

**Long Term Storage Effect of 0.2M Dithiothreitol on Red
Cell Antigen Integrity on Stored Reagent Red Blood Cells**

ABSTRACT

BACKGROUND: Treating reagent red cells with 0.2M dithiothreitol (DTT) removes drug interference due to daratumumab and allows for the detection of underlying alloantibodies.

This study aimed to investigate the effect of DTT-treatment on reagent red cell antigen integrity over a period of time.

STUDY DESIGN AND METHODS: Twelve aliquots of human sourced plasma, each containing an antibody of a single, known specificity, were tested against untreated and 0.2M DTT-treated reagent screen cells for a 28 day period. Hemolysis and serological antibody reaction strengths were observed.

RESULTS: Red cell antigen integrity remained serologically detectable on the DTT-treated cells throughout the entire 28 day study, despite a greater degree of observed hemolysis.

CONCLUSION: Long term storage of 0.2M DTT-treated reagent red cells does not appear to compromise antigen integrity. Advanced DTT-treatment and storage of a large aliquot of screen cells may serve to increase efficiency in the transfusion service.

INTRODUCTION

Targeted drug therapy involving the use of monoclonal antibodies to treat malignancies is becoming increasingly popular; such drugs are highly specific to the intended target cell. One such drug, daratumumab (DARA), is currently being used to treat patients with refractory or relapsed multiple myeloma. DARA works by binding to CD38 on plasma cells and inducing apoptosis—programmed cell death—via a variety of immune mediated mechanisms.^{1;2-3}

Human CD38 contains six disulfide bonds and is strongly expressed on plasma cells.

Additionally, smaller, varying amounts of CD38 are also expressed on red cells; in cancer patients, this expression is also increased.⁴ Considering the variation in density expression of CD38 on red cells, it is believed that anti-CD38 drugs, such as DARA, only bind to those cells sufficiently expressing surface CD38.⁵ However, anti-CD38 drugs pose a problem for the transfusion service in that they may cause a number of drug-induced false positives, including positive direct antiglobulin tests (DAT) and panreactivity in eluates and in indirect antiglobulin tests (IAT). Such results can prolong compatibility testing and delay delivery of suitable blood products for patients requiring transfusion.^{1-2;4-5}

Currently, there are several methods available to resolve this panagglutination. The anti-CD38 in the patient's plasma can be neutralized with recombinant human soluble CD38 or mouse anti-DARA idiotypic prior to testing against reagent red cells.⁴ Alternatively, reagent red cells can be treated with sulfhydryl reagents, such as 0.2M dithiothreitol (DTT) or 2-mercaptoethanol (2-ME). These chemicals cleave the disulfide bonds in the CD38 molecule and denature protein, resulting in complete suppression of CD38 catalytic activity. One side effect of DTT-treating reagent red cells to remove the CD38 activity is that blood group systems

containing disulfide bonds will equally be destroyed. Another option is the use of trypsin, which also disrupts the extracellular domain of CD38. However, it is less efficient than DTT and also disrupts more blood group antigens than does DTT.^{4;6;7} This study focused on DTT treatment of reagent red cells in order to eliminate panagglutination in the IAT phase of compatibility testing. Specifically, we wanted to see if long-term storage of DTT-treated reagent red cells would affect antigen reactivity strength over time.

Currently, our transfusion service's protocol for patients with a recent history of receiving DARA and whose plasma demonstrates panagglutination is to treat small quantities of screen cells with 0.2M DTT immediately prior to use and retest the patient's plasma against the DTT-treated screen cells in order to confirm the presence or absence of underlying alloantibodies. Since each treatment of screen cells requires a 30-45 minute incubation prior to use for testing, the transfusion service in question would potentially like to DTT treat a larger batch of screen cells in advance of testing that can be stored for a specified period of time (i.e. 7 days). However, there is currently no documentation regarding the serological detection of antigens on DTT-treated reagent red cells undergoing extended storage. In order to validate the use of such cells, it is necessary to demonstrate that the various antigens on reagent red cells will be serologically detectable at 2+ or greater reactivity using current testing methods for the proposed extended time frame. Having a previously prepared set of 0.2M DTT-treated screen cells immediately available to technologists would eliminate the need to DTT-treat small quantities of cells for each antibody identification, reduce antibody identification turnaround time by 30-45 minutes, and increase efficiency in the transfusion service.

MATERIALS AND METHODS

Study Length

Both DTT treated and untreated cells were tested daily for 28 days.

Plasma specimens

Human sourced plasma was used to test against both untreated and 0.2M DTT-treated reagent cells. Plasma sources included both donor plasma and hospitalized patients containing a single antibody specificity. Plasma containing antibodies of single specificities previously identified and confirmed by serological means were collected and stored at -20°C prior to the start of the study. For less commonly identified antibodies, plasma from multiple sources, including both patients and donors, were pooled to ensure an adequate amount of plasma was available for the duration of the study.

A volume of 12 mL of plasma was required to perform 28 days of daily quality control and testing against DTT-treated and untreated red cells. A deadline was given for finding plasmas containing the various antibody specificities. After this deadline, those specificities that were not located or did not meet the required quantity were eliminated from further consideration in the study. When pooling plasmas, ABO blood group was ignored since only group O reagent cells were used for quality control and the study itself. Each plasma specimen was divided into small, 0.5mL aliquots, appropriately labeled, and stored frozen at -20°C prior to the start of the study.

Once enough plasma of a single, antibody specificity was collected, positive and negative controls were performed to confirm that adequate reactivity levels of antibody were detected in each plasma specimen and no reactivity was detected when that same plasma was

tested against a cell negative for the antigen. If an antibody in a plasma specimen was found to be nonreactive with the positive control, new plasma from a different source of the same antibody specificity was collected and controls rerun. If a plasma specimen was found to be reactive with a negative control, antibody identification was performed on it to confirm that it did not contain another clinically significant antibody to a common antigen. Antibody strength was initially tested against Immucor reagent cells positive and negative for the specified antigen. Positive controls consisted of a presumed single dose of the antigen being tested, while negative controls lacked the antigen specific to the antibody in the plasma. Controls were performed using a Polyethylene Glycol (PeG) enhancement technique and IAT, since this was the only method with which the antigen reactivity in the DTT-treated and untreated cells were to be studied.

Storage of Plasma Specimens

Once a plasma specimen passed this initial control testing, 28 small aliquots were prepared for frozen storage at -20°C. Each aliquot contained enough plasma (0.5mL) to conduct one day's worth of quality control and reagent red cell testing. The purpose of the aliquots was to avoid daily thawing and freezing of each antibody, which might contribute to antibody integrity degradation.⁸

Antigens studied

Plasma containing antibodies produced against antigens from four of the nine classic independent blood group systems were used for the study: D, C, E, c, e; M, S, s; Fy^a, Fy^b; Jk^a, Jk^b. No plasma containing antibodies to the Kell blood group system were tested since 0.2M DTT destroys these antigens.⁷

DTT treatment of reagent RBCs

Panoscreen I, II, and III reagent screen cells (Immucor, Norcross, Georgia, USA) were used for the study. These cells consisted of R_1R_1 , R_2R_2 , and rr Rh phenotypes as well as presumed double dose examples of M, N, S, s, Fy^a , Fy^b , Jk^a , and Jk^b . An adequate volume of 0.2M DTT—enough to treat the total volume of reagent red cells that were used for the duration of the study—was prepared according to methodology described in the AABB Technical Manual.⁹ A separate set of screen cells from the same lot of reagent screen cells was left untreated and tested in parallel with the 0.2M DTT-treated cells. The 0.2M DTT was created by dissolving 1 gram of DL-1,4-Dithiothreitol, 99% (Acros Organics, Fisher Scientific, Fair Lawn, New Jersey, USA) into 32mL of phosphate buffered saline (PBS) with a pH of 8.0.

Since a large aliquot of reagent red cells was treated all at once, the volume was split over several tubes so that the entire volume of cells in each tube could incubate properly in the heating block. After incubation was complete, cell aliquots were washed one time with PBS, removing as much of the supernatant as possible without disturbing the cells, and four volumes of 0.2M DTT were added, mixed, and incubated at 37°C for 45 minutes. After incubation, the cells were washed at least four times with PBS. All treated aliquots of cells were returned to the original manufacturer's bottle from which they came, and prepared as a 2-5% suspension using Red Blood Cell (RBC) Storage solution (Immucor, Norcross, Georgia, USA) as the diluent. Monoclonal anti-K (Immucor, Norcross, Georgia, USA) was used to confirm the absence of the K antigen from K positive DTT-treated cells and thus confirm successful treatment of the reagent cells.

Storage of reagent RBCs

Upon successful treatment with 0.2M DTT, reagent screen cell aliquots were stored as a 2-5% cell suspension in RBC Storage Solution at 2-4°C. The untreated reagent cells were also stored in the RBC storage solution at 2-4°C for consistency. Hemolysis of treated and untreated cells was noted prior to daily testing. Daily testing was performed regardless of the degree of hemolysis present, since the tubes were tested using a PeG enhancement technique and only read at the IAT phase.

Detection of RBC antigen

For each plasma aliquot, quality control was performed daily prior to testing against the DTT-treated and untreated reagent cells. Quality control consisted of testing each plasma specimen against a presumed single dose expression of the antigen corresponding to that antibody specificity (positive control) as well as testing against a cell negative for the antigen corresponding to each plasma specificity (negative control). Reactivity strength of 1+ or greater in the positive control and no reactivity in the negative control was considered acceptable for the plasma to be used for that day's testing. Plasma not meeting acceptable quality control criteria was discontinued from further testing in the study.

Upon acceptable daily quality control results, each plasma specimen was tested against a presumed double dose of antigen for both the untreated and treated reagent cells using the PeG IAT method described in the AABB Technical Manual. All cells were incubated for 15 minutes at 37°C and then washed four times prior to adding monoclonal anti-IgG (Immucor, Norcross, Georgia, USA). Negative reactions were confirmed by the addition of IgG coated red cells (Immucor, Norcross, Georgia, USA).

Detection of Hemolysis

In order to provide a somewhat objective and qualitative measurement of hemolysis observed, a color comparator chart (Figure 1) was used each day prior to performing quality control testing to “grade” the degree of hemolysis observed in the diluent of the untreated and DTT-treated cells. Baseline readings on the first day of testing (day 0) were graded a 4 for both the untreated and DTT-treated cells as this color on the chart most closely resembled the diluent color. Considering the untreated cells were never altered from how the manufacturer produced them and considering the manufacturer has quality standards of its own in place, the baseline grade of 4 was considered to be the equivalent of no visible hemolysis in the diluent.

RESULTS

The goal of this study was to assess the surface antigen integrity of various red cell antigens on red cells treated with 0.2M DTT over a period of time. Twelve different red cell antigens were evaluated for reactivity strength against untreated and 0.2M DTT-treated red cell antigens. The general trend among all plasma tested was that antibody reactivity persisted on DTT-treated cells with all antigens tested for the duration of the study. Additionally, antibody reactivity with DTT-treated cells demonstrated reactivity strength that was either equal to or stronger than the reactivity strength of the untreated cells. The results for each plasma specificity are documented in Figures 3 through 14.

Overall, there was very little difference in reactivity strength between untreated and DTT-treated cells for antigens not affected by DTT. In most instances, the DTT-treated cells reacted slightly more strongly. There was noticeably more hemolysis with the DTT-treated cells

over time compared to the untreated cells (Table I). None of the antibodies produced reactivity strengths of less than 1+ with the untreated or DTT-treated cells during the study.

Hemolysis Observed

Both sets of screen cells maintained a grade of 4 (no visible hemolysis) through day 5 (Table I). On day six, very slight hemolysis was observed in DTT-treated screen cell II and this was given a grade of 5, since the diluent most closely resembled a 5 color shade on the color comparator chart. DTT-treated screen cells I and III continued to be graded a 4 on this day. For each day, the average of the three screen cells determined the overall hemolysis grade given (Figure 2). Up until day six, the averages of the three untreated screen cells and three DTT-treated screen cells were 4 for both sets. For day six, the average for the untreated screen cells remained a 4, but the average for the three DTT-treated screen cells was $(4+5+4) \div 3 = 4.67$, which was rounded up to 5. On day six, both DTT-treated screen cells I and II were given a 5 grade and on day 8, all three DTT-treated screen cells were at 5, making the average a 5 as well.

The untreated screens did not begin to show very slight visible hemolysis (grade 5) until day 15, 22 days before the manufacturer's documented expiration date. By this point, the DTT-treated cells were a grade 6. By day 28, nine days before manufacturer's documented expiration date, both the untreated screen cells and the DTT-treated screen cells were a grade 6, which was the equivalent of visual gross hemolysis.

Cell Button Size

Cell button size was, not surprisingly, directly proportional to the degree of hemolysis observed. A noticeably smaller button size was first observed in the DTT-treated cells on day eight (grade 5.) By day 11, the button size was obviously smaller. However, since controls

continued to perform as expected, the cells continued to be tested using this assumed lighter suspension. No attempt was made to remove some of the diluent so as to bring the cell suspension up to 2-4%.

By day 15, there was noticeable red cell stroma surrounding cell buttons, to the point that the buttons appeared to consist of 50% stroma and 50% intact red cells. At this point, it was decided to add two drops cell suspension to two drops plasma and two drops PeG. Adding the second drop of cells caused the cell button to have a cell button size resembling that of the 2-4% suspension that was still being observed in the untreated cells. Daily controls continued to work as expected despite this addition of a second drop of red cell suspension and no false negatives were observed.

Plasma Antibody Reactivity Strength in Quality Control Testing

The anti-Jk^b plasma reactivity demonstrated a 1+ reaction on the first day of testing (day 0) with the positive control when incubated with PeG for 15 minutes at 37°C against a single dose of Jk^b antigen. When anti-Jk^b control testing was performed in this same manner on day 1, the reaction strength was less than 1+. As a result, incubation time for anti-Jk^b was increased from 15 minutes to 30 minutes at 37°C. The increased incubation time resulted in the positive control reacting 1+ and was thus acceptable for continued use in the study, so long as the incubation time for the untreated and DTT-treated cells was also 30 minutes at 37°C.

On days 14 and day 15, respectively, the anti-e and anti-M plasma demonstrated reaction strengths weaker than 1+ with the positive control and the same procedure that was used with the weak reacting anti-Jk^b plasma was used with the weak reacting anti-e and anti-M plasma. After a 30 minute incubation at 37°C, the reaction strength of the positive control for

each plasma at IAT was 1+ and thus the both plasma specimens were acceptable for use with the test cells.

Plasma Antibody Reactivity with the Study Cells

The anti-D plasma reacted consistently with the untreated cells for the duration of the study with an average reaction strength of 3+ and occasional 2+ reactions (Figure 3). Anti-D reactivity with the DTT-treated cells also demonstrated a 3+ reactivity strength average with occasional 2+ reactions, but on five different days, 4+ reactions were observed, with three of those 4+ reactions occurring during the last five days of the study and thus revealing an upward trend.

As with the anti-D plasma, the anti-M plasma also reacted consistently with the untreated cells with an average reactivity strength of 2+ (Figure 8). However, anti-M reactivity with the DTT-treated cells demonstrated a slightly downward trend. This may be partially due to a combination of the DTT treatment and the fact that the M antigen can deteriorate in storage.⁷

Anti-Jk^b was the weakest-reacting antibody out of all the plasma specimens used in the study. This might be due to a combination of this particular plasma being a weak example of the antibody and the fact that antibodies to Kidd antigens tend to deteriorate in storage.^{7;10} Anti-Jk^b reactivity with untreated cells never exceeded a 2+ reaction (Figure 14) whereas, anti-Jk^b reactivity with DTT-treated cells occasionally reacted 3+. On days where there was a difference in the reaction strength with anti-Jk^b between the untreated and DTT-treated cells, the DTT-treated cells always reacted stronger than the untreated cells.

DISCUSSION

The treatment of disease using monoclonal antibodies appears to be a promising new technology for patients with conditions where such a treatment is currently an option. However, as with the case of drug interference due to DARA, these drugs are still capable of binding to other cells never intended to be the targeted cell of interest simply because they contain small amounts of the drug's targeted cell marker. This drug-cell marker interaction results in interference with compatibility testing in the transfusion service. Depending on the cell marker's biochemical structure, the interference can be removed via chemical manipulation of the red cells prior to testing with patient plasma. In the case of DARA, treating reagent red cells with 0.2M DTT destroys the disulfide bonds that make up the CD38 marker, rendering it nonfunctional and thus not recognizable by anti-CD38, and alleviating interference with compatibility testing. The long term effects of DTT treatment on red cell antigen integrity, however, has received little investigation, most likely because red cells are typically tested immediately following DTT treatment and acceptable quality control testing.

This study demonstrated that long term storage of 0.2M DTT treated reagent red cells does not compromise antigen red cell integrity. In fact, when a PeG enhancement technique is used, the antibody reacts more strongly with the DTT-treated red cells than with untreated cells. However, since DTT-treatment does destroy some blood group antigens (namely Kell blood group system), this method opens up the possibility of missing an antibody to a clinically significant high prevalence antigen associated with one of these destroyed blood groups. This issue can be somewhat resolved by testing the patient's neat plasma with human umbilical cord blood that has been confirmed positive (serologically or molecularly) for a high prevalence

antigen associated with one of these DTT-destroyed blood groups, such as k (KEL2) or Kp^b (KEL4). Cord blood contains small enough amounts of CD38 that it does not result in interference with DARA.¹¹ For other, lower incidence antigens within these blood group systems, such as K (KEL1), antigen negative blood can easily be acquired from the donor population and be given to those patients who are capable of making the antibody. Ideally, the patient should be molecularly genotyped prior to beginning DARA treatment.^{4;11}

One aspect that was not taken into consideration for this study was a cost analysis of treating cells on an “as needed” basis using individually prepared aliquots of 0.2M DTT-treated cells versus using 0.2M DTT-treated cells that were prepared in advance of testing as part of a much larger batch and stored appropriately prior to use. For an immunohematology reference laboratory or a transfusion service in a large hospital setting, preparing DTT-treated cells in advance and storing them until needed could potentially increase the efficiency of laboratorian time. DTT treatment of red cells requires a 30-45 minute incubation at 37°C.^{9;12} Spending 30-45 minutes preparing a fresh batch of DTT-treated screen cells each time they are required versus spending 30-45 minutes periodically making a large quantity of DTT treated cells that can be accessed multiple times throughout the week could prove to be more efficient in a transfusion service that frequently requires such cells.

In conclusion, this study showed that red cell antigens resistant to 0.2M DTT treatment persisted on the red cell surface for 28 days with little to no weakening when a PeG enhancement technique was used. Plasma reactivity strength of 2+ or greater with the DTT-treated cells was achieved for at least 7 days. This finding introduces the possibility of the transfusion service storing previously prepared DTT-treated reagent screen cells for a defined

period of time until needed for antibody identification. Hemolysis was greater in DTT-treated cells that were the same age as untreated cells, but this aspect is negligible if the cells are tested using a PeG enhancement technique. Treating a large batch of cells with 0.2M DTT offers an opportunity to increase the efficiency of antibody identification by reducing time spent performing special techniques on individual specimens.

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Figure 1. Color Comparator Chart (Haemonetics, Braintree, Massachusetts, USA)

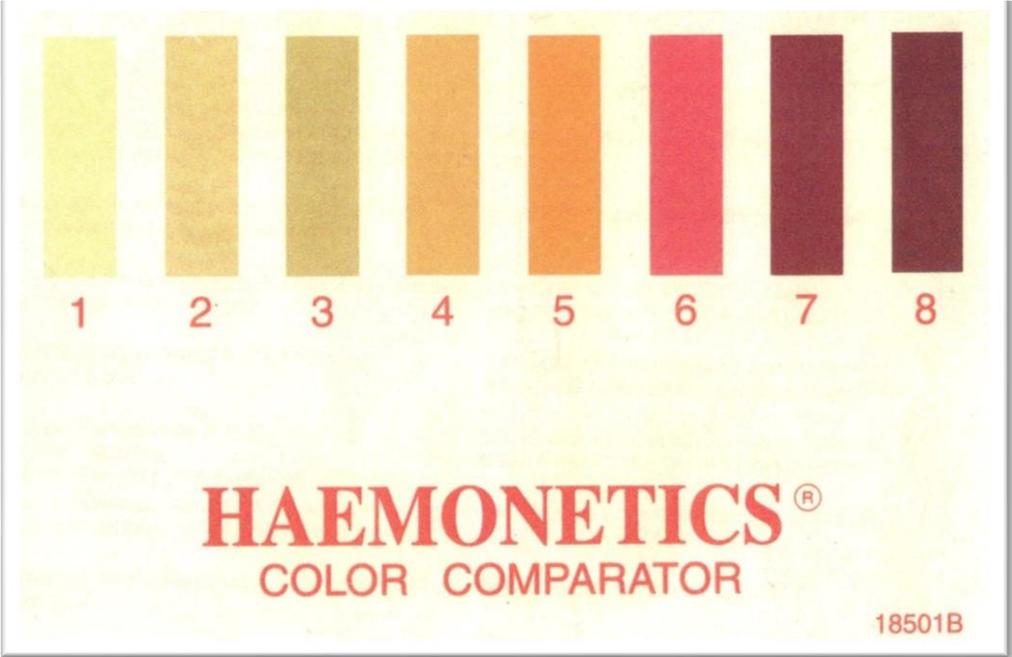


Figure 2. Comparison of hemolysis between untreated and 0.2M DTT-treated cells over a 28 day period

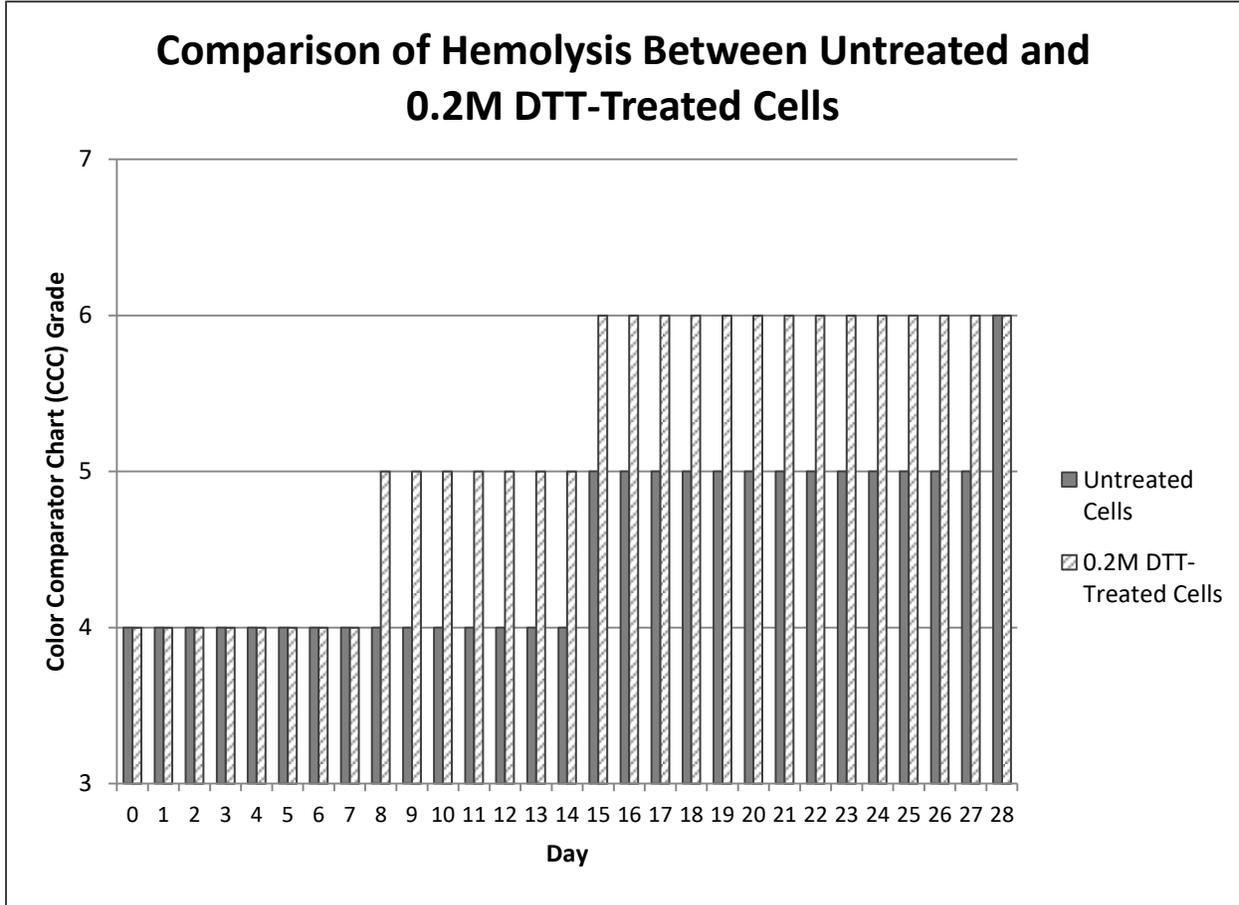


Figure 3. Reaction strengths of Anti-D with untreated and 0.2M DTT-treated screen cells

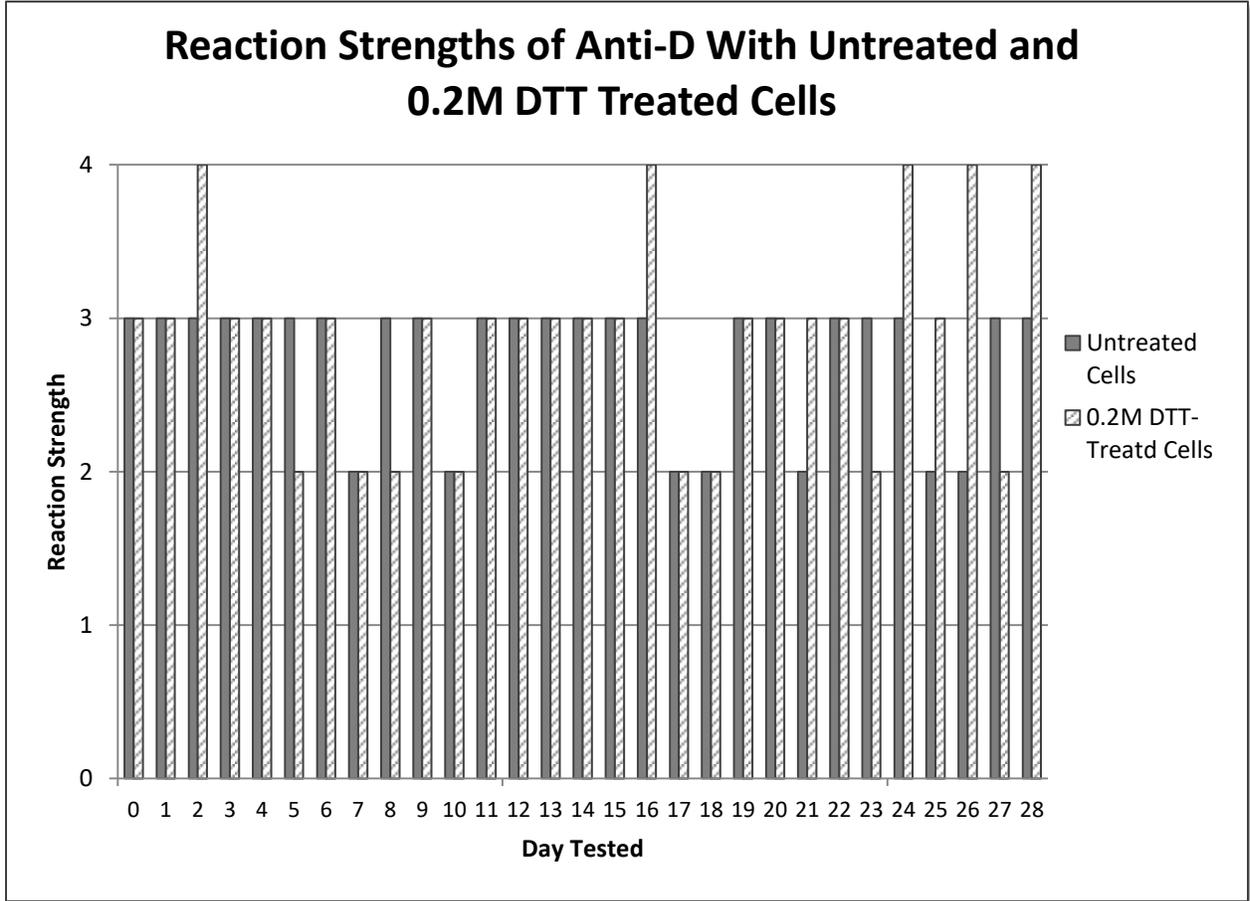


Figure 4. Reaction strengths of anti-C with untreated and 0.2M DTT-treated screen cells

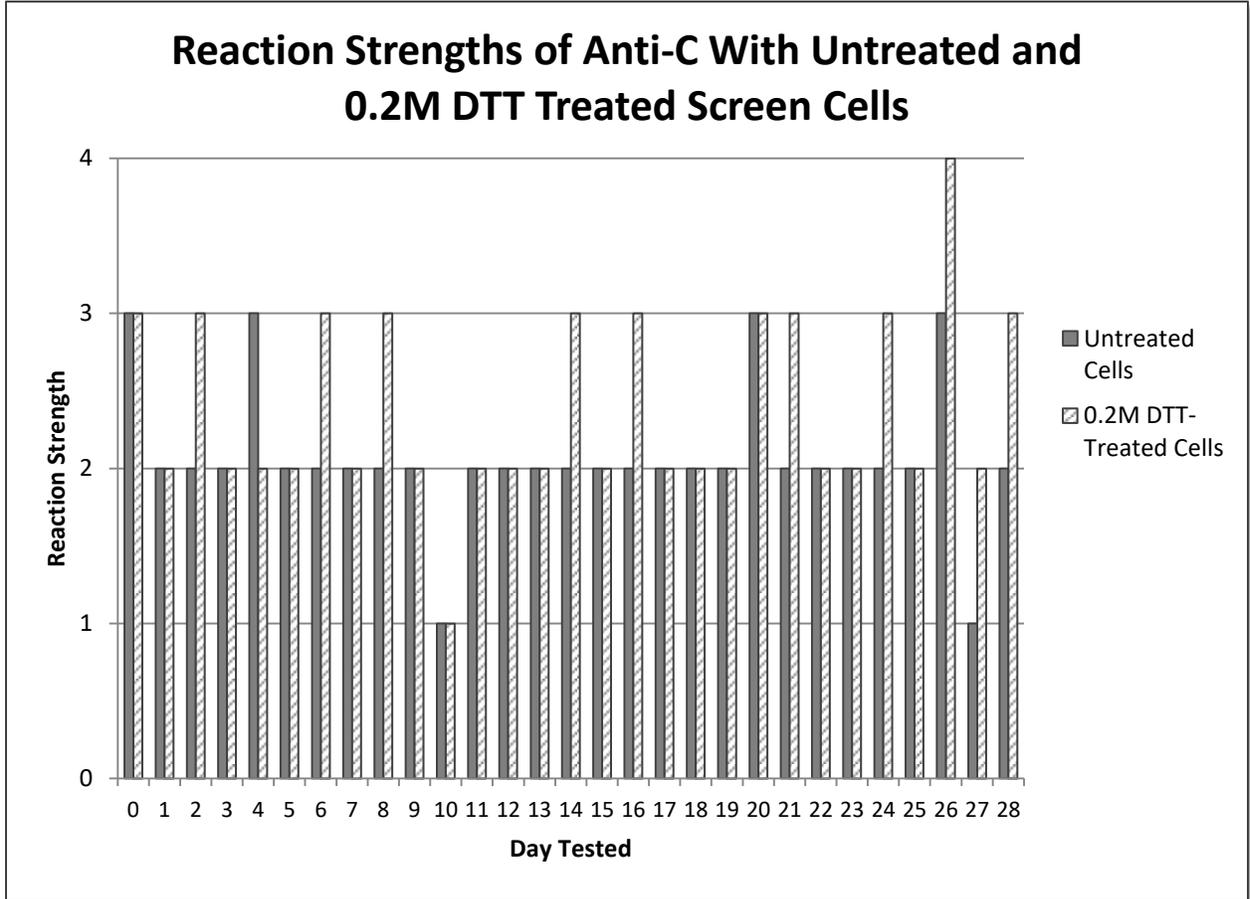


Figure 5. Reaction strengths of anti-c with untreated and 0.2M DTT-treated screen cells

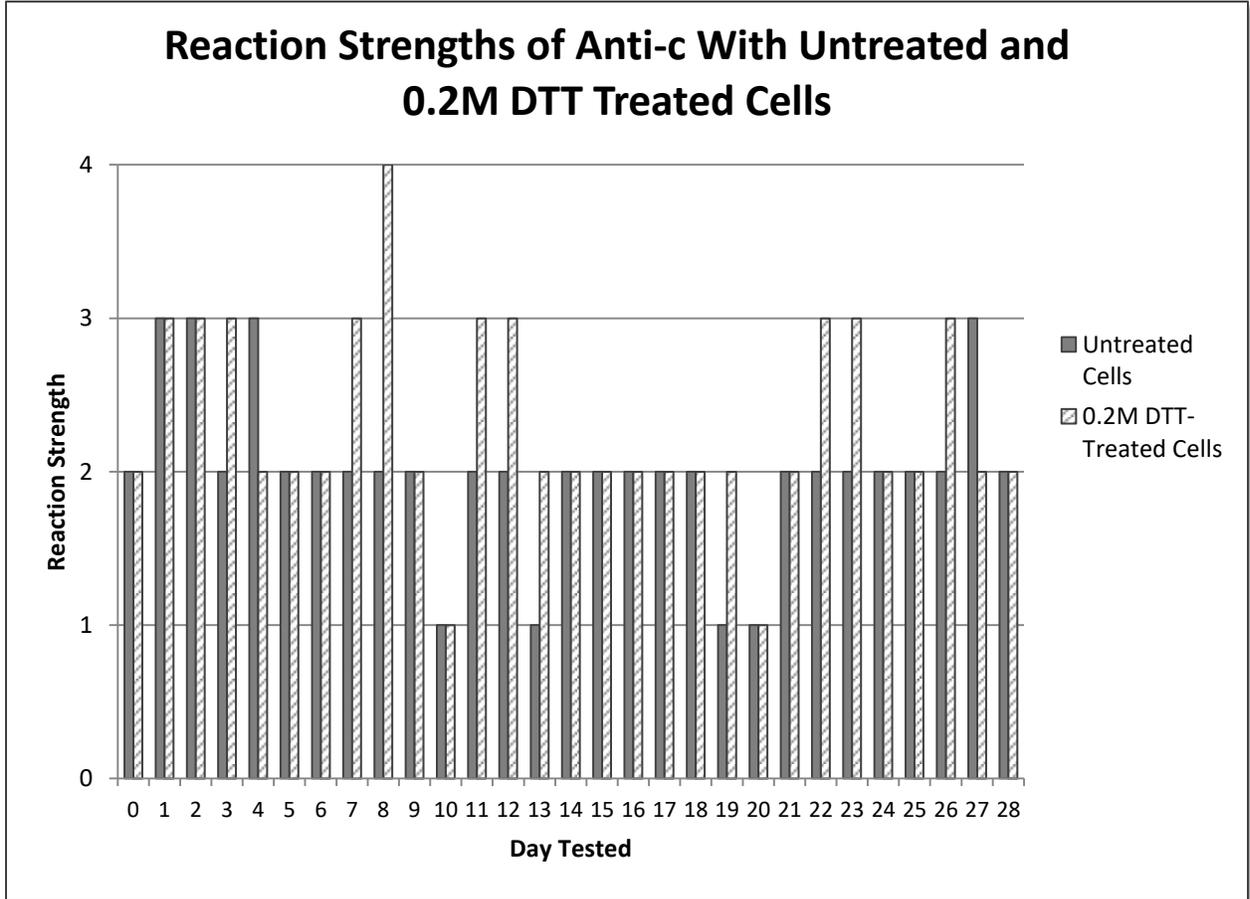


Figure 6. Reaction strengths of anti-E with untreated and 0.2M DTT-treated screen cells

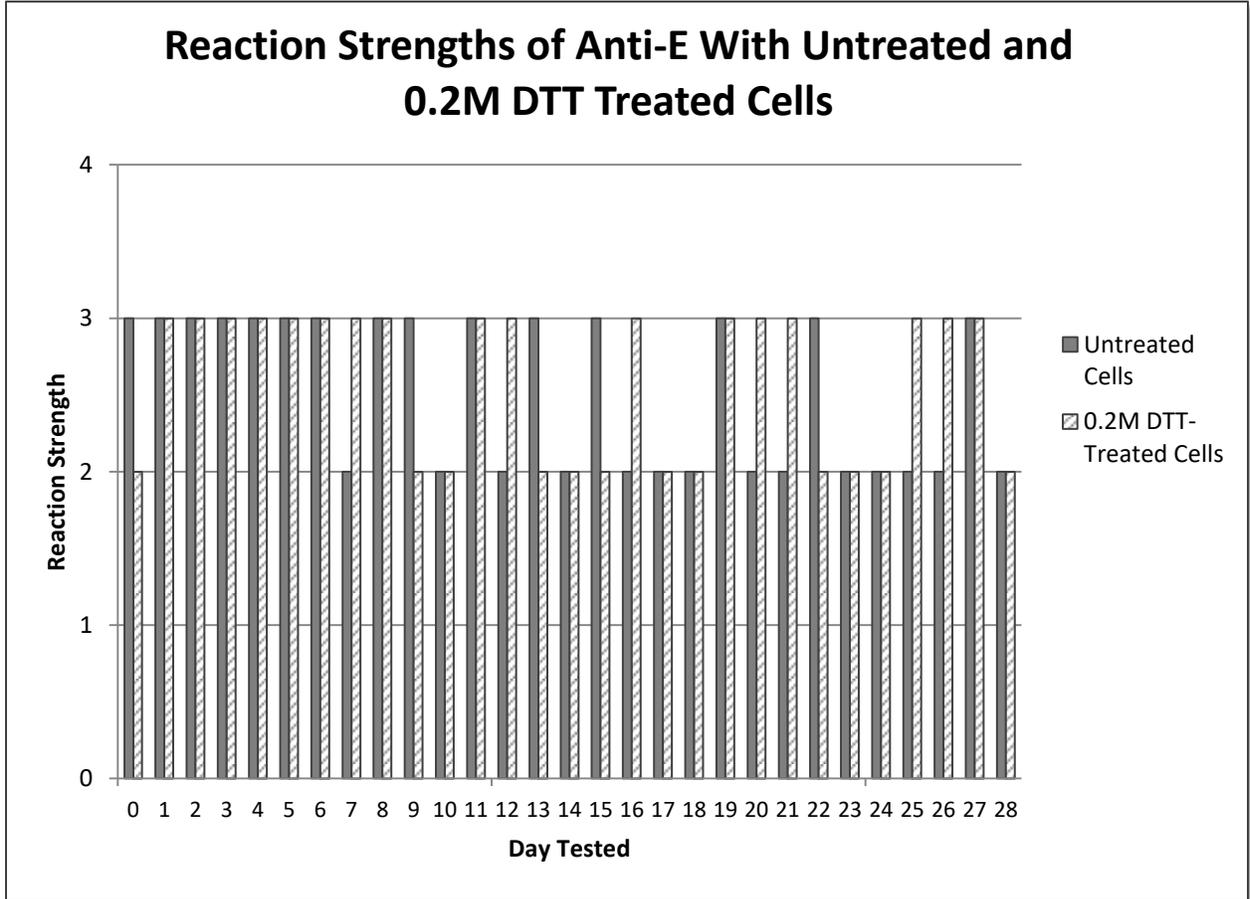


Figure 7. Reaction strengths of anti-e with untreated and 0.2M DTT-treated screen cells

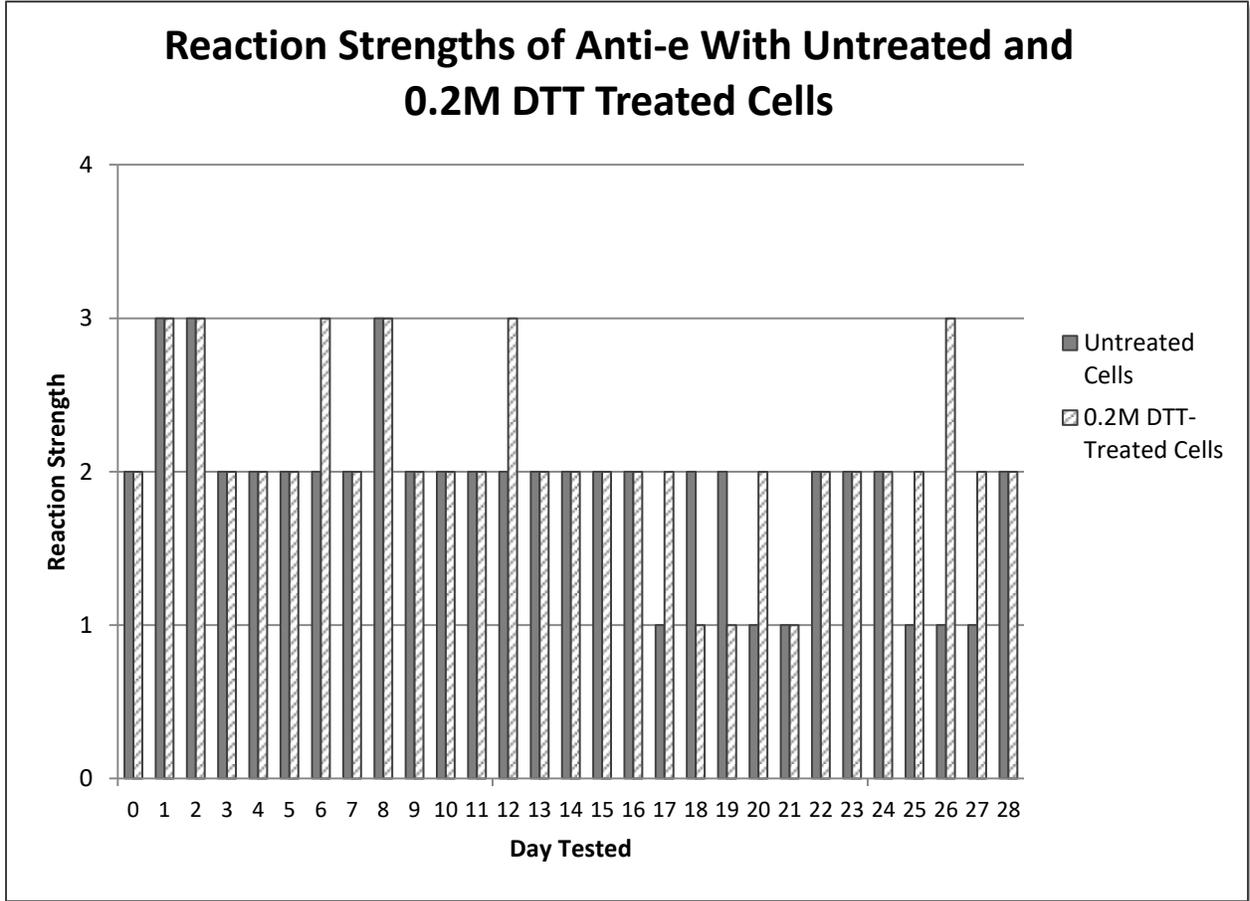


Figure 8. Reaction strengths of anti-M with untreated and 0.2M DTT-treated screen cells

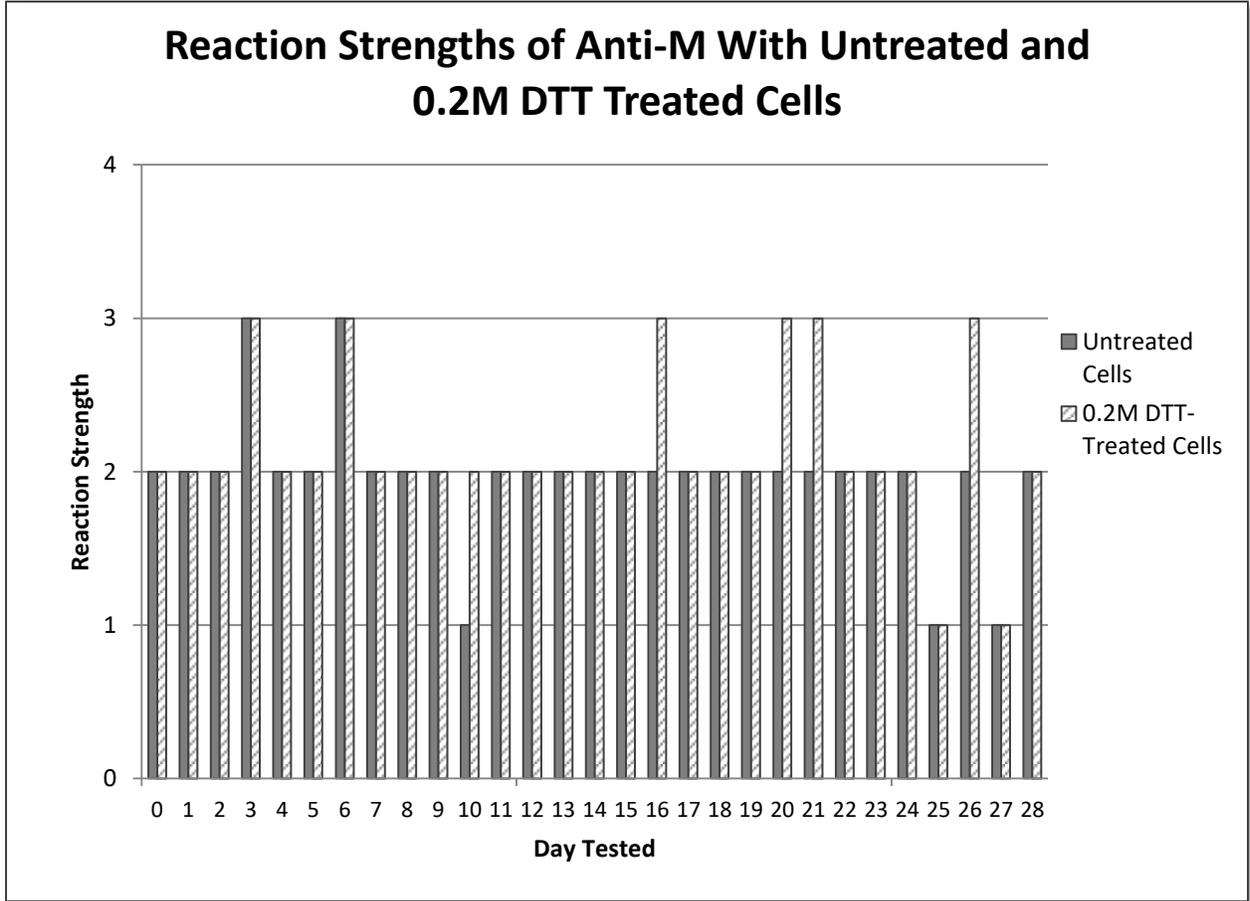


Figure 9. Reaction strengths of anti-S with untreated and 0.2M DTT-treated screen cells

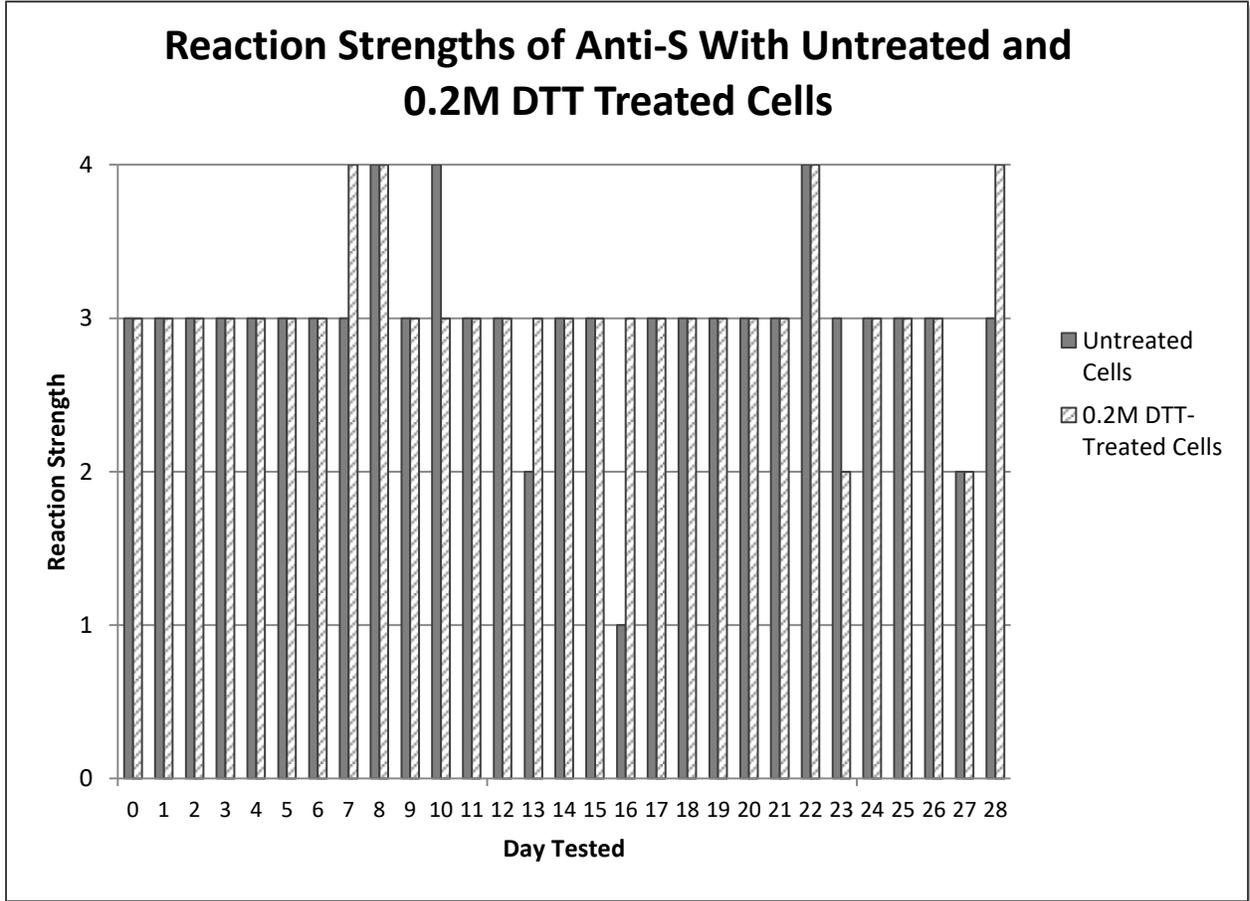


Figure 10. Reaction strengths of anti-s with untreated and 0.2M DTT-treated screen cells

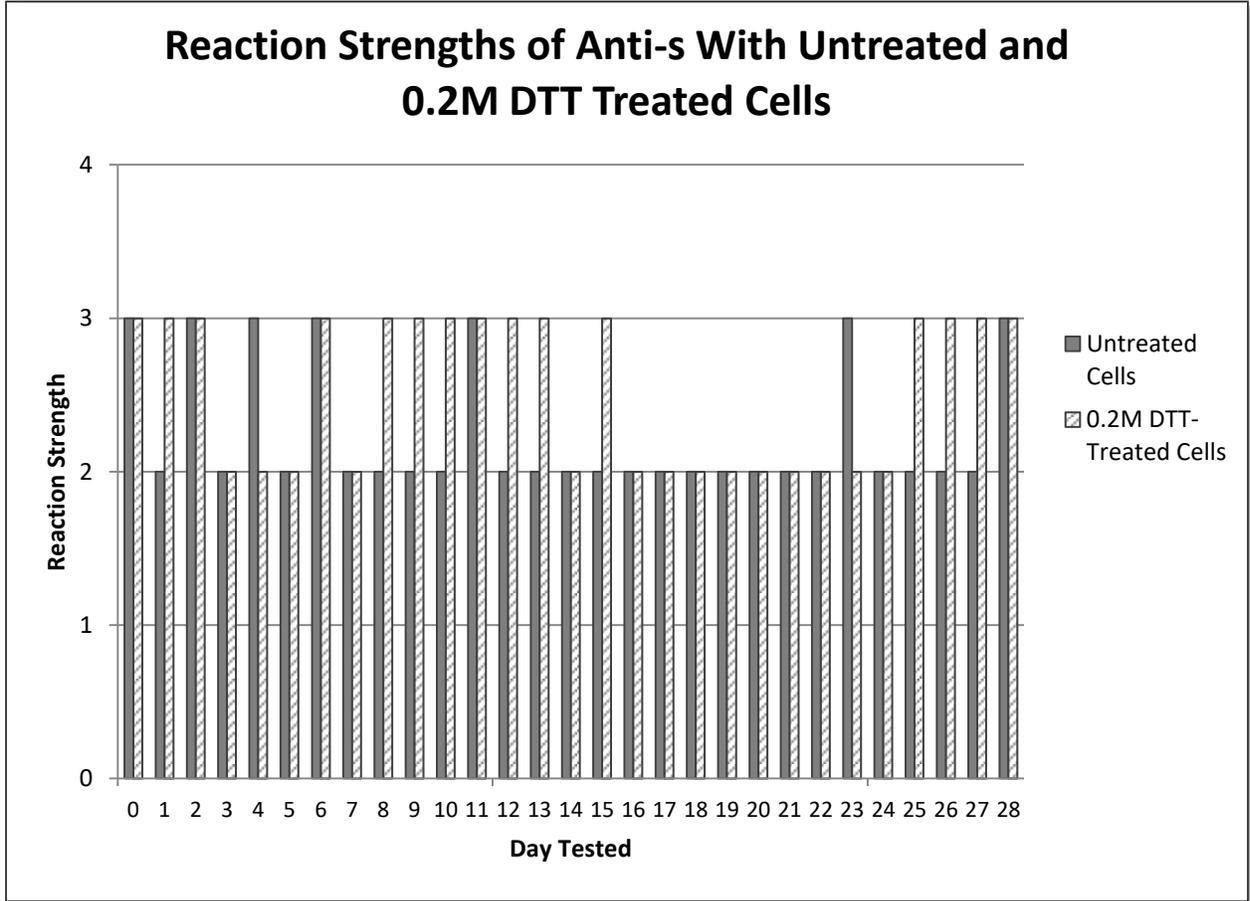


Figure 11. Reaction strengths of anti-Fy^a with untreated and 0.2M DTT-treated screen cells

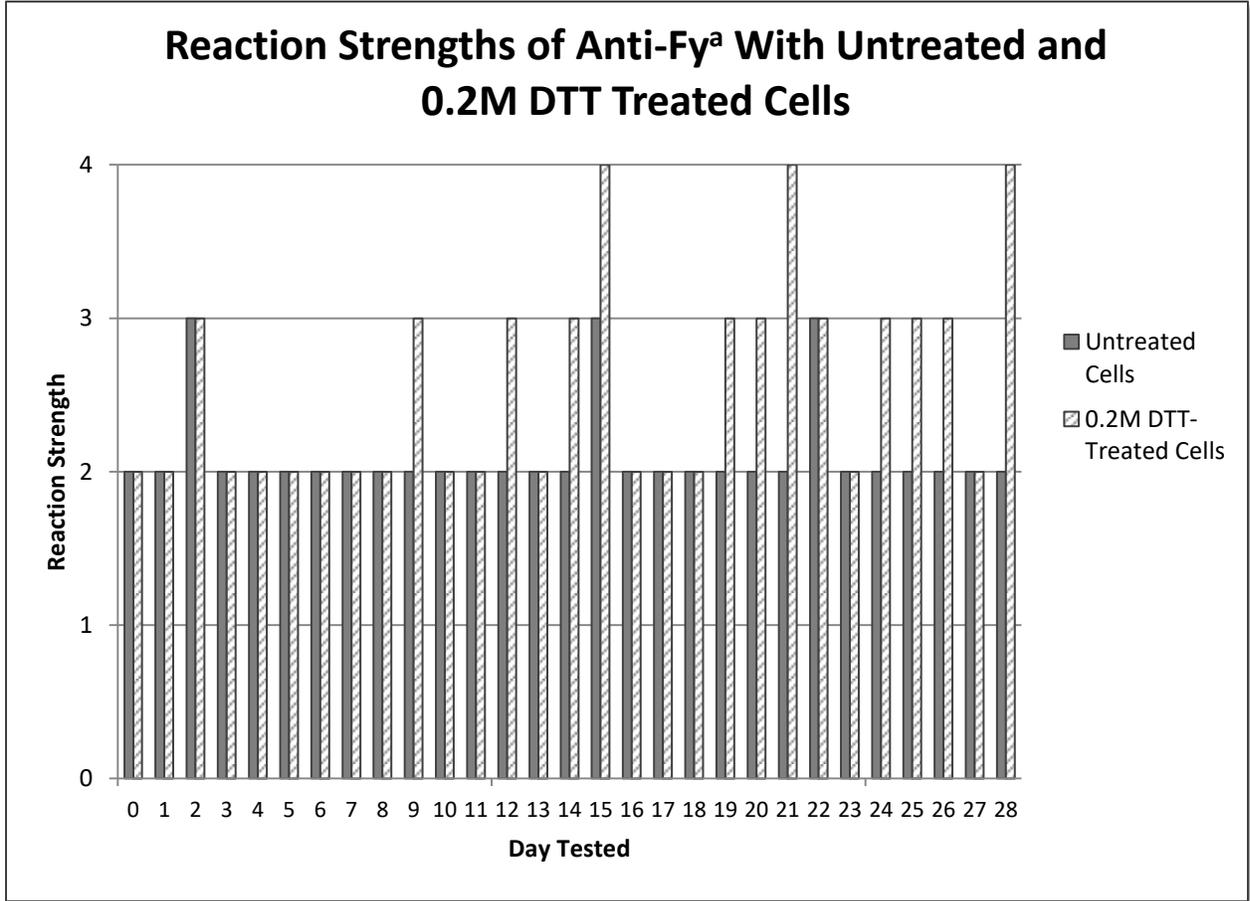


Figure 12. Reaction strengths of anti-Fy^b with untreated and 0.2M DTT-treated screen cells

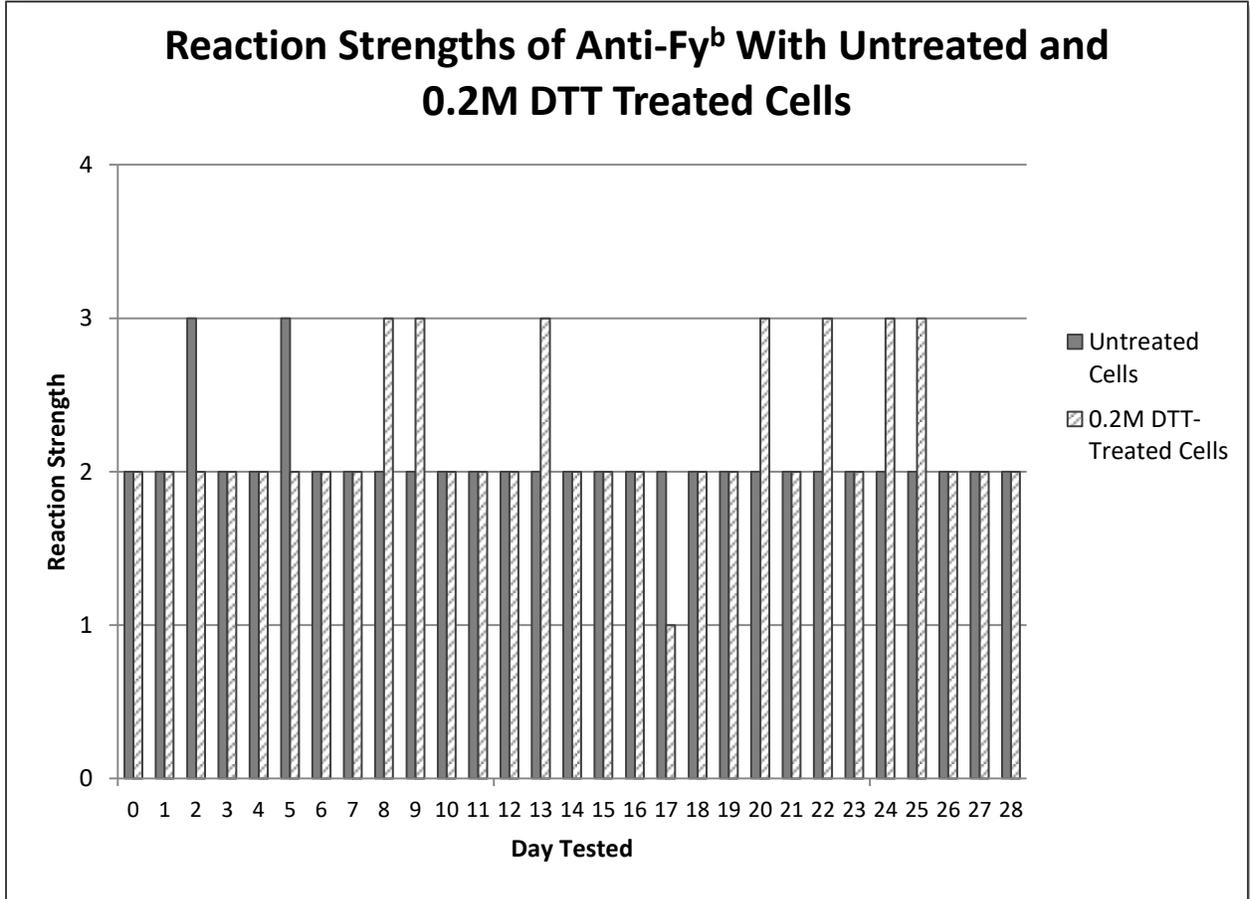


Figure 13. Reaction strengths of anti-Jk^a with untreated and 0.2M DTT-treated screen cells

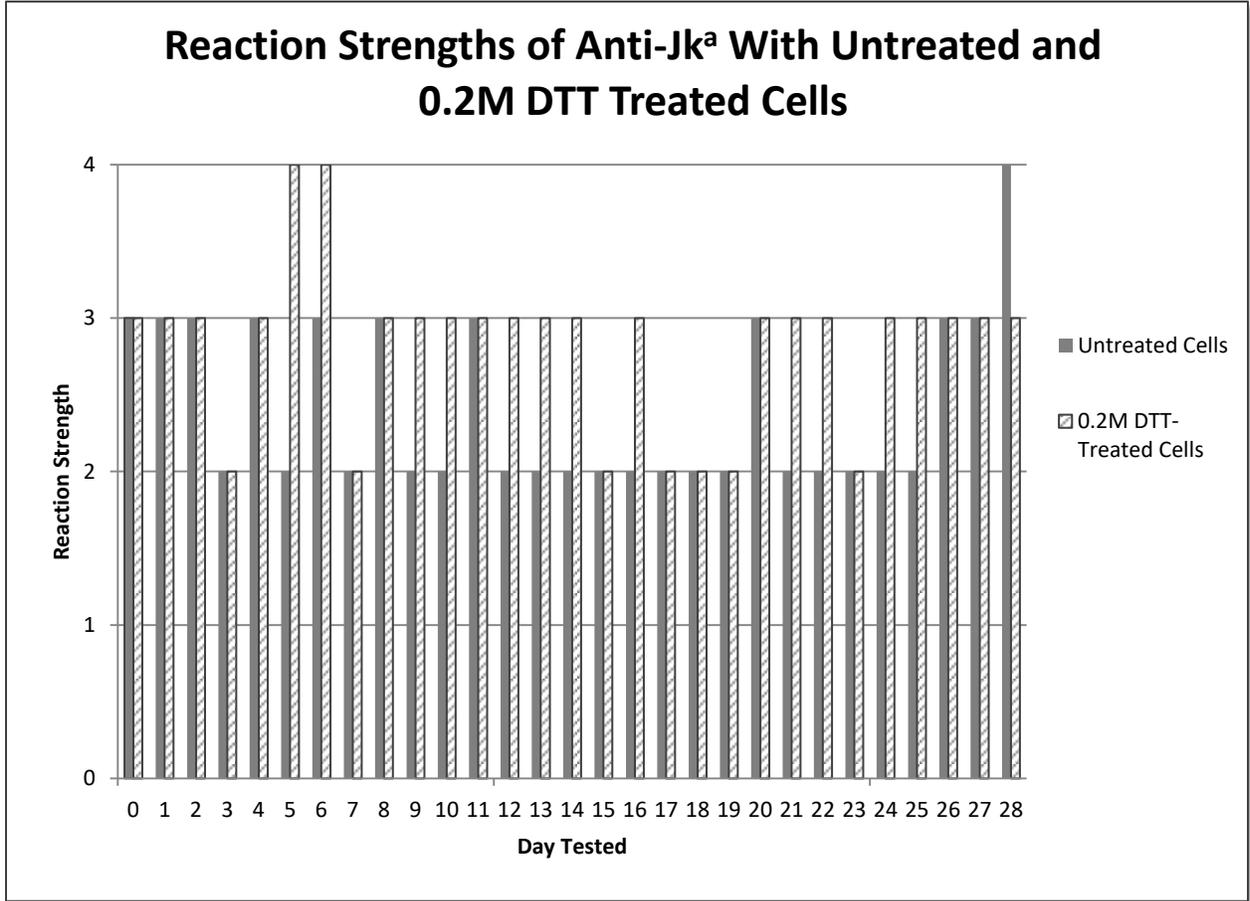


Figure 14. Reaction strengths of anti-Jk^b with untreated and 0.2M DTT-treated screen cells

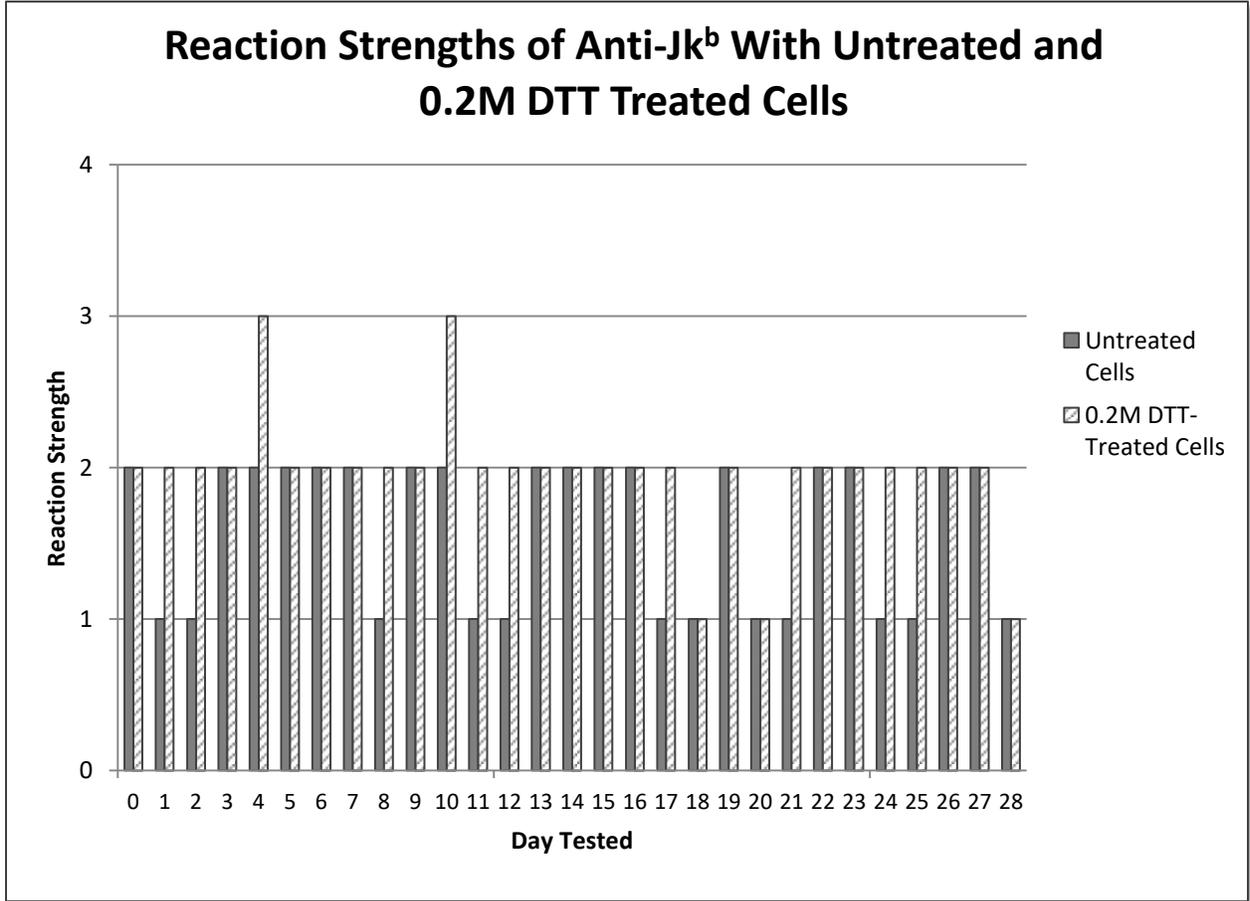


Table I. Hemolysis grade of untreated and 0.2M DTT-treated cells based on Haemonetics color comparator chart for each day tested

Cell	Day Tested																												
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Untreated Immucor 28834 SC I	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5	5	6
Untreated Immucor 28834 SC II	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5	5	6
Untreated Immucor 28834 SC III	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	6	6	6	6	6	6
0.2M DTT-treated Immucor 28834 SC I	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6
0.2M DTT-treated Immucor 28834 SC II	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6
0.2M DTT-treated Immucor 28834 SC III	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6