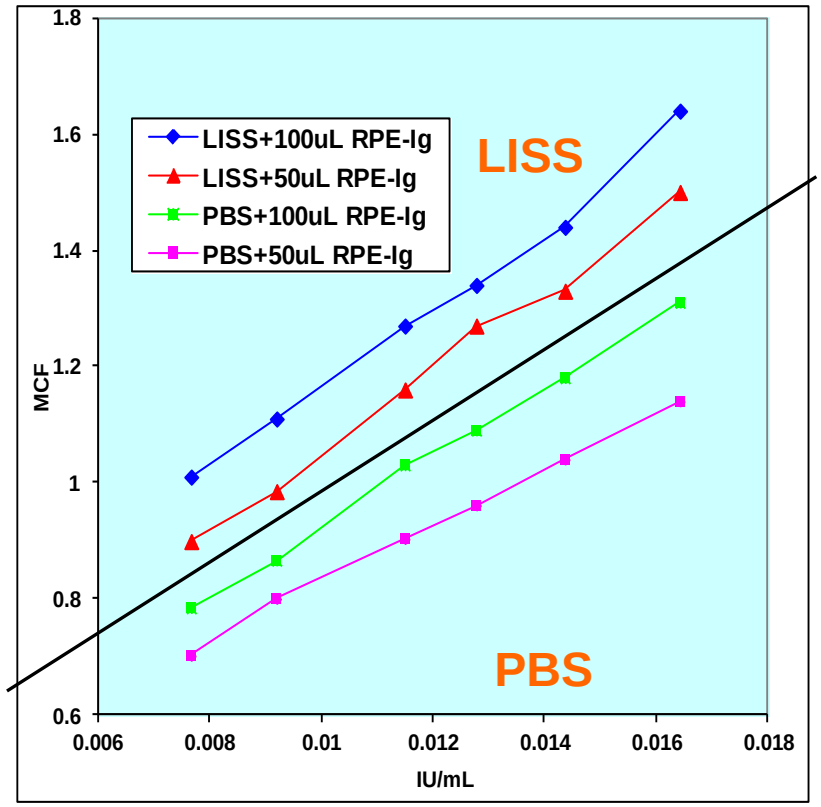
High and Low anti-D controls also analysed



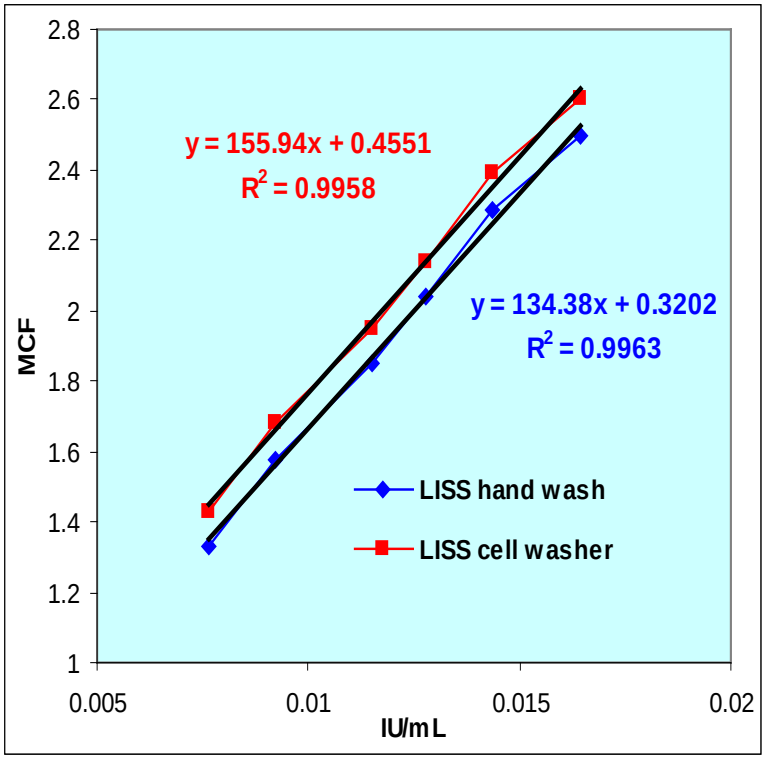
LISS vs PBS

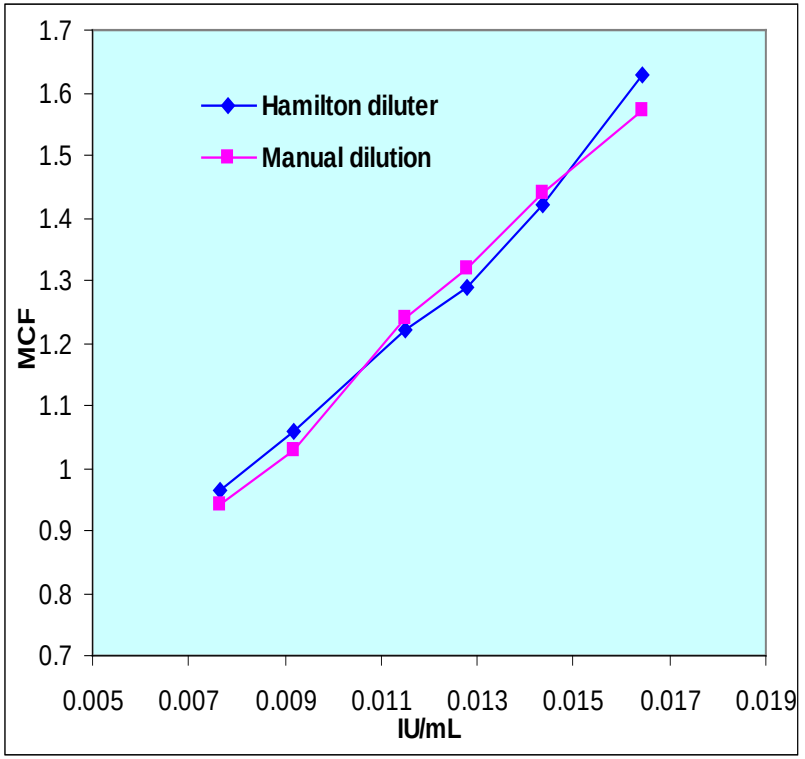
Bromelain-treated pooled red cells
O R1R1 K-

Standard:
NIBSC: 73/515 (0.23 IU/mL)
Range: 0.007667-0.01643 IU/mL



Cell washer and Hamilton Diluter





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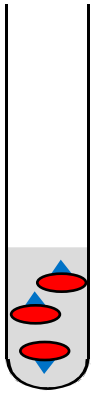
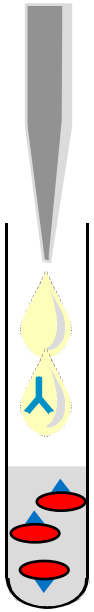
Definitive Method

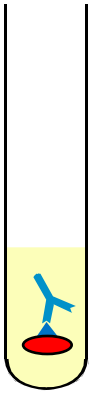
Enzyme treated OR1R1 or Orr 0.5% cell suspension in LISS/0.5% BSA



Add plasma diluted using Hamilton Diluter in LISS/0.5% BSA

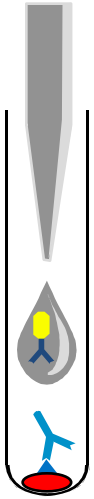


Mix thoroughly & incubate at 37°C for 30 min

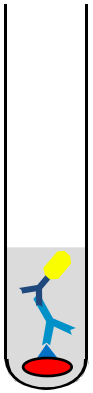


Wash x 2 using LISS in cell washer

RPE Conjugated F(ab')$_2$ anti-HuIgG, Fc$_\gamma$ in LISS/0.5% BSA



Mix thoroughly & incubate for 30 mins at 4°C



Wash x 1 with LISS using cell washer. Mix thoroughly, resuspend in LISS Read in flow cytometer

Method Development

- **147 samples from 103 patients for anti-D quantification**

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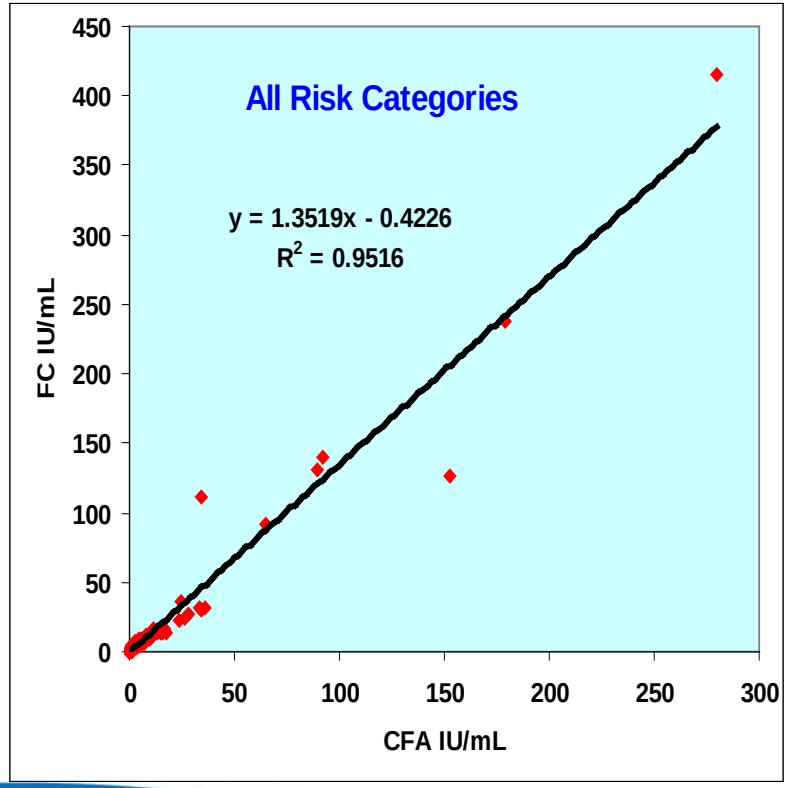
Anti-D Results



147 samples from 103 patients

Anti-D level	HDFN Risk Category
<4.0 IU/mL	Low
4.0-15.0 IU/mL	Moderate
>15.0 IU/mL	High

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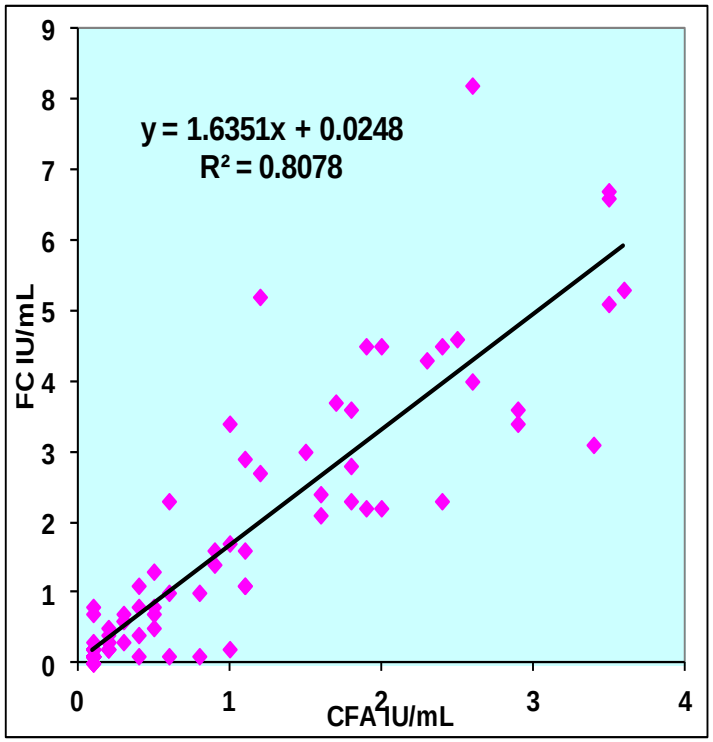
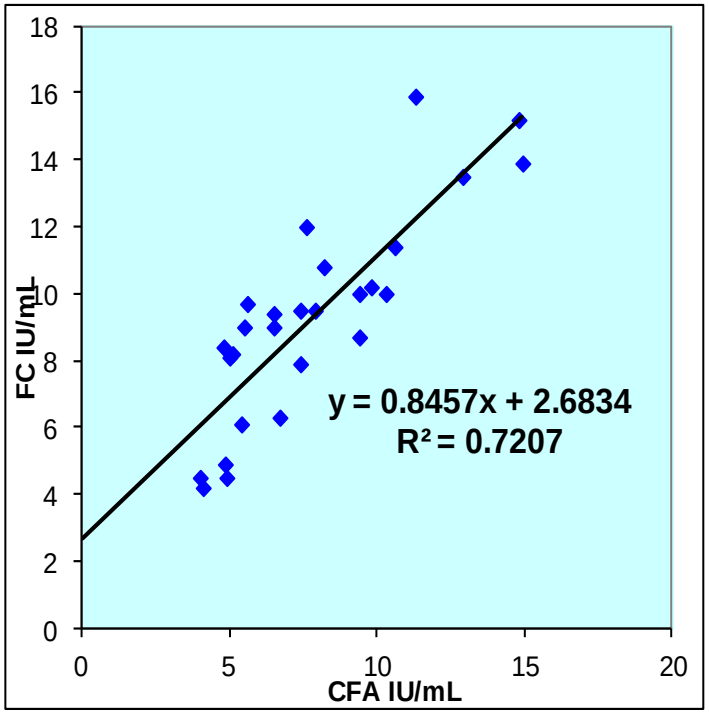
Low Risk



102 samples from 74 patients

Medium Risk



27 samples from 17 patients

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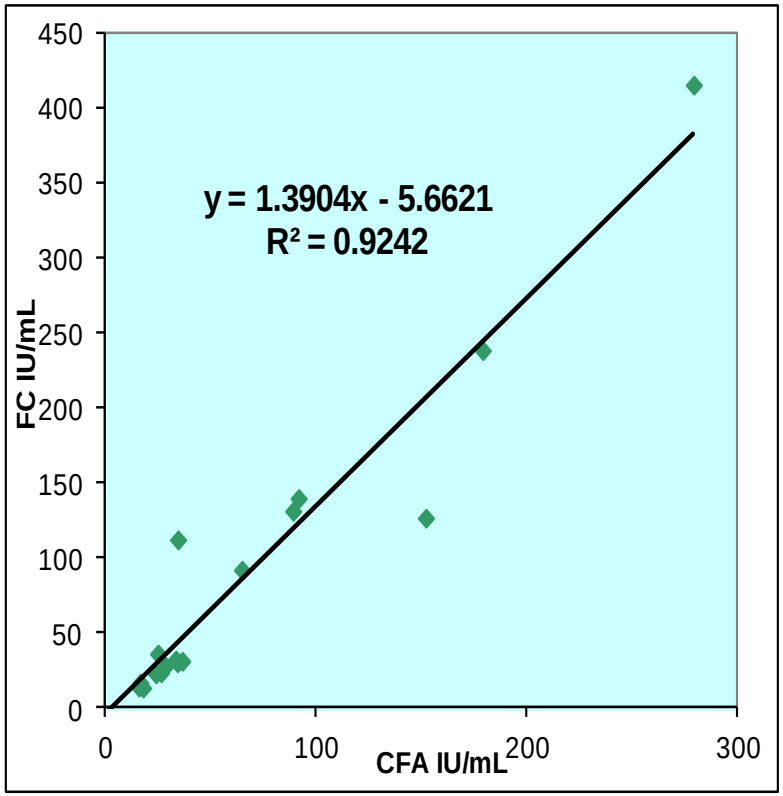
High Risk



17 samples from 14 patients

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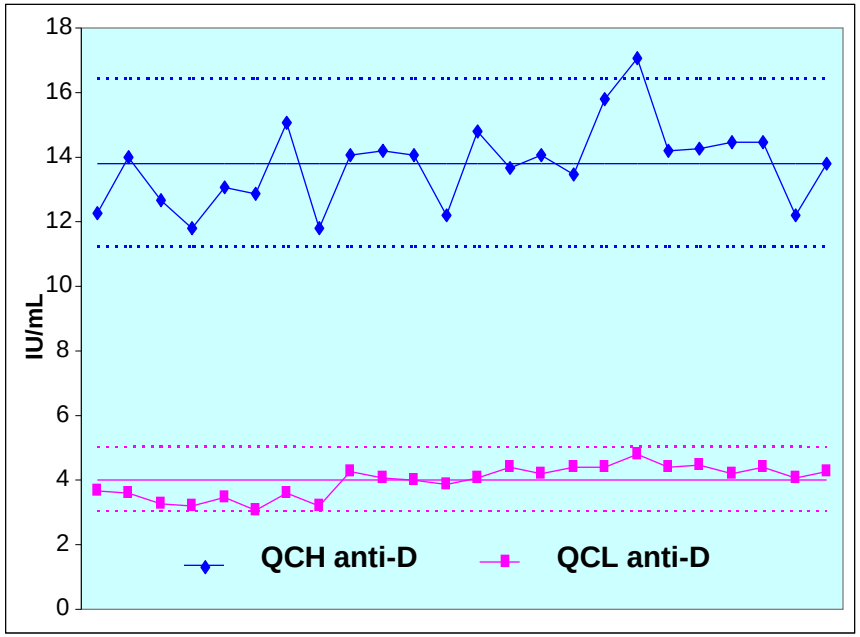
90.0 % agreement between the two technologies in allocating Risk Category

Anti-D level	HDFN Risk Category
<4.0 IU/mL	Low
4.0-15.0 IU/mL	Moderate
>15.0 IU/mL	High

		FC			
		Low	Mod	High	%
CFA	Low	90	12		69.4
	Mod		27	1	19.0
	High		2	15	11.6
	%	61.2	27.9	10.9	89.7

15 anomalous results from 10 patients

Control samples – Inter-assay Variability



Anti-D high control: QCH: N0521005
Anti-D low control: QCL: N0513006

COMMENTS

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- This data suggests that FC could be used as an alternative to CFA for anti-D quantification
  - as expected, some variation was observed between the two different technologies. In the moderate risk group for anti-D, the FC gave higher results than CFA, possibly due to better detection of low affinity antibodies.
  - When PBS is used instead of LISS, the IU/mL level for the anomalous samples is reduced to closer to that of CFA results.
- Pregnancy monitoring and outcome
  - The increased sensitivity of FC may detect rising anti-D levels earlier in the pregnancy
  - For the small number of pregnancy outcomes available for this data set, the FC result was more predictive of outcome

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New Project – Comparison of quantification, titre scores and flow cytometry for estimation of maternal anti-D and anti-c

First telecom – October 29th 2013

Purpose: The feasibility of running a joint collaborative project between Newcastle and Filton RCI

Project aim: To establish a viable alternative method to the current method (CFA) in order to determine the potential risk of HDN due to maternal anti-D and anti-c. This project would compare results from the CFA with FC and titres scores

Study design: To include input from and enlist support of fetal medicine consultants to obtain clinical outcome of the affected pregnancies

Titre Scores : a pilot study has been completed and published (Transfusion Medicine; 2013, **23**, 36-41).

First Stage - Pilot Study

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- 88 anti-D samples
- 41 anti-c samples

→ sent to Newcastle for titre scores

- Requires input from a statistician to determine study sample size

First Stage - Pilot Study

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Agreement of quantification results by CFA with FC and titre score (TS)

	FC <4IU/mL	FC >4IU/mL	TS <=70	TS >=70	TS <=60	TS >=60
CFA quantification <4IU/mL	44	12	53	2	48	7
CFA quantification >4IU/mL	0	31	11	18	4	26
FC <4IU/mL			45	0	39	5
FC >4IU/mL			22	21	14	28

First Stage - Pilot Study

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If we use FC

- **Compared to both CFA and TS there would be considerably more pregnancies referred to an obstetric unit as potentially "at risk of HDN"**

If we use TS (< 70 "no risk of HDN" and > "70 risk of HDN")

- **Compared to both CFA and FC there would be significantly fewer pregnancies classified as "at risk of HDN" and therefore potentially under referral. It should be noted that the majority of these would be for pregnancies where the CFA result is between 4 to 6 IU/mL so the clinical impact, it could be argued, would be negligible.**

⟶ **Further analysis to determine TS boundaries**

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Preliminary Data Analysis for FC

Data from 108 anti-D samples tested between April 2014 and July 2014

- **Assignment of risk category**
- **Comparison between CFA and FC**

Anti-D antibody CFA quantification result versus FC result, by risk group

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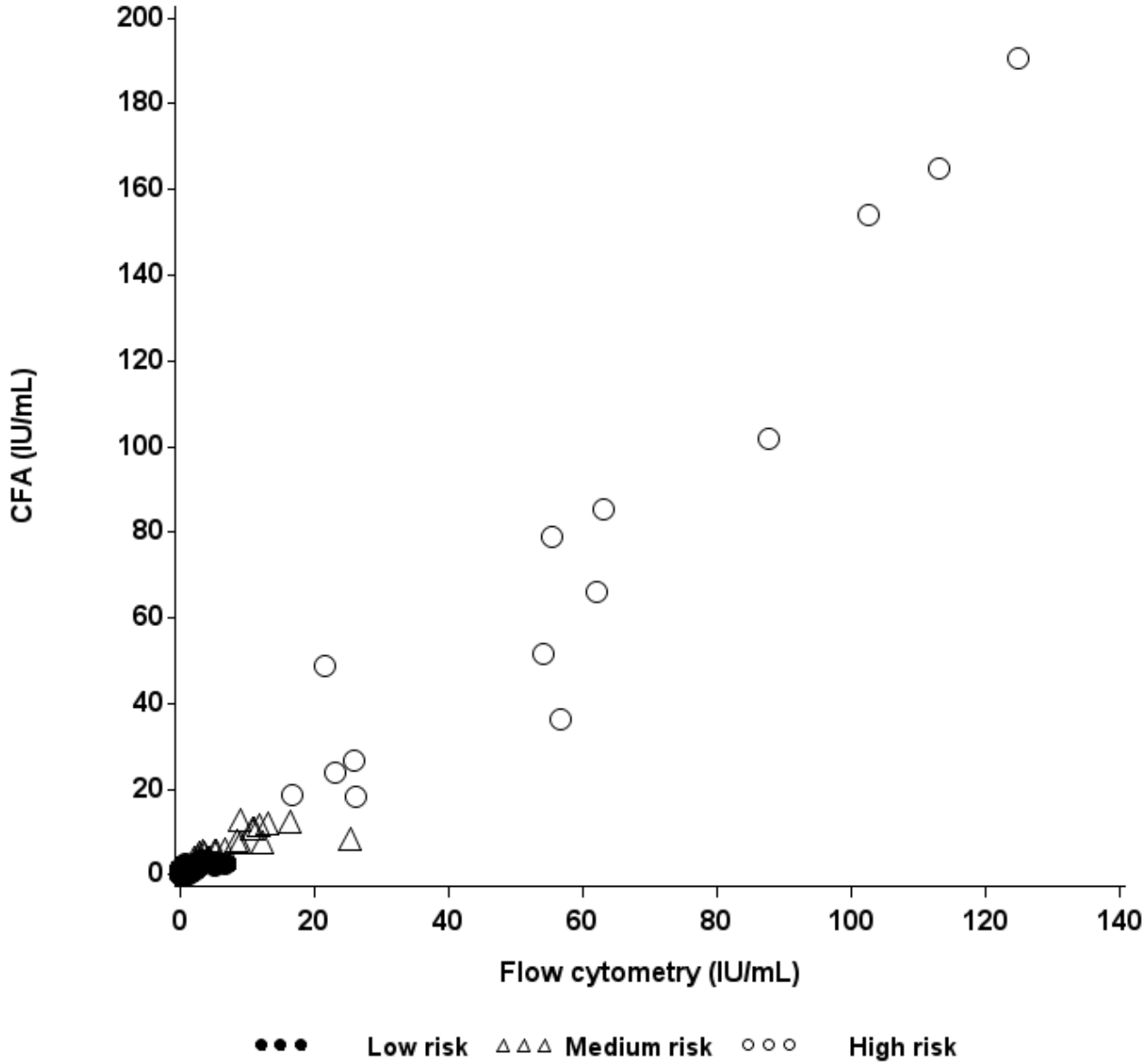
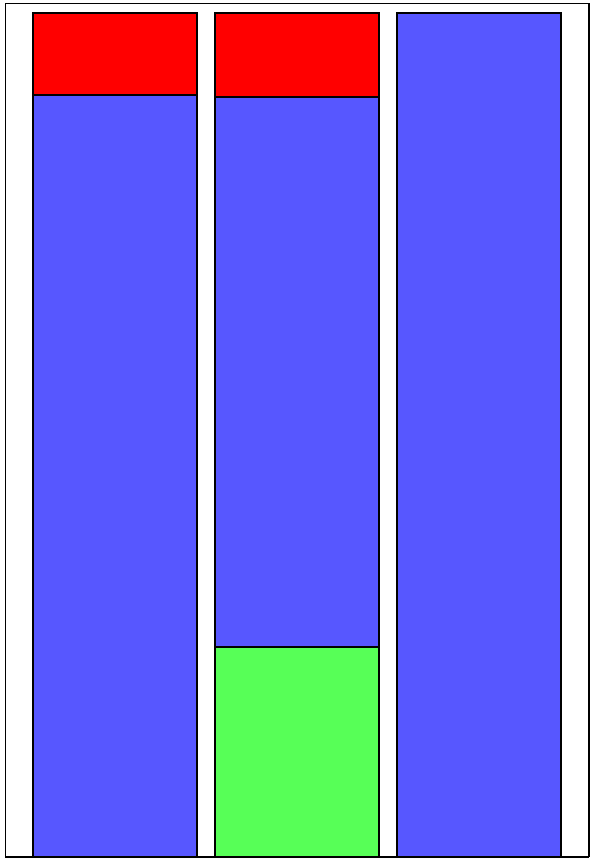


CFA vs FC - percentage in agreement with CFA, based on data to end of July 2014.



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% concordance of assigned risk categories between the three methods

		FC			
		Low	Mod	High	%
CFA	Low	91	9		
	Mod	26	57	17	
	High		5	95	
% Concordance between CFA & FC					81

		TS			
		Low	Mod	High	%
CFA	Low	92	8		
	Mod	27	31	42	
	High			100	
% Concordance between CFA & TS					74

		TS			
		Low	Mod	High	%
FC	Low	94	6		
	Mod	10	40	50	
	High	5	5	90	
% Concordance between FC & TS					75

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Mitigation strategy and long term planning

Costs???

COMMENTS

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- This data suggests that either TS or FC could be used as an alternative to the CFA for anti-D quantification
  - Must realise that as technologies are different, there will be some variation in the assignment of risk category
  - Decision by RCI on which technology to use if required
  - Project extension – collaboration with the fetal medicine units to assess pregnancy outcomes and the assignment of risk category

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Thanks

Any questions please