EXERCISE 8 & 9

Exercise 8  Direct Antiglobulin Test (DAT)
Exercise 9  Elution Study

Task
To perform the DAT and elution procedure with correct interpretation of results.

Objectives
1. State what the Direct Antiglobulin Test (DAT) is specifically used for.
2. State 4 conditions which this test is useful in diagnosing.
3. State the procedure which may be performed when a patient has a positive DAT.
4. State three crucial pieces of information which must be obtained when an adult patient has a positive DAT.
5. List the three types of hemolytic disease of the fetus and newborn including severity and specificity of antibody(ies) involved.
6. State the purpose (principle) of the elution procedure.
7. State how the specificity of an eluate is determined.
8. Describe “Wharton’s jelly” and the problems it may cause in the transfusion service.
9. State the course of action which must be performed to remove Wharton’s jelly from a cord blood cell sample.
10. Recognize ABO discrepancies due to Wharton’s jelly and take appropriate action in resolving the problem.
11. State why a reverse type cannot be performed on a cord blood sample.
12. Given the results of a maternal Type and Screen and cord blood DAT perform an appropriate elution procedure.
13. State the most common cause of a false positive elution test.
14. State what must be done when false positive reactions occur in an elution procedure.
15. Carefully follow the written procedure to accurately perform a DAT, Type and Screen and elution procedure on the appropriate samples with 95% accuracy.

Introduction
DAT

The direct antiglobulin test (DAT) is used to detect \textit{in vivo red blood cell sensitization}. Washed red blood cells from the patient are directly tested with antiglobulin serum. The specimen of choice for the DAT is an EDTA specimen. If a clotted specimen is used a false positive result may be obtained due to non-specific binding of complement to the red blood cells in-vitro. EDTA binds calcium which is necessary for complement activation. The focus of this lab is to determine sensitization of baby’s red blood cells, but the DAT is a procedure which may also be performed on other types of patients as well as donor units.
The DAT is utilized in the blood bank to aid in the diagnosis of a variety of conditions such as:

1. **Diagnosis of hemolytic disease of the newborn (HDFN).** A pregnant woman may form IgG antibodies directed against antigens on baby's cells while it is in utero. These may have the ability to cross the placenta and attach to the baby's red blood cells. IgG is the only antibody class capable of crossing the placenta.

2. **Diagnosis of autoimmune hemolytic anemia.** A patient forms antibodies directed against their own red blood cells causing decreased cell survival.

3. **Investigation of red blood cell sensitization caused by drugs.** Drugs sometimes induce the formation of antibodies, either against the drug itself or against intrinsic red blood cell antigens. Drugs act as haptens eliciting antibodies only after they have been firmly bound to a protein carrier. Once formed, the antibodies can react with the small hapten independent of any protein attachment. Drug-induced antibodies may cause positive DATs and sometimes hemolytic anemias.

4. **Investigation of transfusion reactions.** A patient may form antibodies against transfused cells, causing the transfused cells to be coated with antibodies in-vivo.

If a patient has a positive DAT, an elution procedure should be performed. A critical part of the elution procedure includes the patient history. The patient's diagnosis, transfusion and pregnancy history, and recent/current medications are vital pieces of information in working up a positive DAT.

**Elution**

The purpose of an elution is to cause the release of antibody molecules from the red blood cell membrane. Once free in solution the eluted antibodies are tested against reagent red blood cells to determine if an immune antibody specificity is present. Antibody specificity is expected in cases of HDFN, transfusion reactions due to immune antibodies and some types of autoimmune hemolytic anemias. Eluates are usually non-reactive in cases of drug induced DATs.

Antibody release (elution) can be accomplished either by disrupting the antibody or by changing conditions to favor dissociation of antibodies from antigen. Many techniques are available. No single method is best in all situations. If an eluate prepared by one technique gives unsatisfactory results, it may be helpful to prepare another eluate by a different technique. Negative results in an antibody screening test done on an eluate does not mean an antibody is absent; the antibody may react with a low incidence antigen or with a drug.

The next two (2) laboratories are blood bank tests used in the evaluation of hemolytic disease of the newborn (HDFN). Immune antibodies are stimulated as a result of exposure to red blood cell antigens which an individual does not possess. If a woman has been exposed to foreign red cell antigens (most commonly as a result of transfusion or previous pregnancy), she may form immune (IgG) antibodies capable of crossing the placenta and attaching to antigen positive fetal cells. This causes the baby's red blood
cells to be coated with antibody and destroyed. The resulting effects on the child are collectively termed hemolytic disease of the newborn (HDFN). The severity of the HDFN varies and is dependent upon the antibody involved. The most severe HDFN is due to D negative mothers who have formed immune anti-D that crosses the placenta and destroys the D positive cells of her D positive fetus. If the HDFN is severe, the baby will need an exchange transfusion with blood compatible with the baby and the mother.) The mildest and most common form of HDFN is due to maternal antibodies. This is detected in group A or B infants of group O mothers. Phototherapy is the treatment of choice for ABO HDFN. Antibodies to “other” blood group antigens may cause varying degrees of HDFN.

**Principle**

**Part 1** of this lab is to determine whether an infant's red blood cells have been sensitized by maternal antibody by performing the direct antiglobulin test (DAT). Please remember that the DAT is a general procedure that is performed on babies, adults and donor units. The purpose of the test in this laboratory session is to detect sensitization of baby cells by maternal antibody, but this test is also applicable in many other situations.

The DAT procedure detects in-vivo sensitization of red blood cells. If a negative result is obtained, no sensitization has occurred. A positive result indicates in-vivo sensitization has occurred and further testing is required.

The DAT procedure is routinely performed on the cord bloods of newborns.

The specimen for testing is obtained from the baby's umbilical cord immediately following birth. A syringe is used to withdraw a specimen to prevent contamination with Wharton's jelly. Wharton's jelly is a gelatinous substance which coats the umbilical cord. If the cord specimen becomes contaminated with this substance, it will cause non-specific agglutination of the cells (false positives). False positive reactions are usually discovered when the ABO/D typing is performed. All forward typing tubes are positive, an Rh control is run, and it is also positive, invalidating the test. It is a water soluble substance, and once it is discovered as a contaminant, can be removed by 8-10 washes with large volumes of saline. The cord specimen will be contaminated if the specimen is collected by cutting the cord and allowing the blood to drip in the tube or if the blood is “milked” into the tube.

When a positive cord blood DAT is obtained a series of steps are required to identify the offending antibody. The first step in determining the identity of the coating antibody is to perform a Type and Screen on the mom and a ABO/D typing on the infant. Please remember that a reverse type cannot be performed on babies, as the ABO antibodies present in their serum/plasma is of maternal origin. The results of the antibody screen on the mother will determine which type of elution to perform, as some elution procedures are best at recovering alloantibodies, while others are best at recovering immune ABO antibodies. If the mother's antibody screen is positive, a panel study is done on the mother's serum. An acid elution must be performed on the infant's cord cells to verify that the antibody in the mother's serum is, indeed, coating the baby's cells. If the mom's screens are negative and she is ABO incompatible with her infant then a heat elution is performed on the infant's cord cells to check for an ABO antibody.
Part 2 of this lab will involve the performance of an elution on the infant's cord cells to determine the specificity of the maternal antibody coating the infant's red blood cells. In an elution procedure, the cells are washed free of all unbound antibody and a procedure is performed on the cells to cause the release of the antibody molecules from the cells into a solution. The eluate is then tested against reagent red cells to determine the specificity. Remember that any antibody recovered in the eluate prepared from the infant's cells is of maternal origin.

Today DATs will be performed on cordbloods. If a positive result is obtained, perform a Type and Screen on the mom and an ABO/D typing on the cord cells. From this information determine the type of elution to be performed. Next week the elution procedure will be done and the eluate tested to determine the specificity of the antibody coating the cells.

Please keep the following flow chart handy as you perform the cordblood DAT testing.

```
<table>
<thead>
<tr>
<th>DAT results on cord cells</th>
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<tbody>
<tr>
<td>NEGATIVE</td>
</tr>
<tr>
<td>No elution necessary</td>
</tr>
<tr>
<td>POSITIVE</td>
</tr>
<tr>
<td>Perform Type and Screen on mom</td>
</tr>
<tr>
<td>Perform ABO/D typing on cordblood</td>
</tr>
</tbody>
</table>
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If mom's screens are positive
- Perform acid elution on cord cells and test eluate against panel cells
- Test mom's serum against panel cells

If mom's screens are negative and infant is ABO incompatible with mom
- Perform heat elution on cord cells; test eluate and last wash against A cells, B cells and screen cells
DAT

Procedure

1. Label a tube for each cord specimen using the mother's full name and hospital number.

   **NOTE:** As soon as you have labeled the tube place immediately pick up the appropriate cord blood and place the rbc's in the labeled tube. Placing the cells in the tube after verifying it is the correct sample immediately after labeling will help prevent you from putting the wrong cells in the wrong tube.

2. Prepare a washed 2-5% suspension of cord cells in a properly labeled test tube.

3. Place one (1) drop of cell suspension to be tested in a properly labeled test tube.

4. Wash three (3) times with saline, decanting completely after each wash.

5. After last wash, blot tube with gauze to obtain a dry cell button.

6. Add two (2) drops of AHG reagent.

7. Gently shake tube to mix and centrifuge for 10-15 seconds.

8. Examine for agglutination macroscopically and microscopically.

9. Add one drop of IgG – sensitized red blood cells (check cells) to any tubes which have been recorded as negative.

10. Gently mix, centrifuge for 15 seconds.

11. Resuspend the cell button, grade and record results and interpretation.

   **NOTE:** If the initial washing adequately removed all unbound globulin, the antiglobulin serum will still be reactive and capable of agglutinating the sensitized control red blood cells. Thus, the negative test result obtained for the patient can be considered valid. This is an important control because it is the only way to monitor the adequacy with which each test is washed.

Interpretation

1. Negative, no in-vivo sensitization detected.

2. Positive, in-vivo sensitization detected; elution studies should be performed. The type of elution done will be determined by the results of the mother's type and screen. *At this time, perform the Type and Screen on the maternal specimen according to your previous exercise.*

   While the type and screen on the mother is incubating, perform an ABO/D on the baby's cells. A reverse type cannot be performed on an infant since all antibodies in the baby are of maternal origin. *After all results are in, look at the flow chart to determine which eluate will be performed next week.*
Heat Elution

Introduction

The red blood cells for any elution technique must be thoroughly washed to remove all antibody except that bound to the cells. Six to eight washes with large volumes of saline are usually sufficient. *Adequacy of washing is determined by testing saline from the last wash against reagent screen cells for the presence of antibody. *If positive reactions are obtained with the last wash this is an indication that the red blood cells were not washed sufficiently and that there are still contaminating serum antibodies present.* This would invalidate the results of the eluate and the procedure would need to be repeated using a more thoroughly washed rbc sample.

The heat elution technique is used to remove antibodies from red blood cells coated in-vivo. It is used primarily to recover and identify ABO antibodies in cases of hemolytic disease of the newborn (HDFN) and in patients who have been transfused with non-ABO-identical blood or blood components. The coated red blood cells are thoroughly washed, an albumin solution is added and the cells are heated to 56°C. This amount of heat will cause disruption of the antigen-antibody bond, the antibody will “pop off” the coated rbcs into the albumin solution. After sufficient heating the solution is centrifuged and the supernatant fluid (eluate) is removed. The supernatant will be *very* hemolyzed and you may need a lamp to provide a lighted background to view the eluate/rbc interface. Contaminating the eluate with rbc may cause false positive reactions in the test. The supernatant fluid will contain the antibodies that were coating the cells. The eluate is tested against reagent red blood cells to determine the specificity.
**A. HEAT ELUTION**

**Reagents**

1. See page 1
2. 6% bovine albumin, prepared by diluting 22% or 30% bovine albumin with saline
3. Cord blood red blood cells from previous lab
4. 56°C water bath

**Preparing Eluate**

1. Centrifuge the sample with the positive DAT. Transfer the supernatant serum or plasma to a separate, properly labeled tube.
2. Place 20 drops of the red cells in a properly labeled 12 x 75 mm tube.
3. Wash the cells eight (8) times with large volumes of saline.

**NOTE:** DO NOT decant the saline off the cells, the volume of cells used is critical. Remove the supernatant saline with a pipet.

4. **Mix cells vigorously between washes** by adding a small volume of saline and thumping the tube vigorously with finger or covering the tube with parafilm and inverting the tube until all cells are resuspended off the bottom of the tube. Then, complete filling of tube, adding saline vigorously (do not overfill, may need to parafilm top of tube).

**NOTE:** If cells are not completely resuspended between washes antibody may be trapped in between the packed rbcs, this will cause a false positive reaction in the last wash as well as the eluate and the procedure will need to be repeated.

5. Put the saline from wash #8 in a tube labeled with patient name and “LAST WASH”.

6. Add 20 drops of 6% bovine albumin to the washed packed red blood cells.

7. Place the tubes at 56°C for 10 minutes, agitating the tube constantly during this time with two applicator sticks.

8. Centrifuge the tube at 1,000 X g for five (5) minutes.

9. Immediately transfer the supernatant eluate into a clean test tube, and test in parallel with the final wash supernatant.
Testing the Eluate

1. Label 10, 12 x 75 test tubes (eluate (EL) = A₁, B, S₁, S₂, and S₃; last wash (LW) = A₁, B, S₁, S₂, and S₃) and patient initials.
2. Add one (1) drop of the reagent red cells to each of the properly labeled tubes.
3. To the five (5) tubes labeled “EL”, add three (3) to four (4) drops of the eluate.
4. To the five (5) tubes labeled “LW”, add the same number of drops of the last wash as eluate, IE, if only 3 drops of eluate were added, place only 3 drops of last wash in the LW tubes.
5. Mix and incubate at 37°C for 30 minutes.
6. Remove from heat block and immediately wash three (3) times with saline, decanting thoroughly between washes and blotting the tubes after the last wash.
7. Add two (2) drops of antiglobulin reagent to each tube, mix gently and centrifuge for 15 seconds.
8. Gently dislodge cell button completely.
9. Read macroscopically and negative tubes micro.
10. Record results; grading reactions.
11. Add one (1) drop Coombs control cells to all negative tubes, centrifuge for 15 seconds and read for agglutination. If no agglutination is present, test must be repeated.
Interpretation

1. If “last wash” tubes are negative, any agglutination of reagent cells with eluate indicates recovery of antibodies from the original cells.

2. If the last wash is positive with any of the test cells, the original cell sample was probably underwashed and the elution procedure should be repeated on a more thoroughly washed cell sample.

3. Lack of agglutination of test cells by the eluate indicates absence of antibodies specific for the antigens on the test cells.

4. In the case of cordblood, the interpretation should be reported as follows:

<table>
<thead>
<tr>
<th>Reactions with Cells</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1  B  S1  S2  S3</td>
<td></td>
</tr>
<tr>
<td>+  O  O  O  O</td>
<td>Immune anti-A eluted</td>
</tr>
<tr>
<td>O  +  O  O  O</td>
<td>Immune anti-B eluted</td>
</tr>
<tr>
<td>+s +w  O  O  O</td>
<td>Immune anti-A and anti-A,B eluted</td>
</tr>
<tr>
<td>+w +s  O  O  O</td>
<td>Immune anti-B and anti-A,B eluted</td>
</tr>
<tr>
<td>O  O  (+ on any cell)</td>
<td>Unexpected antibody. Perform panel on maternal serum</td>
</tr>
</tbody>
</table>

If the mother is group O and the baby is group A or B, it is not unusual to obtain positive reactions with both the A and B cells. This reactivity is due to the anti-A,B present in the group O mother’s serum which has coated the baby’s rbcs. This antibody will react with both A and B cells.

Use the interpretation chart above along with your common sense to interpret your data. If the mom is group O and the baby is group A, it is impossible to elute an immune anti-B from the baby cells. Think your interpretation through carefully.

For the example below the mom is group O, antibody screen negative and the baby is group A with a positive DAT. The following elution results were obtained.

<table>
<thead>
<tr>
<th></th>
<th>A(^1) cells</th>
<th>B cells</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluate</td>
<td>4+</td>
<td>2+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Last Wash Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Interpretation: Immune anti-A and -A,B eluted from cord cells.
B. LUI Rapid Freeze/Thaw

Purpose

The Lui rapid freeze/thaw elution technique is used to remove antibodies from red blood cells coated in vivo. It is used primarily to recover and identify ABO antibodies in cases of Hemolytic Disease of the Newborn and in patients who have been transfused with non-ABO-identical blood or components.

Principle

Antibodies are dissociated from the red cell membrane when it is disrupted by a freezing and thawing process.

Limitations of Procedure

1. This technique is best for ABO antibodies, but may work for others. However, other techniques are more proven for recovering irregular IgG antibodies.
2. This eluate is best for antibodies detected by antiglobulin techniques, since the eluate is heavily hemoglobin-tinged and direct agglutination reading is impossible.

Specimen Required

1. A properly labeled cord blood specimen, preferably an EDTA tube.
2. A Typenex labeled EDTA tube if the procedure is to be performed on an adult patient.

Procedure

1. Reagents - No special reagents required

2. If your sample has been agitated, centrifuge it. Add 16 drops of packed red blood cells to your initial cell suspension. Wash the red blood cells 6 times with large volumes of saline, mixing cells vigorously between washes to remove trapped globulin. **DO NOT decant the saline by inverting the tube, use a pipet to remove the supernatant saline after each wash.** Save an aliquot of the last wash as a control. **HINT:** The purpose of this control is to assure that antibody present in the eluate has been derived from a bound state on the original cells, and is not unbound antibody remaining as the result of inadequate washing.

3. Add two (2) drops saline, mix and stopper with small, purple plastic cap.

4. Rotate the tube to coat the entire inside with cells.

5. Place the tube on its side in the Revco Freezer (-6 to -30°C) for ten (10) minutes (minimum).

6. Thaw the cells rapidly by holding the tube under running water.

7. Centrifuge the hemolysate for 1-2 minutes. Harvest the clear, cherry-red eluate into a clean labeled tube.

8. The eluate is now ready to be tested against a panel of cells. Test the last wash in parallel.

9. Procedure for testing the eluate

10. Label ten (10) 12 x 75 test tubes. One set of five for testing the eluate (A1, B, S1, S2, and S3) and another set of five for testing the last wash control labeled in the same manner. Add one (1) drop of the appropriate reagent cell to each tube.
11. Add two (2) drops of eluate or last wash to each tube (increase to 3 drops if possible). Mix and incubate for 30 minutes at 37°C.
12. Wash three (3) times with saline, decanting thoroughly and blotting the tubes after the last wash.
13. Add two (2) drops of antiglobulin serum and centrifuge for the amount of time specified for the Coombs spin on your centrifuge.
14. Gently dislodge the cell button and read macroscopically and microscopically.
15. Record graded results in the appropriate places on the antibody workup sheets.
16. Confirm all apparently negative tests by adding one (1) drop of check cells, mixing, spinning, and reading. Agglutination at this point indicates that the Coombs serum was active at the time the test was originally interpreted as negative.

**Interpretation**

1. If the last wash is negative, agglutination of reagent cells with the eluate indicates recovery of antibody from the original cells.
2. If the last wash is positive with any of the test cells, the original cell sample was probably underwashed and the elution procedure must be repeated on a more thoroughly washed cell sample.
3. Lack of agglutination of test cells by the eluate indicates absence of antibody specific for the antigens on the test cells.
4. In the case of cord bloods, the interpretation should be reported as follows:

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>Immune Anti-A eluted</td>
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<tr>
<td>O</td>
<td>+</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>Immune Anti-B eluted</td>
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<tr>
<td>+s</td>
<td>+w</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>Immune Anti-A and Anti-A,B eluted</td>
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<tr>
<td>+w</td>
<td>+s</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>Immune Anti-B and Anti-A,B eluted</td>
</tr>
<tr>
<td>O</td>
<td>O</td>
<td>+</td>
<td></td>
<td></td>
<td>Unexpected antibody. Perform panel on maternal serum.</td>
</tr>
</tbody>
</table>

**NOTE:** The +s versus +w means that the +s reaction is stronger than the +w reaction. This designation is helpful when anti-A,B is present in the eluate, as it usually reacts weaker than the anti-A or anti-B when present.
Exercise 9: DAT
Recording Results Form

<table>
<thead>
<tr>
<th>Mother's Name</th>
<th>ID #</th>
<th>Maternal ABO/D</th>
<th>Anti-A</th>
<th>Anti-B</th>
<th>Anti-D</th>
<th>D Ctrl *</th>
<th>D⁺</th>
<th>D⁺ C⁺</th>
<th>Interp</th>
<th>DAT</th>
<th>RhIg?</th>
<th>Completion</th>
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DAT and Elution MLAB 2431 •97
**Elution Studies**

**Recording Results**

Mother's Name ________________________________

Hospital Number ______________________________

Baby's DAT ________________________________

Baby's ABO/D Typing __________________________

Mother's ABO/D Typing __________________________

Mother's Antibody Screen __________________________

<table>
<thead>
<tr>
<th>Cells</th>
<th>Reactions with Eluate</th>
<th>Reactions with Last Wash Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S₁</td>
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<td>S₂</td>
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<td></td>
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<tr>
<td>S₃</td>
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</tbody>
</table>

Interpretation of elution results: ________________________________________________
Exercise 8  
Direct Antiglobulin Test (DAT)  
Study Questions

1. What does the DAT procedure detect? (1 point)

2. List four (4) clinical conditions for which the DAT is useful. (2 points)

3. What is the specimen of choice for the DAT? Why? (1 point)

4. What is the source of the specimen used for the DAT on newborns?

5. What is Wharton's jelly? Specifically, what problem(s) does it cause? (2 points)
6. List the three types of HDFN from mildest to most severe and state the antibody specificity involved in each. (3 points)

7. What antibody class is involved in HDFN? (0.5)

8. State the additional testing which must be performed when an infant has a positive DAT. Be sure to state which test is performed on mom and which on baby. (1.5 points)

9. What must be done for the infant if the HDFN is severe at birth? (1 point)

10. Why can’t a reverse type be performed on infants? (1 point)
Exercise 9

Elution Procedure

Study Questions

1. What is the *principle* of the elution procedure? (1 point)

2. List four (4) pieces of information that are useful in the workup of a positive DAT. (2 points)

3. What determines which type of elution procedure to use for an HDFN workup? (1 point)

4. What type of elution is used for ABO-HDFN? (0.5)

5. In the elution procedure, why must the cells be thoroughly washed? (1 point)

6. What is the most probable cause of positive results with the last wash control? (1 point)
7. What must be done if the last wash control gives positive reactions? (1 point)

8. What does lack of agglutination of the test cells by the eluate indicate? (0.5)

9. Interpret the following results obtained in a heat eluate. (1 point)

**Baby is A positive**
**Mom is 0 positive, screens are negative**

<table>
<thead>
<tr>
<th></th>
<th>A1 cells</th>
<th>B cells</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluate</td>
<td>4+*</td>
<td>2+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Last Wash</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Interpretation:

10. When the mother is group O and the baby is A or B why is it common to get reactions in the eluate with both the A and B cells? (1 point)