Basis of Immunology and immunophysiopathology of infectious diseases

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Practical workshop

on

Cytokines quantification

- Quantification of cytokine gene expression (RT-PCR, evaluation of mRNA)
- Measurement of cytokine concentration (ELISA)

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The purpose of this practical workshop is to determine the cytokine levels by two different methods: mRNA quantitative RT-PCR and ELISA technique.
The objective of this 3-day lab session is to demonstrate the power and the limits of these two approaches, which can be applied in both basic and clinical research.

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

I - Isolation of mouse splenocytes

1. Mice are killed.
2. In a hood, the animals are dissected and the spleen is taken.
3. The spleens are dilacerated in a Petri dish containing 5 ml of RPMI and 5% FCS using two sterile polished glass slides.
4. The cell suspension is transferred to a 15 ml tube containing 5 ml of RPMI and 5% FCS.
5. After 5 minutes on ice, the cell suspension is decanted to a new 15 ml tube.
6. Centrifuge (5 minutes at 300 x g) at 4° C.

II - Lysis of erythrocytes

1. Decant the supernatant.
2. Disrupt the cell pellet by “racking” the tube.
3. Resuspend the cells in M-lyse buffer that has been diluted to 1X strength with sterile distilled water and quickly vortex the tube.

Note: We recommend using 2 ml of 1X M-Lyse solution per processed spleen (approximately 1 ml of 1X H-Lyse per 25 million splenocytes).

4. Incubate the cells at room temperature until red cell lysis is complete (10 minutes).

This is easily observed by a darkening in colour of the fluid and clearing of turbidity. Exposure to M-lyse Buffer for longer periods (i.e. 30 minutes) will reduce cellular viability and decrease the total yield of leukocytes.

5. Wash the cells by filling the tube with 1X Wash Buffer

Note: Wash Buffer must be diluted with sterile water to 1X strength prior to use.

6. Centrifuge the cells for 10 minutes at 200 x g. Resuspend the cells in 1ml of 1X column wash buffer.

III - Isolation of the T cells

1. For each column to be used, prepare 20 ml of 1X column wash buffer by mixing 2.0 ml of 10X column wash buffer with 18 ml of sterile distilled water.

Columns have a maximum cell loading capacity of 100 x 10^6 total cells.

2. The column is placed in a column rack or ring stand. The top cap of the column is removed first to avoid drawing air into the bottom of the column. Next, the bottom cap is removed. The fluid within the column is allowed to drain into a waste receptacle. During this process the outside of the column tip should be rinsed with 70% alcohol to ensure sterile column processing.

3. The column content is then washed with a total of 6 ml of 1X column wash buffer and this eluate is also allowed to drain into the waste receptacle. The column is now ready to be loaded with cells.

4. The waste receptacle is replaced with a sterile 15 ml centrifuge tube.

5. After the column buffer has drained down to the level of the white filter, the 1 ml cell suspension is applied to the top of the column. The cells will enter the column and displace the wash buffer contained in the column, which is collected in the sterile centrifuge tube.

6. The cells, now suspended within the column, are incubated at room temperature for 10 minutes.

7. After the incubation step, T cells are eluted from the column with 4 aliquots (2 ml each) of 1X column wash buffer.

8. The collected T cells are centrifuged at 250 x g for 5 minutes.

9. Discard the supernatant and resuspend the pellet in 10 ml of RPMI containing 5% FCS.

10. Count cells on a hemocytometer using Trypan blue.

IV - Culture of the T cells

1. Add 10 ml of cells suspension at 10^6/ ml in a culture flask.

2. Add PMA (Phorbol ester) at 10 ng/ml final concentration and Ionomycin at 500 ng/ml final concentration.

3. Incubate at 37°C with 5% CO_2 for either 4 hours (for RT-PCR method) or overnight (for ELISA method).

V - RNA preparation

1. After 4 hr incubation, count cells on a hemocytometer using Trypan blue.
2. Calculate the volume of the suspension corresponding to 10 millions cells.
3. Pour the suspension in a sterile 15 ml tube.
4. Centrifuge the suspension for 5 minutes at 1200 rpm.

Special precautions must be observed to avoid degradation of mRNA by RNases. Reagents and plastic- or glassware that contact the RNA sample should first be treated with diethyl pyrocarbonate (DEPC) to inactivate any contaminating RNases and then autoclave according to standard procedures. Gloves must be worn to avoid sample contamination with nucleases shed from the skin. Filter pipette tips must be used.

5. Discard the supernatant.
6. Resuspend the pellet in 1ml Trizol and pour the suspension into a 1.5 ml Eppendorf tube.
7. Keep the tube at room temperature for 5 minutes.
8. Add 200 µl chloroform; use a chemical hood and new gloves.
9. Mix vigorously the tube for 15 seconds.
10. Keep for 2-3 minutes on the bench.
11. Centrifuge for 15 minutes at 4° C at maximum speed of 12 000 g.
12. Pipette the clear superior aqueous phase (about 500-600 µl) into a new tube, keep the first tube.
13. Add 500 µl isopropanol into the new tube, mix and keep at room temperature for 10 minutes.
14. Centrifuge for 10 minutes at +4° C, 12 000 g maximum.
15. Transfer the supernatant in an another Eppendorf tube and wash the pellet by adding 1ml Ethanol (75 % in 0.1 % DEPC treated water) onto the pellet. Mix.
16. Centrifuge for 5 minutes at +4° C, 7 500 g.
17. Transfer the supernatant in another Eppendorf tube and the pellet is then dried under vacuum for 5 - 10 minutes.
18. Resuspend the pellet in 50 µl DEPC treated water
19. Put the tube in a heating block (55° C – 60° C) for 5 – 10 minutes.
20. Quantify the RNA using a spectrophotometer (260 nm). Blank the spectrophotometer with water. Read the RNA sample at 260 nm and 280 nm. Calculate the purity of the sample by dividing the 260 reading by the 280 reading. An $A_{260/280}$ ratio of at least 1.8 is required. Multiply the 260 reading by 37 to obtain the concentration of the RNA in µg/ml.

21. The RNA preparation are stored frozen at – 20° C or preferably at – 70° C.

VI - cDNA synthesis

MMLV reverse transcriptase is used in the first-strand cDNA reaction to generate cDNA from mRNA. Prior to first-strand cDNA synthesis the mRNA suspended in RNase-free water is heated to remove any secondary structure which could hinder the reaction.

1. Use 200 ng to 1 µg RNA for the first-strand reaction.

Prepare the following reaction in a 200 µl microcentrifuge tube.

mRNA	X µl
RNase free water	X µl
Oligo dT primers (100 µM)	0.5 µl
Total Volume	32.6 µl

2. Heat the mRNA at 65° C for 5 minutes and cool immediately on ice. Start the first-strand cDNA reaction (bellow) within 2 minutes of placing on ice.

3. During the incubation make the master solution using the table below.

dNTP (25 mM)	0,4 µl
5X buffer	10 µl
DTT 0.1M	5 µl
RNASin	1 µl
RT-MMLV	1 µl
Total Volume	17,4 µl

Mix the solution by inversion, then spin the tube at 1000 x g for 5 seconds.

4. Transfer 17.4 µl of the master solution to the first-strand reaction tube.
5. Incubate for 70 minutes at 37° C.
6. Stop the reaction by incubating 15 minutes at 70° C. Then store the tube at 4° C.

Alternatively the whole reaction can be carried out using a thermal cycler. Use the following programme:

1 cycle of: 65° C for 5 minutes
Pause
1 cycle of: 37° C for 70 minutes, 70° C for 15 minutes, hold at 4° C.

VII - PCR Amplification

The first-strand cDNA is used as a template for PCR amplification to generate cytokine specific DNA fragment. IFNγ mRNA will be the target mRNA during the workshop (Fig. 1, Fig. 2).

It may be a good idea to quantitate a house-keeping gene mRNA as well as target mRNA. This will correct for unequal amounts of mRNA in total RNA. Good house-keeping genes are β-actin, APRT, HPRT or glucose-6-phosphate dehydrogenase.
Note: β-actin and G6PD are high expression, HPRT and APRT are low expression. Standardize with a housekeeping gene which is similar to the expression of the gene of interest.

Since there may be some variability in the quality of RNA extraction from day-to-day, try to extract all samples to be analyzed in the same run, if possible.

During the workshop, we will use G3PDH mRNA as housekeeping gene mRNA (Fig. 3).

1. Label one tube as IFNγ, a second tube as positive control, a third tube as negative control and a fourth tube as G3PDH.
2. Add the reagents shown below to the appropriate tubes.

IFNγ

Sterile distilled water	39.6 µl
10X PCR buffer (without $MgCl_2$)	5 µl
$MgCl_2$ (50 mM)	1.5 µl
dNTP (25 mM)	0.4 µl
Primers 1 et 2 (7.5 µM each)	2 µl
Taq DNA polymerase (5 units/µl)	0.5 µl
First-strand reaction	1 µl
Total Volume	50 µl

Positive control

Sterile distilled water	39.6 µl
10X PCR buffer (without $MgCl_2$)	5 µl
$MgCl_2$ (50 mM)	1.5 µl
dNTP (25 mM)	0.4 µl
Primers 1 et 2 (7.5 µM each)	2 µl
Taq DNA polymerase (5 units/µl)	0.5 µl
dsDNA positive control	1 µl
Total Volume	50 µl

Negative control

Sterile distilled water	40.6 µl
10X PCR buffer (without $MgCl_2$)	5 µl
$MgCl_2$ (50 mM)	1.5 µl
dNTP (25 mM)	0.4 µl
Primers 1 et 2 (7.5 µM each)	2 µl
Taq DNA polymerase (5 units/µl)	0.5 µl
Total Volume	50 µl

G3PDH

Sterile distilled water	33.4 µl
10X PCR buffer (without $MgCl_2$)	5 µl
$MgCl_2$ (50 mM)	2 µl
dNTP (25 mM)	0.1 µl
Primer 1 (10 µM), Fig. 4	2 µl
Primer 2 (10 µM), Fig. 4	2 µl
Taq DNA polymerase (5 units/µl)	0.5 µl
First-strand reaction	5 µl
Total Volume	50 µl

3. Mix gently with a micropipette and spin the tubes at 1000 x g for 5 seconds. Overlay each reaction with 50 µl of mineral oil only if the addition of mineral oil is a requirement for your thermal cycler.
4. Place the tube in a thermal cycler and immediately run the following program:

IFNγ

1 cycle of:	94° C for 4 minutes
30 cycles of:	94° C for 45 seconds
	55° C for 45 seconds
	72° C for 45 seconds
1 cycle of:	72° C for 10 minutes
Hold at 4°C	

G3PDH

1 cycle of:	94° C for 2 minutes
30 cycles of:	94° C for 30 seconds
	61° C for 45 seconds
	72° C for 45 seconds
1 cycle of:	72° C for 10 minutes
Hold at 4°C	

VIII - Agarose gel electrophoresis of PCR amplified DNA fragment

Agarose gel electrophoresis of aliquots of the PCR products alongside a known amount of a standard PCR fragment provides a visual estimate of the relative amounts of the IFNγ or G3PDH fragments based on their band intensities in an ethidium bromide-stained gel.
It is important that the tubes with PCR products are not opened in the room where the PCR is set-up. Take tubes to a separate room.

1. Prior to use, prepare a 1.2 % agarose gel in 1X TBE containing 0.5 µg/ml ethidium bromide with wells sufficient to accomodate 15 µl samples.

2. Add 2 µl of the 6X loading dye to 10 µl of each amplification reaction.

3. Load each sample and 5 µl of Smart Ladder into separate wells of the agarose gel and electrophorese at 100 V for 1 hour.

4. Take a photograph with film that produces a positive image (Polaroid Type 66).

5. Proceed with data analysis (see example Fig. 5).

	Size of the PCR fragment
IFNγ DNA fragment	384 bp
G3PDH DNA fragment	450 bp
Positive control	300 bp

Figure 1: Mouse Interferon γ mRNA sequence.

```
LOCUS       XM_125899               1207 bp    mRNA    linear   ROD 24-FEB-2003
DEFINITION  Mus musculus interferon gamma (Ifng), mRNA.
ACCESSION   XM_125899
VERSION     XM_125899.2  GI:28501526
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 1207)
  AUTHORS   NCBI Annotation Project.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-FEB-2003) National Center for Biotechnology
            Information, NIH, Bethesda, MD 20894, USA
COMMENT     MODEL REFSEQ:  This record is predicted by automated computational
            analysis. This record is derived from an annotated genomic sequence
            (NT_039501) using gene prediction method: BLAST, supported by mRNA
            evidence.
            Also see:
                Documentation of NCBI's Annotation Process

            On Feb 24, 2003 this sequence version replaced gi:20866206.
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                     /strain="C57BL/6J"
                     /db_xref="taxon:10090"
                     /chromosome="10"
     gene            1..1207
                     /gene="Ifng"
                     /db_xref="LocusID:15978"
                     /db_xref="MGI:107656"
     CDS             109..576
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                     /codon_start=1
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                     /protein_id="XP_125899.1"
                     /db_xref="GI:20866207"
                     /db_xref="LocusID:15978"
                     /db_xref="MGI:107656"
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                     EEKSLFLDIWRNWQKDGDMKILQSQIISFYLRLFEVLKDNQAISNNISVIESHLITTF
                     FSNSKAKKDAFMSIAKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSRC"
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                     /gene="Ifng"
                     /note="IFN-gamma; Region: Interferon gamma"
                     /db_xref="CDD:pfam00714"

       1 atagctgcca tcggctgacc tagagaagac acatcagctg atcctttgga ccctctgact
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     121 cactgcatct tggctttgca gctcttcctc atggctgttt ctggctgtta ctgccacggc
     181 acagtcattg aaagcctaga aagtctgaat aactatttta actcaagtgg catagatgtg
     241 gaagaaaaga gtctcttctt ggatatctgg aggaactggc aaaaggatgg tgacatgaaa
     301 atcctgcaga gccagattat ctctttctac ctcagactct ttgaagtctt gaaagacaat
     361 caggccatca gcaacaacat aagcgtcatt gaatcacacc tgattactac cttcttcagc
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     481 caggtccagc gccaagcatt caatgagctc atccgagtgg tccaccagct gttgccggaa
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     661 tttaaaacta tttatatgga gaatctattt tagatgcatc aaccaaagaa gtatttatag
     721 taacaactta tatgtgataa gagtgaattc ctattaatat atgtgttatt tataatttct
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    1021 agacagcact cgaatgtgtc aggtagtaac aggctgtccc tgaaagaaag cagtgtctca
    1081 agagacttga cacctggtgc ttccctatac agctgaaaac tgtgactaca cccgaatgac
    1141 aaataactcg ctcatttata gtttatcact gtctaattgc atatgaataa agtatacctt
    1201 tgcaacc
```

Figure 2: Comparison between mouse and human Interferon γ sequences.

```
       S1
Mouse  MNATHCILALQLFLMAVS-GCYC
Human  .KY.SY...F..CIVLG.L....

       1       10                                              50
Mouse  HGTVIESLES LNNYFNAGHS DV-EEKSLFLD IWRNWQKDGD MKILQSQIIS
Human  QDPYVKEA.N .KK...SSGI ..ADNGT...G .LK..KEES. R..M....V.

                                                             100
Mouse  FYLRLFEVLK DNQAISNNIS VIESHLITTF FSNSKAKKDA FMSIAKFEVN
Human  ..FK..KNF. .D.S.QKSVE T.KEDMNVK. .NSN.K.R.D .EKLTNYS.T

              110
Mouse  NPQVQRQAFN ELIRVVHQLL PESSLRKRKR SRC
Human  DLN...K.IH ...Q.MAE.S .AAKTG.... .QM LFRGRRASQ
```

Figure 3: Mouse G3PDH gene sequence.

```
LOCUS       MUSGAPDH                1228 bp    mRNA    linear   ROD 12-JUN-1993
DEFINITION  Mouse glyceraldehyde-3-phosphate dehydrogenase mRNA, complete cds.
ACCESSION   M32599
VERSION     M32599.1  GI:193423
KEYWORDS    glyceraldehyde-3-phosphate dehydrogenase.
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 1228)
  AUTHORS   Sabath,D.E., Broome,H.E. and Prystowsky,M.B.
  TITLE     Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major
            interleukin 2-induced transcript in a cloned T-helper lymphocyte
  JOURNAL   Gene 91 (2), 185-191 (1990)
  MEDLINE   91007274
   PUBMED   2145197
COMMENT     Original source text: Mouse (strain C57BL/10J) adult lymphocyte,
            cDNA to mRNA, clones B7 and A3.
            Draft entry and computer-readable sequence for [Gene (1990) In
            press] kindly submitted
            by D.E.Sabath, 05-MAR-1990.
FEATURES             Location/Qualifiers
     source          1..1228
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                     /mol_type="mRNA"
                     /strain="C57BL/10J"
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                     /dev_stage="adult"
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     CDS             47..1048
                     /note="glyceraldehyde-3-phosphate dehydrogenase"
                     /codon_start=1
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                     /db_xref="GI:309243"
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                     FQYDSTHGKFNGTVKAENGKLVINGKPITIFQERDPTNIKWGEAGAEYVVESTGVFTT
                     MEKAGAHLKGGAKRVIISAPSADAPMFVMGVNHEKYDNSLKIVSNASCTTNCLAPLAK
                     VIHDNFGIVEGLMTTVHAITATQKTVDGPSGKLWRDGRGAAQNIIPASTGAAKAVGKV
                     IPELNGKLTGMAFRVPTPNVSVVDLTCRLEKPAKYDDIKKVVKQASEGPLKGILGYTE
                     DQVVSCDFNSNSHSSTFDAGAGIALNDNFVKLISWYDNEYGYSNRVVDLMAYMASKE"
```

```
ORIGIN
        1 acagccgcat cttcttgtgc agtgccagcc tcgtcccgta gacaaaatgg tgaaggtcgg
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      181 ccagtatgac tccactcacg gcaaattcaa cggcacagtc aaggccgaga atgggaagct
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     1141 tccatcccag acccccataa taacaggagg ggcctaggga gccctcccta ctctcttgaa
     1201 taccatcaat aaagttcgct gcacccac
```

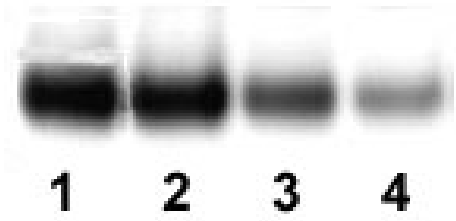
Figure 4: Primers used for G3PDH PCR amplification

primer GR:
TCCACCACCCTGTTGCTGTA

primer GF:
ACCACAGTCCATGCCATCAC

The size of the PCR fragment is 450 bp (566 - 1016).

Figure 5: Measurement of a RNA sample. The four bands are the standard bands. Lane 1: 250 pg; lane 2: 100 pg; lane 3: 50; lane 4: 25 pg.



The band intensities can be analyzed by ImageQuant software (Molecular Dynamics). A regression line is calculated or drawn.

Quantification of INFγ by Enzyme Linked Immunosorbent Assay (ELISA)

1. After overnight culture, centrifuge the T cells suspension for 5 minutes at 1200 rpm at 4° C.

2. Transfer the supernatant in sterile 15 ml tubes. The tubes are stored at 4° C until quantification step.

Plate Preparation:

3. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 µl per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.

4. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher.

5. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.

6. Block plates by adding 300 µl of Block Buffer to each well. Incubate at room temperature for a minimum of 1 hour.

7. Repeat the aspiration/wash as in step 4. The plates are now ready for sample addition.

Assay Procedure :

8. Add 100 µl of sample or standards in Reagent Diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.

9. Repeat the aspiration/wash as in step 4 of Plate Preparation.

10. Add 100 µl of the Detection Antibody, diluted in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.

11. Repeat the aspiration/wash as in step 2 of Plate Preparation.

12. Add 100 µl of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

13. Repeat the aspiration/wash as in step 4.

14. Add 100 µl of Substrate Solution to each well.

15. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

16. Add 50 µl of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

17. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

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Quantitative RT-PCR. Methods and Applications Book 3; Clontech Laboratories, Inc. 1993.

PCR quantitative. Techniques en Immunologie. Société Française d'Immunologie. Sonia Berrih-Aknin, ed. 2000.

Main materials required

Equipment

UV spectrophotometer.

Thermal cyclers.

Microcentrifuge with variable-speed control.

Microtiter plate reader.

Reagents and buffers

All water used in these protocols should be sterile, deionized, distilled water.
All reagents, plastic- and glassware should be sterile

Ethanol (75 %) keep at - 20°C.

Isopropanol à 80 %.

Taq DNA Polymerase (GeneAmp, Perkin-Elmer Cetus).

10 X TBE buffer: 0.89 M Tris; 0.89 M boric acid; 20 mM EDTA (pH ≈ 8.3).

Smart Ladder (Eurogentec).

Agarose NA (17-0554-01, -02, -03).

6X loading dye: 10 mM Tris-HCl (pH 7.6) containing 0.25 % bromophénol blue and xylene cyanol and 30 % glycerol.

$MgCl_2$ 25 mM (sterile aqueous solution).

dNTP (25 mM each dNTP in sterile water).

10X PCR buffer: 100 mM Tris-HCl, pH 8.3 (25°C), 500 mM KCl 0.01% (w/v) gelatine (Sigma, n°G2500).

TE buffer: 10 mM Tris-HCl (pH 8.0) et 0.1 mM EDTA.

Trizol (Invitrogen)

Microtiter paltes Maxisorb (Nunc, Danemark).

PBS: 0.15 M Sodium chloride containing 0.01 M phosphate buffer pH 7.4.

PBS-Tw: 0.15 M Sodium chloride containing 0.01 M phosphate buffer pH 7.4 and 0.05 % Tween 20.

PBS-Tw-BSA: PBS-Tw solution containing 0.1 % bovine albumin.

Enzyme-antibody conjugate diluted in PBS-BSA-Tw.

Peroxidase substrate: Tetramethyl benzidine and hygrogen peroxide.