US/EG Bulletin 2079

Non-Protein A Based Purification of MAb 414 From Ascites Fluid

Joseph E. Machamer, Andrew J. Stapleton, Tracy King and Krishna Balakrishnan*, Bio-Rad Laboratories, Hercules, CA 94547. *Berkeley Antibody Company, Richmond, CA 94804-4609*

Abstract

The monoclonal antibody 414 (MAb 414) is a Nuclear Pore Complex (NPC) recognizing antibody commercially available from the Berkeley Antibody Company (BAbCO). MAb 414 is currently produced by BAbCO in ascites fluid and it is purified by Protein A chromatography. In many cases Protein A purification of MAbs is a very good technique. However, due to the high cost, harsh elution conditions required, leakage of Protein A ligand, and the inability to separate different populations of IgG, many laboratories are seeking alternatives to Protein A based purifications. In order to investigate alternative methods of MAb 414 purification from ascites fluid, MAb 414 was purified using cation exchange and ceramic hydroxyapatite chromatography. This application note discusses the methods development strategy which led to the use of cation exchange and ceramic hydroxyapatite chromatography and compares the results of this purification with the Protein A based purification.

Materials and Methods

Tris-HCl, Bis-Tris propane, MES, HEPES, and sodium acetate were purchased from Sigma Chemical Co., St. Louis, MO. Sodium phosphate was purchased from Spectrum Chemical Manufacturing Corporation, Gardena, CA. Ammonium sulfate was purchased from Aldrich, Milwaukee, WI, and sodium chloride was purchased from J. T. Baker, Phillipsburg, NJ. Protein A purified MAb 414 and MAb 414 in ascites fluid were provided by BAbCO. All chromatography was performed on the BioLogic Chromatography Workstation from Bio-Rad Laboratories, Hercules, CA. All SDS-PAGE gels were run according to Laemmli[1] using 12% Ready Gels and a PowerPac 3000 from Bio-Rad Laboratories and Coomassie staining. Bio-Scale S2, Bio-Scale Q2, Bio-Scale DE2, Bio-Scale CHT I-2, and Econo-Pac® t-Butyl columns were obtained from Bio-Rad Laboratories, Hercules, CA. Macro-Prep® high S and Macro-Prep ceramic hydroxyapatite, Type I, 40 µm bulk supports were from Bio-Rad.

Support and pH Screening Runs

Twenty chromatographic screening runs were performed by loading and eluting 5 ml of 1:100 MAb 414 ascites fluid in equilibration buffer using the columns and conditions shown in Table 1.

All of the columns screened, except for the Econo-Pac t-Butyl columns, were 2 ml pre-packed Bio-Scale columns containing 10 µm beads. This column format was chosen for scouting runs because the high resolution afforded by these 10 µm beads allows one to visualize, in detail, changes in chromatographic behavior as a function of pH.

Furthermore, the information obtained from these high resolution runs is then directly applicable to scale-up on Macro-Prep 50 µm beads because the 10 µm and the 50 µm beads are both

Table 1. Conditions and Supports Screened for MAb 414 Purification from Ascites Fluid.

Columns	Column Volume (ml)	Particle Size (µm)	pH	Buffer A	Buffer B
Bio-Scale Q2 (Strong anion exchange)	2	10	6.0, 7.0, 8.0, 9.0	20 mM Tris-HCl/Bis-Tris Propane	Buffer A + 1 M NaCl
Bio-Scale DE2 (Weak anion exchange)	2	10	6.0, 7.0, 8.0, 9.0	20 mM Tris-HCl/Bis-Tris Propane	Buffer A + 1 M NaCl
Bio-ScaleS2 (Strong cation exchange)	2	10	7.5, 6.5, 5.5, 4.5	20 mM HEPES/MES/Na Acetate	Buffer A + 1 M NaCl
Bio-Scale CHTI-2 (Ceramic hydroxyapatite, type I)	2	10	8.0, 7.5, 7.0, 6.5	10 mM Sodium Phosphate	500 mM Sodium Phosphate
Econo-Pac HIC t-butyl	1	50	7.5, 6.5, 5.5, 4.5	20 mM HEPES/MES/Na Acetate + 1.7 M Ammonium Sulfate	20 mM HEPES/MES/Na Acetate

All gradients were over ten column volumes, from 0 to 350 mM buffer B, except for the HIC run which was from 1.7 M ammonium sulfate to 0 over ten column volumes.

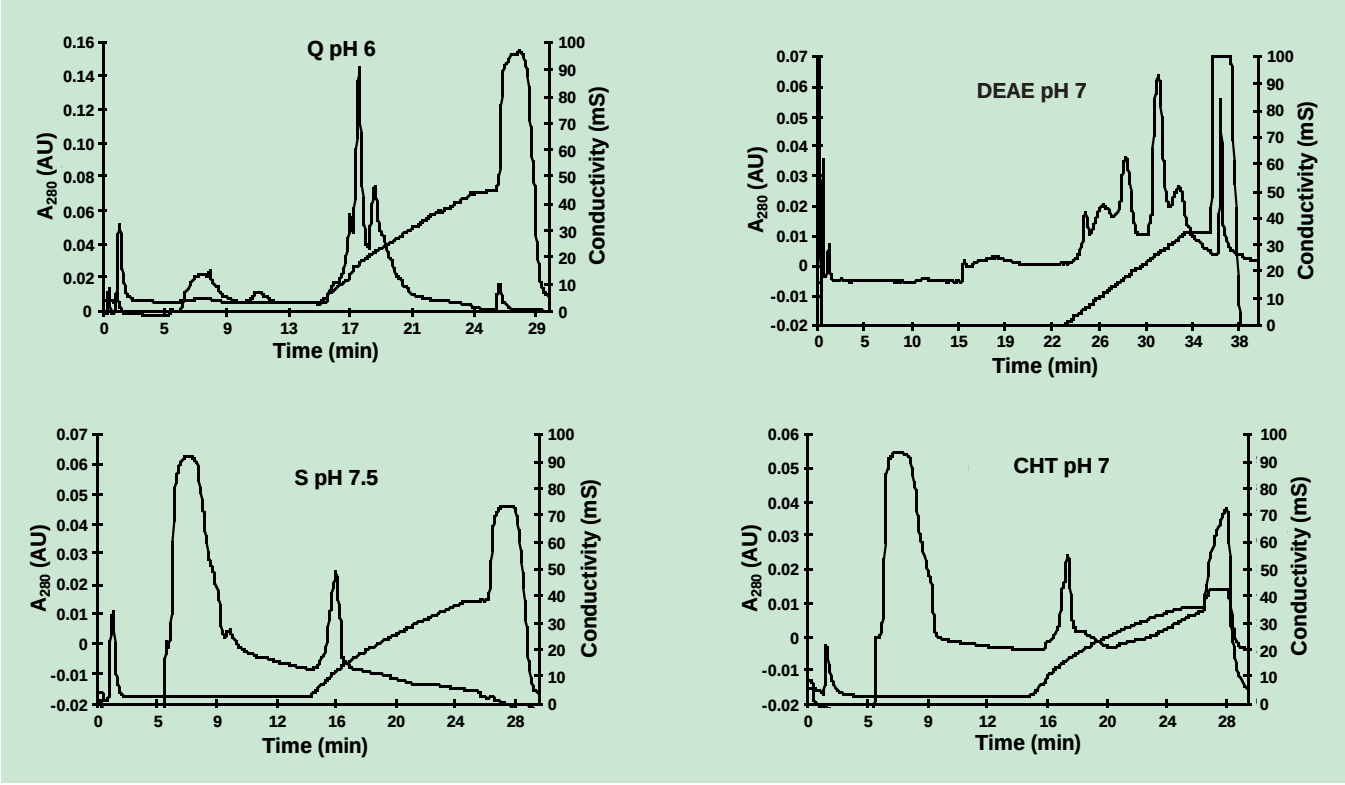

Fig. 1. Examples of chromatograms showing majority of proteins binding (Q and DEAE) and chromatograms showing more selective binding of IgG (S and CHT).

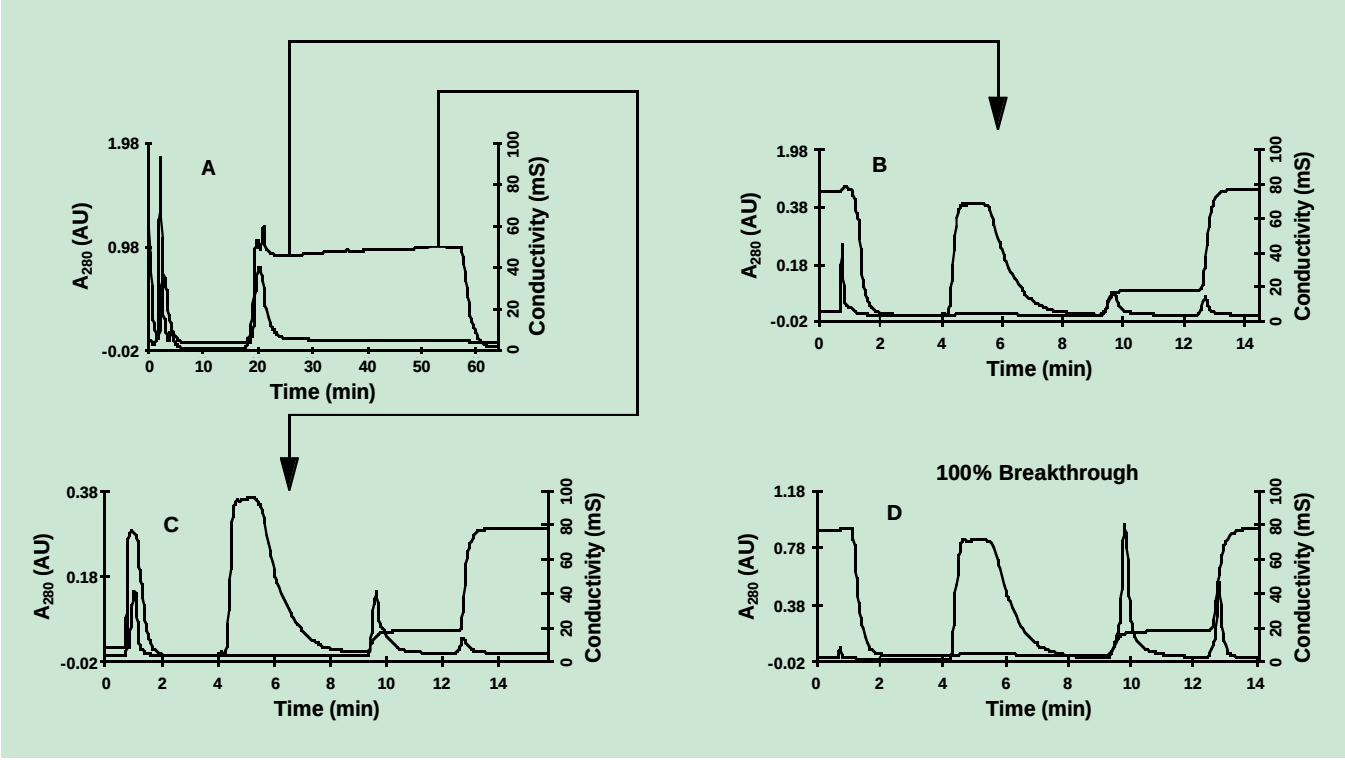

Fig. 2. Loading study of ascites MAb 414 on a 2 ml Macro-Prep high S column (A) and analytical runs of fractions represented by arrows on Bio-Scale S2 column (B, C, and D).

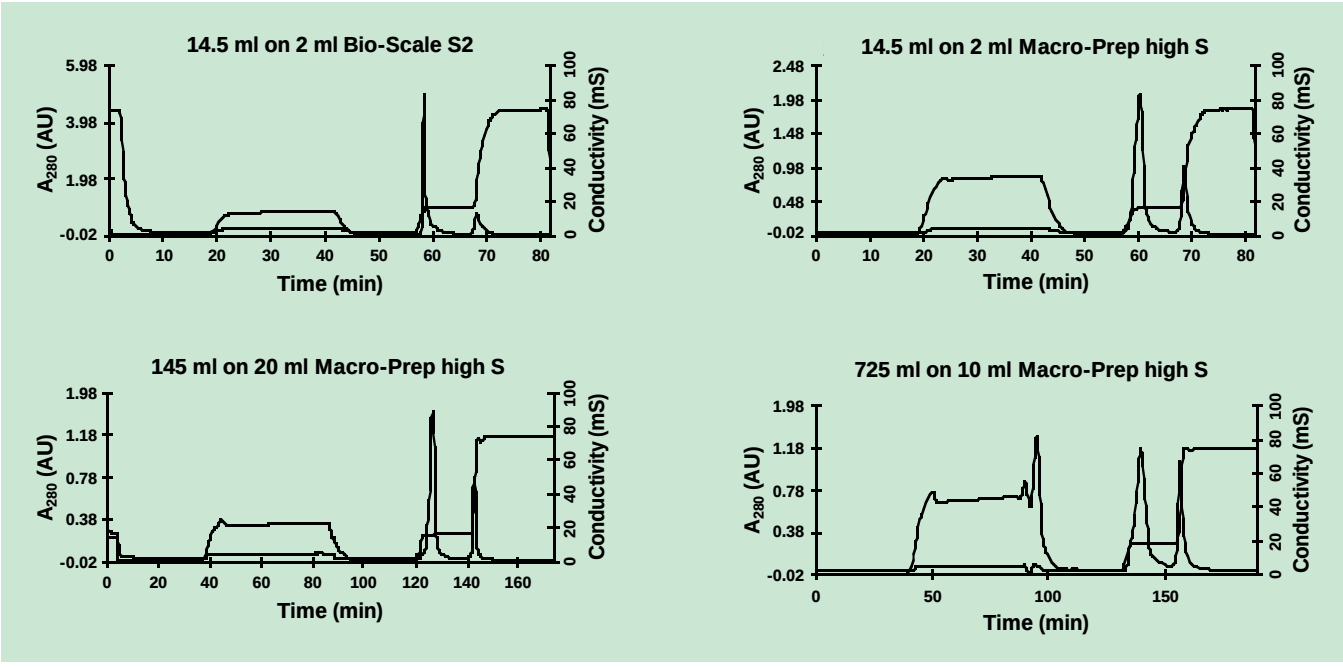

Fig. 3. 50 fold scale-up from 2 ml Bio-Scale S2 column to a 100 ml Macro-Prep high S column.

50 µm beads because the 10 µm and the 50 µm beads are both based on the same bead chemistry.

All of the buffers, except for the sodium phosphate buffers, used for screening the effect of pH on the separation, were made by off line mixing of 5x concentrates of the highest and lowest pH buffers in the proper ratio to give the desired pH, followed by dilution to 20 mM.

The strong cation exchange Bio-Scale S2, pH 7.5, and the ceramic hydroxyapatite Bio-Scale CHTI-2, pH 7.0, runs provided the best separations. Under these conditions, the MAb 414 did bind, but the majority of the contaminating proteins did not. Therefore, the 50 µm Macro-Prep high S and the 40 µm Macro-Prep ceramic hydroxyapatite Type I supports were chosen for the scale-up. Figure 1 shows typical examples of conditions under which the majority of the proteins bound and the more selective Bio-Scale S2, pH 7.5, and Bio-Scale CHT I-2, pH 7.0, runs.

The Macro-Prep high S support was chosen as the capture step of the purification followed by polishing on the Macro-Prep ceramic hydroxyapatite Type I support to take advantage of an effect shown by Lindenberg *et al*.[2] The effect seen by Lindenberg is that while low levels of NaCl affect chromatographic behavior of proteins, overall retention is not compromised. At concentrations below 100 mM, NaCl is a weak eluting salt for proteins loaded on ceramic hydroxyapatite. Therefore, the MAb eluted with 100 mM NaCl from the Macro-Prep high S column had the potential of binding to the Macro-Prep ceramic hydroxyapatite Type I column without any dilution or buffer exchange.

Figure 2 shows the loading study of the capture of MAb 414 on the 50 µm Macro-Prep high S support. Chromatogram A shows 2.5 ml of ascites fluid diluted to 25 ml with 10 mM phosphate buffer, pH 7, loaded at 100 cm/hr on to a 2 ml column packed with Macro-Prep high S support. three ml fractions were collected and analyzed by injecting 5 ml of a 1:1 dilution of each fraction with 10 mM phosphate pH 7 on a 10 µm Bio-Scale S2 column. The arrows from chromatogram A to chromatograms B and C show the fractions and corresponding analytical runs from fractions 4 and 9 respectively. Chromatogram D shows the analysis of a 100% breakthrough fraction. The peak height of the MAb 414 peak from fraction 4 is approximately 10% of the MAb 414 peak from the 100% breakthrough run. Therefore, the 12 ml load volume (6 ml of 1:10 ascites/ml Macro-Prep high S) represented by fraction 4 was taken as the approximate volume equal to a 10% breakthrough capacity for ascites fluid diluted 1:10 and loaded on to a 2 ml column.

For the purposes of the 50 fold scale-up, 7.25 ml of 1:10 ascites/ml of Macro-Prep high S was used as the 10% breakthrough capacity. Figure 3 shows the scale-up of the capture step

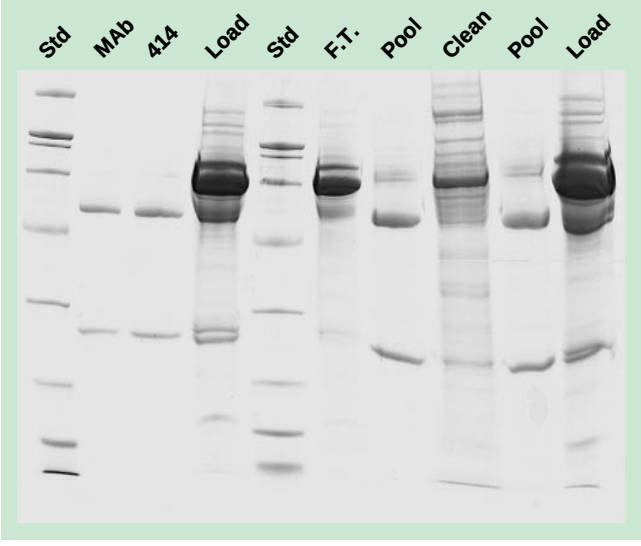

Fig. 4. SDS-PAGE analysis of fractions from the 725 ml of 1:10 ascites fluid run on the 100 ml Macro-Prep high S column.

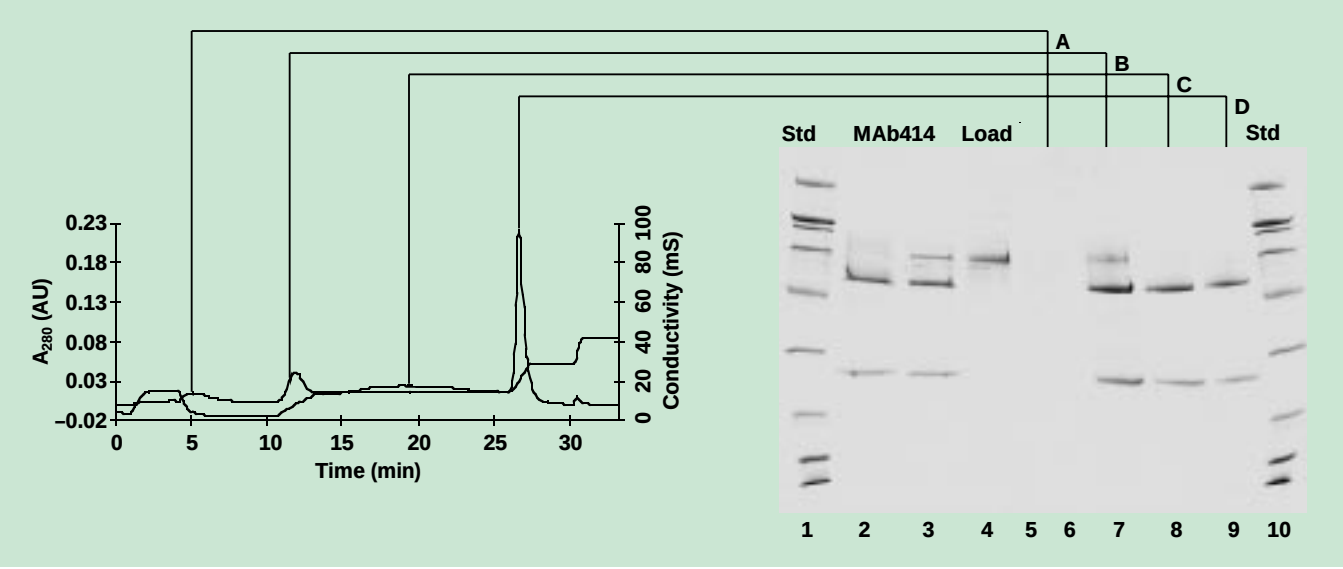

Fig. 5. 10 ml of "Pool" fraction from Macro-Prep high S 100 ml column run directly on a 2 ml CHT Type I, 40 µm column.

from a 2 ml Bio-Scale S2 column packed with 10 µm beads to a 100 ml column packed with 50 µm Macro-Prep high S support.

Figure 4 is the SDS-PAGE analysis of fractions from the 725 ml of 1:10 ascites fluid run on the 100 ml Macro-Prep high S column. The lanes labeled MAb 414 are the Protein A purified Ab. The lane labeled F.T. is the flow through fraction, the lane labeled "Pool" contains the MAb 414 from the Macro-Prep High S run, and the lane labeled "Clean" is from the column cleaning step. As can be seen from this gel, the Pool lane indicates a fraction which has been significantly purified relative to the load. The remaining contaminant appears to be albumin.

A 10 ml aliquot of the Pool fraction which eluted from the Macro-Prep High S in 100 mM NaCl was loaded directly on to a 2 ml column packed with Macro-Prep ceramic hydroxyapatite Type I, 40 µm. The column had been equilibrated in 10 mM sodium phosphate buffer pH 7. The column was eluted at 500 cm/hr with a series of steps to 500 mM sodium phosphate, pH 7. As the chromatogram in Figure 5 shows, almost all of the material loaded from the 100 mM NaCl elution from the cation exchange step bound to the CHT column even though the NaCl concentration had not been reduced through dilution or buffer exchange.

The SDS-PAGE analysis of the fractions from this chromatogram show the presence of albumin in the peak at 12 minutes, a mixture of albumin and IgG in the peak at 20 minutes, and highly purified IgG in the peak at 26 minutes. Lanes 2 and 3 on this gel show the purity of Protein A purified ascites fluid.

Conclusion

The combination of capture of MAb 414 from ascites fluid on the strong cation exchange Macro-Prep high S followed by polishing on the Macro-Prep ceramic hydroxyapatite Type I support is a viable alternative to purification by Protein A chromatography. Furthermore, the ability of Macro-Prep ceramic hydroxyapatite to bind protein in the presence 100 mM NaCl allows one to develop two step methods in which a protein eluted from an ion exchange column can be directly loaded on to a ceramic hydroxyapatite column without any dilution or buffer exchange step.

References

1. Laemmli, U. K., *Nature,* **277**, 68 (1970).
2. Lindenberg, J., Srichaiyo, T. and Hjerten, *S., J. Chromatogr.*, **499**, 153–163 (1990).

Presented at the IBC MAb Purification Conference, in La Jolla, CA, May 16–17, 1996.

Visit our web site at http://www.bio-rad.com for more information on Bio-Rad's complete line of process chromatography supports and other products for life science research and production.

BIO-RAD

Bio-Rad Laboratories

Life Science Group

Bio-Rad Laboratories Main Office, *2000 Alfred Nobel Drive, Hercules, California 94547, Ph. (510) 741-1000, Fx. (510) 741-5800*
Also in: Regents Park, Australia*, Ph. 02-9914-2800, Fx. 02-9914-2889* ***Wien, Austria****, Ph. (1) 877 89 01, Fx. (1) 876 56 29* ***Nazareth, Belgium****, Ph. 09-385 55 11, Fx. 09-385 65 54*
Mississauga, Canada*, Ph. (905) 712-2771, Fx. (905) 712-2990* ***Beijing, China****, Ph. (86-10) 2046622, Fx. (86-10) 2051876* ***Kybebhavn Ø, Denmark****, Ph. 39 17 9947, Fx. 39 27 1698*
Espoo, Finland, *Ph. 90 804 2200, Fx. 90 804 1100* ***Ivry sur Seine Cedex, France****, Ph. (1) 43 90 46 90, Fx. (1) 46 71 24 67* ***München, Germany****, Ph. 089 31884-0, Fx. 089 31884-100*
New Delhi, India*, Ph. 91-11-461-0103, Fx. 91-11-461-0765* ***Milano, Italy****, Ph. 02-21609.1, Fx. 02-21609.399* ***Tokyo, Japan****, Ph. 03-5811-6270 Fx. 03-5811-6272* ***Veenendaal, The Netherlands****, Ph. 031318-540666, Fx. 0313 18-542216* ***Auckland, New Zealand****, Ph. 09-443 3099, Fx. 09-443 3097* ***Kowloon, Hong Kong****, Ph. 7893300, Fx. 7891257* ***Singapore,*** *Ph. (65) 272-9877, Fx. (65) 273-4835* ***Sundbyberg, Sweden****, Ph. 46 (0) 8 627 50 00, Fx 46 (0) 8 627 54 00* ***Madrid, Spain****, Ph. (91) 661 70 85, Fx. (91) 661 96 98* ***Glattbrugg, Switzerland****, Ph. 01/809 55 55, Fx. 01/809 55 00* ***Hemel Hempstead, United Kingdom****, Ph. 0800 181134, Fx. 01442 259118*