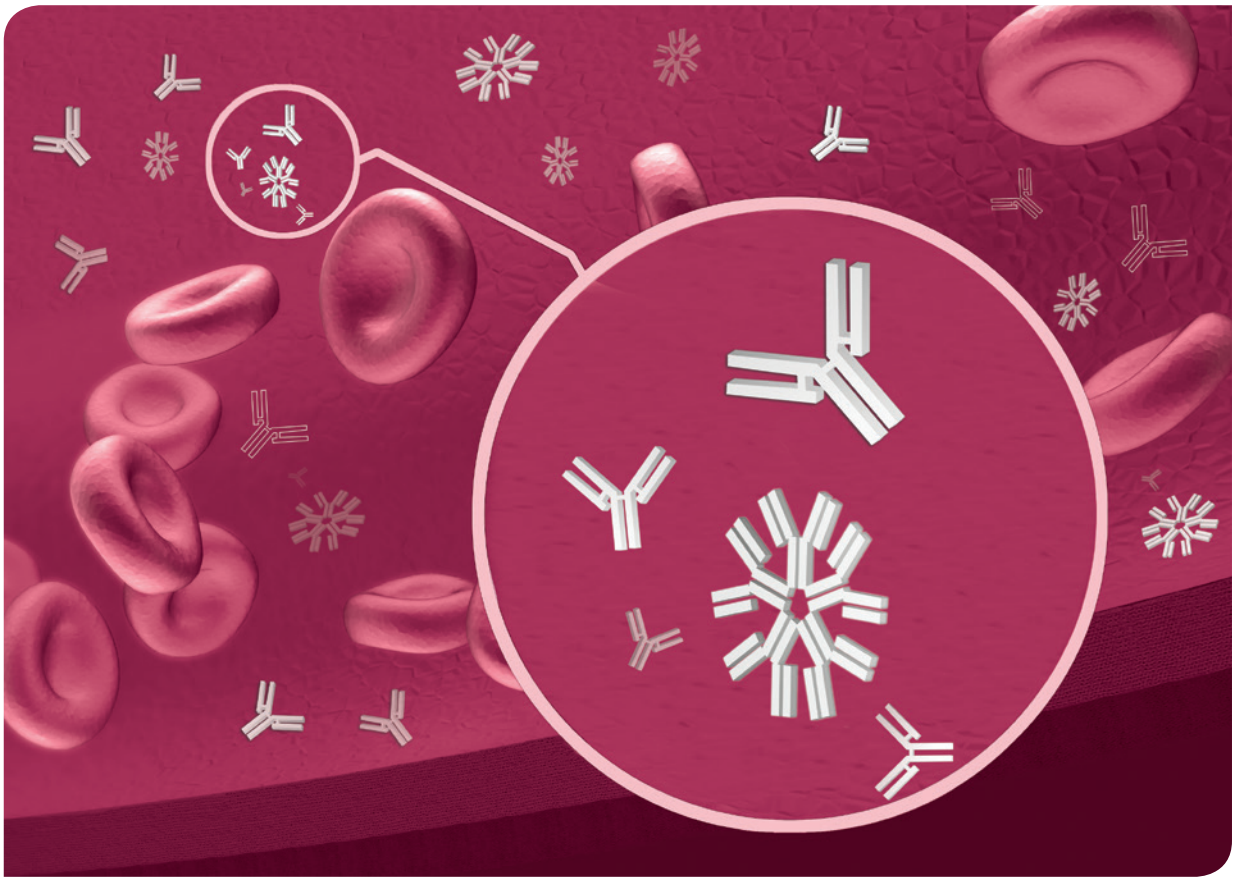

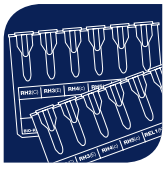

Titration Procedures

A Guide to Good Laboratory Practices

BIO-RAD

Identification

Document

Titration Procedures - A Guide to Good Laboratory Practices
2019/10

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Titration Procedures

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1 ▪ Introduction

Antibody titration is a semiquantitative method of determining antibody concentration; it may be undertaken for a number of reasons:

- To monitor alloantibodies of potential importance in pregnant women. This is the most common reason for antibody titration.
- Titration of ABO antibodies is undertaken to allow clinical assessment of the feasibility of ABO mismatched transplant, and monitoring of treatment to reduce antibody titre in preparation for ABO mismatched transplant.
- To investigate so-called High Titre Low Avidity “HTLA” antibodies. For example, antibodies now known to belong to the Knops blood group system.
- To determine the relative specificity of autoantibodies in Warm Antibody Immune Hemolytic Anemia (WAIHA), e.g. in an eluate.
  In Cold Agglutinin Syndrome (CAS), titration may help in elucidating the specificity of autoanti-I exhibiting a broad thermal range.
- To determine the antigen status of direct antiglobulin test (DAT) positive cells when reagents are unavailable or unsuccessful in removing IgG from the cells to be typed.
- To assess the antigen site density of red cell reagents, although this has largely been superseded by more accurate methods such as flow cytometry.
- To assess the characteristics of a new antiserum or compare a new batch with a previous one.

2 ▪ Good Laboratory Practices

Titration usually is described as an “inherently imprecise procedure”.

As for any other laboratory method, standardization is therefore imperative in order to minimize variables that may interfere with an accurate and meaningful result. The results also depend on the equilibrium constant of the antibody itself in addition to its concentration, making control of the test parameters very important. Key factors to consider include the following items.

2.1 Preparation of Dilutions

Titration uses a two-fold serial dilution of the sample from neat (undiluted), 1:2, 1:4, … to 1:2048 (12 tubes). The number of dilutions prepared can be adapted to what is most convenient for the method to be followed.

Titrations are more accurate when larger volumes are used when preparing the dilutions.

The tubes - as contained in the racks in the ID-System - should be unused, clean and large enough to allow proper and thorough mixing of each dilution in the series.

A properly calibrated and regularly maintained volumetric pipette with a clean tip should be used to prepare each of the dilution in the series.

On IH-500, a dedicated ID-Titration Rack is used. The instrument contains a “properly calibrated and maintained pipette” and the needles are properly washed when making the serial two-fold dilutions.

2.2 Controls

It is recommended to titrate a standard antibody of known concentration or better with an “expected” titre to validate the titration procedure (see tables in the examples on next pages). This can be performed as a routine control of the method as well. It will help to minimize variability affecting the technical performance.

When the titration is being performed in the context of pregnancy follow-up, the first sample - usually from around the 12th week - establishes the base line titre. All subsequent titrations should be carried out in parallel with the previous sample. Again this will minimize variation inherent to the technique and it will provide for a more accurate reflection of the antibody status.

Samples should be stored frozen (at -20°C or lower). Special attention should be taken when thawing samples: concentration gradients are produced during thawing, as the concentrated solution melts first and then runs down inside the tube along the inner wall.

After thawing, the tube should be inverted several times. Before testing, the tube should be examined for presence of any undissolved material. When present, it usually helps to carefully warm the sample at 37°C to redissolve this[1].

[1] *Young DS. Effects of Preanalytical Variables on Clinical Laboratory Tests. 2nd ed. Washington, DC: AACC Press; 1997: 4- 180, 181,533*

2.3 Choice of the Red Cell Reagents

Whenever possible, the same antigen source should be used each time, as there is great heterogeneity of antigen expression even among cells of apparently the same phenotype.

For instance, R^1R^2 cells (DCcEe, RH:1,2,3,4,5) can have between 23,000 and 31,000 copies[2] of the D-antigen. Because it is impossible for most laboratories to have the same red cells available for the follow-up during a whole pregnancy, it is recommended to use a pool of three cells (volume by volume) of the same phenotype. Together with performing the titration in parallel with the previous sample, this minimizes variability.

The selected cells should be prepared accurately according to the test method used, e.g. 0.8% in the appropriate diluent for the ID-System. The appropriate volumes should be dispensed using a calibrated volumetric pipette.

Whether the red cells used for titration should be single or double dose, in particular during pregnancy, is a debate. Most but not all countries would recommend the use of single dose cells (heterozygous expression). The reasoning behind this is that the fetus of an allo-immunized mother will never be homozygous for the gene in question. Therefore the use of single dose cells will more closely mimic the in vivo situation.

Two things are clear:

- Laboratories should use the cells recommended by their local guidelines
- The key to standardization is to use the same phenotype as a standard procedure each time

2.4 Antibody Should Be Identified Prior to Titration

It does not make sense to perform titration just on the basis of a positive antibody screening test.

As explained in section 2.3, a proper choice of the red cell reagents can only be made when the antibody is properly identified.

In addition there may be multiple antibodies present, and if not properly identified they may interfere with the titration result.

If more than one antibody is present, the selection of the red cell reagents for titration must be done carefully, to ensure that the titre of each antibody is assessed independently.

For example, for a mixture of anti-K+Fy^a (anti-KEL1+FY1), two independent titrations should be done, the first using K-;Fy(a+b+) / (KEL:-1;FY:1,2) red cells and the second using K+k+;Fy(a-) / (KEL:1.2;FY.-1) red cells.

2.5 Results Interpretation

When dispensing the properly prepared dilutions, it is recommended to start with the highest dilution ending with the neat undiluted sample, to prevent any possibility of carry over due to a strong antibody.

Clear endpoints should be defined. Usually the last dilution that produces a 1+ (+ in the ID-System) is considered to be the endpoint. The reciprocal of this defined end point is then the titre of the antibody in the sample: if this dilution is 1:32 than the titre is 32 (and not 1:32).

There usually are weaker reactions observed beyond this dilution showing a 1+ (+ in ID) reaction: these are looked at when reporting a score instead of a titre (see tables in the examples on next pages).

[2] *Daniels, G., 2013. Human Blood Groups. 3rd ed. Chichester, UK: John Wiley & Sons*

2.6 Scoring and Reporting of Results

Titres are often simply reported as the reciprocal of the define endpoint, e.g. a titre of 64. However, this does not always reflect the true strength of an antibody or point to a significant rise in titre. Scoring the reactions may help to overcome this problem. Scoring methods differ, some using scores from 12 to 0 (most commonly), others more simply from 5 to 0. The actual method is not important, as long as the same system is always used. The scoring system should be incorporated in the laboratory SOP, together with the definition of what is a significant rise in titre, a significant rise in score.

2.7 Examples

Example 1 ▪ Titres and Scores

		Sample Dilution												
		Neat	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Titre	Endpoint	Score
Sample 1	Strength	3+	3+	3+	2+	2+	2+	1+	+/-	+/-	-	64		
	Score	10	10	10	8	8	8	5	2	2	0		1:256	63
Sample 2	Strength	4+	4+	4+	3+	3+	3+	2+	1+	+/-	-	128		
	Score	12	12	12	10	10	10	8	5	2	0		1:256	81
Sample 3	Strength	1+	1+	1+	1+	+/-	+/-	+/-	+/-	+/-	-	8		
	Score	5	5	5	5	2	2	2	2	2	0		1:256	30

In the above example, all three samples are showing reactions up to and including dilution 1:256.

The last dilution showing a clear 1+ reaction is different for each one of them, leading to different titres reported for each sample.

Assigning scores to all of the individual reaction strengths leads three different scores.

Sample 3 is an example of an HTLA-antibody (see example 4 at page 10).

Example 2 ▪ Interpretation: Significant Rise in Titre e.g. in Pregnancy

	Titre of "today's" sample	Titre of "previous" sample	Titre of standard, expected titre = 32	Conclusion
a	4	4	32 › ✓	No titre increase Titre of 4
b	32	4	32 › ✓	Significant titre increase from 4 to 32
c	16	16	64 › ✓	No titre increase Titre of 16
d	8	4	32 › ✓	No titre increase 1 step is no increase

It is necessary for a laboratory to establish a baseline titre using a standard with an expected titre specific to the method.

In the above example the expected titre of the standard is 32; this titre should be reproducible within ±1 dilution, i.e. 16-32-64 are acceptable titres, as illustrated.
Note that the "previous" sample, stored frozen, is titrated again in parallel to the newly drawn sample (e.g. 2 weeks later).

Sample a: the standard shows a titre of 32 › ✓; both the previous sample and the newly drawn sample show a titre of 4.

Conclusion: no titre increase.

Sample b: the standard shows a titre of 32 › ✓; the previous sample shows a titre of 4, the new sample has a titre of 32.

Conclusion: a significant (more than 2 dilutions) titre increase.

Sample c: the standard shows a titre of 64 › ✓; both the previous sample and the newly drawn sample show a titre of 16.

Conclusion: no titre increase.

Sample d: the standard shows a titre of 32 › ✓; the first sample shows a titre of 4, the new sample has a titre of 8.

Conclusion: a 1 step increase is not significant, no titre increase.

Note: In UK[3], some antibodies are quantified (IU/ml) rather than titred.

- Anti-D (RH1) less than 4 IU/ml › HDFN unlikely
- Anti-D (RH1) between 4-15 IU/ml › Moderate risk of HDFN
- Anti-D (RH1) More than 15 IU/ml › High risk of hydrops fetalis

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- Anti-c (RH4) less than 7.5 IU/ml › continue to monitor
- Anti-c (RH4) between 7.5 to 20 IU/ml › Risk of moderate HDFN, refer to specialist unit
- Anti-c (RH4) more than 20 IU/ml › Risk of severe HDFN, refer to specialist unit

[3] *https://www.transfusionguidelines.org/red-book (accessed Jan 24, 2018)*

Example 3 ▪ ABO Titration in the context of ABO incompatible (ABOi) organ transplants (kidneys and others). ABOi haemopoetic stem cell transplants and ABO HDFN

In most countries there is a lack of available kidneys for transplantation. The use of ABOi kidneys was investigated as a possible solution: generally speaking HLA matching is more important than ABO matching and there is now a consensus that "*ABOi-Kidney Transplant (KT) outcome is comparable to ABO compatible KT*"[4]. Assessment of anti-A/B antibody titre is crucial in ABOi-KT. It guides the effectiveness of operative preconditioning and determines the period to permit transplantation. In addition, post-transplant monitoring helps early detection of antibody-mediated rejection by antibody rebound.

Transplant protocols are different between transplantation centres, between regions, between countries. Efforts are on-going to establish minimum transplant candidate ABO titres. Overall titres are ranging from 32 to 128 (Gel method). When the titres are above these limits, patients are treated to "desensitize" to ABO with medication, antigen-specific immunoadsorption, plasma exchange, or double-filtration plasmapheresis.

When performing titrations on ABO antibodies, it is important to realize that samples will always contain mixtures of IgM and IgG. Most laboratories will perform the titrations in both at room temperature (usually referred to as DRT, Direct agglutination at Room Temperature) and in IAT.

It is imperative to understand that:

- DRT will preferentially detect IgM, but that some IgG will occasionally directly agglutinate
- IAT will preferentially detect IgG, but occasionally there will be some agglutination by IgM

Some laboratories want to specifically measure IgG. In that case, the sample should be treated with DTT (or 2-ME). These molecules will inactivate IgM molecules without affecting the IgG molecules. At the negative side, it needs to be understood the method itself introduces another cause of variability to the system.

Reports from exercises in the UK (NEQAS reference to be added October 2014 workshop) are showing:

- A standard technique is recommended using ID-Cards IAT and Neutral:
  - it allows more reproducible results
  - the results show a tighter range, i.e. closer to method median
- IAT is more reproducible than DRT
- DTT introduces variability
- It is recommend to develop a standard

That standard has been developed and is available at NIBSC (High titre anti-A and anti-B in serum - WHO Reference Reagent 14/300[5]).

The testing of replicate dilution series of both 14/300 and the samples is recommended to take into account intra-laboratory variation in repeat titrations.

Finally, it is important to make sure an antibody detections test is done. Any alloantibody present may interfere with the ABOi titration should the antigen toward which the antibody is directed be present on the cells used for titration.

For example, when an alloanti-K (KEL1) is present, one should make sure the red cell reagents are K-, when titrating using the IAT:

For example, when a cold reactive alloanti-P1 (P1PK1) is present, one should make sure the red cell reagents are P1- (P1PK:-1), when titrating using DRT.

[4] *Muramatsu M et al., 2014. ABO incompatible renal transplants*
[5] *http://www.nibsc.org/products/brm_product_catalogue/detail_page.aspx?catid=14/300 (accessed Jan 24, 2018)*

Example 4 ▪ HTLA (High Titre, Low Avidity)

The "term" HTLA is a colloquial statement intended to describe "rather roughly" the serological results of this group of antibodies and not intended to define antibody specificity (quote from the late JJ Moulds).

Common characteristics are: weak, variable reactions in IAT and in some cases hard to reproduce. The antigens are of high frequency, so almost always the identification panel shows weak reactions in IAT with all cells, making it difficult to draw conclusions.

Most of the antibodies in this "group" are clinically insignificant. Specificities include anti-Ch, anti-Rg, anti-JMH, anti-Csa, anti-Yka, and others.

Titrations can provide a clue to whether the antibody is likely to be one that exhibits HTLA characteristics, i.e. usually 1+ reactions (Low Avidity) in IAT and titres > 32.

Dilution	Neat	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Low Titre Low Avidity	+/-	0	0	0	0	0	0	0
Low Titre High Avidity	2+	2+	1+	0	0	0	0	0
High Titre Low Avidity	1+	1+	1+	+/-	+/-	+/-	+/-	0
High Titre High Avidity	3+	3+	3+	2+	2+	1+	1+	+/-

In this table, the HTLA characteristic (dark blue) is clearly demonstrated: it is "unexpected" to observe that a weakly reactive antibody shows reactions up to the dilution 1:64.

Example 5 Autoantibodies[6] ▪ Reactions of a Typical Auto-Anti-I associated with CAS

		Titre											
Temp	Cells from	2	4	8	16	32	64	128	256	512	1024	2048	4096
4°C	Adult	4+	4+	4+	4+	4+	4+	4+	3+	3+	2+	1+	0
	Cord	4+	4+	4+	4+	4+	4+	3+	3+	2+	1+	0	0
RT	Adult	4+	4+	3+	3+	3+	2+	1+	0.5+	0	0	0	0
	Cord	1+	1+	0.5+	0	0	0	0	0	0	0	0	0
37°C	Adult	1+	0.5+	0	0	0	0	0	0	0	0	0	0
	Cord	0	0	0	0	0	0	0	0	0	0	0	0

In this example, 6 titrations are performed at three different temperatures, using adult ($I+^{s}$, $i+^{w}$) and cord ($I+^{w}$, $i+^{s}$) cells.

The titres with both cells are highest at 4°C, lower at RT, and lowest at 37°C. At each of the different temperatures, the titre versus the adult cells is higher than the titre versus the cord cells.

[6] *Petz, LD and Garratty G, 2004. Immune Hemolytic Anemias. 2nd ed. Philadelphia: Churchill Livingstone*

Example 6 ▪ Reactions of an Eluate or Serum/Plasma showing Anti-e (RH5) "Relative Specificity"

	Titre							
Rh Phenotype	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
dce/dce rr RH:-1,-2,-3,4,5	4+	3+	3+	2+	2+	**1+**	0	0
DCe/DCe R_1R_1 RH:1,2,-3,-4,5	4+	3+	3+	2+	2+	**1+**	0	0
DcE/DcE R_2R_2 RH:-1,-2,3,4,-5	3+	2+	**1+**	0	0	0	0	0

In this example, the sample is titrated versus E-e+ (rr and R_1R_1) and E+e- (R_2R_2) cells.
The results can be interpreted as showing "relative specificity" for the e (RH5) antigen.

Such reaction should confirmed by testing against more examples of, in this case, e+ (RH:5) and e- (RH:-5) cells.

Example 7 ▪ Phenotyping DAT+ Red Blood Cells when using IAT Reactive Reagents

As a first step, laboratories would try to dissociate the IgG autoantibodies e.g. using chloroquine. Sometimes such treatment will be unsuccessful.

In such cases, typing can be carried out by measuring the amount of specific antibody left in the typing reagent after adsorption with the patient's red blood cells, and comparing it using adsorptions on known single and double dose red blood cells.

	Dilutions of adsorbed anti-Fy^a reagent						
Red blood cells used to adsorb anti-Fy^a reagent	**Neat**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**Score**
Fy(a+b+) / Fy:1,2	2+	**1+**	**1+**	+/-	0	0	21
Fy(a+b-) / Fy:1,-2	**1+**	**1+**	0	0	0	0	10
Fy(a-b+) / Fy:-1,2	3+	3+	2+	2+	**1+**	0	41
Patient	2+	**1+**	0	0	0	0	13

These results indicate that the patient is probably Fy(a+) / (Fy:1).

Example 8 ▪ Microtitration of Anti-D (RH1) expressed in ng/ml

Microtitration of anti-D (RH1) allows to distinguish allo-anti-D from passively acquired anti-D (RH1) after injection of Ig anti-D (prophylaxis).

Particularly in France, this microtitration is indicated when pregnant women or women who recently delivered present with:

- No decrease in reaction strength between two consecutive antibody detection test (antibody screening)
- No information available on a possible antenatal injection with Ig anti-D (RH1)
- An incoherent reaction strength relative to the date of injection of the Ig anti-D (RH1) (e.g. > 3+ for more than 1 month, > 2+ if more than 50 days post-injection, > 1+ if more than 70 days post-injection)

In this method, both a known standard, usually 28 ng/ml is titrated along with the sample.

Calculations are then performed based on Brossard et al.[7].

					Calculations		
	Dilutions	**1:4**	**1:8**	**1:16**	**A x**	**B =**	**C**
Standard 28 ng/ml	**Concentration (ng/ml)**	7	3.5	1.75	Reciprocal of the last reactive dilution of the sample (< ++++)*	Concentration of the standard showing the same reaction strength as (A)	Concentration of anti-D (RH1) in the sample
	Strength	++++	+++	+			
Sample	**1**	+	0	0	4	1.75	7 ng/ml
	2	++++	++++	+++	16	3.5	56 ng/ml
	3	++++	++++	++++	> 16	7	> 112 ng/ml
	4	+++	+	0	8	1.75	14 ng/ml

- Note, that in this procedure the samples and the standard are not tested to the normal titration endpoint of +; the reaction strength of the last reactive dilution of the sample is related to the concentration of the dilution of the standard exhibiting the same reaction strength.
  - **Sample 1**: the "last" reactive dilution is 1:4 with a reaction strength of + and the concentration of the dilution of the standard exhibiting the same reaction strength of + is 1.75 ng/ml.
    Logically, the concentration in the sample is:
    4 x 1.75 ng/ml = 7 ng/ml.
  - **Sample 3**: reaction strengths of the sample dilution are ++++. To observe reaction < ++++, the sample would have to be diluted further. That is not done; the conclusion is simply > 16, and the calculation than becomes: > 16 x 7 ng/ml = > 112 ng/ml.
- The calculated values are interpreted using tables with the expected concentrations of anti-D (RH1), taking into account:
  - The concentration of the anti-D (RH1) injected, e.g. 200 µg or 300 µg
  - The number of doses injected
  - The delay (days) between the date of injection and the draw date of the sample

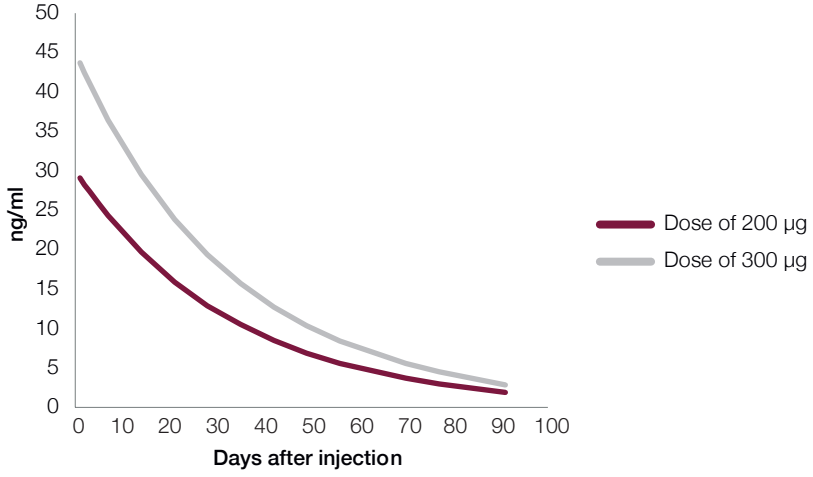
[7] *Brossard, Y, 2002. M. Diagnostic et suivi prénatals des allo-immunisations érythrocytaires. Feuill Biol, 43, 11-17*

- The graph (Figure 1) below represents the expected values post-injection after 1 dose of anti-D (RH1) of 200 µg (dark red) and 300 µg (grey).
- Possible interpretations:
  - If the concentration of anti-D (RH1) in the sample is clearly < the expected value, then it is quite probable that the anti-D (RH1) is the passively acquired antibody
  - If the concentration of anti-D (RH1) in the sample is clearly > the expected value, a new sample should be tested a few weeks later (usually 2 weeks), because an allo-immunisation anti-D (RH1) may be taking place

In case there is no information on the exact date of injection of the prophylactic dose, care should be taken on how the result is reported. Ultimately a new sample - with proper information on the dates - should be tested.

- Finally, as indicated in chapter 2.4, it is important to make sure the presence of any other alloantibody is tested

Figure 1



3 ▪ Frequently Asked Questions (FAQ)

Questions	Answers
Will I obtain different titres when performing titration on IH-500 diluting the samples with the ID-Titration Solution?	It is good laboratory practice to review and redefine the "normal" values when there is a significant change in a test procedure. There may implications on the actions the treating physicians may decide on. This accounts for titrations as well. This means that critical titres for antibodies detected during pregnancy, or ABO titres in case of an ABOi mismatch, must be reviewed and communicated to the clinicians.
Why in a context of pregnancy is it so important to compare the titres from previous sample and the new one?	The titration of the previous sample in parallel with the new one must be done to verify that the change in titre is not due to the variability in the method.
Why two titres obtained in different test conditions cannot be compared?	As explained before, the variability in titration is reality, both between and within techniques used. Titres obtained in different tests are inherently biased and should not be compared.
Why should I reset my threshold values if I change my titration method?	The technology used will impact the titre; as titres obtained with different test methods should not be compared, threshold titres (or critical titres) should be reestablished.
If I want to reestablish my threshold titres, how should I proceed?	Titration should be carried out with a significant number of antibodies with the old and the new method. Care should be taken to properly select the antibodies by Ig class, IgG or IgM (if relevant like in ABOi transplants). It is also important to include antibodies with titres close to the threshold titre.
Why should I perform a new antibody identification on each new sample?	Immunized patients are more prone to producing new antibodies. These newly formed antibodies may interfere with the titration as explained in section 2.4 on page 6; therefore it is important to confirm or exclude their presence or absence.

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