INSTITUT PASTEUR

The 1st PSU-IP International
Teaching Platform on Cytokines

Jointly organized by
Prince of Songkla University and Institut Pasteur

September 9 – 14, 2002
At The Department of Biomedical Sciences
Faculty of Medicine, Prince of Songkla University,
Hat Yai, Songkhla, Thailand

Lab Course Handout
(September 12-14, 2002)

Eliane Bourreau, Institut Pasteur de la Guyane Française
Adrien Six, Université Pierre et Marie Curie et Institut Pasteur

Cytokine production determined by ELISA and RT-PCR methods

The purpose of this practical study is to determine the cytokine levels by two different methods, the ELISA and the RT-PCR methods. The objective of this 3-day lab course session is to demonstrate the ease of use and the power of these two techniques which can be applied in both basic and clinical research.

In this handout, you will find a detailed protocol for the experimental steps that will be carried out during the next days, as well as some reference information on the techniques, protocols, reagents and equipments.

I. Cytokine secretion by T-cell populations

Peripheral blood mononuclear cells (PBMC) were obtained from venous blood using the method of density-gradient centrifugation on Ficoll-Hypaque solution (d=1.077, Sigma Ref.1077-1) (Figure 1). PBMC were washed thrice and resuspended in RPMI medium (Sigma, Ref. R6504) supplemented with 10% human heat-inactivated serum from AB positive blood donors. Cell cultures for cytokine production (10^6 cells/ml of culture medium) were incubated in RPMI with phytohemagglutinin (PHA-L, Sigma, Ref. L2769) on flat-bottom 24-well plates. Control wells received RPMI only.

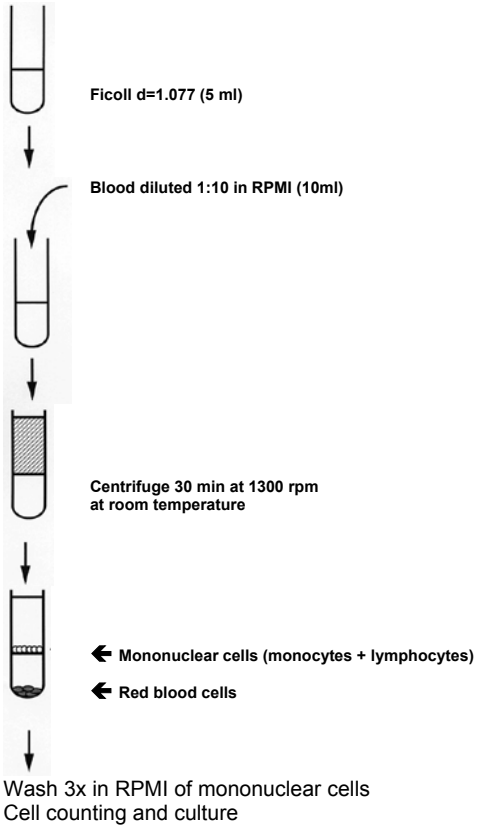


Figure 1 : Peripheral blood mononuclear cell isolation

II. Reverse Transcription-Polymerase Chain Reaction

Analysis of messenger RNA (mRNA) levels using cDNA obtained by reverse transcription coupled with the polymerase chain reaction (PCR) provides a powerful tool for studying cytokine regulation in cellular immunology. Detection of mRNA can now be performed on as few as one cell ("single-cell PCR technique"). The schematic of PCR is shown on Figure 2.

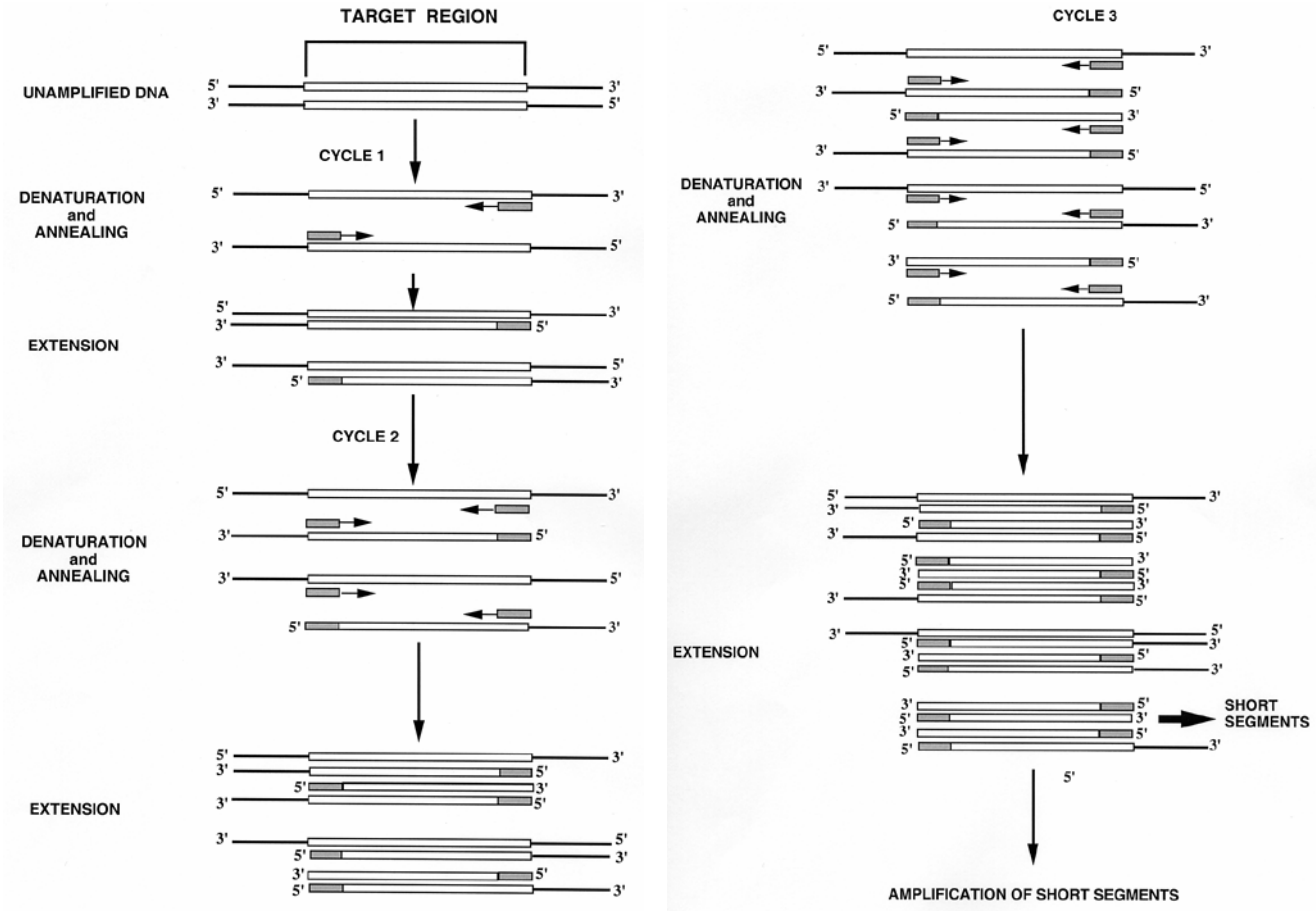


Figure 2: Schematic representation of PCR

To standardize this assay, the PCR step is controlled by the amplification in parallel of housekeeping genes. Due to the exponential nature of amplification, standard PCR protocol is not quantitative. In order to be (semi-)quantitative, we use the simultaneous amplification of competitive genes of known concentration within the same PCR reaction. These competitors can be amplified with the same primers but differ from the target DNA by length. The amplified PCR products from target and competitor DNA can thus be differentiated following their electrophoresis through agarose gel (see below, page 6).

We can assume here that the PCR yield is identical for both target and competitor (this is an approximation) since they do not differ extensively and are amplified with the same primers. The ratio of target amplified product to competitor product is thus conserved after

amplification. Therefore, knowing the concentration of the competitor, we can measure the initial amount of target DNA by measuring the ratio of target to competitor after amplification. A representation of the experimental procedure is shown on Figure 3.

1. RNA extraction

The method of RNA extraction is based on the use of guanidinium thiocyanate (GCN) (a strong inhibitor of RNAses) and acid phenol (Chomczynski and Sacchi, Anal. Biochem.162:156-1987).

Note that RNA is very sensitive to degradation by RNAses. Therefore all reagents and material should be RNAse-free certified, kept and handled carefully wearing gloves throughout experimentation. Water should be treated with DEPC (diethylpyrocarbonate) and sterilized, which degrades RNAses.

Buffers and solutions:

GCN (for 50ml): Mix 23.6 g guanidinium thiocyanate, 1.6 ml 0.75M sodium citrate, 2.45 ml 10% Sarkosyl and 27.9 ml H_2O. Heat to 65°c to dissolve. *Before use*, add 0.36 ml of β-mercaptoethanol.

Sodium acetate: 2M sodium acetate acidified with acetic acid to pH 4.5.

Note that GCN, Phenol and Chloroform are toxic and shall be manipulated carefully wearing gloves; wastes must be handled carefully according to local regulations.

Protocol:

- Dissolve the cell pellet in 500 µl of GCN + β-mercaptoethanol.
- Add 50 µl of 2M sodium acetate. Mix well.
- Add 500 µl of acid phenol. Mix well.
- Add 200 µl of chloroform. Vortex vigorously for about 10s.
- Leave on ice for 15 min.
- Centrifuge for 20 min at 10,000 g at 4 °C.
- Transfer the aqueous phase (top layer) to a clean Eppendorf tube.
- Add 40 µl of 7.5M ammonium acetate and 1 ml of ethanol.
- Mix well and leave at least 30 min at –20°C for nucleic acid precipitation.
- Spin out precipitate at 10,000 g for 10 min.
- Discard supernatant.
- Dissolve pellet in 500 µl of GCN.
- Add 40 µl of 7.5M ammonium acetate and 1 ml of ethanol.

- Leave at –20°C for nucleic acid precipitation (2h).
- Spin out precipitate at 10,000 g for 10 min.
- Discard supernatant.
- Wash pellet with 70% ethanol.
- Dry and dissolve pellet in a small volume of RNAse-free H_2O (50 μl per $20x10^6$ cells).
- RNA is ready. Store at –20°C before cDNA synthesis.

2. cDNA Preparation

First-strand cDNA synthesis is performed on total RNA by using a first-strand cDNA synthesis kit (Pharmacia Biotech – Ref. 27-9261-01), as described by the manufacturer (see Annex A). Two oligonucleotides are available to prime cDNA synthesis by the reverse transcriptase enzyme:

- oligo-dT, which hybridizes to 3'poly-A tails found on the vast majority of eukaryotic mRNA
- pd(N)6, a mix of random hexamer primers which can hybridize "anywhere" along the RNA molecule causing the initiation of reverse transcription

In this experiment, we use the pd(N)6 random primer. First-strand cDNA will be used directly as a template for PCR (in the presence of competitor).

Protocol:

- Place RNA sample in a 1,5 ml Eppendorf tube and bring volume to 20 μl RNAse-free H_2O.
- Heat RNA to 65°C for 10 min, then chill on ice.
- Mix the following components for first-strand reaction:

5 μl	Bulk RT mix
1 μl	pd(N)6 primer
1 μl	DTT
8 μl	RNA

- Incubate at 37°C for 1 hour.
- cDNA is now ready for PCR amplification.

3. Semi-quantitative Reverse Transcription-PCR

Here we use the competitor plasmid constructs:

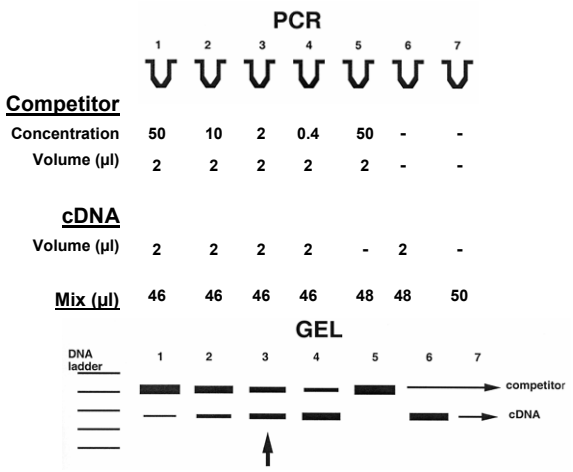
- pQA-1 for IFN-γ
- pQB-3 for IL-10 and β-actin (a housekeeping gene).

The competitor templates contain the identical primer binding sites as the wild-type cDNA.

For each PCR reaction, mix the following components before adding template (PCR mix):

5 µl	Buffer (10x)
3 µl	$MgCl_2$ (25 mM)
2 µl	dNTP (20 pmol/µl)
1 µl	Primer-1 (20 pmol/µl)
1 µl	Primer-2 (20 pmol/µl)
1 U	Taq polymerase (5 U/µl)
33.5 µl	DEPC H_2O

Prepare 0.2 ml PCR tubes by adding 2 µl cDNA and/or competitor to PCR mix according to what is shown on Figure 3:



1-4: semi-quantitative RT-PCR in the presence of decreasing amounts of competitor
5: competitor alone
6: cDNA alone
7: negative control

Figure 3: Experimental procedure for competitive PCR

PCR tubes are incubated on a 96-well thermocycler with the following cycling conditions:

1. Denaturation at 94°C for 5 min
2. 30 cycles of denaturation at 94°C for 30s
   hybridization at 50°C for 30s
   extension at 72°C for 30s
3. Final extension at 72°C for 5 min

Amplified PCR products are then separated by electrophoresis on agarose gel and stained with ethidium bromide:

- Pour a 1.5 % agarose gel in 1x Tris/Borate/EDTA buffer (TBE) added with 1 µg/ml of ethidium bromide. *Note that ethidium bromide is toxic (mutagen) and shall be manipulated carefully wearing gloves; ethidium bromide waste must be handled carefully according to local regulations.*
- After cooling down agarose, set the gel into the electrophoresis tank and fill up with TBE 1x migration buffer.

- Load samples (5 µl of PCR products + 2 µl of loading solution (20 ml glycerol, 2 ml 0.5 M EDTA pH 8, 125 mg bromophenol blue, H_2O qsp 50 ml). Do not forget to load "100 bp ladder" DNA markers (100 ng per mm of well width).
- Run electrophoresis at 80 V ; note that bromophenol blue migrates approximately as a 300 pb fragment.
- Take a photograph of the gel.

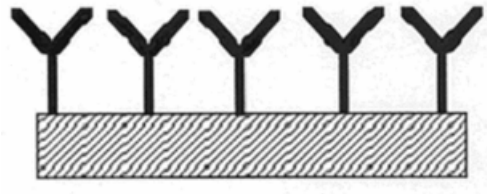
III. ELISA method applied to PBMC culture supernatants

The IFN-γ and IL-10 cytokine level is measured with the OptEIA Human IFN-γ and IL-10 kits (Pharmingen, Ref. 555142 and 555157), as described by the manufacturer (see Annex E and Annex F).

- Add 100 µl of 1:250 diluted capture antibody (Ab) to each well (dilution in 0.1 M carbonate, pH 9.5 coating buffer). Incubate overnight at 4°C.
- Wash x3 with PBS-0.05% Tween.
- Block wells with 200 µl of PBS-10% FCS. Incubate for 1 hour at room temperature (RT).
- Wash x3 with PBS-0.05% Tween.
- Add 100 µl of samples or standards in each well (see standard preparation procedure in the manufacturer technical sheet). Incubate for 2 hours at RT.
- Wash x5 with PBS-0.05% Tween.
- Add 100 µl/well of detection antibody (biotinylated anti-human cytokine mAb), diluted in PBS-10% FCS (1:250). Incubate for 1 hour at RT.
- Wash x5 with PBS-0.05% Tween.
- Add 100 µl/well of enzyme reagent (avidin-horseradish peroxidase conjugate), diluted in PBS-10% FCS (1:250). Incubate for 1 hour at RT.
- Wash x5 with PBS-0.05% Tween.
- Add 100 µl/well of substrate solution (3,3',5,5'-Tetramethylbenzidine + H_2O_2, TMB solution, Sigma, Ref. T8665). Incubate 30 min at RT.
- Stop reaction with 50 ml/well of H_2SO_4 0.1 M
- Estimate O.D. plates at 450 nm with a spectrophotometer for 96-well plates

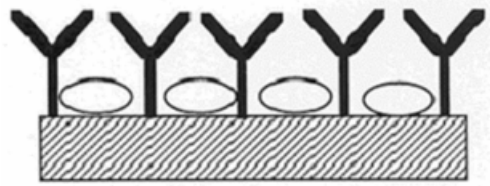
These steps are visualized on Figure 4, Figure 5 and Figure 6:

STEP 1: Coating of capture antibody (100µl/ well) in coating buffer (0.1M carbonate pH 9.5) incubation 4°C O.N.



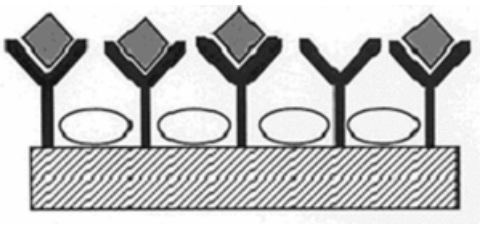
STEP 2: Aspirate and wash x3 (PBS-0.05% Tween)

STEP 3: Block plates with PBS+10% FSC 1h RT



STEP 4: Aspirate and wash x3

STEP 5: Add samples or standards (100µl/well) 2h RT



STEP 6: Aspirate and wash x5

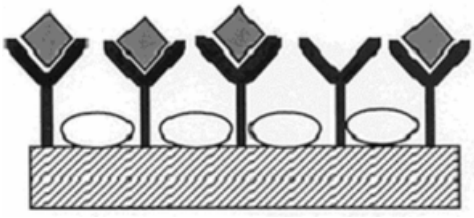
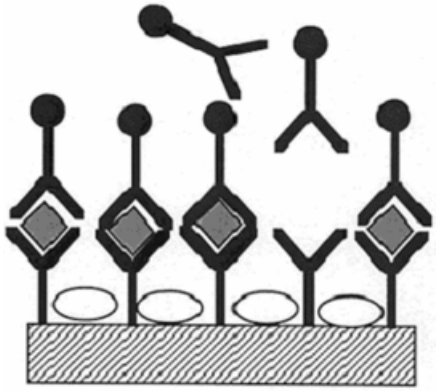
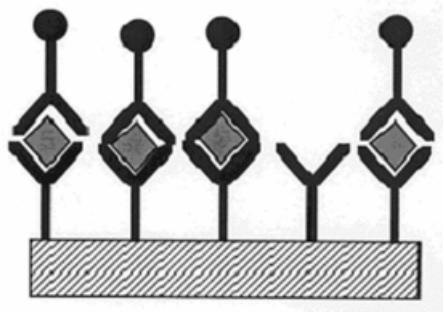


Figure 4: ELISA procedure (1)
(O.N., overnight; RT, room temperature)

STEP 7: Add detection antibody (100µl/well)
biotinylated anti-human monoclonal Ab
1h RT



STEP 8: Aspirate and wash x5



STEP 9: Add enzyme reagent (100µl/well)
avidin-horseradish peroxidase conjugate
1h RT

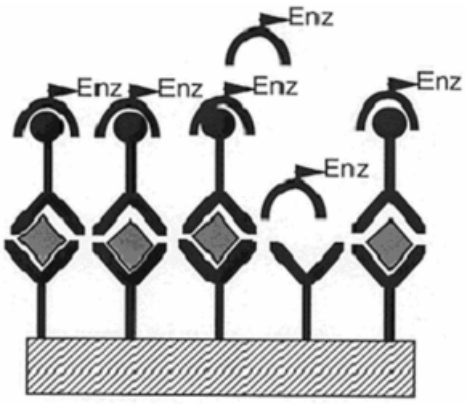
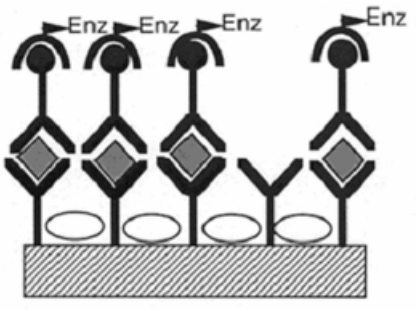
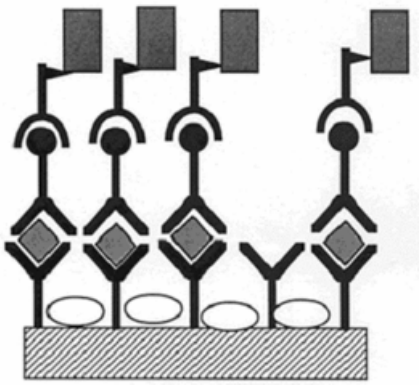


Figure 5: ELISA procedure (2)

STEP 10: Aspirate and wash x5



STEP 11: Add substrate solution (100µl/well)
Tetramethylbenzidinze (TMB)+H202
30min RT



STEP 12: add stop solution (50µl/well)
and read at 450 nm

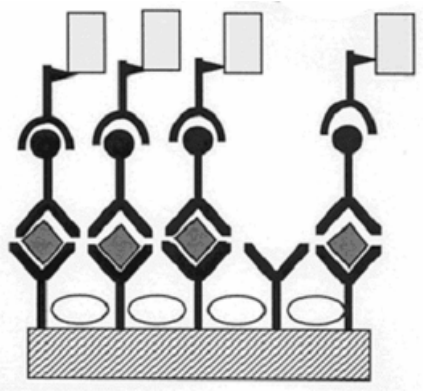


Figure 6: ELISA procedure (3)

IV. Analysis of Results and Discussion

V. Conclusion

More and more research findings demonstrate the importance of studying cytokine up- and down-regulation in immunocompetent leucocytes.

Studies in humans clearly show that Th1 dominated responses are effective in protection against several intracellular microorganisms and usually drive their complete clearance or at least contribute to reduce their load.

The important physiologic function of Th2 cells may be the regulation or suppression of some immune responses (*e.g.* regulation of inflammatory processes in non-lymphoid tissues, B-cell coactivation in secondary lymphoid organs).

Th1 and Th2 predominance in various physiological and pathological conditions

Th1	Th0	Th2
Lyme arthritis		
Hepatitis C virus-induced chronic hepatitis		
Multiple sclerosis		
Recurrent abortion		
	Hepatitis B virus chronic hepatitis	
	Progression to AIDS in HIV infection	
	Systemic lupus erythematous	
		Successful pregnancy
		Allergic diseases
		Helminth infections

Adapted from d'Elios and Del Prete (1998) *Transplant. Proceed.*, 30 :2373

VI. Tentative schedule

Date	***Activity***
Sept. 9	- *PBMC isolation on Ficoll* - *Start of cell culture with PHA by Eliane*
Sept. 12, Morning	- Introduction - PBMC isolation on Ficoll and counting - Start Cell culture with PHA (EB)
Sept. 12, Afternoon	- Collection of Day 3 PHA cell culture and supernatants - RNA prep (O.N. precipitation at -20°C) - Coating of Elisa plates
Sept. 13, Morning	- Finish RNA extraction - cDNA synthesis - Start RT-PCR - Saturation of ELISA plate
Sept. 13, Afternoon	- Finish ELISA and read plates on spectrophotometer - Start analysis of results - Agarose gel preparation - Exercise session on cytokines (if time allows)
Sept. 14, Morning	- Analyze PCR products on agarose gels - Analyze PCR results - Analyze ELISA results - Exercise session on cytokines (if time allows)
Sept. 14, Afternoon	- Short presentation of results by each group - Discussion of results and conclusion

VII. Annexes

Annex A: First-Strand cDNA Synthesis Kit Instructions
Pharmacia Biotech – Ref. 27-9261-01

Annex B: *Taq* Polymerase data sheet
Roche Diagnotics – Ref. 1 146 173

Annex C: OptEIA™ Reagent Set A Technical Data Sheet
BD PharMingen – Ref. 550536

Annex D: OptEIA™ Reagent Set B Technical Data Sheet
BD PharMingen – Ref. 550534

Annex E: OptEIA™ Human IFN-γ Set
BD PharMingen – Ref. 555142

Annex F: OptEIA™ Human IL-10 Set
BD PharMingen – Ref. 555157