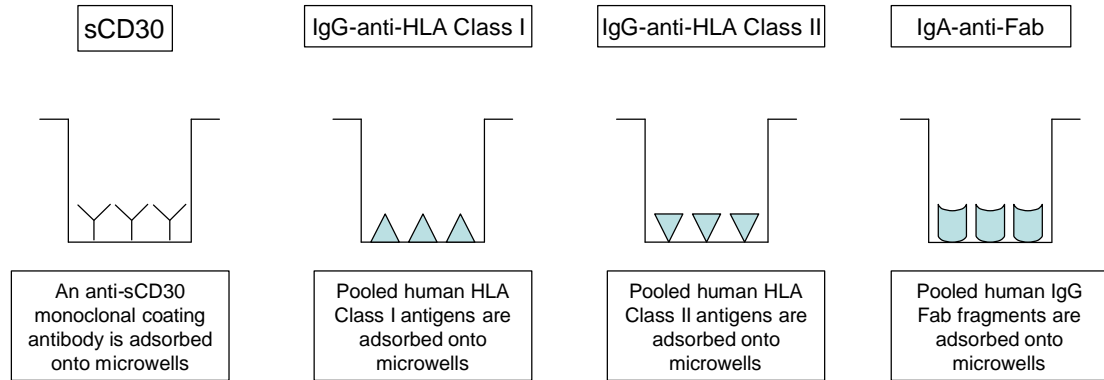


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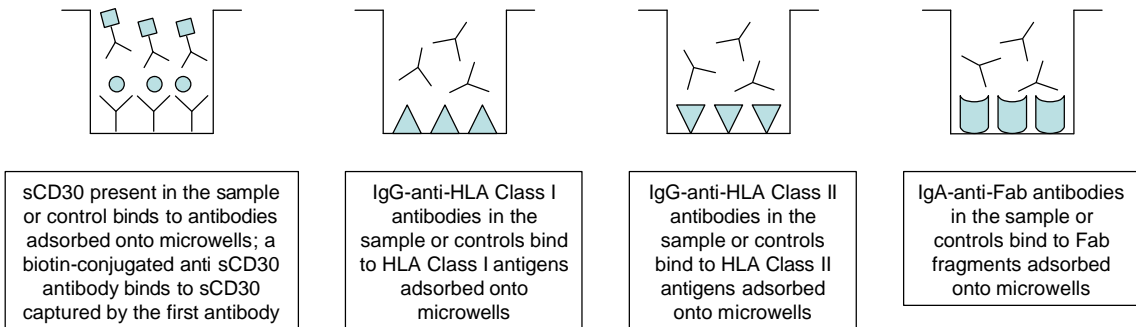
4 – ELISA Kit

Manual

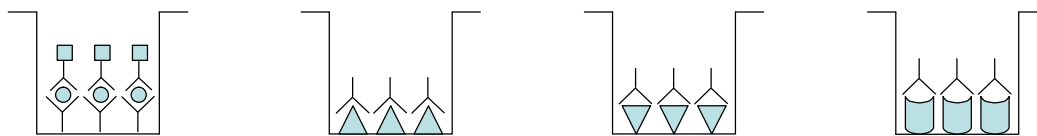
1. PRINCIPLES OF THE ASSAY



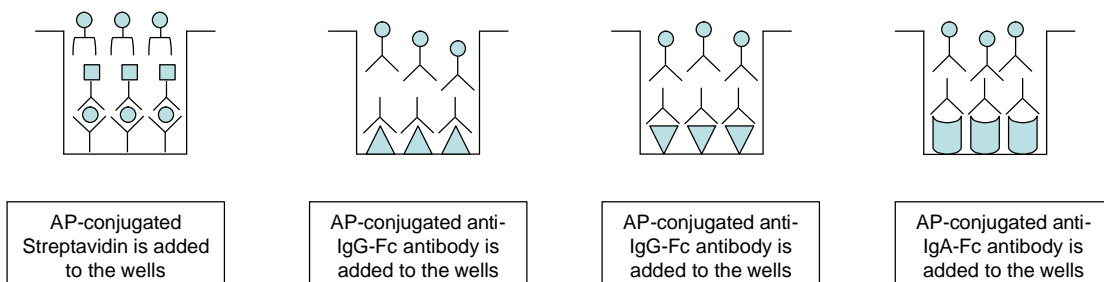
First Incubation (2 hours at room temperature)



Following incubation, unbound materials are removed during a wash step.



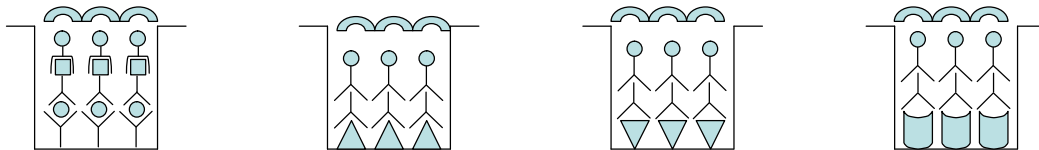
Second Incubation (1 hour at room temperature)



Following incubation, unbound materials are removed during a wash step.



Third Incubation (about 10 minutes at room temperature)



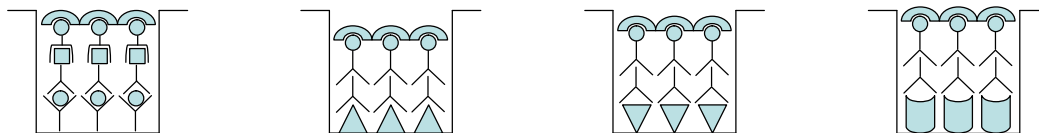
A substrate solution reactive with AP-conjugated Streptavidin is added to the wells

A substrate solution reactive with AP-conjugated anti-IgG-Fc antibody is added to the wells

A substrate solution reactive with AP-conjugated anti-IgG-Fc antibody is added to the wells

A substrate solution reactive with AP-conjugated anti-IgA-Fc antibody is added to the wells

During the third incubation



A colored product is formed in proportion to the amount of sCD30 present in the sample

A colored product is formed in proportion to the amount of IgG-anti-HLA Class I antibodies present in the sample

A colored product is formed in proportion to the amount of IgG-anti-HLA Class II antibodies present in the sample

A colored product is formed in proportion to the amount of IgA-anti-Fab antibodies present in the sample

After 10 minutes of incubation, read Optical Density [OD] on an ELISA reader at regular intervals (every 5 minutes) until the Positive and Negative Controls have reached the values given in this Manual.

2. REAGENTS PROVIDED

A. 1 Aluminium Pouch with a Microwell Plate containing:

- ? 3 strips coated with Monoclonal Antibody to sCD30 (marked in blue)
- ? 3 strips coated with HLA Class I Antigens (marked in green)
- ? 3 strips coated with HLA Class II Antigens (marked in pink)
- ? 3 strips coated with IgG Fab fragments (marked in yellow)

B. 1 bottle (50ml) Wash Buffer Concentrate 20x

C. 1 Aluminium Pouch containing Sample Diluents:

- ? 1 vial (2.2 ml) sCD30 Sample Diluent (ready to use; colored blue)**
- ? 1 vial (1.5 ml) IgG-anti-HLA Class I Sample Diluent (ready to use; colored green)**
- ? 1 vial (1.5 ml) IgG-anti-HLA Class II Sample Diluent (ready to use; colored pink)**
- ? 1 vial (3 ml) IgA-anti-Fab Sample Diluent (ready to use; colored yellow)**

D. 1 Aluminium Pouch containing Positive and Negative Controls:

- ? 1 vial (105 µl) sCD30 Positive Control (ready to use; colored blue)**
- ? 1 vial (55 µl) IgG-anti-HLA Class I Positive Control (ready to use; colored green)** and 1 vial (55 µl) IgG-anti-HLA Class I Negative Control (ready to use; colored green)**
- ? 1 vial (55 µl) IgG-anti-HLA Class II Positive Control (ready to use; colored pink)** and 1 vial (55 µl) IgG-anti-HLA Class II Negative Control (ready to use; colored pink)**
- ? 1 vial (55 µl) IgA-anti-Fab Positive Control (ready to use; colored yellow)** and 1 vial (55 µl) IgA-anti-Fab Negative Control (ready to use; colored yellow)**

E. 1 Aluminium Pouch containing Conjugates and Assay Buffers:

- ? 1 vial (10 µl) Anti-sCD30-Biotin Conjugate concentrate (colored blue)** and 1 vial (2 ml) Assay Buffer (ready to use) for dilution (colored blue)**
- ? 1 vial (9 µl) Streptavidin-Alkaline-Phosphatase Conjugate concentrate for detection of sCD30 (colored blue)** and 1 vial (4 ml) Assay Buffer (ready to use) for dilution (colored blue)**
- ? 1 vial (10 µl) Anti-human-IgG-Alkaline-Phosphatase Conjugate concentrate (colored green) for detection of IgG-anti-HLA Class I antibodies** and 1 vial (2 ml) Assay Buffer (ready to use) for dilution (colored green)**

- ? 1 vial (10 µl) Anti-human-IgG-Alkaline-Phosphatase Conjugate concentrate (colored pink) for detection of IgG-anti-HLA Class II antibodies** and 1 vial (3.5 ml) Assay Buffer (ready to use) for dilution (colored pink)**
- ? 1 vial (10 µl) Anti-human-IgA-Alkaline-Phosphatase Conjugate concentrate (colored yellow) for detection of IgA-anti-Fab antibodies** and 1 vial (2 ml) Assay Buffer (ready to use) for dilution (colored yellow)**

F. 1 Aluminium Pouch containing the Substrate System:

- ? 1 Tablet pNPP Substrate (silver foil) and 1 Tablet Tris Buffer (gold foil)

G. 3 Adhesive Plate Covers

**** It is recommended to spin vials in microcentrifuge before use.**

3. STORAGE INSTRUCTIONS

Store at 2° to 8°C:

Microwell Strips and Wash Buffer

Store at -20°C:

Sample Diluents, Conjugates, Assay Buffers, sCD30 Positive Control, and Positive and Negative Controls for IgG-anti-HLA Class I and Class II, IgA-anti-Fab, and Substrate Tablet I / II

Expiry of the kit and reagents is stated on labels.

The expiry of the kit components can only be guaranteed if the components are stored properly.

4. SPECIMEN COLLECTION

Human serum is suitable for use in the assay. Remove serum from the clot as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Blood samples should be kept at 2° to 8°C and serum should be separated fairly rapidly before storing at -20°C to avoid loss of bioactive sCD30, IgG-anti-HLA Class I, IgG-anti-HLA Class II, and IgA-anti-Fab antibodies. If samples are to be run within 24 hours, they may be stored at 2° to 8°C.

Avoid repeated freeze-thaw cycles.

Serum samples should not be heat-inactivated, as this may give false positive or negative results.

5. MATERIALS REQUIRED BUT NOT PROVIDED

- ? 1 to 10 μ l adjustable single channel micropipette with disposable tips
- ? 10 to 100 μ l adjustable single channel micropipette with disposable tips
- ? 100 to 1000 μ l adjustable single channel micropipette with disposable tips
- ? 50 to 300 μ l adjustable multichannel micropipette with disposable tips
- ? Multichannel micropipette reservoir
- ? Beakers, flasks, cylinders necessary for preparation of reagents
- ? Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- ? ELISA reader capable of reading at 405 nm and 492 nm
- ? Glas-distilled or deionized water
- ? 96 well Dilution Plate

6. PRECAUTIONS

- ? Do not mix or substitute reagents with those from other lots or other sources.
- ? Do not use kit reagents beyond expiration date on label.
- ? Do not expose kit reagents to strong light during storage or incubation.
- ? Do not pipette by mouth.
- ? Do not eat or smoke in areas where kit reagents or samples are handled.
- ? Avoid contact of skin or mucous membranes with kit reagents or specimens.
- ? Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- ? Reagents containing preservatives may be toxic if ingested.
- ? Avoid contact of substrate solutions with oxidizing agents and metal.
- ? Avoid splashing or generation of aerosols.
- ? In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test, use disposable pipette tips and/or pipettes.
- ? Use clean dedicated reagent trays for dispensing substrate reagents.
- ? Exposure to lye or acids will inactivate the conjugates.
- ? Glass-distilled water or deionized water must be used for the preparation of substrate solution.

7. PREPARATION OF REAGENTS

A. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm gently until they have completely dissolved.

Pour entire contents (50 ml) of the Wash Buffer Concentrate into a clean 1000 ml graduated cylinder. Bring final volume to 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. **The pH of the final solution should adjust to 7.4.** pH values above or below 7.4 will result in incomplete washing of the wells.

Transfer to a clean wash bottle and store at 2° to 8°C. Please note that the Wash Buffer is stable for 2 weeks.

B. Anti-sCD30-Biotin Conjugate

- ? The Anti-sCD30-Biotin Conjugate must be diluted with Assay Buffer.
- ? Spin vial of Biotin Conjugate and Assay Buffer in microcentrifuge before use.
- ? Mix Biotin Conjugate well by pipetting and transfer **6 µl** directly into the vial of the supplied Assay Buffer.
- ? Mix well, the solution is now ready to use.

Please note that the Biotin Conjugate should be used within 30 minutes after dilution.

C. Streptavidin-Alkaline-Phosphatase Conjugate

- ? The Streptavidin-Alkaline-Phosphatase Conjugate must be diluted with Assay Buffer.
- ? Spin vial of Streptavidin-Alkaline-Phosphatase Conjugate and Assay Buffer in microcentrifuge before use.
- ? Mix Streptavidin-Alkaline-Phosphatase Conjugate well by pipetting and transfer **8 µl** directly into the vial of the supplied Assay Buffer.
- ? Mix well, the solution is now ready to use.

Please note that the Streptavidin-Alkaline-Phosphatase Conjugate should be used within 30 minutes after dilution.

D. Anti-human-IgG-Alkaline-Phosphatase Conjugate for detection of IgG-anti-HLA Class I antibodies

- ? The Anti-human-IgG-Alkaline-Phosphatase Conjugate must be diluted with Assay Buffer.
- ? Spin vial of Anti-human-IgG-Alkaline-Phosphatase Conjugate and Assay Buffer in microcentrifuge before use.

- ? Mix Anti-human-IgG-Alkaline-Phosphatase Conjugate well by pipetting and transfer **5 µl** directly into the vial of the supplied Assay Buffer.
- ? Mix well, the solution is now ready to use.

Please note that the Anti-human-IgG-Alkaline-Phosphatase Conjugate should be used within 30 minutes after dilution.

E. Anti-human-IgG-Alkaline-Phosphatase Conjugate for detection of IgG-anti-HLA Class II antibodies

- ? The Anti-human-IgG-Alkaline-Phosphatase Conjugate must be diluted with Assay Buffer.
- ? Spin vial of Anti-human-IgG-Alkaline-Phosphatase Conjugate and Assay Buffer in microcentrifuge before use.
- ? Mix Anti-human-IgG-Alkaline-Phosphatase Conjugate well by pipetting and transfer **5 µl** directly into the vial of the supplied Assay Buffer.
- ? Mix well, the solution is now ready to use.

Please note that the Anti-human-IgG-Alkaline-Phosphatase Conjugate should be used within 30 minutes after dilution.

F. Anti-human-IgA-Alkaline-Phosphatase Conjugate for detection of IgA-anti-Fab antibodies

- ? The Anti-human-IgA-Alkaline-Phosphatase Conjugate must be diluted with Assay Buffer.
- ? Spin vial of Anti-human-IgA-Alkaline-Phosphatase Conjugate and Assay Buffer in microcentrifuge before use.
- ? Mix Anti-human-IgA-Alkaline-Phosphatase Conjugate well by pipetting and transfer **4 µl** directly into the vial of the supplied Assay Buffer.
- ? Mix well, the solution is now ready to use.

Please note that the Anti-human-IgA-Alkaline-Phosphatase Conjugate should be used within 30 minutes after dilution.

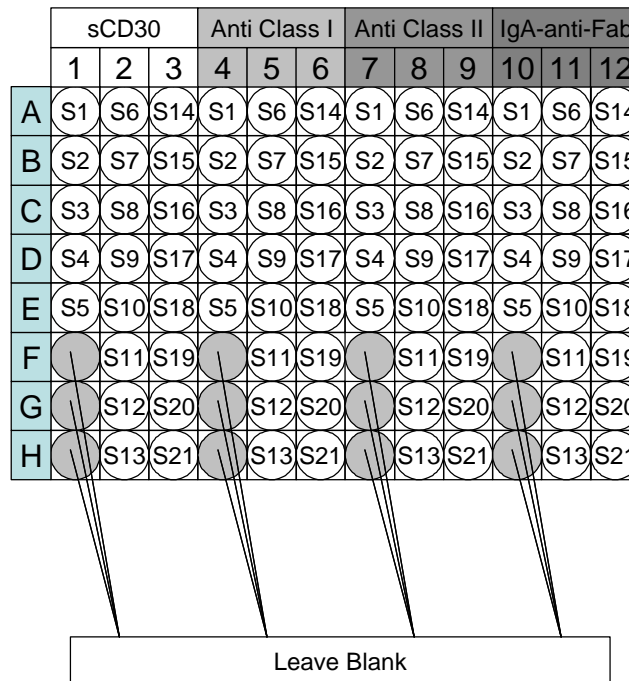
G. Substrate Solution

- ? After pipetting the AP-Conjugates, remove Substrate Tablets from the freezer.
- ? Incubate the Tablets for 30 minutes at room temperature.
- ? Open the pNPP tablet package (silver foil) and Tris Buffer tablet package (gold foil) and drop the tablets into an appropriate **light protected container** containing 20 ml distilled or deionized water.
- ? **Do not touch the tablets.**
- ? Vortex until tablets are completely dissolved.

? For best results, the solution should be used within 30 minutes.

8. TEST PROTOCOL

- ? Prepare Wash Buffer (according to preparation of reagents section 7A).
- ? Remove Sample Diluents, Conjugates, Assay Buffers, and Positive and Negative Controls from the freezer and incubate at room temperature for approximately 60 minutes.
- ? Arrange a 96-well Dilution Plate (not delivered) according to Figure 1.
- ? Figure 1. Arrangement of Samples (S1 to S21) and Blanks in the Dilution Plate (not delivered).

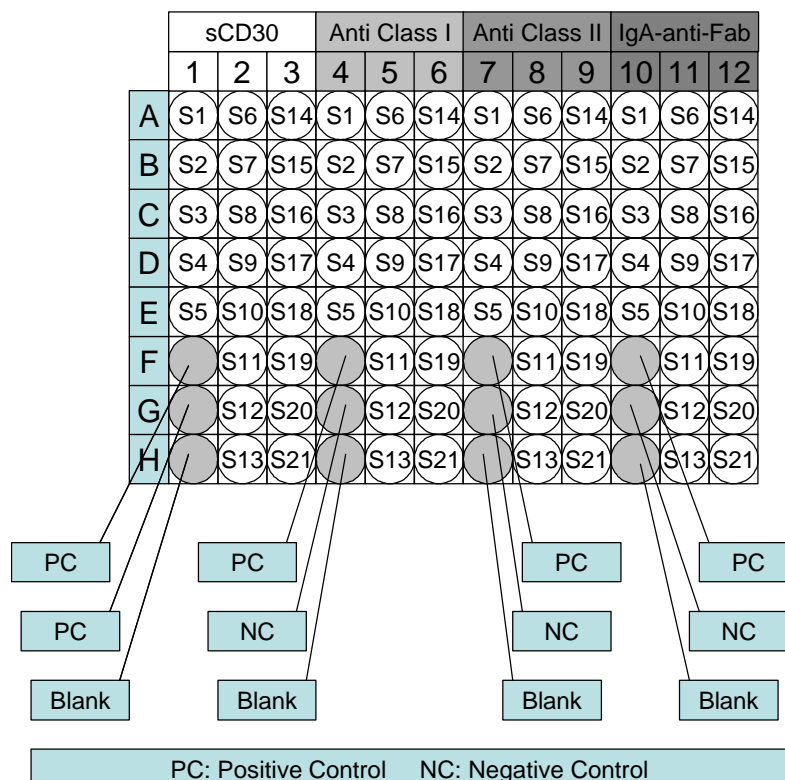


E. Dilute Samples as stated below in Table 1.

F. Table 1. Preparation of Sample Dilutions in the Dilution Plate according to Figure 1

Parameter	Sample	Sample Diluent
sCD30 (1:4)	15 µl	45 µl
IgG-anti-HLA Class I (1:2)	30 µl	30 µl
IgG-anti-HLA Class II (1:2)	30 µl	30 µl
IgA-anti-Fab (1:32)	4 µl	124 µl

- G. Prepare Anti-sCD30-Biotin Conjugate (according to preparation of reagents section 7B).
- H. Remove coated Microwell Strips from the foil bag.
- I. Pipette 300 µl of Wash Buffer into each well of the coated Microwell Strips and incubate for 10 minutes. **Take care not to scratch the surface of the microwells.**
- J. After 10 minutes of incubation, empty the wells by decanting and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. **Use the microwell strips immediately after washing. Do not allow wells to dry.**
- K. Mix the contents of the Dilution Plate by aspiration and ejection. Transfer 50 µl of the reaction mixture from the Dilution Plate to the coated Microwell Strips in the same scheme as prepared on the Dilution Plate.
- L. Pipette 50 µl of each Positive and Negative Control into the coated Microwell Strips according to the scheme shown in Figure 2. **Leave the Blank wells empty.**
- M. Figure 2. Arrangement of Controls and Blanks in the coated Microwell Strips.



- N. Add 50 µl of diluted Anti-sCD30-Biotin Conjugate to the microwell strips coated with monoclonal antibody to human sCD30 (A1 to H3), **excluding the Blank well H1.**

- O. Cover with a Plate Cover and incubate at room temperature for 2 hours, if available on a rotator set at 100 rpm.
- P. Prepare Alkaline-Phosphatase Conjugates a few minutes prior to use (according to preparation of reagents sections 7C-F).
- Q. Remove plate cover and empty the wells by decanting. Wash microwell strips 4 times with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells. After the last wash, empty the wells by decanting and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. **Use the microwell strips immediately after washing.**
- R. Add 100 μ l of diluted Streptavidin-Alkaline-Phosphatase Conjugate to the microwell strips coated with monoclonal antibody to human sCD30 (A1 to H3), **excluding the Blank well H1.**
- Add 50 μ l of diluted Anti-human-IgG-Alkaline-Phosphatase Conjugate for detection of IgG-anti-HLA Class I antibodies to the microwell strips coated with human HLA Class I antigens (A4 to H6), **excluding the Blank well H4.**
- Add 50 μ l of diluted Anti-human-IgG-Alkaline-Phosphatase Conjugate for detection of IgG-anti-HLA Class II antibodies to the microwell strips coated with human HLA Class II antigens (A7 to H9), **excluding the Blank well H7.**
- Add 50 μ l of diluted Anti-human-IgA-Alkaline-Phosphatase Conjugate for detection of IgA-anti-Fab antibodies to the microwell strips coated with human Fab fragments (A10 to H12), **excluding the Blank well H10.**
- S. Cover with a Plate Cover and incubate at room temperature for 1 hour, if available on a rotator set at 100 rpm.
- T. Immediately after pipetting the AP-Conjugates, prepare Substrate Solution (according to preparation of reagents section 7G).
- U. After 1 hour of incubation with AP-Conjugates, remove plate cover and empty the wells by decanting. Wash microwell strips 4 times with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells. After the last wash, empty the wells by decanting and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. **Use the microwell strips immediately after washing.**
- V. Pipette 200 μ l of prepared Substrate Solution to all wells, **including the Blank wells.** Incubate the microwell strips in the dark at room temperature for about 10 minutes.

- W. After 10 minutes of incubation read Optical Density [OD] at regular intervals (every 5 minutes) until the Positive (and Negative) Controls have reached the **values given in the lot-specific Manual delivered with the product.**
- X. Read Optical Density on an ELISA reader using 405 nm as the primary wave length and 492 nm as the reference wave length. Blank the plate reader according to the manufacturers instructions by using the blank wells.

9. INTERPRETATION OF RESULTS

	Increased Risk of Graft Rejection
sCD30	OD > Mean OD of sCD30 Positive Controls
IgG-anti-HLA Class I	OD > 0.300
IgG-anti-HLA Class II	OD > 0.300
IgA-anti-Fab	OD < 0.060

OD = Optical Density

	Decreased Risk of Graft Rejection
sCD30	OD < Mean OD of sCD30 Positive Controls
IgG-anti-HLA Class I	OD < 0.300
IgG-anti-HLA Class II	OD < 0.300
IgA-anti-Fab	OD > 1.000

OD = Optical Density

10. LIMITATIONS

- ? Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- ? Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

- ? Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

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If you have any particular questions concerning this kit, please do not hesitate to contact us under the following numbers:

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CTS reagents are provided exclusively for investigational use within the Collaborative Transplant Study