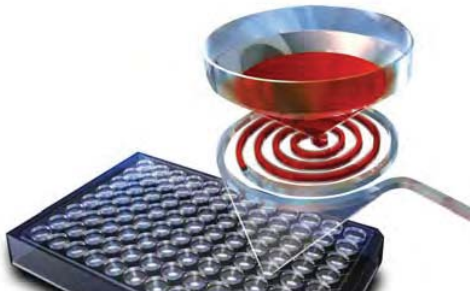
ANIARA

Manufactured By: U-CyTech Biosciences

Instruction Manual
CytoMax ELISA kit
(extended version)



2- or 5-plate format

Read this manual attentively
before using the kit for the first time.

For research use only.
Not for use in diagnostic or therapeutic procedures.

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U-CyTech CytoMax ELISA kits

(please find below the catalogue number of the CytoMax ELISA kit)

	Species				
Analyte	Human	Old World Monkey	New World Monkey	Mouse	Rat
IFN-γ	ACT920	ACT945	ACT970	ACT980	ACT992
IL-2		ACT947		ACT981	
IL-4		ACT948		ACT982	ACT993
IL-6	ACT925	ACT950		ACT984	
IL-10				ACT985	
IL-13	ACT929	ACT954	ACT971		
TNF-α	ACT936	ACT959	ACT972		

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Contents

Abbreviations

ANSI	American National Standards Institute
APC	Antigen presenting cells
CCL	Chemokine C-C motif ligand
CD	Cluster of differentiation
ELISA	Enzyme-linked immunosorbent assay
HRP	Horse Radish Peroxidase
IFN	Interferon
IL	Interleukin
L	Liter
MHC	Major Histocompatibility Complex
min	minute(s)
RFU	Relative Fluorescence Units
RT	Room temperature
SBS	Society for Biomolecular Sciences
SPP	Streptavidin-HRP
Th	T helper subset
TNF	Tumor necrosis factor

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1. Introduction

U-CyTech's CytoMax ELISA kits offer a rapid, sensitive and specific chemifluorescent-based immunoassay for the measurement of cytokines using exceptionally small sample volumes. The speed, sensitivity and small sample requirements are enabled by the unique microfluidic design of the CytoMax ELISA plate in which all reactions, including coating and detection, occur within a 4.5 µl microfluidic reaction chamber. The distinctive microchannel geometry and small reaction volume favor rapid reaction kinetics. The CytoMax assay utilizes only 5 µl sample and each reaction step is completed in 5 to 20 min. Including wash time, substrate incubation and read time, a typical CytoMax ELISA assay can be completed within two hours and ten minutes. The CytoMax ELISA plate is compatible with all standard fluorescence plate readers, robotic sample processors and other equipment used in conducting traditional 96-well microplate-based ELISA assays.

1.1 CytoMax ELISA plate

The CytoMax ELISA is a revolutionary new plate format. With an ANSI/SBS compliant 96-well layout, the plate by using Optimiser™ technology integrates the power of microfluidics for small volume and rapid immunoassay protocols. Figure 1 shows schematically the CytoMax ELISA plate with magnified view of one well. Each well of the plate contains one loading part (for the addition of reagents), which is connected to one microfluidic reaction chamber. Reagents and samples are pipetted in the loading well and via capillary action transported through the reaction chamber to an integrated absorbent pad. The unique design of the CytoMax ELISA plate allows the well to be drained but each liquid is trapped in the reaction chamber by capillary forces. As the next liquid volume is added, the capillary barrier is broken and the liquid within the chamber is drawn out by the absorbent pad and replaced by the new reagent. All assay reactions occur on the inner surface of the microfluidic reaction chamber. Small flow rate variations (time to empty well) do not affect assay results.

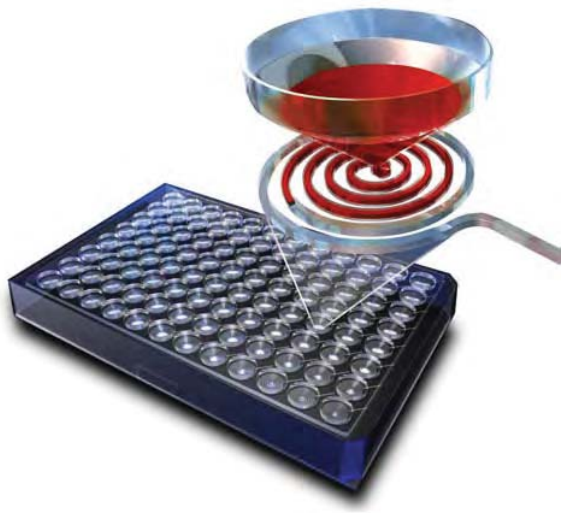

Figure 1.
The CytoMax ELISA plate and magnified view of one well.

1.2 Assay principle

The CytoMax ELISA procedure is an immunoassay in which traditional ELISA reactions take place within the unique plate. Briefly, capture antibody is immobilized on the inner surface of the plate's microfluidic reaction chamber. Next, the unreacted sites are blocked twice with a blocking buffer. Subsequently, standards and samples are dispended to the wells. The immobilized coating antibody on the surface of the reaction chamber will specifically bind cytokines present in samples and standards. Following another wash, a biotinylated detection antibody is added to the wells. The biotinylated

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antibody will bind to the cytokine, thus 'sandwiching' the cytokine between the coating and detection antibody. Following another wash, streptavidin-HRP (SPP) conjugate is added to the wells. The SPP conjugate binds specifically to the biotin moiety of the detection antibody in the complexes formed. Following two additional washes, a chemifluorescent substrate is applied. If HRP has been captured on the microchannel surface during the sequence of reactions cited above, the enzyme will react with the substrate solution and will yield a fluorescent signal when excited at the appropriate wavelength. The light signal emitted will be directly proportional to the concentration of cytokine in standards and samples and will be quantifiable when the plate is read using a fluorescence plate reader.

1.3 Cytokines

Cytokines are a group of regulatory proteins critically involved in various physiological processes such as immune recognition, cell differentiation and cell proliferation. They have been identified in many vertebrate species and are produced by a variety of different cell types. Cytokines are usually produced transiently and locally, acting in a paracrine or autocrine manner. They interact with high affinity cell surface receptors specific for each cytokine or cytokine group and are active at very low concentrations mostly in the picogram to femtogram range. Currently, it is well known that the type of an antigen-specific immune response largely depends on the selection or preferential activation of defined $CD4^+$ T cell subsets (i.e. Th1, Th2 and Th17). Activation of these subsets is characterized by the secretion of distinct patterns of cytokines. Th1, but not Th2 cells, primarily secrete IFN-γ; while Th2, but not Th1 cells, produce IL-4, IL-5, IL-9 and IL-13. Other cytokines, such as TNF-α, are produced by both Th subsets. More recently, two novel subsets were found to differentiate from naïve $CD4^+$ T cells, namely T regulatory cells (Treg) and Th17 cells. T regulatory cells are characterized by the production of TGF-ß, IL-10 and IL-35, while Th17 cells can produce IL-17A, IL-17F, IL-17AF, IL-21, IL-22, IL-26, GM-CSF, TNF-α and CCL20 (MIP-3α).

Additionally, different critical cytokines are required for the differentiation of the various cell types. The production of IL-12 and IL-10, produced by antigen presenting cells (APC) such as macrophages and dendritic cells, critically contributes to the preferential expansion of Th1- or Th2-type of cells. For instance, early production of IL-12 is considered essential for the development of Th1 cells. On the other hand, the absence or low concentrations of IL-12 and IFN-γ in the early phase of an immune response and concomitant production of IL-4 by cells of the mast cell/basophil lineage or T cells themselves is known to favor the development of Th2 cells. The differentiation of Treg and Th17 cells is interlinked through TGF-ß, indicating potential plasticity between these two types of cells. The ultimate T cell subset is determined by the concentration of TGF-ß along with the concentrations of IL-6 and IL-23.

In addition to cytokine regulatory effects on Th subset differentiation, the cytokines released by the different types of Th cells also produce distinct effector functions. For instance, IL-4 and IFN-γ have differential or antagonistic activities on immunoglobulin isotype selection or MHC class II expression. Therefore, the properties of an immune response can be best studied by determining the amounts of cytokines produced by the responding T cells and APC.

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2. Contents of the kit

All reagents and items provided with the kit have been developed and/or validated for the CytoMax ELISA.

Note:
For proper functioning of the assay, do not use alternate buffers/reagents or solvent-containing samples.

Items	Quantity (2-plate format)	Quantity (5-plate format)	Storage conditions
Coating antibodies*	1 vial	1 vial	4°C (39°F)
Cytokine standard*	2 vials	5 vials	4°C (39°F)
Biotinylated detection antibodies*	1 vial	1 vial	4°C (39°F)
Coating buffer (ready-to-use)	1 vial (5 ml)	1 vial (7.5 ml)	4°C (39°F)
Blocking buffer (ready-to-use)	1 vial (25 ml)	2 vials (2x 25 ml)	4°C (39°F)
Wash buffer (ready-to-use)	1 vial (30 ml)	2 vials (2x 30 ml)	4°C (39°F)
SPP conjugate*	1 vial	2 vials	≤-20°C (≤-4°F)
Substrate solution A**	1 vial (2.5 ml)	1 vial (5 ml)	4°C (39°F)
Substrate solution B**	1 vial (2.5 ml)	1 vial (5 ml)	4°C (39°F)
Substrate solution C**	1 vial (0.5 ml)	1 vial (1 ml)	4°C (39°F)
CytoMax ELISA plates***	2	5	RT
96-well U-bottom Transfer plate	2	none	RT
Adhesive cover slips	5	10	RT

* Lyophilized

** Substrate solutions are manufactured by Thermo Fisher Scientific, Inc.

*** This item is manufactured by Siloam Biosciences, Inc.

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Hazard information

Warning:
Substrate solution A is classified as dangerous according to Regulation (EC) no. 1272/2008 and Directive 67/548/EEC and its amendments:

Reproductive toxicity (Category 1B)

Hazard statements:
H360: May damage fertility or the unborn child.

Precaution statements:
P201: Obtain special instructions before use.
P308 + P313: IF exposed or concerned: Get medical advice/attention.

In case of contact with skin, wash with soap and water and remove contaminated clothing and shoes. Upon ingestion or contact with eyes, rinse mouth (if person is conscious; do NOT induce vomiting) or eyes with copious amounts of water for at least 15 min. Assure adequate flushing by separating the eyelids. Consult a physician.

Warning:
Substrate solution B is classified as dangerous according to Regulation (EC) no. 1272/2008 and Directive 67/548/EEC and its amendments:

Acute toxicity, Dermal (Category 4)

Hazard statements:
H312: Harmful in contact with skin.

Precaution statements:
P280: Wear protective gloves/protective clothing/eye protection/face protection.
P302 + P352: IF ON SKIN: wash with plenty of soap and water.

In case of contact with skin, wash with soap and water and remove contaminated clothing and shoes. Upon ingestion or contact with eyes, rinse mouth (if person is conscious; do NOT induce vomiting) or eyes with copious amounts of water for at least 15 min. Assure adequate flushing by separating the eyelids. Consult a physician.

Other kit components are not classified as dangerous according to Regulation (EC) no. 1272/2008 and Directive 67/548/EEC or 1999/45/EC and their amendments.

Please find the Material Safety Data Sheet on www.aniara.com.

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2.1 Working solutions

- Coating antibodies and buffer

The supplied Coating buffer is mandatory for CytoMax ELISA assays and ready-to-use. The coating antibodies are supplied in lyophilized form. The contents in the vial should be reconstituted in 200 µl (2-plate format kit) or 300 µl (5-plate format kit) sterile distilled water and allowed to stand for 5 min at RT before further dilution in Coating buffer. Keep the solution sterile during storage at 4°C.

- Cytokine standards

The kit contains several vials with cytokine standard. The contents of one vial should be reconstituted in 0.5 ml sterile distilled water and allowed to stand for 5 min at RT. Thereafter, the stock solution is placed on ice and diluted in Blocking buffer (preferentially use this standard solution within one hour). Use vials with cytokine standards only once.

The total amount of cytokine standard is indicated on the label of the vial (ng/vial). After reconstitution in 0.5 ml water, the concentration (ng/ml) will become twice the amount on the label [e.g. when amount on label is 4.8 ng/vial; after reconstitution, the concentration becomes 9.6 ng/ml = 9600 pg/ml].

- Detection antibodies

The biotinylated detection antibodies are supplied in lyophilized form. The contents of the vial should be reconstituted in 200 µl (2-plate format kit) or 300 µl (5-plate format kit) sterile distilled water and allowed to stand for 5 min at RT before further dilution in Blocking buffer. Keep the solution sterile during storage at 4°C.

- SPP conjugate

The concentration and activity of the SPP conjugate have been characterized and optimized for use with the CytoMax ELISA plate system. Use of alternate SPP conjugates may lead to low signals and/or high backgrounds. The vial with lyophilized SPP should be reconstituted in sterile distilled water (200 µl per vial) and allowed to stand for 5 min at 4°C in the dark before further dilution in Blocking buffer. It is strongly recommended that after reconstitution, the solution is divided into small aliquots for single use and stored at ≤-20°C.

- Substrate

The substrate should be prepared no more than 30 min before loading into the CytoMax ELISA plate. The substrate is a mixture of 50:50:1 ratio from solution A, solution B and solution C, and should be gently vortexed.

Note:
Substrate solution C should be completely thawed before mixing. Warm the reagent in a 37°C water bath or by holding the vial in your hands.

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2.2 Storage kit reagents

The vials with lyophilized coating and biotinylated detection antibodies can be safely stored at 4ºC. After reconstitution in sterile water, antibodies are stable for at least 6 months at 4°C when kept sterile. However, it is strongly recommended to divide the reconstituted antibody preparations into small aliquots for single use. These aliquots should be stored at ≤-20°C (stable for at least two years).

The vial(s) with lyophilized SPP conjugate can be best stored at ≤-20°C. After reconstitution in sterile water, it is recommended to divide the solution into small aliquots for single use. These aliquots should be stored at ≤-20°C in the dark (stable for at least one year).

The vials with lyophilized cytokine standard can be safely stored in a refrigerator for at least one year. For maximum recovery, the contents of the vial should be reconstituted in 0.5 ml distilled water and allowed to stand for 5 min at RT and thereafter placed on ice. Subsequently, the reconstituted cytokine standard should be diluted in Blocking buffer and used within one hour.

The ready-to-use Coating, Blocking and Wash buffer and substrate solutions (A, B and C) should be stored at 4°C until the expiry date (indicated on the vials). After opening, these solutions are stable for minimal one year when kept sterile.

3. Materials and equipment required but not supplied

3.1 Materials

- Eppendorf or similar microtubes for centrifugation of reagent or sample dilutions.
- Sterile distilled water for reconstitution of lyophilized cytokine standards, antibodies and SPP conjugate.
- Reagent reservoirs (e.g. 96-well U-bottom Transfer plate) for the preparation of reagent working solutions to be transferred to the CytoMax ELISA plates (96-well U-bottom Transfer plates are supplied only with the 2-plate kit).
- Pipette tips for the accurate delivery of volumes in the ranges of 1-10 µl, 10-100 µl, and 100-1000 µl (see also paragraph 3.2).

3.2 Equipment

- Electronic multi-channel pipette for precise and accurate delivery of small volumes.

  Note:
  We recommend the Biohit eLINE® 8-channel Electronic pipette (volume range 5-120 µl), Model 730340.

- Compatible pipette tips to transfer solutions to the wells.

  Note:
  We recommend the use of the Biohit Optifit tips (volume range 0.5-300 µl) (Cat# 790300/ 790302) in combination with the Biohit eLINE® 8-channel Electronic pipette.

- Fluorescence or multi-mode plate reader with fluorescence reading capability for 96-well microtiter plates (see Paragraph 4.2 Reader setup for more detailed information).

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- Microcentrifuge to remove particulates from samples capable to set at 13,000 x g.
- Vortex mixer for adequate mixing of reagents.
- Timer for exact timing of incubation steps.
- Robotic sample processor (optional) for automation of ELISA procedure.

  Note: We recommend the BioTek Precision™ XS Microplate Sample Processor.

4. Critical information before starting the assay

4.1 Pipetting techniques

Assays on CytoMax ELISA plates require the accurate and precise delivery of 5 µl volumes.

The following guidance is offered to users.

1. Use pipette for which the upper limit of their operating range is ≤10 µl.

2. Use pipette tips appropriate for 5 µl pipetting.

3. To aspirate liquid, hold the pipette near vertical and immerse the pipette tip to a depth of approximately two mm in the liquid. Release the operating button steadily. Wait one second. Withdraw the tip from the liquid.

4. To dispense liquid, hold the pipette nearly vertical. With the pipette tips touching the surface of the well, press the operating button steadily until the liquid is dispensed.

Note:
The pipette tip should make contact with the well surface for proper dispensing (see "RIGHT" frame in Figure 2). Do not pipet into the hole at the bottom of the well (see "WRONG" frame in Figure 2).

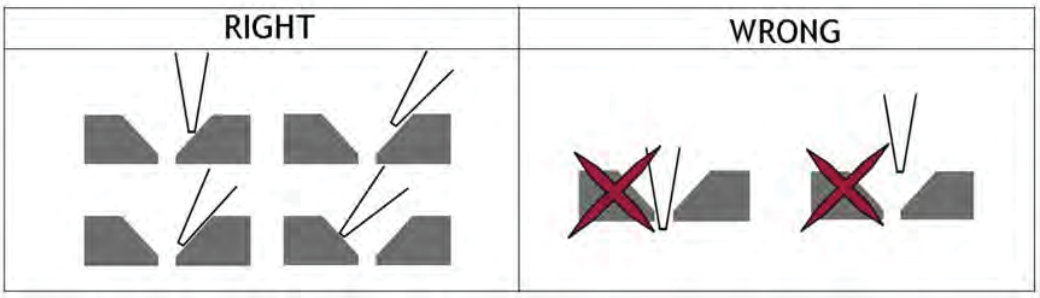

Figure 2. Pipette tip positioning for loading the CytoMax ELISA reaction chambers.

Avoiding Bubbles While Pipetting:

1. Bubbles will compromise the performance of the CytoMax ELISA by interfering with the flow of liquid within the microchannels.

2. Reagents may form bubbles readily with standard pipetting techniques.

3. To avoid complications due to bubbles, U-CyTech recommends the use of the "Reverse Pipetting" technique during all pipetting steps (see Figure 3).

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a. To aspirate liquid, press the operating button of the pipette to the second stop (position 1).

b. Immerse the pipette tip in the liquid to a depth of about 2 mm and steadily release the operating button completely (position 2).

c. Withdraw the tip from the liquid, touching it against the edge of the reservoir to remove excess liquid.

d. Dispense the liquid into the loading well of CytoMax ELISA plate by gently and steadily pressing the pipette's operating button to the first stop (position 3). Briefly hold the operating button in this position.

e. With the button in this position, move the tip from the loading well to the reagent reservoir, immerse the tip in the liquid and aspirate (position 4).

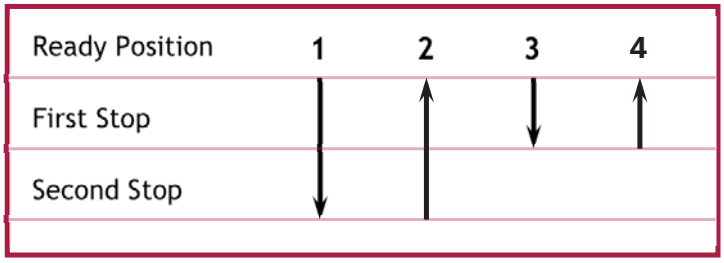

Figure 3. Reverse pipetting technique.

Note:
An electronic 8-well multi-channel pipette is ideally suited for use with CytoMax ELISA plates since (a) it eliminates possibility of injecting bubbles and (b) can be used for convenient repetitive loads with single aspiration step for rapid reagent transfers.

General setup for using an electronic 8-well multi-channel pipette:

- Select pipette capable of delivery 5 µl and 30 µl (e.g. with volume range of 5-120 µl).
- Choose "Reverse Pipetting" in function setting.
- Use "Multiple Dispensing" mode to transfer the solution into the CytoMax ELISA plate.
- For example, to transfer coating antibody solution to a plate, set the program for 12 times dispensing, 5 µl per dispensing step. In this way, the pipette will first automatically aspirate more than 60 µl of solution (a few µl extra will be aspirated to avoid dispensing air) and dispense the preselected volume 12 times. Users will not need to move pipette back and forth to transfer solution.

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4.2 Reader setup

The CytoMax ELISA is compatible with standard fluorescence plate readers and multi-mode plate readers with fluorescence reading capability (e.g. Synergy HT from BioTek Instruments, Inc.). In this section, we present a general guidance for setting up the plate readers.

For further assistance, please contact Aniara's technical support at info@aniara.com.

Step 1: Selecting the wavelength for excitation and emission light:

The CytoMax ELISA includes a substrate, which can be detected using 530-575 nm for excitation and 585-630 nm for emission (Figure 4). Quantitation does not require filters that precisely match the excitation/emission maxima. However, a non-overlapping filter set with a bandpass that includes the excitation/emission spectra is required.

Below examples for different types of readers:

Filter-based readers: Install 528/20 nm (or similar) filter for excitation and 590/35 nm (or similar) filter for emission.

Monochromator-based readers: Set excitation at 544 nm and emission at 590 nm.

Readers with pre-configured optical set: Select the wavelength setting for Rhodamine or Cy3.

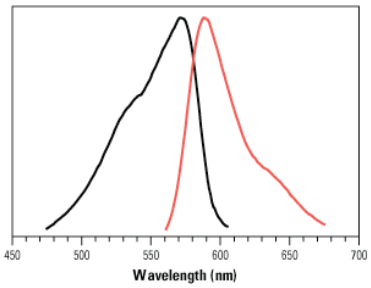

Figure 4. Normalized absorption (left) and emission (right) spectra of the HRP substrate.

Step 2: Selecting the plate type:

CytoMax ELISA plate fits 96-well SBS standard in all specifications. Please use "96-well standard" or similar in plate type setting.

Step 3: Selecting the probe direction:

Select "top reading" for probe direction. When the probe height in the reader is adjustable, the most optimal probe height should be selected in a test run (for more information, please contact info@aniara.com).

Step 4: Selecting the sensitivity/gain:

When defining reading parameters for fluorescence analysis, setting the Photomultiplier tube (PMT) sensitivity (or "gain" in some types of fluorescence reader) is important for obtaining useful measurements. A manual sensitivity/gain setting is recommended for reading CytoMax ELISA plates. The procedure is as described below:

1. In a clean plastic tube, add 50 µl of Substrate solution A, 50 µl of Substrate solution B, 1 µl of Substrate solution C and 1 µl of supplied SPP conjugate, mix well and wait for 2 min. The substrate will be fully developed and stable for hours.
2. Load 4 µl of mixture into one well of the CytoMax ELISA plate and wait until the well is empty.
3. Read that well in the plate reader with various gain setting.

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4. Select the gain, which gives the RFU closest to 11,000.
5. Use the same gain setting and read one empty well of the plate. The readout should be less than 50. Save or record this gain setting.
6. This gain defines the maximum reading (RFUmax) that CytoMax ELISA based assays can reach with this reader gain/sensitivity setting.

Note:
The gain will be valid for all following CytoMax ELISA experiments. Repeat step 1-5 if a) changing the plate reader or b) changing the optical unit such as light bulb, filters, etc.

The following readers are compatible with CytoMax ELISA plates. If your reader is not listed below, please contact technical support info@aniara.com for assistance.

Reader Name	Optic Setting	Plate Type	Gain / Sensitivity	Z-position adjustment	Other Instruction
BioTek Flx800	529/20 & 590/35	96-well plate	Manual	N/A	N/A
BioTek Synergy HT	530/25 & 590/35	96-well plate	Manual	N/A	10 mm Probe height
Tecan Infinite® M200	544/590	96-well, Clear, Round	Manual	Manual	Define well depth under Plate Type
BMG FLUOStar® Omega	544/590	SBS Standard 96-well	Manual	N/A	N/A
Promega GloMax®	Green Optical Kit	N/A	N/A	N/A	N/A
Molecular Devices SpectraMax® M series	529/590	96-well	Auto	N/A	Define plate dimension under Plate Type
PerkinElmer VICTOR™ X Series	531/25 & 590/10	Generic 8x12; 96-well	N/A	N/A	Define properties in new fluorometry file. Define plate dimensions in protocol editor

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5. General procedure

To ensure optimal assay performance, all individual assay reagents should be transferred to the CytoMax ELISA plate within 1 min. However, transfer of standards and samples may take longer (5 to 10 min) without adverse effects on assay readings. To facilitate rapid, yet accurate and precise transfer, the 2-plate kit contains two 96-well U-bottom Transfer plates in which the standards, controls, samples, blanks and assay reagents can be prepared prior to their transfer to the CytoMax ELISA plate using an (electronic) multi-channel pipette capable of the accurate and precise delivery of 5 and 10 µl volumes. The buffers and reagents provided with the kit are specifically formulated for compatibility with the microfluidic design of the plate.

Bring all reagents to RT before use and prepare all necessary dilutions before beginning the assay procedure. The incubation times for the CytoMax ELISA range from 5 to 20 min. Preparing all reagents, samples and standards in advance will allow proper timing. Always prepare ~25 µl extra volume in each well of the 96-well U-bottom Transfer plate for easy transferring. Careful pipetting with a mono-channel pipette may reduce the extra sample volume, when sample is very limited or precious.

5.1 Preparation of Transfer plate

- Coating antibodies

See for reconstitution of the coating antibodies instructions on page 7. To coat all wells of one CytoMax ELISA plate, 30 µl is pipetted out of the vial (or use a frozen aliquot; see *"Storage kit reagents"*) and added to 720 µl Coating buffer. Mix the solution gently but thoroughly for approximately 15 seconds and allow it to stand for 1 min at RT. Distribute this volume over 8 wells (90 µl/well) of a 96-well U-bottom Transfer plate (for example see Figure 5). Now, all wells of one plate can be coated with 5 µl coating solution/well with use of a multi-channel pipette.

- Blocking

Wells are blocked twice with 5 µl Blocking buffer. For blocking, a total of 1.5 ml Blocking buffer is required for one CytoMax ELISA plate. Distribute this volume over 16 wells (90 µl/well) of a 96-well U-bottom Transfer plate (Figure 5).

- Standards

The standard stock solution should be diluted in Blocking buffer to the highest concentration cytokine to be used in the standard range. For example, if the standard concentrations range from 2.5 to 160 pg/ml, the stock solution should first be diluted to 160 pg/ml. The standards are now obtainable in a series of two-fold dilutions in Blocking buffer starting from 160 to 2.5 pg/ml. Always, include a blank control (Blocking buffer only) in the standard range.

The preparation of the cytokine standard of 160 pg/ml can be performed in a plastic tube. Using a column of the 96-well U-bottom Tranfer plate, load 50 µl of Blocking buffer in wells B-H (Figure 5). Load 100 µl of 160 pg/ml standard into the first well (A), transfer 50 µl of top standard to well B, change tip and mix with the Blocking buffer to create two-fold dilution. Continue this process till well G; do not transfer a diluted standard to well H (blank).

Note: It is recommended to test each dilution of standard in triplicate.

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- Samples

For measuring cytokines in cell culture supernatant, serum or plasma, samples should be diluted at least 1:1 in Blocking buffer. Particulates in samples may block liquid flow through the microchannels. It is therefore important to centrifuge (10 min at 13,000 x g) or filtrate (0.2 µm) serum/plasma samples or serum-containing tissue culture supernatants prior to testing. When the assay is performed in triplicate, a total volume of at least 20 µl (with use of a mono-channel pipette) or 45 µl (with use of a multi-channel pipette) is required for each sample (5 µl sample per well).

- Biotinylated detection antibodies

See for reconstitution of the biotinylated detection antibodies instructions on page 7. For one CytoMax ELISA plate, 30 µl is pipetted out of the vial (or use a frozen aliquot; see *"Storage kit reagents"*) and added to 720 µl Blocking buffer. Mix the solution gently but thoroughly for approximately 15 seconds and allow it to stand for 1 min at RT. Distribute the solution over 8 wells (90 µl/well) of the 96-well U-bottom Transfer plate (Figure 5)**.** Now, all wells of one CytoMax ELISA plate can be loaded with 5 µl detection solution/well with use of a multi-channel pipette.

- SPP conjugate

See for reconstitution of the SPP conjugate instructions on page 7. For one CytoMax ELISA plate, 30 µl is pipetted out of the vial (or use a frozen aliquot; see *"Storage kit reagents"*) and added to 720 µl Blocking buffer. Mix the solution for approximately 15 seconds and allow it to stand for 1 min at RT. Distribute over 8 wells (90 µl/well) of the 96-well U-bottom Transfer plate (Figure 5). Now, all wells of one CytoMax ELISA plate can be loaded with 5 µl SPP conjugate/well with use of a multi-channel pipette.

- Substrate

The substrate solution should be prepared no more than 30 min before loading into the CytoMax ELISA plate. For one plate, mix 700 µl Substrate solution A, 700 µl Substrate solution B and 14 µl Substrate solution C. Mix thoroughly by gently vortexing. Distribute over 8 wells (150 µl/well) of the 96-well U-bottom Transfer plate (Figure 5). Now, all wells of one CytoMax ELISA plate can be loaded with 10 µl substrate solution/well with use of a multi-channel pipette.

- Wash buffer

Wash buffer is a ready-to-use solution and wells are washed 2 times with 5 µl and 2 times with 30 µl during the procedure. A total of 7.5 ml Wash buffer is required for one CytoMax ELISA plate. This volume can be added to a separate reagent reservoir (or part of the buffer can be added to 16 wells in the Transfer plate and filled up during the procedure).

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Examples of plate layouts

	1	2	3	4	5	6	7	8	9	10	11	12
	Coating antibodies	Blocking buffer		Standards	Samples			Detection antibodies	SPP conjugate			Substrate
A				St_1	S1	S9	S17					
B				St_2	S2	S10	S18					
C				St_3	S3	S11	S19					
D				St_4	S4	S12	S20					
E				St_5	S5	S13	S21					
F				St_6	S6	S14	S22					
G				St_7	S7	S15	S23					
H				St_8	S8	S16	S24					

Figure 5. Example of layout of the U-bottom Transfer plate (for testing in triplicate).
St_1 to St_8: serial dilutions of standard [160 (St_1) to 2.5 (St_7) pg/ml and blank (St_8)]; Sx: sample x.

	1	2	3	4	5	6	7	8	9	10	11	12
A	St_1	St_1	St_1	S1	S1	S1	S9	S9	S9	S17	S17	S17
B	St_2	St_2	St_2	S2	S2	S2	S10	S10	S10	S18	S18	S18
C	St_3	St_3	St_3	S3	S3	S3	S11	S11	S11	S19	S19	S19
D	St_4	St_4	St_4	S4	S4	S4	S12	S12	S12	S20	S20	S20
E	St_5	St_5	St_5	S5	S5	S5	S13	S13	S13	S21	S21	S21
F	St_6	St_6	St_6	S6	S6	S6	S14	S14	S14	S22	S22	S22
G	St_7	St_7	St_7	S7	S7	S7	S15	S15	S15	S23	S23	S23
H	St_8	St_8	St_8	S8	S8	S8	S16	S16	S16	S24	S24	S24

Figure 6. Example of layout of the CytoMax ELISA plate (for testing in triplicate).
St_1 to St_8: serial dilutions of standard [160 (St_1) to 2.5 (St_7) pg/ml and blank (St_8)]; Sx: sample x.

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5.2 Protocol

Note: all following incubation steps are performed at RT.

1. Add 5 µl of the diluted coating antibody solution to the required number of wells of the CytoMax ELISA plate and incubate for 20 min.
2. Add 5 µl of Blocking buffer to each well and incubate for 5 min.
3. Once more, add 5 µl of Blocking buffer and incubate for 10 min.
4. Add 5 µl of diluted standards and samples to appropriate wells and incubate for 20 min.

   Note: see example on the previous page (Figure 6).
5. Wash the wells by adding 5 µl of Wash buffer to each well and incubate for 5 min.
6. Add 5 µl of the diluted detection antibody solution to each well and incubate for 10 min.
7. Wash the wells by adding 5 µl of Wash buffer to each well and incubate for 5 min.
8. Dispense 5 µl of diluted SPP conjugate solution to each well and incubate for 15 min.
9. Wash the wells by adding 30 µl of Wash buffer and incubate for 10 min.
10. Repeat washing step 10.

    Note: within this 10 min incubation step prepare the substrate solution.
11. Dispense 10 µl substrate solution into each well.
    a. Observe the wells during this last incubation step. Step 12a will be completed at the end of the 20 to 30 min substrate incubation time.
    b. The substrate produces a soluble fluorescent product that is pink in color and can be read with a fluorescent or multi-mode plate reader at wavelengths at 530-575 nm for excitation and 585-630 nm for emission.
12. Place the CytoMax ELISA plate in the reader chamber of the fluorescence plate reader. Read the plate at the conclusion of 20 to 30 min incubation time.

    Note: for Flow diagram see Figure 7 on the next page.

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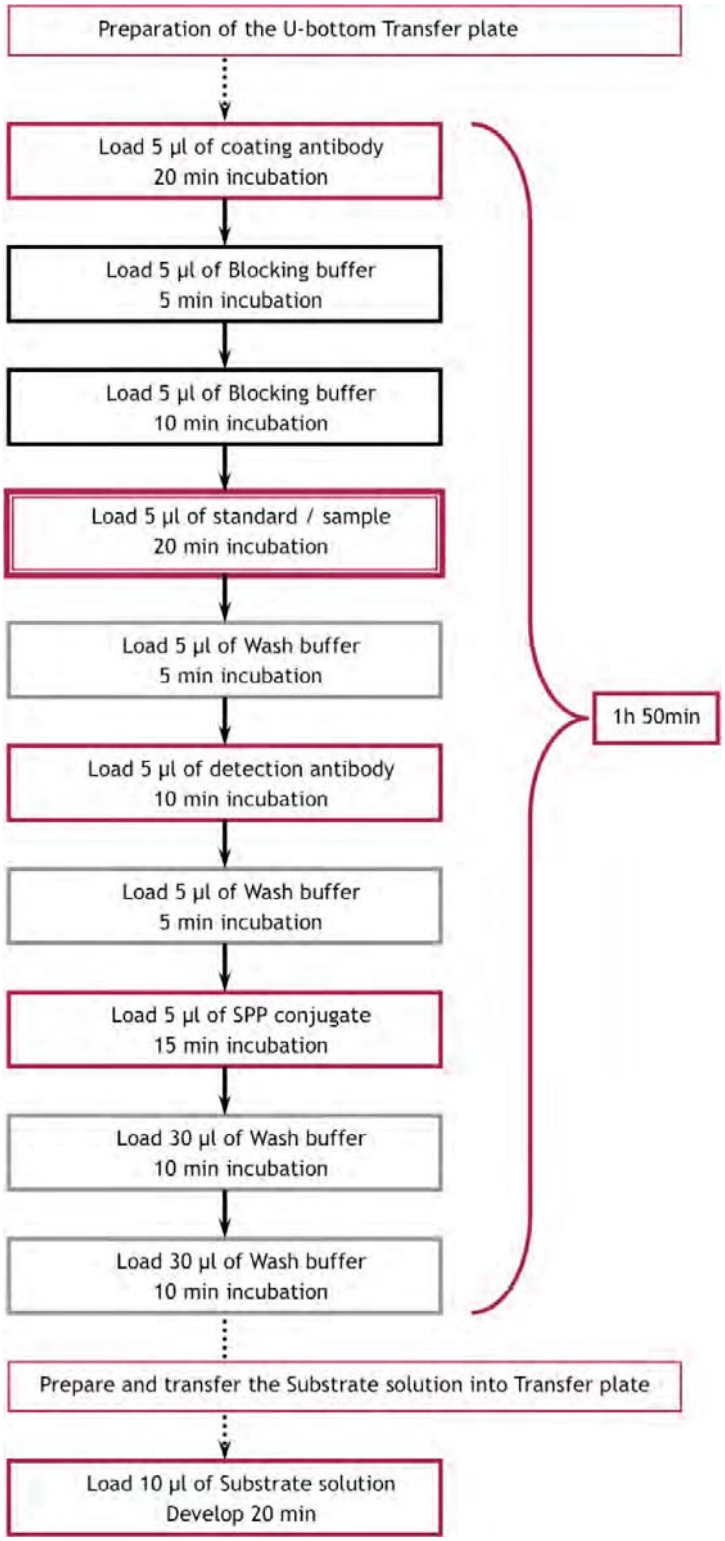

Figure 7. Flow diagram of CytoMax ELISA.

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6. Calculation and interpretation of results

Drawing of standard curve

Calculate the mean of RFU values recorded for the standards (e.g. 0.625 to 40 pg/ml) and the negative control (blank). Subtract the mean of the blank from all mean RFUx values (= RFU_{40}, RFU_{20}, etc.). Plot these RFU values of the different standards versus the cytokine concentration (in pg/ml). Draw the standard curve, using a linear or polynomial regression curve (see an example of a polynomial regression curve in Figure 8). On www.aniara.com, you can find the specific standard curve of different CytoMax ELISA kits.

Example of monkey IL-13 CytoMax ELISA

IL-13 (pg/ml)	RFUx values			Mean	SD	CV	Mean RFUx minus mean blank
	1	2	3				
40	2910	2915	2906	2910	5	0.2%	2732
20	1949	2062	2235	2082	144	6.9%	1904
10	1149	1048	1098	1098	51	4.6%	920
5	584	603	610	599	13	2.2%	421
2.5	360	361	375	365	8	2.3%	187
1.25	282	295	290	289	7	2.3%	111
0.625	242	256	250	249	7	2.8%	71
blank	176	171	187	178	8	4.6%	0

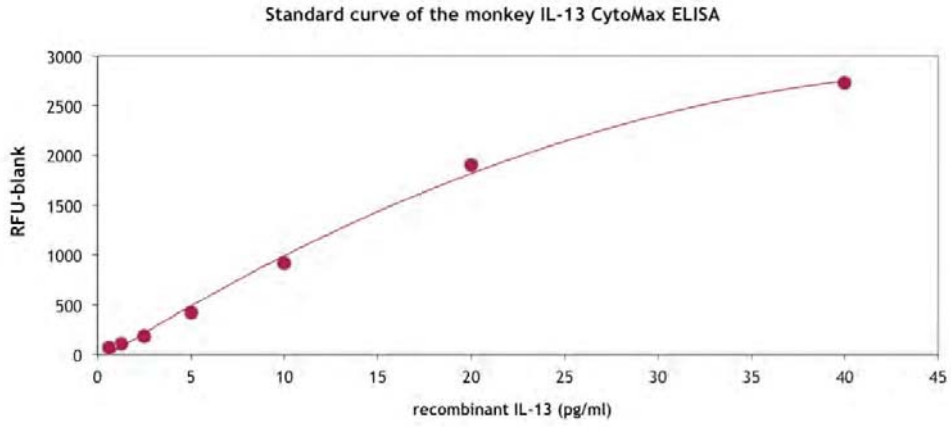

Figure 8. Example of a standard curve for the monkey IL-13 CytoMax ELISA.
This standard curve is for demonstration only. A standard curve should be run with each assay.

Determination of cytokine concentration in samples

Calculate the mean of RFUx values recorded for your samples. Subtract the mean of the blank from all RFU_x values. Plot the calculated RFU values of the different samples on the standard curve. The cytokine level can now be read from the abscissa of the standard curve (or calculated using the equation of the standard curve). The results should be multiplied by the dilution factor to obtain the final concentration in the sample.

Samples showing an RFU value below the cut-off value of the Standard curve (= $RFU_{0.625} - RFU_{blank}$) should be re-analyzed at a lower sample dilution. Samples showing an RFU value that exceeds $RFU_{40} - RFU_{blank}$ should be re-analyzed at a higher sample dilution.

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7. Frequently Asked Questions

1. **How is the CytoMax ELISA plate different from a conventional 96-well plate?**

   All assay reactions in CytoMax ELISA plates occur in the microfluidic chamber at the base of each well. The wells simply serve as loading ports for samples and reagents, which are subsequently drawn into the microchannels. These microchannels have a significantly higher surface to volume ratio compared to the wells of a traditional plate. This fact, coupled with significant reductions in diffusion distance results in significant improvements in assay kinetics, performance and workflow compared to the traditional 96-well microtiter plates.

2. **What are the dimensions of the microchannel?**

   The microchannel has a dimension of 200 µm x 200 µm.

3. **Do I need special pipettes to work with the CytoMax ELISA plate?**

   Only multi-channel pipette(s) capable of accurately and precisely delivering of 5 µl and 30 µl should be used for transferring liquid into the CytoMax ELISA plate.

4. **Is there a particular way of loading liquids in the CytoMax ELISA plate?**

   Reverse pipetting technique should be used for transferring liquid into the plate (see Section 4.1, “Pipetting techniques”, page 9 in the manual).

5. **Why does the manual advise users to touch the bottom surface of the well with pipette tip when loading reagent?**

   In the CytoMax ELISA plate, all assay reactions occur within the microchannel. Hence, touching the pipette tip on the loading well of the plate has absolutely no effect on the assay performance. For most dispensing steps in the CytoMax ELISA, users are dispensing only 5 µl volumes. If the pipette tip does NOT touch the wells surface, the dispensed volume may stick to the end of the tip. The well geometry of the plate is engineered to ensure smooth filling of well and microchannel provided the liquid is dispensed steadily and directly on the well surface.

6. **Why should all materials be transferred to the CytoMax ELISA plate within 1 min at each step in the assay procedure?**

   All incubation steps are from 10 to 20 min in length. Longer time to transfer material will cause time difference between each well in incubation, which may affect the assay accuracy. However, exceptions are the standards and samples. When bound and free analyte have reached equilibrium, a 5 to 10 min longer incubation time does not affect assay accuracy.

7. **How critical is the accuracy of 5 µl dispense volume?**

   The optimizer is designed such that the 5 µl volume represents a slight excess compared to the microchannel internal volume. Provided that the dispense volume is greater than 4.5 µl, slight (even up to 10%) dispense volume variations will not affect assay results.

8. **What should I do when the liquids do not flow properly?**

   Check if all solutions are clear of particulates (e.g. supernatants from cell lysate or serum/plasma).

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Only the buffers that are provided in the CytoMax ELISA kits should be used with the CytoMax ELISA plates. These buffers are specifically developed for and validated for use with these plates.

Our CytoMax ELISAs have been validated with aqueous solutions only. DO NOT use solvent-containing samples or reagents containing surfactants.

Rarely, you will notice that liquid has not drained out even after ~10 min. This indicates a flow failure and signal from that well should not be used for analysis.

9. What happens if there are differences in the flow rate (time required for different wells to empty)?

Most surface binding reactions in CytoMax ELISA plates saturate in ~5 min. The flow sequence of the plate is designed such that differences in flow rates of individual wells do not affect the overall assay performance. The assay results will be valid if the liquid does empty from the well within 5 to 10 min.

10. Do I need a special reader to detect signals in the CytoMax ELISA?

Fluorescence or multi-mode (with fluorescence) 96-well plate readers can be used to read the CytoMax ELISA plates. Please note that absorbance (colorimetric) readers **cannot** read these plates.

11. Is it possible to incubate the plate for more than 30 min or at 37°C?

No, we strongly recommend applying the incubation periods as stated in this manual. Incubating beyond 30 min or at 37°C, will cause evaporative losses.

12. Can I use cell lysate supernatants or other biological fluids such as serum or urine?

The flow does work in some circumstances even with particulates in the solution. However, this flow may not be very repeatable. For these fluids, we recommend using supernatant after centrifuging at 13,000 x g for 10 min or pass through 0.2 µm filter. Whole blood cannot be used as sample in the CytoMax ELISA. Always use clear serum or plasma.

13. Can we combine sample/standard with detection antibody?

Yes, it is possible to pre-incubate sample/standard with detection antibody at least 35 min before dispensing it on the plate. This will reduce assay time with about 15 min. However, it is recommended to first perform a feasibility test by comparing ELISA readings with those of the standard procedure or contact us at info@aniara.com.

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