*Oxis*Research™

A Division of OXIS Health Products, Inc.

BIOXYTECH® MDA-586™

Spectrophotometric Assay for Malondialdehyde
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number 21044

INTRODUCTION

The Analyte

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds. These include reactive carbonyl compounds, of which the most abundant is malondialdehyde (MDA). Therefore, measurement of malondialdehyde is widely used as an indicator of lipid peroxidation (1). Increased levels of lipid peroxidation products have been associated with a variety of chronic diseases in both humans (2, 3) and model systems (4, 5). MDA reacts readily with amino groups on proteins and other biomolecules to form a variety of adducts (1), including cross-linked products (6). MDA also forms adducts with DNA bases that are mutagenic (7, 8) and possibly carcinogenic (9). DNA-protein cross-links are another result of the reaction between DNA and MDA (10). The TBARS method is commonly used to measure MDA in biological samples (11). However, this reaction is relatively nonspecific; both free and protein-bound MDA can react. The MDA-586 method is designed to assay free MDA or, after a hydrolysis step, total MDA (i.e., free and protein-bound Schiff base conjugates). The assay conditions serve to minimize interference from other lipid peroxidation products, such as 4-hydroxyalkenals.

PRINCIPLES OF THE PROCEDURE

The MDA-586 method[1] (12) is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1, NMPI), with MDA at 45°C. One molecule of MDA reacts with 2 molecules of NMPI to yield a stable carbocyanine dye as shown in **Figure 1** (13).

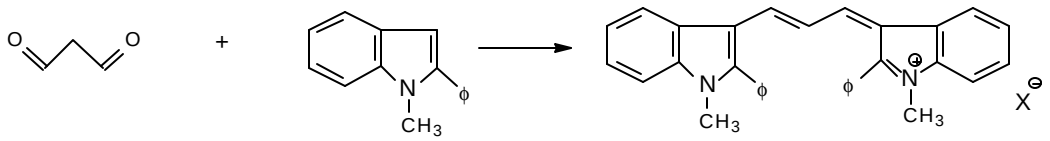

Figure 1. N-methyl-2-phenylindole (NMPI) reacts with malondialdehyde to form an intensely colored carbocyanine dye with a maximum absorption at 586 nm.

[1] US Patent No. 5726063

The MDA-586 method is specific for MDA because 4-hydroxyalkenals do not produce significant color at 586 nm under the conditions of the assay (14). **Figure 2** shows the absorption spectra of the reaction products of MDA under the standard MDA-586 reaction conditions. The reaction is carried out in hydrochloric acid (provided) and with the addition of Probucol (provided), an antioxidant, to further minimize the reaction of 4-hydroxyalkenals. Under these conditions, there is little absorbance at 586 nm from HNE, the most common 4-hydroxyalkenal produced in cells subjected to lipid peroxidation.

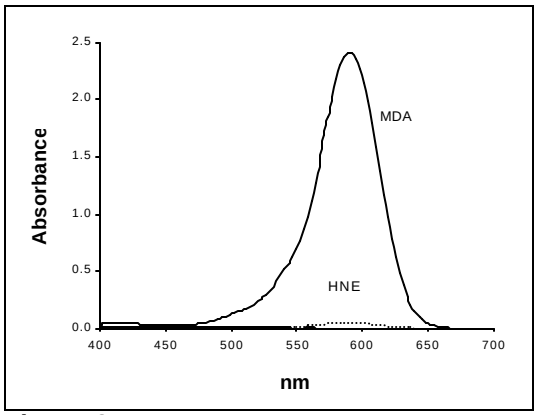

Figure 2. Absorption spectra obtained from the reaction of NMPI with MDA (21 µM) or HNE (19 µM) in the presence of HCl.

In the MDA-586 assay, a calibration curve is prepared using the MDA standard provided. The [MDA] in an unknown and is determined from the absorbance of the unknown at 586 nm in the MDA-586 assay and the standard curve. For colored samples, a sample blank is run (omitting the NMPI) and any absorbance at 586 nm is subtracted from the sample absorbance to give the true absorbance due to the carbocyanine dye.

REAGENTS

Materials Provided (for 100 tests)

- Reagent R1 — N-methyl-2-phenylindole, in acetonitrile, 3 X 18 mL
- Reagent R2 — Concentrated hydrochloric acid, 1 X 16.5 mL
- MDA Standard — 1,1,3,3-tetramethoxypropane (TMOP) in Tris-HCl, 1 X 1 mL
- BHT — BHT (butylated hydroxytoluene) in acetonitrile, 1 X 2 mL
- Probucol — Probucol in methanol, 1 X 1.1 mL
- Methanol — 1 X 30 mL

Materials Required But Not Provided

- Spectrophotometer
- Spectrophotometric cuvettes with a 1 cm optical path length (glass, quartz, or polystyrene)
- Water bath or heat block set to control temperature at 45 ± 1°C
- Disposable tubes and stoppers (glass or polypropylene)
- Microcentrifuge

Warnings and Precautions

- **Acetonitrile** (R1 and BHT) is a flammable liquid and is harmful if swallowed, inhaled, or absorbed through the skin. Causes irritation. Use with adequate ventilation. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.
- **Hydrochloric acid** is corrosive and may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.
- **Methanol** (Probucol) is a flammable liquid and is harmful if swallowed, inhaled, or absorbed through the skin. Use with adequate ventilation. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.
- **BHT** is harmful if swallowed, inhaled, or absorbed through the skin. Risk of serious eye injury. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
- **Probucol** is harmful if swallowed, inhaled, or absorbed through the skin. In case of contact with skin or eyes, rinse immediately with plenty of water and seek medical advice.

Reagent Storage and Handling

- It is good practice to transfer the desired volume of reagents for an experiment to a clean glass test tube or other vessel and return the stock reagent bottles to 4°C storage.
- Do not allow the capped reagent bottles to sit at room temperature for long periods of time. When not in use, place the bottles at 4°C.
- Unopened reagents are stable until the indicated expiration date.

PROCEDURE

Reagent Preparation

Dilution of the R1 solution for use in the assay. Add one volume (6 mL) of 100% methanol to three volumes (18 mL) reagent R1. This solution (diluted R1) is stable for two days at 4°C. Do not leave the R1 reagent bottle uncapped (open to the atmosphere).

Preparing TMOP (MDA) Standard

A MDA Standard is provided as tetramethoxypropane (TMOP) because MDA is not stable. The TMOP is hydrolyzed during the acid incubation step at 45°C, which will generate MDA. The TMOP Standard is provided as a 10 mM stock solution. Just prior to use, dilute the stock 1/500 (v/v) in water to give a 20 µM stock solution. Place at 0-4°C until use.

For the standard curve, pipet the the indicated volumes of Standard and water (or buffer) to the reaction tube to give a total of 200 µL. The Final Concentration value is the [MDA] in the MDA-586 reaction mixture (1000 µL total volume).

Recommended addition table for the MDA-586 standard curve.

Volume of 20 µM Standard, µL	0	25	50	100	150	200
Volume of water, µL	200	175	150	100	50	0
Final Concentration, µM	0	0.5	1.0	2.0	3.0	4.0

Sample Preparation

Note: Please read the appropriate **NOTES** sections before starting sample preparation procedure.

Assay

1. Add 10 µL of probucol to each assay tube.[+]
2. Add 200 µL of sample or standard to the respective assay tubes.
3. Add 640 µL of diluted R1 reagent to each tube.
4. Mix by briefly vortexing each tube.
5. Add 150 µL of R2.
6. Stopper each tube and mix well by vortexing.
7. Incubate at 45°C for 60 minutes.
8. Centrifuge turbid samples (e.g., 10,000 X g for 10 minutes) to obtain a clear supernatant.
9. Transfer the clear supernatant to a cuvette.
10. Measure absorbance at 586 nm.*

[+] Some of the probucol may precipitate but this will not cause a problem. Any precipitate formed is removed in Step 8.

*The color is stable for at least two hours at room temperature (13).

Calculations

1. Using the standard data, perform a linear regression of A_{586} on [MDA] :

$$A_{586} = a[MDA] + b$$

2. Calculate the concentration of analyte in a sample:

$$[MDA] = \frac{A_{586} - b}{a} \bullet df$$

Where [MDA] = Concentration of MDA in the sample
A_{586} = Absorbance at 586 nm of sample
a = Regression coefficient (slope)
b = Intercept
df = Sample dilution factor

Example

To illustrate the calculations, consider the following experiment: Three 200 µL aliquots of a biological sample were assayed along with a set of MDA standards, also in triplicate. The average A_{586} value for the zero concentration standard was subtracted from the average A_{586} values of the other standards and the average sample A_{586} value to give corrected absorbances (A_{586} corr). A plot of A_{586} corr vs. [MDA] for the standards was constructed (**Figure 3**, see below).

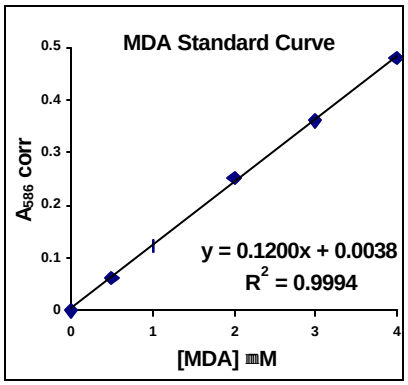

Figure 3. Plot of A_{586} corr vs. [MDA] for the standards in the MDA-586 reaction

The equation for calculating [MDA] is thus:

$$[MDA] = \frac{(\text{Sample } A_{586} \text{ corr}) - b}{a} \bullet df$$

The sample had an average A_{586} corr = 0.2362. Steps in the calculation of sample [MDA] are:

1. Use the equation to calculate [MDA] in the reaction mixture.

$$[MDA] = \frac{0.2362 - 0.0038}{0.1200} = 1.937\ \mu M$$

2. Correct for the dilution of the sample.

200 µL of sample was used and the final reaction volume was 1000 µL:

$$df = \frac{1000}{200} = 5.000$$

[MDA] in biological sample = 1.937 µM x 5.000 = 9.69 µM

PERFORMANCE CHARACTERISTICS

Linearity

The absorbance at 586 nm is a linear function of the MDA concentration over the range from 0.5 µM to 20 µM. Properly stored and accurately diluted standard should have a slope within 10% of the expected extinction coefficient; between 0.110 and 0.130 A_{586}/µM MDA (**Figure 3**).

Specificity

The specificity of the MDA-586 method was determined by measuring the absorbance ratios at 586 nm of MDA and HNE at a constant concentration (13).

Substance	% Recovery
MDA	100
4-HNE	0.46

Precision

Precision of the MDA-586 method was estimated by measuring three levels of MDA, in buffer, each day for 10 days (15). Precision results are given in µM.

Concentration	**Low**	**Medium**	**High**
Days	10	10	10
Mean	1.0384	1.9864	4.7936
SD Within Run	0.0352	0.0232	0.0776
CV Within Run (%)	3.4	1.2	1.6
SD Total	0.0384	0.0328	0.0800
CV Total (%)	3.7	1.6	1.7

Sensitivity

The lower limit of detection is defined as 5.185 standard deviations (n=10) from the blank absorbance at 586 nm. The sensitivity of the MDA-586 method for MDA is shown below.

Average A_{586}	0.0102
Standard deviation, A_{586}	0.0017
Detection limit, A_{586}	0.0088
Detection limit, ìM (in reaction mixture)	0.0801

NOTES

Interference

- Reducing Sugars. Sucrose or fructose, at concentrations of 50 mM or greater in the sample, will cause a high bias in the assay (16).
- Antioxidants. Glutathione (200 µM), ascorbate (100 µM), probucol (1 mM), and BHT do not interfere. (13).
- Others. Hydrogen peroxide at 50 µM will reduce MDA by 13% (13).

Sample Preparation

- Sample Oxidation. OXIS recommends that butylated hydroxytoluene (BHT) be added to a final concentration of 5 mM prior to homogenization of tissue or cells. BHT is provided as a 500 mM solution in acetonitrile. If no antioxidant is added, new lipid peroxidation can occur during homogenization, resulting in biased values (11).
- Total MDA**.** The standard MDA-586 method is designed to measure free MDA. Determination of total MDA requires hydrolysis of the sample in the presence of BHT, at pH 1-2 at 60°C for

80 minutes. The hydrolysis yield is markedly reduced at pH less than 1 and pH greater than 2 (13).

- Tissue. Sample homogenates should be as concentrated as possible, (i.e., approximately 20% - 30% or 200-300 mg tissue per mL of buffer). It is recommended that 0.2 mL of a homogenate containing 15-60 mg/mL of protein, should be assayed for initial studies of a previously untested biological sample. Homogenates not assayed immediately must be stored at –70°C or lower (17, 18).
- Cell culture. Cells should be washed to removed protein and other constituents from the media, then lysed by 2 or 3 freeze/thaw cycles or by sonication. Cell debris are then removed by centrifugation at 3000 x *g* for 10 minutes at 4°C. It is recommended that a lysate from 10^7 cells be added to the MDA-586 reaction mixture (i.e., 0.2 mL of 5 X 10^7 cells per mL). Lysates not assayed immediately must be stored at –70°C or lower (17, 18).
- Plasma. The concentration of free MDA in normal plasma is below the limit of quantitation of the MDA-586 method (16). Bound MDA, which represents over 80% of the plasma MDA (17), can be hydrolyzed in the presence of BHT, at pH 1-2 at 60°C for 80 minutes. The hydrolysis yield is markedly reduced at pH less than 1 and pH greater than 2 (13). See below for protocol. Because MDA has a room temperature half-life in plasma of approximately two hours, samples must be kept cold and assayed as soon as possible after hydrolysis (1). Plasma not assayed immediately must be stored at –70°C or lower (17, 18). Detection of MDA in plasma requires 3rd derivative spectroscopy (Below).

Limitations

- Normal Plasma. MDA is at the detection limit in normal plasma.
- Normal Tissue. Normal tissues have very low levels of free malondialdehyde, typically 10-100 pmol/mg protein (11, 19). Assuming the lower value, an assay of a 0.2 mL sample containing 10 mg of protein derived from normal tissue will give an absorbance value at 586 nm of 0.012 in the MDA-586 assay. Caution must be taken not to interpret very low absorbance values (near zero) as an accurate reflection of analyte concentrations in biological samples.

Assay Performance

- Sample *O*xidation**.** The kinetics of color development in the sample should be followed in comparison with that of the TMOP supplied with the reagent set. The A_{586} of the sample should reach a plateau and then remain stable. Continual increase in the A_{586} indicates non-MDA reactivity (interference) or oxidation is occurring in the reaction mixture. This could also be due to slow hydrolysis of protein-bound MDA.
- Turbidity. A wavelength scan from 400 to 700 nm should be performed on the clarified sample reaction mixture and compared to that obtained with the TMOP standard. The lack of a peak at 586 nm or a continuous increase in the baseline would suggest interference or non-specific reactivity in the sample (16).
- Sample Blank. Turbid or hazy samples will give biased values in the assay resulting from a contribution to the absorbance at 586 nm from light scattering. If the reaction mixtures do not clarify upon centrifugation, an individual sample blank should be prepared. The Sample Blank is made by substituting 650 µL of 75% acetonitrile to 25% methanol (i.e., 3 volumes of acetonitrile plus 1 volume of methanol) for the R1 reagent in the sample reaction mixture. Correct for any A_{586} contribution due to the sample by subtracting the Sample Blank from the sample (R1 reaction).

Third Derivative Spectroscopy

- Derivative Spectroscopy. Specificity and sensitivity can be improved using 3rd derivative spectroscopy to analyze the MDA-586 data. Derivative spectroscopy helps to eliminate or reduce the effects from drifting baseline and absorption from endogenous substances in biolological samples. Most modern spectrophotometers include derivative spectroscopy in the application software. Contact OXIS Technical Service Department for further details.

Determination of Total MDA in Plasma

- Hydrolysis of simple Schiff base MDA adducts in plasma can be carried out at pH 1-2, and heating at 60° C for 80 minutes. This procedure will not hydrolyze more complex adducts involving cross-linking of MDA to protein lysine residues. The protocol involves an acid hydrolysis step prior to the MDA-586 assay . Because of the low concentration of the A_{586} chromophore is expected, a set of low concentration standards are run under identical conditions. A spectra of both standards and plasma samples are recorded. 3rd derivative spectroscopy is used to quantitate the MDA content of the solutions. The assay is carried out as follows:

1. Dilute R1 as in the regular assay procedure.
2. Using a 100 µM TMOP solution (10 µL of S1 (10 mM TMOP) + 990 µL water), prepare the following MDA standards according to the table listed below: of 0.000, 0.250, 0.500, 0.800, and 1.00 µM. Use water for the 0.000 standard.

Plasma Dilution of TMOP Standard for Total MDA Assay

Volume 100 µM standard (µL)	5.0	10.0	8.0	10.0
Volume water (µL)	1995	1990	992	990
Concentration (µM)	0.250	0.500	0.800	1.00

3. Use four replicates for each sample and standard. Pipet 11 µL of BHT (500 mM) into each microfuge tube.
4. Add 210 µL of plasma sample or standard to each tube.
5. Add 5.3 µL concentrated HCl to each tube, cap and mix well. Incubate at 60° C for 80 minutes.
6. Cool to room temperature and add 680 µL of diluted R1 to each tube. Mix well.
7. Centrifuge at 13,000 x *g* for 5 minutes.
8. Transfer 660 µL of clear supernatant to new microfuge tubes.
9. Add 115 µL of concentrated HCl to each tube, cap and mix well.
10. Incubate at 45° C for 60 minutes.
11. Centrifuge at 13,000 x *g* for 5 minutes.
12. Transfer as much of the supernatant as possible to a cuvette and record the visible spectrum from 400 – 700 nm promptly in 0.5 nm intervals (use water to do a background correction). If spectra cannot be recorded within one hour, store samples on ice. Spectra should be recorded within the same day.
13. Obtain the 3rd derivative of each spectrum (see above). For each spectrum, multiply the value of the 3rd derivative at 572 nm ($\mathbf{d^3A_{572}/dl^3}$) by (-1 x 10^7) to obtain a more convenient set of numbers. Calculate the mean $\mathbf{d^3A_{572}/dl^3}$ for each sample and standard by averaging the individual values. Plot the mean $\mathbf{d^3A_{572}/dl^3}$ vs the original [MDA] (Table above) for each of the standards and determine the equation of the linear regression line. Use the equation of the line and the mean $\mathbf{d^3A_{572}/dl^3}$ for the samples to calculate the [MDA] in each. Use of the standard curve and calculations are analogous to the example given earlier for free MDA. There is no dilution factor in this calculation.

Determination of Free MDA in Plasma

- Free MDA in plasma may be determined using the standard protocol with the more dilute standards given in the procedure for total MDA in plasma above. 3rd derivative spectroscopy should be employed to analyze samples, as described above.

Reagent Preparation

- R1 Dilution. Failure to dilute the R1 reagent with methanol will result in the formation of a 2 phase reaction mixture.
- Assay Buffers. Buffers should not contain amino groups (e.g., Tris) since these can react with MDA to form Schiff bases, which may hydrolyze only slowly under the MDA-586 reaction

conditions. Potential interference in the assay should be assessed by adding MDA (hydrolyzed TMOP) to aliquots of buffer (water as a control) and assaying for free MDA after an appropriate incubation period, which is determined by the experimenter's own protocol for tissue preparation. A suggested protocol follows:

1. Prepare 0.2 M HCl solution by mixing 17 µL of R2 with 983 µL of water.
2. Combine 50 µL MDA Standard and 50 µL of 0.2 M HCl. Stopper, vortex, and allow it to incubate at room temperature for two hours. This will hydrolyze the TMOP, forming 5 mM MDA.
3. Dilute 20 µL of the MDA to a final volume of 10 mL with the buffer being tested ([MDA] = 10 µM)
4. Prepare an identical sample using water instead of buffer.
5. Allow both samples to incubate at room temperature. Remove 200 µL aliquots at appropriate times, determined by the experimenter's own sample preparation protocol, and analyze using the MDA-586 assay. Phosphate buffers do not react with MDA and are recommended for sample preparation.

REFERENCES

1. Esterbauer, H. et. al., (1991) Chemistry and Biochemistry of 4-Hydroxynonenal, Malondialdehyde and Related Aldehydes, Free Rad. Biol. Med. **11**, 81-128.
2. De Maria, N. et. al., Association Between Reactive Oxygen species and Disease Activity in chronic Hepatitis C, (1996) Free Radicals Biol. Med. **21**, 291-295.
3. Browne, S.E. et. al., (1999) Oxidative Stress in Huntington's Disease, Brain Pathol. **9**, 147-163.
4. Vento, R. et. al., (2000) Induction of Apoptosis by Arachidonic Acid in Human Retinoblastoma Y79 Cells: Involvement of Oxidative Stress, Exp. Eye Res. **70**, 503-517.
5. Valerio, L.G. and Petersen, D.R., (1998) Formation of Liver Microsomal MDA-Protein Adducts in Mice with Chronic Dietary Iron Overload, Toxicol. Lett. **98**, 31-39.
6. Beppu, M. et. al., (1988) Interaction of Malondialdehyde-Modified Bovine Serum Albumin and Mouse Peritoneal-Macrophages, Chem. Pharm. Bull. **36**, 4519-4526.
7. Mao, H., et. al., (1999) Solution Structure of an Ologonucleotide Containing the Malondialdehyde Deoxyguanosine Adduct N2-(3-oxo-1-propenyl)-dG (ring-opened M1G) Positioned in a (CpG)3 Frameshift Hotspot of the Salmonella typhimurium hisD3052 Gene, Biochemistry **38**, 13491-13501.
8. Marnett, L.J., (1999) Chemistry and Biology of DNA Damage by Malondialdehyde, IARC **No. 150** 17-27.
9. Marnett, L.J., (1999) Lipid Peroxidation-DNA Damage by Malondialdehyde, Mutat. Res. **424**, 83-95.
10. Voitkun, V and Zhitkovich, A., (1999) analysis of DNA-Protein Crosslinking Activity of Malondialdehyde in vitro, Mutat. Res. **424**, 97-106.
11. Botsoglou, N.A., (1994) Rapid, Sensitive, and Specific Thiobarbituric Acid Method for Measuring Lipid Peroxidation in Animal Tissue, Food and Feedstuff Samples, J. Agric. Food Chem. **42**, 1931-1937.
12. Gérard-Monnier, et. al., (1998) Method of Colorimetric Analysis of Malonic Dialdehyde and 4-Hydroxy-2-enaldehydes as Indexes of Lipid Peroxidation, Kits for Carrying Out Said Method, Substitued Indoles for Use in Said Method and Their Preparation, US Patent No. US5726063.
13. Gérard-Monnier, et.al., (1997) Reactions of N-Methyl-2-phenlindole with Malondialdehyde and 4-Hydroxyalkenals. Analytical Applications to a Colorimetric Assay of Lipid Peroxidation, Chemical Research in Toxicology **11**:10, 1176-1183.
14. Erdelmeier, I., et. al., (1997) Reactions of N-Methyl-2-phenlindole with Malondialdehyde and 4-Hydroxyalkenals. Mechanistic Aspects of the Colorimetric Assay of Lipid Peroxidation, Chemical Research in Toxicology **11**:10, 1184-1194.

15. NCCLS Tentative Guideline. (1992) EP5-T2, 2nd edition. Evaluation of Precision Performance of Clinical Chemistry Devices. Villanova, PA. National Committee for Clinical Laboratory Standards, **12**:4.
16. *OXIS*Research, unpublished data.
17. Carbonneau, M.A. et. al., (1991) Free and Bound Malondialdehyde Measured as Thiobarbituric Acid Adduct by HPLC in Serum and Plasma, Clin. Chem. **37**, 1423-1429.
18. Bull, A.W. and Marnett, L.J. (1985) Determination of Malondialdehyde by Ion-Pairing High-Performance Liquid Chromatography, Analyt. Biochem. **149**, 284-290.
19. Liu, J. et. al., (1997) Assay of Aldehydes from Lipid Peroxidation: Gas Chromatography-Mass Spectrometry Compared to Thiobarbituric Acid, Analyt. Biochem. **245**, 161-166.
20. Sun, J., *OXIS*Research, unpublished observations.

*OXIS*Research™
6040 N. Cutter Circle, Suite 317
Portland, OR 97217-3935 U.S.A.
503-283-3911 or 800-547-3686 Fax: 503-283-4058
Last revision June 2004

Made in the U.S.A.