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**INTERNATIONAL STANDARD FOR MINIMUM POTENCY OF
ANTI-D BLOOD GROUPING REAGENTS**

**REPORT OF THE INTERNATIONAL COLLABORATIVE STUDY TO EVALUATE
A PROPOSED WHO INTERNATIONAL STANDARD FOR MINIMUM POTENCY
OF ANTI-D BLOOD GROUPING REAGENTS**

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Summary

The candidate international minimum potency reference reagent for anti-D blood grouping reagents, code 99/836, was evaluated against a wide range of commercial anti-D blood grouping reagents in an international collaborative study involving 20 laboratories in 13 countries. Laboratories titrated 99/836 in parallel with as many commercial anti-D blood grouping reagents as were available to them according to specified haemagglutination methodology. The ratios of the mean endpoint titres of the reagents to that of 99/836 within each laboratory were calculated. By international consensus, a 1 in 3 dilution of the reconstituted contents of 99/836 was deemed appropriate to define the minimum acceptable potency of low protein (i.e. monoclonal IgM) anti-D blood grouping reagents. A 1 in 8 dilution of the reconstituted contents of 99/836 was deemed appropriate to define the minimum acceptable potency of high protein (e.g. polyclonal) anti-D blood grouping reagents.

Introduction

The quality of blood grouping reagents is clearly an important factor for safe blood transfusion, yet there is currently no appropriate international standardization of anti-D, anti-A or anti-B blood grouping reagents. Although WHO has International Standards for anti-A, anti-B and anti-D blood typing sera, they were prepared many years ago and represent grouping reagents available at that time i.e. sera from immunized donors. A review conducted by WHO recommended replacement of these materials [1]. Appropriate international reference reagents are needed to in particular to ensure minimum standards of potency of current grouping reagents, particularly as CE marking for *in vitro* diagnostic products is now mandatory under Directive 98/79/EC. Reagents used for typing the major blood groups are listed in Annex II of the Directive, which defines those products which are considered to present a serious health risk in the event of failing to perform as intended, although potency requirements are not specifically stated.

The aim of the present collaborative study was to evaluate a lyophilized monoclonal IgM anti-D preparation using haemagglutination titrations in tubes, to determine, by international consensus, an appropriate dilution of the reconstituted contents to specify the minimum acceptable potency of anti-D blood grouping reagents in tube tests (a preliminary decision to include a range of test methods e.g. tube, slide, microplate [1], was subsequently dropped as it was deemed too difficult to carry out [2]). The candidate minimum potency reagent is not intended to determine specificity. Specificity was considered to be best addressed by cell panels [2].

Materials and methods

Candidate International Minimum Potency Reference Reagent

Culture supernatant containing a high avidity IgM monoclonal anti-D RUM-1 was kindly donated by Serologicals Inc., Livingston, UK. At NIBSC, the supernatant was dispensed into glass ampoules (~1ml/ampoule), lyophilized, subjected to secondary desiccation to remove residual moisture, and coded 99/836. Full details are summarized below:

	<i>Anti-D 99/836</i>
<i>Mean weight of the dispensed solution (number of fill weights measured)</i>	1.0075g (132)
<i>Imprecision of the filling (coefficient of variation)</i>	0.12%
<i>Residual moisture</i>	1.4%
<i>Number of ampoules for distribution as WHO reference reagent</i>	3000

Collaborative study participants

A total of twenty laboratories in thirteen countries worldwide participated in the study (Appendix 1). Each was assigned a code number, which does not reflect the order of listing. The participants included manufacturers of blood grouping reagents and expert immunohaematology laboratories.

Methods

Participants were requested to perform haemagglutination tests in tubes according to detailed methodology protocols provided. Separate protocols were provided for testing of low protein reagents and high protein reagents (Appendices 2 and 3).

Study design

Each participant was provided with 6 ampoules of the candidate international minimum potency reference reagent 99/836 (3 ampoules for testing in each of the low protein and high protein reference methods). Participants were requested to perform haemagglutination titrations of reconstituted 99/836 in parallel with as many anti-D blood grouping reagents as were available to them. They were asked to use the low protein reference method for parallel testing against monoclonal or low protein anti-D reagents and the high protein reference method for parallel testing against high protein anti-D reagents. For each method, participants were requested to test two independent doubling dilution series from neat of reconstituted 99/836 and each reagent in three assays on each of three days using a fresh ampoule of 99/836 each day. Participants were requested to record the grade of the haemagglutination reaction at each dilution on the results sheets provided according to specified criteria, and return the results together with their own estimate of the endpoint titres of 99/836 and reagents.

Statistical analysis

The reciprocal endpoint titres were used as the basis for analysis. All references to 'titre' in this report refer to the reciprocal endpoint titre. For each reagent tested by each laboratory, a geometric mean titre was calculated, by calculating the geometric mean across replicates on each day, and calculating a geometric mean across the three days, giving a single mean titre for each laboratory and reagent. Geometric means were used as the endpoint titration method is based on a doubling dilution scale e.g. the geometric mean of 1:8 and 1:32 is 1:16, the mid-dilution point between them; a simple arithmetic mean would give $(8 + 32)/2 = 20$. The ratio of the mean titre of each reagent to that of 99/836 was then calculated for each laboratory.

Results

Data received

Data were received from twenty laboratories. These laboratories tested 45 different low protein (monoclonal or monoclonal blends) reagents, along with the candidate international minimum potency reference reagent 99/836, using the low protein method. Laboratory 3 tested a total of ten reagents in 3 separate series of tests. Each was treated as if performed by separate laboratories and are referred to as 03A, 03B and 03C. Ten of the laboratories additionally used the high protein method to test ten high protein (mostly polyclonal) reagents. The reagents are listed in Appendices 2 and 3.

The last dilution that gave 1+ grade haemagglutination was taken as the endpoint titre for analysis where the grades were recorded for all dilutions tested, as it was clear from the participants' own reported endpoint titres that some laboratories used other criteria to define endpoint, e.g. the first negative dilution or the last dilution giving +/- grade.

Mean titres of 99/836 and low protein reagents in the low protein reference method

The mean titres obtained by the participants using 99/836 and low protein reagents (monoclonal or monoclonal blends) in the low protein method are shown in Table 1, for each laboratory and each reagent. The mean titres are also plotted in histogram form, where each box represents the titre obtained by a particular laboratory with a particular reagent, in Figure 1. The boxes are labelled with the laboratory code number, and the reagent code. The results for 99/836 are coloured coded differently from the other reagents.

The mean titres for each reagent tested were compared to the concurrently tested candidate international minimum potency reference reagent 99/836 by calculating the ratio of the mean titre of the reagent to that of 99/836. For example, if a reagent had a mean titre of 256, and 99/836 had a mean titre of 128, the ratio is 2. If a reagent had a mean titre of 128, and 99/836 had a mean titre of 128, the ratio is 1. If a reagent had a mean titre of 64, and 99/836 had a mean titre of 128, the ratio is 0.5. The resulting ratios are shown in Table 2 in ascending order of size of ratio, and plotted in histogram form in Figure 2.

Mean titres of 99/836 and high protein reagents in the high protein reference method

The mean titres obtained by the participants using high protein anti-D grouping reagents (e.g. polyclonal reagents) in the high protein method are shown in Table 3, for each laboratory and each reagent. The mean titres are also plotted in histogram form, where each box represents the titre obtained by a particular laboratory with a particular reagent, in Figure 3.

The ratios of the mean titres of high protein reagents to the mean titres of 99/836 are shown in Table 3 and illustrated in histogram form in Figure 4.

Stability of 99/836

Haemagglutination

Ampoules stored at -70°C, -20°C, +4°C +20°C and +37°C for 52 months were assayed concurrently in 6 assays from each of 2 replicate ampoules. The results are given in Table 5. The result of each assay is a titre (eg 128, 256, 512 etc) which is a semi-quantitative measurement. The raw assay data are therefore not suitable for analysis using the usual Arrhenius model of accelerated degradation. However, from the overall geometric mean titres (GMT's) it can be seen that the potency (titre) of the samples stored at +20°C for 52 months has not dropped below the potency of those stored at -70°C, suggesting that the material will be adequately stable at -20°C.

Quantitative anti-D potency assays

Ampoules stored at -70°C, -20°C, +4°C +20°C and +37°C for 55 months were assayed using a competitive enzyme-linked immunoassay (competitive EIA) in which anti-D standard and samples compete with a biotinylated monoclonal anti-D to bind to D-positive red cells. Binding of the biotinylated monoclonal anti-D is then detected using an alkaline phosphatase-labelled ExtrAvidin preparation and carrying out the enzyme reaction. The method was developed at NIBSC for potency assays of therapeutic anti-D immunoglobulin (IgG) products and is a European Pharmacopoeia reference method. Two assays were performed, using 99/836 stored at -70°C as the 'standard'. The potencies of 99/836 stored at the remaining temperatures are expressed as a percentage of the potency of 99/836 stored at -70°C, in Table 6. It can be seen that the potency estimates of 99/836 rise with increased storage temperature with material stored at +37°C having 145% of the activity of material stored at -70°C. This effect may be due to aggregation of the 99/836 which prevents the biotinylated monoclonal anti-D binding through steric hindrance. An alternative explanation is that the 99/836 IgM pentamer has fragmented at +37°C e.g. to monomeric IgM, with individual monomeric units (i.e. IgG-like molecules) or fragments each being capable of binding to the D sites on the red cells and inhibiting binding of the biotinylated monoclonal anti-D. This effect is not seen with the haemagglutination tests as direct haemagglutination of native red cells requires the presence of intact IgM anti-D.

These results clearly show that the stability of 99/836 cannot be estimated or monitored using the potency assay.

Comparison of 99/836 with existing British Minimum Potency Reference Reagent, 91/592

A comparison was made between 99/836 and 91/592 because the latter preparation is also a monoclonal IgM anti-D lyophilizate, and defines an already agreed-upon minimum potency specification for anti-D blood grouping reagents in the UK. Haemagglutination titrations performed at NIBSC from the reconstituted contents of 99/836 and 91/592 showed that the two preparations are of similar potency (Table 7). A three-fold dilution of reconstituted 91/592 defines the recommended British minimum potency specification.

Discussion

From the plots shown in Figures 1 and 3, it is clear that there is considerable variation in haemagglutination endpoint titres for both the low protein and high protein reagents between laboratories (512 fold for each). However, considering the titres for the candidate international minimum potency reference reagent 99/836 alone, there is still wide variation between laboratories (128 fold for the low protein method; 64 fold for the high protein method), suggesting that, despite the use of detailed reference methods, varying sensitivity of the titration tests is the primary source of variability, rather than differences between the reagents tested.

The inter-laboratory variation was not unexpected as haemagglutination tests are notoriously difficult to standardize, and a previous collaborative study to evaluate a candidate EU Community Bureau of Reference (BCR) anti-D reference preparation (subsequently established as the British Minimum Potency Reference Preparation, 91/592) also showed wide inter-laboratory variability [3]. However, as the candidate minimum potency reference reagent 99/836 and the grouping reagents being tested were subjected to the same conditions within each laboratory, any effect of possible variables e.g. red cells, centrifugation speeds, on titres would apply equally to both reference reagent and grouping reagent. For example,

using the low protein method, laboratory 14 has high titres for all reagents tested, including the candidate international minimum potency reference reagent 99/836. Therefore, the ratio of reference reagent titre to grouping reagent titre should be a more reliable indicator of true differences in potency between grouping reagents than reagent titre alone, although it should be noted that the manufacturers' recommended conditions of use were not necessarily exactly the same as the prescribed conditions for the collaborative study.

The plots of the titration ratios (Figures 2 and 4), show much closer agreement between laboratories and reagents, with all ratios falling between 0.125 and 2.5 (except for 3 high protein outliers). However, these figures are based on means over replicates and days. For individual titrations there will be some additional assay variability.

The ratios of the mean titres of the high protein reagents to the mean titre of 99/836 within a laboratory fell within 0.125 and 1 for 11 out of the 14 values obtained from the high protein reagents tested (Figure 4). It was therefore proposed to the participants and scientific advisors that an eight-fold dilution of reconstituted 99/836 should define the minimum potency of high protein anti-D grouping reagents. Eight of the 10 different high protein reagents tested met this specification. All of the participants and scientific advisors who expressed an opinion (14/14) agreed.

The ratios of the mean titres of the low protein reagents to the mean titre of 99/836 within a laboratory fell between 0.25 and 2 for 43 of the 45 low protein anti-D reagents tested (67/69 values; Figure 2). This means that the potencies of the low protein reagents compared to 99/836 were between a 1 in 4 dilution of 99/836 to twice as potent as 99/836. The single most frequent ratio was 1 i.e. the most common mean endpoint titre amongst the low protein reagents tested was the same as that of 99/836.

Almost all the low protein anti-D grouping reagents tested would therefore meet a minimum potency specification of a four-fold dilution of reconstituted 99/836. However, adoption of this specification might encourage many manufacturers to dilute their reagents more than they do so at present. A minimum potency specification corresponding to a three-fold dilution of reconstituted 99/836 would be more in line with the current quality of most low protein anti-D reagents tested. The opinions of the participants and scientific advisors was sought. Of the 18 who expressed an opinion, five were in favour of a four-fold dilution, 12 were in favour of a three-fold dilution, one was in favour of a 16-fold or 32-fold dilution. The haemagglutination data indicate that 99/836 will be adequately stable at -20°C.

However, ongoing real-time haemagglutination studies will be essential to monitor the stability of 99/836. It is proposed to carry out haemagglutination titrations on ampoules of 99/836 stored at -20°C every 6 months and on ampoules stored at -70°C and the elevated temperatures annually to look for developing trends in absolute and relative haemagglutination titres. As 2-fold differences between individual titrations are not necessarily significant, at least 12 replicate titrations will be performed on each sample to look for a consistent drop in overall mean titre resulting from 2-fold or 4-fold drops in individual titrations. In addition, the value of SE-HPLC and/or SDS-PAGE in detecting changes in the structural integrity of the antibody e.g. fragmentation, over time will be investigated.

Although it was pointed out in preliminary discussions that WHO preparations are customarily labelled in IU, and that 'attempts should be made to determine the concentration of anti-D in IU/vial' [2], IU have not been assigned to 99/836 for the following reasons: firstly, potency estimates of monoclonal anti-D are method-dependent; secondly, such an assignment is not only irrelevant, but could be misleading to potential users of the preparations.

Recommendation

It is recommended that 99/836 is established by WHO as the 1st International Standard for Minimum Potency of anti-D blood grouping reagents (in tube tests). With the agreement of all the participants and scientific advisors who expressed an opinion (14/14) it is recommended that an eight-fold dilution of reconstituted 99/836 should define the minimum potency of high protein anti-D blood grouping reagents.

With the agreement of the majority of participants and scientific advisors who expressed an opinion (12/18) it is recommended that a three-fold dilution of reconstituted 99/836 should define the minimum potency of low protein anti-D blood grouping reagents.

It is intended that manufacturers ensure their anti-D blood grouping reagents are at least as potent as 99/836 when reconstituted and diluted as described above, in parallel haemagglutination titrations in tubes. Preparation 99/836 is not intended to determine specificity.

Stocks of 99/836 have been shared with CBER/FDA for distribution as the US Minimum Potency Reference Reagent.

Note.

The British Minimum Potency Reference Reagent, 91/592, will be discontinued upon establishment of the candidate International Minimum Potency Reference Reagent, 99/836.

Acknowledgements

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References

1. Review and replacement of the current WHO International Standards for blood grouping reagents: CBER/FDA, USA, September 1998.
2. Review and update of the WHO International Standards for blood grouping reagents: Amsterdam, the Netherlands, October 1999.
www.who.int/bloodproducts/publications/en/
3. Report on an interlaboratory trial and workshop to produce specifications for BCR reference materials for major blood grouping reagents (anti-A, anti-B and anti-D monoclonals). Provided by Professor ML Scott, IBGRL, Bristol, UK.

Table 1 Laboratory mean titres using low protein reagents in the low protein reference haemagglutination method

Reagent NIBSC code)	01	02	03 A,B,C	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20
99/836	102	512	A:456 B:246 C:287	81	81	1625	276	2048	59	406	813	40	128	5161	246	256	53	161	128	203
abo						813														
brtc																	78			
bsb													161							
bsms													51							
bsnlp													16							
bt38			B:51																	
bt39			A:196																	
bts226											575								45	
bts32						512														
clbm			B:47								342									
clbmx			B:181								181									
csl														8192						
dde																				
dgs1													51	128					39	
dgs2													81							
dgto		512						3251					81							
dmj								3649												
dnc								2299											219	
ds31	98																			
ds36	84																			
ds39	128																			
ds41	106																			
g15-2				81																
g45-3				114			203				323								45	
hconl																				64
i004										323										
i4					114		299										256			
i5					81		228													
ibgrl						1024													287	
irap											2048						474			
irc																				144
ll1			A:98																	
ll2			A:94																	
llde			A:98					256												
m201																144				
mco																138				
mer										406										
obc									28											
obc3									149											
sang		256																		
spr																				51
srbm		256	C:621											8192						64
srgg			C:439										128							323
srtf			C:128											5161						228
tul														5793						

Table 2 Ratios of the mean titres of low protein reagents to the mean titres of 99/836 (ascending order) using the low protein reference haemagglutination method

<i>Reagent</i>	<i>Lab</i>	<i>Ratio</i>	<i>Reagent</i>	<i>Lab</i>	<i>Ratio</i>	<i>Reagent</i>	<i>Lab</i>	<i>Ratio</i>
lldc	08	0.13	hconl	19	0.50	dnc	08	1.12
bsnlp	13	0.13	mco	15	0.56	irc	19	1.12
clbm	03B	0.19	m201	15	0.58	srtp	20	1.12
L12	03A	0.21	ibgrl	06	0.63	tul	14	1.12
bt38	03B	0.21	bts226	11	0.71	bsb	13	1.26
ds41	16	0.21	clbmx	03B	0.73	dgs1	12	1.26
L11	03A	0.21	g45-3	07	0.73	ds39	01	1.26
lldc	03A	0.21	ddc	17	0.73	g45-3	04	1.41
clbmx	11	0.22	i004	10	0.79	i4	05	1.41
bts226	18	0.28	ds36	01	0.82	brtc	17	1.47
G45-3	18	0.28	i5	07	0.82	srgg	03C	1.53
spr	18	0.31	dnc	16	0.86	csl	14	1.59
bts32	06	0.31	ds31	01	0.96	srbm	14	1.59
srbm	20	0.31	dgto	02	1.00	dgto	08	1.59
G45-3	11	0.40	g15-2	04	1.00	srgg	20	1.59
bsms	13	0.40	i5	05	1.00	dmj	08	1.78
clbm	11	0.42	mer	10	1.00	ibgrl	18	1.78
bt39	03A	0.43	dgs1	13	1.00	irap	16	1.85
srtp	03C	0.45	srgg	13	1.00	dgs2	12	2.00
obc	09	0.47	srtp	14	1.00	dgto	12	2.00
sang	02	0.50	i4	16	1.00	srbm	03C	2.16
srbm	02	0.50	ds41	01	1.04	irap	11	2.52
abo	06	0.50	i4	07	1.08	obc3	09	2.52

Table 3 Laboratory mean titres using high protein reagents in the high protein reference haemagglutination method

<i>Reagent (NIBSC code)</i>	<i>Laboratory</i>									
	<i>04</i>	<i>05</i>	<i>06</i>	<i>07</i>	<i>09</i>	<i>11</i>	<i>15</i>	<i>16</i>	<i>17</i>	<i>19</i>
99/836	72	64	4096	256	81	2580	149	219	62	128
G240	9			161						
i2k		20		110				31		
mc			1024							
oslide					47					
clben						1218				
diam						3			2	
gd23						25				
iGm							26			
min							33			
hconh										128

Table 4 Ratios of the mean titres of high protein reagents to the mean titres of 99/836 (ascending order) using the high protein reference haemagglutination method

<i>Reagent</i>	<i>Lab</i>	<i>Ratio</i>
diam	11	0.00
gd23	11	0.01
diam	17	0.02
G240	04	0.13
i2k	16	0.14
iGm	15	0.18
min	15	0.22
mc	06	0.25
i2k	05	0.31
i2k	07	0.43
clben	11	0.47
oslide	09	0.58
G240	07	0.63
hconh	19	1.00

Table 5 Stability of the candidate International Minimum Potency Reference Reagent for anti-D blood grouping reagents, 99/836

<i>Storage Temperature:</i>	<i>Titre with OR_{1r} cells</i>									
	<i>-70°C</i>		<i>-20°C</i>		<i>+4°C</i>		<i>+20°C</i>		<i>+37°C</i>	
<i>Assay Number</i>	<i>Ampoule</i>		<i>Ampoule</i>		<i>Ampoule</i>		<i>Ampoule</i>		<i>Ampoule</i>	
	<i>1</i>	<i>2</i>	<i>1</i>	<i>2</i>	<i>1</i>	<i>2</i>	<i>1</i>	<i>2</i>	<i>1</i>	<i>2</i>
1	256	128	128	256	512	256	128	256	128	64
2	256	256	256	128	256	256	256	128	64	64
3	128	256	512	128	256	256	256	128	64	64
4	256	256	256	256	256	128	256	256	64	32
5	256	128	256	256	256	256	256	256	64	64
6	128	256	256	256	256	256	256	256	64	64
Geometric Mean Titre	203	203	256	203	287	228	228	203	72	57
Overall Geometric Mean Titre	203		228		256		215		64	

Table 6 Competitive EIA potency assays of the candidate International Minimum Potency Reference Reagent for anti-D blood grouping reagents, 99/836, stored at varying temperatures

<i>Storage temperature</i>	<i>Potency expressed as a % of potency of 99/836 stored at -70°C</i>	<i>Fiducial limits</i>
-20	99	93-104
+4	110	104-116
+20	109	103-116
+37	145	136-155

Table 7 Comparison of the candidate International Minimum Potency Reference Reagent for anti-D blood grouping reagents, 99/836, with the British Minimum Potency Reference Reagent, 91/592

<i>Assay number</i>	<i>Titre with OR_{1r} cells</i>			
	<i>91/592</i>		<i>99/836</i>	
	<i>Ampoule</i>		<i>Ampoule</i>	
	<i>1</i>	<i>2</i>	<i>1</i>	<i>2</i>
1	256	256	128	256
2	256	512	256	128
3	128	512	512	128
4	256	256	256	256
5	256	256	256	256
6	256	256	256	256
Geometric mean titre	228	323	256	203
Overall geometric mean titre	271		228	

Figure 1 **Laboratory mean titres of the candidate international minimum potency reference reagent 99/836 and monoclonal or low protein anti-D blood grouping reagents using the low protein reference method**

Figure 2 Ratios of the mean titres of low protein anti-D blood grouping reagents to the mean titres of the candidate international minimum potency reference reagent (CMPRP) 99/836 using the low protein reference method

Figure 3 Laboratory mean titres of the candidate international minimum potency reference reagent 99/836 and high protein anti-D blood grouping reagents using the high protein reference method

Figure 4 Ratios of the mean titres of high protein anti-D blood grouping reagents to the mean titres of the candidate international minimum potency reference reagent (CMPRP) 99/836 using the high protein reference method

Appendix 1 Participants of the collaborative study

(listed in alphabetical order of country)

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Appendix 2 LOW PROTEIN REFERENCE METHOD

1. GENERAL INFORMATION

This method is one of two methods to be used by participants in the WHO collaborative study to assess a proposed new minimum potency reference preparation for anti-D blood grouping reagents. This method should be used for parallel testing of the candidate minimum potency reference preparation 99/836 against **monoclonal IgM or low-protein anti-D reagents**.

2. MATERIALS SUPPLIED BY WHO COLLABORATING CENTRES

- 3 ampoules of candidate WHO minimum potency reference preparation for anti-D, 99/836.

3. MATERIALS TO BE SUPPLIED BY PARTICIPANTS

- Water (deionized or distilled)
- Buffered saline (buffered 0.85 – 0.9% NaCl)
- Buffered saline containing 2% (w/v) bovine serum albumin (BSA)
ie 2 grams BSA per dL of buffered saline, or 1 mL of 30% BSA plus 14 mL buffered saline, or 2 mL of 22% BSA plus 20 mL buffered saline

NOTE: *BSA should not be deliberately polymerized*

- Glass test tubes (10-12 mm diameter x 70-80 mm length)
- Pipettes (transfer, graduated, & semi-automatic with changeable tips)
- R_{1r} red blood cells, pooled from 4 different donors
- As many commercial monoclonal IgM or low protein anti-D blood grouping reagents as are available to you.

4. PREPARATION OF DILUTIONS

Prepare 2 independent series of two-fold serial (doubling) dilutions (1 in 2, 1 in 4, etc) from neat of the reconstituted candidate minimum potency reference preparation, 99/836, and each of the low protein anti-D reagents under test, continuing to 1 in 1024.

- Use buffered saline containing 2% bovine serum albumin as diluent.
- Test tubes should be of a size that facilitates adequate mixing of the contents
- A separate, clean pipette or pipette tip should be used for each dilution to avoid carryover of higher reagent concentrations.

5. PREPARATION OF RED BLOOD CELL SUSPENSIONS

- a) Prepare a pool consisting of equal parts of packed red blood cells from four different donors of the R_{1r} phenotype.
- b) Wash the red cells at least three times in buffered saline or until a clear supernatant is obtained.
- c) Resuspend packed cells to a 2% (v/v) suspension in buffered saline containing 2% BSA e.g., 1 ml packed red blood cells plus 49 ml 2% BSA.

6. THE TEST

- a) Place 1 volume of neat and each of the dilution series, in replicate, of 99/836 and each reagent in a separate, clean test tube.
- b) Add 1 volume of the 2% red cell suspension to each test tube.
- c) Mix the contents of each tube thoroughly and incubate the test tubes at room temperature (19-25 °C) for 5

minutes.

- d) Centrifuge for 1 minute at approximately 1000 rpm (100-125 rcf) or 15 seconds at approximately 3400 rpm (900-1000 rcf) or at a time and speed appropriate for the centrifuge being used or, in the case of a reagent, for the shortest period of time at the lowest speed recommended in the manufacturer's package insert.
 - e) Resuspend the cell button by gentle agitation.
 - f) Macroscopically grade the reactions as described below and record the results on the accompanying results sheet.
- All titrations should be carried to a negative endpoint. The last tube should contain diluent only and serve as a diluent control.

7. INTERPRETATION OF THE TEST

REACTION STRENGTH	GRADE	OBSERVATION
++++	4+/C	Cell button remains in one clump
+++	3+	Cell button dislodges into several large clumps
++	2+	Cell button dislodges into many small clumps
+	1+	Cell button dislodges into granular but definite clumps
±/(+)	D	Cell button dislodges into fine small granules
0	0	Cell button dislodges to give a homogeneous suspension of red blood cells

NB The dilution caused by the addition of the red blood cells should not be considered as contributing to the dilution of the reagent.

Test results should show at least one tube with no agglutination after the endpoint. The diluent control tube should be negative.

Appendix 3 HIGH PROTEIN REFERENCE METHOD

1. GENERAL INFORMATION

This method is one of two methods to be used by participants in the WHO collaborative study to assess a proposed new minimum potency reference preparation for anti-D blood grouping reagents. This method should be used for comparative testing of the candidate minimum potency reference preparation 99/836 against **high protein anti-D reagents that require the use of an Rh control.**

2. MATERIALS SUPPLIED BY WHO COLLABORATING CENTRES

- 3 ampoules of candidate WHO minimum potency reference preparation for anti-D, 99/836.

3. MATERIALS TO BE SUPPLIED BY PARTICIPANTS

- Water (deionized or distilled)
- Buffered saline (buffered 0.85 – 0.9% NaCl)
- Buffered saline containing 2% (w/v) bovine serum albumin (BSA)
ie 2 grams BSA per dL of buffered saline, or 1 mL of 30% BSA plus 14 mL buffered saline, or 2 mL of 22% BSA plus 20 mL buffered saline

NOTE: BSA should not be deliberately polymerized

- Glass test tubes (10-12 mm diameter x 70-80 mm length)
- Pipettes (transfer, graduated, & semi-automatic with changeable tips)
- R_{1r} red blood cells, from 4 different donors
- As many commercial high protein anti-D blood grouping reagents and their respective manufacturer-specified Rh controls as are available to you.

4. PREPARATION OF DILUTIONS OF 99/836

Prepare 2 independent series of two-fold serial (doubling) dilutions (1 in 2, 1 in 4, etc) from neat of the reconstituted candidate minimum potency reference preparation, 99/836, continuing to 1 in 1024.

- Use **buffered saline containing 2% bovine serum albumin as diluent.**
- Test tubes should be of a size that facilitates adequate mixing of the contents.
- A separate, clean pipette or pipette tip should be used for each dilution to avoid carryover of higher reagent concentrations.

5. PREPARATION OF DILUTIONS OF ANTI-D REAGENTS

Prepare 2 independent series of two-fold serial (doubling) dilutions (1 in 2, 1 in 4, etc) beginning with the neat reagent and continuing to 1 in 1024.

- Use the **manufacturer's specified Rh control or buffered saline containing 22% (w/v) BSA as the diluent.**
- Test tubes should be of a size that facilitates adequate mixing of the contents.
- A separate, clean pipette or pipette tip should be used for each to avoid carryover of higher reagent concentrations.

6. PREPARATION OF RED BLOOD CELL SUSPENSIONS

- a) Prepare a pool consisting of equal parts of packed red blood cells from four different donors of the R_{1r}

phenotype.

- b) Wash the red cells at least three times in buffered saline or until a clear supernatant is obtained.
- c) Resuspend packed cells to a 2% (v/v) suspension in buffered saline containing 2% BSA e.g., 1 ml packed red blood cells plus 49 ml 2% BSA.

7. THE TEST

- a) Place 1 volume of neat and each of the dilution series, in replicate, of 99/836 and each reagent in a separate, clean test tube.
 - b) Add 1 volume of the 2% cell suspension to each test tube.
 - c) Mix the contents of each tube thoroughly and incubate the test tubes at 37 ± 1 °C for 15 minutes.
 - d) Centrifuge for 1 minute at approximately 1000 rpm (100-125 rcf) or 15 seconds at approximately 3400 rpm (900-1000 rcf) or at a time and speed appropriate for the centrifuge being used or, in the case of a reagent, for the shortest period of time at the lowest speed recommended in the manufacturer's package insert.
 - e) Resuspend the cell button by gentle agitation.
 - f) Macroscopically grade the reactions as described below and record the results on the accompanying results sheet.
- All titrations should be carried to a negative endpoint. The last tube should contain diluent only and serve as a diluent control.

8. INTERPRETATION OF THE TEST

REACTION STRENGTH	GRADE	OBSERVATION
++++	4+/C	Cell button remains in one clump
+++	3+	Cell button dislodges into several large clumps
++	2+	Cell button dislodges into many small clumps
+	1+	Cell button dislodges into granular but definite clumps
±/(+)	D	Cell button dislodges into fine small granules
0	0	Cell button dislodges to give a homogeneous suspension of red blood cells

NB The dilution caused by the addition of the red blood cells should not be considered as contributing to the dilution of the reagent.

Test results should show at least one tube with no agglutination after the endpoint. The diluent control tube should be negative.

**Appendix 4 Low protein anti-D grouping reagents
(in alphabetical order of code; details supplied by participants)**

Code	Manufacturer	Product name	Clone	Reagent type*	Labs
abo	ABO Limitada	Anti-D	BS 225 (IgM)	LPM	6
brtc	Bio-rad	Transclone anti-D FAST M (IgM) 86370	B9 A4-B2 A6 A6 A1.A1	LPM	17
bsb	Bioscot	AV1TYPE	TH-28 MS-26	LPMB	13
bsms	“	AV1 TYPE anti-D TP	MS-201	LPM	13
bsnlp	“	AV1 TYPE	NELP-4	LPM	13
bt38	Biotec Labs Ltd	Anti-D (IgG+IgM) blend 1/038i	IgG-ESD1 IgM-LDM3	LPMB	3
bt39	“	Anti-D IgM 1/039i	TH-28	LPM	3
bts226	Biotest	Seroclona anti-D (RH1) 226	BS226	LPM	11, 18
bts32	“	Seraclone anti-D 802032	BS 221/H41 11B7 (IgG) BS 232 (IgM)	LPMB	6
clbm	CLB Reagents (Sanquin products)	Pelikloon anti-D IgM K1155	MS-201	LPM	3, 11
clbmx	“	Pelikloon anti-D mix (IgG/IgM) K1157	IgG-MS-26 IgM-TH-28	LPMB	3, 11
csl	CSL Biosciences	Anti-D (IgM) Rhesolve	D-D7-F2-F4 (RUM-1)	LPM	14
ddc	Diamed	DiaClon Anti-D complete (IgM) 11270	Not stated	LPM	17
dgs1	Diagast	Anti-D (RH1) IgM S1 71114	P3X61	LPM	12, 13
dgs2	“	Anti-D IgMS2	HM10	LPM	12
dgto	“	Anti-D(RH1)totem IgM+IgG 71104	P3X61 P3X21223B10 P3X290 P3X35	LPMB	2, 8, 12
dmj	DiaMond-Jordan	Anti-D (monoclonal) complete	Not stated	LPM	8

		monoclonal IgM, IgG-Abs			
dnc	Dominion Biologicals Ltd	Novaclone Anti-D (IgM+IgG) monoclonal blend 0350	D175-2 (IgM) D415-1E4 (IgG)	LPMB	8, 16
ds31	Diagnostics scotland	Anti-D alpha Z031	LDM1	LPM	1
ds36	“	Anti-D beta Z036	LDM3	LPM	1
ds39	“	Anti-D optimum Z039	LDM1 and ESD1M	LPMB	1
ds41	“	Anti-D blend Z041	LDM3 and ESD1	LPMB	1, 16
G15-2	Gamma Biologicals Inc.	Anti-D (monoclonal) by slide, tube or microwell test	GAMA401	LPM	4
G45-3	“	Anti-D (monoclonal blend) by slide, tube or microwell test 420501	GAMA401 (IgM) F8D8 (IgG)	LPMB	4, 7, 11, 18
hconl	National Institute of Infectious Diseases	NIID control anti- D antibody, low protein method (house control)	Monoclonal blend, low protein	LPMB	19
i004	Immucor Inc	Anti-D IgM+IgG IMC004 6/99	?85-2 (IgM) 415-?F4 (IgG)	LPMB	10
i4	“	Anti-D series 4 006413	MS-201 (IgM) MS-26 (IgG)	LPMB	5, 7, 16
i5	“	Anti-D series 5 006415	TH-28 (IgM) MS-26 (IgG)	LPMB	5, 7
ibgrl	IBGRL	Anti-D	RUM-1	LPM	6, 18
irap	Immucor Medizinische Diagnostik GmbH	Immucor anti-D rapid D00381.01.1	RUM-1	LPM	11, 16
irc	International Reagents Corporation	Anti-D antibody (IgM) 11450	Monoclonal	LPM	19
Ll1	Lorne Labs Ltd	Anti-D (IgM) clone 1 730010	RUM-1	LPM	3
Ll2	“	Anti-D (IgM) clone 2 710010	MS-201	LPM	3
lldc	“	Anti-D Duoclone	IgM-RS1126	LPMB	3, 8

		740010	IgG-MS-26		
M201	Medion Diagnostics GmbH	IgM anti-D	MS-201	LPM	15
mco	“	Combi anti-D	LDM3 ESD1	LPMB	15
mer	Meridian Diagnostics Europe GmbH	Gullclone anti-D blended	TH-28 (IgM) MS-26 (IgG)	LPMB	10
obc	Ortho-Clinical Diagnostics Inc	Anti-D (Anti- Rho)(Monoclonal- polyclonal blend) BioClone 717380	MAD-2 blend with polyclonal	LPMPB	9, 19
obc3	“	Anti-D (Anti- RH1) IgM+IgG monoclonal BioClone	D7B8 (IgM) H1121G6 (IgG) LORIFA (IgG)	LPMB	9
sang	Elitech	A-D,M/G blend	SANG-1505	LPMB	2
spr	Spin react SA	Anti-D	Monoclonal blend	LPMB	18
srbm	Serologicals	Monoclonal IgM+IgG blend, BM	TH-28/MS-26	LPMB	2, 3, 14
srgg	“	Monoclonal IgM anti-D GG	RUM-1	LPM	3, 13
srtp	“	Monoclonal IgM TP	MS-201	LPM	3, 14
tul	Tulip Diag- nostics Ltd	Eryclone anti-D (Rho) IgM	Not stated	LPM	14

* LPM = low protein monoclonal; LPMB = low protein monoclonal blend; LPMPB = low protein monoclonal/polyclonal blend

**Appendix 5 High protein anti-D grouping reagents
(in alphabetical order of code; details supplied by participants)**

Code	Manufacturer	Product name	Reagent type	Labs
clben	CLB Reagents (Sanquin products)	Pelikloon anti-D enhanced (IgG/IgM) K1153	Enhanced monoclonal blend (IgG – MS-26 IgM – TH-28)	11
diam	Diamed	Anti-RH1 (D) 11210	Polyclonal	11, 17
G240	Gamma Biologicals Inc.	Anti-D by slide or modified tube test 720201	Polyclonal	4, 7
gd23	“	Anti-D	Polyclonal	11
hconh	National Institute of Infectious Diseases	NIID control anti-D antibody, high protein method	Monoclonal blend, high protein	19
i2k	Immucor Inc	Anti-D slide, rapid tube and microplate (high protein) 204-5, 002289	Polyclonal	5, 7, 16
iGm	Immucor Medizinische Diagnostik GmbH	Anti-D incomplete	Polyclonal high protein	15
mc	MediCuba	Anti-D MD 40025	Polyclonal	6
min	Medion Diagnostics GmbH	Anti-D incomplete	Polyclonal high protein	15
oslide	Ortho-Clinical Diagnostics Inc	Anti-D (Anti-Rho) for slide and modified tube test 712380	Polyclonal high protein	9

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