

How slow can you be when performing a direct antiglobulin test?

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While continuous performance is preferred for any assay, transfusion services are particularly susceptible to interruptions that may interfere with testing. If these interruptions occur after the red blood cell (RBC) suspension is made or after the last wash is decanted, direct antiglobulin test (DAT) results may be affected. This study aimed to assess if delays at these two procedural steps compromise DAT results. Samples with positive DATs (up to 2+) using anti-IgG (IgG-DAT) were included. RBC suspensions (2–5%) were prepared from unwashed RBCs or RBCs washed once. These suspensions were used to perform tube IgG-DATs without delays to obtain baseline results. IgG-DATs were repeated with delays after preparation of the RBC suspension (1, 5, 15, 30, and 60 minutes) or after the last wash decant (1, 5, 15, or 30 minutes). Three anti-IgG reagents were used: Anti-IgG (Murine Monoclonal) Gamma-clone; Werfen, Barcelona, Spain; Anti-IgG (Rabbit); QuidelOrtho, Raritan, NJ; and AHG Anti-IgG; Bio-Rad, Hercules, CA. Samples were considered impacted if their IgG-DAT strength differed by two or more reaction grades from baseline. The analysis included 25 samples. Samples with baseline IgG-DAT results of 1+^s and 2+ were determined to not be impacted. Three samples were weakened by delays after RBC suspension preparation. One sample was weakened when the addition of anti-IgG was delayed. While most samples with weakly positive baseline IgG-DAT results were not impacted, the samples with the weakest baseline IgG-DAT results were impacted more frequently than those with stronger baseline IgG-DAT results. Because it is impossible to predict which samples will not be impacted by delays in these testing steps, DAT procedures should be performed with minimal interruption. *Immunohematology* 2026;42:35–41. doi: 10.2478/immunohematology-2026-007.

Key Words: direct antiglobulin test, stability, delay

The direct antiglobulin test (DAT) is used to detect IgG antibodies or complement components bound to the surface of red blood cells (RBCs) *in vivo*.¹ Among other clinical indications, this test is primarily used to detect autoantibodies, alloantibodies bound to transfused RBCs, and maternal alloantibodies bound to neonatal RBCs. When the DAT detects

IgG on the surface of the RBCs, laboratories often elute the antibody for subsequent identification. Testing the resulting eluate can identify the offending agent in cases of autoimmune hemolytic anemia, antibody-mediated transfusion reactions, and hemolytic disease of the fetus and newborn (HDFN). Using the conventional test tube method, the general procedure of the DAT is to (1) prepare a 2–5 percent suspension of RBCs in saline from a well-mixed sample of whole blood or packed RBCs collected in EDTA; (2) add one drop of the RBC suspension to each tube in which a specific antihuman globulin (AHG) reagent will be added to test the RBCs; (3) wash the RBCs three to four times with saline to remove any unbound antibody and complement present; (4) add the specific AHG reagent to each tube's dry RBC button after the last wash is decanted; and (5) mix the tubes, centrifuge, and examine with an optical aid to detect agglutination in each tube.²

Many preanalytical and analytical variables are known to impact the accuracy of DAT results, including improper sample collection, transport, or storage; contaminated sample, supplies, or reagents; delays in testing; and improper testing technique.^{2–6} Two steps within the DAT procedure are frequently mentioned when discussing the occurrence of false-negative results. One step is at the beginning when a fresh 2–5 percent suspension of RBCs is prepared. The other critical step is at the end with the addition of AHG after the last wash. It is noted that prolonging the time antibody-coated RBCs remain in saline could cause the antibody to elute off the RBCs and cause false-negative DAT results using anti-IgG (IgG-DAT).^{2,3} However, no published literature could be found assessing *how long* RBCs can remain in saline before IgG-DAT results are impacted. This study was designed to assess the impact of delays after 2–5 percent RBC suspension preparation and after the delayed addition of anti-IgG on IgG-DAT results when interrupting the testing process.

Materials and Methods

Sample Selection

The study protocol was reviewed and approved by the study site institutional review board. Samples were selected from residual EDTA samples from adult patients and/or donors collected less than 48 hours before initiation of testing. No additional diagnostic or medical information was to be generated; therefore, consent was waived. These samples were tested previously by the American Red Cross Detroit Immunohematology Reference Laboratory (IRL) and were known to be weakly (<2+) or moderately (2+) IgG-DAT+. Samples were blinded once accepted into the study.

IRL samples with DATs that required warm washing or 0.01 M dithiothreitol treatment to obtain valid results and, to avoid introducing bias into the results, samples from previously included patients or donors were excluded from this study.

Sample Handling

The IRL manager recorded the date of the most recent transfusion, labeled glass tubes with unique alphanumeric identifiers, and aliquoted RBCs from selected samples into the labeled tubes. The aliquots were then transferred to the principal investigator (PI). The IRL manager did not have access to test results obtained by the PI, and the PI did not have access to any other information other than the most recent transfusion date. All testing was performed by conventional test tube methodology using glass test tubes and anti-IgG reagents only (Anti-IgG [Murine Monoclonal] Gamma-clone; Werfen, Barcelona, Spain; Anti-IgG [Rabbit]; QuidelOrtho, Raritan, NJ; and AHG Anti-IgG; Bio-Rad, Hercules, CA).

Because 2–5 percent RBC suspensions are commonly prepared from washed-once or unwashed RBCs, RBCs from individual aliquots were used to make suspensions by each of the following methods:

- **Method 1** (unwashed 2–5 percent RBC suspension): 0.85 percent buffered saline (Thermo Fisher Scientific, Middletown, VA) was added to RBCs to form a 2–5 percent RBC suspension.
- **Method 2** (washed-once 2–5 percent RBC suspension): 0.85 percent buffered saline (Thermo Fisher Scientific) was used to fill the test tube containing the RBCs. The test tube was centrifuged for the calibrated time for washing RBCs. The saline was decanted, and fresh 0.85 percent buffered saline (Thermo Fisher Scientific) was added to the washed RBCs to form a 2–5 percent RBC suspension.

Both 2–5 percent RBC suspensions (washed-once and unwashed) were tested in the same manner, with identical reagents, equipment, and variables outlined in later sections: **Baseline Testing, Delays After 2–5 Percent RBC Suspension, and Delays After Last Wash.**

Anti-IgG Selection

Anti-IgG may be produced by a hybridoma cell line that secretes a monoclonal antibody (Anti-IgG [Murine Monoclonal] Gamma-clone; Werfen; and Anti-Human Globulin Anti-IgG; Grifols, Barcelona, Spain).^{4,7} This method produces an anti-IgG reagent containing an antibody directed against a single antigenic site on the IgG protein. Anti-Human Globulin Anti-IgG (Grifols) is formulated to detect all four IgG subclasses, and Anti-IgG (Murine Monoclonal) Gamma-clone (Werfen) is formulated to detect IgG1, IgG2, and IgG3. Anti-IgG may also be produced by immunizing rabbits with purified human IgG and subsequently purifying the antibody produced by the rabbits (Anti-IgG [Rabbit]; Alba Bioscience, Edinburgh, UK; Anti-IgG [Rabbit]; QuidelOrtho; and AHG Anti-IgG; Bio-Rad).^{5,6,8} This method produces an anti-IgG reagent containing IgG antibodies directed against many antigenic sites on the IgG protein and is expected to detect all four subclasses of IgG. Since different preparations of reagent anti-IgG could produce differing results when used to perform the IgG-DAT, multiple sources were chosen to evaluate potential impacts.

Experimental Design

Progression of testing using the 2–5 percent RBC suspensions is summarized in Figure 1.

BASILINE TESTING

A baseline result was obtained using Method 3-14 in the AABB *Technical Manual*, 21st Edition.² Immediately after making the 2–5 percent RBC suspension, one drop of the suspension was dispensed into clean, labeled test tubes. An automatic cell washer (Helmer UltraCW Cell Washing System; Helmer Scientific, Noblesville, IN) was programmed to wash the tubes three times in accordance with the manufacturer's directions for all three anti-IgG reagents used. Once washing was completed, two drops of each anti-IgG reagent were immediately added to the corresponding test tube, including a saline control. Tubes were mixed, centrifuged, and read macroscopically for agglutination. Macroscopic agglutination was graded as described in *Judd's Methods in Immunohematology*.³ An abbreviated table is shown in Table 1. Tubes with no macroscopic agglutination were examined

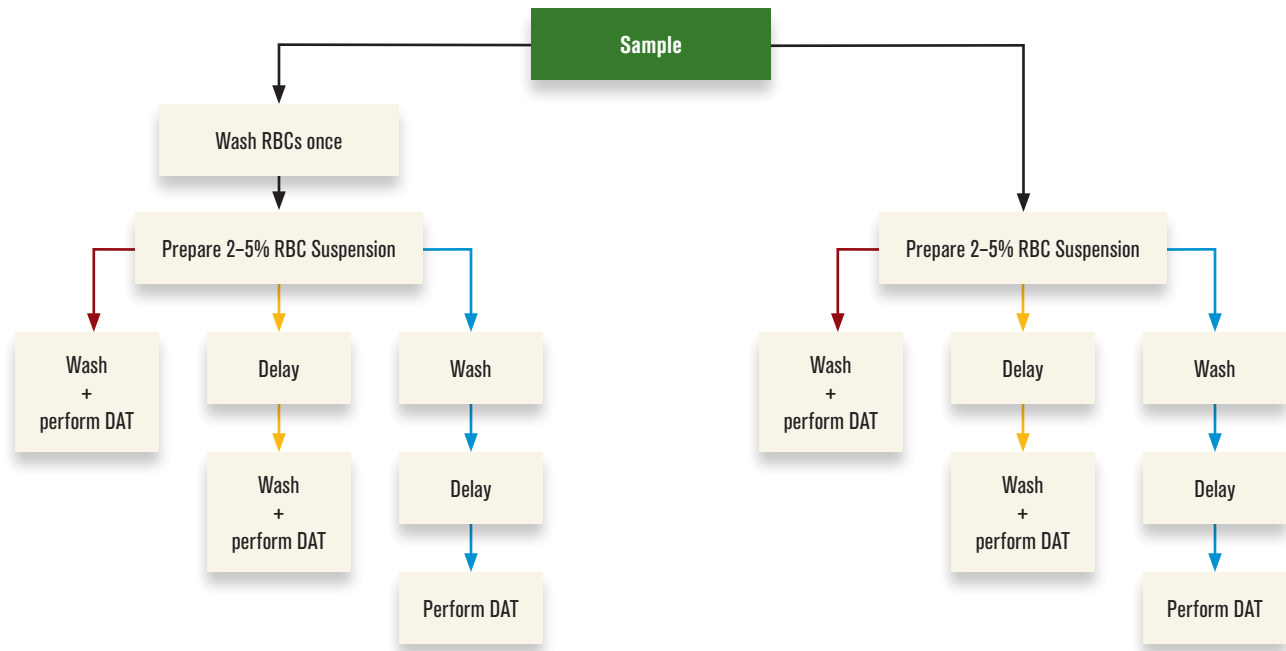


Fig. 1 Outline of experimental design. Red arrows indicate baseline testing process. Yellow and blue arrows correspond to the processes outlined in sections: **Delays After 2–5% RBC Suspension** and **Delays After Last Wash**, respectively. RBCs = red blood cells; DAT = direct antiglobulin test.

microscopically for agglutination. Tubes with microscopic agglutination were graded m+. One drop of IgG-coated control check RBCs was added to all AHG-containing nonreactive tubes, mixed, centrifuged, and observed for agglutination to confirm addition of anti-IgG and adequate washing.

DELAYS AFTER 2–5 PERCENT RBC SUSPENSION

After the 2–5 percent RBC suspension was made, the suspension remained in its test tube at room temperature for intervals of 1, 5, 15, 30, and 60 minutes. After the delay

period, one drop of the suspension was dispensed into clean, labeled test tubes. The remainder of testing was completed as described in the section: **Baseline Testing**.

DELAYS AFTER LAST WASH

Immediately after making the 2–5 percent RBC suspension, one drop of the suspension was dispensed into clean, labeled test tubes and washed three times using the automatic cell washer (Helmer UltraCW Cell Washing System). Once the last wash was decanted, the washed RBCs remained in their test tubes at room temperature for intervals of 1, 5, 15, or 30 minutes. After each delay period, anti-IgG from each manufacturer was added to its respectively labeled test tube, and testing was completed as described in the section: **Baseline Testing**.

Data Analysis

All results were recorded in a spreadsheet (Microsoft Excel). Results following each delay period after 2–5 percent RBC suspension preparations were compared with baseline results for each sample with each anti-IgG reagent tested. Results that varied by at least two reaction grades from baseline qualified as impacted. Similarly, results following each delay period after the last wash was decanted were compared

Table 1. Macroscopic agglutination grading

Grade	Appearance
2+	Moderate reaction: large agglutinates in a sea of smaller agglutinates; no unagglutinated RBCs
1+ ^s	Intermediate between 1+ and 2+
1+	Weak reaction: many agglutinates of up to 20 RBCs with some smaller agglutinates and unagglutinated RBCs
w+	Granular reaction: scattered small agglutinates with many unagglutinated RBCs
+/-	Trace reaction: small agglutinates of 3–4 RBCs with many unagglutinated RBCs
0	No reaction

RBCs = red blood cells.

with baseline results for each sample with each anti-IgG reagent tested. Again, results that varied by at least two reaction grades from baseline qualified as impacted.

Results

Twenty-eight samples were selected for testing. Two samples (DAT-05 and DAT-07) could not be tested before they exceeded the sample age requirements. Another sample (DAT-12) had baseline IgG-DATs of m+ with all three reagents tested, could not become two reaction grades weaker, and was therefore excluded from the analysis. Distribution of baseline results for the remaining 25 samples according to manufacturer and 2–5 percent RBC suspension preparation method used is outlined in Figure 2. During baseline testing, uniform results were not obtained across anti-IgG manufacturers and 2–5 percent RBC suspension preparation methods for each sample. However, these intrasample variations were, at most, plus or minus one reaction grade and thus were not classified as impacted by merely changing the 2–5 percent RBC suspension preparation method or anti-IgG manufacturer. No reactive saline controls were observed, indicating that test results were valid.

DELAYS AFTER 2–5 PERCENT RBC SUSPENSION

IgG-DAT results for samples with delays after 2–5 percent RBC suspension preparation are detailed in Table 2. Using Method 1 (unwashed 2–5 percent RBC suspension), a single sample (DAT-27), with a baseline of 1+ using monoclonal anti-IgG and a baseline of w+ using rabbit anti-IgG reagents, changed from its baseline result at 30 minutes after preparation of the 2–5 percent RBC suspension using AHG Anti-IgG (Bio-Rad) only. At the 30-minute delay, the sample was only microscopically reactive. This sample result changed with all three anti-IgG reagents after a 60-minute delay, was only microscopically reactive with AHG Anti-IgG (Bio-Rad) and Anti-IgG (Murine Monoclonal) Gamma-clone (Werfen), and was nonreactive with Anti-IgG (Rabbit) (QuidelOrtho).

Similarly, IgG-DAT results were also impacted by testing delays when using Method 2 (washed-once) of 2–5 percent RBC suspension. One sample (DAT-06), with a baseline of w+ with all anti-IgG reagents tested, changed using Anti-IgG (Murine Monoclonal) Gamma-clone (Werfen) at the 30-minute delay mark, when agglutination could only be detected microscopically. After the 60-minute delay, this same sample was nonreactive with Anti-IgG (Murine Monoclonal) Gamma-clone (Werfen) only. Another sample (DAT-27) changed with

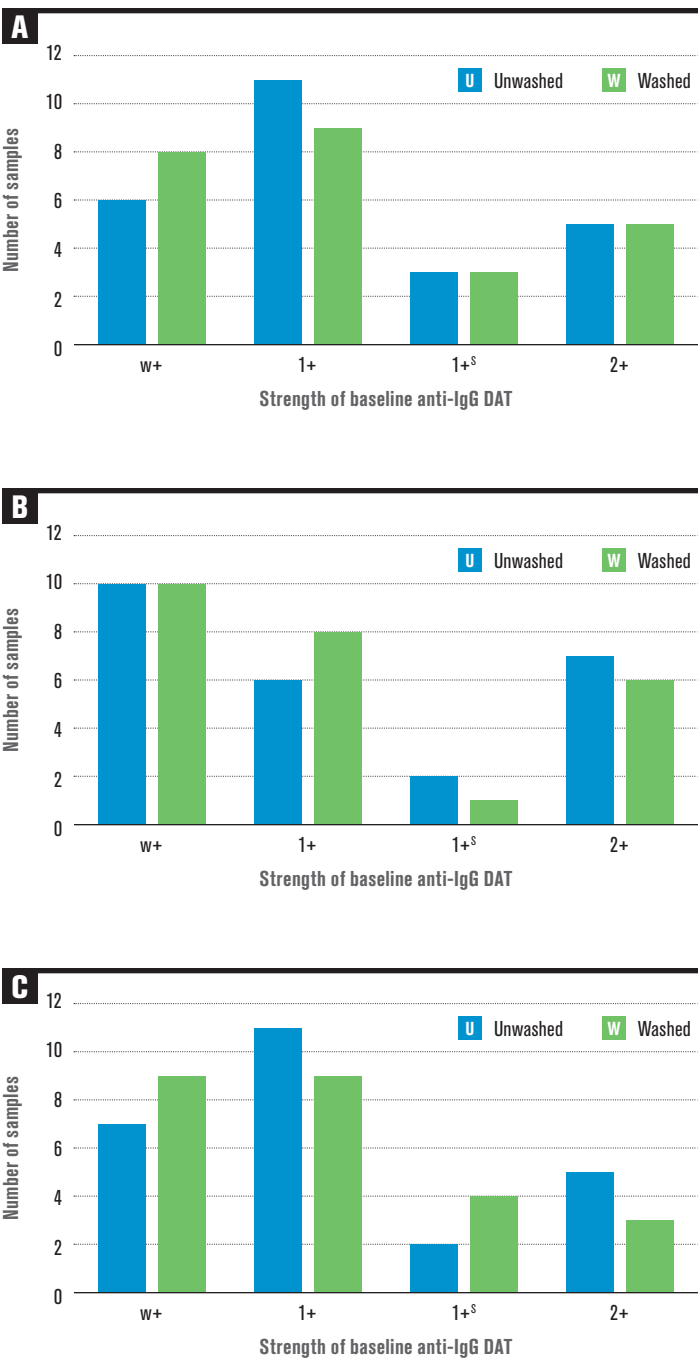


Fig. 2 Distribution of baseline results with anti-IgG using washed and unwashed red blood cell (RBC) suspensions. (A) Werfen. (B) QuidelOrtho. (C) Bio-Rad. DAT = direct antiglobulin test.

only Anti-IgG (Murine Monoclonal) Gamma-clone (Werfen) (1+ to +/-) after 60 minutes. A third sample (DAT-04), with a baseline of w+ with all three anti-IgG reagents tested, changed with all three anti-IgG reagents to microscopically reactive only after a 60-minute delay.

Table 2. Results from delays after 2–5% RBC suspension preparation

Sample number	Method 1 (unwashed) 2–5% RBC suspension preparation, delay period (minutes)						Method 2 (washed once) 2–5% RBC suspension preparation, delay period (minutes)					
	0 (Baseline)	1	5	15	30	60	0 (Baseline)	1	5	15	30	60
Werfen*												
DAT-04	w+	w+	w+	w+	w+	w+	w+	w+	w+	w+	w+	m+
DAT-06	w+	w+	w+	+/-	+/-	+/-	w+	w+	+/-	+/-	m+	0✓
DAT-27	1+	1+	1+	1+	w+	m+	1+	1+	1+	w+	w+	+/-
QuidelOrtho*												
DAT-04	w+	w+	w+	w+	w+	w+	w+	w+	w+	w+	w+	m+
DAT-06	w+	w+	w+	w+	w+	w+	w+	w+	w+	w+	+/-	+/-
DAT-27	w+	w+	w+	w+	+/-	0✓	w+	w+	w+	w+	+/-	+/-
Bio-Rad*												
DAT-04	1+	1+	1+	1+	1+	w+	w+	w+	w+	+/-	+/-	m+
DAT-06	w+	w+	w+	w+	w+	w+	w+	w+	w+	w+	w+	w+
DAT-27	w+	w+	w+	w+	m+	m+	w+	w+	w+	+/-	+/-	+/-

Reaction grades of IgG-DATs performed on samples that changed from baseline using different anti-IgG reagents when testing was delayed after preparation of the 2–5% RBC suspension. Highlighted reaction grades indicate at which delay period the testing impact (change of two or more reaction grades from baseline) occurred.

*Anti-IgG reagent used. RBC = red blood cell; DAT = direct antiglobulin test.

DELAYS AFTER LAST WASH DECANT

Similar to samples tested with delays after preparing the 2–5 percent RBC suspension, all samples in which a change was documented were at least two reaction grades weaker than baseline; again, no increases were observed.

A single sample (DAT-22), tested using an unwashed 2–5 percent RBC suspension with a baseline of w+, was nonreactive at 15 and 30 minutes after the last wash was decanted using Anti-IgG (Murine Monoclonal) Gamma-clone (Werfen) only. The same sample, tested using a washed 2–5 percent RBC suspension, was only microscopically reactive at 5 minutes and was nonreactive at 15 and 30 minutes after the last wash was decanted using Anti-IgG (Murine Monoclonal) Gamma-clone (Werfen) only. The IgG-DAT results of this sample are

detailed in Table 3. Interestingly, this sample was not one of those influenced by delays after preparation of the 2–5 percent RBC suspension. No other samples were impacted by delays after decant of the last wash.

INDIVIDUAL TRANSFUSION HISTORY

Of the individuals from the 25 samples studied, 17 had received prior transfusions, 15 of which were transfused in the past 90 days, including six individuals being transfused within the last 2 weeks. Of the remaining eight individuals, four had no history of transfusion and four had an unknown transfusion history. Two impacted samples (DAT-04 and DAT-06) were from individuals with no history of transfusion, one sample (DAT-22) was from an individual with unknown

Table 3. Results from delays after last wash decant

Sample number	Method 1 (unwashed) 2–5% RBC suspension preparation, delay period (minutes)					Method 2 (washed once) 2–5% RBC suspension preparation, delay period (minutes)				
	0 (Baseline)	1	5	15	30	0 (Baseline)	1	5	15	30
DAT-22										
Werfen*	w+	w+	w+	0✓	0✓	w+	+/-	m+	0✓	0✓
QuidelOrtho*	w+	w+	w+	w+	w+	w+	w+	w+	w+	w+
Bio-Rad*	w+	w+	w+	w+	+/-	w+	w+	w+	w+	w+

Reaction grades of IgG-DATs performed on the sample (DAT-22) that changed from baseline when the addition of anti-IgG was delayed after last wash decant. Highlighted reaction grades indicate at which delay period the testing impact (change of two or more reaction grades from baseline) occurred.

*Anti-IgG reagent used. RBC = red blood cell; DAT = direct antiglobulin test.

transfusion history, and the last sample (DAT-27) was from an individual most recently transfused 3 months before sample testing.

Discussion

The DAT is one of the more frequently performed tests in the transfusion service department of the clinical laboratory. Like all other areas of the laboratory, the transfusion service must handle many interruptions from phone calls; however, they must also handle more time-consuming and urgent interruptions when emergency-released blood components are requested. These interruptions can delay the DAT before it is started or at any time during testing. This protocol was designed to simulate delays during IgG-DAT performance at two points—after the preparation of the 2–5 percent RBC suspension and after the last wash is decanted—and to assess how long the delays can be before results are compromised.

Based on the results of this study, IgG-DAT results can be impacted quickly in some samples, and even those that would otherwise be 1+ may decrease in strength enough to escape the untrained eye. Results of this study suggest that samples with 1+^s and 2+ IgG-DAT results are more likely to be detected even if the procedure is interrupted, while samples with weaker strengths ($\leq 1+$) could potentially be missed, particularly if a microscopic reading is not performed. Results that are impacted and weakened by testing delays can decrease in reaction strength within 5 minutes, depending on when the delay occurs and which anti-IgG reagent is used, and may even become nonreactive as delay time increases.

Looking closer at delays in testing after making the 2–5 percent suspension prepared from washed RBCs, testing with monoclonal IgM anti-IgG (Werfen) was impacted earlier and affected two more samples than IgG-DATs performed using rabbit anti-IgG (QuidelOrtho and Bio-Rad). Testing with monoclonal IgM anti-IgG (Werfen) was also the only method to be influenced by delays after decant of the last wash. This finding may be due to a specific component of the reagent's formulation or a principle specific to monoclonal antibodies. Definitive conclusions regarding type of anti-IgG cannot be drawn from such small differences; however, the observation is worthy of note and could be evaluated in future studies.

The nature of the antibody causing the positive IgG-DAT may also play a role in the sample's vulnerability to testing delays. Of the three impacted samples from individuals with known transfusion histories, none were from individuals recently transfused, raising the question of

whether alloantibodies and autoantibodies confer increased or decreased resistance to an interrupted IgG-DAT because of different affinities for their respective antigens. A study assessing the correlation of antibody identification and eluate results to IgG-DAT testing delays could be conducted to investigate this question.

There are some limitations to this study that should be noted. A study with a larger sample size of weakly positive IgG-DATs at baseline would be better at determining how quickly results can be impacted. Preanalytical factors including sample storage and transport time before arriving at the testing laboratory may have influenced results. Other DAT methods were not assessed, and tube washing was performed using a single cell washer model. Results cannot be extrapolated to other cell washers or manual cell washing because varying amounts of residual saline may remain in the tubes after the last wash is decanted. Other washing methods may allow more or less flexibility in testing timing.

Conclusions

Current teachings stress the importance of using a fresh 2–5 percent RBC suspension for performing the IgG-DAT and stress that the anti-IgG must be added immediately after the last wash is decanted. This teaching stems from the thought that IgG antibodies can elute off the RBCs and cause a decrease in IgG-DAT strength or an outright false-negative IgG-DAT. A missed positive IgG-DAT could result in a case of missed autoimmune hemolytic anemia, antibody-mediated transfusion reaction, or HDFN. In addition, subsequent elution performance would likely be omitted and may result in a newly forming clinically significant alloantibody to go undetected. Results from this study indicate that samples with 1+^s and 2+ IgG-DATs are generally stable in the face of testing delays at the two time points evaluated. Regardless of anti-IgG manufacturer or method of preparation, 2–5 percent RBC suspensions give reliable IgG-DAT results even if they were prepared 15 minutes before. With the exception of Anti-IgG (Murine Monoclonal) Gamma-clone (Werfen), a delay of up to 30 minutes after the last wash is decanted does not appear to impact IgG-DAT results. This study intended to be pragmatic and assess the effects of common interruptions that would otherwise compel the laboratory scientist to make a new RBC suspension and start the DAT procedure all over again. For laboratories looking to improve IgG-DAT procedures, the avoidance of using a 2–5 percent RBC suspension more than 15 minutes from preparation should be emphasized. To be safe

and ensure that samples with weakly positive IgG-DATs are not missed, the current practice of minimizing interruptions should be continued. While a minority of the samples were impacted by the delays assessed, capturing all positive IgG-DATs is the goal for good laboratory practices. Minimizing delays in the IgG-DAT procedure supports accurate reporting of all IgG-DATs, not merely most of them.

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