Complement Titration Tray

Class I and II

Instructions for Use

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Instructions for Use

For Research Use Only

The Invitrogen™ Complement Titration Tray, Class I and II, is a serological based method designed to determine the strength and effectiveness of each individual lot of complement. The strength of the complement, with respect to two specific Class I or Class II antibodies, is determined by diluting the complement and testing against a positive and negative cell. By observing total cell death at the various dilutions, the optimal complement titration can be chosen. A new lot of complement is tested as compared to the previous lot of complement to ensure the identical efficacy of complement in microlymphocytotoxicity testing.

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1 Kit Components:

	Description	Quantity	Storage
1.1	72 well trays containing 1 µl/well of antisera or medium, overlaid with mineral oil	6	-55° C or colder in a **NON-FROST FREE FREEZER**
1.2	Certificate of Analysis	1	---------
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2 Materials, Reagents, and Equipment not Supplied:

2.1 A donor cell positive for antibodies contained on tray

2.2 A donor cell negative for antibodies contained on tray

2.3 Microcentrifuge tubes

2.4 Serological pipettes

2.5 Disposable transfer pipettes

2.6 37°C water bath

2.7 RPMI-1640 with 5% fetal calf serum

2.8 Hemocytometer

2.9 Dry ice and Styrofoam container

2.10 Multiple dispensing syringes

2.11 Cell plater

2.12 Eosin Y - Solution 5% or CFDA

2.13 Hanks Balanced Salt Solution (HBSS)

2.14 12-37% Neutralized Formalin - pH 7.0 ±0.2 or Propidium iodide in quenching solution

2.15 Glass coverslide

2.16 Inverted Phase Contrast or Inverted Fluorescent Microscope

3 Sample Setup:

3.1 Thaw two Complement Titration Trays for each lot of complement to be tested.

3.2 Select an HLA typed donor cell(s) that is positive for both specific antibodies contained on the tray (see worksheet) and an HLA typed donor cell that is negative for both specific antibodies contained on the tray (see worksheet).

3.3 Thaw the cells, check the cell viability, count cells, and adjust the cell counts to 2-3 X 10^6 cells/ml.

3.4 Plate 1 µl of negative cell suspension into each well of one thawed tray using a cell plater or 50 µl multiple dispensing syringe. Plate 1 µl of positive cell suspension in the same manner into the remaining thawed tray.

3.5 Incubate the trays at room temperature (22°C ± 3°C).

Class I (dye exclusion)	30 min
Class I (fluorescence)	30 min
Class II (fluorescence)	45 min

3.6 Reconstitute the complement if it is lyophilized or thaw the complement according to standard laboratory procedure.

3.7 For each lot of complement to be tested, add 200 µl of RPMI- 1640 with 5% fetal calf serum to a set of five microcentrifuge tubes. Label the tubes with intended dilution factors, for example, 1:2, 1:4, 1:8, 1:16, and 1:32.

3.8 Using a 250 µl multiple dispensing syringe, transfer 200 µl of test complement to the first tube labeled 1:2 and mix well. Transfer 200 µl of 1:2 complement to the tube labeled 1:4. Continue with serial dilutions up to 1:32.

3.9 If different dilution factors are used, such as 1:3, 2:3, etc., calculate the volume of diluent (RPMI 1640 with 5% fetal calf serum) and the volume of complement required to make a final volume of 200 µl. Using a 250 µl multiple dispensing syringe, add the calculated volume of diluent to each labeled microcentrifuge tube. Using a 250 µl multiple dispensing syringe, add the calculated volume of complement to each labeled microcentrifuge tube.

3.10 Place the diluted complement in a rack and keep samples cold at all times during the test.

3.11 Using a 50 µl multiple dispensing syringe, add 5 µl of undiluted test complement and test complement dilutions to the tray. It is recommended to add the undiluted test complement into wells in column A, the first diluted complement into wells in column B, etc (see the following table for an example). Current working complement may be added to the last row or alternatively to a separate tray.

		COMPLEMENT DILUTIONS					
		UND	***1:2***	***1:4***	***1:8***	***1:16***	***1:32 or Current working C′***
SERUM							
		A	B	C	D	E	F
RPMI 1640	1						
NEG. CONTROL	2						
T-CELL CONTROL	3						
POS. CONTROL	4						
A2	5						
A2 (1:2)	6						
A2 (1:4)	7						
A2 (1:8)	8						
B8	9						
B8 (1:2)	10						
B8 (1:4)	11						
B8 (1:8)	12						

3.12 Incubate the trays at room temperature (22°C ± 3°C).

Class I (dye exclusion)	60 min
Class I (fluorescence)	50 min
Class II (fluorescence)	60 min

3.13 Using a 50 µl or 100 µl multiple dispensing syringe, add 2 µl of filtered 5% aqueous Eosin Y to each well and incubate at room temperature (22°C±3°) for 3 to 5 minutes.

3.13.1 If using a fluorescein based assay, add 5 µl of propidium iodide in quenching ink to each well.

3.14 Using a 50 µl or 250 µl multiple dispensing syringe, add 5 µl of filtered 37% neutralized formalin to each well.

3.14.1 If using a fluorescent assay, trays are read using an excitation wavelength of 493nm and emission wavelength 635nm for propidium iodide.

3.15 Place a 2 x 3 inch coverslide over the tray.Allow trays to stand at room temperature for an hour before reading the reactions, or trays stored at 2-10°C can be read the following day. Observe the test microscopically at 150x magnification using phase contrast illumination. It is recommended that the tray be read within 48 hours. Trays stained with fluorescein dye can be read after 30 minutes or trays stored at 2-10°C can be read the following day.

4 Interpretation:

Dead cells (those possessing the antigen for which the antibody is specific) absorb the dye, appear enlarged and darkened and show distinct nuclear detail. Viable cells (those lacking the antigen), exclude the dye, appear slightly brighter and smaller in size as compared to dead cells. Fluorescently labeled viable cells are green and nonviable cells are red. After correcting for percent dead cells in negative control wells, the test is graded as follows:

% Dead Cells	Score	Interpretation
0-10	1	Negative
11-20	2	Doubtful Negative
21-50	4	Weak Positive
51-80	6	Positive
81-100	8	Strong Positive
--	0	Unreadable

4.1 Complete the top half of Complement Titration Tray worksheets for each tray to be read. Record the complement dilutions for each column in the gray cells of the result table.

4.2 Read and record results on the result table of worksheets.

4.3 Examine the Complement Titration trays and determine the highest titer (dilution) at which the complement gives 6 or 8 reactions against the undiluted antibody.

4.4 The recommended complement working titer is the dilution one step back determined in step 4.3. For example, if the highest titer determined in step 4.3 is 1:4, the working titer would be 1:2. Record results on worksheets.

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For country-specific contact information visit our website at

www.invitrogen.com

Invitrogen Corporation

9099 N. Deerbrook Trail

Brown Deer, WI 53223 USA

Tel. 1-800-955-6288

Fax 1-800-331-2286

Email: catalog@invitrogen.com