

## INTERPRETING DISCREPANT RhD TYPING RESULTS

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### OVERVIEW

The presence or absence of RhD antigen (Rh-positive or Rh-negative) is determined by mixing anti-D reagent with red cells. If RhD antigen is present, Anti-D will bind to D antigen and cause agglutination of the red cells. Although this appears simple, there are many variables impacting Rh typing results:

- RhD antigen expression
- *RHD* gene mutations
- Reagent differences
- Method variability

Rh typing is further complicated by the fact that interpretation is dependent upon why an individual is being tested:

- Obstetrical patient
- Transfusion recipient
- Cord blood
- Blood donor

This paper provides explanation for discrepant RhD typing and the variable interpretation that accompanies each group of individuals being tested. Discrepant RhD typing cannot be avoided but understanding why it occurs and how to interpret the testing are key to understanding whether to call an individual Rh-positive or Rh-negative.

RhD typing today is a challenge due to several variables that can affect the outcome of the test including the D protein itself and the reagents and methods available to detect D antigen. Discrepant results with the patient's Rh type on file are a relatively common occurrence. The following scenarios may sound familiar.

- Patient has record of being typed several years ago as Rh-positive in your facility but now appears Rh-negative.
- Patient has record of being typed several years ago as Rh-negative in your facility but now appears Rh-positive.
- Sample types Rh-negative in your facility but has a donor card that states they are Rh-positive.
- Sample types Rh-positive in your facility but clinic/hospital laboratory typed them Rh-negative.
- Sample types Rh-negative in your facility but clinic/hospital laboratory typed them Rh-positive.
- Sample types Rh-positive but has anti-D.

Nearly everyone performing Rh typing has encountered discordance with previous typing. The challenge is in deciding what to report as the final Rh type to the patient's physician. Simply stated, Rh typing determines the presence of the RhD protein on red blood cells. Rh-negative individual's red blood cells lack the RhD protein in most cases. Traditionally, blood bankers determine the presence or absence of RhD antigen by mixing anti-D reagent with red cells. If RhD antigen is present, Anti-D will bind to D antigen and cause agglutination of the red cells. Although this appears simple, there are many variables impacting Rh typing results. These include RhD antigen variability, genetic variations, reagent differences and method variability. Each of these will be reviewed in greater detail.

Rh typing is further complicated by the fact that interpretation is different depending upon why an individual is being tested; obstetrical patient, transfusion recipient, cord blood or blood donor. Differences in interpretation in each of these groups will also be discussed.

## **RhD ANTIGEN**

Individuals who inherit one or two *RHD* genes, which result in expression of RhD protein, are considered Rh-positive. The RhD protein crosses the cell membrane 12 times making it an integral part of the RBC membrane. Only small loops of Rh proteins are exposed on the outside surface of the RBC. These small loops are subject to conformational/shape changes which explain some of the variations seen in typing with different anti-D reagents.

The protein on which D antigen resides is composed of at least 30 different epitopes (antigenic determinants) to which an anti-D reagent may bind. The difficulty this creates in typing will

be discussed later with reagent variation. Not only are there inherent challenges in detecting “normal” RhD protein just described, there are those who inherit variant *RHD* genes. These cause changes in expression of RhD protein resulting in weaker than normal expression of RhD.

In addition to the *RHD* gene(s), two *RHCE* genes are inherited, one from each parent. The *RHCE* gene is responsible for most other Rh antigens including C, E, c and e. The position and presence or absence of the other antigens can affect the ability to detect the RhD antigen. This is important when variant D antigen expression is discussed.

### VARIATIONS OF D ANTIGEN EXPRESSION

Rh-positive RBC samples are expected to show strong positive reactivity with anti-D reagents using test tube methods. However, some individual’s RBCs possess D antigen that requires an indirect antiglobulin test (IAT) for detection. It has been common practice to refer to these individuals as having weak D because their RhD antigen is not immediately detected.

Individuals classified as being weak D positive can be categorized into the following defined types:

- Weakened D due to C in trans to *RHD*
- Genetic Weak D (different than the general description)
- Partial D
- D<sub>el</sub>
- D Epitopes on RhCE Protein

Also included in this section is a rare group of individuals who possess D Epitopes on the RhCE protein. These individuals may be typed as Rh positive with some anti-D reagents.

### **C in Trans to *RHD***

The first mechanism that may result in weakened expression of D antigen was originally described as position effect or gene interaction effect. The allele carrying *RHD* is *trans* to the allele carrying C, for example, **D**ce/d**C**e. The Rh antigen on the RBC is normal and complete, but the steric arrangement of the C antigen in relationship to the D antigen appears to interfere with the expression of D antigen. Serologically, it is not possible to distinguish genetic weak D from the position effect weak D. These individuals can receive D-positive RBCs and will not make anti-D. In most cases, reactivity with anti-D reagents is not significantly reduced.

### Genetic Weak D

The second mechanism which may result in a weakened expression of D antigen results from inheritance of *RHD* genes that code for weakened expression. Mutations in the *RHD* gene occurs causing conformational changes in the protein on the outside of the RBC. The D antigen expressed appears to be complete but in fewer numbers. See Figure 1. Individuals with this weak D phenotype very rarely make anti-D and will generally show weak (1-2+) or no reactivity with many anti-D reagents at immediate spin (IS) in tube testing.

### Partial D

The third mechanism that may result in weakened expression of D antigen may occur when one or more D epitopes within the entire RhD protein is missing and/or altered. Partial D antigens result from portions of the *RHD* gene being replaced with portions of *RHCE*. These individuals with partial D, who type D-positive, rarely produce an anti-D that reacts with all normal D-positive samples except their own. If an individual lacks one (or more) pieces, or epitopes, of the total D antigen, alloantibody can be made to the missing portion(s) if exposed to RBCs that possess the complete D antigen. See Figure 1.

Tippett and Sanger worked with RBCs and sera of partial-D individuals to classify these antigens. Seven categories were recognized (I through VII), based on testing anti-D from different D-positive individuals. Partial DVI is the most common type found in the Caucasian population and the most challenging to detect as it is missing portions of the RhD protein along with fewer exposed epitopes making it difficult to detect by monoclonal anti-D reagents.

Some partial D types may show normal typing with reagent anti-D on direct agglutination (immediate spin). Other individual's red cells with partial-D antigen may type weaker than expected; or may not react at all at immediate spin with most commercial anti-D reagents as is the case in partial DVI.

Identification of a person with a partial-D routinely occurs after the person begins producing anti-D unless there are discrepant RhD typings. This discovery should prompt collection of additional samples to be sent to an immunohematology reference laboratory for further RhD classification.

### D<sub>el</sub>

Another mechanism for weakened D expression is the D<sub>el</sub> phenotype. This phenotype occurs in individuals whose red blood cells possess an extremely low number of D antigen sites which most anti-D reagents are unable to detect. Adsorbing and eluting anti-D from the individual's red cells is often the only way to detect the D antigen. Molecular studies can detect a mutant *RHD* gene that alters expression of the RhD protein. This phenotype is relatively common in D-negative individuals of Asian ethnicity occurring in 10% to 30% of the population. It is rare in Caucasians and individuals of African descent.

### D Epitopes on RhCE Protein

RhD epitopes can be expressed on RhCE protein and be detected by some monoclonal anti-D. Examples of these unusual phenotypes include DHAR and ceCF (Crawford).

$R_0^{\text{Har}}$ , also known as DHAR results from a hybrid gene *RHCE-RHD-RHCE* where only a small portion of *RHD* is inserted into the *RHCE* gene. These individuals should be classified as RhD-negative since they essentially lack the RhD protein.

The Crawford (ceCF) phenotype results from a specific amino acid change in the *RHce* gene resulting in RhD epitope on the Rhce protein. These individuals are non-reactive with the majority of anti-D reagents available.

In addition to this variability in RhD, the variability of reagents and methods used when testing in different labs (i.e. reference lab versus a hospital transfusion service) causes the need to understand reagent and methodology variability in order to best guide and communicate to the clinicians the differences in typing results.

### REAGENT VARIABILITY

Anti-D in reagents produced by different manufacturers can be human source (polyspecific) or monoclonal. Slide and Modified Tube Anti-D is the traditional reagent consisting of the human source anti-D. This Anti-D is generally polyspecific, IgG in nature and may be able to recognize many epitopes on the RhD protein. In contrast, monoclonal Anti-D is generally more targeted. By definition, a monoclonal antibody recognizes only one epitope. To ensure that monoclonal Anti-D reagents are able to detect a wide range of D positive and weak D positive samples, a combination or blend of monoclonal IgM and oligoclonal (human source) IgG antibody or monoclonal IgM and IgG anti-D is used to manufacture the reagent.

There are several anti-D reagents on the market that are immediate spin only reagents. These are monoclonal only anti-D reagents and will not detect partial DVI antigen. Awareness of the type of anti-D reagent being used in the laboratory by all individuals performing typing is important to ensure the manufacturer's directions are followed.

Today, there are many anti-D reagents available to the laboratory, each with its unique characteristics. Table 1 lists common anti-D reagents on the market today and summarizes specific clone(s) in each reagent as well as their reactivity with partial D phenotypes. This clone information and reagent characteristics, (i.e. how the reagent is expected to perform with different RhD variants) are always available in the Instructions for Use that comes with each anti-D reagent. It is important to review these when selecting new reagents as well as in resolving discrepant typing.

Table 1

Reactivity of Anti-D Blood Grouping Reagents with Partial D Samples

Partial D Phenotypes <sup>1</sup>	Anti-D Reagents																				
	Immucor								Alba Bioscience (ALBAclone®)								Biotest (Seracclone®)		Ortho		
	Polyclonal Slide/Tube			Series 4 (MS201/MS26)		Series 5 (TH28/MS26)		Gamma-clone (401/F8D8)	Alpha Monoclonal IgM (LDM1)		Beta Monoclonal IgM (LDM3)		Delta Monoclonal IgM/IgM (LDM1, ESD1M)		Blend Monoclonal IgM/IgM (LDM3, ESD1)		Monoclonal Blend IgG/IgM (BS232, BS221, H41 11B7)		Monoclonal-Polyclonal Blend (BioClone®) (MAD2)		Monoclonal IgM (MS201)
	IS	37°C	IAT	IS	IAT	IS	IAT	IS	IAT	IS	IS	IS	IAT	IS	IAT	IS	IAT	IS	IAT	Gel	
II	+	/	/	+	/	+	/	+	/	/	/	/	/	/	/	/	/	+	/	+	
IIIa	+	/	/	+	/	+	/	+	/	V	V	+	/	+	/	+	/	+	/	+	
IIIb	V	+	+	+	/	+	/	+	/	/	/	/	/	/	/	/	/	+	/	+	
IIIc	+	/	/	+	/	+	/	+	/	/	/	/	/	/	/	/	/	/	/	/	
IVa	+	/	/	+	/	+	/	+	/	V	V	+	/	V	/	+	/	+	/	+	
IVb	+	/	/	+	/	+	/	+	/	/	/	/	/	/	/	/	/	/	/	/	
Va	V	+	+	+	/	+	/	+	/	+	+	+	/	+	/	+	/	+	/	0	
Vb	V	+	+	0	+	0	+	+	/	/	/	/	/	/	/	/	/	/	/	/	
VI	0	V	+	0	+	0	+	0	+	0	0	V	V	0	+	0	+	0	+	0	
VII	0	0	+	+	/	+	/	+	/	0	0	/	/	/	/	/	/	/	/	/	
DFR	0	0	+	0	+	0	+	+	/	0	0	0	0	0	+	0	+	0	+	0	
R <sub>0</sub> <sup>HAR</sup>	0	0	0	+	/	+	/	+	/	0	0	0	+	0	+	0	+	0	0	V	
Crawford	0	0	0	0	0	0	0	+	/	/	/	/	/	/	/	/	/	0	0	0	

<sup>1</sup> = Where possible, at least 2 examples of each of the partial D phenotypes were tested.  
V = variable reactivity.  
/ = Not specifically indicated in Instructions for Use or described in published reports.

Source: Manufacturer’s Instructions for Use and personal communications.

## METHOD VARIABILITY

### Tube testing

Both manual and automated methods add additional variability to RhD typing results. Spin tube testing is the “gold standard” for D typing. In most cases normal expression of the D antigen is detected when agglutination is observed directly after centrifugation (immediate spin). However, some samples require the indirect antiglobulin test to detect the weakened expression of the D antigen. Variability in the ability of the person performing the test to consistently and correctly resuspend the red cells can greatly affect the outcome of the test. Variability of this type of skill within laboratories has been demonstrated in numerous surveys. When it comes to resolving discrepant typing, test tubes is the method of choice due to the number of different anti-D reagents available for testing and the ability to do the IAT allowing detection of partial DVI.

### Automation

Just as in tube testing, automated methods will show variable positive reactivity with RBCs from individuals possessing weakened expression of RhD. One individual’s red cells may show strong positive reactivity with test tube anti-D reagent but weaker on an automated platform or vice versa. Many automated platforms encourage or require the use of two Anti-D reagents to determine RhD status. Table 2 lists typical reactivity of anti-D reagents used in automated test methods available in the North American market today.

Table 2 Typical Reactivity of Anti-D Blood Grouping Reagents Used in Automated Methods with Partial D Samples

Partial D Phenotypes	Galileo®, Echo®, Neo® Immunor		Provue® Micro Typing Systems	Tango® Solidscreen II Biorad	Tango® Erytype S ABD+Rev.A <sub>1</sub> B Biorad		Olympus® PK® Systems		
	Series 4 Monoclonal Blend (MS201/MS26)	Series 5 Monoclonal Blend (TH28/MS26)	Monoclonal IgM (MS201)	Monoclonal Blend* (BS221/H4 11B7)	Monoclonal IgM (BS226)	Monoclonal IgM (BS232)	Monoclonal Blend (P3X61, P3X21223B10, P3X290, P3X35)	Monoclonal IgM (PK 1)	Monoclonal IgM (PK 2)
II	+	+	+	/	/	/	/	/	/
IIIa	+	+	+	/	/	/	/	/	/
IIIb	+	+	+	/	/	/	/	/	/
IIIc	+	+	/	/	/	/	/	/	/
IVa	+	+	+	/	/	/	/	/	/
IVb	+	+	/	/	/	/	/	/	/
Va	+	+	+	/	/	/	/	/	/
Vb	0	0	/	/	/	/	/	/	/
VI	0	0	0	+	0	0	+	0	0
VII	+	+	/	+	V	V	/	/	/
DFR	0	0	0	/	/	/	/	/	/
R <sub>0</sub> <sup>HAR</sup>	+	+	+	0	/	/	/	/	/
Crawford	0	0	/	/	/	/	/	/	/

\*Used to test donor samples which have been tested negative with IgM anti-D Erytype S in Tango optimo.

/ Not specifically indicated in Instructions for Use or described in published reports.

Source: Manufacturer's Instructions for Use, Instrument Operator Manuals and personal communications.

Automated methods may utilize microplates as a receptacle of reagent and cells, much like a series of small test tubes. Others utilize microplates containing dried pre-dispensed reagents for ABO and RhD typing and bromelin-treated red cells. Following an incubation period, traditional agglutination reactions result and the agglutination patterns are captured and interpreted by the instrument. While others utilize column agglutination where anti-D is incorporated into gel or glass beads. Patient RBCs are added to the microcolumn and if the D antigen is present the RBCs will agglutinate and become trapped in the gel or beads.

To add to the method variation, the manufacturer of each automated method encourages use of their own anti-D reagent; each having a unique clone. Some manufacturers utilize the same anti-D clones in their instrumentation licensed for test tube methods which reduces variation with historical records if previously typed using tube methods. However, due to variability in equipment, there may not be equivalency in the reaction strength of results of automated versus manual test methods.

## INTERPRETATION OF TESTING

It is no surprise that the amount of variability in typing clearly causes confusion in final interpretation of patient/donor Rh type. How do we interpret results? In actual fact, it depends on the population being tested. Whether the individual being typed is a transfusion recipient, obstetrical patient, infant or donor, they should be classified simply as Rh-positive or Rh-negative to avoid confusion for the clinician.

### Transfusion Recipients

Regulatory (FDA) and accrediting agencies (AABB and CAP) do not require routine testing for weak D in blood recipients; however, performing the test for weak D is debatable in the laboratory and regardless of the final interpretation, can help resolve discordant results seen in testing. Because blood recipients with C in *trans* to *RHD* and those with the genetic weak D clearly have the complete D antigen and do not produce alloanti-D, Rh-positive blood may be transfused. It must be remembered however, that very rare individuals with a partial-D phenotype can form alloanti-D when exposed to D-positive RBCs. Some believe the number of individuals homozygous for the partial-D gene is so low that the risk of sensitizing a partial-D individual is very low. Some laboratories are moving toward having the ability to define the type of weakened RhD an individual possesses. However, until methods are available that will easily differentiate between the types of weakened RhD antigen expression, policy regarding transfusion of weak-D positive transfusion recipients is established individually within each transfusion service.

### Obstetrical Patient

Determining the RhD status of obstetric patients is critical. Determining weak D status is not required in prenatal patients. For those patients with partial D antigen it is more prudent to interpret them Rh-negative on direct agglutination. Until routine testing allows determining with certainty which individuals possess partial D from other forms of weakened D antigen it is felt all individuals negative on direct agglutination should be considered candidates for Rh immune globulin. The most conservative approach is to interpret any individual typing <1+ on direct agglutination with anti-D in tube testing or <2+ in gel testing be considered Rh-negative.<sup>1</sup> Several automated instruments also offer assays to detect weak D as part of their test method.

At delivery, when the mother is Rh-negative and the newborn is typed Rh-negative, the weak-D status of the mother must be determined to avoid problems in testing for excessive fetal-maternal hemorrhage. A rosette screening test is routinely performed on Rh-negative mothers who have delivered Rh-positive infants to detect excessive fetomaternal hemorrhage (FMH). This test uses anti-D as its main component to detect Rh-positive fetal RBCs. When a mother's RBCs possess weak D antigen the screening test will show a false positive reaction due to the reagent anti-D binding to mom's RBCs versus fetal RBCs. An alternative method for detecting FMH is required.

Rarely, when typing a Rh-negative mother at delivery the anti-D IAT may be weakly positive due to FMH. Even more uncommon would be observing positive reactivity on direct agglutination. Both instances would likely show mixed field agglutination due to fetal bleed. A method to quantify FMH such as the Kleihauer-Betke or flow cytometry would be required.

Weak D status of the newborn must be determined to assess the likelihood of maternal sensitization and the need for Rh immune globulin prophylaxis for the mother.

### Blood Donors

Determining D status of a donor, regardless of the type of D antigen expression, is required when testing donor bloods. Regardless of the method used to type donors it must be known to



detect weakened D antigen expression. If the donor sample tests positive in any phase of RhD testing, the donor is considered Rh-positive. This explains why discordant results are sometimes noted between facilities typing an individual as a patient versus a donor. As a patient only a direct test may have been performed by the lab as opposed to both direct and indirect testing on the donor sample from the same individual.

For further discussion on the types of anti-D reagents to consider as well as when to consider molecular testing, readers are referred to a recent publication in the J Obstet Gynaecol Can 2007;29(9):746–752 by Flegel, Denomme and Yazer.<sup>2</sup>

The following table provides guidelines in interpreting discordant results. *It is the responsibility of the laboratory director to make final interpretation.*

**Table 3 Guideline for Interpreting Discordant Rh Typing Results<sup>3</sup>**

**Rh typing results are evaluated at immediate spin (direct agglutination) and Rh typing is repeated with identical results.**

If individual types...	And individual is a....	And...	Then...
Rh-negative	Transfusion recipient	Donor record is Rh-positive	Interpret Rh-negative
Rh-negative	Obstetrical patient	Donor record is Rh-positive	Interpret Rh-negative
Rh-negative	Post delivery	Donor record is Rh-positive	Perform anti-D IAT*
Rh-negative	Transfusion recipient	Facility history is Rh-positive	Interpret Rh-negative
Rh-negative	Obstetrical patient	Facility history is Rh-positive	Interpret Rh-negative
Rh-negative	Post delivery	Facility history is Rh-positive	Perform anti-D IAT*
Rh-positive	Transfusion recipient	Rh Negative at another facility	Type with different anti-D reagent
Rh-positive	Obstetrical patient	Rh Negative at another facility	Type with different anti-D reagent
Rh-positive	Post delivery	Rh Negative at another facility (regardless of history)	Type with different anti-D reagent Assess for mixed field agglutination Test for Fetal Bleed

\*Additional testing may include a weak D test i.e. incubating the patient RBCs with reagent anti-D at 37C followed by an indirect antiglobulin test (IAT) or type with additional sources of anti-D that possess different clones. See Tables 1 and 2 for a summary of the different anti-D reagents available today.

Finally, some laboratory directors choose to add additional comments when an Rh discrepancy has been recognized in the records. For example, the following verbiage or some derivation of this could be used in a final report to describe the different approach to interpretation by patient type.

*For transfusion purposes and candidacy for Rh immune globulin (RhIg) at the testing facility, patient is considered "Rh-negative" (patient to receive Rh-negative blood products and RhIg prophylaxis recommended.) As a blood donor this individual is considered Rh-positive.<sup>4</sup>*

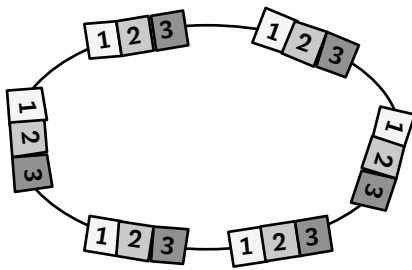
### **Conclusion**

Discrepant results in Rh typing are inevitable. It is up to the transfusion medicine specialists and medical laboratory scientists to determine their best course of action to interpret the results. Numerous factors for variability seen in typing have been outlined and are summarized in Table 4. Finally, guidance in interpretation of this important testing has been provided. One must keep in mind that D typing challenges will remain despite our best efforts to improve methods and to improve our understanding of the RhD protein and the molecular basis of the *RH* genes. Perhaps routine sequencing of the *RHD* gene will one day provide the ultimate determination required to designate someone as Rh-positive or Rh-negative.

FIGURE 1

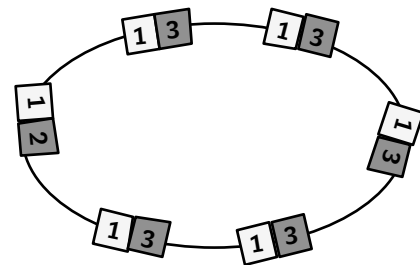
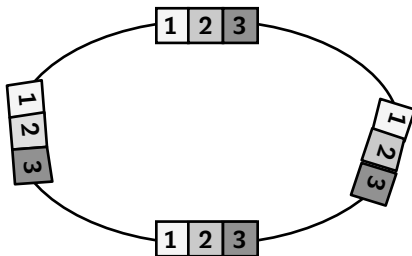
**Weak D versus Partial D**

Modified from Flegel WA, Denomme GA, Yazer MH, J Obstet Gynaecol Can 2007;29(9):746-752<sup>2</sup>. Permission to reprint these figures has been provided courtesy of the Society of Obstetricians and Gynaecologists of Canada.



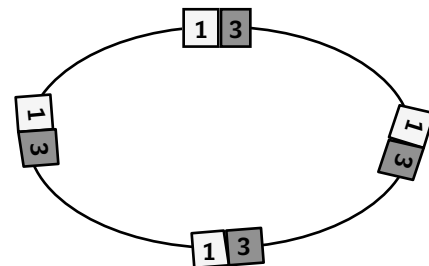
**Normal RhD Positive (all epitopes 1, 2, 3 present)**

Anti-D – 3-4+



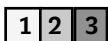
**Partial D - missing epitope 2**

Anti-D – 0 – 4+, Anti-D IAT – 3-4+



**Weak D Type 1-53 (fewer RhD proteins)**

Anti-D – 0 – 2+, Anti-D IAT – 2-3+



**Normal RhD protein – each block depicts normal epitopes**

“Typical” reactivity with Anti-D Reagent in Test Tubes - this will vary depending on reagent used and each individual typed.

**Partial DVI (missing 2 & fewer in number)**

Anti-D – 0, Anti-D IAT – 2-3+

Table 4 Variables Impacting Rh Typing

CONTRIBUTORS OF VARIABILITY	VARIABLES				
<b>RHD Gene</b>	Weak D	C in <i>Trans</i> to RHD	Partial D	D <sub>el</sub>	
<b>D epitopes on RhCE Protein</b>	ceCF	R <sub>0</sub> <sup>HAR</sup> or DHAR			
<b>Anti-D Reagents</b>	Polyspecific Slide and Modified Tube Human IgG	Monoclonal IgG	Monoclonal IgM	Monoclonal IgM Human IgG	Monoclonal Blends
<b>Testing Platform</b>	Test Tubes IS & IAT	Column Agglutination	Solid Phase	Liquid Microtiter	
<b>Individual being Rh Typed</b>	Transfusion Recipient	Obstetrical Patient	Cord Blood	Donor Blood	

### References

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