The 1st KKU International Teaching Platform "Effect of Natural Products on Cancer Cell and Cytokine Production"

Jointly organized by

Khon Kaen University, Université Pierre et Marie Curie (Paris 6) and Institut Pasteur

July 17 – 22, 2006

At the Department of Microbiology

Faculty of Medicine, Khon Kaen University,

Khon Kaen, Thailand

Lab Course Handout

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Effect of Natural Products on Cancer Cell and Cytokine Production

Introduction

The purpose of this 6-day training is to study the effect of a natural substance on the production of cytokines and growth of cancer cells. We have chosen to study the action of different products, known or unknown, on cell proliferation and induction of apoptosis. For this purpose, the preceding lectures give a theoretical and technological background on the topics of cytokine production and apoptosis. After going through this background, as well as the different available biomaterial, reagents and experimental protocols, you will be asked to implement a true scientific process: problem definition, choice of strategy, experimental design and implementation, data collection and analysis, interpretation and discussion.

Due to time constraints, it is suggested that the participants arrange in groups of six people and focus their attention on a limited number of tests as indicated by your instructors. It is recommended that each group consults with the other groups in order to address different and complementary questions. At the end of the week, the methodological approach and results of the different groups will be presented for group discussion. This should allow all participants to familiarize with standard and up-to-date techniques and procedures commonly used in immunology (flow cytometry & quantitative PCR), as well as allow them to make a better and critical use of the theoretical concepts and techniques learnt during training or from the literature.

This handout gives, in a first part, a presentation of the subject which should lead you to hypotheses which you will try to answer through experiments. In the second part, you will find a series of experimental protocols describing the techniques which you can implement as well as the list of the available biologic material and reagents. It will be up to you to choose among these protocols, by adapting them if necessary.

Consequently, you will take care to properly document your strategy, experimental design and implementation. This will allow you to report to other groups at the end of the week so that critical comparison of selected strategies and techniques can be made. The objective of this reporting is to achieve capacity to approach the problem, to set hypotheses, to implement experiments to test these hypotheses, to analyze and to discuss the obtained results.

Introductory background

Each participant shall refer to the lecture handouts on the Immunoregulatory effect of natural products, and apoptosis. A number of molecules derived from natural products, in particular those found in herbal and traditional medicine, exhibit immunomodulatory properties: they can either activate or inhibit proliferation of cancer cells or induce apoptosis.

We have selected different compounds as proliferation or apoptosis inducers and you will have the opportunity to test your own natural compounds. Based on the available biomaterial and reagents (Annexe B), as well as technique protocols presented in Annexe C, you will design relevant experiments in order to characterize the properties of these compounds. A tentative schedule for the following days is provided in Annexe A; it will be completed during the first with your instructors.

For additional information on relevant techniques:

- Annexe I : Few notions in flow cytometry and intracellular cytokine detection – Sylvie Garcia
- Annexe J : Quantitative PCR – Sophie Dulauroy and Adrien Six

Annexe A : Tentative schedule

Date	***Activities***
July 19, Morning	- Workshop presentation and objectives (0h30) - Problem analysis and rationale, choice of strategy (1h) - Experimental design and planning (1h)
July 19, Afternoon	- Experimental design and planning (1h) - Experiments (cell culture and RT-PCR)
July 20, Morning	- Brief review of Experimental design and planning - Experiments
July 20, Afternoon	- Experiments
July 21, Morning	- FACS acquisition - End of experiments
July 21, Afternoon	- Interpretation of real time RT-PCR - Combine data & interpretation
July 22, Morning	- Presentation of data from individual group - Summary discussion of overall experiments
July 22, Afternoon	- Discussion on potential research collaboration - Question & answers

Annexe B : Reagents, solutions, disposable and equipment

Note: Please use care to keep the biological reagents at -20°C or +4°C as indicated.

Culture media, solutions and reagents

Note: The protocols presented in this handout have been designed with the following reagents; it is possible to use alternate brands or references with appropriate adjustments and optimization.

- 7-AAD (Sigma Ref. A9400-1)
- AD (Sigma Ref. A1410)
- C2-ceramide (Sigma Ref. A1410)
- CFSE (Invitrogen, 25mg, Ref. C1157 or Fluka Ref. 21888)
- CO_2 (for cell incubator)
- Cycloheximide (Sigma Ref. C4859)
- Demineralised water
- DMSO (Sigma Ref. D4540)
- Etoposide (Sigma Ref. E1383)
- FACS buffer (PBS – 2% FCS – 0,01% NaN_3)
- Fetal calf serum (ATGC Biotechnologies Ref. 04-001-1B)
- Hepes buffer 1M solution in distilled water
- NaN_3 (Sigma Ref. S-2002)
- Paraformaldehyde (Sigma Ref. P6148)
- PBS (50 tablets Sigma Ref. P4417-50 or 1X Invitrogen Ref. 14190094)
- Penicillin-Streptomycin 10000U/10 mg/mL (Invitrogen)
- RPMI 1640 culture medium with glutamax (Invitrogen Ref. 61870-010)
- Trypan blue (Invitrogen-Gibco Ref: 15250-06) (for cell counting)

Molecular biology solutions and reagents

Note: The protocols presented in this handout have been designed with the following reagents; it is possible to use alternate brands or references with appropriate adjustments and optimization.

- Bromo-Chloro-Propane (RNA grade)
- dNTP (Promega Ref. C1141)
- Ethanol 70% (Prolabo Réf : UN1170)
- Glycogen (Sigma Ref. G1767)
- H_20 PCR
- H_20 RNAse-free
- Isopropanol (RNA grade)
- Oligo-dT (17mer)

- RNAsin (Promega Ref. N2511)
- RT-AMV (Roche Ref. 1495062)
- SYBR green mix (Gene system Ref. 4309155 or Sigma Ref. S4438)
- Trireagent (Invitrogen Ref. 15596-026)

Primers for quantitative PCR (housekeeping, pro- & anti-apoptotic genes)

Note: For more information regarding the primer list see the references provided in Annexe K.

- Bax 01 DO (5'-ACAAAGATGGTCACGGTCTGCC-3')
- Bax 01 UP (5'-ACCAAGAAGCTGAGCGAGTGTC-3')
- Bax 02 DO (5'-GGCGGCAATCATCCTCTG-3')
- Bax 02 UP (5'-TGCTTCAGGGTTTCATCCAG-3')
- BCL2 01 DO (5'-GGAGAAATCAAACAGAGGCC-3')
- BCL2 01 UP (5'-GTGAACTGGGGGAGGATTGT-3')
- BCL2 02 DO (5'-GCCGGTTCAGGTACTCAGTCA-3')
- BCL2 02 UP (5'-CATGTGTGTGGAGAGCGTCAA-3')
- BCL2 04 DO (5'-GCTCAGTTCCAGGACCAGGC-3')
- BCL2 04 UP (5'-AGGAAGTGAACATTTCGGTGAC-3')
- BCL-XL 01 DO (5'-GCGATCCGACTCACCAATAC-3')
- BCL-XL 01 UP (5'-CCCAGAAAGGATACAGCTGG-3')
- BCL-XL 02 DO (5'-GGTCGCATTGTGGCCTTT-3')
- BCL-XL 02 UP (5'-TCCTTGTCTACGCTTTCCACG-3')
- Bim DO (5'-GACAATGTAACGTAACAGTCG-3')
- Bim UP (5'-GAGAAGGTAGACAATTGCAG-3')
- B2M DO (5'-TCTCTGCTCCCCACCTCTAAGT-3')
- B2M UP (5'-TGCTGTCTCCATGTTTGATGTATCT-3')
- BA DO (5'-AAGGGACTTCCTGTAACAATGCA-3')
- BA UP (5'-CTGGAACGGTGAAGGTGACA-3')
- EF1a DO (5'-GCCGTGTGGCAATCCAAT-3')
- EF1a UP (5'-CTGAACCATCCAGGCCAAAT-3')
- GAPDH DO (5'-GGCATGGACTGTGGTCATGAG-3')
- GAPDH UP (5'-TGCACCACCAACTGCTTAGC-3')
- HPRT DO (5'-GGTCCTTTTCACCAGCAAGCT-3')
- HPRT UP (5'-TGACACTGGCAAAACAATGCA-3')

Disposables

Note: Brand and reference is mostly indicative.

- Aluminum foil

- Benchcoat (Whatman Benchkote for example)
- Culture plate [6wells]
- Detergent
- Dry ice
- FACS Tube (Falcon Ref. 352052)
- Filter bottle 0.2 µm (for medium sterilization)
- Gloves L
- Gloves M
- Gloves S
- H_20 Demineralised
- Hand towel
- Marker
- Parafilm
- PCR Plate (Gene system Ref. N801-0560)
- PCR Plate cover (Gene system Ref. 4313663)
- Pipette [10mL]
- Pipette [5mL]
- Saran wrap
- Soap
- Tip [10µL] (Bioactive Ref. 37660-10R)
- Tip [1000µL] (Bioactive Ref. 10130-10R)
- Tip [200µL] (Bioactive Ref. 15370-10R)
- Tip filter [10µL] (Bioactive Ref. SRS38000-01R)
- Tip filter [1000µL] (Bioactive Ref. SRS???)
- Tip filter [200µL] (Bioactive Ref. SRS37850-01R)
- Tissue culture flask [75mL]
- Tube [1,5mL]
- Tube [15mL] (Gibthai Ref.)
- Tube [2mL]
- Tube [50mL]

Equipment

- Bench bin
- Benchtop centrifuge (for 2.0/1.5 mL tubes)
- Benchtop centrifuge refrigerated (for 2.0/1.5 mL tubes)
- Cell culture CO_2 incubator
- Cell culture hood

- Centrifuge refrigerated (for 15/50 mL tubes)
- Chemical hood
- Dessicator
- Electric pipetor
- Flow cytometer
- Freezer -20°C
- Glassware
- Hemocytometer
- Ice bucket
- Labcoat
- Microfuge tube rack
- Micro-pipettes 1000 µL
- Micro-pipettes 20 µL
- Micro-pipettes 200 µL
- Microscope
- Microtube rack
- Multi-channel pipettes
- Propipette
- Protection glass
- Refrigerated centrifuge
- Vortex
- Water bath [37°C]
- Water bath [42°C]

Annexe C : Detailed protocols

Analysis of T cell activation
Measure of proliferation and cytokine detection

a. Jurkat T-cell line culture

a-1. Jurkat T-cell line is diluted with 10 mL of complete RPMI culture medium (500 mL RPMI w/Glutamate supplemented with 50 mL fetal calf serum, 5 mL streptomycin/penicillin antibiotic solution, 5 mL Hepes 1M) in a tissue culture flask.

a-2. Incubate flasks at 37°C, 5% CO_2.

b. Cell culture and activation

This section describes the cell culture step that you will implement in order to test the effect of your molecule with regard to cell activation or apoptosis induction. It is therefore dependent upon your choice of experiment, as well as the nature and number of controls.

This cell culture step will be performed on 6-well cell culture plates in 1-2 mL culture medium for each well, as follows:

b-1. Dilute cells with RPMI culture medium to 2.10^6 cells/mL ("2X" concentration).

b-2. Distribute 1-2 mL of cells per culture well.

b-3. Depending on the conditions to be tested, add 1-2 mL medium with or without "additives" at 2X in order to obtain the appropriate final concentration:

- Etoposide (10 µM)
- Cycloheximide (20 µg/mL)
- Your product(s) (typical concentration: 1 µg/mL)

b-4. Incubate flasks at 37°C, 5% CO_2.

c. Measure of proliferation by CFSE staining

CFSE (carboxyfluorescein diacetate, succinimidyl ester) is a fluorescent dye that is widely used for the analysis of cell generation and proliferation. This fluorescent SE (succinimidyl ester) covalently couples to both intracellular and cell-surface proteins by reaction with lysine side chains and other available amine groups. When cells divide, the SE labeling is distributed equally between the daughter cells, which are, therefore, half as fluorescent as the parents. As a result, each successive generation in a population of proliferating cells is marked by a halving of cellular fluorescence intensity that is readily followed by flow

cytometry. Using flow cytometric analysis of CFSE labeling, one can resolve up to ten successive generations of lymphocytes (see Figure 1).

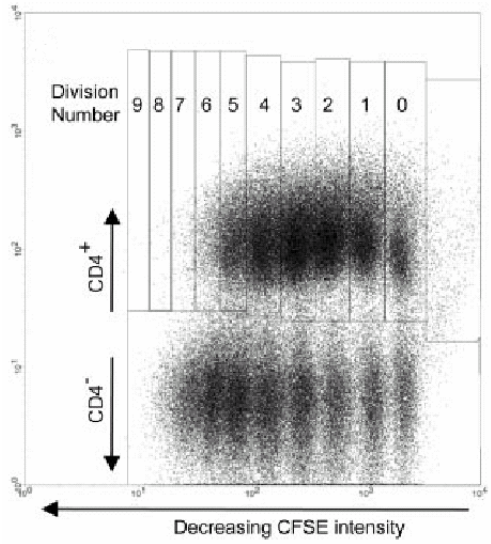


Figure 1: Mitogen-induced proliferative responses measured using CFSE incorporation.

See Annexe I : Few notions in flow cytometry and intracellular cytokine detection – Sylvie Garcia for further information.

c-1. Prepare the stock solution of CFSE in DMSO at 5 mM. Aliquot in small volume and keep it frozen at –20°C.

c-2. Resuspend the cells at 2.10^7/mL in PBS (make sure that the PBS is suitable to the cell survival – good pH).

c-3. Add the same volume of CFSE at 5 µM (2X=1/1000) and mix gently.

c-4. The final concentration of cells will be 10^7/mL and of CFSE 2.5 µM.

c-5. Incubate for 10 min at room temperature, agitating gently from time to time.

c-6. Wash once in a big volume of cold complete medium (5 % FCS).

c-7. Resuspend the cells at 2.10^6/mL of complete medium and incubate overnight in the presence or not of the adequate drugs.

c-8. After cell culture with the appropriate stimuli (see above), cells can be analyzed for CFSE staining with a flow cytometer for evaluation of proliferation.

NB: Check by Facs that the totality of the cells is stained as an homogeneous peak.

c-9. Induction of apoptosis can be evaluated by different techniques:

- 7AAD labelling (see section d)
- Quantitative RT-PCR of pro- and anti-apoptotic genes (see section e and following)

d. Measure of apoptosis induction by flow cytometry

The principle of this technique is to detect and measure apoptotic cells by fluorescence staining and flow cytometric analysis. In the present experiment, we will detect apoptosis by labeling cells with 7AAD. See Annexe I : Few notions in flow cytometry and intracellular cytokine detection – Sylvie Garcia for further information.

d-1. Experimental procedure for 7AAD staining:

- Transfer cells for staining in 15 mL.
- Cells are washed once in "Facs buffer" (PBS 1X, 2% fetal calf serum, 0.1% NaN_3) and centrifuged 5 minutes at 1500 rpm.
- Resuspend the cell pellet in 100 µL of Facs buffer containing 20µg/ml 7AAD.
- Incubate for 20 min on ice in the dark.

Note: longer time will cause "unspecific" staining.

- Wash twice with 1 mL Facs buffer (PBS 1X, 2% fetal calf serum, 0.1% NaN_3) and centrifugation microfuge tube 5 min at 2000 rpm.
- Resuspend cells in 500 µL of Facs buffer containing 20µg/ml AD and run the sample on the flow cytometer as soon as possible.

d-2. Flow cytometer: acquisition and analysis

- The use of the flow cytometer will be demonstrated by an instructor for acquisition and analysis of data.
- Results are expressed as a percentage of positive cells after normalization with the appropriate controls and gating on the relevant cell population.

e. Quantitative RT-PCR – RNA extraction

Note: All reagents and plasticware must be of RNA-grade and performer must wear gloves at all time in order to prevent contamination with RNAse. All steps must be performed under a chemical hood.

e-1. Resuspend up to 1.10^6 cells in 1 mL of Trizol/TriReagent [See Annexe F for manufacturer's recommendations].

e-2. Add 200 µL/ml of BCP.

e-3. Vortex to mix the 2 phases.

e-4. Incubate 5 min at room temperature.

e-5. Centrifuge 15 min at 4°C.

e-6. Add 1 µL of glycogen (20 et 35 mg/mL) in an empty new tube.

e-7. Transfer the aqueous phase (be careful) and add to this new tube.

e-8. Add 500 µL of isopropanol/mL of Trizol.

e-9. Incubate in dry ice 20 to 30 min for precipitation.

e-10. Incubate 5 min at room temperature.

e-11. Centrifuge 10 min at 4°C (10000 tr/min).

e-12. Remove the supernatant carefully with a pipette.

e-13. Wash twice :

- Wash the pellet in 1 mL of EtOH 70%, without dissolving the pellet (do not vortex!)
- Centrifuge 10 min at 4°C (10000 tr/min)
- Remove the EtOH (turn the tube upside down + hand towel then P20)

e-14. Evaporate the EtOH under vacuum for 15 min in a dessicator.

e-15. Dissolve the RNA pellet in 10 µL of RNA-grade water

e-16. Keep at -80°C or –20°C before retrotranscription (see section f)

f. Quantitative RT-PCR – cDNA synthesis

f-1. Thaw the following reagents ahead of time:

- 5X dNTP 5mM / oligo-dT 25µM mix
- 5X RT buffer (vortex and quick spin before use)
- RNA (10 µL; see section a above)

f-2. Switch on water bath at 42°C and drying oven at 72°C.

f-3. Add 4 µL of the 5X dNTP/ oligo-dT mix in the tube with RNA.

f-4. Incubate 10 min at 70°C.

f-5. Chill on ice.

f-6. Centrifuge briefly 2 s.

f-7. Prepare a mix (n tubes + 2) :

→ for 1 tube, mix :

- 4 µL of 5X RT buffer
- 1 µL of RNasin (Recombinant RNasin® Ribonuclease Inhibitor)

- 1 µL of AMV RT (Deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase [RNA-directed] from avian myeloblastosis virus)

f-8. Distribute 6 µL per tube.

f-9. Homogenize reagents.

f-10. Incubate 1h at 42°C.

f-11. Centrifuge briefly 2 s.

f-12. Keep at -20°C.

g. Quantitative RT-PCR – Amplification

Each RNA/cDNA sample of interest will be tested by quantitative PCR for a maximum of 12 control or pro-/anti-apoptotic genes. The table below represents a suggested experimental design on a 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	β A	B 2 M	G A P D H	H P R T	E F - 1 α	B C L 2	B C L 2	B C L 2	B A X	B A X	B C L - X L	B C L - X L
B												
C												
D												
E												
F												
G												
H												

Note: The sequence of available primers is provided in Annexe B.

g-1. Dilute cDNA to an equivalent of 25 000 cells per µL.

g-2. Distribute 2 µL of cDNA in each well.

g-3. Prepare a mix as follows:

→ For 1 PCR point:

	1 well
SybrGreen 2X	12,5 µL
H_20	5,5 µL
Primer Reverse 10 µM	2,5 µL
Primer forward 10 µM	2,5 µL
cDNA	2,0 µL

Master Mix:

	1 well	100 wells
SybrGreen	12.5 µL	1250 µL
Water	5.5 µL	550 µL
	Total:	1800 µL

1. In 1 tube, mix SybrGreen and water.

2. Distribute 150 µL of this mix in 12 tubes.
3. Add 25 µL of each of the corresponding forward and reverse primers for each gene to be quantified.

g-4. Distribute 23 µL of this mix in each well of the corresponding column.

g-5. Set the plate on the real-time quantitative PCR machine using the following programme:

1. Incubate at 95°C for 00:05:00
2. Incubate at 95°C for 00:00:45
3. Incubate at 55°C for 00:00:45
4. Incubate at 72°C for 00:01:00
5. Incubate at 79°C for 00:00:01
6. Plate read
7. Go to line 2 and repeat for 39 more times
8. Incubate at 72°C for 00:30:00
9. Melting curve from 65°C to 95°C read every 0.5°C hold 01 sec
10. End

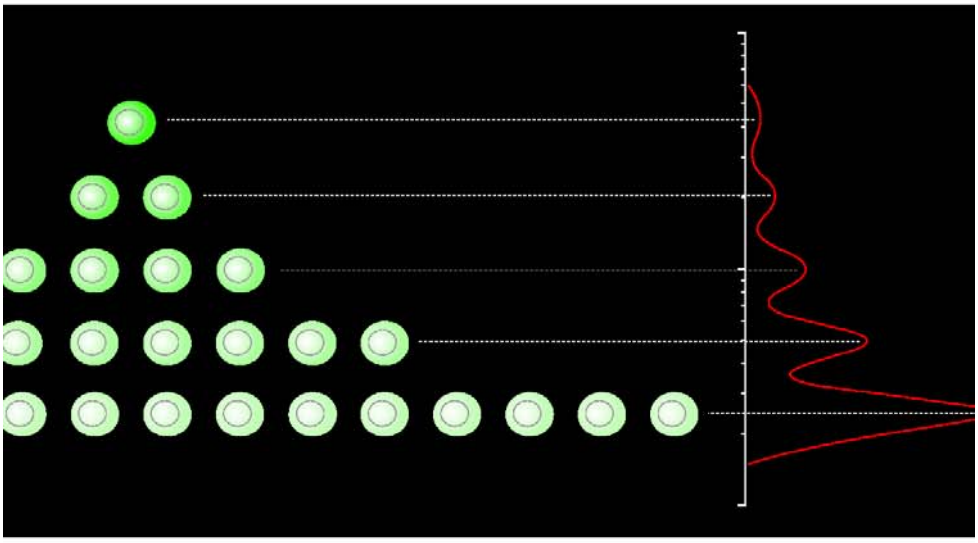
Note: The PCR programme may need some adjustments depending on the Sybr Green kit and brand/model of real-time thermocycler.

g-6. After PCR cycling and real-time plate measurement, results must be analysed with the thermocycler manufacturer's software in order to determine ΔCt values for each gene of interest, corresponding to cycle number to which signal increases over background, which will be used for analysis.

Annexe D : CFSE recommendations

Carboxyfluorescein Diacetate, Succinimidyl Ester (CFDA, SE)

Cell Proliferation Tracking by Flow Cytometry



- Direct detection of cell division via fluorescent label partition
- Capable of resolving 8 or more generations without data deconvolution
- Validated alternative to BrdU or [^{3}H]-thymidine incorporation
- Diagnostic tool for T cell function assessment
- Combine with R-PE immunolabeling for phenotype analysis

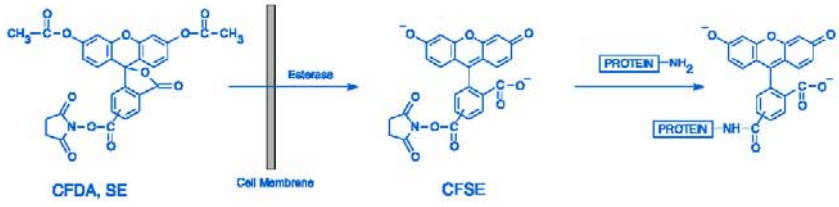


Figure 1. *Mechanism of cellular labeling by CFDA, SE. CFDA, SE is a nonpolar molecule that spontaneously penetrates cell membranes and is converted to anionic CFSE by intracellular esterases. Amine-reactive coupling of CFSE to proteins results in stable long-term intracellular retention.*

Molecular Probes

Technical Information

Properties

Carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE) spontaneously and irreversibly couples to cellular proteins by reaction with lysine sidechains and other available amines (Figure 1). After an initial period of equilibration (~24 hours), the fluorescence of resting cells labeled with CFDA, SE is stable over periods of several months and can be analyzed by flow cytometry using excitation at 488 nm and the FL1 detection channel. When cells divide, CFDA, SE labeling is distributed equally between the daughter cells, which are therefore half as fluorescent as their parents. Consequently, each successive generation in a population of proliferating cells is marked by specific two-fold decrements in cellular fluorescence intensity that are readily discriminated by flow cytometry (Figure 2). Maximum resolution of division cycles is obtained when the distribution of initial cellular fluorescence intensities is narrow and autofluorescence background levels are low. CFDA, SE produces more uniform cellular labeling and consequently better inter-generation resolution than other cell tracking dyes such as the membrane marker PKH26.[1] Poor resolution can lead to erroneous conclusions such as cells that have divided several times being misidentified as undivided. Flow cytometric analysis of CFDA, SE labeling has the demonstrated capacity to directly resolve eight to ten successive generations of lymphocytes.[2,3]

Applications

CFDA, SE labeling has been validated relative to standard proliferation analysis techniques such as [^{3}H]-thymidine incorporation[4] and BrdU labeling,[2,3] while also providing much higher-level information content. For example, an entire population of cells dividing once will incorporate the same amount of [^{3}H]-thymidine as one third of the population dividing twice. These quite different division patterns are readily distinguished by CDFA, SE labeling analysis.[2] Furthermore, multiplex analysis of CFDA, SE and other markers can be implemented to correlate cell division status with phenotype identification and functional parameters.[2,4-6] Although current applications of CFDA, SE labeling are centered on lymphocyte proliferation, the technique is also applicable to other cell types, including fibroblasts, NK cells and even bacteria.[1] CFDA, SE labeling has recently been used to show that transplantable hematopoietic cells proliferate *in vitro* in response to stimulation by a growth factor cocktail.[7] This observation has provided previously lacking direct evidence that the hematopoeitic potential of cultured stem cells is limited by homing activity and not by proliferative capacity.

Products

CFDA, SE is available as a standalone reagent in 25 mg units (C-1157) or in our Vybrant CFDA SE Cell Tracer Kit (V-12883). Each kit includes ten single-use vials of CFDA, SE, as well as high-quality anhydrous DMSO and a complete protocol.

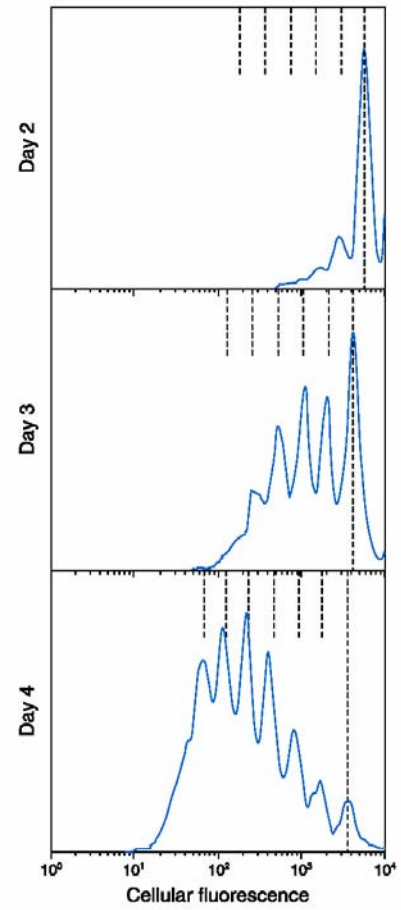


Figure 2. *Resting B lymphocytes labeled with CFDA, SE were stimulated under conditions designed to reproduce T cell–dependent activation. 2, 3 and 4 days after stimulation, cells were harvested and analyzed by flow cytometry. Peaks from right to left in the cellular fluorescence histogram represent successive generations. The observation of multiple peaks on days 3 and 4 is indicative of highly asynchronous division. Adapted with permission from J Exp Med 184, 277 (1996).*

References

1. Immunol Cell Biol 77, 499 (1999); **2.** J Immunol Meth 171, 131 (1994); **3.** Immunol Cell Biol 77, 509 (1999); **4.** Immunol Cell Biol 77, 559 (1999); **5.** Immunol Cell Biol 77, 530 (1999); **6.** J Exp Med 184, 277 (1996); **7.** Blood 94, 2161 (1999).

For further information contact

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Annexe E : 7-AAD recommendations

SIGMA®

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Saint Louis, Missouri 63103 USA
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Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

7-Aminoactinomycin D

Product Number **A 9400**
Storage Temperature 2-8 °C

Product Description
Molecular Formula: $C_{62}H_{87}N_{13}O_{16}$
Molecular Weight: 1,270
CAS Number: 7240-37-1
Synonym: 7-AAD
Fluorescent Properties

Free form:
Excitation: 503 nm (0.01 M phosphate buffer, pH 7.0 containing 0.1 mM EDTA)[1]; 550 nm[2]
Emission: 675 nm (0.01 M phosphate buffer, pH 7.0 containing 0.1 mM EDTA)[1]; 672 nm[2]

DNA Complex:
Excitation: 543 nm[1]; 555 nm[2]
Emission: 655 nm[1]; 665 nm[2]

7-AAD is used in flow cytometry analysis of viable cells. Cell surface markers were stained by FITC and phycoerythrin-conjugated antibody. After surface staining, cells were further stained with 10 µg/ml of 7-AAD in PBS on ice for 30 minutes. After washing with PBS twice, the cells were fixed in 1% paraformaldehyde supplemented with 50 µg/ml actinomycin D. Non-apoptotic cells are 7-AAD negative.[3]

This material like its parent molecule, Actinomycin D, is a DNA-intercalator with growth-inhibitory properties.[4,5]

This product has been tested for its labeling properties on transformed thymocytes that are scanned by FACS. It was possible to distinguish diploid, triploid and tetraploid sub-populations and % mitosis. When tested at fixed intervals of time, it was possible to calculate generation time.

Precautions and Disclaimer
For Laboratory Use Only. Not for drug, household or other uses.

Preparation Instructions
7-AAD is souble in chloroform (1 mg/ml) and produce a clear, dark red solution. One milligram of 7-AAD is souble in 50 µl of absolute methanol. A further addition of 950 µl of 1x PBS with Ca^{2+} and Mg^{2+} will achieve a concentration of 1 mg/ml.[6]

Storage/Stability
A solution prepared by adding 1mg of 7-AAD to 50 µl of absolute methanol followed by a further addition of 950 µl of 1x PBS with Ca^{2+} and Mg^{2+} was stable for several months when stored tightly closed and protected from light at 4 °C.[6]

References

1. Gill, J.E., et al., 7-Aminoactinomycin D as a Cytochemical Probe. I. Spectral Properties. J. Histochem. Cytochem., **23(11)**, 793-799 (1975).
2. Sengupta, S.K., et al., 7-substituted Actinomycin D analogs. Chemical and Growth-inhibitory Studies. J. Med. Chem., **18(12)**, 1175-1180 (1975).
3. Su, X., et al., J. Immunology, **156**, 4198 (1996).
4. Cancer Chemotherapy Reports, **58**, 35 (1974).
5. Madhavarao, M.S., et al., N7-Substituted 7-aminoactinomycin D Analogues. Synthesis and Biological Properties. J. Med. Chem., **21(9)**, 958-961 (1978).
6. http://cyto.mednet.ucla.edu/7aad%20Staining%20for%20dead%20cells.htm

HLD/RXR 8/03

Annexe F : Tri Reagent/Trizol recommendations

Invitrogen™
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TRIZOL® Reagent

Cat. No. 15596-026 **Size: 100 ml**
Store at 2 to 8°C.

WARNING: Toxic in contact with skin and if swallowed. Causes burns. After contact with skin, wash immediately with plenty of detergent and water. If you feel unwell, seek medical advice (show label where possible). Phenol (108-95-2) and Other Components (NJTSRN 80100437-5000p).

TRIZOL has demonstrated stability of 12 months when stored at room temperature. However, we recommend storage at 2 to 8°C for optimal performance.

Description:
TRIZOL Reagent (U.S.Patent No.5,346,994) is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1). During sample homogenization or lysis, TRIZOL Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation (2). Precipitation with ethanol yields DNA from the interphase, and an additional precipitation with isopropyl alcohol yields proteins from the organic phase (2). Copurification of the DNA may be useful for normalizing RNA yields from sample to sample.

This technique performs well with small quantities of tissue (50-100 mg) and cells (5×10^6), and large quantities of tissue (≥1 g) and cells (>10^7), of human, animal, plant, or bacterial origin. The simplicity of the TRIZOL Reagent method allows simultaneous processing of a large number of samples. The entire procedure can be completed in one hour. Total RNA isolated by TRIZOL Reagent is free of protein and DNA contamination. It can be used for Northern blot analysis, dot blot hybridization, poly (A)$^+$ selection, in vitro translation, RNase protection assay, and molecular cloning. For use in the polymerase chain reaction (PCR®), treatment of the isolated RNA with amplification grade DNase I (Cat. No. 18068) is recommended when the two primers lie within a single exon.

TRIZOL Reagent facilitates isolation of a variety of RNA species of large or small molecular size. For example, RNA isolated from rat liver, electrophoresed on an agarose gel, and stained with ethidium bromide, shows discrete bands of high molecular weight RNA between 7 kb and 15 kb in size, (composed of mRNA's and hnRNA's) two predominant ribosomal RNA bands at ~5 kb (28S) and at ~2 kb (18S), and low molecular weight RNA between 0.1 and 0.3 kb (tRNA, 5S). The isolated RNA has an A_{260}/A_{280} ratio ≥1.8 when diluted into TE.

Precautions for Preventing RNase Contamination:
RNases can be introduced accidentally into the RNA preparation at any point in the isolation procedure through improper technique. Because RNase activity is difficult to inhibit, it is essential to prevent its introduction. The following guidelines should be observed when working with RNA.

- Always wear disposable gloves. Skin often contains bacteria and molds that can contaminate an RNA preparation and be a source of RNases. Practice good microbiological technique to prevent microbial contamination.
- Use sterile, disposable plasticware and automatic pipettes reserved for RNA work to prevent cross-contamination with RNases from shared equipment. For example, a laboratory that is using RNA probes will likely be using RNase A or T1 to reduce background on filters, and any nondisposable items (such as automatic pipettes) can be rich sources of RNases.
- In the presence of TRIZOL Reagent, RNA is protected from RNase contamination. Downstream sample handling requires that nondisposable glassware or plasticware be RNase-free. Glass items can be baked at 150°C for 4 hours, and plastic items can be soaked for 10 minutes in 0.5 M NaOH, rinsed thoroughly with water, and autoclaved.

Other Precautions:
- Use of disposable tubes made of clear polypropylene is recommended when working with less than 2-ml volumes of TRIZOL Reagent.
- For larger volumes, use glass (Corex) or polypropylene tubes, and test to be sure that the tubes can withstand 12,000 × *g* with TRIZOL Reagent and chloroform. Do not use tubes that leak or crack.
- Carefully equilibrate the weights of the tubes prior to centrifugation.
- Glass tubes must be sealed with parafilm topped with a layer of foil, and polypropylene tubes must be capped before centrifugation.

INSTRUCTIONS FOR RNA ISOLATION:
Caution: When working with TRIZOL Reagent use gloves and eye protection (shield, safety goggles). Avoid contact with skin or clothing. Use in a chemical fume hood. Avoid breathing vapor.

Unless otherwise stated, the procedure is carried out at 15 to 30°C, and reagents are at 15 to 30°C.

Reagents required, but not supplied:
- Chloroform
- Isopropyl alcohol
- 75% Ethanol (in DEPC-treated water)
- RNase-free water or 0.5% SDS solution [To prepare RNase-free water, draw water into RNase-free glass bottles. Add diethylpyrocarbonate (DEPC) to 0.01% (v/v). Let stand overnight and autoclave. The SDS solution must be prepared using DEPC-treated, autoclaved water.]

1. HOMOGENIZATION (see notes 1-3)
   a. *Tissues*
   Homogenize tissue samples in 1 ml of TRIZOL Reagent per 50-100 mg of tissue using a glass-Teflon® or power homogenizer (Polytron, or Tekmar's TISSUMIZER® or equivalent). The sample volume should not exceed 10% of the volume of TRIZOL Reagent used for homogenization.
   b. *Cells Grown in Monolayer*
   Lyse cells directly in a culture dish by adding 1 ml of TRIZOL Reagent to a 3.5 cm diameter dish, and passing the cell lysate several times through a pipette. The amount of TRIZOL Reagent added is based on the area of the culture dish (1 ml per 10 cm^2) and not on the number of cells present. An insufficient amount of TRIZOL Reagent may result in contamination of the isolated RNA with DNA.
   c. *Cells Grown in Suspension*
   Pellet cells by centrifugation. Lyse cells in TRIZOL Reagent by repetitive pipetting. Use 1 ml of the reagent per 5-10 × 10^6 of animal, plant or yeast cells, or per 1 × 10^7 bacterial cells. Washing cells before addition of TRIZOL Reagent should be avoided as this increases the possibility of mRNA degradation. Disruption of some yeast and bacterial cells may require the use of a homogenizer.

   OPTIONAL: An additional isolation step may be required for samples with high content of proteins, fat, polysaccharides or extracellular material such as muscles, fat tissue, and tuberous parts of plants. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12,000 × *g* for 10 minutes at 2 to 8°C. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. In samples from fat tissue, an excess of fat collects as a top layer which should be removed. In each case, transfer the cleared homogenate solution to a fresh tube and proceed with chloroform addition and phase separation as described.
2. PHASE SEPARATION
   Incubate the homogenized samples for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at 15 to 30°C for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 × *g* for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization.
3. RNA PRECIPITATION
   Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of DNA or protein is desired. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at no more than 12,000 × *g* for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.
4. RNA WASH
   Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 7,500 × *g* for 5 minutes at 2 to 8°C.
5. REDISSOLVING THE RNA
   At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an $A_{260/280}$ ratio < 1.6. Dissolve RNA in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C. (Avoid SDS when RNA will be used in subsequent enzymatic reactions.) RNA can also be redissolved in 100% formamide (deionized) and stored at -70°C (5).

RNA Isolation Notes:
1. Isolation of RNA from small quantities of tissue (1 to 10 mg) or Cell (10^2 to 10^4) Samples: Add 800 µl of TRIZOL to the tissue or cells. Following sample lysis, add chloroform and proceed with the phase separation as described in step 2. Prior to precipitating the RNA with isopropyl alcohol, add 5-10 µg RNase-free glycogen (Cat. No 10814) as carrier to the aqueous phase. To reduce viscosity, shear the genomic DNA with 2 passes through a 26 gauge needle prior to chloroform addition. The glycogen remains in the aqueous phase and is co-precipitated with the RNA. It does not inhibit first-strand synthesis at concentrations up to 4 mg/ml and does not inhibit PCR.
2. After homogenization and before addition of chloroform, samples can be stored at -60 to -70°C for at least one month. The RNA precipitate (step 4, RNA WASH) can be stored in 75% ethanol at 2 to 8°C for at least one week, or at least one year at –5 to -20°C.
3. Table-top centrifuges that can attain a maximum of 2,600 × *g* are suitable for use in these protocols if the centrifugation time is increased to 30-60 minutes in steps 2 and 3.

INSTRUCTIONS FOR DNA ISOLATION:
After complete removal of the aqueous phase, as described in the RNA isolation protocol, the DNA in the interphase and phenol phase from the initial homogenate may be isolated. Following precipitation and a series of washes, the DNA is solubilized in 8 mM NaOH. Full recovery of DNA from tissues and culture cells permits the use of TRIZOL Reagent for the determination of the DNA content in analyzed samples (2). Simultaneous extraction of genomic DNA allows for normalization of the results of Northern analysis per genomic DNA instead of the more variable total RNA or tissue weight. (Depending on the source, the DNA pellet obtained may require additional purification (e.g., phenol extraction) prior to other applications.

Reagents required, but not supplied:
- Ethanol
- 0.1 M Sodium citrate in 10% ethanol
- 75% Ethanol
- 8 mM NaOH

This product is distributed for laboratory research use only. CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

For technical questions about this product, call the Invitrogen TECH-LINE℠ 800 955 6288

Unless otherwise stated, the procedure is carried out at 15 to 30°C.

1. DNA PRECIPITATION
   Remove the remaining aqueous phase overlying the interphase, and precipitate the DNA from the interphase and organic phase with ethanol. Add 0.3 ml of 100% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization, and mix samples by inversion. Next, store the samples at 15 to 30°C for 2-3 minutes and sediment DNA by centrifugation at no more than 2,000 × *g* for 5 minutes at 2 to 8°C.
   Careful removal of the aqueous phase is critical for the quality of the isolated DNA.
2. DNA WASH
   Remove the phenol-ethanol supernatant, and if desired, save it for protein isolation. Wash the DNA pellet twice in a solution containing 0.1 M sodium citrate in 10% ethanol. Use 1 ml of the solution per 1 ml of TRIZOL Reagent used for the initial homogenization. At each wash, store the DNA pellet in the washing solution for 30 minutes at 15 to 30°C (with periodic mixing) and centrifuge at 2,000 × *g* for 5 minutes at 2 to 8°C. Following these two washes, suspend the DNA pellet in 75% ethanol (1.5-2 ml of 75% ethanol per 1 ml TRIZOL Reagent), store for 10-20 minutes at 15 to 30°C (with periodic mixing) and centrifuge at 2,000 × *g* for 5 minutes at 2 to 8°C.
   An additional wash in 0.1 M sodium citrate-10% ethanol solution is required for large pellets containing > 200 µg DNA or large amounts of a non-DNA material.
3. REDISSOLVING THE DNA
   Air dry the DNA 5 to 15 minutes in an open tube. (DO NOT DRY UNDER CENTRIFUGATION; it will be more difficult to dissolve.) Dissolve DNA in 8 mM NaOH such that the concentration of DNA is 0.2 – 0.3 µg/µl. Typically add 300 – 600 µl of 8 mM NaOH to DNA isolated from 10^7 cells or 50 – 70 mg of tissue. Resuspending in weak base is HIGHLY recommended since isolated DNA does not resuspend well in water or in Tris buffers. The pH of the 8 mM NaOH is only ~9 and should be easily adjusted with TE or HEPES once the DNA is in solution. At this stage, the DNA preparations (especially from tissues) may contain insoluble gel-like material (fragments of membranes, etc.) Remove the insoluble material by centrifugation at >12,000 × *g* for 10 minutes. Transfer the supernatant containing the DNA to a new tube. DNA solubilized in 8 mM NaOH can be stored overnight at 4°C; for prolonged storage, samples should be adjusted with HEPES to pH 7-8 (see table) and supplemented with 1 mM EDTA. Once the pH is adjusted, DNA can be stored at 4°C or –20°C.

Quantitation and Expected Yields of DNA

Take an aliquot of the DNA preparation solubilized in 8 mM NaOH, mix it with water and measure the A_{260} of the resulting solution. Calculate the DNA content using the A_{260} value for double-stranded DNA. One A_{260} unit equals 50 µg of double-stranded DNA/ml. For calculation of cell number in analyzed samples, assume that the amount of DNA per 1×10^6 diploid cells of human, rat, and mouse origin equals: 7.1 µg, 6.5 µg, and 5.8 µg, respectively (3).

Applications:

Amplification of DNA by PCR:

After redissolving the DNA in 8 mM NaOH, adjust the pH to 8.4 with 0.1 M HEPES (see table). Add 0.1 to 1.0 µg of the DNA sample to your PCR reaction mixture and perform the standard PCR protocol.

Restriction endonuclease reactions:

Adjust the pH of the DNA solution to a required value using HEPES (see table). Alternatively, samples may be dialyzed against 1 mM EDTA, pH 7 to pH 8.0. Use 3-5 units of enzyme per microgram of DNA. Use the conditions recommended by the manufacturer for the particular enzyme, and allow the reaction to proceed for 3 to 24 h. In a typical assay, 80-90% of the DNA is digestible.

pH Adjustment of DNA Samples Dissolved in 8 mM NaOH:

(For 1 ml of 8 mM NaOH use the following amounts of 0.1 M or 1 M HEPES, free acid.)

Final pH	0.1 M HEPES (µl)	Final pH	1 M HEPES (µl)
8.4	86	7.2	23
8.2	93	7.0	32
8.0	101		
7.8	117		
7.5	159		

DNA Isolation Notes:

1. The phenol phase and interphase can be stored at 2 to 8°C overnight.
2. Samples suspended in 75% ethanol can be stored at 2 to 8°C for months.
3. Samples dissolved in 8 mM NaOH can be stored overnight at 2 to 8°C. For long-term storage, adjust the pH to 7-8, and adjust the EDTA concentration to 1 mM.

INSTRUCTIONS FOR PROTEIN ISOLATION:

Proteins are isolated from the phenol-ethanol supernatant obtained after precipitation of DNA with ethanol (step 1, DNA PRECIPITATION). The resulting preparation can be analyzed for the presence of specific proteins by Western blotting (2).

Reagents required, but not supplied:

- Isopropyl alcohol
- 0.3 M Guanidine hydrochloride in 95% ethanol
- Ethanol
- 1% SDS

1. PROTEIN PRECIPITATION
   Precipitate proteins from the phenol-ethanol supernatant (approximate volume 0.8 ml per 1 ml of TRIZOL Reagent) with isopropyl alcohol. Add 1.5 ml of isopropanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Store samples for 10 minutes at 15 to 30°C, and sediment the protein precipitate at 12,000 × *g* for 10 minutes at 2 to 8°C.
2. PROTEIN WASH
   Remove the supernatant and wash the protein pellet 3 times in a solution containing 0.3 M guanidine hydrochloride in 95% ethanol. Add 2 ml of wash solution per1 ml of TRIZOL Reagent used for the initial homogenization. During each wash cycle, store the protein pellet in the wash solution for 20 minutes at 15 to 30°C and centrifuge at 7,500 × *g* for 5 minutes at 2 to 8°C. After the final wash, vortex the protein pellet in 2 ml of ethanol. Store the protein pellet in ethanol for 20 minutes at 15 to 30°C and centrifuge at 7,500 × *g* for 5 minutes at 2 to 8°C.
3. REDISSOLVING THE PROTEIN PELLET
   Vacuum dry the protein pellet for 5-10 minutes. Dissolve it in 1% SDS by pipetting. Complete dissolution of the protein pellet may require incubating the sample at 50°C. Sediment any insoluble material by centrifugation at 10,000 × *g* for 10 minutes at 2 to 8°C, and transfer the supernatant to a fresh tube. The sample is ready for use in Western blotting or may be stored at -5 to -20°C for future use.

Protein Isolation Notes:

1. The protein pellet suspended in 0.3 M guanidine hydrochloride-95% ethanol or in ethanol can be stored for at least one month at 2 to 8°C, or for at least one year at -5 to -20°C.
2. The following protocol is an alternative approach that allows for more efficient recovery of proteins. Dialyze the phenol-ethanol supernatant against three changes of 0.1% SDS at 2 to 8°C. Centrifuge the dialyzed material at 10,000 × *g* for 10 minutes. Use the clear supernatant for Western blotting.
3. Proteins may be quantified by the Bradford method as long as the concentration of SDS is low enough (<0.1%) so that it will not interfere. Methods that do not have detergent-interface problems, and that do not rely on A_{260}/A_{280} measurements may be used (traces of phenol may cause overestimation of protein concentrations).

Troubleshooting Guide:

RNA ISOLATION

•Expected yields of RNA per mg of tissue or 1×10^6 cultured cells
  Liver and spleen, 6-10 µg
  Kidney, 3-4 µg
  Skeletal muscles and brain, 1-1.5 µg
  Placenta, 1-4 µg
  Epithelial cells (1×10^6 cultured cells), 8-15 µg
  Fibroblasts, (1×10^6 cultured cells) 5-7 µg

•Low yield
  Incomplete homogenization or lysis of samples.
  Final RNA pellet incompletely redissolved.

•A_{260}/A_{280} ratio <1.65
  RNA sample was diluted in water instead of TE prior to spectrophotometric analysis. Low ionic strength and low pH solutions increase absorbance at 280 nm (6,7).
  Sample homogenized in too small a reagent volume.
  Following homogenization, samples were not stored at room temperature for 5 minutes.
  The aqueous phase was contaminated with the phenol phase.
  Incomplete dissolution of the final RNA pellet.

•RNA degradation
  Tissues were not immediately processed or frozen after removal from the animal.
  Samples used for isolation, or the isolated RNA preparations were stored at -5 to-20°C, instead of -60 to -70°C.
  Cells were dispersed by trypsin digestion.
  Aqueous solutions or tubes were not RNase-free.
  Formaldehyde used for agarose-gel electrophoresis had a pH below 3.5.

•DNA contamination
  Sample homogenized in too small a reagent volume.
  Samples used for the isolation contained organic solvents (e.g., ethanol, DMSO), strong buffers, or alkaline solution.

• Proteoglycan and polysaccharide contamination
  The following modification of the RNA precipitation (step 3) removes these contaminating compounds from the isolated RNA. Add to the aqueous phase 0.25 ml of isopropanol followed by 0.25 ml of a high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) per 1 ml of TRIZOL Reagent used for the homogenization. Mix the resulting solution, centrifuge and proceed with the isolation as described in the protocol. The modified precipitation effectively precipitates RNA while maintaining polysaccharides and proteoglycans in a soluble form. A combination of the modified precipitation with an additional centrifugation of the initial homogenate (note 2, RNA isolation protocol) is required to isolate pure RNA from plant material containing a very high level of polysaccharides.

DNA ISOLATION

•Expected yields of DNA per mg of tissue or 1×10^6 cultured cells
  Liver and kidney, 3-4 µg
  Skeletal muscles, brain, and placenta 2-3 µg
  Cultured human, rat, and mouse cells (1×10^6), 5-7 µg
  Fibroblasts, 5-7 µg

•Low yield
  Incomplete homogenization or lysis of samples.
  Final DNA pellet incompletely redissolved.

•$A_{260/280}$ ratio <1.70
  DNA sample was diluted in water instead of TE prior to spectrophotometric analysis.
  Phenol was not sufficiently removed from the DNA preparation. Wash the DNA pellet an additional time with 0.1 M sodium citrate in 10% ethanol.

•DNA degradation
  Tissues were not immediately processed or frozen after removal from the animal.
  Samples used for isolation, or the isolated RNA preparations were stored at -5 to-20°C, instead of -60 to -70°C.
  Samples were homogenized with a Polytron or other high speed homogenizer.

•RNA contamination
  Incomplete removal of aqueous phase.
  DNA pellet insufficiently washed with 0.1 M sodium citrate in 10% ethanol.

•Other applications
  Prior to use in PCR amplification, adjust the pH to 8.4.
  For digestion of the DNA with restriction endonucleases, adjust the pH to the desired value, use 3-5 units of enzyme per µg of DNA, and allow the reaction to go for 3-24 hours under optimal conditions for the particular enzyme. Typically 80-90% of the DNA is digested.

PROTEIN ISOLATION

•Low yield
  Incomplete homogenization or lysis of samples.
  Final DNA pellet incompletely redissolved.

•Protein degradation
  Tissues were not immediately processed or frozen after removing from the animal.

•Band deformation in PAGE
  Protein pellet insufficiently washed.

References:

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Cat No. 15596-026

Annexe G : AMV Reverse Transcriptase recommendations

For general laboratory use.
FOR *IN VITRO* USE ONLY.

Reverse Transcriptase, AMV

From avian myeloblastosis virus
Deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase (RNA-directed), EC 2.7.7.49

Cat. No. 11 495 062 001 500 U
Cat. No. 10 109 118 001 1000 U

Version August 2004
Store at −15 to −25° C

Product description

Storage buffer	200 mM potassium phosphate; 2 mM dithiothreitol; Triton X[1]-100, 0.2% (v/v); glycerol, 50% (v/v); pH ca. 7.2.
Volume activity	> 20 U/µl. One unit is the enzyme activity which incorporates 1.0 nmol of [^{3}H]TMP into acid insoluble products in 10 min at 37 ° C with poly(A) · $(dT)_{15}$ as substrate. For lot-specific values, see data label.
Specific activity	> 50 U/µg. For lot-specific values, see data label.
Stability	The undiluted enzyme is stable when stored at −15 to −25 ° C. Repeated freezing and thawing should be avoided.
Supplied buffer for cDNA synthesis (1st strand)	Incubation buffer for AMV reverse transcriptase, 5 × conc.: 250 mM Tris-HCl; 40 mM $MgCl_2$; 150 mM KCl; 5 mM dithiothreithol; pH 8.5 (20 ° C).

Properties and application

AMV reverse transcriptase is a gene product of the RNA genome of avian myeloblastosis virus. The enzymatically active forms of the purified enzyme are α,ββ and αβ. The molecular weight of the α-subunit is 68 000, that of the β-subunit 92000. The mature αβ form, the most active form of AMV reverse transcriptase, includes a RNA-directed DNA polymerase, a DNA-dependent DNA polymerase, a RNase H, and an unwinding activity (1, 2). AMV reverse transcriptase is used for cDNA synthesis, for synthesis of first strand cDNA for use in subsequent amplification reactions (3, 4) and dideoxy DNA sequencing (5, 6).
The enzyme can also be used for RNA sequencing (7), 3′end labeling of DNA fragments, and the generation of ss probes for genomic footprints (8).

Standard assay for 1st strand cDNA synthesis

Pipette in a microfuge tube on ice, mix and make up to a final volume of 20 µl : 4 µl 5 × incubation buffer; 2 µg poly(A)$^+$ RNA [e.g. neo poly(A), ca. 1 kb]; 20 µCi [α-^{32}P]dCTP (3000 Ci/mMol ≙ 110 TBq/mMol); 40 mA_{260} units primer Poly(A) x $(dT)_{15}$*; 20 nMol each of dATP*, dCTP*, dGTP* and dTTP*, 25 units RNase inhibitor* and 40 units AMV reverse transcriptase. Incubate at 42°C for 60 min. The resulting first strand cDNA can be easily used for second strand synthesis, hybridization or amplification by the polymerase chain reaction (see note).

After the incubation, incorporation rates are > 2 × 10^5 cpm (Cerenkov). This corresponds to 15–30% conversion to cDNA, and depends on the quality of the template RNA. The obtained transcripts are > 90% "full length". A protocol for the preparation of 2nd strand cDNA is supplied with the cDNA Synthesis Kit * or (3).

Quality control

See data label for lot-specific values.

Absence of endonucleases

Supercoiled or linearized pBR322 DNA is incubated for 1 h at 37° C with increasing amounts of AMV reverse transcriptase. The number of enzyme units which do not change the band pattern is stated under "Endo/Exo".

Absence of ribonuclease

5 µg of MS2 RNA are incubated with reverse transcriptase for 4 h at 37° C in a final volume of 50 µl. The number of units which show no degradation of MS2 RNA is stated under "RNase".

Absence of exonuclease activity

Approx. 5 µg [^{3}H] labeled calf thymus DNA are incubated with 3 µl reverse transcriptase, AMV for 4 h at 37° C in a total volume of 100 µl 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, pH approx. 7.5. The release of radioactivity is calculated as a percentage value of liberated to input radioactivity per unit of enzyme (stated under "Exo").

cDNA synthesis

Reverse Transcriptase, AMV is function-tested in the cDNA Synthesis Kit *.

The incorporation rate in the 1st strand assay [20 µl with 2 µg poly(A)$^+$ RNA] with 20 µCi [α-^{32}P]dCTP, (3000 Ci/mMol ≙ 110 TBq/mMol), after 1 h incubation at 42 ° C is > 2 × 10^5 cpm (Cerenkov).

For 2nd strand synthesis, the products from a non-radioactive 1st strand assay are incubated with 20 µCi [α^{32}P]dCTP, (3000 Ci/mMol ≙ 110 TBq/mMol). The incorporation rate is > 2 × 10^5 cpm (Cerenkov). The efficiency of the second strand synthesis is in the range of 80–90% of the input first strand.

Function testing in RT-PCR

Reverse Transcriptase, AMV is function tested using 1 µg of total human skeletal muscle RNA and specific dystrophin reverse primer 5′-AAT-GTT-ACT-GCC-CCC-AAA-GGA-TGC-AAC-TTC-A-3′. In the following PCR reaction with forward primer 5′-AAG-AAG-TAG-AGG-ACT-GTT-ATG-AAA-GAG-AAG-3′ and reverse primer 5′-CGT-CCC-GTA-TCA-TAA-ACA-TTC-AGC-AGC-3′ over 30 cycles a 9 556 bp fragment is visible after agarose gel electrophoresis and ethidium bromid staining.

0804.10885 029®

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* available from Roche Applied Science
[1] Triton is a trademark of Rohm & Haas Company, Philadelphia, PA, USA.

Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our homepage http://www.roche-applied-science.com and our Special Interest Sites including:

- PCR - Innovative Tools for Amplification: http://www.roche-applied-science.com/sis/amplification/

Product	Pack size	Cat. No
PCR Nucleotide Mix	100 reactions 10 x 200 µl (1000 reactions)	11 581 295 001 11 814 362 001
PCR Nucleotide Mix Plus	2 x 100 µl	11 888 412 001
Set of Deoxynucleotides, PCR Grade	4 x 25 µmol	11 969 064 001
dATP, PCR Grade	25 µmol 125 µmol,	11 934 511 001 11 969 013 001
dCTP, PCR Grade	25 µmol 125 µmol,	11 934 520 001 11 969 021 001
dGTP, PCR Grade	25 µmol 125 µmol	11 934 538 001 11 969 030 001
dTTP, PCR Grade	25 µmol 125 µmol,	11 934 546 001 11 969 048 001
dUTP, PCR Grade	25 µmol 125 µmol	11 934 554 001 11 969 056 001
Poly (A) x $(dT)_{15}$	5 A_{260} U	10 108 677 001
RNAse inhibitor	10 000 U 2000 U	10 799 025 001 10 799 017 001
cDNA Synthesis Kit	1 kit (transcription of 25 µg RNA) 1 kit (transcription of 50 µg RNA)	11 117 831 001 11 013 882 001
First Strand cDNA Synthesiis Kit for RT-PCR (AMV)	1 kit (30 reactions)	11 483 188 001
Reverse Transcriptase M- MuLV	500 U,	11 062 603 001
Reverse Transcriptase HIV-1	10 µg (50 U)	11 465 333 001
NEW Transcriptor First Strand cDNA Synthesis Kit	50 reactions	04 379 012 001
Transcriptor Reverse Transcriptase	2000 U (4 x 500 U) for 200 reactions 500 U for 50 reactions 250 U for 25 reactions	03 531 287 001 03 531 295 001 03 531 317 001

How to contact Roche Applied Science

www.roche-applied-science.com
to order, solve technical queries, find product information, or contact your local sales representative.

www.roche-applied-science.com/pack-insert/11495062001a.pdf

Please visit our new Online Technical Support Site under www.roche-applied-science.com/support

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Annexe H : Sybr Green recommendations

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sigma-aldrich.com

ProductInformation

SYBR® Green JumpStart™ Taq ReadyMix™

Product Code S 4438
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

SYBR® Green JumpStart™ Taq ReadyMix™ combines the performance enhancements of JumpStart Taq antibody for hot start PCR with SYBR® Green I and the convenience of an easy-to-use ReadyMix solution. Since the ReadyMix includes a fluorescent dye and the reagents for PCR, this is the ideal solution for performing high-throughput quantitative PCR. This ready-to-use mixture of SYBR Green I, JumpStart Taq DNA polymerase, 99% pure deoxynucleotides and reaction buffer is provided in a 2× concentrate for ease of use. Simply add 25 µl of the 2× mix to DNA template, primers and water. The JumpStart Taq antibody inactivates the DNA polymerase at room temperature. When the temperature is raised above 70 °C in the first denaturation step of the cycling process, the complex dissociates and the polymerase becomes fully active. There are no special preparation or protocol changes required to activate this hot start.

- The perfect ReadyMix for high throughput, quantitative PCR applications.
- SYBR Green I is ideal for quantifying any DNA sequence.[1] The dye binds to double-stranded DNA and detection is monitored by measuring the increase in fluorescence throughout cycling.
- The hot start mechanism, using JumpStart Taq antibody, prevents non-specific product formation and allows assembled PCR reactions to be placed at room temperature up to 2 hours without compromising performance.
- Internal Reference Dye is provided for reaction normalization. Maximum excitation of this dye is 586 nm and maximum emission is 605 nm.
- When performing large numbers of PCR reactions, the SYBR Green JumpStart Taq ReadyMix can save a significant amount of preparation time, reduce the risk of contamination from multiple pipetting steps, and provide consistent batch-to-batch and reaction-to-reaction performance.

Reagents

Sufficient for 100 or 500 PCR reactions (50 µl reaction volume)

- SYBR Green JumpStart Taq ReadyMix, Product Code S 9939, containing 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 7 mM $MgCl_2$, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), stabilizers, 0.05 unit/µl Taq DNA Polymerase, JumpStart Taq antibody, and SYBR Green I.
- Internal Reference Dye, Product Code R 4526, 100X dye. Provided as a 0.3 ml vial.

Materials and Reagents Required but not Provided

- Water, PCR reagent, Product Code W 1754
- Primers
- DNA template
- Thermal cycler for quantitative PCR

Storage/Stability

SYBR Green JumpStart Taq ReadyMix can be stored at 2-8 °C for up to 3 months; there is no waiting for the reaction components to thaw. It can also be stored at –20 °C for up to one year. There was no detectable loss of performance after 10 freeze-thaw cycles.

Precautions and Disclaimer

SYBR Green JumpStart Taq ReadyMix is for R & D laboratory use only. Not for drug, household or other uses.

Procedure

Note: Because SYBR Green I binds to all double-stranded DNA, it is important to test primers and cycling conditions to insure that the PCR product is a single band, or the results will be uninterpretable. It is best to insure PCR specificity by checking the reaction on a normal (non-quantitative) thermocycler and analyzing the result using agarose gel separation.[2]

Optimal concentrations of template DNA, $MgCl_2$, KCl and PCR adjuncts as well as pH are often target specific. Optimization may be needed for specific template and primers. Additional components ($MgCl_2$, dNTP, betaine, etc.) may be added to the template/primer mixture, although this is not required for most applications. The following procedure serves as a reference.

1. Add the following reagents to the proper tube or plate for thermocycling.

Amount	Component
25 µl	SYBR Green JumpStart Taq ReadyMix
(0.5 µl)	Internal Reference Dye (optional)
- µl	Forward primer, 0.2 µM final concentration
- µl	Reverse primer, 0.2 µM final concentration
- µl	Template DNA
- µl	Water
50 µl	Total volume

Note: A template-primer master mix is recommended when performing multiple PCR reactions.

2. Mix gently by vortexing and briefly centrifuge to collect all components at the bottom of the tube.

3. Optimum cycling parameters vary with PCR composition and thermal cycler. It may be necessary to optimize the cycling parameters to achieve maximum product yield and/or quality.

Typical cycling parameters for 100 bp – 600 bp fragments:

Initial denaturation	94 °C	2 min
40 cycles:		
Denaturation	94 °C	15 sec
Annealing	60 °C	1 min
Extension	72 °C	1 min
Hold	4 °C	

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1. Morrison, T. B., *et al*., Quantification of Low-Copy Transcripts by Continuous SYBR® Green I Monitoring during Amplification. *BioTechniques,* **24**: 954-962 (1998).
2. Sambrook, J. *et al*. *Molecular Cloning: A. Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press, New York (2000). (Product Code M 8265)

† The PCR process is covered by patents owned by Hoffman-LaRoche, Inc.

Troubleshooting Guide

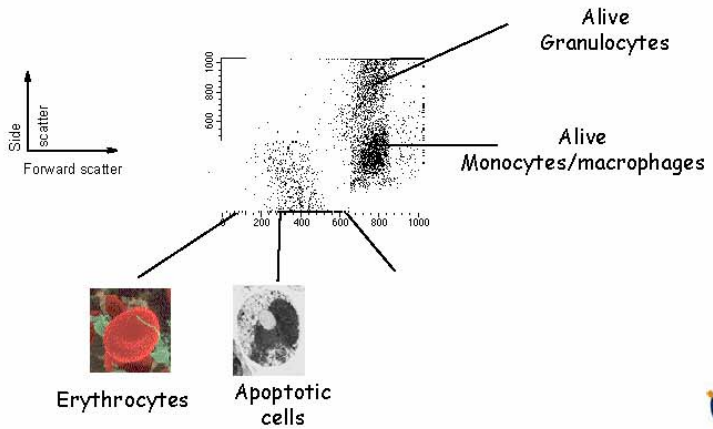
Symptom	Possible Cause	Solution
No PCR product (signal) is observed	A PCR primer is missing or degraded.	A positive control should always be run to insure components are functioning. A checklist is also recommended when assembling reactions.
	Too few cycles are performed.	Increase the number of cycles.
	The annealing temperature is too high.	Decrease the annealing temperature in 2-4 °C increments.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information. If the primers are less than 27 nucleotides long, try to lengthen the primer to 27-33 nucleotides. If the primer has a GC content of less than 45%, try to redesign the primer with a GC content of 45-60%.
	There is not enough template.	After increasing the number of cycles has shown no success, repeat the reaction with a 10-fold higher concentration of the template.
	The template is of poor quality.	Evaluate the template integrity by agarose gel electrophoresis. It may be necessary to repurify template using methods that minimize shearing and nicking.
	Target template is difficult.	In most cases, inherently difficult targets are due to unusually high GC content and /or secondary structure. Betaine has been reported to help amplification of high GC content templates at a concentration of 0.8-1.3 M. [Rees, W. *et al*., *Biochemistry*, **32**, 137-144 (1993)]
Signal is independent of template dilution (multiple products or smeared products)	The annealing temperature is too low.	Increase the annealing temperature in increments of 2-3 °C.
	The primers are not designed optimally.	Confirm the accuracy of the sequence information. If the primers are less than 27 nucleotides long, try to lengthen the primers to 27-33 nucleotides. If the primer has a GC content of less than 45%, try to redesign the primers with a GC content of 45-60%.
	The template concentration is too high.	Reduce the concentration of the template in the PCR reaction.
	The primer concentration is too high.	Reduce the primer concentrations in a series of two-fold dilutions (i.e. 0.1 µM, 0.05 µM, 0.025 µM and 0.0125 µM) and subject these trial reactions to PCR.

Annexe I : Few notions in flow cytometry and intracellular cytokine detection – Sylvie Garcia

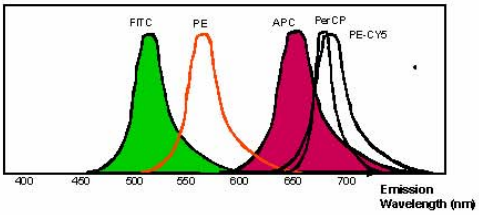
Few notions in flow cytometry and intracellular cytokine detection

Sylvie Garcia, Institut Pasteur, Paris

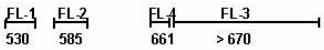
Detection of the blood subsets using SSC/FCS parameters



Usual dye coupled with antibodies

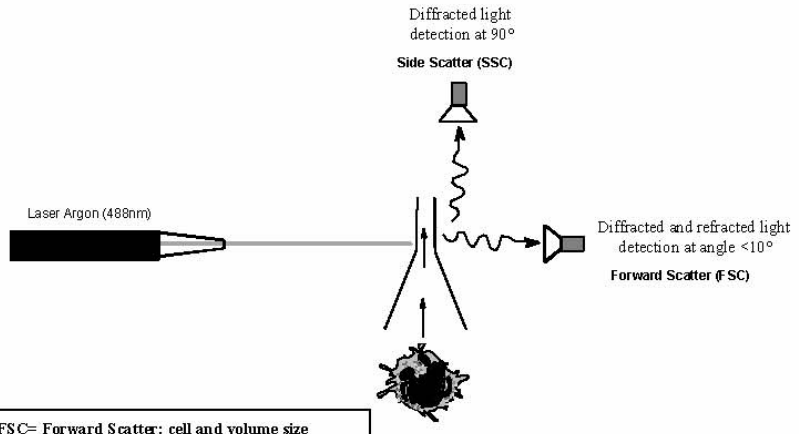


Channel:



FITC : Fluorescein Isothiocyanate
PE: Phycoerythrin
APC: Allophycocyanin (*)
perCP: Peridinin Chlorophyll Protein
PE-Cy5: PE + Cyanine-5

Size (FCS)-granularity (SSC) detection



FSC= Forward Scatter: cell and volume size
SSC= Side scatter: cell complexity and granularity

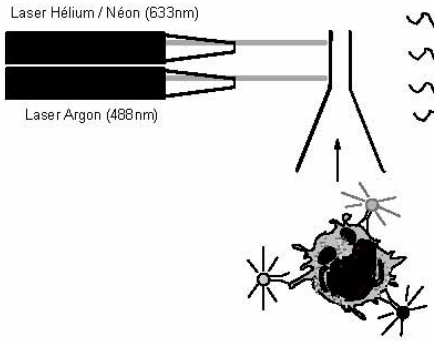
Subsets detectable by flow cytometry

	Human	Mouse
- Leukocytes:	CD45	CD45 (2 alleles: Ly5.1, Ly5.2)
- T Lymphocytes :	CD2, CD3, CD4, CD8, $\alpha\beta$, $\gamma\delta$ (different chains)	
- NK:	NK: CD16, CD56, KIR	NK: CD56, NK1.1, KIR
- B Lymphocytes:	CD19, CD20	
- Monocytes / macrophages:	CD4, CD44	CD11b, CD14
- Granulocytes:	CD15	Anti-GR1
- DC:	CD11c	

Detection of activation/differentiation by cytometry

	Human	Mouse
Naïve/Memory	CD45RA,CD45RO, CD62-L, CCR7	CD45RB,CD44, CD62-L
Resting/Effector	CD69, CD25, HLA-DR	CD69, CD25

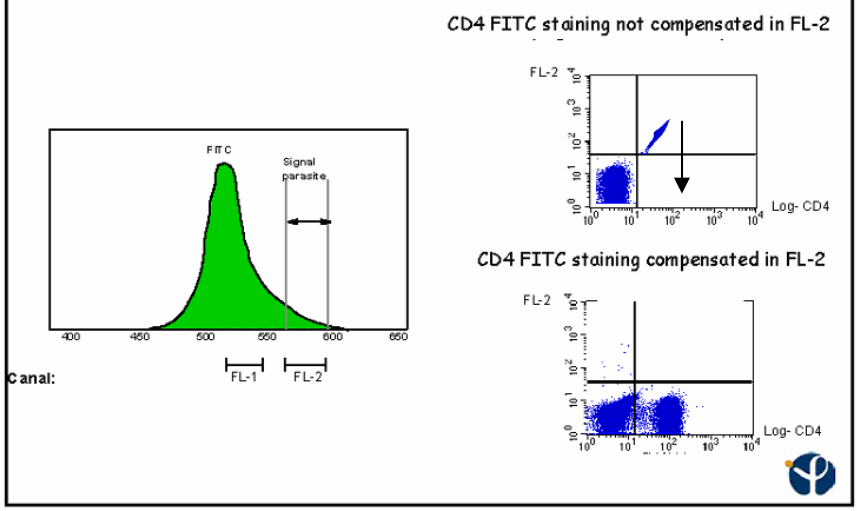
Fluorescence detection



Cytokines/Cytokine receptors detected by flow cytometry

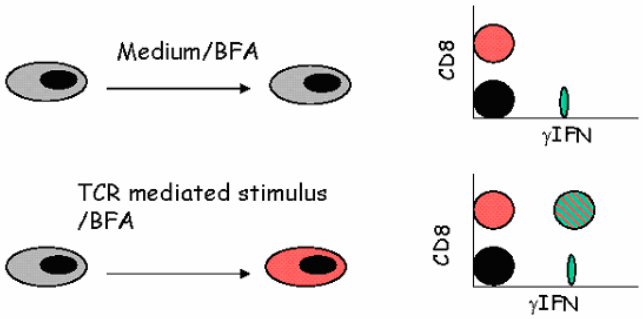
	Human	Mouse
Cytokines	IL-1, IL-2, IL-4, IL-6, IL-8, IL-13, TNFα, γIFN	IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, GM-CSF, γIFN, TNFα
Cytokine receptors	G-CSF-R, GM-CSF-R, IL-2R, IL-3R, IL-5R, γc, IL-10R, IL-12R,	IL-4Rα, IL-3R, IL-6R IL-7Rα, IL-12Rβ, γ IFN, TNF-R, IL2R, γc, IL-15R, IL-10R

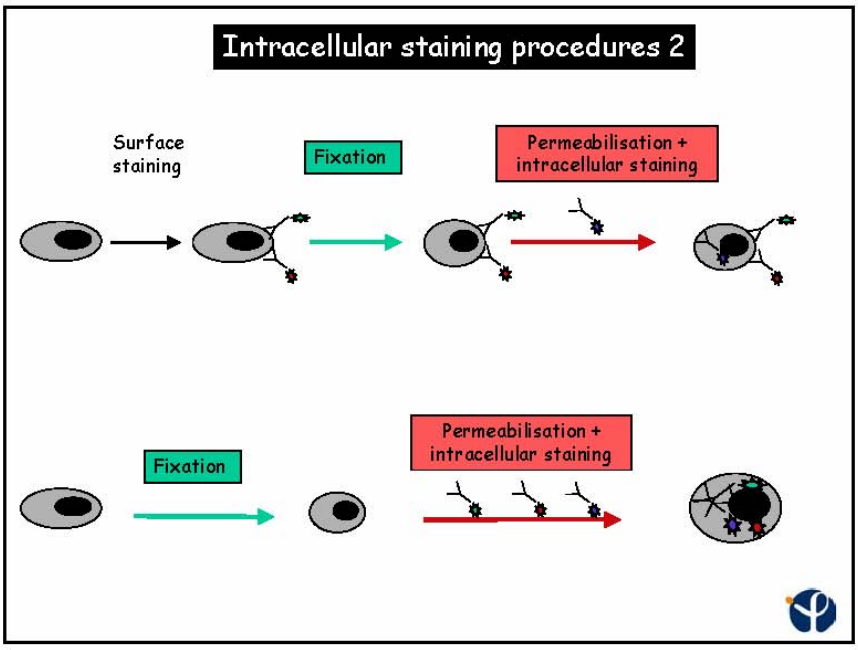
Settings: the compensation problem



Intracellular staining procedures 1

4-ON culture step:
cytokine accumulation for further detection




Intracellular staining procedures 2
Surface staining
Fixation
Permeabilisation + intracellular staining
Fixation
Permeabilisation + intracellular staining

Annexe J : Quantitative PCR – Sophie Dulauroy and Adrien Six

Quantitative Real-Time PCR

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Sophie Dulauroy (sophie.dulauroy@pasteur.fr)
Institut Pasteur & Université Pierre et Marie Curie

Real-Time PCR: principle

1. The real time PCR is based on the detection and the quantification of a fluorescent transmitter during the process of amplification.

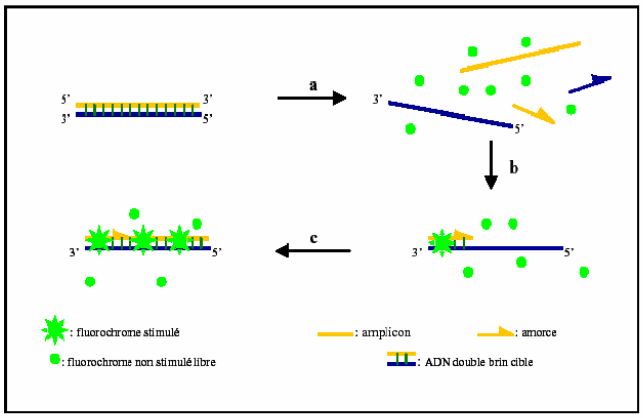
2. The increase in the fluorescent signal is directly proportional to the quantity of amplicons produced during the reaction.

3. Two general principles for the quantitative detection of amplicons:
   - agents binding to the double-stranded-DNA (SybrGreen I)
   - fluorescent probes (FAM, TAMRA, JOE, ROX,…)

4. For the fluorescent probes, there are 4 main technologies:
   - probe hydrolysis
   - hybridisation of 2 probes
   - molecular beacons
   - scorpion primer

   ] Equivalent sensitivity, Different Specificity

Agents binding to the double-stranded-DNA (SYBR Green I)

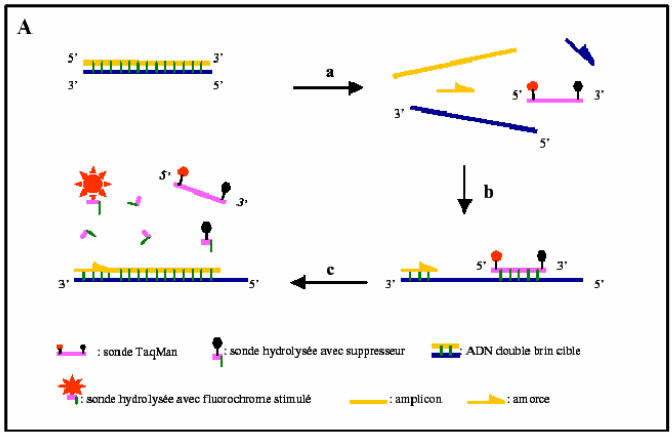


a. The free SYBR Green exhibits little fluorescence at the time of the denaturation.

b. With the temperature of pairing, some molecules bind to the nascent double-stranded-DNA.

c. During the polymerisation step, more and more of molecules bind to the nascent strand and the increase in fluorescence can be followed in real time.

Hydrolysis probes (Taqman)

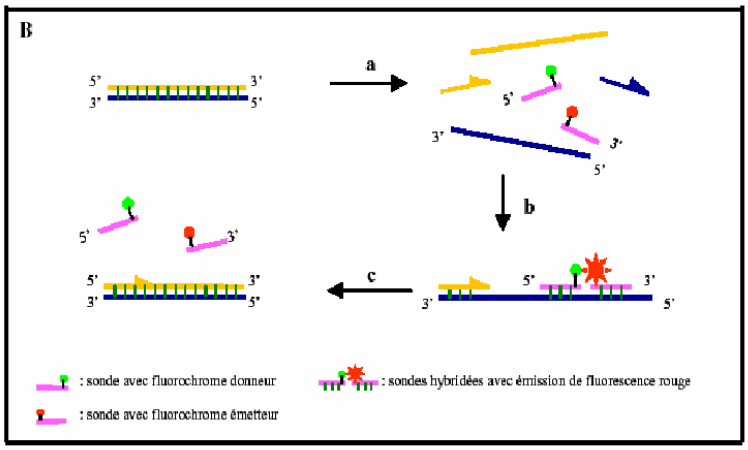


a. During the denaturing step, the probe is free on solution.

b. During the annealing step, both probes hybridise to their target sequence. The proximity of the fluorochrome allows the inhibition of fluorescence.

c. The polymerase moves and hydrolyses the probe. The transmitting fluorochrome is released from the environment of the suppressor thus allowing the emission of fluorescence.

Hybridisation probes (HybProbes)

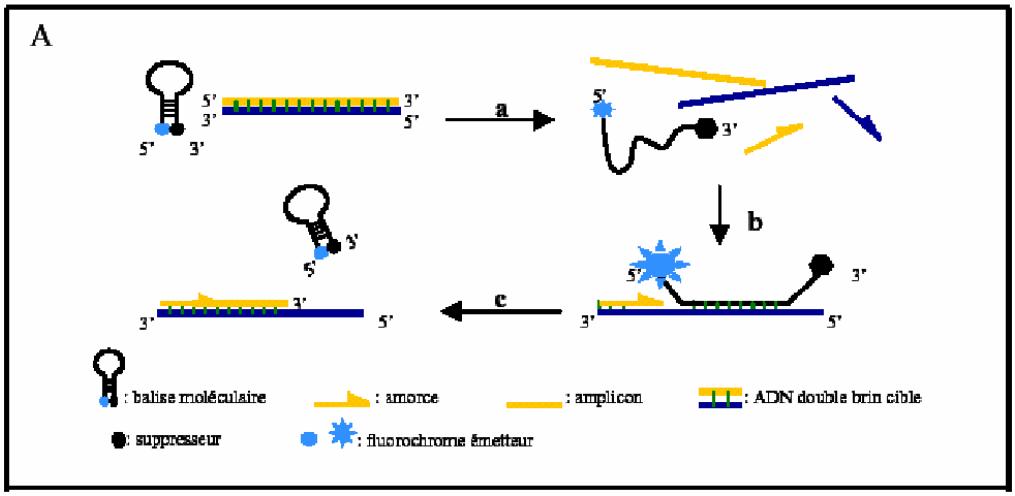


a. During the denaturing step, in solution the 2 probes are apart.

b. During the annealing step, both probes hybridise to their target sequence. The proximity of the fluorochrome allows the red emission of fluorescence.

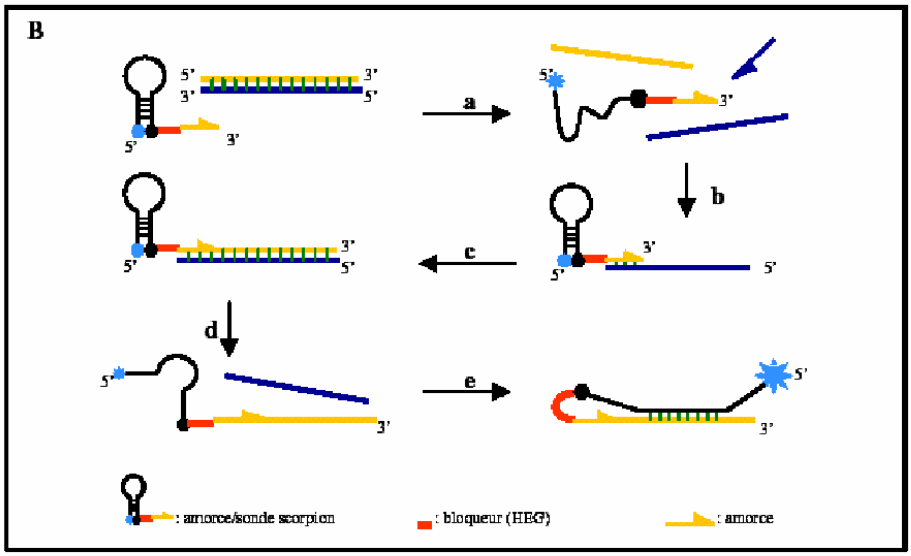
c. The probes turn over free in solution.

Molecular beacons



- High specificity + high precision
- When T°C<Tm, hairpin structure → no hybridisation and no fluorescence

Scorpion primer



- Suppressor HEG prevents the replication of the molecular beacon
- One of the best method

The SYBR Green: advantages and disadvantages

1.Advantages:

- Economic
- Easy to use
- Has more sensibility than the ethidium bromide (another intercalating agent)
- Does not inhibit the reaction of amplification
- Does not require any fluorescent probe, thus does not require any particular expertise for the design of the probes
- Is not affected by mutations in the target DNA

2.Disadvantages:

- Impossible to make sure of specificity of amplicons
- Bad pairing can lead to positive forgeries or an over-estimate of the quantification
- The emission of fluorescence can be skewed by the molecular mass of the DNA amplified by a longer amplicon which will fix more fluorescent molecules compared to a shorter amplicon in the same reaction
- Still unspecified mutagen capacity

Hydrolysis probes: advantages and disadvantages

1. Advantages:

- Increased specificity: the specificity of hybridisation between the fluorescent probe and the sequence of DNA significantly reduces the emission of non-specific fluorescence due to bad pairings or primers dimers.

- Better capacity of multiplexing: reactions multiplex can be elaborate by using distinct transmitting fluorochromes related to different probes in a PCR reaction.

2. Disadvantages:

- Taqman technology is less effective and less flexible device that other technologies in real time for the detection of specific mutations.

- To respect the principles of design of the probes

Hybridisation probes: advantages and disadvantages

1. **Advantages:**

   - High specificity
   - High flexibility for probe design
   - As the probes are not hydrolysed, they are used at each cycle

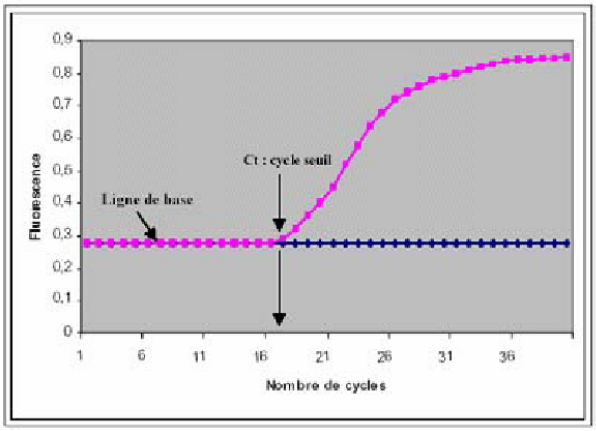
2. **Disadvantages:**

   - Taqman probe design

Threshold cycle = Ct (1)

-The concept of the threshold cycle is at the heart of accurate and reproducible quantification using fluorescence-based PCR.

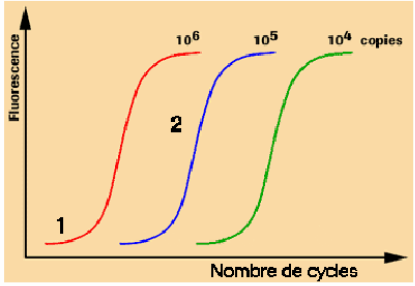
-It corresponds to the cycle from which one observes a statistically significant increase in standardized fluorescence



Threshold cycle = Ct (2)

- The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point for which the fluorescence signal is first recorded as statistically significant above background. This point is defined as the Ct.

- The threshold cycle will always occur during the exponential phase of amplification.



Threshold cycle = Ct (3)

-Quantification is not affected by any reaction components becoming limited in the plateau phase
-The Ct value can be translated into a quantitative result by constructing a standard curve

$$R = 2^{-\Delta Ct1/\Delta Ct2}$$

ΔCt1 = ΔCt target gene = Ct target gene with treated sample – Ct same gene with sample calibrator

ΔCt2 = ΔCt standardizing gene = Ct standardizing gene with treated samples – Ct same gene with sample calibrator

⇒ The difference between two samples is considered as significant from one Ct.

Annexe K : Bibliography

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