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A WHO Reference Reagent to standardise haemagglutination testing for anti-A and anti-B in serum and plasma

Report of the international collaborative study to evaluate candidate preparations

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **14 September 2015** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr M Nübling** at email:-nueblingc@who.int

Summary

A lyophilised serum preparation with high anti-A and anti-B titres, 14/300, was evaluated for its suitability to serve as a WHO Reference Reagent to standardise and control haemagglutination titrations for anti-A and anti-B in serum and plasma in an international collaborative study. Two plasma-based reserve preparations, 14/304 (high titre anti-A) and 14/208 (high titre anti-B), were also included in the study.

Twenty-three laboratories in 12 countries tested 14/300, 14/304 and 14/208 using haemagglutination methodology. Twenty-one laboratories performed the recommended haemagglutination methodology: direct agglutination at RT using DiaMed neutral (DRT) gel cards (measuring predominantly IgM) and indirect antiglobulin test (IAT) using Diamed anti-IgG and/or LISS/Coombs gel cards (measuring both IgM and IgG). One laboratory used Grifols IAT gel cards (no DRT performed), and one laboratory used Immucor Galileo Neo DRT and IgG-specific solid phase (included with IAT results) methodology. There was up to 64-fold variation in reported titres per preparation.

Using DRT, anti-A and anti-B titres ranged from 32-2048 and 32-1024, respectively, for 14/300, and 32-1024 and 8-256, respectively, for 14/304. Using IAT, anti-A and anti-B titres ranged from 64-2048 and 64-1024, respectively, for 14/300, and 64-1024 and 16-128, respectively, for 14/304. DRT and IAT anti-B titres for 14/208 ranged from 64-1024 and 16-512, respectively. No reactivity with group A red cells was reported for 14/208. None of the 3 preparations reacted with group O red cells.

For 14/300, the candidate Reference Reagent, the most common DRT titres were 128 for both anti-A and anti-B (48.5% and 63.6%, respectively, of all tests); the most common IAT titres were 256 for both anti-A and anti-B (66.4% and 60.0% of all tests, respectively). After exclusion of non-DiaMed tests, the frequency of anti-A and anti-B DRT titres of 128 rose to 50.8% and 66.7%, respectively, and the frequency of anti-A and anti-B IAT titres of 256 rose to 71.1 and 65.6%, respectively.

The results show that haemagglutination tests can exhibit wide inter-laboratory variation, even when using a common procedure, and demonstrate the need for international reference reagents with ‘nominal’ titres to facilitate inter-laboratory comparisons and allow sample titres to be reported relative to the reference titres. Data are presented to illustrate the value of a reference reagent.

Establishment of 14/300 as a WHO Reference Reagent for high titre anti-A and anti-B in serum, with nominal anti-A and anti-B titres of 128 for DRT, and nominal anti-A and anti-B titres of 256 for IAT, will facilitate global standardisation of haemagglutination testing for anti-A and anti-B in patient samples, and allow identification of more consistent ‘cut-off’ titres for various applications such as ABO-incompatible renal transplants.

Introduction

Testing for high titre anti-A and anti-B in serum/plasma is important to:

- Minimise the risk of causing clinically significant haemolysis when blood components rich in plasma containing high titre anti-A/B are transfused to patients of blood groups A, B or AB e.g., platelet concentrates.
- Facilitate mismatched kidney transplants from living donors. These can be performed successfully if the recipient has sufficiently low levels of anti-A and anti-B. Patients may be considered for admission to ABOi transplant programmes if anti-A/anti-B titres are within nominal cut-off values.
- Identify high titre anti-A/B plasma for exclusion from manufacture of blood products such as IVIG, where passive transfer of IgG anti-A/B to recipients can cause haemolysis.

However, anti-A and anti-B titrations are notoriously inconsistent across laboratories [1, 2]. The availability of a reference preparation with nominal anti-A and anti-B titres should ensure greater standardisation of methodology and results, with a later possibility of re-defining cut-off limits for transplant and other purposes when it has become clearer what they should be.
The aim of the present study was to evaluate lyophilised serum and plasma preparations to determine their suitability to serve as WHO Reference Reagent(s). As previous studies have shown considerable variation in haemagglutination titres even when using a specified procedure, it was decided to recommend the use one of the most common commercial haemagglutination methods, DiaMed gel card technology, to facilitate the identification of appropriate 'mode' titres for assignment.

Materials and Methods

Candidate reference materials

Liquid (stored at 4°C until use) and frozen plasma packs were received from the NHSBT. Packs were sampled aseptically for haemagglutination titrations (frozen packs were first thawed at +37°C). A total of 46 plasma packs were titrated for anti-A and anti-B using DiaMed IAT gel card titrations and those with the highest titres of at least 1+ at 1 in 128 dilution for both anti-A and anti-B were selected for defibrination and possible pooling to produce the candidate 'High titre anti-A and anti-B in serum Reference Reagent'. Defibrination of individual packs was carried out as follows: thrombin was reconstituted in 100 mM $CaCl_2$ to give 500 IU/ml and added, with stirring, to plasma to give final concentrations of 5 IU/ml thrombin and 1 mM $CaCl_2$, respectively. Following incubation overnight at 4°C, the defibrinated plasma was recovered by squeezing the clots in synthetic cheesecloth. The defibrination process was repeated and the clots were squeezed out as before. The defibrinated plasma (serum) was tested and found negative for residual clotting activity using RecombiPlastin 2G before filtration through sterile Nalgene 0.45µm SFCA membranes into sterile plastic containers and re-testing. A serum bulk pool of 4.5 litres was prepared from the 17 defibrinated packs with the highest titres. A 'High titre anti-A plasma' pool was prepared from the remaining 29 packs, whilst a 'High titre anti-B plasma' pool was prepared from additional plasma packs from blood group A donors. Fill details (~1 mL/ampoule) are shown below:

Preparation	Mean weight of dispensed solution (coefficient of variation)	Dry weight	Moisture	Residual oxygen	No. ampoules available
14/300	1.008g (0.16%)	0.08g	0.37%	0.17%	4261

14/304	1.009g (0.13%)	0.08g	0.34%	0.09%	7595
14/208	1.008g (0.11%)	0.08g	0.71%	0.16%	5452

Participants
Twenty-three laboratories located in 12 countries contributed results in the collaborative study. Each laboratory is referred to in this report by an arbitrarily assigned number, not necessarily representing the order of listing in Appendix 1. The participants included laboratories in blood transfusion centres and manufacturers of blood typing systems.

Samples provided
Each participant was provided with:

- 7 ampoules of the candidate reference reagent 'High titre anti-A and anti-B in serum', 14/300
- 7 ampoules of reserve preparation 1, 'High titre anti-A in plasma', 14/304
- 7 ampoules of reserve preparation 2, 'High titre anti-B in plasma', 14/208

Participants were advised to store all ampoules at -20^{o}C or below until reconstitution and use.

Assay methods and study design
Participants were requested to titrate the study samples using DiaMed gel cards (or an equivalent system) by DRT using neutral cards (predominantly for IgM antibodies) and by IAT (for additional IgG reactivity) using anti-IgG cards (or an equivalent system) at 37^{o}C, against pooled A_1 and B cells (from at least 3 donors), according to a detailed protocol provided. For each assay, participants were instructed to test serial doubling dilutions in saline or PBS, from neat, of each of the reconstituted ampoule contents, in 2 independent runs on each of three days (preferably consecutive). Participants were asked to include group O cells as a negative control using only the neat plasma and serum, and use a fresh ampoule of each preparation for each day's runs (surplus ampoules were provided to ensure sufficient quantities).
Participants were asked to record the haemagglutination reaction score for each dilution tested on results sheets provided.

Analysis of data
The last reciprocal dilution at which haemagglutination was recorded (e.g., score of weak 0.5-2) was tabulated for each sample and assay, for each laboratory, to give a total of 6 titres for each preparation per laboratory. Where scores of 'very weak 0.5' was reported, the previous dilution was taken as the unambiguous end-point.

Results
Test data
Twenty-three laboratories in 12 countries returned data. Eighteen laboratories performed the recommended haemagglutination methodology provided (using DiaMed neutral and anti-IgG IAT gel cards); one laboratory tested 14/300 on both DiaMed anti-IgG IAT cards (referred to as lab 8a) and IAT LISS/Coombs (referred to as lab 8) cards on the first day, and then completed the study using the LISS/Coombs cards (lab 8); two further laboratories used DiaMed IAT

LISS/Coombs gel cards throughout the study (labs 15 and 17); one laboratory used Grifols IAT gel cards only (no DRT; lab 23); one laboratory used Immucor Galileo Neo DRT and IgG-specific solid-phase 'Capture' methodology (lab 4). The latter tests are included with the IAT tests in this report although they are specific for IgG anti-A and anti-B and do not detect IgM anti-A and anti-B. Laboratory mean titres for each preparation and titration method are shown in Figures 1-10.

Overall titres

The total number of tests resulting in a particular titre against A or B cells across all laboratories was counted for each preparation. The results are shown in Tables 1-6, along with the % of tests giving a particular titre. The results are also illustrated in histogram form in Figures 11-16.

For 14/300, the most common DRT anti-A and anti-B titres were 128 (48.5% of tests) and 128 (63.6% of tests), respectively. For 14/300, the most common IAT anti-A and anti-B titres were 256 (66.4% of tests) and 256 (60.0% of tests), respectively. Considering only the DiaMed tests, the frequency of anti-A and anti-B DRT titres rose to 50.8% and 66.7%, respectively, and the frequency of anti-A and anti-B IAT titres rose to 71.1 and 65.6%, respectively.

For 14/304, the most common DRT anti-A and anti-B titres were 128 (49.2% of tests) and 64 (65.2% of tests), respectively, while the most common IAT titres were 256 (68.8% of tests) and 64 (52.2% of tests) for anti-A and anti-B, respectively.

For 14/208, the most common DRT anti-B titre was 256 (51.5% of tests) and the most common IAT anti-B titre was 128 (61.6% of tests). No anti-A was detected in 14/208 by any laboratory. None of the 3 preparations reacted with group O red cells using either DRT or IAT titrations.

Intra- and inter-laboratory variability

The geometric mean titres reported by each laboratory for each sample are shown in Tables 7-9. Variability within a laboratory was expressed as the maximum fold-range in titres for each sample. Most laboratories reported titres with no variability or within a maximum 2-fold range for each sample and method. Intra-laboratory 4-fold ranges were reported for 0-21% of tests for each sample, and laboratory 9 reported 8-fold ranges for DRT anti-B and IAT anti-A. Overall, the range of titres for each preparation (fold range) was as follows:

	DRT (all laboratories)		IAT (all laboratories)		DiaMed DRT only		DiaMed IAT only	
	Anti-A	Anti-B	Anti-A	Anti-B	Anti-A	Anti-B	Anti-A	Anti-B
14/300	32-2048 (64)	32-1024 (32)	64-2048 (32)	64-1024 (16)	64-2048 (32)	64-1024 (16)	128-2048 (16)	64-1024 (16)
14/304	32-1024 (32)	8-256 (32)	64-1024 (16)	16-128 (8)	32-1024 (32)	8-256 (32)	64-1024 (16)	16-128 (8)
14/208	0	64-1024 (16)	0	16-512 (32)	-	64-1024 (16)	-	64-512 (8)

When the titres using DiaMed only were considered, the ranges were reduced in 4 cases, as highlighted above.

Fitness of purpose of candidate Reference Reagent 14/300

The data presented thus far clearly show that even when using a single commercial haemagglutination method, titres of the same preparation vary considerably between laboratories, and even within some laboratories. An inspection of the graphs in Figures 17 and 18, illustrating the relationship between titres reported for preparations 14/300 and 14/304 suggests a degree of consistency in relative titres between preparations, and that by assigning a nominal titre to a preparation, relative titres can be determined. To test this, the ratios of the mean geometric titres of 14/304 and 14/208 relative to the mean geometric titres of 14/300 (shown in Tables 7-9) were calculated for each laboratory and method. By multiplying these ratios by the nominal titre assignments of 128 (DRT) and 256 (IAT) to 14/300, relative titres were obtained. These are illustrated in Figures 19-30 and summarised below:

	DRT		IAT	
	Anti-A	Anti-B	Anti-A	Anti-B
14/304 Actual mean titre range	32-644	16-204	102-724	25-80
14/304 Relative mean titre range	91-161	32-64	128-323	28-102
14/208 Actual mean titre range		64-815		16-256
14/208 Relative mean titre range		114-256		51-202

In 5 of the 6 cases above (highlighted), the mean titre range was significantly reduced when titres of 14/304 and 14/208 were expressed in terms of the titres of 14/300.

The candidate Reference Reagent also has the potential to define cut-off values. For example, if a 1 in 2 dilution of 14/300 (with an assigned titre of 256) defined the cut-off for IAT anti-A (i.e., a titre of 128), preparation 14/304 would 'fail' in 22/23 laboratories as shown in Figure 31, despite the overlapping ranges of titres (32-1024 for a 1 in 2 dilution of 14/300; 64-1024 for 14/304). In only one laboratory were the titres the same resulting in a 'pass'. Based on actual titres, this laboratory would have 'failed' 14/304, but 14/304 would have 'passed' in other 2 laboratories. However, it should be borne in mind that the majority of laboratories performed DiaMed titrations and only by collection of relative titration data from multiple methodologies and comparison with clinical outcome will the true value of a reference reagent be established.

Stability

Long term stability data are not yet available for the candidate Reference Reagent for high titre anti-A and anti-B, 14/300, as ampoules for accelerated degradation studies have been stored at elevated temperatures for only a few months. However, an indication of the long term intrinsic stability of anti-A and anti-B was provided by examining ampoules of previous fills of anti-A and anti-B, albeit in different matrices.

(i) Stability of IgM anti-A and anti-B:

Ampoules of the British Minimum Potency Preparations for anti-A and anti-B were stored at -20°C, +4°C and +20°C for nearly 16 years. Geometric mean titres from a total of 12 independent titrations from 2 ampoules are shown below:

		-20°C	**+4°C**	**+20°C**	**+37°C**
Overall Geometric Mean Titre	**Anti-A**	1218	1085	967	Not tested
	Anti-B	483	483	456	456

(ii) Stability of IgG anti-A and anti-B
Ampoules of the WHO Reference Reagent to standardise and control haemagglutination tests for anti-A and anti-B in IVIG, 07/306, were stored at a range of temperatures for 8 years. Titration data using the direct haemagglutination method using papain-treated red cells (replicate plates) are shown below:

	Red cell phenotype		
Temperature °C	A	B	O
-70	64/64	32/32	<2/<2
+20	64/64	32/32	<2/<2
+4	64/64	32/32	<2/<2
+20	64/32	32/32	<2/<2
+37	32/64	32/32	<2/<2
+45	32/32	16/32	<2/<2

Titration data using DiaMed IAT gel cards are shown below:

	Red cell phenotype		
Temperature °C	A	B	O
-70	32	16	<2
+20	32	16	<2
+4	32	16	<2
+20	32	16	<2
+37	16	16	<2
+45	16	16	<2

Although the data are semi-quantitative and thus unsuitable for analysis using the usual Arrhenius model of accelerated degradation, both IgM and IgG anti-A and anti-B titres appear extremely stable. Thus there is no evidence to suggest that the anti-A and anti-B titres in 14/300 will not be sufficiently stable at -20°C for many years.

Participant feedback

All of the participants agreed with the recommendation.

Instructions for Use

Draft Instructions for Use to accompany this material are provided in Appendix 3.

Discussion

This study demonstrates up to 64-fold variability in haemagglutination titres of serum and plasma preparations between laboratories, and up to 32-fold variability even when using a specified method i.e., DiaMed gel cards, and pooled red cells to minimise variability due to differences in antigen concentration on red cells. This titre range is comparable to the variability encountered in previous collaborative studies involving haemagglutination methodology (up to 32-fold excluding extreme values), and shows that setting a 'limit' or 'cut-off' value in terms of a titre in the absence of a reference preparation is meaningless. Thus there is a need for reference preparations to be used in parallel titrations with samples to take into account variations in actual titres between tests and laboratories.

Whilst preparation 14/304 had broadly comparable anti-A titres to those of 14/300, the latter preparation had higher anti-B titres, equivalent to those of anti-A. Preparation 14/208, whilst having high titre anti-B, did not contain anti-A. 14/300 is therefore the best suited preparation to serve as a reference reagent with DRT titres of 128 against both A and B red cells, and IAT titres of 256 against both A and B red cells.

We conclude that the availability and use of 14/300 would help overcome inter-laboratory variability in haemagglutination titres and allow laboratories to report titres of patient samples relative to 14/300 to facilitate comparability of results between laboratories and methodology, including international harmonisation. The results presented in this report show, firstly, the feasibility of calculation of relative geometric titres from repeated titrations, and secondly, the use of 14/300 as a cut-off indicator in parallel titrations with patient samples e.g., neat for DRT and ½ dilution for IAT, to ensure greater consistency in the identification of high titre plasma or serum samples.

Recommendation

It is recommended that preparation 14/300 is established by WHO ECBS as a Reference Reagent for high titre anti-A and anti-B with nominal DRT titres of both anti-A and anti-B of 128, and nominal IAT titres of both anti-A and anti-B of 256, for use in standardising haemagglutination testing methodology for anti-A and anti-B in plasma and serum, and for establishing consistent cut-off values where appropriate.

Acknowledgements

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References

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Table 1 Number of tests resulting in a particular titre against A, B and O cells for the candidate Reference Reagent, 14/300, using DRT gel cards or equivalent methodology

	Number of tests				**% of tests**		
Titre	**A cells**	**B cells**	**O cells**	**Titre**	**A cells**	**B cells**	**O cells**
0	0	0	62	0	0	0	100
2	0	0	0	2	0	0	0
4	0	0	0	4	0	0	0
8	0	0	0	8	0	0	0
16	0	0	0	16	0	0	0
32	5	6	0	32	3.8	4.6	0
64	5	5	0	64	3.8	3.8	0
128	64	84	0	128	48.5	63.6	0
256	50	32	0	256	37.9	24.2	0
512	5	2	0	512	3.8	1.5	0
1024	2	3	0	1024	1.5	2.3	0
2048	1	0	0	2048	0.7	0	0
Total number of tests	132	132	62	% of tests	100	100	100

Table 2 Number of tests resulting in a particular titre against A, B and O cells for the candidate Reference Reagent, 14/300, using IAT gel cards or equivalent methodology

	Number of tests				**% of tests**		
Titre	**A cells**	**B cells**	**O cells**	**Titre**	**A cells**	**B cells**	**O cells**
0	0	0	65	0	0	0	100
2	0	0	0	2	0	0	0
4	0	0	0	4	0	0	0
8	0	0	0	8	0	0	0
16	0	0	0	16	0	0	0
32	0	0	0	32	0	0	0
64	2	6	0	64	1.4	4.3	0
128	13	30	0	128	9.3	21.4	0
256	93	84	0	256	66.4	60.0	0
512	26	16	0	512	18.5	11.4	0
1024	5	4	0	1024	3.6	2.9	0
2048	1	0	0	2048	0.8	0	0
Total number of tests	140	140	65	% of tests	100	100	100

Table 3 Number of tests resulting in a particular titre against A, B and O cells for reserve preparation 1, 14/304, using DRT gel cards or equivalent methodology

	Number of tests				**% of tests**		
Titre	**A cells**	**B cells**	**O cells**	**Titre**	**A cells**	**B cells**	**O cells**
0	0	0	60	0	0	0	100
2	0	0	0	2	0	0	0
4	0	0	0	4	0	0	0
8	0	1	0	8	0	0.8	0
16	0	7	0	16	0	5.3	0
32	7	23	0	32	5.3	17.4	0
64	6	86	0	64	4.6	65.2	0
128	65	11	0	128	49.2	8.3	0
256	47	4	0	256	35.6	3.0	0
512	5	0	0	512	3.8	0	0
1024	2	0	0	1024	1.5	0	0
2048	0	0	0	2048	0	0	0
Total number of tests	132	132	60	% of tests	100	100	100

Table 4 Number of tests resulting in a particular titre against A, B and O cells for reserve preparation 1, 14/304, using IAT gel cards or equivalent methodology

	Number of tests				**% of tests**		
Titre	**A cells**	**B cells**	**O cells**	**Titre**	**A cells**	**B cells**	**O cells**
0	0	0	63	0	0	0	100
2	0	0	0	2	0	0	0
4	0	0	0	4	0	0	0
8	0	0	0	8	0	0	0
16	0	4	0	16	0	2.9	0
32	0	54	0	32	0	39.1	0
64	3	72	0	64	2.2	52.2	0
128	17	8	0	128	12.3	5.8	0
256	95	0	0	256	68.8	0	0
512	19	0	0	512	13.8	0	0
1024	4	0	0	1024	2.9	0	0
2048	0	0	0	2048	0	0	0
Total number of tests	138	138	63	% of tests	100	100	100

Table 5 Number of tests resulting in a particular titre against A, B and O cells for reserve preparation 2, 14/208, using DRT gel cards or equivalent methodology

	Number of tests				**% of tests**		
Titre	**A cells**	**B cells**	**O cells**	**Titre**	**A cells**	**B cells**	**O cells**
0	130	0	63	0	100	0	100
2	0	0	0	2	0	0	0
4	0	0	0	4	0	0	0
8	0	0	0	8	0	0	0
16	0	0	0	16	0	0	0
32	0	0	0	32	0	0	0
64	0	9	0	64	0	6.8	0
128	0	45	0	128	0	34.1	0
256	0	68	0	256	0	51.5	0
512	0	6	0	512	0	4.6	0
1024	0	4	0	1024	0	3.0	0
2048	0	0	0	2048	0	0	0
Total number of tests	130	132	63	% of tests	100	100	100

Table 6 Number of tests resulting in a particular titre against A, B and O cells for reserve preparation 2, 14/208, using IAT gel cards or equivalent methodology

	Number of tests				**% of tests**		
Titre	**A cells**	**B cells**	**O cells**	**Titre**	**A cells**	**B cells**	**O cells**
0	136	0	64	0	0	0	100
2	0	0	0	2	0	0	0
4	0	0	0	4	0	0	0
8	0	0	0	8	0	0	0
16	0	8	0	16	0	5.8	0
32	0	4	0	32	0	2.9	0
64	0	24	0	64	0	17.4	0
128	0	85	0	128	0	61.6	0
256	0	16	0	256	0	11.6	0
512	0	1	0	512	0	0.7	0
1024	0	0	0	1024	0	0	0
2048	0	0	0	2048	0	0	0
Total number of tests	138	138	64	% of tests	100	100	100

Table 7 Intra-laboratory variability and geometric mean titres for the candidate Reference Reagent, 14/300

Lab	14/300							
	DRT				IAT			
	A		B		A		B	
	max fold-range[1]	geo-metric mean	max fold-range[1]	geo-metric mean	max fold-range[1]	geo-metric mean	max fold-range[1]	geo-metric mean
1	1	256	1	256	2	455	1	256
2	1	128	1	128	1	256	1	256
3	2	144	1	128	1	256	1	128
4	2	36	1	32	1	128	2	80
5	1	128	1	128	2	288	1	128
6	4	288	2	181	4	724	4	512
7	1	256	2	204	2	362	2	288
8	1	128	1	128	1	256	1	256
8a	-	-	-	-	1	512	1	256
9	2	80	2	72	4	288	4	288
10	1	128	1	128	1	256	4	228
11	1	128	1	128	1	256	1	256
12	1	256	2	204	1	256	2	407
13	2	144	1	128	2	228	2	181
14	2	228	2	181	2	228	2	228
15	1	128	1	128	2	288	4	228
16	2	181	2	181	2	322	2	288
17	2	204	2	181	2	228	2	181
18	2	204	1	128	1	256	1	256
19	4	815	4	644	4	724	4	644
20	1	256	2	204	1	512	1	256
21	2	144	2	144	1	256	1	256
22	2	204	1	256	1	256	1	256
23	-	-	-	-	4	128	2	102

[1] across number of dilution steps

Table 8 Intra-laboratory variability and geometric mean titres for reserve preparation 1, 14/304

Lab	14/304							
	DRT				IAT			
	A		B		A		B	
	max fold-range[1]	geo-metric mean	max fold-range[1]	geo-metric mean	max fold-range[1]	geo-metric mean	max fold-range[1]	geo-metric mean
1	2	228	1	64	2	322	2	80
2	1	128	1	64	1	256	1	64
3	2	144	2	57	1	256	2	25
4	1	32	1	16	1	128	1	32
5	1	128	1	32	2	204	1	32
6	4	256	4	64	2	572	2	80
7	2	228	1	64	2	322	1	64
8	2	114	2	51	1	256	2	40
9	2	57	8	25	8	181	2	72
10	2	144	2	45	1	256	2	31
11	1	128	1	32	1	256	1	32
12	2	228	2	102	1	256	1	64
13	2	181	1	64	1	256	1	32
14	2	228	2	72	1	256	2	40
15	2	144	1	64	1	256	4	72
16	2	161	1	64	2	322	2	57
17	2	181	2	72	2	181	2	51
18	2	161	2	57	2	322	2	51
19	2	644	2	204	2	724	2	72
20	1	256	1	64	1	256	1	64
21	1	128	1	64	1	256	1	64
22	2	204	2	80	1	256	2	36
23	-	-	-	-	2	102	4	32

[1] across number of dilution steps

Table 9 Intra-laboratory variability and geometric mean titres for reserve preparation 2, 14/208

Lab	14/208							
	DRT				IAT			
	A		B		A		B	
	max fold-range[1]	geo-metric mean	max fold-range[1]	geo-metric mean	max fold-range[1]	geo-metric mean	max fold-range[1]	geo-metric mean
1	1	0	1	256	1	0	2	144
2	1	0	1	256	1	0	1	128
3	1	0	2	204	1	0	1	64
4	1	0	1	64	1	0	1	16
5	1	0	1	128	1	0	1	64
6	1	0	4	256	1	0	4	228
7	1	0	2	288	1	0	2	114
8	1	0	1	128	1	0	1	128
9	1	0	2	91	1	0	2	161
10	1	0	1	128	1	0	2	80
11	1	0	1	128	1	0	1	128
12	1	0	1	256	1	0	1	128
13	1	0	2	204	1	0	1	64
14	1	0	2	228	1	0	1	128
15	1	0	2	161	1	0	2	181
16	1	0	2	228	1	0	2	144
17	1	0	1	256	1	0	1	128
18	1	0	1	256	1	0	1	128
19	1	0	2	815	1	0	1	256
20	1	0	2	288	1	0	1	128
21	1	0	1	128	1	0	1	128
22	1	0	1	256	1	0	2	114
23	-	-	-	-	1	0	2	25

[1] across number of dilution steps

Figure 1 Laboratory mean titres: 14/300 DRT anti-A

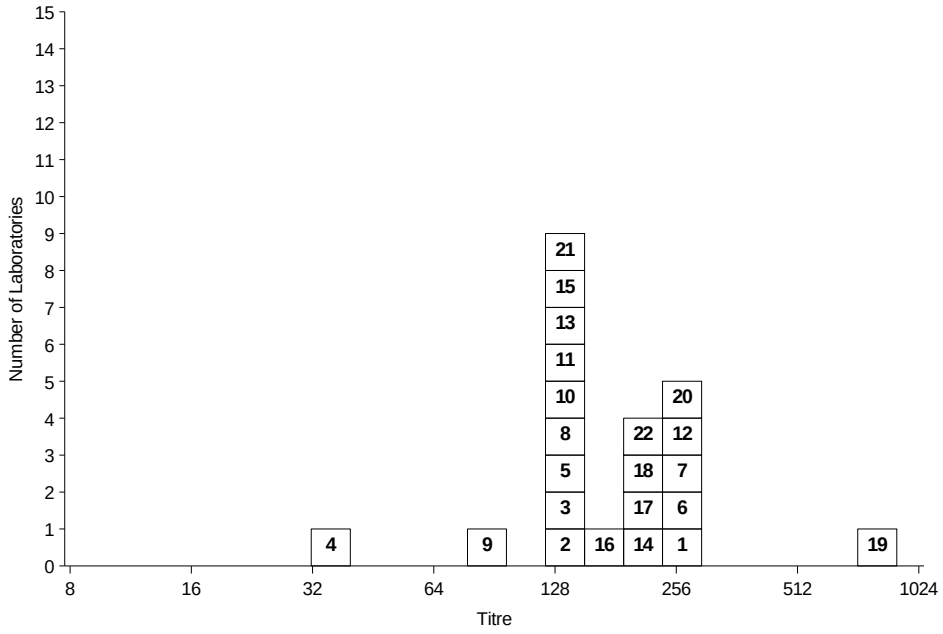


Figure 2 Laboratory mean titres: 14/300DRT anti-B

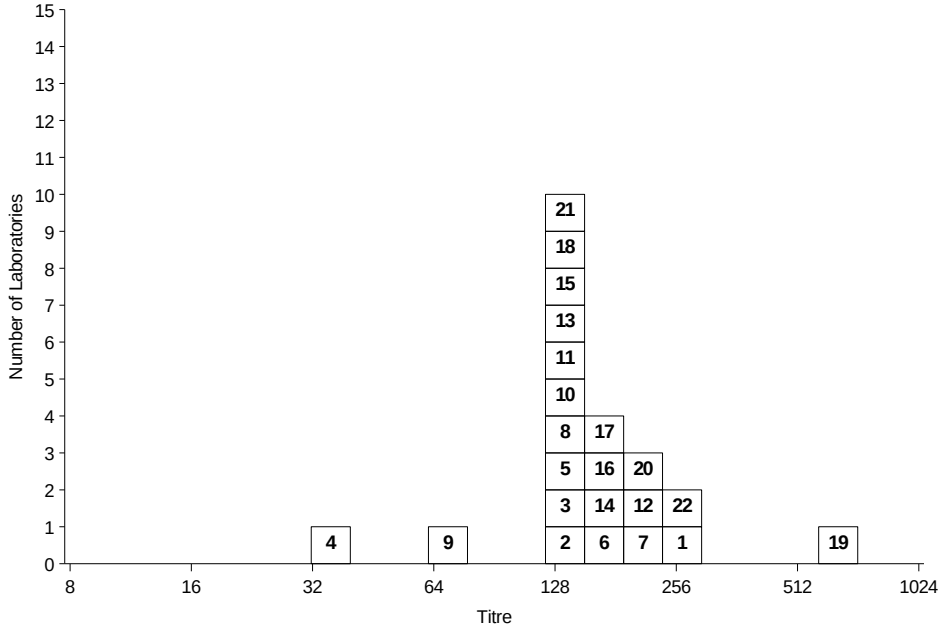


Figure 3 Laboratory mean titres: 14/300 IAT anti-A

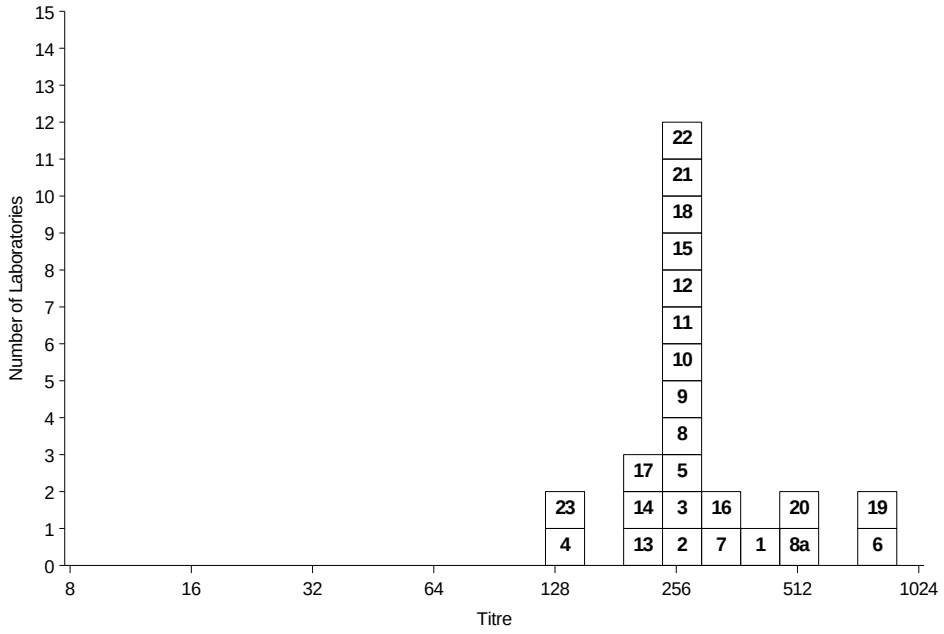


Figure 4 Laboratory mean titres: 14/300 IAT anti-B

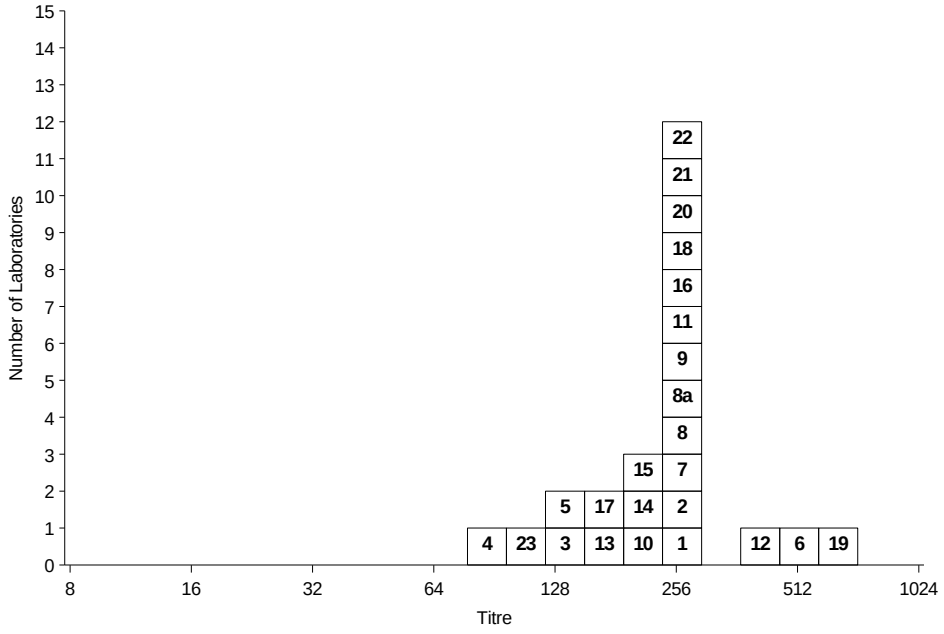


Figure 5 Laboratory mean titres: 14/304 DRT anti-A

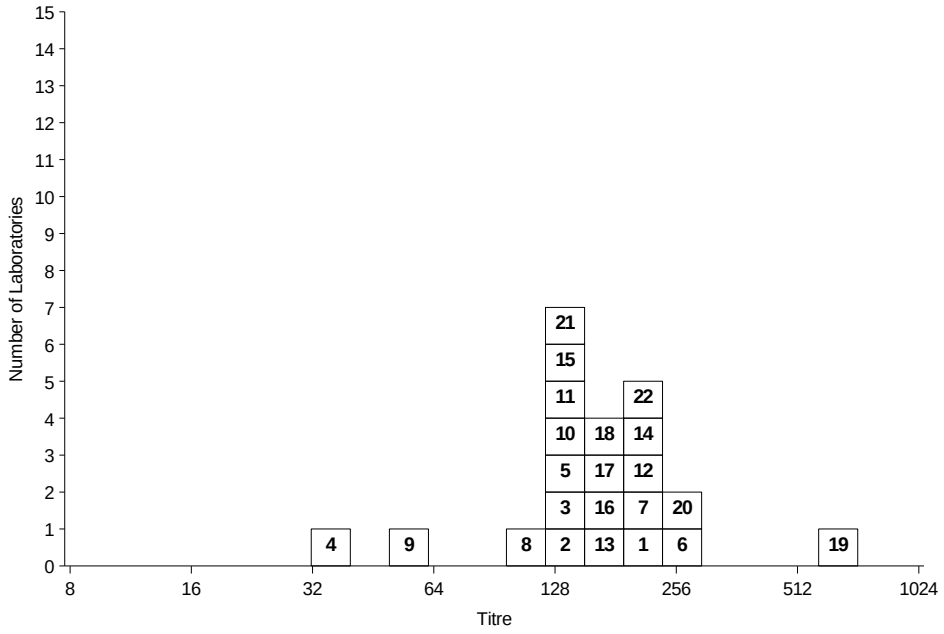


Figure 6 Laboratory mean titres: 14/304 DRT anti-B

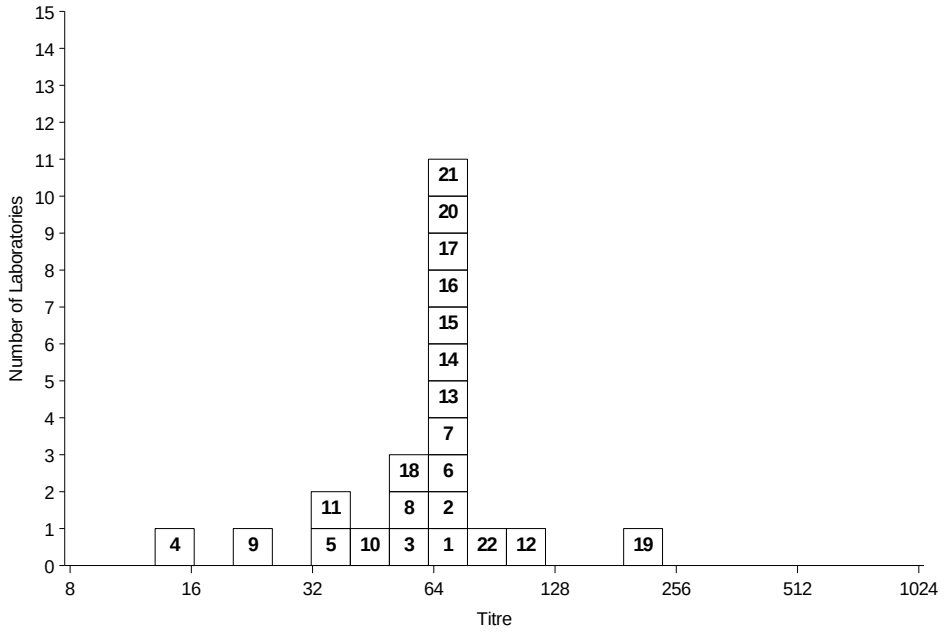


Figure 7 Laboratory mean titres: 14/304 IAT anti-A

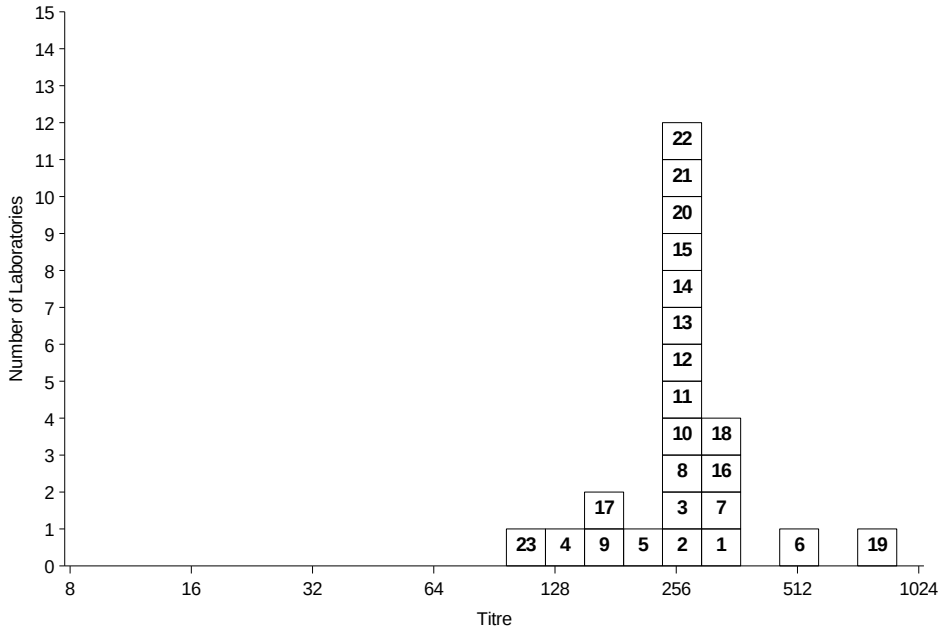


Figure 8 Laboratory mean titres: 14/304 IAT anti-B

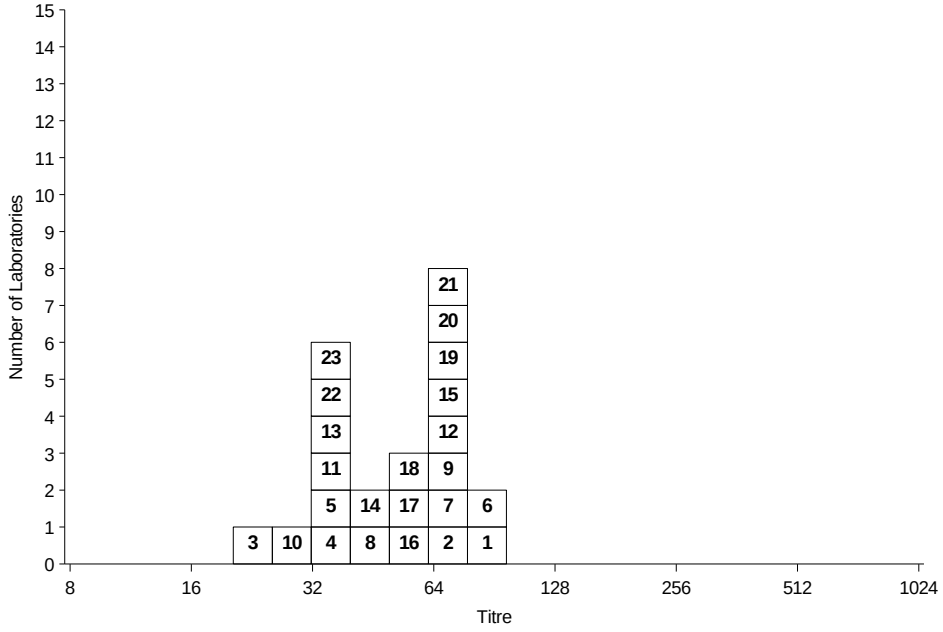


Figure 9 Laboratory mean titres: 14/208 DRT anti-B

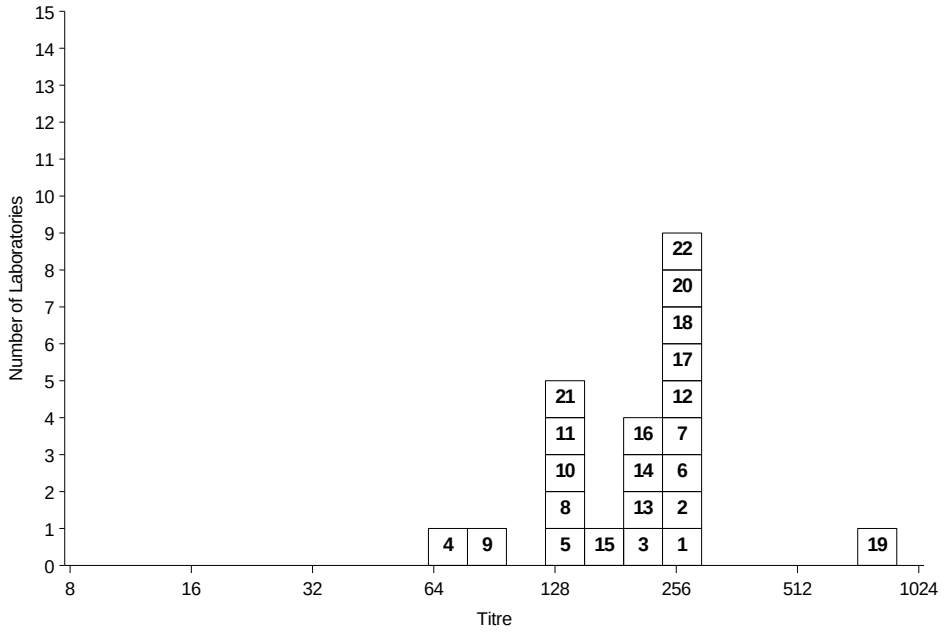


Figure 10 Laboratory mean titres: 14/208 IAT anti-B

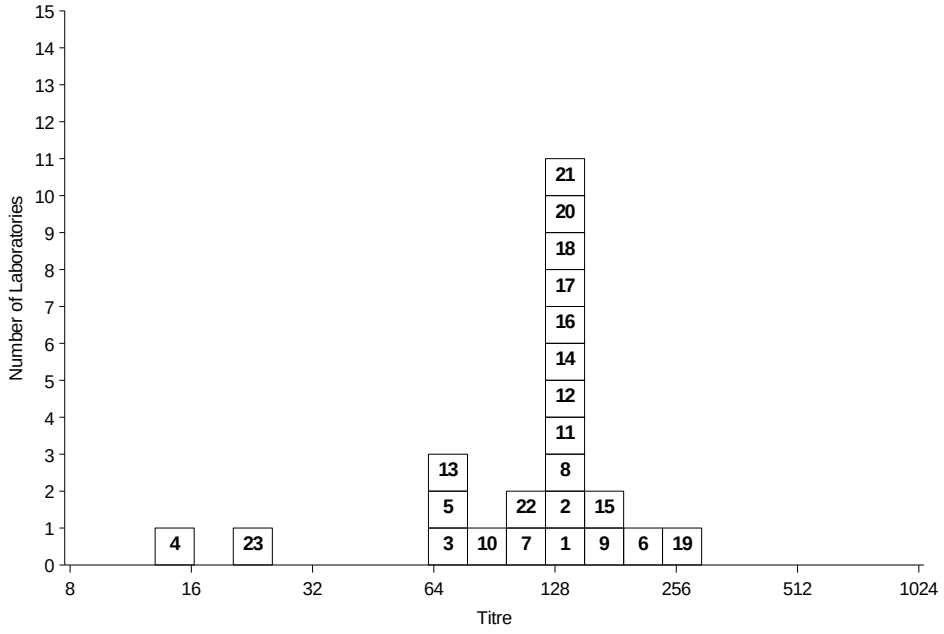


Figure 11 14/300: all titres using DiaMed DRT or equivalent methodology

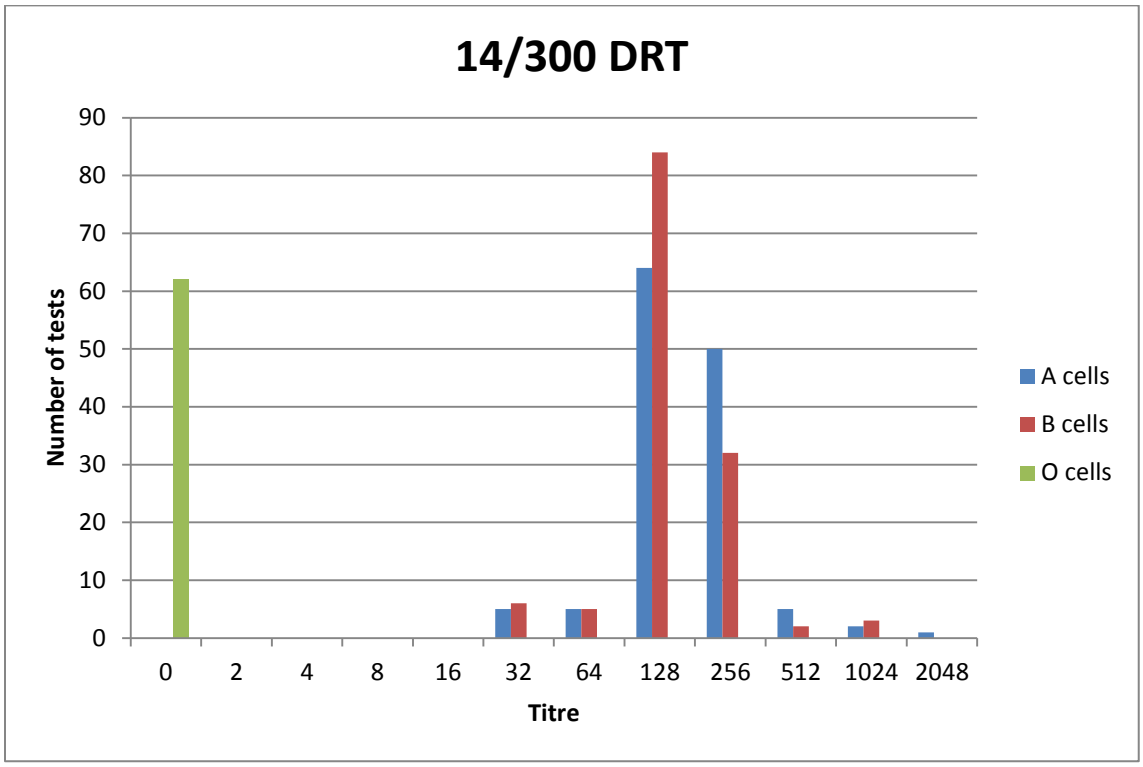


Figure 12 14/300: all titres using DiaMed IAT or equivalent methodology

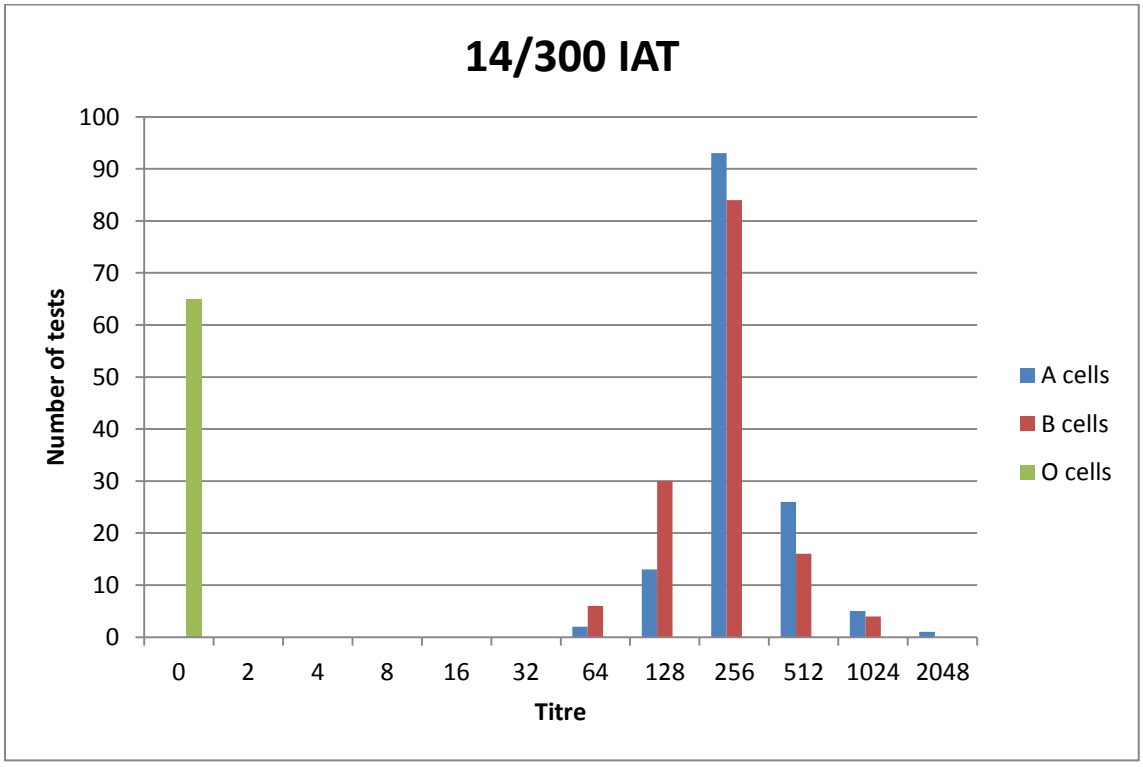


Figure 13 14/304: all titres using DiaMed DRT or equivalent methodology

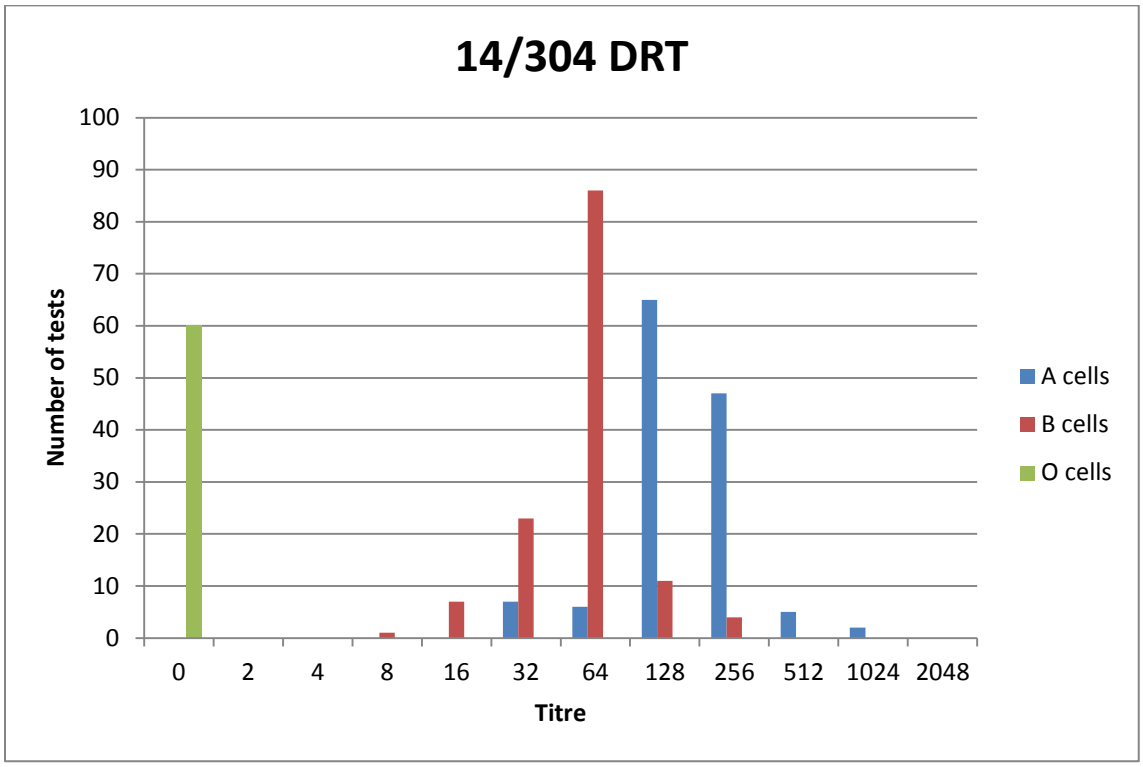


Figure 14 14/304: all titres using DiaMed IAT or equivalent methodology

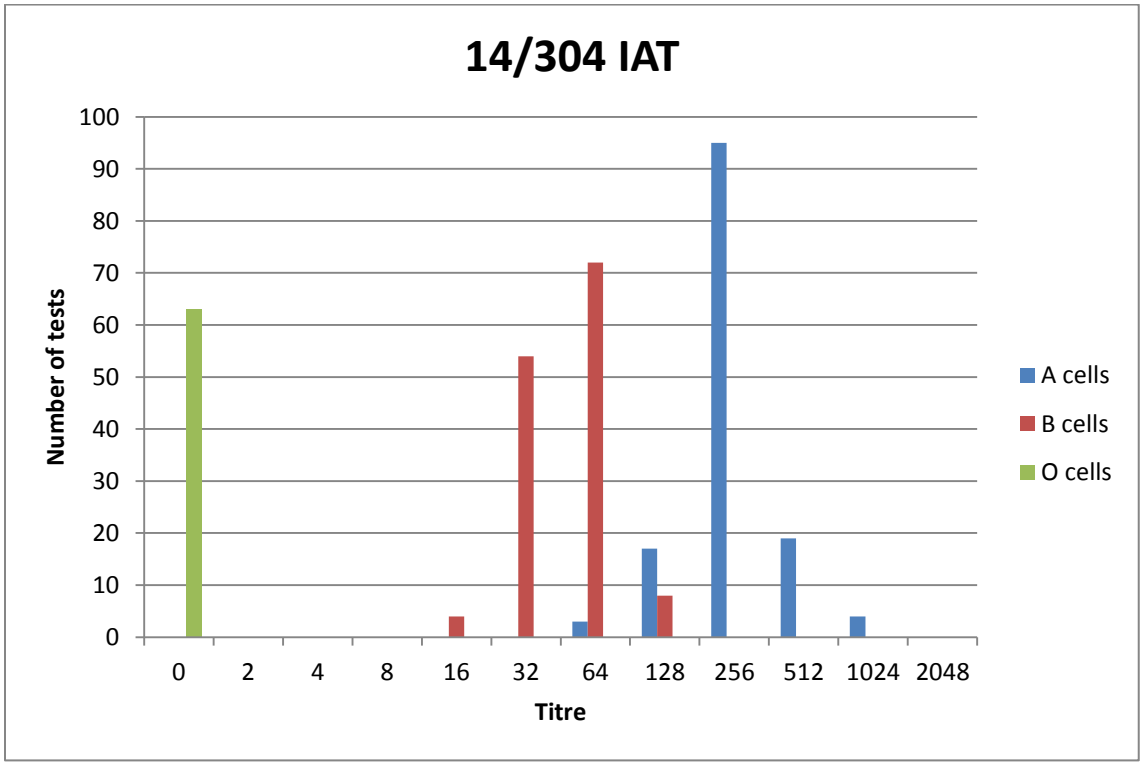


Figure 15 14/208: all titres using DiaMed DRT or equivalent methodology

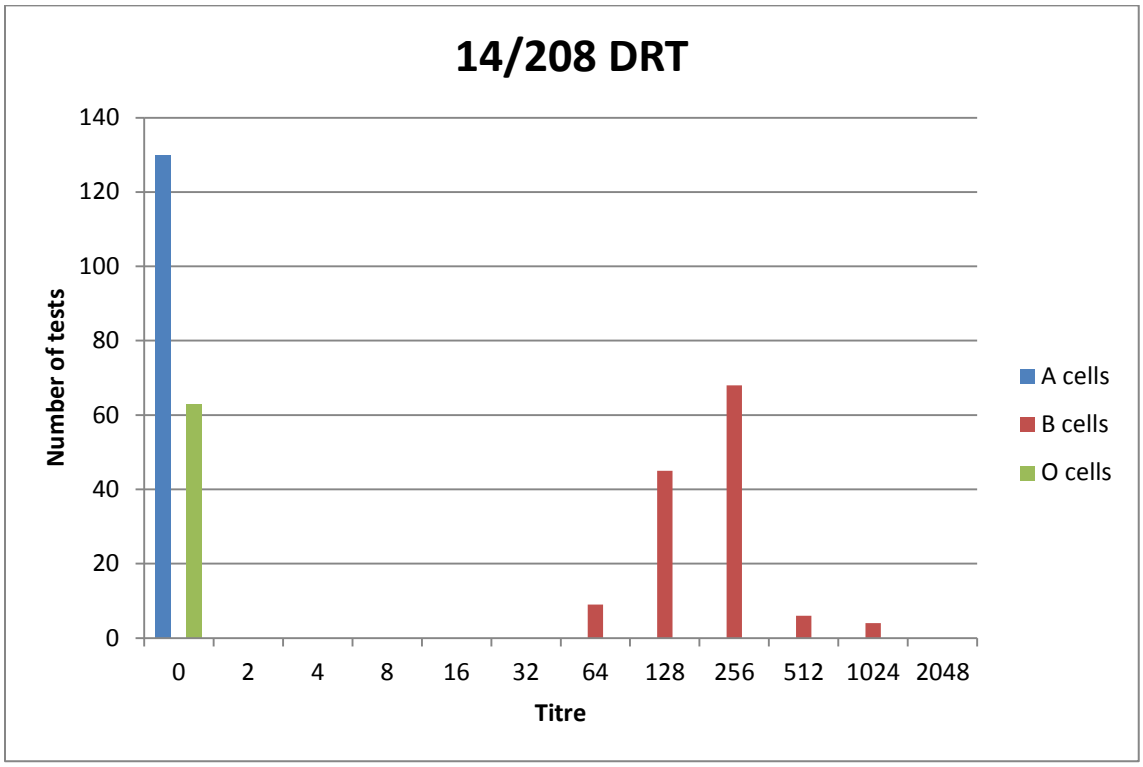


Figure 16 14/208: all titres using DiaMed IAT or equivalent methodology

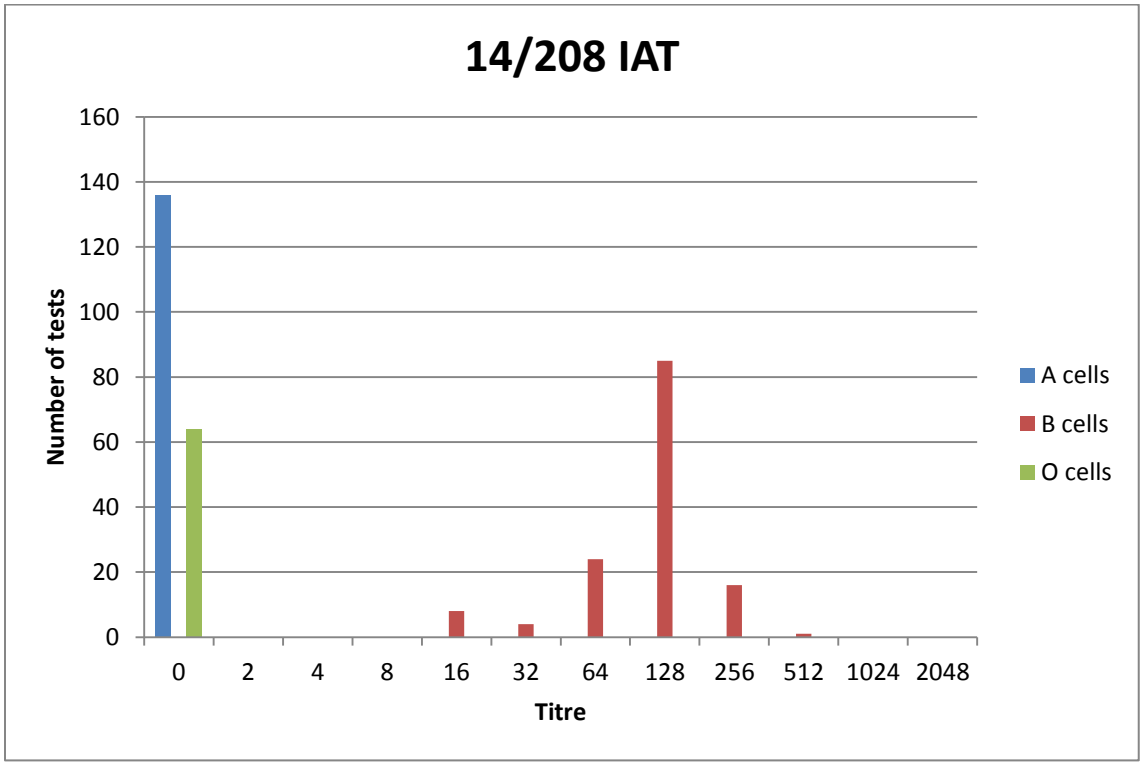


Figure 17 Comparison of DRT anti-A titres of 14/300 and 14/304

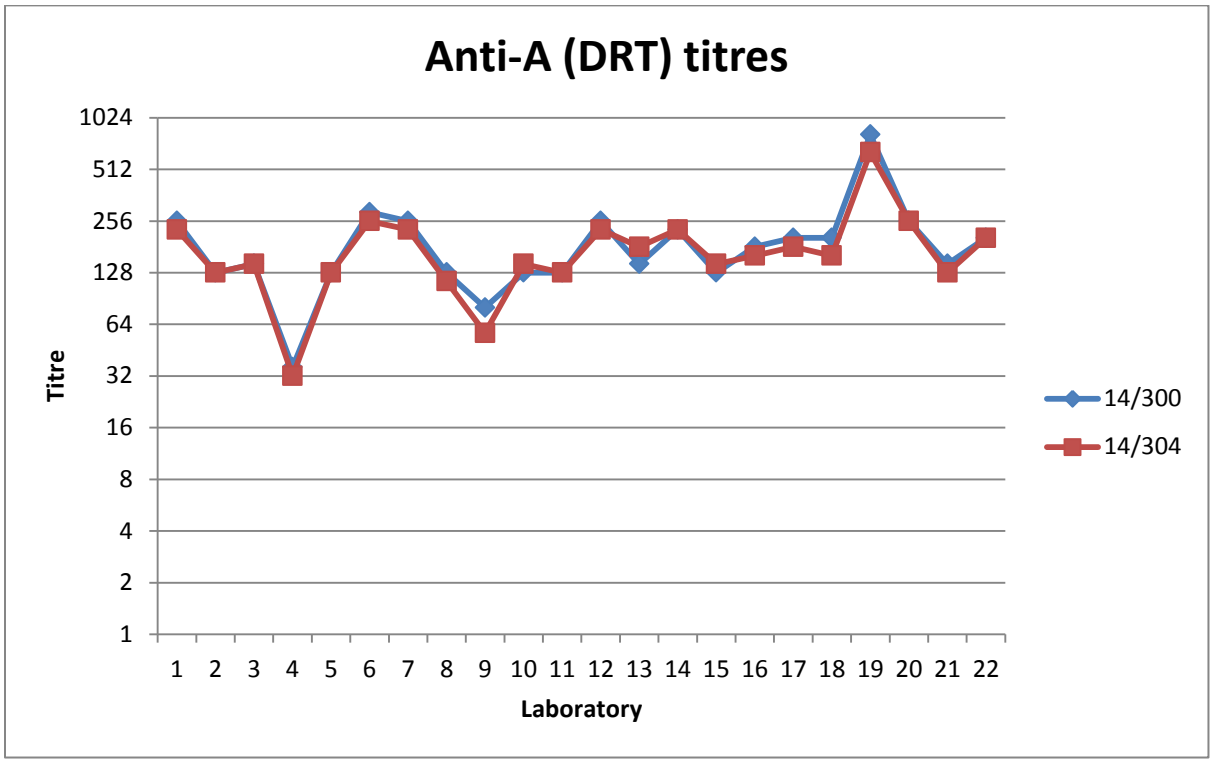


Figure 18 Comparison of DRT anti-B titres of 14/300 and 14/304

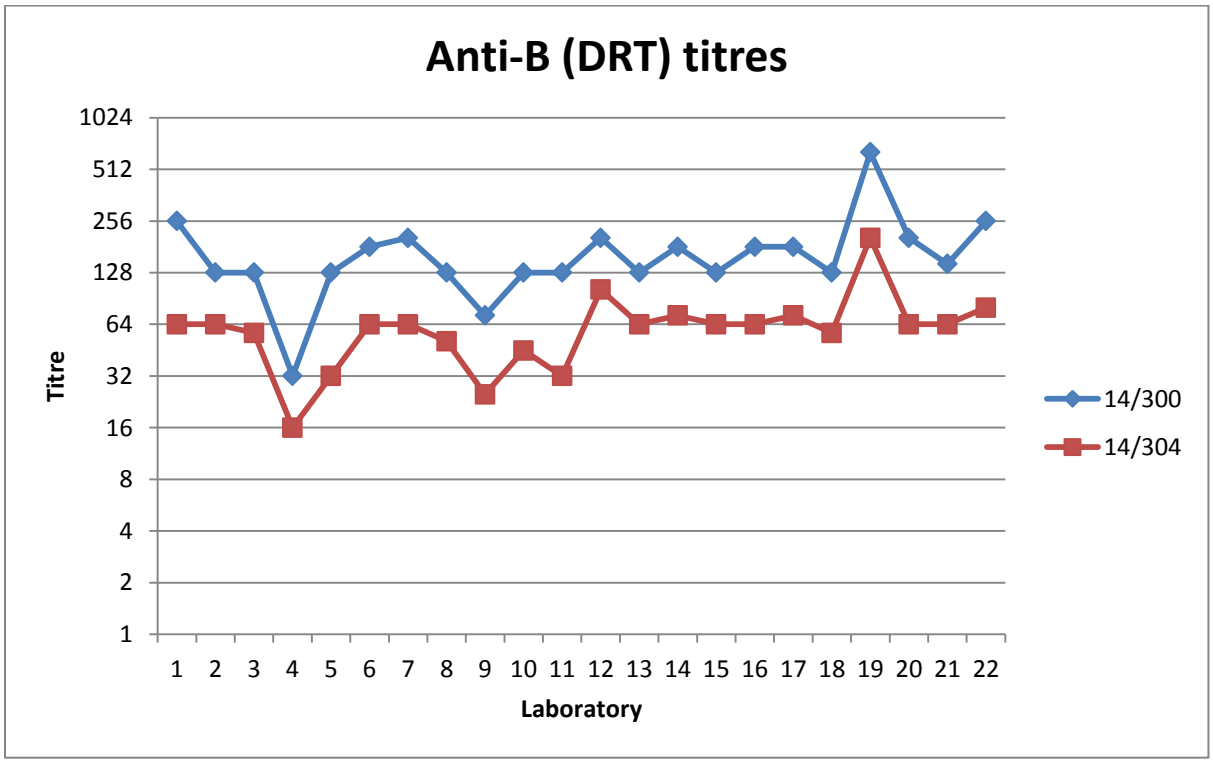


Figure 19 Laboratory mean titres relative to those of 14/300 for 14/304 DRT anti-A

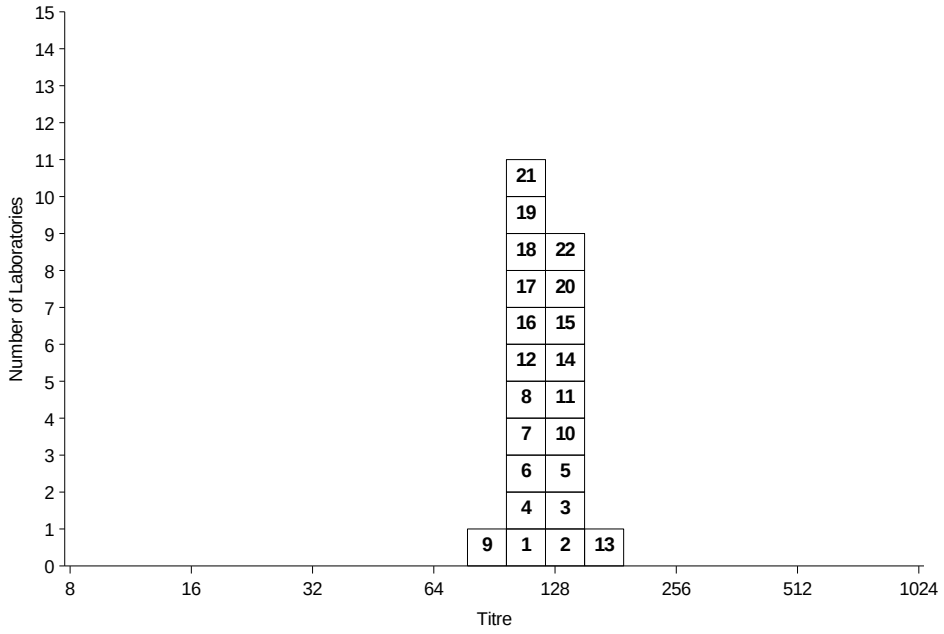


Figure 20 Laboratory mean titres relative to those of 14/300 for 14/304 DRT anti-B

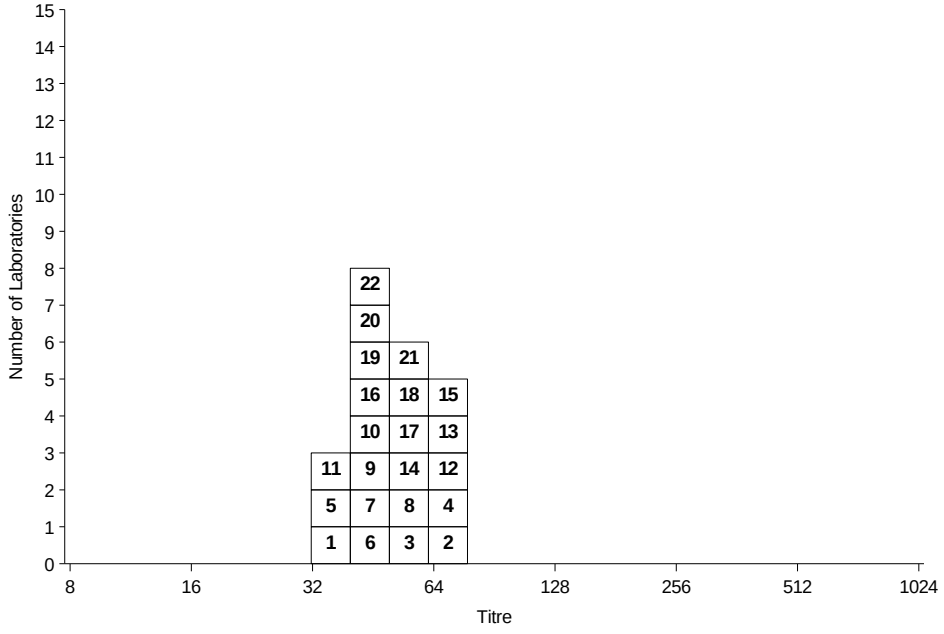


Figure 21 Laboratory mean titres relative to those of 14/300 for 14/304 IAT anti-A

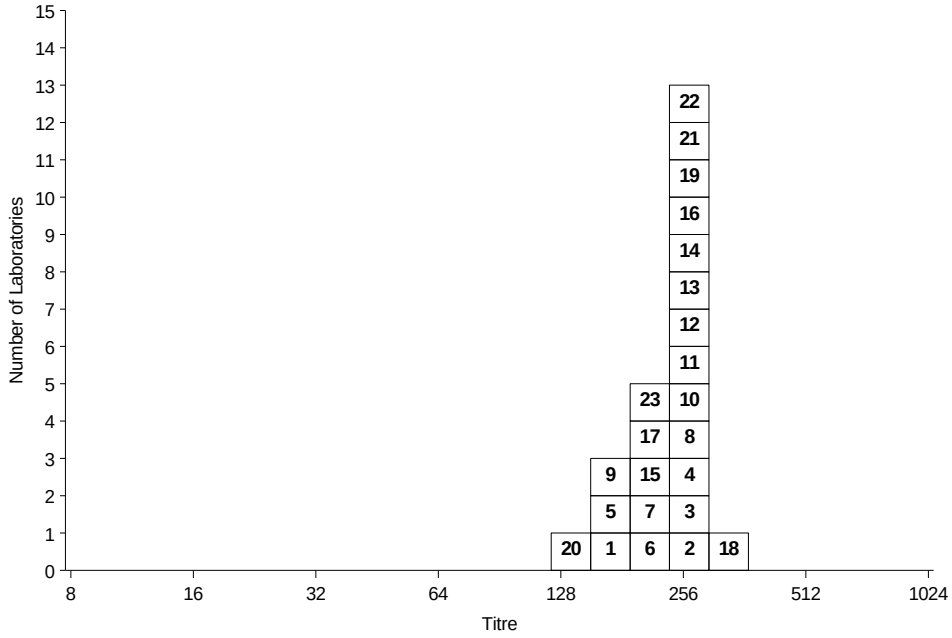


Figure 22 Laboratory mean titres relative to those of 14/300 for 14/304 IAT anti-B

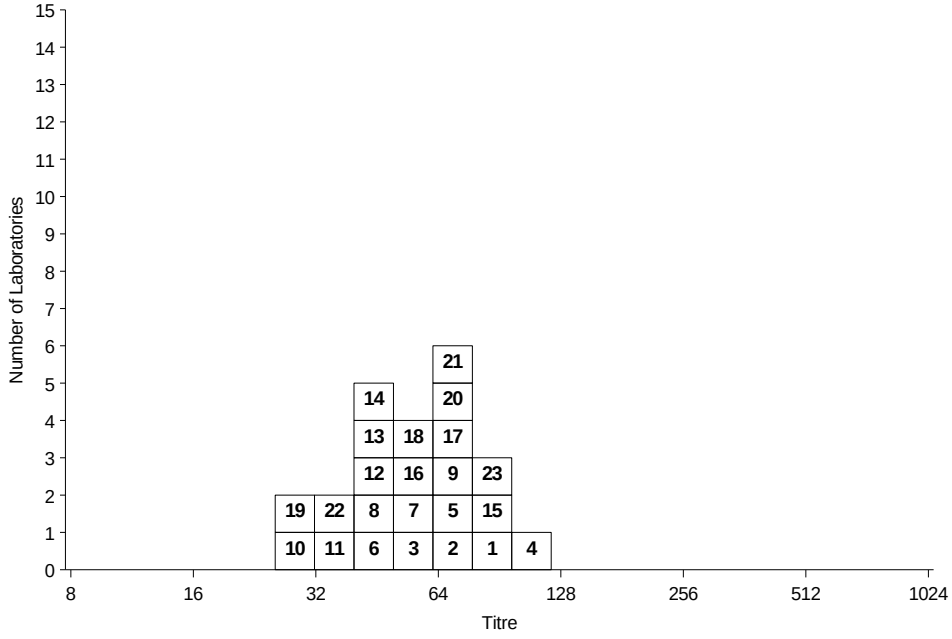


Figure 23 Laboratory mean titres relative to those of 14/300 for 14/208 DRT anti-B

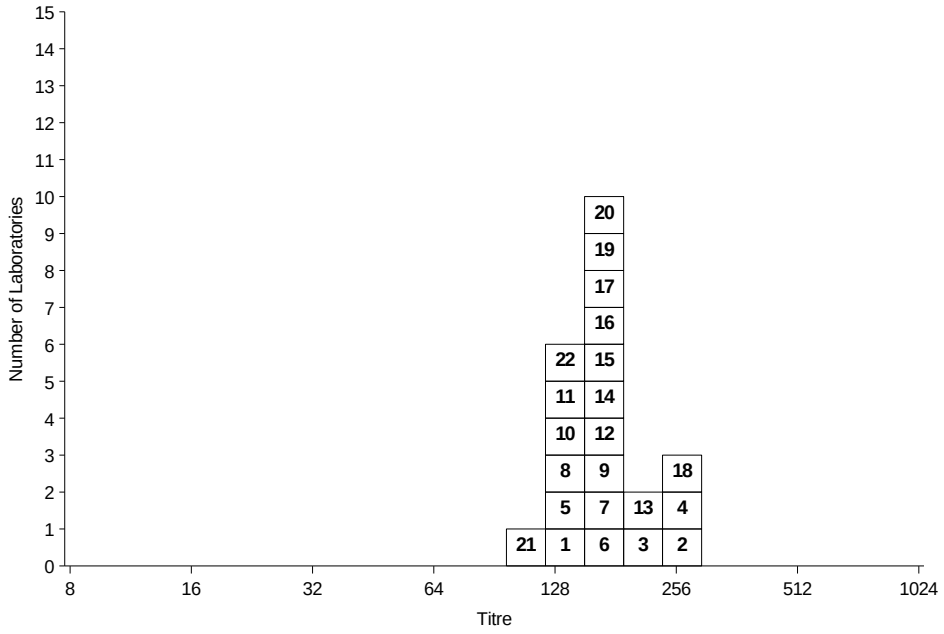


Figure 24 Laboratory mean titres relative to those of 14/300 for 14/208 IAT anti-B

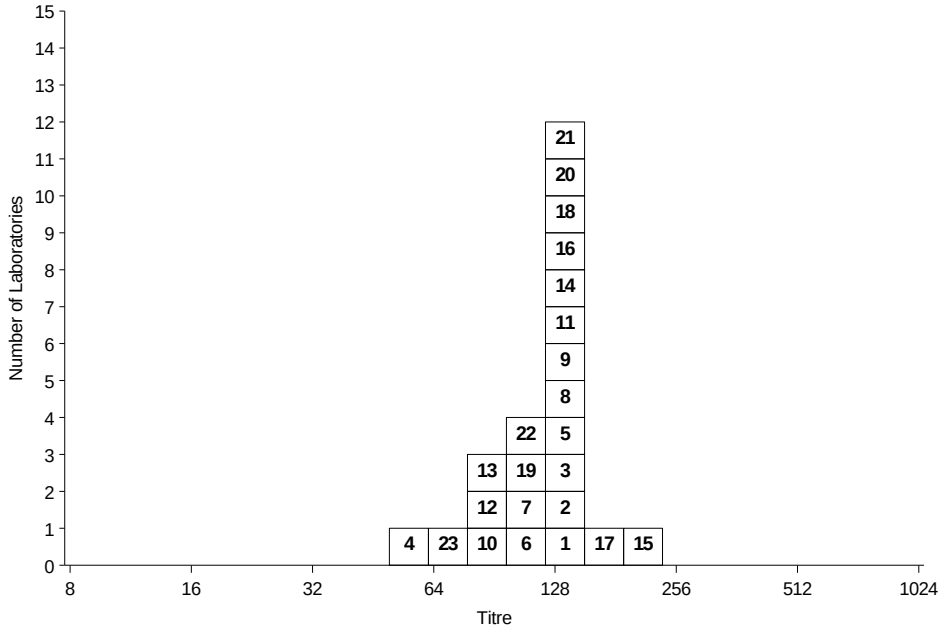


Figure 25 Actual and relative anti-A DRT titres of 14/304

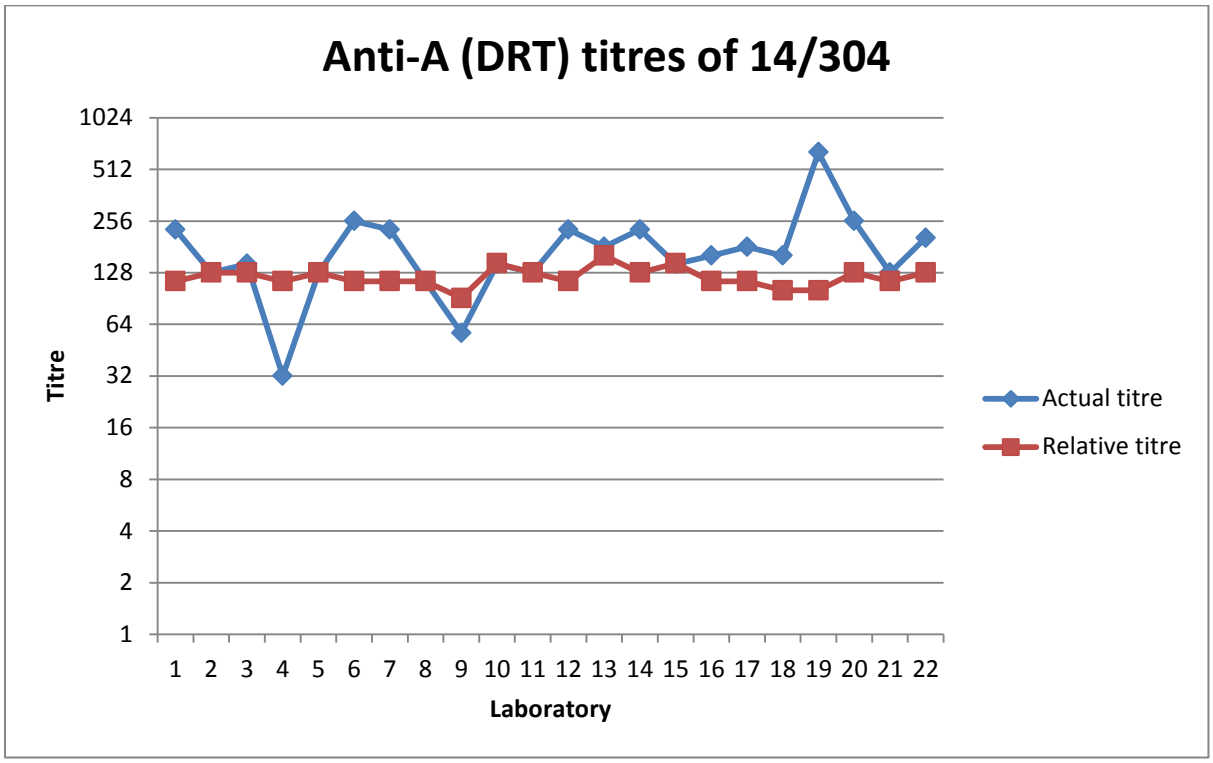


Figure 26 Actual and relative anti-B DRT titres of 14/304

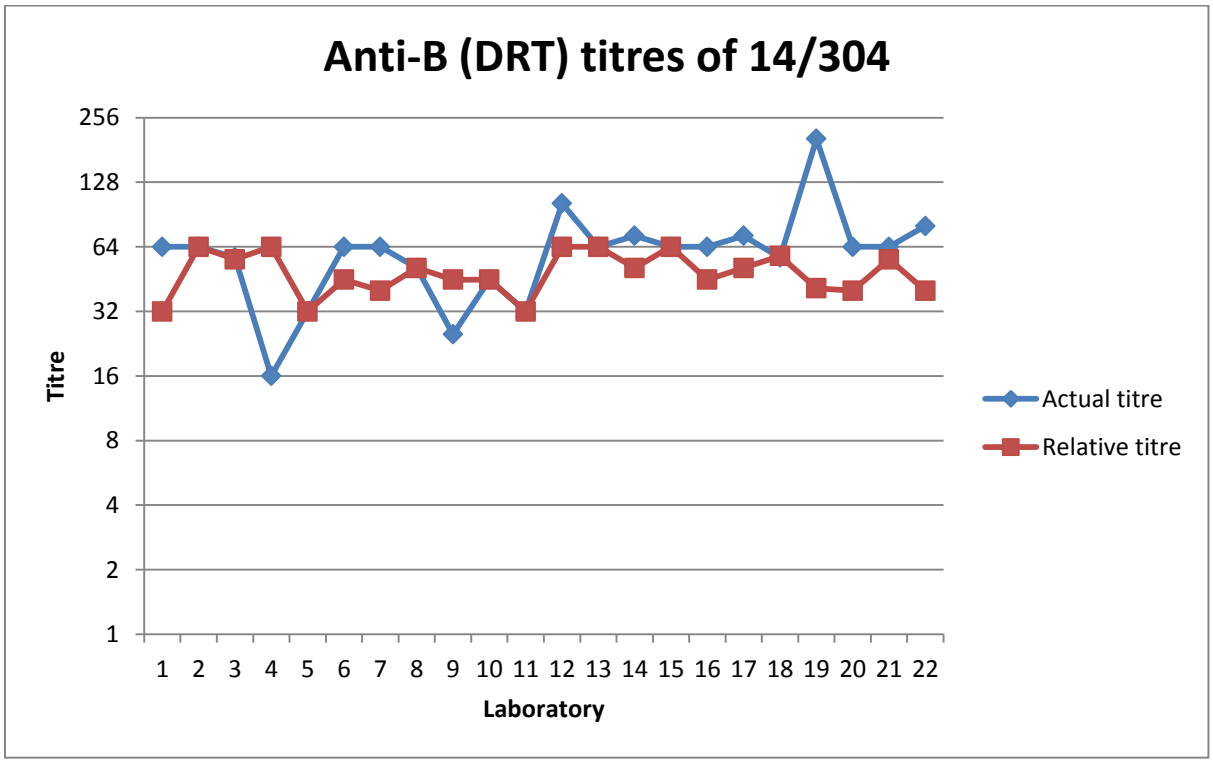


Figure 27 Actual and relative anti-A IAT titres of 14/304

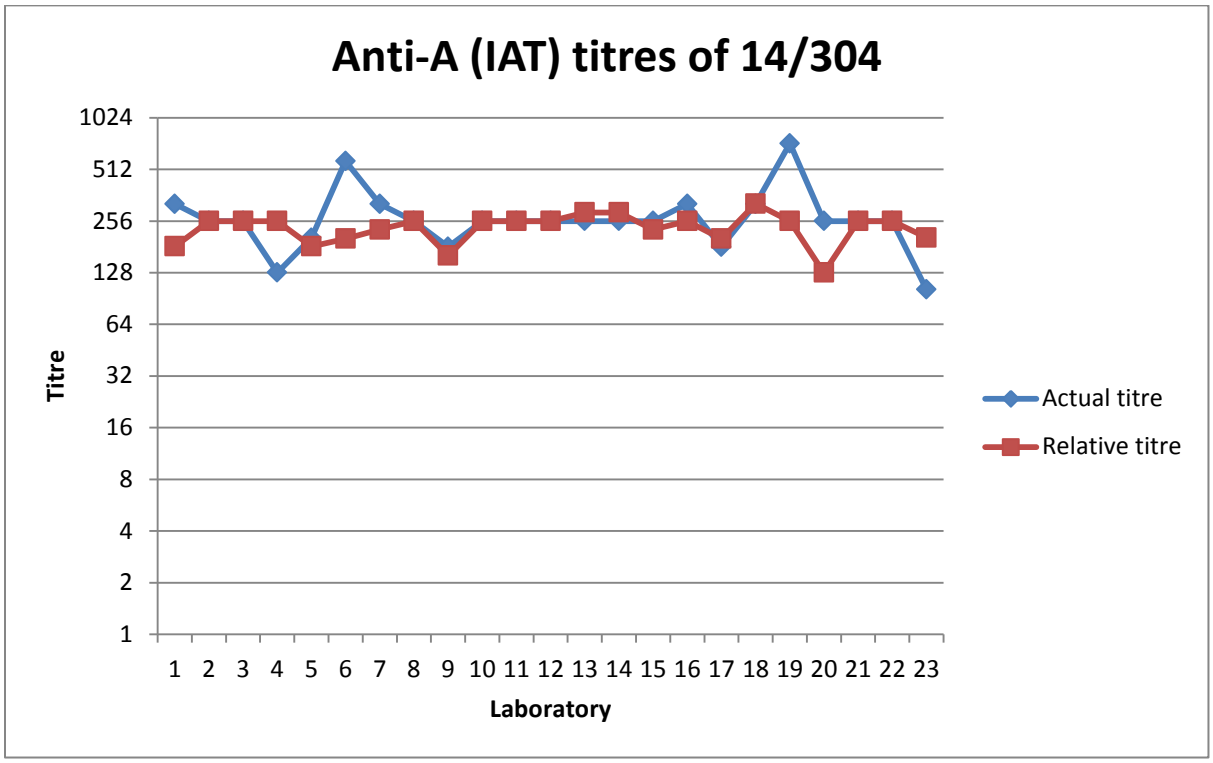


Figure 28 Actual and relative anti-B IAT titres of 14/304

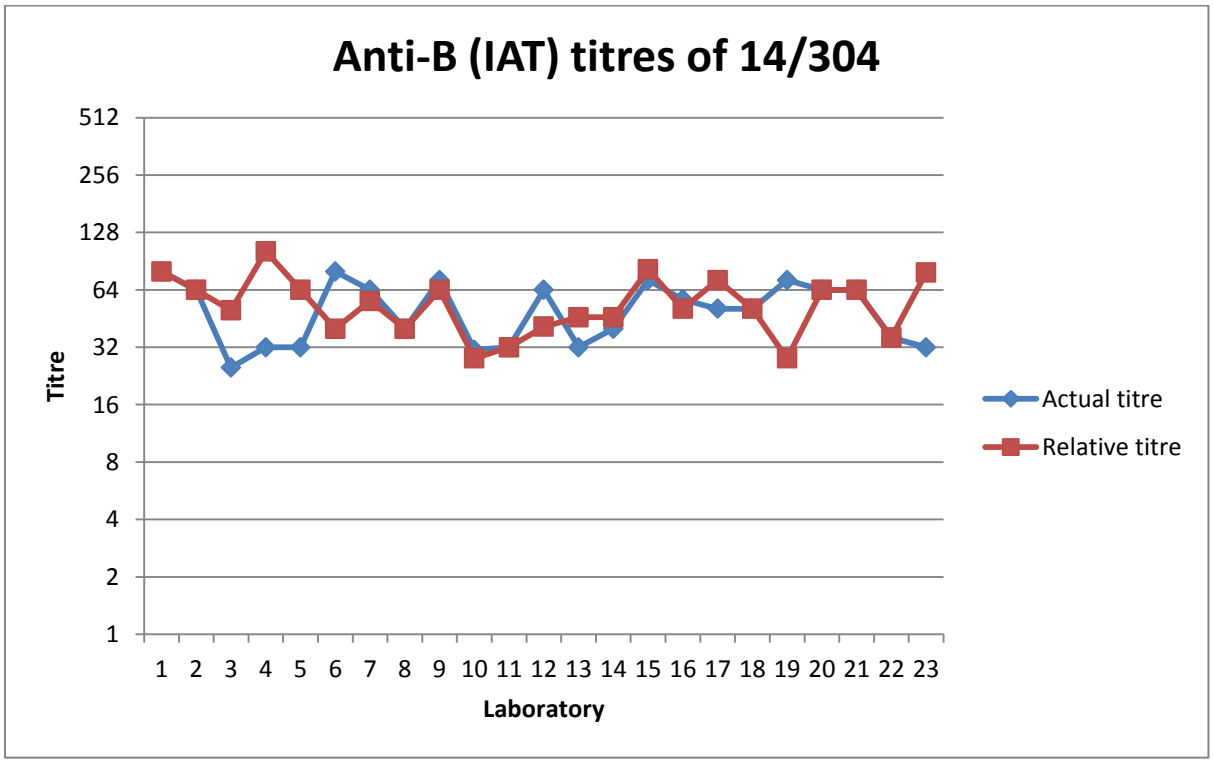


Figure 29 Actual and relative anti-B DRT titres of 14/208

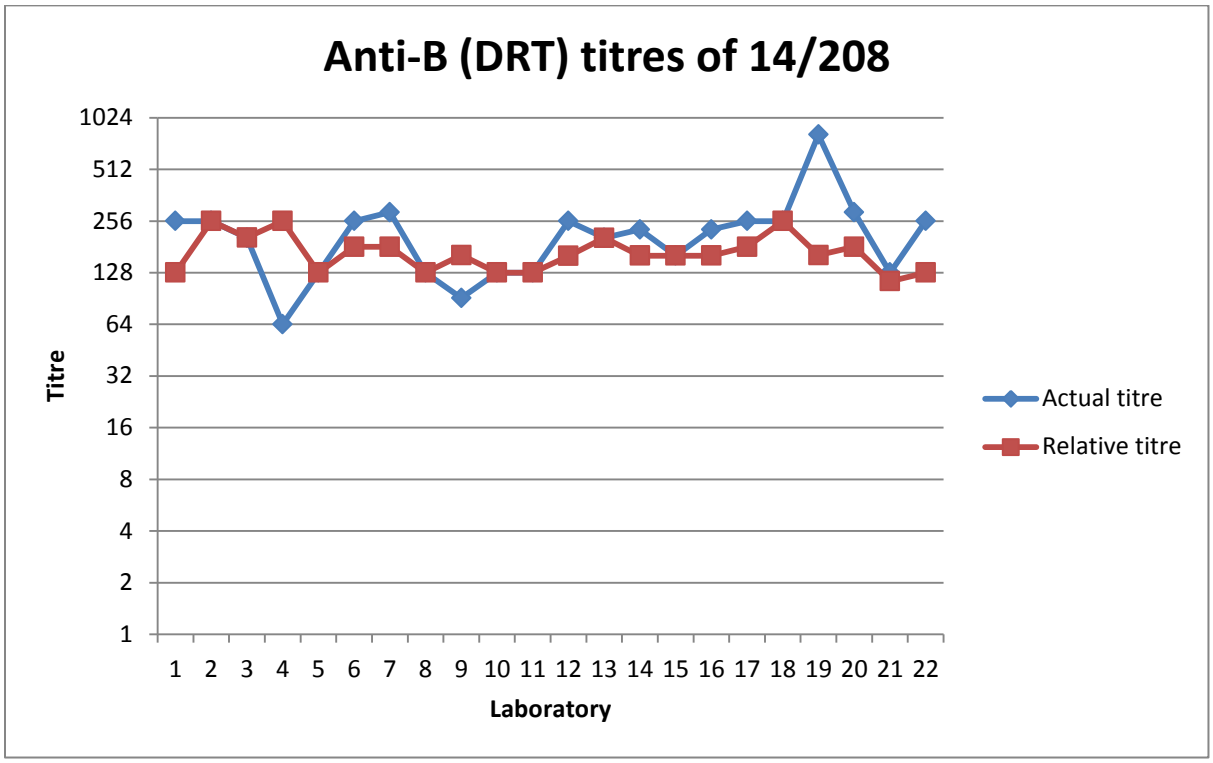


Figure 30 Actual and relative anti-B IAT titres of 14/208

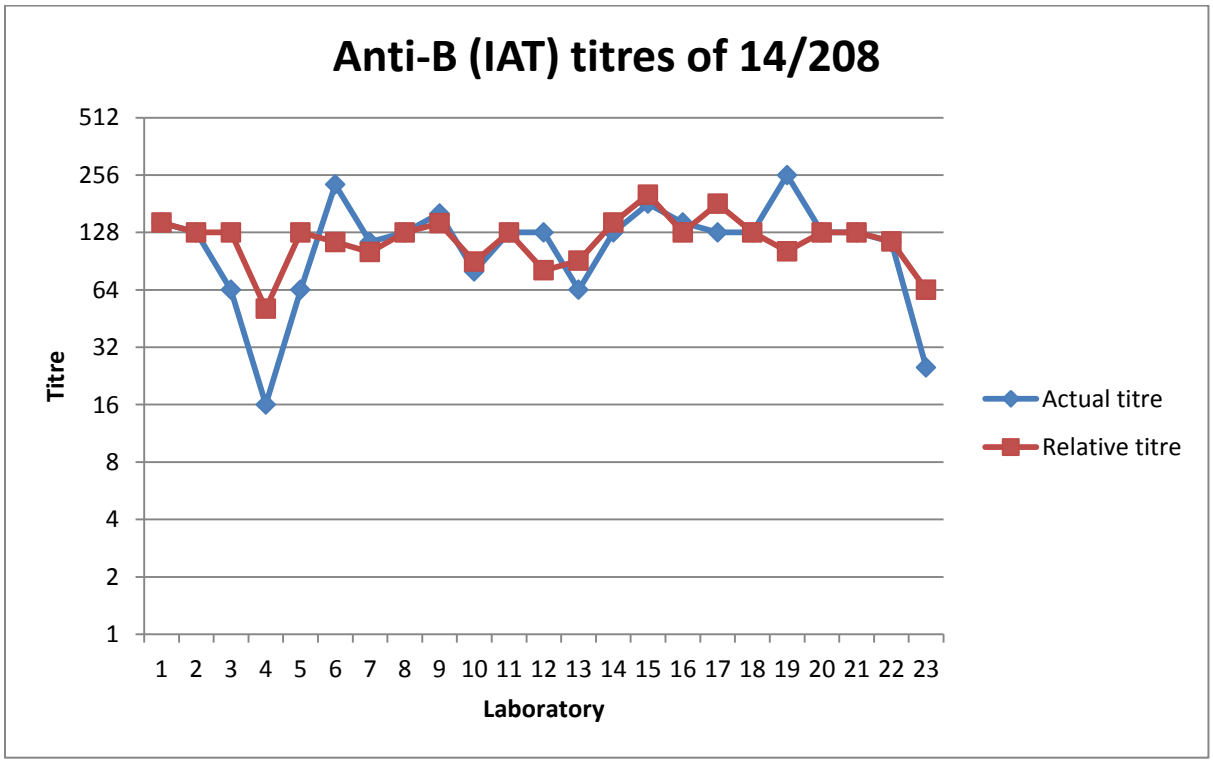
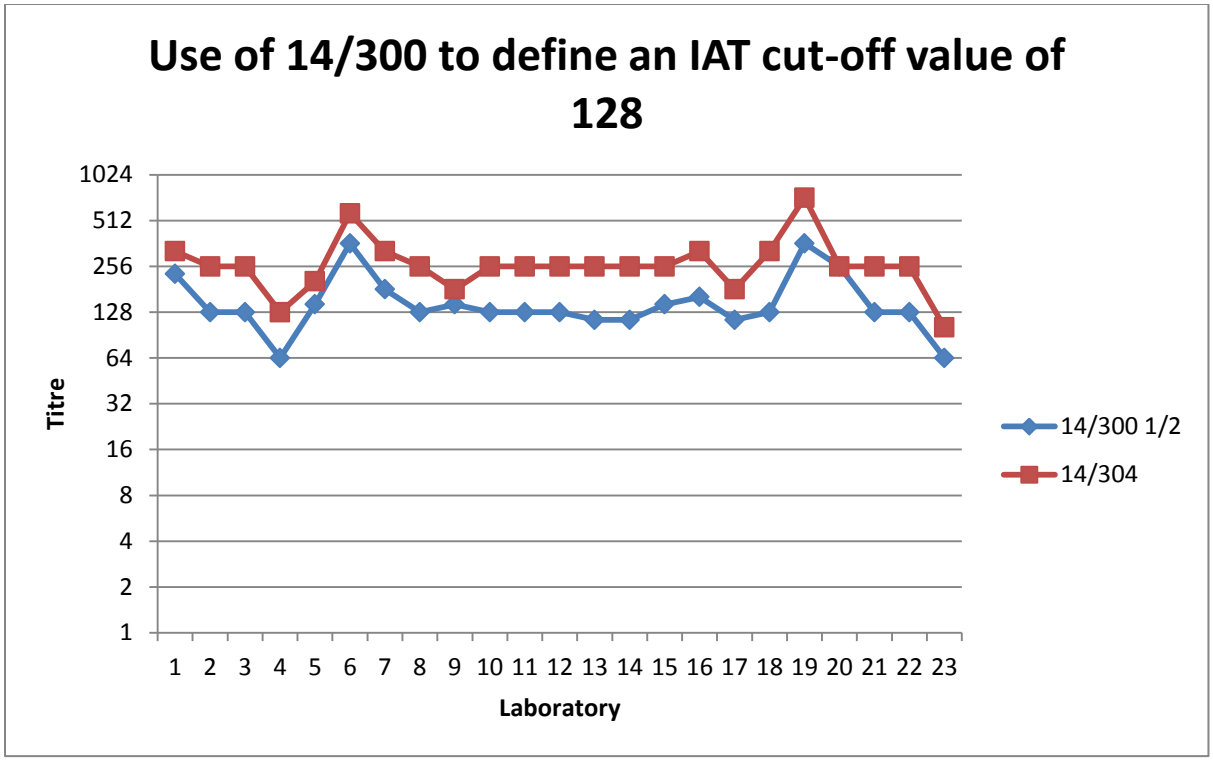


Figure 31 The use of 14/300 to define an IAT cut-off value of 128 (i.e., a 1 in 2 dilution of 14/300 with assigned titre of 256). Preparation 14/304 has a higher titre in 22/23 laboratories.



Appendix 1 Study participants (listed in alphabetical order of country)

Anne Vanhonsebrouck, Shu Win Lee and Anne-Sophie Heroes, Red Cross, Sango Reference Laboratory Immunohematology, Belgium
Ana Hećimović, Croatian Institute of Transfusion Medicine, Croatia
Malla Kuosmanen, Taru Jäske, Sari-Anne Kuula and Suvi Natunen, Finnish Red Cross Blood Service, Finland
Anri Tienhaara and Johanna Salminen, Turku University Central Hospital, Finland
Véronique Deneys, Transfusion, Blood Bank, CHU Dinant Godinne, France
Stuart Blincko and Ellen Preuss, Immucor Medizinische Diagnostik, Germany
CY Tong, Hong Kong Red Cross Blood Transfusion Service, Hong Kong
Antonella Matteocci, Laboratorio di Immunoematologia Eritro-Piastrinica, A.O. S. Camillo-Forlanini, Rome, Italy
Maria Vicarioto, Cinzia Vio and Giustina De Silvestro, Azienda Ospedaliera di Padova, Servizio Immunotrasfusionale, Italy
Séidou Konate and Bamory Dembele, CNTS, Ivory Coast
Haruka Momose, Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, Japan
Lorna Wall and Leigh Mosen, New Zealand Blood Service, New Zealand
Bogumila Michalewska and Justyna Bednarz, Institute of Hematology and Transfusion Medicine, Poland
Anabela Sousa Brites, Imuno-hematologia, Hospital de Santa Maria, Portugal
Margarida Miranda and Luís Miguel Rebelo, Laboratório de Imunohematologia, Portugal
Marjeta Maček Kuanka and Sasa I Trcek, RBC Reference Laboratory, Blood Transfusion Centre of Slovenia, Slovenia
Sabria Nasser Khalfan Al-Hashami, Central Blood Bank, Department of Blood Services, Sultanate of Oman
David Bruce, National Blood Service, RCI Newcastle, UK
Marie McQuade and Lindsay Fergus, West of Scotland Blood Transfusion Service, Clinical Services Laboratories, UK
Ian Skidmore and Jasbir Jutlla, National Blood Service, RCI Birmingham, UK
Susan Galloway and Julie Sim, Product Support Laboratory, Quotient, UK
Tracey Tomlinson, National Blood Service, RCI Colindale, UK
Arnold Mavurayi, UK NEQAS BTLP, UK

Appendix 2 The collaborative study protocol

Reference Reagents to standardise testing for high titre anti-A and anti-B in serum/plasma

COLLABORATIVE STUDY PROTOCOL

1. BACKGROUND

Testing for high titre anti-A and anti-B in serum/plasma is important to:

- Minimise the risk of causing clinically significant haemolysis when blood components rich in plasma containing high titre anti-A/B are transfused to patients of blood groups A, B or AB e.g., platelet concentrates.
- Facilitate mismatched kidney transplants from living donors. These can be performed successfully if the recipient has sufficiently low levels of anti-A and anti-B. Patients may be considered for admission to ABOi transplant programmes if anti-A/anti-B titres are within nominal cut-off values.
- Identify high titre anti-A/B plasma for exclusion from manufacture of blood products such as IVIG, where passive transfer of IgG anti-A/B to recipients can cause haemolysis.

However, anti-A and anti-B titrations are not consistent across laboratories. The availability of reference preparations with nominal anti-A and anti-B titres should ensure greater standardisation of methodology and results, with a later possibility of re-defining cut-off limits for transplant and other purposes when it has become clearer what they should be. NIBSC and UK NEQAS BTLP are collaborating on the production and evaluation of such reference materials.

2. AIM

The aim of the study is to evaluate lyophilised serum and plasma preparations to determine their suitability to serve as WHO Reference Reagent(s), and whether 'mode' titres can be identified from titrations across laboratories.

3. MATERIALS PROVIDED

- 7 ampoules of candidate reference reagent 'High titre anti-A and anti-B in serum', 14/300
- 7 ampoules of reserve preparation 1, 14/304
- 7 ampoules of reserve preparation 2, 14/208

Store all ampoules at -20^{o}C until reconstitution and use.

4. CAUTION

These preparations are not for administration to humans. They consist of lyophilised serum or plasma, tested and found negative for HBsAg, HCV, and anti-HIV. However, as with all materials of biological origin, these preparations should be regarded as potentially hazardous to health. They should be used and discarded according to your own laboratory's safety procedures. Such safety procedures will probably include the wearing of protective gloves and avoiding the generation of aerosols.

5. RECONSTITUTION

Do not reconstitute until the day of assay.
Tap the ampoule gently to collect the material at the bottom end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule.
Care should be taken to avoid cuts and projectile glass fragments that might enter one's eyes. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure.
Reconstitute the ampoule contents with 1.0 ml distilled/deionized water on the day of assay. Allow several minutes, with occasional vortexing, for reconstitution. Transfer the reconstituted contents to a capped tube.
The reconstituted contents are equivalent to neat plasma or serum.

6. TESTS

SUMMARY

Titrate the reconstituted contents of each preparation in 2 independent runs on each of three days (preferably consecutive) using the DiaMed* technique described below, in neutral cards at room temperature (DRT) and by indirect antiglobulin test (IAT) at 37^{o}C, against pooled A_1 and B cells (from at least 3 donors). Include group O cells as a negative control using only the neat plasma and serum. Use a fresh ampoule of each preparation for each day's runs.

PROCEDURE

Titrate the reconstituted contents of each preparation by DRT and IAT using the following procedures:

Prepare doubling dilutions (up to 4096 or to at least two tests with negative results in the titration) from each of the three reconstituted preparations. Make these dilutions in saline (PBS or NaCl) with a minimum volume of 200µl, using an automatic pipette. Use a new tip to dispense each dilution. The volume of each dilution should be sufficient to test against A_1 and B cells.

Using a volumetric pipette, prepare a 0.8 - 1% suspension of pooled A_1 cells and B cells in CellStab (use ID-diluent 2 if CellStab is not available).

*or equivalent gel card system

DRT using NaCl cards

a) Add 50µl of cells suspended in CellStab to each microtube
b) Add 50µl of each plasma or serum dilution to the corresponding microtube
c) Incubate at RT for 15 minutes
d) Centrifuge for 10 minutes in DiaMed centrifuge

IAT using IgG cards

a) Add 50µl of cells suspended in CellStab to each microtube
b) Add 25µl of each plasma or serum dilution to the corresponding microtube
c) Incubate at 37^{o}C for 15 minutes
d) Centrifuge for 10 minutes in DiaMed centrifuge

Test each neat preparation against group O cells, as a negative control, using the techniques described.

DAY 1

Reconstitute and titrate one ampoule of each of 14/300, 14/304 and 14/208 from neat using neutral and IgG DiaMed gel cards (replicate 1).
Repeat the testing in a second, independent assay run with a **fresh dilution series** (replicate 2).
NOTE: If you have insufficient neat volume left to prepare a fresh dilution series, please reconstitute a fresh ampoule.

DAY 2

Reconstitute and titrate one ampoule of each of 14/300, 14/304 and 14/208 from neat using neutral and IgG DiaMed gel cards (replicate 1).
Repeat the testing in a second, independent assay run with a **fresh dilution series** (replicate 2).

DAY 3

Reconstitute and titrate one ampoule of each of 14/300, 14/304 and 14/208 from neat using neutral and IgG DiaMed gel cards (replicate 1).
Repeat the testing in a second, independent assay run with a **fresh dilution series** (replicate 2).

Please note that an additional ampoule of each preparation has been provided in case of losses.

7. RECORDING RESULTS

Please record your results on the accompanying results sheets and return electronic copies to Dr Susan Thorpe (susan.thorpe@nibsc.org) and Mrs Clare Milkins (clare.milkins@whht.nhs.uk).

PLEASE RETURN YOUR RESULTS BY 29TH MAY 2015

Collaborative Study to assess the candidate WHO Reference Reagent 14/300

RESULTS SHEET FOR DAY ……. AMPOULE……. (please copy this sheet as necessary)

Name **Laboratory** **Method**

Date **Red Cell Phenotype/number of donors in pool**

Please record grade of each reaction (0, 0.5, 1, 2, 3, 4)

DRT:DiaMed neutral gel cards – at RT

Serum	neat	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	
A_1 cells Replicate 1														
A_1 cells Replicate 2														
B cells Replicate 1														
B cells Replicate 2														
O cells		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

IAT:DiaMed IAT (IgG) gel cards – at 37^{o}C

Serum	neat	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	
A_1 cells Replicate 1														
A_1 cells Replicate 2														
B cells Replicate 1														
B cells Replicate 2														
O cells		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Collaborative Study to assess reserve preparation 1, 14/304

RESULTS SHEET FOR DAY ……. AMPOULE……. (please copy this sheet as necessary)

Name **Laboratory** **Method**

Date **Red Cell Phenotype/number of donors in pool**

Please record grade of each reaction (0, 0.5, 1, 2, 3, 4)

DRT:DiaMed neutral gel cards – at RT

Plasma	**neat**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**	**1/4096**	
A_1 cells Replicate 1														
A_1 cells Replicate 2														
B cells Replicate 1														
B cells Replicate 2														
O cells		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

IAT:DiaMed IAT (IgG) gel cards – at 37^{o}C

Plasma	**neat**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**	**1/4096**	
A_1 cells Replicate 1														
A_1 cells Replicate 2														
B cells Replicate 1														
B cells Replicate 2														
O cells		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Collaborative Study to assess reserve preparation 2, 14/208

RESULTS SHEET FOR DAY ……. AMPOULE……. (please copy this sheet as necessary)

Name **Laboratory** **Method**

Date **Red Cell Phenotype/number of donors in pool**

Please record grade of each reaction (0, 0.5, 1, 2, 3, 4)

DRT:DiaMed neutral gel cards – at RT

Serum	neat	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	
A_1 cells Replicate 1														
A_1 cells Replicate 2														
B cells Replicate 1														
B cells Replicate 2														
O cells		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

IAT:DiaMed IAT (IgG) gel cards – at 37^{o}C

Serum	neat	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	
A_1 cells Replicate 1														
A_1 cells Replicate 2														
B cells Replicate 1														
B cells Replicate 2														
O cells		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Appendix 3 Draft Instructions for Use

International Ref. Reagent
High titre anti-A and anti-B in serum
NIBSC code: 14/300
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

Testing for high titre anti-A and anti-B in serum/plasma is important to:

- Minimise the risk of causing clinically significant haemolysis when blood components rich in plasma containing high titre anti-A/B are transfused to patients of blood groups A, B or AB e.g., platelet concentrates.
- Facilitate mismatched kidney transplants from living donors. These can be performed successfully if the recipient has sufficiently low levels of anti-A and anti-B. Patients may be considered for admission to ABOi transplant programmes if anti-A/anti-B titres are within nominal cut-off values.
- Identify high titre anti-A/B plasma for exclusion from manufacture of blood products such as IVIG, where passive transfer of IgG anti-A/B to recipients can cause haemolysis.

However, anti-A and anti-B titrations are notoriously inconsistent across laboratories.

Preparation 14/300 was validated in an international collaborative study. The mode anti-A and anti-B titres were both 128 using neutral gel cards at RT, and the mode anti-A and anti-B titres were 256 using IAT gel cards at 37°C. Preparation 14/300, with these nominal titre assignments, is intended to be used in parallel titrations with serum and plasma samples to facilitate inter-laboratory comparisons of titre data, allow sample titres to be reported relative to the reference titres, and allow the establishment of consistent cut-off titres for various applications such as ABO-incompatible renal transplants. The testing of replicate dilution series is recommended to take into account intra-laboratory variation.

2. CAUTION

This preparation is not for administration to humans.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

Preparation 14/300 has nominal anti-A and anti-B titres of 128 for neutral gel cards at RT/IgM titrations, and nominal anti-A and anti-B titres of 256 for IAT gel cards/IgG titrations at 37°C.

4. CONTENTS

Country of origin of biological material: United Kingdom.

Each ampoule contains the lyophilised residue of 1 mL defibrinated plasma (serum), pooled from plasma packs containing high titre anti-A and anti-B.

5. STORAGE

Store unopened ampoules at -20°C or below.

Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.

Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.

Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution

Reconstitute the ampoule contents with 1.0 mL distilled or deionised water (containing 0.02% (w/v) sodium azide for storage at 4°C).

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

9. REFERENCES

N/A

10. ACKNOWLEDGEMENTS

This preparation was produced in collaboration with UK NEQAS for Blood Transfusion Laboratory Practice.

We are grateful to the UK National Blood Service for providing high titre plasma donations.

11. FURTHER INFORMATION

Further information can be obtained as follows;

This material: enquiries@nibsc.org

WHO Biological Standards:
http://www.who.int/biologicals/en/

JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/

Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx

Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_questions.aspx

NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties			
Physical appearance: Lyophilisate		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	Unknown
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	Contains human serum: See caution, Section 2		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.1g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached:** No