CYTODETECT™ Kit

Cell stimulation and permeabilization kit

for cell surface and intracellular antigen detection

by flow cytometry analysis

IQ Products
bright fluorescence

Manual

REF IQP-366

50 tests

RUO For Research Use Only

IQ Products BV, The Netherlands

CYTODETECT™ KIT

Contents

Introduction

The CYTODETECT™ kit is composed of a complete set of reagents for stimulation of leukocytes to enhance production of specific intracellular antigens, like cytokines. In parallel immune reagents are available which are composed of fluorochrome-labeled specific monoclonal antibodies, selected for detection of either cell-surface antigens or intracellular antigens.

Intended Use

The CYTODETECT™ kit is intended for stimulation of leukocytes prior to analysis of intracellular antigens like cytokines and immunophenotyping of corresponding cells. Detection is performed using monoclonal antibodies selected for specific reactivity towards these antigens. Monoclonal antibodies are fluorochrome-conjugated for easier, direct application in flow cytometry analysis. This procedure allows detection of different subpopulations of lymphocytes or leukocytes based on both immunophenotype and functional activity with respect to intracellular production of specific cytokines. Possibly, discrimination of different types of leukocytes based on their cytokine profile allows assignment of functional activity, as has been suggested for T helper 1 and T helper 2 cells. Distinction may be helpful in determination of immunological presentation of activated cells in disease-related immuno-monitoring.[1, 2]

Summary and Explanation

In response to penetration of foreign antigens in humans, a balanced network between humoral and cellular immune responses will be elicited. The immune system reacts to the antigen by activation of the network and subsequently the activated condition will be down-regulated to a balanced situation. Control of the immune-response requires efficient communication between the different cells involved in this response. This interaction is provided by cell-cell contact and by a complex array of cytokines, soluble factors produced by these cells.
Cytokines can act on other cells locally or distantly, but can be even auto-regulating. Cytokines can behave stimulatory or inhibitory, or can even perform both activities, depending on the (pre)activation stage of the target cell.[3, 4]

Lymphocytes play an important role in antigen-specific immune responses. Much interest is focusing on the activity of T helper cells, which can further be devided in T_H1 cells and T_H2 cells. T_H1 cells seem to be involved in cell-mediated immune responses, while T_H2 cells appear to have an important role in humoral immune responses. Both T helper cell types express CD3 and CD4 antigens, and no further differentiation can be made immune phenotypically.

However, functionally, these cells can be distinguished based on the cytokines produced by each subtype.[5, 6, 7] Production of a selected number of cytokines by T cells is illustrated in table 1.

Table 1 - Production of cytokines by human T helper cells type 1 and 2 [8, 9]

Cytokine	*T helper 1 cells*	*T helper 2 cells*
IL-2	++	-
IFN-γ	++	-
TGF-ß	++	-
TNF-α	++	-
IL-4	-	++
IL-5	-	++
IL-3	+/-	+
IL-6	+/-	+
IL-10	+/-	+
IL-13	-	+/-

Both T_H1 cells and T_H2 cells seem to be derived from progenitor T_H0 cells, which can produce IL-2, IFN-γ, IL-4 and IL-5.

IL-1ß is produced by monocytes and macrophages.
IL-12 is produced by monocytes, macrophages, B cells, dendritic cells, neutrophils, Langerhans cells and keratinocytes.

Clinically, a number of important applications has been put forward. As stated above, T_H1 cells appear to be involved in cell-mediated immune responses like in bacterial infections, development of auto-immune diseases and transplant rejection. Whereas, T_H2 cells appear to be involved in immune protection in response to allergens, but may also lead to allergenic reactivity.[9, 10] Furthermore, development of progressive disease in patients with HIV infection may be accompanied by a shift from a T_H1 cell response to a T_H2 cell response. These findings can be important for the therapeutic approach of HIV during disease.[11]

Detection of cytokine-producing cells using flow cytometry

The technique to detect intracellular cytokines using flow cytometry is a well established method to analyze the production of specific cytokines on single cell level.[1, 2, 5, 12]

The clinical significance of this development is increasing and develops in at least three fields of application, i.e. transplant patients,[13, 14] atopic disease[15, 16] and HIV/AIDS[17, 18] patients. More applications of detection of intracellular cytokines will develop based on the need to monitor various diseases involving an imbalance of the immune system.

Principles of the Procedure

The level of most of the cytokines produced by immune unstimulated cells is too low to be detected by flow cytometry analysis[19].

Therefore, a method has been developed to analyze cells which have been stimulated before detection of these intracellularly expressed cytokines. The CYTODETECT™ kit provides reagents for cell stimulation, fixation and permeabilization, according to the sequential steps as listed below.

Three procedures are provided, a *General Test Procedure* which can be followed for immunostaining of e.g. CD3-positive or CD8-positive cells, an *Alternative Test Procedure* developed for immunostaining of CD4-positive cells and a slightly modified *General Test Procedure* for detection of TGF-ß. The *Alternative Test Procedure* results in a better discrimination of CD4-positive from CD4-negative cells, and thereby facilitating gate setting for flow cytometry analysis. A summary of the essential steps in each procedure is given below.

I. General Test Procedure

1) Whole blood sample
2) Separation of blood leukocytes

3) Stimulation of cells
4) Fixation
5) Immunodetection of cell surface antigens
6) Permeabilization
7) Immunodetection of intracellular antigens
8) Analysis by flow cytometry

II. Alternative Test Procedure

1) Whole blood sample
2) Separation of blood leukocytes
3) Immunodetection of cell surface antigens
4) Stimulation of cells
5) Fixation

6) Permeabilization
7) Immunodetection of intracellular antigens
8) Analysis by flow cytometry

Reagents provided in the CYTODETECT™ kit allow processing of 50 blood samples. Each sample can be further analyzed for five (5) different intracellular antigens using one step labeled immune conjugates, followed by flow cytometry.

Cell Stimulation and Permeabilization Reagents supplied in the CYTODETECT™ Kit

Item	Description	Amount
Reagent A	Stimulation reagent (contains PMA)	1 vial containing 250 µl Reconstitute before use
Reagent B	Stimulation reagent (contains Ionomycin)	1 vial containing 75 µl Reconstitute before use
Reagent C	Accumulation reagent (contains Monensin)	1 vial containing 250 µl Reconstitute before use
Reagent D	HBSS, Wash solution (10x)	1 vial containing 100 ml Dilute before use
Reagent E	Fixative solution (contains paraformaldehyde)	1 vial containing 25 ml
Reagent F	Permeabilization buffer (10x)	1 vial containing 75 ml Dilute before use
Reagent G	Permeabilization reagent (100x) (Additive for permeabilization solution; contains Saponin)	1 vial containing 7.5 ml Dilute before use

Reagents for detection of intracellular antigens or cell surface antigens

Detection of intracellular antigens or cell surface antigens is performed using conjugated monoclonal antibodies. One or more vials of monoclonal antibodies directed against the following human leukocyte cell-surface antigens or intracellular antigens can be bought separately and used in combination with the CYTODETECT™ kit:

Table 2 - Monoclonal antibodies directed against human intracellular antigens, which can be used in combination with the CYTODETECT™ kit

Specificity	*Clone*	*Isotype*	*Tests*	*Product code (R-PE)*	*Product code (FITC)*	*Product code (APC)*
IFN-γ	45-14	IgG1	50 tests	IQP-160R	IQP-160F	IQP-160A
TNF-α	B-C7	IgG1	50 tests	IQP-163R		
TGF-ß	B-A15	IgG1	50 tests	IQP-169R		
IL-1ß	N7-48A	IgG2a	50 tests	IQP-167R		
IL-2	N7-48A	IgG2a	50 tests	IQP-161R		
IL-4	8F-12	IgG1	50 tests	IQP-162R		
IL-6	B-E8	IgG1	50 tests	IQP-164R		
IL-10	B-N10	IgG1	50 tests	IQP-165R	IQP-165F	
IL-12	B-P24	IgG1	50 tests	IQP-168R		
IL-13	B-B13	IgG1	50 tests	IQP-166R		

Table 3 - Examples of monoclonal antibodies directed against human cell surface antigens, which can be used in combination with the CYTODETECT™ kit. Other specificities of monoclonal antibodies are available, please contact us for more information

Specificity	*Clone*	*Isotype*	*Tests*	*Product code (FITC)*	*Product code (R-PE)*	*Product code (APC)*	*Product code (CyQ)*
CD3	UCHT1	IgG1	100 tests	IQP-519F	IQP-519R	IQP-519A	IQP-519C
CD4	Edu-2	IgG1	100 tests	IQP-535F	IQP-535R	IQP-535A	IQP-535C
CD8	MCD8	IgG1	100 tests	IQP-104F	IQP-104R	IQP-104A	IQP-104C

Table 4 - Isotype control antibodies for intracellular staining

Specificity	*Clone*	*Tests*	*Product code (FITC)*	*Product code (R-PE)*	*Product code (APC)*	*Product code (CyQ)*
Isotype control IgG1	MCG1	100 tests	IQP-191F	IQP-191R	IQP-191A	IQP-191C
Isotype control IgG2a	MCG2a	100 tests	IQP-192F	IQP-192R	IQP-192A	IQP-192C

Storage
Reagents stored according to stated storage instructions, are stable until the expiration date indicated on the label.

Warnings and Precautions ⚠

Caution
All reagents should be handled in accordance with good laboratory practices using appropriate precautions. In addition, handle all patient samples with appropriate precautions. Do not pipette by mouth and wear gloves during the procedure.

Reagents containing Sodium Azide may react with lead or copper plumbing to form explosive metal azides. On disposal, flush with large amounts of water to prevent azide build-up.

Reagent A contains Phorpol 12-myristate 13-acetate, a highly toxic and potentially carcinogenic reagent, which should be handled in accordance with good laboratory practices using appropriate precautions. Avoid skin or eye contact or inhalation. Wash thoroughly after handling.

Reagent B contains Ionomycin, a hazardous reagent, which should be handled in accordance with good laboratory practices using appropriate precautions. Avoid contact with eyes, skin or clothing. Wash thoroughly after handling.

Reagent C contains Monensin, a highly toxic allergenic reagent, which should be handled in accordance with good laboratory practices using appropriate precautions. Avoid skin or eye contact or inhalation. Wash thoroughly after handling.

Reagent E contains Formaldehyde, a highly toxic allergenic and potentially carcinogenic reagent, which should be handled in accordance with good laboratory practices using appropriate precautions. Avoid skin or eye contact.

Reagent G contains Saponin, an irritating reagent, which should be handled in accordance with good laboratory practices using appropriate precautions. Avoid contact with eyes, skin or clothing. Wash thoroughly after handling.

Processing of reagents supplied in the* CYTODETECT™ *kit

Cell Stimulation and Permeabilization Reagents

Reagent A: Stimulation reagent
Dissolve reagent A in 250 µl of DMSO. Aliquot and store at -20 °C (Stock solution). Before use in stimulation experiments, dilute one aliquot 1000x in RPMI-1640 medium. This working solution should be prepared freshly and is not stable at -20 °C.

Reagent B: Stimulation reagent
Dissolve reagent B in 75 µl of DMSO. Aliquot and store at -20 °C. Before use in stimulation experiments, dilute one aliquot 50x in RPMI-1640 medium. This working solution should be prepared freshly and is not stable at -20 °C.

Reagent C: Accumulation reagent
Dissolve reagent C in 250 µl of Ethanol (98%) and store at -20 °C. Before use dilute an aliquot 100x in RPMI-1640 medium. This working solution should be prepared freshly and is not stable at -20 °C.

Reagent D: HBSS, Wash solution
Stock solution 10x. Dilute the HBSS 10x with demineralized water and store at room temperature (2 to 8 °C).

Reagent E: Fixation solution
Ready to use. Store at 2 to 8 °C in the dark.

Reagent F: Permeabilization buffer
Stock solution 10x. Dilute the stock solution 10x with demineralized water. Store at 2 to 8 °C in the dark.

Reagent G: Permeabilization reagent
Stock solution 100x. Dilute the stock solution 100x in working solution of reagent F. The resulting solution is the **permeabilization solution**. Store at 2 to 8 °C in the dark.

Reagents not supplied in the CYTODETECT™ *kit*

- PBS (Phosphate Buffered Saline) w/o Ca^{2+}/Mg^{2+}
- RPMI-1640 fully supplemented for cell culture w/o Phenol Red
- Isopaque-Ficoll

Test Procedure

Note no. 1:
Application of reagents provided in the CYTODETECT™ kit has been developed for intracellular staining of cytokines combined with cell surface antigen detection. Reagents provided in the CYTODETECT™ kit should be used according to the *General Test Procedure*.

Alternatively, a procedure can be used which has been optimized especially for combined intra- and extracellular immunostaining of CD4-positive cells. Although the general procedure can be used as well for this detection, the alternative procedure results in a better discrimination of CD4-positive cells from CD4-negative cells, and thereby facilitates gate setting (see page 15). This modified procedure is designated *Alternative Test Procedure (A)* and is presented below as well.

Note no. 2:
Intracellular staining with anti-TGF-ß needs a slightly modified general procedure. This modified procedure is designated *TGF-ß Test Procedure* and is presented below as well.

Note no. 3:
Unless stated otherwise in the test procedures, reagents (including PBS) should be at room temperature when used.

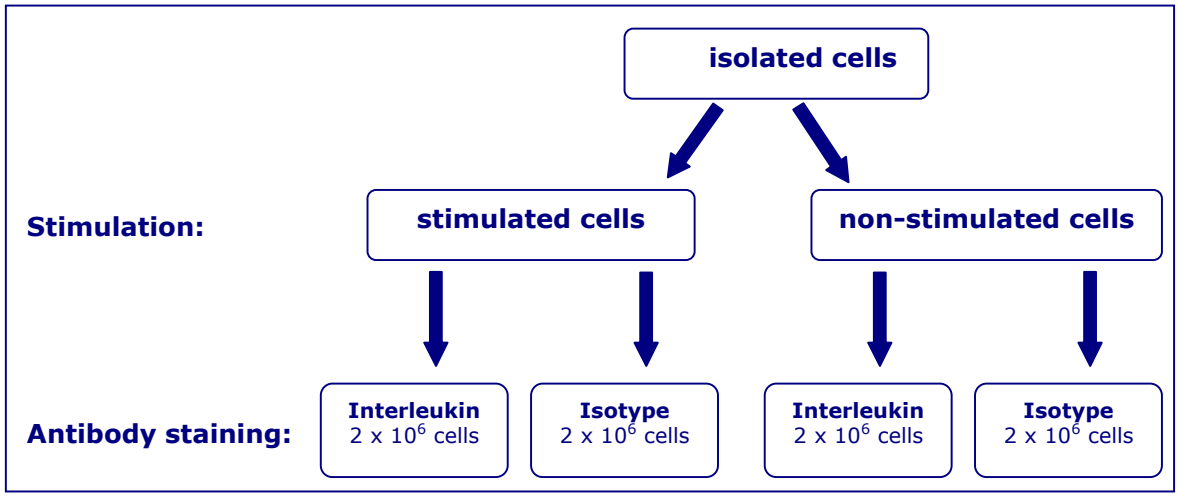
I. General Test Procedure

1. Isolation of blood lymphocytes

- Collect 5 - 10 ml venous blood into a heparinized tube or EDTA-treated tube, using aseptic venipuncture technique. Process the blood sample within 6 hours of sample collection. In patients with severe neutropenia (absolute neutrophil count less than 200/mm^3) at least 10 ml of blood may be required.
- Dilute blood sample 1:1 with PBS (Phosphate Buffered Saline).
- Add Ficoll-Paque (5 ml) in a centrifuge tube.
- Carefully layer 5 ml of the diluted blood sample on 5 ml of Ficoll-Paque.
- Centrifuge at 600 g for 20 minutes.

- Transfer the lymphocyte layer to a clean centrifuge tube.
- Add 5 ml of HBSS (Reagent D) and centrifuge at 400 g for 15 minutes.
- Remove the supernatant and add 10 ml of RPMI-1640.
- Centrifuge at 300 g for 10 minutes and remove the supernatant.
- Resuspend the cells in RPMI-1640 to a concentration of 2 x 10^6 cells/ml.
- 1 ml of cell suspension is sufficient for intracellular detection of 5 different cytokines, see scheme below to calculate the amount of samples needed for stimulated cells and unstimulated controls.

Scheme 1 - Calculation of samples needed stimulated cells and unstimulated controls



2. Stimulation of cells

- Put 1 ml of the cell suspension into a 24 wells culture plate and add 10 µl of Reagent A, 10 µl of Reagent B and 10 µl of Reagent C. Mix carefully with a pipette.
- Incubate for 5 hours at 37 °C, 5% CO_2.

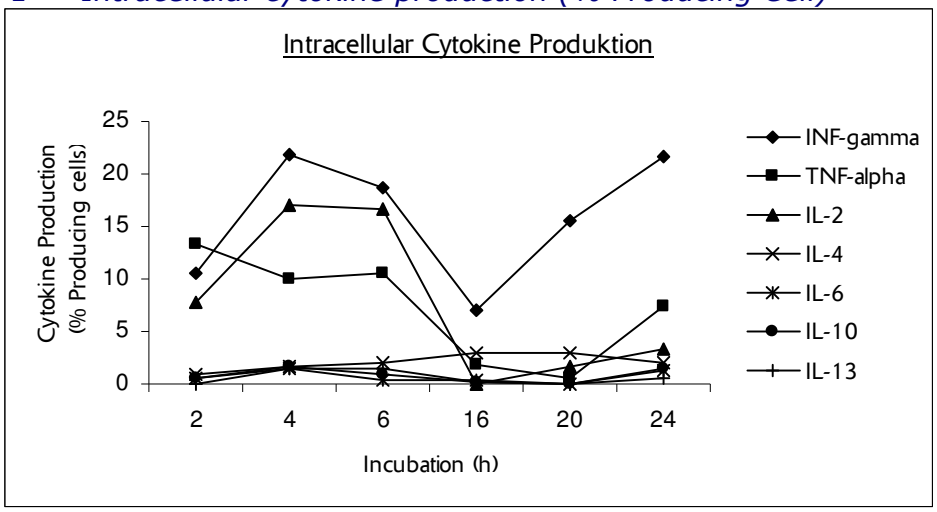
Note: For the CYTODETECT™ Kit we recommend a stimulation time of 5 hours for every interleukin to be tested. 5 hours of incubation for the CYTODETECT™ Kit are an average; optimum stimulation times are found in the table below. The optimum can differ because of the state of cells and kind of cells. Please take these figures as suggestions to find your own optimum.

Table 5 - Optimum of stimulation times concerning different interleukins

Interleukin	*Optimum of Stimulation (h)*
IFN-γ	4
TGF-ß	24*
TNF-α	5
IL-2	4-6
IL-4	4-6 (16-20)
IL-6	4-6
IL-10	4-6
IL-13	5

*mind the different protocol

Figure 1 - Intracellular cytokine production (% Producing Cell)



Note: IL-1ß and IL-12 need different stimulation protocols and vary enormously in expression per person.

- After stimulation transfer the cells to a centrifuge tube and add 5 ml of Reagent D.
- Centrifuge at 300 g for 10 minutes and remove the supernatant.

3. Fixation of cells

- Add 500 µl of cold (2 to 8 °C) Reagent E and incubate for 10 minutes at room temperature.
- Add 9 ml of Reagent D and centrifuge at 300 g for 10 minutes.
- Remove the supernatant and resuspend the cells in 1 ml of Reagent D.
- Cells can be stored overnight at 2 to 8 °C.

4. *Staining of cell surface antigens*

- After fixation of the cells, add 5 ml of Reagent D and centrifuge at 300 g for 10 minutes.
- Remove the supernatant and resuspend the cells in 500 µl of Reagent D. This cell suspension is sufficient for five separate experiments.
- Add 10 µl of monoclonal antibody-conjugate of choice against surface antigen to a 3 ml tube.
- Add 100 µl of cell suspension to the tube and mix well by vortexing, and incubate for 20 minutes at room temperature in the dark.

5. *Permeabilization of cells*

- Add 1.5 ml of permeabilization solution (diluted Reagent F combined with Reagent G).
- Centrifuge at 200 g for 5 minutes.
- Remove the supernatant and resuspend the cells in 500 µl of permeabilization solution; use 100 µl for staining of intracellular antigens.

6. *Staining of intracellular antigens*

- Add 10 µl of conjugated monoclonal antibody against intracellular antigens to the reagent tube, and mix well by vortexing.
- Incubate for 20 minutes at 2 to 8 °C in the dark.
- Add 1.5 ml of permeabilization solution and centrifuge at 200 g for 5 minutes.
- Remove the supernatant and resuspend the cells in 100 - 300 µl of HBSS (Reagent D).

7. *Analysis by flow cytometry*

- Analyze the cells by flow cytometry.
- Use appropriate controls for immunostaining and data processing.

II. Alternative Test Procedure

A1. Isolation of blood lymphocytes

- Collect 5 - 10 ml venous blood into a heparinized tube or EDTA-treated tube, using aseptic venipuncture technique. Process the blood sample within 6 hours of sample collection. In patients with severe neutropenia (absolute neutrophil count less than 200/mm^3) at least 10 ml of blood may be required.
- Dilute blood sample 1:1 with PBS (Phosphate Buffered Saline).
- Add Ficoll-Paque (5 ml) in a centrifuge tube.
- Carefully layer 5 ml of the diluted blood sample on 5 ml of Ficoll-Paque.
- Centrifuge at 600 g for 20 minutes.
- Transfer the lymphocyte layer to a clean centrifuge tube
- Add 5 ml of HBSS (Reagent D) and centrifuge at 400 g for 15 minutes.
- Remove the supernatant and add 10 ml of RPMI-1640.
- Centrifuge at 300 g for 10 minutes and remove the supernatant.
- Resuspend the cells in RPMI-1640 to a concentration of $2x10^6$ cells/ml. 1 ml of cell suspension is sufficient for intracellular detection of 5 different cytokines, see scheme on page 9 to calculate the amount of samples needed for stimulated cells and unstimulated controls.
- Transfer 1 ml of the cell suspension per centrifuge tube.

A2. Staining of CD4

- Add 100 µl of monoclonal CD4 FITC antibody-conjugate before stimulation and incubate for 20 minutes at room temperature in the dark.
- Add 10 ml of RPMI-1640 per 2 x 10^6 cells and centrifuge at 300 g for 10 minutes.
- Remove the supernatant and resuspend the cells in 1 ml of RPMI-1640.

A3. Stimulation of cells

- Put 1 ml of the cell suspension into a 24 wells culture plate and add 10 µl of Reagent A, 10 µl of Reagent B and 10 µl of Reagent C. Mix carefully with a pipette.
- Incubate for 5 hours at 37 °C, 5% CO_2. For optimal incubation time see also page 10.
- 1 ml of cell suspension is sufficient for intracellular detection of 5 different cytokines.
- After stimulation transfer the cells to a centrifuge tube and add 5 ml of Reagent D.
- Centrifuge at 300 g for 10 minutes and remove the supernatant.

A4. Fixation of cells

- Add 500 µl of cold (2 to 8 °C) Reagent E and incubate for 10 minutes at room temperature.
- Add 9 ml of Reagent D and centrifuge at 300 g for 10 minutes.
- Remove the supernatant and resuspend the cells in 1 ml of Reagent D.
- Cells can be stored overnight at 2 to 8 °C.

A5. Permeabilization of cells

- After fixation the cells, add 5 ml of Reagent D and centrifuge at 300 g for 10 minutes.
- Remove the supernatant and resuspend the cells in 1.5 ml of permeabilization solution.
- Centrifuge at 200 g for 5 minutes.
- Remove the supernatant and resuspend the cells in 500 µl of permeabilization solution, use 100 µl for staining of intracellular antigens.

A6. Staining of intracellular antigens

- Add 10 µl of conjugated monoclonal antibody against intracellular antigens (anti-IFN-γ PE, anti-TGF-ß PE, etc.) to the reagent tube.
- Mix well by vortexing and incubate for 20 minutes at 2 to 8 °C in the dark.
- Add 1.5 ml of permeabilization solution and centrifuge at 200 g for 5 minutes.
- Remove the supernatant and resuspend the cells in 100 - 300 µl of HBSS (Reagent D).

A7. Analysis by flow cytometry

- Analyze the cells by flow cytometry.
- Use appropriate controls for immunostaining and data processing.

III. TGF-ß Test Procedure

T1. Isolation of blood lymphocytes

- Collect 5 - 10 ml venous blood into a heparinized tube or EDTA-treated tube, using aseptic venipuncture technique. Process the blood sample within 6 hours of sample collection. In patients with severe neutropenia (absolute neutrophil count less than 200/mm^3) at least 10 ml of blood may be required.
- Dilute blood sample 1:1 with PBS (Phosphate Buffered Saline).
- Add Ficoll-Paque (5 ml) in a centrifuge tube.
- Carefully layer 5 ml of the diluted blood sample on 5 ml of Ficoll-Paque.
- Centrifuge at 600 g for 20 minutes.
- Transfer the lymphocyte layer to a clean centrifuge tube.
- Add 5 ml of HBSS and centrifuge at 400 g for 15 minutes.
- Remove the supernatant and add 10 ml of RPMI-1640.
- Centrifuge at 300 g for 10 minutes and remove the supernatant.
- Resuspend the cells in RPMI-1640 to a concentration of 2 x 10^6 cells/ml.
- 1 ml of cell suspension is sufficient for intracellular detection of 5 different cytokines, see scheme on page 9 to calculate the amount of samples needed for stimulated cells and unstimulated controls.

T2. Stimulation of cells

- Put 1 ml of the cell suspension into a 24 wells culture plate and add 20 µl of Reagent A, 10 µl of Reagent B and 10 µl of Reagent C. Mix carefully with a pipette.
- Incubate for 24 hours at 37 °C, 5% CO_2.
- After stimulation transfer the cells to a centrifuge tube and add 5 ml of Reagent D.
- Centrifuge at 300 g for 10 minutes and remove the supernatant.

T3. Fixation of cells

- Add 500 µl of cold (2 to 8 °C) Reagent E and incubate for 10 minutes at room temperature.
- Add 9 ml of Reagent D and centrifuge at 300 g for 10 minutes.
- Remove the supernatant and resuspend the cells in 1 ml of Reagent D.
- Cells can be stored overnight at 2 to 8 °C.

T4. Staining of cell surface antigens

- After fixation of the cells, add 5 ml of Reagent D and centrifuge at 300 g for 10 minutes.
- Remove the supernatant and resuspend the cells in 500 µl of Reagent D. This cell suspension is sufficient for five separate experiments.
- Add 10 µl of monoclonal antibody-conjugate of choice against surface antigen to a 3 ml tube.
- Add 100 µl of cell suspension to the tube and mix well by vortexing, and incubate for 20 minutes at room temperature in the dark.

T5. Permeabilization of cells
- Add 1.5 ml of permeabilization solution.
- Centrifuge at 200 g for 5 minutes.
- Remove the supernatant and resuspend the cells in 500 µl of permeabilization solution, use 100 µl for staining of intracellular antigens.

T6. Staining of intracellular antigens
- Add 10 µl of R-PE conjugated monoclonal antibody against intracellular antigens (anti-TGF-ß PE) to the reagent tube, and mix well by vortexing.
- Incubate for 20 minutes at 2 to 8 °C in the dark.
- Add 1.5 ml of permeabilization solution and centrifuge at 1000 rpm for 5 minutes.
- Remove the supernatant and resuspend the cells in 100 - 300 µl of HBSS.

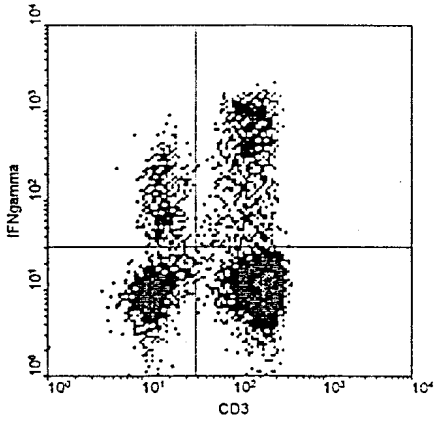
T7. Analysis by flow cytometry
- Analyze the cells by flow cytometry.
- Use appropriate controls for immunostaining and data processing.

Results

Typical results are shown below of analysis by flow cytometry of intracellular antigens and cell membrane antigens using the CYTODETECT™ kit. These results are presented as cytograms showing dual staining of lymphocytes obtained from normal human volunteers. Blood samples were stimulated according to protocol for 5 hours, and labeled for detection of expression of cell surface antigens or intracellular cytokines. Subsequently, samples were analyzed by flow cytometry.

Cells obtained from a human volunteer was stimulated with PMA in the presence of ionomycin and monensin, were analyzed for identification of subpopulations of T cells. Detection of IFN-γ (IQP-160R) expressing CD3-positive T cells (IQP-519F) is shown in figure 2.

Figure 2



Results of detection of CD4-positive T cells (IQP-535F) according to the General Test Procedure (figure 3a) or detection before stimulation of cells according to the Alternative Test Procedure (figure 3b). Analysis of CD4 was combined with detection of IFN-γ (IQP-160R). Although both procedures of detection can be followed, a more pronounced discrimination between IFN-γ producing CD4-positive and CD4-negative cells can be found using incubation with CD4 FITC before stimulation (Alternative Test Procedure) allowing facilitated gate setting.

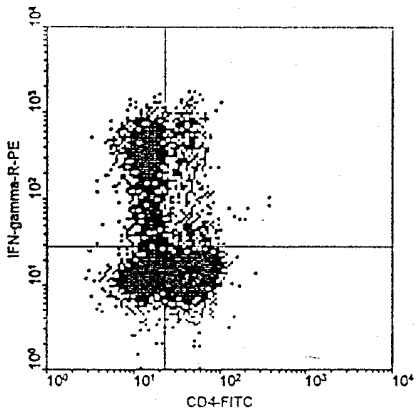


Figure 3a

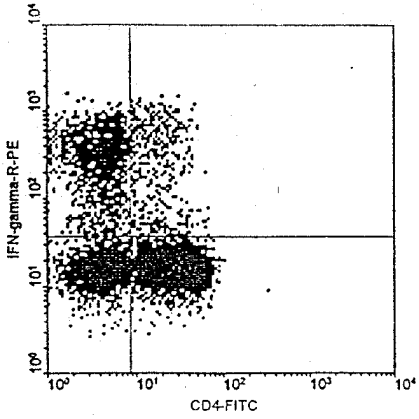
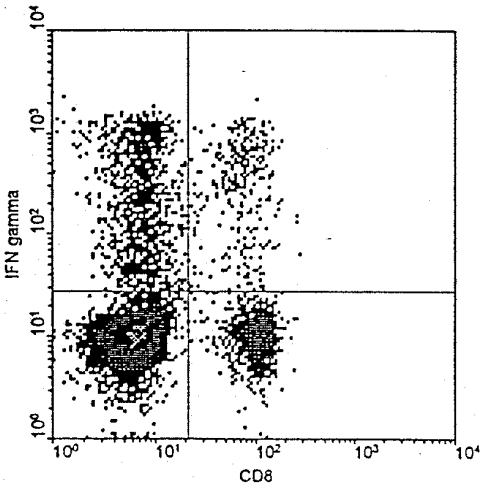


Figure 3b

Figure 4 illustrates blood cells stimulated with PMA in the presence of ionomycin and monensin. They were analyzed for identification of CD8 sub-populations of T cells (IQP-104F). Detection of IFN-γ (IQP-160R) expressing CD8-positive cells is shown.

Figure 4



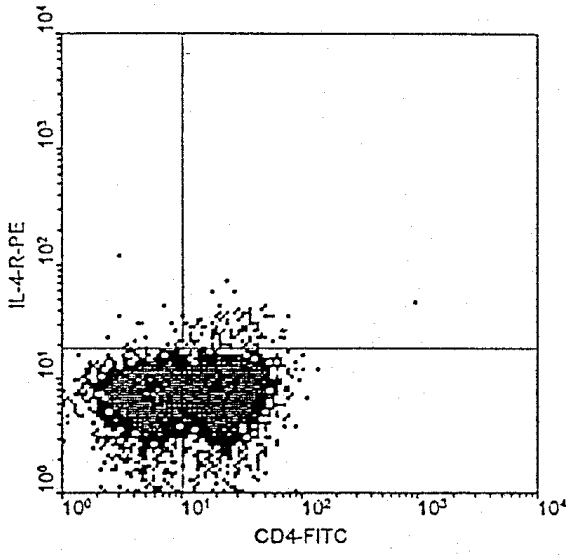
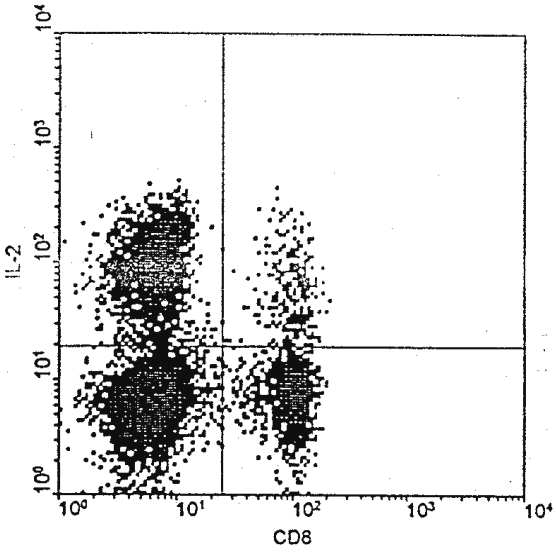


Figure 5 shows stimulated cells with PMA in the presence of ionomycin and monensin. Analysis was done for identification of CD4 sub-populations of T cells (IQP-535F). In this picture detection of IL-4 (IQP-162R) expressing CD4-positive cells using the alternative test procedure is shown.

Figure 5

Lymphocytes, obtained from a healthy volunteer stimulated with PMA in the presence of ionomycin and monensin, were analyzed for identification of CD8 (IQP-104F) sub-population of T cells. Detection of IL-2 (IQP-161R) expressing CD8-positive cells is shown in figure 6.

Figure 6



Cells stimulated with PMA in the presence of ionomycin and monensin were analyzed for identification of CD4 sub-populations of T cells (IQP-535F). Detection of TNF-α (IQP-163R) expressing CD4-positive cells using the Alternative Test Procedure is shown in figure 7.

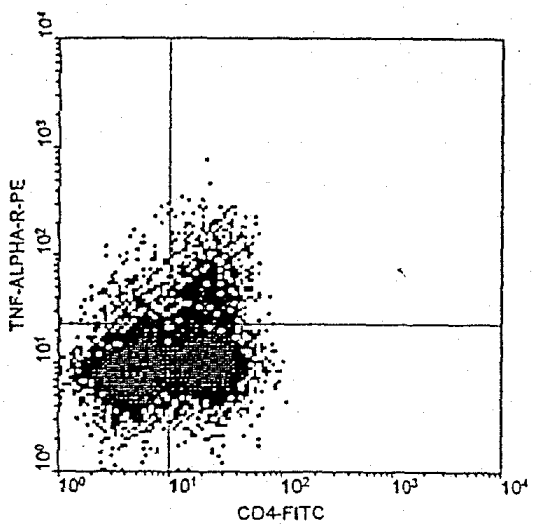


Figure 7

Stimulated human lymphocytes cells were analyzed for production of TGF-ß using the TGF-ß test procedure. The results are shown in figure 8.

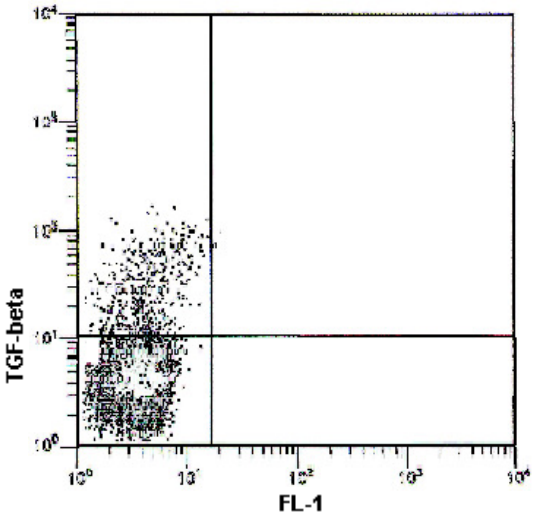


Figure 8

Stimulation of cells with PMA in the presence of ionomycin and monensin were analyzed using isotype control antibodies (IQP-191R and IQP-192R). Results are shown in figures 9a, and 9b respectively.

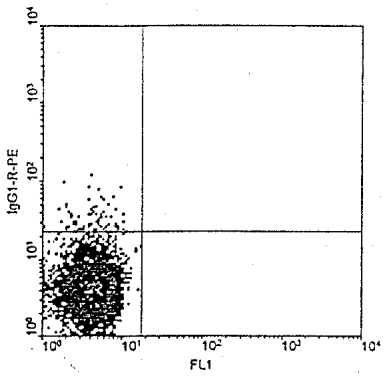


Figure 9a

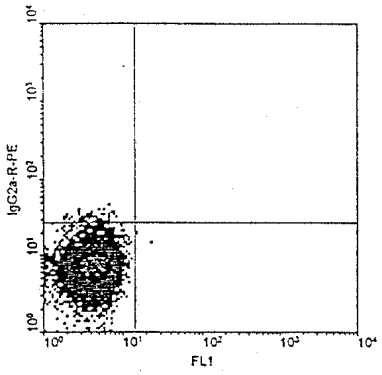


Figure 9b

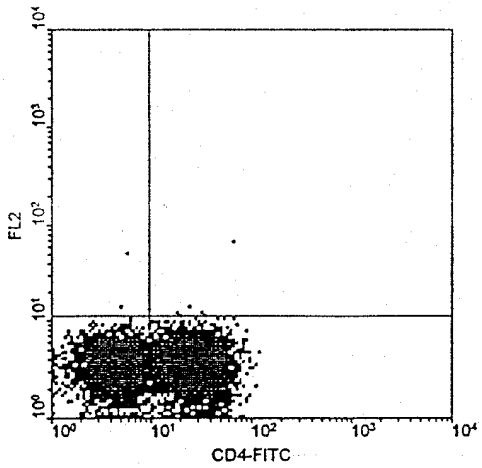


Figure 10

An additional valuable control is analysis of (PMA-) stimulated cells in the presence of ionomycin and monensin, in combination with cell surface staining, without detection of intracellular antigens after processing the sample according to protocol. Analysis of CD4-positive cells (IQP-535F) using the Alternative Test Procedure is illustrated in figure 10.

Expression of intracellular cytokines by immune cells obtained from normal human volunteers or patient samples, may be too low for analysis by flow cytometry. Therefore protein levels of intracellular cytokines can be increased by short time stimulation (e.g. five hours) and using PMA as a general stimulating reagent. A comparison of non-stimulated and stimulated CD3-positive T cells has been made using the CYTODETECT™ kit, to show this difference in expression level as measured by a relative difference in fluorescence intensity of PE signal. Analysis of normal human CD3-positive cells (IQP-519F) without (figure 11a), or with stimulation using PMA (figure 11b) for intracellular expression of IFN-γ (IQP-160R).

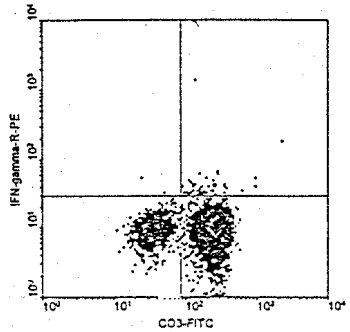


Figure 11a

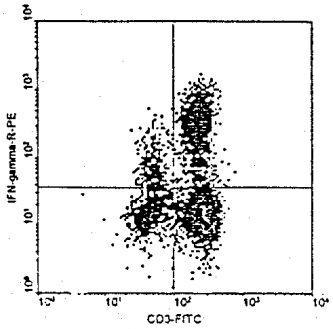


Figure 11b

Expected values

A limited number of blood samples obtained from healthy volunteers were processed using the CYTODETECT™ kit, according to protocol. Stimulation of blood lymphocytes by PMA was performed for five hours, and samples were further treated as indicated in the text. Flow cytometric analysis was performed of detection of four intracellular antigens, i.e. IL-2 (IQP-161R), IL-4 (IQP-162R), IFN-γ (IQP-160R), and TNF-α (IQP-163R) using PE-conjugated antibodies. In table 6 results are shown of a study of a limited number (n=8) of blood samples processed using the CYTODETECT™ kit.

Table 6 – values achieved using CYTODETECT™ kit

Cytokine	*Product code*	*Clone*	*Positive cells (mean %, n=8)*	*Positive cells (range %)*
IL-2	IQP-161R	N7 48A	35.5%	25.0% - 41.5%
IL-4	IQP-162R	8F12	1.6%	0.7% - 2.2%
IFN-γ	IQP-160R	45-14	24.9%	18.0% - 31.7%
TNF-α	IQP-163R	B-C7	23.3%	13.6% - 35.9%

The percentage of positive cells obtained from different donors found for a specific cytokine, showed a broad range as illustrated in table 6. This variation is not uncommon, based on immunological differences and immuno competence which differs from person to person and may show (small) day to day variations.

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Explanation of used symbols

	Consult instructions for use
REF	Catalogue number
	Sufficient for
IVD	In Vitro Diagnostic medical device
	Caution, consult accompanying document
	Keep away from (sun)light
	Biological risks
	Temperature limitation (°C)
RUO	For Research Use Only
LOT	Batch code
	Use by yyyy-mm-dd
	Manufacturer
EC REP	Authorized Representative in the European Community
CE	Conformité Européenne (European Conformity)

		Label - tandem	**Ex -max (nm)**	**Em -max (nm)**
P	PURE	purified material	-	-
F	FITC	FITC	488	519
R	R-PE	PE	488, 532	578
C	CyQ	PE-Cy5.18	488, 532	667
A	APC		595, 633, 635, 647	660
PC	PerCP		488, 532	678
PCC	PerCP-Cy5.5		488, 532	695

Warranty

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