LumiMax Superoxide Anion Detection Kit

Instruction Manual

Catalog #204525

Revision B

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204525-12

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LumiMax Superoxide Anion Detection Kit

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LumiMax Superoxide Anion Detection Kit

MATERIALS PROVIDED

Material provided	Concentration	Quantity
Enhancer solution	5.0 mM	0.5 ml[a]
Luminol solution	4.0 mM	0.5 ml[a]
Xanthine solution	1.0 mM	0.1 ml[b]
Xanthine oxidase	0.02 U/µl	20 µl[b]
Superoxide dismutase (SOD)	25 U/µl	20 µl[b]
SOA assay medium	—	25 ml
Xanthine assay medium	—	25 ml

[a] This quantity is sufficient for 100 reactions.

[b] This quantity is sufficient for 20 control reactions.

STORAGE CONDITIONS

Superoxide dismutase: –20°C
All other components: 4°C

ADDITIONAL MATERIALS REQUIRED

Supplemented growth medium (see *Preparation of Medium*)
Human γ-interferon
Dimethyl sulfoxide (DMSO)
Phorbol 12-myristate 13-actetate (PMA)[||]
(PMA is an activator of the intracellular enzyme protein kinase C and is a potent stimulator of NADPH oxidase, which synthesizes superoxide anions.[1, 2] Another stimulator of NADPH oxidase can be substituted for PMA.)
Luminometer
Polystyrene round-bottom tubes

[||] PMA is available from L C Laboratories in Woburn, Massachusetts.

Revision B

INTRODUCTION

Superoxide anion ($O_2^{-}\bullet$) is a short-lived radical of molecular oxygen that plays key roles in the immune system and intracellular functions. The enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase—a cytochrome-b-558-containing, plasma-membrane-bound enzyme complex—synthesizes superoxide anion by transferring an electron to molecular oxygen. Superoxide anion is a potent oxidant, which is released by leukocytes (such as monocytes, macrophages, and polymorphonuclear leukocytes)[3] to damage infectious organisms. Superoxide anion is also implicated in oxidative stress damage,[4] tumor promotion,[5] and cell growth and DNA synthesis,[6] which superoxide anion affects through the cell signaling pathway of Ras (the protein encoded by the protooncogene *ras*). The enzyme superoxide dismutase (SOD) suppresses the activity of superoxide anion by catalyzing the dismutation of $O_2^{-}\bullet$ into O_2 and H_2O_2.

The LumiMax superoxide anion detection kit qualitatively detects the presence of superoxide anions and superoxide dismutase in cell cultures with a higher sensitivity than the standard luminol-based assay.[7] The principles for detecting superoxide anion using the enhanced assay are the same as those for the standard assay: superoxide anion oxidizes luminol in a reaction that produces photons of light that are readily measured with a standard luminometer.[8, 9, 10] The LumiMax superoxide anion detection kit contains an enhancer that increases the sensitivity of the assay by amplifying the chemiluminescence, which allows the measurement of lower concentrations of superoxide anion than the standard assay. Unlike some enhancing reagents such as the phenol-based compound iodophenol, the enhancer can be used to assay living cells because it is nontoxic and does not denature components of the subcellular systems.

The LumiMax superoxide anion detection kit can be used to detect superoxide anion activity in any preparation that contains a suspected generator of superoxide anions. The protocols in this manual are optimized for tissue culture cells. Figure 1 illustrates this unique, rapidly performed method for detecting superoxide anions in cell culture.

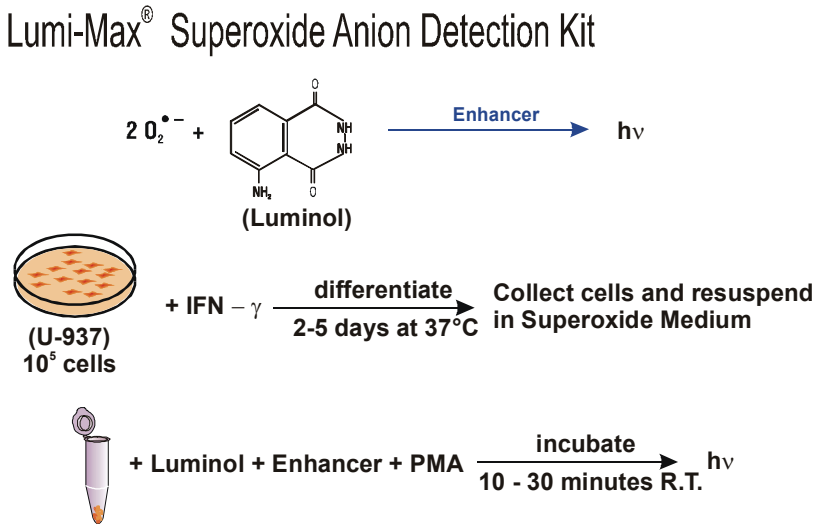

Figure 1 LumiMax superoxide anion detection kit is based on the oxidation of luminol by superoxide anion, which is a chemiluminescent reaction. The unique feature of this system is the noncytotoxic and nondenaturing enhancement reagent that amplifies the chemiluminescent signal by several fold. The protocol is simple: The cells are harvested, then luminol, PMA (a stimulating reagent), and the enhancer are added to the cells. After a short incubation period, the chemiluminescent signal is recorded using a standard luminometer. *IFN-γ*, human γ-interferon (IFN-γ is a differentiator that stimulates NADPH oxidase activity); *h ν*, light (chemiluminescence); *RT*, room temperature.

TISSUE CULTURE CONDITIONS

1. Seed 25 ml of supplemented growth medium with ~1.25 × 10^7 cells (resulting in a concentration of ~5.0 × 10^5 cells/ml).

2. Add 500–1000 U (20–40 U/ml) of human γ-interferon (a differentiator) to the flask.

   Note *If human γ-interferon is not an appropriate differentiator for the cell line of interest, use an appropriate differentiator (e.g., DMSO or retinoic acid).*

3. Incubate the cells for 2–5 days in a 37°C incubator with 5% carbon dioxide to allow the cells to differentiate. The differentiation process is different for each cell line. To maintain the health of the cells, replace the growth medium with fresh supplemented growth medium that contains human γ-interferon twice per week.

ASSAY FOR SUPEROXIDE ANION

Preparing the Tissue Culture Cells

1. Collect ~5.0 × 10^6 cells in a conical tube by spinning the cell suspension in a centrifuge at 3000 × *g* for 5 minutes.

2. Resuspend the cells in 1 ml of fresh supplemented growth medium to increase the reactivity of the cells.

3. Incubate the cells for an additional 30 minutes at 37°C.

4. Aliquot ~5.0 × 10^5 cells (100 µl) in a microcentrifuge tube (As few as 1 × 10^5 cells can be assayed successfully.)

5. Spin the tube in a microcentrifuge at 1600 rpm for 2 minutes. Remove and discard the supernatant.

6. Resuspend the cells in 100 µl of SOA assay medium.

Preparing the Reagents for the Superoxide Anion Assay

Warning *PMA is a potent carcinogen. Follow the manufacturer's safety recommendations.*

Note *Prepare and assay one reaction at a time. Store all the provided solutions on ice while preparing and assaying the reactions.*

1. Dilute 5.0 µl of 4.0 mM luminol solution with 89 µl of SOA assay medium.

2. Add 5.0 µl of 5.0 mM enhancer solution to the mixture of luminol and SOA assay medium.

3. Prepare a 1 mg/ml solution of PMA in DMSO and vortex it vigorously until the PMA dissolves. To prevent PMA hydrolysis in water, dilute this solution to a concentration of 20 µg/ml PMA with chilled 150 mM NaCl. Vortex the diluted PMA solution for 15 seconds. The diluted PMA solution should be stored at room temperature and used within 1 hour. Add 1 µl of the diluted PMA solution to the mixture of luminol, enhancer, and SOA assay medium.

   Notes *This solution of luminol, enhancer, and PMA in SOA assay medium is referred to as the SOA assay medium–reagent mixture. The concentrations of luminol, enhancer, and PMA in this 100-µl mixture are 200 µM, 250 µM, and 200 ng/ml, respectively.*

   The effective concentration of the enhancer ranges from 0.01 to 2.5 mM. The enhancer may require titration within this range to optimize the chemiluminescence.

   Another stimulator of NADPH oxidase can be substituted for PMA. The concentration of the substituted NADPH oxidase stimulator will require optimization.

Superoxide Anion Assay Protocol

Note *For comparison, unenhanced control samples and control samples without PMA should be assayed along side of the enhanced samples. The enhanced samples should emit light at an intensity several fold higher than the control samples. To prepare the unenhanced control samples, replace the 5.0 µl of enhancer with 5.0 µl of SOA assay medium. To prepare the control samples without the PMA, replace the 1 µl of PMA with 1 µl of SOA assay medium.*

1. Add 100 µl of the SOA assay medium–reagent mixture to the cells suspended in 100 µl of SOA assay medium.

   Note *The final volume of the reaction is 200 µl, comprised of 100 µl of 5 × 10^5 cells suspended in SOA assay medium and 100 µl of the SOA assay medium–reagent mixture. The final concentrations of luminol, the enhancer, and PMA in the 200-µl reaction are 100 µM, 125 µM, and 100 ng/ml, respectively.*

2. Incubate the sample for 10–30 minutes at room temperature.

   Note *The length of the incubation may need to be optimized. Some types of cells or generators of superoxide anions may require a longer incubation.*

3. Transfer the sample to the bottom of a polystyrene round-bottom tube. Avoid introducing bubbles into the sample during pipetting; bubbles in the sample can result in inconsistent measurements.

4. Place the sample in a luminometer and record the light emission at regular intervals.

Results Expected from the Assay for Superoxide Anion

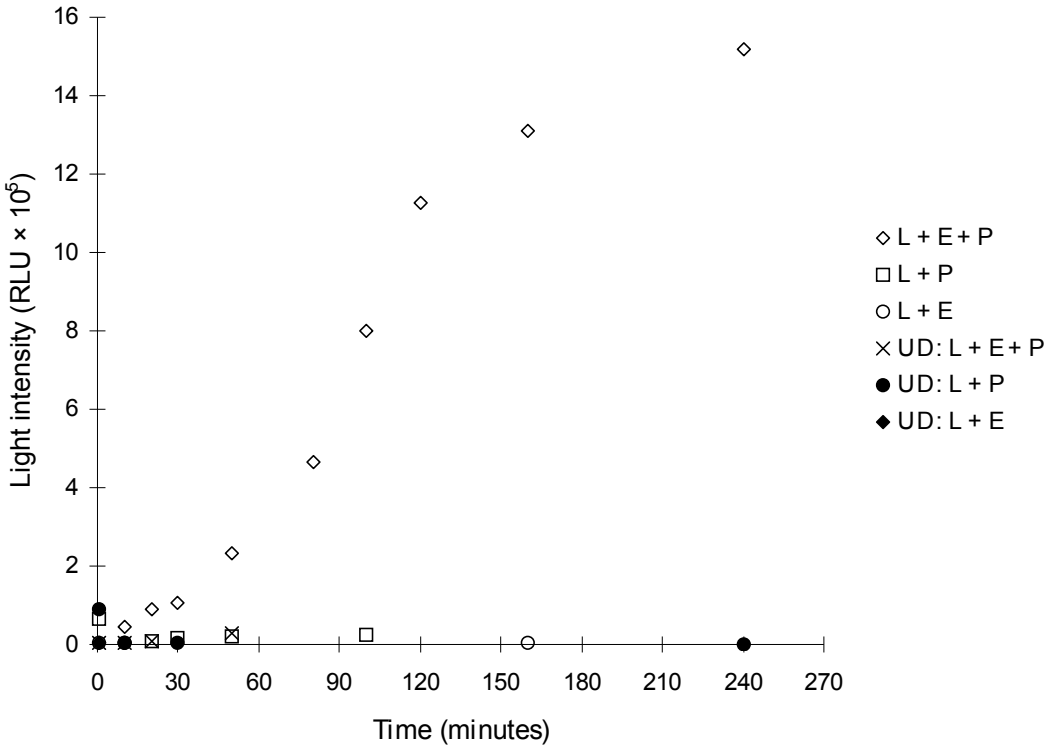

Figure 2 The light detected in the assay for superoxide anion activity during a four-hour period. *RLU*, relative light units; *L*, luminol; *E*, enhancer; *P*, PMA; *UD*, undifferentiated. (The light intensities were 64157, 8065, 15091, 19359, and 22727 RLU for the *L+P* assay at 0.5, 20, 30, 50, and 100 minutes, respectively; 2709, 2869, and 2578 RLU for the *L+E* assay at 0.5, 10, and 160 minutes, respectively; 2654, 2870, 9071, and 27190 RLU for the *UD:L+E+P* assay at 0.5, 10, 20, and 50 minutes, respectively; 90617, 4802, 2869, and 1585 RLU for the *UD:L+P* assay at 0.5, 10, 30, and 240 minutes, respectively; and 2709 and 2869 RLU for the *UD:L+E* assay at 0.5 and 10 minutes, respectively.)

Methods U-937 cells were assayed for superoxide anion activity after incubating for 2–5 days in supplemented growth medium that contained human γ-interferon. After two days, superoxide anion activity was minimal. After three days, the U-937 cells demonstrated superoxide anion activity in response to stimulation with PMA (100 ng/ml).

ASSAY FOR THE ACTIVITY OF SUPEROXIDE DISMUTASE

Superoxide dismutase catalyzes the dismutation of $O_2^{-}\bullet$ into O_2 and H_2O_2. This protocol can be used to assay cell supernatant suspected to contain SOD or to produce a positive control reaction for superoxide dismutase activity, for which SOD is provided. The addition of SOD to the xanthine oxidase–xanthine–luminol reaction results in a decrease of the superoxide anion concentration, which in turn leads to a decrease in oxidation of luminol and reduced chemiluminescence.

Preparing the Reagents

Note *Prepare and assay one reaction at a time. Store all the provided solutions on ice while preparing and assaying the reactions.*

1. Add 1 µl of xanthine oxidase and 5 µl of 4.0 mM luminol solution to 94 µl of xanthine assay medium.

2. Prepare a 50 µM xanthine solution by adding 5.0 µl of the 1.0 mM xanthine solution to 95 µl of xanthine assay medium.

3. Prepare the supernatant of the cells suspected to contain SOD according to an appropriate protocol. The total protein content of the cell supernatant should be 0.8–40.0 µg. Additional information about the preparation of the cell supernatant can be found in references 11, 12, 13.

Superoxide Dismutase Assay Protocol

Note *Avoid introducing bubbles into the sample during pipetting; bubbles in the sample can result in inconsistent measurements.*

1. Add 100 µl of xanthine oxidase–luminol solution to the bottom of a polystyrene round-bottom tube.

2. Add 100 µl of the 50 µM xanthine solution to the tube to initiate the reaction.

   Note *The final concentrations of xanthine, xanthine oxidase, and luminol in the 200-µl reaction are 25 µM, 0.1 mU/µl, and 100 µM, respectively.*

3. Add either 5–40 µl of the cell supernatant that is suspected to contain SOD or 1 µl of the provided SOD to the tube.

4. Place the tube in a luminometer.

5. Record the light emission at 30-second intervals (from the time the reactants are combined) for 2 minutes.

Results Expected from the Assay for Superoxide Dismutase

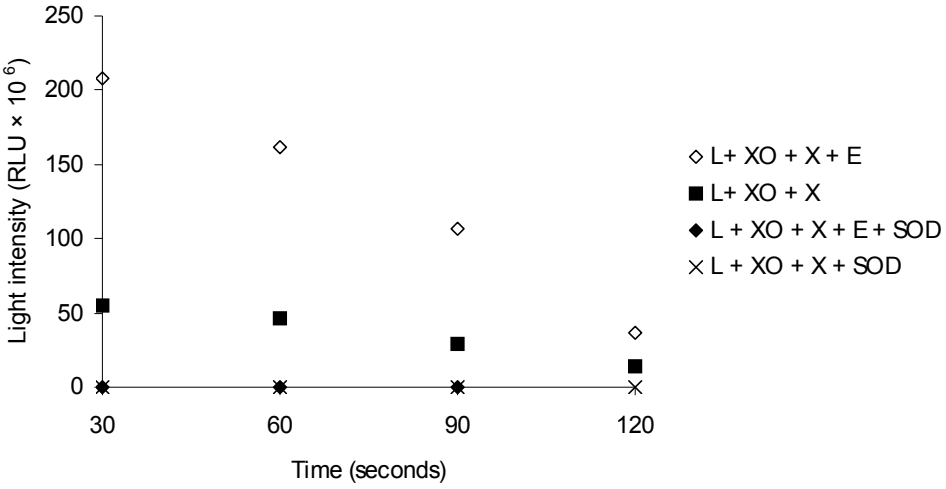

Figure 3 Light emitted as a result of the xanthine–xanthine oxidase reaction. Superoxide dismutase suppresses the activity of the superoxide anions generated in the reaction. *RLU*, relative light units; *L*, luminol; *XO*, xanthine oxidase; *X*, xanthine; *E*, enhancer; *SOD*, superoxide dismutase. (The light intensities were 8413, 4090, and 2223 RLU for the *L+XO+X+E+SOD* assay and 6222, 4870, 4446, and 3996 RLU for the *L+XO+X+SOD* assay at 30, 60, 90, and 120 seconds, respectively.)

OPTIONAL CONTROL EXPERIMENT: XANTHINE–XANTHINE OXIDASE REACTION

The reaction between xanthine and xanthine oxidase generates superoxide anions.[14, 15] The reactants xanthine and xanthine oxidase are provided to produce a positive control reaction that demonstrates superoxide anions oxidize luminol.

Definition *One unit of xanthine oxidase activity converts 1.0 µmol of xanthine to uric acid and forms 2 µmol of superoxide anion per minute.*

Preparing the Reagents for the Xanthine–Xanthine Oxidase Reaction

Note *Prepare and assay one reaction at a time. Store all the provided solutions on ice while preparing and assaying the reactions.*

Control Solutions

1. Prepare a xanthine oxidase–luminol–enhancer solution by adding 1 µl of xanthine oxidase, 5 µl of 4.0 mM luminol solution, and 5 µl of 5.0 mM enhancer solution to 89 µl of xanthine assay medium.

2. Prepare a xanthine oxidase–enhancer solution by adding 1 µl of xanthine oxidase and 5 µl of 5.0 mM enhancer solution to 94 µl of xanthine assay medium.

3. Prepare a xanthine oxidase solution by adding 1 µl of xanthine oxidase to 99 µl of xanthine assay medium.

Xanthine Solution

Prepare a 50 µM xanthine solution by adding 5.0 µl of the 1.0 mM xanthine solution to 95 µl of xanthine assay medium.

Xanthine–Xanthine Oxidase Reaction Protocol

Note *Avoid introducing bubbles into the sample during pipetting; bubbles in the sample can result in inconsistent measurements.*

1. Add 100 µl of a control solution to the bottom of a polystyrene round-bottom tube.

2. Add 100 µl of the 50 µM xanthine solution to the tube of a control solution to initiate the reaction.

   Notes *The concentrations of xanthine, xanthine oxidase, luminol, and enhancer in the 200-µl reaction are 25 µM, 0.1 mU/µl, 100 µM, and 125 µM, respectively.*

3. Immediately place the tube in a luminometer.

4. Record the light emission at 30-second intervals (from the time the reactants are combined) for 2 minutes.

5. Repeat steps 1–4 for each control solution.

Results Expected from the Xanthine–Xanthine Oxidase Reaction

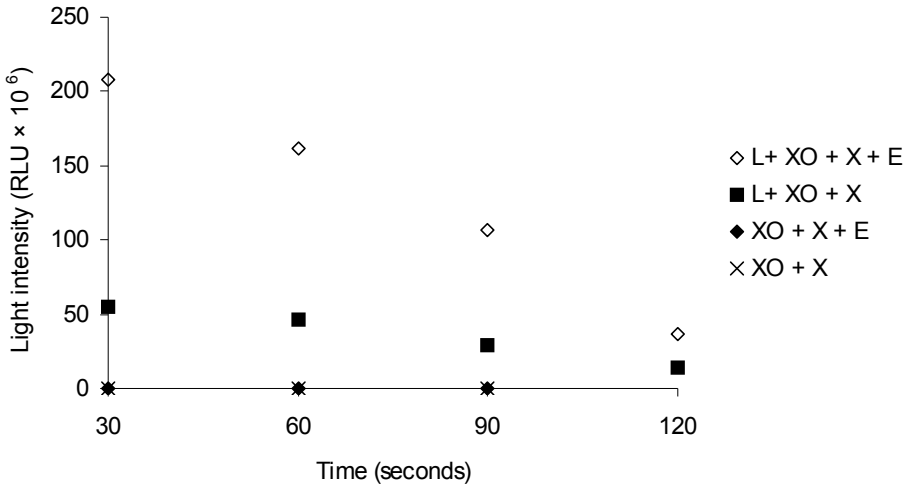

Figure 4 The light emitted as a result of the xanthine–xanthine oxidase reaction. *RLU*, relative light units; *L*, luminol; *XO*, xanthine oxidase; *X*, xanthine; *E*, enhancer. (The light intensities were 9284, 5685, and 2829 RLU for the *XO+X+E* assay and 2222, 1958, and 1853 RLU for the *XO+X* assay at 30, 60, 90, and 120 seconds, respectively.)

PREPARATION OF MEDIA

Supplemented Growth Medium

RPMI 1640 medium with glucose and glutamine
10% fetal bovine serum
50 U/ml penicillin–streptomycin
1 mM sodium pyruvate
0.1 mM non-essential amino acids

TROUBLESHOOTING

Observation	Suggestion
Low light emission	Sample may not generate superoxide anions. Perform a xanthine–xanthine oxidase reaction as a positive control for the assay.
Enhanced assay does not yield a more robust signal than the unenhanced assay	Sample may not generate superoxide anions. Perform a xanthine–xanthine oxidase reaction as a positive control for the assay.
	Oxidation of luminol by superoxide anion is time-dependent. Perform a time-course assay, recording the light emission at 30-second intervals for 30 minutes.
	Concentration of the enhancer may be too low. Titrate the amount of enhancer per assay. The working concentration for the enhancer ranges from 0.01–2.5 mM.
	A reagent (e.g. luminol) may have been omitted. Repeat the assay, verifying that each reagent is added.
Light emission for the unenhanced sample peaks before that of the enhanced sample	Enhancer acts as a catalyst and requires time to act. Observe the light emission during a 30-minute period. The unenhanced sample may peak initially but will readily decrease compared to an enhanced sample, which increases with time.
Xanthine–xanthine oxidase control reaction yields low light emission	A reagent (e.g. luminol) may have been omitted. Repeat the assay, verifying that each reagent is added.
The addition of cell supernatant containing SOD does not decrease the light emission	SOD is degraded if the cell supernatant is not stored properly at –20°C. Perform a xanthine–xanthine oxidase–luminol control reaction with freshly prepared cell supernatant.

REFERENCES

1. Pfefferkorn, L. C., Guyre, P. M. and Fanger, M. W. (1990) *Mol Immunol* 27(3):263-72.
2. Corbisier, P., Houbion, A. and Remacle, J. (1987) *Anal Biochem* 164(1):240-7.
3. Tanaka, M., Sotomatsu, A., Yoshida, T., Hirai, S. and Nishida, A. (1994) *J Neurochem* 63(1):266-70.
4. Stohs, S. J. (1995) *J Basic Clin Physiol Pharmacol* 6(3-4):205-28.
5. Kozumbo, W. J., Trush, M. A. and Kensler, T. W. (1985) *Chem Biol Interact* 54(2):199-207.
6. Irani, K., Xia, Y., Zweier, J. L., Sollott, S. J., Der, C. J. *et al.* (1997) *Science* 275(5306):1649-52.
7. Hoang, D. Q. and Pfefferkorn, L. C. (1998) *Strategies* 11(1):1-2.
8. Allred, C. D., Margetts, J. and Hill, H. R. (1980) *Biochim Biophys Acta* 631(2):380-5.
9. Vilim, V. and Wilhelm, J. (1989) *Free Radic Biol Med* 6(6):623-9.
10. Wang, J. F., Komarov, P., Sies, H. and de Groot, H. (1991) *Biochem J* 279(Pt 1):311-4.
11. Nakano, M., Kimura, H., Hara, M., Kuroiwa, M., Kato, M. *et al.* (1990) *Anal Biochem* 187(2):277-80.
12. Somville, M., Houben, A., Raes, M., Houbion, A., Henin, V. *et al.* (1985) *Mech Ageing Dev* 29(1):35-51.
13. Nebot, C., Moutet, M., Huet, P., Xu, J. Z., Yadan, J. C. *et al.* (1993) *Anal Biochem* 214(2):442-51.
14. van Klaveren, R. J., Roelant, C., Boogaerts, M., Demedts, M. and Nemery, B. (1997) *Thorax* 52(5):465-71.
15. Huu, T. P., Marquetty, C., Pasquier, C. and Hakim, J. (1984) *Anal Biochem* 142(2):467-72.

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