# Development of Clear and Turbid Virtual Serum for Hemolysis and Agglutination Reading on Unexpected Antibody Screening

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## Abstract

The purpose of this study was to develop a virtual serum preparation with a yellow color comparable to real human serum, including clear yellow and turbid yellow, for use in antibody screening in normal saline using the conventional tube test, with positive reactions observable as both hemolysis and agglutination. In this study, distilled water was added to the virtual serum containing anti-D (IgG) to create a hypotonic solution. Antibody screening results showed that the clear yellow and turbid yellow virtual serum diluted with distilled water at ratios of 1:4, 1:5, and 1:6 produced positive hemolysis and agglutination reactions. Absorbance and specific gravity were compared among undiluted samples and those diluted at 1:4, 1:5, and 1:6 using the Kruskal-Wallis test. Significant differences in absorbance were observed between the undiluted and 1:6 dilutions for both clear yellow and turbid yellow virtual serum (p value = 0.013). Specific gravity also differed significantly between the undiluted and 1:6 dilutions (p = 0.010). These findings indicate that the clear yellow and turbid yellow virtual serum can be prepared to yield positive antibody screening results that allow interpretation of both hemolysis and agglutination reactions.

***Keywords*****:** *virtual serum, unexpected antibody screening, hemolysis, agglutination, Transfusion Science education*

## 1. Introduction

Practical instruction in the course Blood Transfusion Science I at the Faculty of Medical Technology, Rangsit University, includes a laboratory topic on antibody screening. This test requires the use of serum samples. However, the preparation of serum samples for practical teaching has several limitations, as a large volume of whole blood is required to obtain sufficient serum for approximately 180 third-year students. Collecting a large volume of blood from human donors is not feasible due to potential risks and harm to the donors. In addition, unexpected antibodies are relatively rare in certain population groups, with reported prevalence ranging from 0.3% to 38.0%, depending on the population studied and the testing methods used (Roback et al., 2008). Furthermore, serum samples pose a risk of transfusion-transmitted infections, such as hepatitis B virus, hepatitis C virus, and human immunodeficiency virus (HBV, HCV, and HIV) (Bihl et al., 2007), which may endanger students when performing antibody screening practice. Yothinarak and Prommano (2017) conducted a preliminary study on the preparation of virtual serum for screening unexpected antibodies in isotonic saline. The findings provided instructors with an alternative to pooled serum or frozen plasma, which carries a potential risk of exposure to human blood–borne pathogens. However, the study reported several issues in antibody screening: some virtual serum preparations that yielded negative results caused red blood cell hemolysis during incubation at room temperature and at 37°C. In 2018, the virtual serum formulation was further developed by supplementing albumin and was prepared as both negative and positive control materials for antibody screening in isotonic saline, low-ionic-strength saline, and papain enzyme media. The results indicated that the virtual serum did not cause red blood cell hemolysis in any of the three media at any stage of the testing procedure (Yothinarak, 2018). In 2025, the amount of albumin, an expensive reagent, was reduced to decrease costs. In addition, the virtual serum was diluted to obtain a range of yellow to pale-yellow colors, enabling the preparation of multiple control materials with different intensities of yellow coloration (Yothinarak & Gatedee, 2025). Later, the preparation of virtual serum was further developed to produce a more turbid formulation that yielded both negative and positive antibody screening results. This improvement was intended to generate a virtual serum that was visually

distinguishable from formulations reported in earlier studies, thereby enabling the preparation of multiple control materials with physical characteristics, particularly color and turbidity, closely resembling those of authentic human serum for practical teaching in antibody screening (Yothinarak et al., 2025). However, a virtual serum that produces a positive antibody screening result specifically observable as red blood cell hemolysis has not yet been developed. Hemolysis is considered a significant indicator of antigen-antibody reactions, particularly in complement-mediated antibody activity. In antibody screening, the presence of hemolysis may indicate clinically significant antibodies and must be interpreted alongside agglutination reactions. Therefore, this study was undertaken as part of a Routine to Research (R2R) approach, which translates problems encountered in routine work into research questions to identify solutions and improve routine practices. This approach requires appropriate planning and rigorous study conducted in accordance with academic standards to ensure the highest possible reliability of the findings (Yimklib et al., 2023).

## 2. Objectives

1) To develop a virtual serum preparation with a clear yellow and turbid yellow appearance that yields positive antibody screening outcomes, detectable by both red blood cell hemolysis and agglutination.

2) To evaluate and compare, within clear yellow and turbid yellow virtual serum preparation demonstrating positive antibody screening outcomes, differences in macroscopic appearance, optical density (OD), and specific gravity (Sp.Gr.).

## 3. Materials and Methods

### *3.1 Preparation of virtual serum for antibody screening, yielding agglutination-positive results at the antiglobulin phase using the conventional tube test*

*3.1.1 Preparation of clear yellow virtual serum formulation*

Six grams of chrysanthemum powder, 0.85 g of NaCl, and 0.1 g of albumin were weighed and transferred to a container. Distilled water was added to a final volume of 100 mL, and the mixture was stirred on a magnetic stirrer for 3 min. Subsequently, 150 µL of egg-yellow food coloring was added and mixed for an additional 3 min until homogenous. Anti-D (IgG) was then diluted with this virtual serum solution at a dilution of 1:8 to obtain a positive reaction grade of 2+ to 3+ at the antiglobulin phase. The physical characteristics (color, clarity, and turbidity) were assessed by visual inspection. Optical density (OD) was measured at 450 nm in triplicate, and specific gravity (Sp.Gr.) was also measured in triplicate.

*3.1.2 Preparation of turbid yellow virtual serum formulation*

A non-dairy creamer stock solution was prepared by weighing 10 g of creamer and adding 10 mL of distilled water. The mixture was heated on a hot plate until boiling and completely dissolved. Then, 6 g of chrysanthemum powder, 0.85 g of NaCl, and 0.1 g of albumin were weighed, and distilled water was added to a final volume of 100 mL. The mixture was stirred on a magnetic stirrer for 3 min. Then, 150 µL of egg-yellow food coloring was added and mixed for an additional 3 min until homogeneous. Next, 150 µL of the mixture was removed and replaced with 150 µL of the non-dairy creamer stock solution. The solution was then mixed on a magnetic stirrer for 3 min until thoroughly combined. Anti-D (IgG) was subsequently diluted with this turbid yellow virtual serum at a dilution ratio of 1:8 to obtain a positive reaction grade of 2+ to 3+ at the antiglobulin phase. Physical characteristics (color, clarity, and turbidity) were assessed by visual inspection. Optical density (OD) at 450 nm and specific gravity (Sp.Gr.) were each measured in triplicate.

### *3.2 Preparation of virtual serum for antibody screening yielding positive results based on hemolysis and agglutination*

The addition of distilled water to the virtual serum produces a hypotonic solution. When this hypotonic virtual serum is used in antibody screening, water enters the screening cells by osmosis, causing red blood cells to swell and subsequently undergo hemolysis. Therefore, virtual serum prepared using the two formulations described in sections 3.1.1 and 3.1.2 was combined with distilled water in a checkerboard dilution scheme (Table 1). The physical characteristics (color, clarity, and turbidity) were assessed by visual

inspection. Optical density (OD) at 450 nm was measured in triplicate, and specific gravity (Sp.Gr.) was also measured in triplicate.

**Table 1** Preparation of virtual serum using a checkerboard design between virtual serum and distilled water

| Virtual serum | Distilled water | | | | | Number of tubes |
|---|---|---|---|---|---|---|
| | 5 mL | 4 mL | 3 mL | 2 mL | 1 mL | |
| 5 mL | 5-5 (1:2) | 5-4 (1:1.8) | 5-3 (1:1.6) | 5-2 (1:1.4) | 5-1 (1:1.2) | 5 |
| 4 mL | 4-5 (1:2.3) | 4-4 (1:2) | 4-3 (1:1.8) | 4-2 (1:1.5) | 4-1 (1:1.3) | 5 |
| 3 mL | 3-5 (1:2.7) | 3-4 (1:2.3) | 3-3 (1:2) | 3-2 (1:1.7) | 3-1 (1:1.3) | 5 |
| 2 mL | 2-5 (1:3.5) | 2-4 (1:3) | 2-3 (1:2.5) | 2-2 (1:2) | 2-1 (1:1.5) | 5 |
| 1 mL | 1-5 (1:6) | 1-4 (1:5) | 1-3 (1:4) | 1-2 (1:3) | 1-1 (1:2) | 5 |
| | | | | | Total number of tubes | 25 |

### *3.3 Antibody screening*

Two drops of virtual serum solution were added to each of three test tubes. One drop of screening cells (O1, O2, and O3) was added to tubes 1, 2, and 3, respectively. The tubes were gently mixed and incubated at room temperature (RT) for 30 min, then centrifuged for 20 sec to evaluate hemolysis. The tubes were subsequently resuspended gently to assess agglutination, and the results were recorded at room temperature (RT). The tubes were then incubated at 37°C for 30 min and centrifuged for 20 sec to evaluate hemolysis. The cell button was resuspended to assess agglutination, and the results were recorded at 37°C. For the indirect antiglobulin test (IAT) phase, red blood cells were washed three times with normal saline solution (NSS), adding NSS to approximately three-quarters of the tube volume each time. After the final wash, the supernatant was completely decanted, and residual saline was removed by blotting. Two drops of anti-human globulin (AHG) reagent were added, mixed thoroughly with the cell button, and centrifuged for 20 sec. Agglutination was read macroscopically; if no reaction was observed, the test was examined microscopically. IAT results were recorded. Each antibody screening test was performed in triplicate. For all negative tubes (after microscopic examination), one drop of 3% Coombs' Control Cells (CCC) was added, followed by centrifugation for 20 sec and interpretation. A positive reaction confirmed that the AHG test was valid, that cell washing was adequate, and that the AHG reagent was active; therefore, the test results were considered interpretable. A negative reaction indicated an invalid test (e.g., inadequate washing or inactive reagent), and the test was repeated. Quality control was performed using positive and negative controls to monitor test validity throughout the antibody screening procedure. Three types of control materials were included as follows:

Positive control: Distilled water was used in place of the virtual serum during antibody screening, resulting in complete hemolysis.

Negative control 1: Normal saline solution (NSS) was used in place of the virtual serum in the antibody screening, yielding negative results at all test phases.

Negative control 2: Virtual serum without anti-D (IgG) was used in the antibody screening, yielding negative results at all test phases.

All control tests were performed in triplicate, following the same antibody screening procedure described in section 3.3.

### *3.4 Interpretation of test results*

1) Interpretation of hemolysis

After centrifugation at room temperature (RT) and at 37°C, the test tubes were not resuspended. Hemolysis was assessed by observing the appearance of the supernatant and the presence or absence of a red blood cell (RBC) button at the bottom of the tube. Results were recorded using the following categories:

PH (partial hemolysis): The supernatant is clear red, with residual RBCs remaining as a cell button at the bottom of the tube**.**

H (complete hemolysis): The supernatant is clear red, with no residual RBCs remaining at the bottom of the tube.

2) Interpretation of agglutination

If no hemolysis was observed, agglutination was evaluated by gently resuspending the cell button and tilting the tube to assess RBC agglutination macroscopically. If no agglutination was detected by visual inspection, the reaction was further examined microscopically**.** Positive agglutination reactions were graded as follows:

4+**:** One large agglutinate; clear supernatant.
3+**:** Several large agglutinates; clear supernatant.
2+**:** Several medium-sized agglutinates; clear supernatant.
1+**:** Several small agglutinates; turbid, reddish supernatant.
w (weak): RBCs appear evenly dispersed with minimal or no visible agglutination macroscopically; agglutination is clearly observed microscopically; turbid, reddish supernatant.
0: No hemolysis and no agglutination reaction.

### *3.5 Data collection and statistical analysis*

Study results were collected and analyzed using SPSS Statistics (version 26.0; IBM Corp., Armonk, NY, USA). Optical density (OD) and specific gravity (Sp.Gr.) were summarized as the median (range). Differences in gross physical appearance, OD, and Sp.Gr. among virtual serum preparations, such as clear yellow and turbid yellow mixed with different volumes of distilled water were evaluated using the Kruskal–Wallis test. A p-value $< 0.05$ was considered statistically significant. For antibody screening outcomes, dilution ratios between virtual serum and distilled water were selected based on those producing positive results, as evidenced by both hemolysis and agglutination reactions.

## 4. Results and Discussion

After diluting anti-D (IgG) at a 1:8 dilution in the clear yellow and turbid yellow virtual serum formulations, visual inspection showed that both preparations were yellow but exhibited different degrees of turbidity, as shown in Figures 1A and 1B. Virtual serum samples prepared by adding different volumes of distilled water exhibited distinct physical characteristics. Specifically, the yellow coloration and turbidity decreased as the volume of distilled water increased.

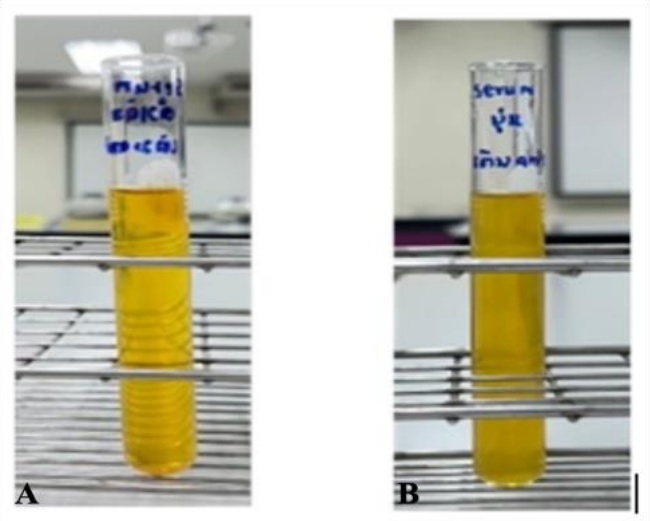


**Figure 1** Appearance of the virtual serum formulations. (A) Clear yellow virtual serum and (B) turbid yellow virtual serum after dilution with anti-D (IgG) reagent, prior to the addition of distilled water for hemolysis induction.

**Table 2** Antibody screening results of the clear yellow and turbid yellow virtual serum

| Dilution factors | Number of tests | O1 | | | | O2 | | | | O3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | RT | 37ºC | IAT | CCC | RT | 37ºC | IAT | CCC | RT | 37ºC | IAT | CCC |
| 1:1.2 - 1:3.5 | 1 | 0 | 0 | 2+ | ND | 0 | 0 | 2+ | ND | 0 | 0 | 2+ | ND |
| | 2 | 0 | 0 | 2+ | ND | 0 | 0 | 2+ | ND | 0 | 0 | 2+ | ND |
| | 3 | 0 | 0 | 2+ | ND | 0 | 0 | 2+ | ND | 0 | 0 | 2+ | ND |
| 1:4 - 1:6 | 1 | PH | PH | 2+ | ND | PH | PH | 2+ | ND | PH | PH | 2+ | ND |
| | 2 | PH | PH | 2+ | ND | PH | PH | 2+ | ND | PH | PH | 2+ | ND |
| | 3 | PH | PH | 2+ | ND | PH | PH | 2+ | ND | PH | PH | 2+ | ND |

ND; not done, PH; partial hemolysis, CCC; Coombs' Control Cells

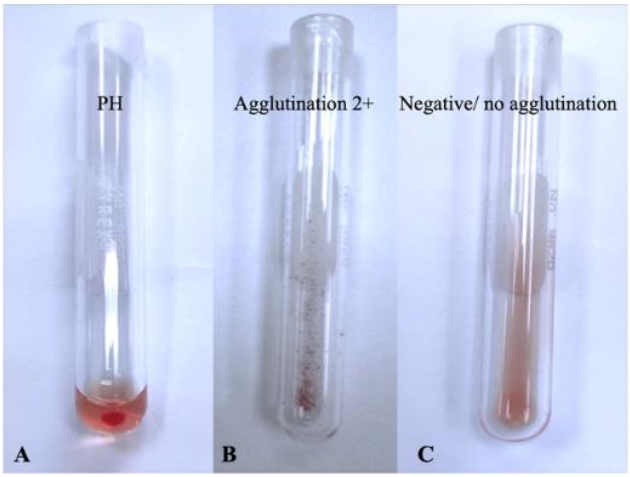


**Figure 2** Antibody screening reactions obtained using the virtual serum model. (A) Partial hemolysis observed after incubation at RT and 37 °C. (B) Positive agglutination reaction (2+) detected in the indirect antiglobulin test (IAT) phase. (C) Negative reaction showing no agglutination.

In the antibody screening, the dilution series of the clear yellow and turbid yellow formulation produced identical test outcomes. Positive reactions were observed at dilutions of 1:4, 1:5, and 1:6**,** characterized by hemolysis during the 37°C incubation phase and 2+ red cell agglutination in the indirect antiglobulin test (IAT) phase**.** At other dilutions (1:1.2-1:3.5), no hemolysis was detected; only agglutination was observed (Figure 2A, 2B, and 2C). This may be attributed to the presence of albumin, which helps maintain red blood cell membrane integrity and reduces osmotic fragility (Reinhart et al., 2015). As a result, the protective effect of albumin may prevent hemolysis at lower dilutions despite the addition of distilled water. Hemolysis occurred only when sufficient distilled water was present to create a hypotonic environment, allowing net water influx into erythrocytes through osmosis, leading to cell swelling and eventual membrane rupture. In this study, this effect became evident at dilutions of 1:4 -1:6 (Table 2). A Hypotonic solution refers to a solution containing a lower amount of solute in comparison to the solute concentration in other solutions, across a semipermeable membrane. Such a solution has a decreased solute concentration and a total movement of water in the cell. This can cause the swollen cell to rupture. When a cell is immersed in a hypotonic solution, water molecules move into the cell from the surrounding solution because of the osmotic gradient. The continuous influx of water leads to cell swelling and may eventually cause cytolysis (rupture of the cell) (Lopez & Hall, 2023).

**Table 3** Optical density (OD) and specific gravity (Sp.Gr.) of dilution factors of the virtual serum solution

| Appearance of virtual serum solution | Dilution | OD median (min, max) | Sp.Gr. median (min, max) |
|---|---|---|---|
| Clear yellow | Undiluted* | 1.580 (1.580, 1.581) | 1.027 (1.027, 1.027) |
| | 1: 4 | 0.421 (0.420, 0.425) | 1.008 (1.008, 1.008) |
| | 1: 5 | 0.340 (0.339, 0.341) | 1.007 (1.007, 1.007) |
| | 1: 6 | 0.281 (0.280, 0.284) | 1.006 (1.006-1.006) |
| Turbid yellow | Undiluted* | 1.635 (1.635, 1.636) | 1.027 (1.027, 1.027) |
| | 1: 4 | 0.453 (0.453, 0.454) | 1.008 (1.008, 1.008) |
| | 1: 5 | 0.366 (0.364, 0.367) | 1.006 (1.006, 1.006) |
| | 1: 6 | 0.303 (0.302, 0.303) | 1.005 (1.005, 1.005) |

* The clear yellow and turbid yellow virtual serum, after dilution with anti-D (IgG) and prior to the addition of distilled water (baseline condition), did not induce hemolysis or agglutination.

**Table 4** Optical density (OD), specific gravity (Sp.Gr.), and p-values of clear yellow and turbid yellow virtual serum

| Appearance of virtual serum | Pair No. | Matched dilution ratios | p-value | |
|---|---|---|---|---|
| | | | OD | Sp.Gr. |
| Clear yellow | 1 | Undiluted - 1: 4 | 1.000 | 1.000 |
| | 2 | Undiluted - 1: 5 | 0.247 | 0.216 |
| | 3 | Undiluted - 1: 6 | 0.013* | 0.010* |
| | 4 | 1: 4 - 1: 5 | 1.000 | 1.000 |
| | 5 | 1: 4 - 1: 6 | 0.247 | 0.216 |
| | 6 | 1: 5 - 1: 6 | 1.000 | 1.000 |
| Turbid yellow | 1 | Undiluted - 1: 4 | 1.000 | 1.000 |
| | 2 | Undiluted - 1: 5 | 0.243 | 0.216 |
| | 3 | Undiluted - 1: 6 | 0.013* | 0.010* |
| | 4 | 1: 4 - 1: 5 | 1.000 | 1.000 |
| | 5 | 1: 4 -1: 6 | 0.243 | 0.216 |
| | 6 | 1: 5 - 1: 6 | 1.000 | 1.000 |

* p-value < 0.05 indicates a statistically significant difference

The median OD and specific gravity (Sp.Gr.) values of dilution ratios that produced hemolysis and agglutination in the antibody screening assay are presented in Table 3. Comparison of OD and Sp.Gr. values of the virtual serum diluted with distilled water at undiluted, 1:4, 1:5, and 1:6 for both the clear yellow and turbid yellow formulations showed that the undiluted and 1:6 conditions differed significantly in OD and Sp.Gr. ($p < 0.05$), indicating that a sufficiently large volume of distilled water is required to produce measurable differences (Table 4). Previous studies have reported the preparation of virtual serum using the clear yellow formulation (Yothinarak, 2018), a light yellow formulation, and a turbid yellow formulation (Yothinarak et al., 2025). However, these formulations were designed as isotonic solutions. In the present study, distilled water was added to convert the simulated serum into a hypotonic solution to enable observation of hemolysis during antibody screening. This approach was intended to support teaching and allow students to directly observe this reaction type during practical laboratory sessions. Nevertheless, hemolysis in antibody screening can also occur as an artifact, for example, when test tubes are not adequately cleaned. In such cases, residual red cells may be absent or insufficient for testing in the antiglobulin phase, resulting in no agglutination and suggesting a false-positive hemolysis result. To ensure that the antiglobulin phase yielded a true positive reaction, anti-D (IgG) was added in this study to generate positive agglutination, enabling students to learn that a positive antibody screening result does not identify the specific unexpected antibody. Therefore, antibody identification is required as the subsequent step to determine the specificity of the unexpected antibody. This study has several limitations. The hemolysis observed in the virtual serum model was induced by osmotic pressure rather than complement-mediated antigen–antibody reactions as observed in clinical samples. Thus, the model is intended primarily for educational simulation rather than for

reproducing the exact immunological mechanisms of transfusion medicine. Furthermore, because the formulation contains food-based ingredients such as chrysanthemum powder and creamer, microbial growth during storage may occur. Future studies should therefore evaluate the long-term stability, shelf-life, and suitable preservation strategies for safe laboratory training use.

## 5. Conclusion

This study successfully developed a virtual serum solution capable of producing positive antibody screening reactions. Both clear yellow and turbid yellow virtual serum diluted with distilled water at ratios of 1:4, 1:5, and 1:6 produced positive hemolysis and agglutination reactions. The developed model can be used as a practical teaching material for the Transfusion Science I course in the Faculty of Medical Technology, Rangsit University.

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