8-iso-Prostaglandin $F_{2\alpha}$ ELISA Kit

Catalog Number: EKS-200

TOLL FREE 1-800-661-4978 FAX 250-744-2877
www.stressgen.com/reagents

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Assay Design

Stressgen's StressXpress™ 8-iso-PGF$_{2\alpha}$ ELISA Kit provides a method to quantitate free 8-iso-Prostaglandin F$_{2\alpha}$ in biological fluids. This kit is a competitive immunoassay. The assay uses a rabbit polyclonal antibody specific for 8-iso-PGF$_{2\alpha}$ to bind competitively to either 8-iso-PGF$_{2\alpha}$ in the sample or to 8-iso-PGF$_{2\alpha}$ covalently attached to alkaline phosphatase. After a simultaneous incubation at room temperature, the excess reagents are washed away and p-nitrophenly phosphate substrate is added. The enzyme reaction is stopped with an acid stop solution which converts the end point colour to yellow. The intensity of the bound yellow color is inversely proportional to the concentration of 8-iso-PGF$_{2\alpha}$ in either standards or samples and is measured on a microplate reader at 405nm. 8-iso-PGF$_{2\alpha}$ concentrations of the samples are quantitated by interpolating absorbance readings from a standard curve generated with the calibrated 8-iso-PGF$_{2\alpha}$ standard provided.

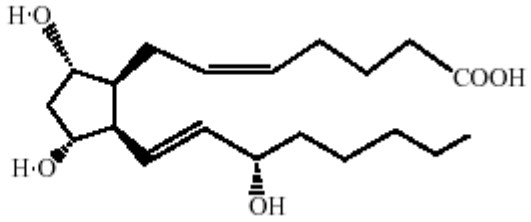
Scientific Overview

8-epimer of Prostaglandin F$_{2\alpha}$ (8-iso-PGF$_{2\alpha}$) is a major isoprostane that is mainly produced by non-enzymatic free radical-induced perioxidation of arachidonic acid (AA) present in phospholipids[1,2,3]. 8-iso-PGF$_{2\alpha}$ is also produced as a minor product of the COX-1 enzyme in human platelets[4] and the COX-2 isoform in human monocytes[5].

8-iso-PGF$_{2\alpha}$ has been found to be a potent constrictor of the renal and pulmonary vasculature and a broncoconstrictor[6,7]. It mediates smooth muscle cell growth, activates platelets[8] and induces derangement of the endothelial cell barrier function[9]. 8-iso-PGF$_{2\alpha}$ has also been suggested to participate as a pathophysiological mediator and to modify the fluidity and integrity of membranes[10].

8-iso-F$_{2\alpha}$ has been shown to circulate in plasma and be excreted in urine[11]. Elevated plasma, serum and urine levels of 8-iso-PGF$_{2\alpha}$ have been shown to be associated with cardiovascular risk factors such as cigarette smoking, hypercholesterolemia[12] and hyperhomocysteinemia[13]. Enhanced urinary excretion of 8-iso-PGF$_{2\alpha}$ has been described in association with both type 1 and type 2 diabetis mellitus and correlated with impaired glycemic control[14]. Elevated plasma content of free 8-iso-F$_{2\alpha}$ has been reported in women with preeclampsia and recent data indicates that 8-iso-PGF$_{2\alpha}$ may have a potential role in reduced trophoblast invasion in this condition[15, 16, 17].

8-iso-Prostaglandin F$_{2\alpha}$



Precautions

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.

- Some components in this kit contain azide, which may react with lead or copper plumbing. Always flush with large volumes of water when disposing of reagents to prevent azide build-up.
- The Stop Solution (part# 200-P8) is a solution of trisodium phosphate. This solution is caustic; please handle with care and keep tightly capped.
- The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions and is also affected by high concentrations of chelators, such as EDTA and EGTA.
- The 8-iso-PGF$_{2\alpha}$ Standard provided (part# 200-P6) is formatted in ethanolic buffer at a pH optimized to maintain 8-iso-PGF$_{2\alpha}$ integrity. Due to the possible known and unknown effects of prostaglandins, please handle this material with care.
- Kit performance has been tested with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
- Please read the complete kit insert before performing this assay.
- The components in each kit lot # have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot #s.

Materials Provided

Stressgen's StressXpress™ 8-iso-Prostaglandin $F_{2\alpha}$ ELISA Kit contains the following components in sufficient quantities for 96 wells.

Part Number	Component	Size	Description
200-P1	Goat anti-Rabbit IgG Microtiter Plate	96 well plate	12x8 removable strips and frame. Pre-coated plate with goat polyclonal antibody specific for rabbit IgG.
200-P2	8-iso-PGF$_{2\alpha}$ ELISAConjugate	5mL	Blue solution of 8-iso-PGF$_{2\alpha}$ conjugated to alkaline phosphatase.
200-P3	8-iso-PGF$_{2\alpha}$ ELISA Antibody	5mL	Yellow solution of Rabbit polyclonal antibody specific for 8-iso-PGF$_{2\alpha}$.
200-P4	Assay Buffer	30mL	Tris buffered saline containing proteins and detergents and sodium azide as a preservative.
200-P5	Wash Buffer Concentrate	30mL	Concentrated solution of Tris buffered saline containing detergents and sodium azide as a preservative.
200-P6	8-iso-PGF$_{2\alpha}$ Standard	0.5mL	1,000,000 pg/mL solution of 8-iso-PGF$_{2\alpha}$.
200-P7	p-Npp Substrate	20mL	Ready to use solution of p-nitrophenyl phosphate in buffer.
200-P8	Stop Solution	5mL	Solution of trisodium phosphate in water. **Caution**: Caustic. Keep tightly capped.
200-P9	Plate Sealer	1	Adhesive sealer to cover plate.

Storage of Materials

All components of this kit are stable at 4°C until the kit's expiration date.

Unused wells of the Goat anti-Rabbit IgG Microtiter Plate should be resealed in the foil bag provided with desiccant and stored at 4°C.

Materials Needed but Not Supplied

- Deionized or distilled water
- Precision pipettes capable for accurately delivering volumes between 5 µL and 1,000 µL
- Repeater pipettes for delivering 50 µL and 200 µL
- A disposable beaker for diluting buffer concentrates
- Graduated cylinders
- A microplate shaker
- Adsorbent paper for blotting
- Microplate reader capable of reading at 405 nm, preferably with correction at between 570 and 590 nm

Critical Assay Parameters and Notes

- This kit contains a pre-coated microtiter plate with removable wells to allow assaying on separate occasions. Unused wells must be kept desiccated at 4°C in the sealed foil bag. The wells should be used in the frame provided.
- Exercise appropriate laboratory safety precautions when performing this assay.
- Allow reagents to warm to room temperature at least 30 minutes prior to opening.
- **Care must be taken to minimize contamination by endogenous alkaline phosphatase**. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
- To avoid cross contamination, change disposable pipette tips between the addition of each standard, samples, and reagents and add the reagents to the side of the wells. Use separate reagent troughs/reservoirs for each reagent.
- This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated and pipette tips are pre-rinsed with the reagent.
- Pipet standards and samples to the bottom of the wells.
- **Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**
- The components in each kit lot # have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot #s. It is recommended that the reagents are not used beyond the kit expiration date.
- Run both standards and samples in duplicate.
- Include a standard curve each time the assay is performed.
- Standards can be made up in either glass or plastic tubes.

Sample Preparation

The StressXpress™ 8-iso-PGF$_{2\alpha}$ Detection Kit can be used to quantitate 8-iso-PGF$_{2\alpha}$ in a wide variety of sample types, if they are diluted using the Assay Buffer provided. Suggested starting dilutions are provided in the Sample Recovery section on page 10.

Note: ***Samples containing rabbit IgG may interfere with the assay.***

Tissue Culture Media

Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted in the tissue culture media instead of Assay Buffer. There will be a small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of 8-iso-PGF$_{2\alpha}$ in the appropriate matrix.

Tissue, Urine and Plasma

For tissue, urine and plasma samples, prostaglandin synthetase inhibitors, such as indomethacin or meclofenamic acid, at concentrations up to 10 µg/mL should be added to either the tissue homogenate or urine and plasma samples. Urine samples may be used in the assay directly by diluting 1:100 in Assay Buffer. Plasma samples normally have very low levels of 8-iso-PGF$_{2\alpha}$ present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below.

Extraction of Samples

Materials Needed

1. High Specific Activity Tritiated Prostaglandin. Activity should be >3.5 TBq/mmol. Sufficient labeled 8-iso-PGF$_{2\alpha}$ should be added to allow extraction efficiency to be accurately determined.
2. 2M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.
3. 200 mg C_{18} Reverse Phase Extraction Columns.

Extraction Protocol

1. Acidify the plasma, urine or tissue homogenate by addition of 2M HCl to pH of 3.5. Approximately 50 µL of HCl will be needed per mL of plasma.
2. Incubate at 4ºC for 15 minutes.
3. Microcentrifuge samples for 2 minutes to remove any precipitate.
4. Prepare the C_{18} reverse phase column by washing with 10 mL of ethanol followed by 10 mL of deionized water.
5. Add the sample to the column under a slight positive pressure to obtain a flow rate of about 0.5 mL/minute
6. Wash the column with 10 mL of water, followed by 10 mL of 15% ethanol, and finally 10 mL hexane.
7. Elute the sample from the column by addition of 10 mL ethyl acetate.
8. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen.
9. Add 50 µL ethanol to the dried samples and reconstitute sample with at least 200 µL of Assay Buffer. If analysis is to be delayed, store samples as the eluted ethyl acetate solutions at -80°C until the immunoassay is to be run.
10. Evaporate the organic solvent under a stream of nitrogen prior to running assay and reconstitute as above. Please refer to references 18-21 for details of extraction protocols.

Reagent Preparation

Diluted Standard should be used within 60 minutes of preparation.

1. 8-iso-PGF$_{2\alpha}$ Standard (Part# 200-P6)

a) Bring the 8-iso-PGF$_{2\alpha}$ Standard solution to room temperature.
b) Label eight 12 x 75 mm glass Tubes #1 through #8.
c) Add 900 µL of standard diluent (Assay Buffer or Tissue Culture Media) into Tube #1.
d) Add 750 µL of standard diluent into Tubes #2 through #8.
e) Add 100 µL of the 8-iso-PGF$_{2\alpha}$ Standard (1,000,000 pg/mL) to Tube #1 (100,000 pg/mL). Vortex thoroughly.
f) Add 250µL of Tube #1 (100,000 pg/mL) to Tube #2 (25,000pg/mL). Vortex thoroughly.
g) Similarly, complete the dilution series to generate the remaining standards (250 µL from Tube #2 to Tube #3, vortex thoroughly, etc) up to and including Tube#8

The concentration of 8-iso-PGF$_{2\alpha}$ in Tubes #1 through #8 will be 100,000, 25,000, 6,250, 1,563, 391, 98, 24 and 6 pg/mL respectively.

2. Wash Buffer (Part# 200-P5)

a) Allow the Wash Buffer Concentrate to warm to room temperature.
b) Dilute the 30mL of Wash Buffer Concentrate with 270mL of deionized water. This can be stored at room temperature for up to 3 months.

Assay Procedure

Allow all reagents to warm to room temperature for at least 30 minutes prior to opening.
All standards and samples should be run in duplicate.

1. Determine the required number of wells to be used. Remove excess wells from the frame and reseal with the desiccant back into the foil pouch provided. Store unused wells at 4°C.

2. Add 100 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the Non-Specific Binding (NSB) and the Bo (0 pg/mL Standard) wells.

3. Add 100 µL of Standards #1 through #8 into the appropriate wells.

4. Add 100 µL of the Samples into the appropriate wells.

5. Add 50 µL of Assay Buffer into the NSB wells.

6. Add 50 µL of blue 8-iso-PGF$_{2\alpha}$ ELISA Conjugate into each well, except the Total Activity (TA) and Blank wells.

7. Add 50 µL of yellow 8-iso-PGF$_{2\alpha}$ ELISA Antibody into each well, except the Blank, TA and NSB wells.

Note: *Every well used should be **Green** in color except the NSB wells which should be **Blue**. The Blank and TA wells are empty at this point and have no color.*

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. Cover the plate with the Plate Sealer provided, if so desired.

9. Empty the contents of the wells and wash by adding 200 µL of wash solution to every well. Repeat the wash 2 more times for a total of **3 Washes.**

10. After the final wash, empty or apsirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

11. Add 5 µL of the blue 8-iso-PGF$_{2\alpha}$ ELISA Conjugate to the TA wells.

12. Add 200 µL of the p-Npp Substrate solution to each well. Incubate at room temperature for 45 minutes without shaking.

13. Add 50 µL of Stop Solution to each well. This stops the reaction and the plate should be read immediately.

14. Blank the plate reader against the Blank wells, read the optical density at 405nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

Calculation of Results

The concentration of 8-iso-PGF$_{2\alpha}$ can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:
   Average Net OD = Average Bound OD - Average NSB OD

2 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

$$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. To generate the standard curve, plot on a log scale the 8-iso-PGF$_{2\alpha}$ Standard Concentration (pg/mL) on the X-axis and the Percent Bound (%) on the Y-axis. Determine the best fit line.

4. Interpolate the sample concentrations of 8-iso-PGF$_{2\alpha}$ from the standard curve.

Note: *Manufacturers of microplate readers usually offer accompanying software programs that will analyze data, plot standard curves and calculate sample concentrations. To set up the program for calculating the results, consult with the software instruction manual or contact the manufacturer of the microplate reader.*

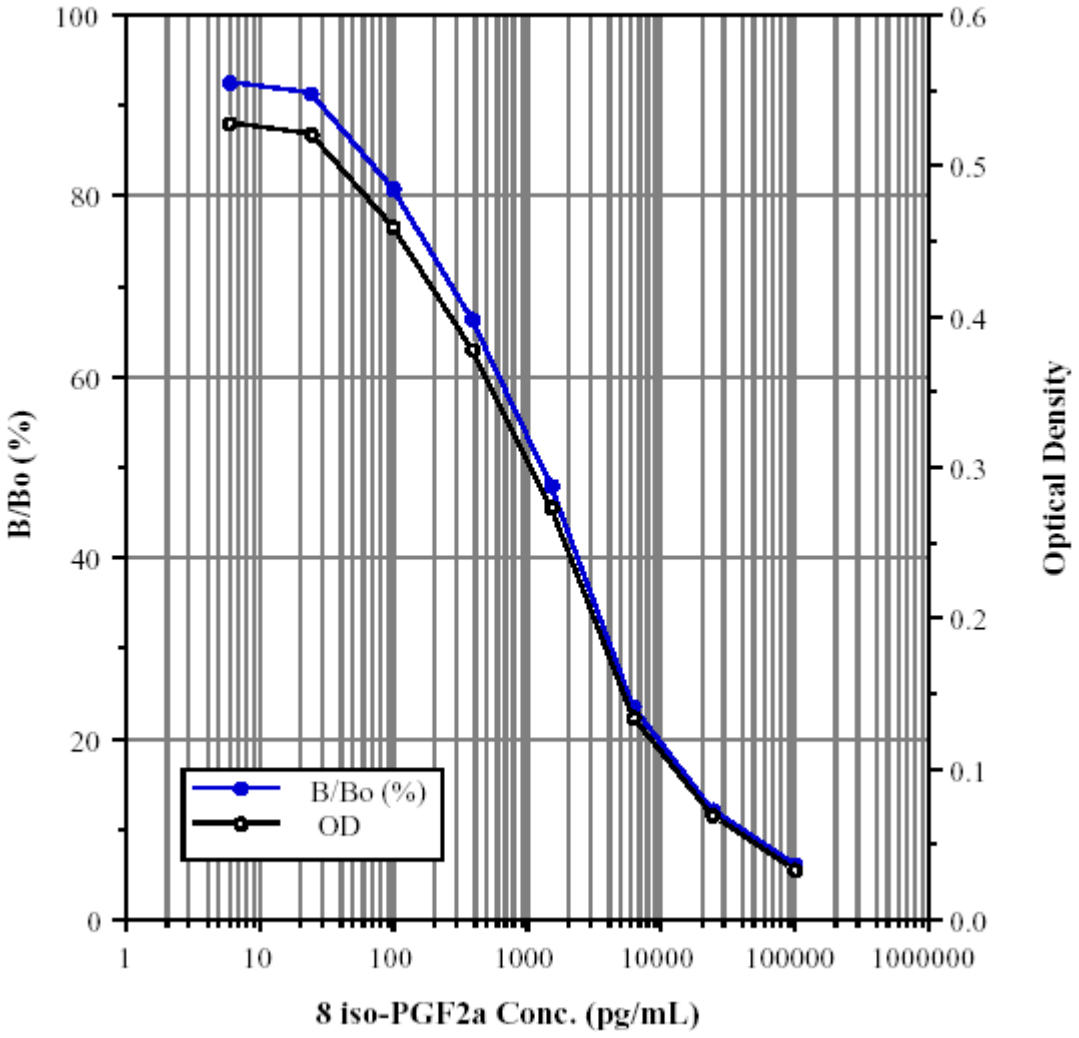
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Mean OD (-Blank)	Average Net OD	Percent Bound	**8-iso-PGF$_{2\alpha}$ (pg/mL)**
Blank OD	(0.073)			
TA	0.341	0.341		
NSB	0.000	0.000	0.00%	
Bo	0.570	0.570	100%	**0**
S1	0.033	0.033	5.7%	**100,000**
S2	0.068	0.068	11.9%	**25,000**
S3	0.133	0.133	23.4%	**6,250**
S4	0.272	0.272	47.8%	**1,562.5**
S5	0.378	0.378	66.3%	**390.6**
S6	0.459	0.459	80.6%	**97.7**
S7	0.520	0.520	91.3%	**24.4**
S8	0.528	0.528	92.6%	**6.1**
Unknown 1	0.100	0.102	26.56%	**10,877**
Unknown 2	0.281	0.283	73.70%	**7,479**

Typical Standard Curve

A typical standard curve is shown below. This standard curve shown below **must not** be used to calculate 8-iso-PGF$_{2\alpha}$ concentrations; each user must run a standard curve for each plate used.



Typical Quality Control Parameters

Total Activity Added	=	0.341 x 10 = 3.41
%NSB	=	0.010%
%Bo/TA	=	1.67%
Quality of Fit	=	0.9999 (Calculated from 4 parameter logistic curve fit)
20% Intercept	=	9,750.0 pg/mL
50% Intercept	=	1,191.2 pg/mL
80% Intercept	=	123.8 pg/mL

Performance Characteristics

Sensitivity
To determine sensitivity of the assay, the mean optical density value for 16 replicates of Bo (0 pg/mL Standard) were compared to the mean optical density for 16 replicates of Standard #8 (6.1 pg/mL). The detection limit was determined as the concentration of 8-iso-$PGF_{2\alpha}$ measured at two standard deviations from the zero along the standard curve.
The sensitivity of this assay was determined to be 16.3pg/mL.

Linearity
To determine linearity, a sample containing 10,000 pg/mL 8-iso-$PGF_{2\alpha}$ was diluted 8 times 1:2 in the Assay Buffer provided and measured in the assay. The data was plotted graphically as actual 8-iso-$PGF_{2\alpha}$ concentration versus measured 8-iso-$PGF_{2\alpha}$ concentration. The line obtained had a slope of 0.650 and a correlation coefficient of 0.999.

Precision
Intra-Assay Precision (Within Run Precision)
To determine intra-assay precision, three samples of low, medium and high 8-iso-$PGF_{2\alpha}$ concentrations were assayed twelve times on one plate. The intra-assay coefficient of variation of the assay was determined to be ≤11%.

Inter-Assay Precision (Between Run Precision)
To determine inter-assay precision, three samples of low, medium and high 8-iso-$PGF_{2\alpha}$ concentrations were measured in eight individual assays. The inter-assay coefficient of variation of the assay was determined to be ≤11%.

	8-iso-$PGF_{2\alpha}$ (pg/mL)	Intra-assay %CV	Inter-assay %CV
Low	232.24	11.0	
Medium	1,127.72	4.4	
High	8,262.98	5.8	
Low	183.50		11.0
Medium	854.76		8.8
High	6,238.63		5.0

Cross Reactivities
To determine the cross reactivities for a number of related eicosanoid compounds with this assay, the cross reactant (purity checked by NMR and other analytical methods) was dissolved in Assay Buffer at concentrations from 100,000 to 6 pg/mL and measured in the 8-iso-$PGF_{2\alpha}$ assay. The measured 8-iso-$PGF_{2\alpha}$ concentration at 50% B/Bo was calculated and compared to the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
8-iso-$PGF_{2\alpha}$	100%
$PGF_{1\alpha}$	4.6%
$PGF_{2\alpha}$	1.85%
PGE_1	0.19%
TXB_2	0.023%
PGB_1	0.02%
PGE_3	0.012%
6-keto-$PGF_{1\alpha}$	0.008%
13,14-dihydro-15-keto-$PGF_{2\alpha}$	0.008%
6,15-keto-13,14-dihydro-$PGF_{1\alpha}$	0.005%
8-iso-PGE_1	<0.001%
PGA_2	<0.001%
PGJ_2	<0.001%
2-Arachidonoylblycerol	<0.001%
Anandamide	<0.001%

Sample Recoveries

To determine sample recovery, 8-iso-PGF$_{2\alpha}$ was spiked into five undiluted sample matrices (tissue culture media, human saliva, urine, serum, plasma), and were diluted into the appropriate diluent and then assayed in the kit. The % recovery was calculated to be >85% for all five sample types.

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	129.9	1:10
Human Saliva	86.4	1:100
Human Urine	91.7	1:100
Human Serum	134.5	1:10
Human Plasma	90.2	None

* See Sample Preparation on page 4 for details.

References

1. Zeldin, D.C. (2001) *J. Biol. Chem*. **276**: 36059-36062.
2. Reilly, M.P., Lawson, J.A. and FitzGerald, G.A. (1998) *J. Nutr*. **128:** 434S-438S.
3. Funk, C. D. (2001) *Science* **294**: 1871-1875.
4. Pratico, D., Lawson, J.A. and FitzGerald, G.A. (1995) *J. Biol. Chem*. **270:** 9800-9808.
5. Pratico, D. and FitzGerald, G.A. (1996) *J. Biol. Chem*. **271**: 8919-8924.
6. Takahashi, K., Nammour, T.M., Fukunaga, M., Ebert, J., Morrow, J.D., Roberts, L.J.II., Hoover, R.L. and Badr, K.F. (1992) *J. Clin. Invest*. **90**:136-141.
7. Kang, K.H., Morrow, J.D., Roberts, L.J.II., Newman, J. and Banerjee, M. (1993) *J. Appl. Physiol*. **74**: 460-465.
8. Patrono, C. and Fitzgerald, G.A. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17**: 2309-2315.
9. Hart, C.M., Karman, R.J., Blackburn, T.L., Gupta, M.P., Garcia, J.G. and Mohler, E.R. (1998) *Prostaglandins Leukot. Essent. Fatty Acids* **58**: 9-16.
10. Yura, T., Fukunaga, M., Grygorczyk, R., Makita, N., Takahashi, K and Badr, K. F. (1995) *Adv. Prostaglandin Thromboxane Leukot. Res*. **23**: 237-239.
11. Helmersson, J. and Basu, S. (2001) *Prostaglandins Leukot. Essent. Fatty Acids* **65**:99-102.
12. Reilly, M.P., Protico, D., Delanty, N., DiMinno, G., Tremoli, E., Rader, D., Kapoor, S., Rokach, J., Lawson, J. and FitzGerald, G.A. (1998) *Circulation* **98**: 2822-2828.
13. Voutilainen, S., Morrow, J.D., Roberts, J. II., Alfthan, G., Alho, H., Nyyssonen, K. and Salonen, J.T. (1999) *Arterioscler. Thromb. Vasc. Biol*. **19**: 1263-1266.
14. Mezzetti, A., Cipollone, F. and Cuccurullo, F. (2000) *Cardiovasc. Res.* **47:** 475-488.
15. Barden, A., Beilin, L.J., Ritchie, J., Croft, K.D., Walters, B.N. and Michael, C.A. (1996) *Clin. Sci.* **91**: 711-718.
16. Staff, A. C., Ranheim, T., Henriksen, T. and Halvorsen, B. (2000) *Hypertension* **35**: 1307-1313.
17. Halvorsen, B., Staff, A. C., Henriksen, T., Sawamura, T. and Ranheim, T (2001) *Hypertension* **37:**1184-1190.
18. Green, K., Granstrom, E., Samuelsson, B. and Axeu, U. (1973) *Anal. Biochem.* **54**: 434-453.
19. Frolich, J.C., Wilson, T.W., Sweetman, B.J., Smigel, M., Nies, A.S., Carr, K., Watson, J.T., Oates, J.A. *J. Clin. Invest*. (1975) **55**: 763-770.
20. Shaw, J.E. and Ramwell, P.W. (1969) *Methods Biochem. Anal*. **17**: 325-371.
21. Green, K. Hamberg, M., Samuelson, B. and Frolich, J.C. *Adv. Prostaglandin Thrombane Res.* (1978) **5:** 15-38.