

Applying the European Pharmacopoeia Capillary Zone Electrophoresis Method for the Separation of Recombinant Human Erythropoietin (EPO) Using the ProteomeLab PA 800 Protein Characterization System

Marcia R Santos
Discovery Products, Beckman Coulter, Inc., Fullerton, CA USA

Introduction

Erythropoietin or EPO is a naturally occurring red blood cell stimulating hormone produced in the kidney and was one of the first therapeutic recombinant glycoproteins commercialized for the treatment of anemia. The first patent on EPO was issued to Amgen in 1987 for the production of EPO in Chinese Hamster Ovary Cells.¹ It is well known that EPO has a complex glycosylation pattern on both the N- and O-terminus of its primary protein sequence and can exist as many isoforms. These isoforms play a critical role in the drug's availability, activity, potency and stability. Proper characterization, therefore, is of paramount importance for any manufacturer of EPO as a therapeutic product.

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has established several guidelines for the characterization of therapeutic proteins. Among these are Q5C and Q1A (R2),² which define parameters for the stability testing of biologicals. Parameters such as identity, purity, activity and general physicochemical properties are assessed under these guidelines.

The European Pharmacopoeia first defined a quantitative method to determine EPO isoform distribution using capillary electrophoresis in 1999.³ Subsequently, collaborative studies among pharmaceutical companies, government bodies and academia around the world have refined this method and also developed an EPO system suitability standard. The most recent update to the pharmacopoeial method occurred in June 2007, and resulted in the installment of BRP (Biological Reference Preparation) Batch No. 3 of EPO for system suitability.

This Application Information Bulletin offers guidance for the analysis of EPO using the ProteomeLab PA 800 Protein Characterization System and describes system set up, sample preparation, data analysis and basic troubleshooting tips.

Experimental

System Set Up and Configuration. All experiments were performed on the PA 800 Protein Characterization System (Beckman Coulter, Inc., Fullerton, CA), equipped with a UV detector and a 214 nm filter, with a data collection rate of 0.5 Hz. The instrument was controlled by 32 Karat v 8.0 software. All separations were carried out using a bare fused silica capillary with 100 cm effective length and 50 μm inner diameter. A 200 x 100 μm aperture was used in the cartridge detection window. The capillary temperature was set at 35°C and the sample storage maintained at 4°C.

Capillary equilibration was performed as follows: 60 min rinse at 20 psi of 0.1 N NaOH followed by a 60 min rinse at 20 psi of running buffer followed by voltage equilibration of 20 kV for 12 hours.

EPO Separation was performed as follows: 10 min rinse at 20 psi with double-deionized (DDI) water, followed by a 5 min rinse at 20 psi with 0.1 N NaOH, followed by a 10 min rinse with CZE running buffer.

Sample was introduced hydrodynamically at 0.7 psi for 20 s.

Separation was performed at 17.5 kV for 70 min (143 V/cm).

Sample Preparation. The EPO – European Pharmacopoeia Reference Standard was purchased from LGC Promochem, Middlesex – UK. Two hundred and fifty microliters of DDI water was added to the lyophilized EPO std, which should dissolve rapidly. The resulting solution was desalted using a Microcon YM-10 (Amicon cat. no. 42407). First the Microcon's membrane was washed by adding 250 µL of DDI water to the concentrator cup and centrifuging at 13,000 *g* for 10 min. Both retentate and eluent were discarded. Two hundred and fifty microliters of reconstituted EPO was dispensed to the concentrator cup and centrifuged at 13,000 *g* for 10 min. The eluent was discarded and 250 µL of DDI water was added to the concentrator cup and centrifuged at 13,000 *g* for 10 min. This procedure was repeated 3 more times. The retentate is the desalted EPO standard. The desalted EPO was recovered by placing the cup upside down into a new microcentrifuge tube as a receptacle and centrifuged at 2,000 *g* for 2 min. After concentration the sample was filtered through a 0.2 µL syringe filter and then stored at 4 °C. The recovered volume was 125 µL.

The protein content was measured spectrophotometrically at 280 nm, as per the European Pharmacopoeia, using a Beckman Coulter DU® 800 Spectrophotometer. The absorptivity of a 1% solution of EPO is 7.43. The concentration of desalted EPO was found to be 1.0 mg/mL.

Buffer Preparation. Instructions as follows:

Buffer Concentrate: The buffer preparation was performed as described by the European Pharmacopoeia. To a 100 mL volumetric flask were added: 0.582 g of NaCl (S1249 - Spectrum), 1.793 g of Tricine (cat. no. T5816 – Sigma), 0.820 g of sodium acetate (cat. no. 3470-01 - J.T. Baker), DDI water was added until 100 mL mark. This solution was filtered through a 0.2 µm membrane using a Nalgene filter (unit MF75, VWR cat. no. 28199-338) and stored at 4 °C.

1M Putrescine solution: A bottle of putrescine (cat. no. 3279 - Sigma) was placed in a water bath at

60 °C and all contents were allowed to melt. This process took between 20 to 30 min. With a disposable transfer pipette, 0.882 g of putrescine was directly weighed into a 10 mL volumetric flask. DDI water was added to the 10 mL mark and the solution was mixed. The solution was split into 500 µL aliquots and stored at 4 °C.

CZE running buffer: To a 50 mL polypropylene tube were added 21 g of urea (cat. no. U1250 – Sigma), 5 mL of CZE buffer concentrate and 125 µL of 1M Putrescine and finally 25 mL of DDI water.

The pH was adjusted to 5.5 at 30 °C, with 2N acetic acid prepared from glacial acetic acid, (cat. no. A6283 – Sigma). This solution was filtered through a 0.2 micron membrane using a Nalgene filter unit MF75 (VWR cat. no. 28199-338) and stored at 4 °C. This buffer has a shelf life of one week.

Setting up the Separation Methods in 32 Karat: Three separate methods were created in 32 Karat v 8.0 software. These include capillary equilibration, EPO separation and shutdown.

The initial conditions are the same for all three methods and were set as follows:

Capillary/ Sample Storage Initial Conditions: Voltage current maximum were set at 30.0 kV and 300 µA respectively. The cartridge temperature was thermostatted at 35.0 °C and sample storage was set to 4 °C. Peak detect parameters were set to a threshold of 2 and peak width of 9. Analog output scaling was set to 1.

UV Detector Initial Conditions: Acquisition enabled was set at a wavelength of 214 nm and data rate of 0.5 Hz. Filter setting was 'Normal' and peak width points were 16-25. Direct absorbance detection was used.

Current: The current generated throughout the separation should be between 5 and 6 µA.

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	60.00 min	BI:A6	BO:A6	forward	0.1 N NaOH rinse
2		Rinse - Pressure	20.0 psi	60.00 min	BI:B6	BO:A6	forward	CZE running buffer rinse
3	0.00	Separate - Voltage	20.0 KV	720.00 min	BI:C6	BO:B6	0.17 Min ramp, normal polarity	Capillary voltage equilibration
4	720.10	Stop data						
5	720.20	End						
6								

Figure 1: Capillary equilibrium time program.

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	10.00 min	BI:B1	BO:B1	forward	Water rinse
2		Rinse - Pressure	20.0 psi	5.00 min	BI:C1	BO:B1	forward	0.1N NaOH rinse
3		Rinse - Pressure	20.0 psi	10.00 min	BI:D1	BO:B1	forward	CZE Running Buffer rinse
4		Inject - Pressure	0.7 psi	20.0 sec	SI:A1	BO:C1	Override, forward	
5	0.00	Separate - Voltage	15.7 KV	70.00 min	BI:E1	BO:C1	0.17 Min ramp, normal polarity	Separation 143 V/cm
6	5.00	Autozero						
7	70.10	Stop data						
8	70.20	End						

Figure 2: EPO separation time program.

	Time (min)	Event	Value	Duration	Inlet vial	Outlet vial	Summary	Comments
1		Rinse - Pressure	20.0 psi	10.00 min	BI:B1	BO:B1	forward	Water rinse
2		Rinse - Pressure	20.0 psi	5.00 min	BI:C1	BO:B1	forward	0.1N NaOH rinse
3	0.00	Separate - Pressure	20.0 psi	10.00 min	BI:A1	BO:A1	forward	Buffer rinse
4	10.10	Stop data						
5	10.20	Lamp - Off						
6	10.30	End						
7								

Figure 3: Shutdown time program.

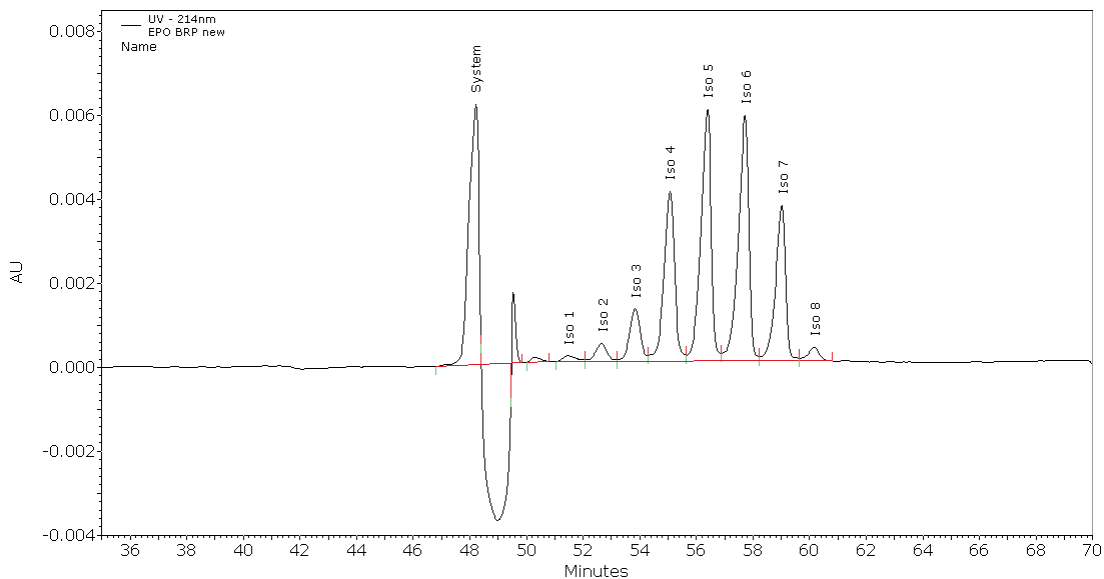


Figure 4: Typical Electropherogram of EPO BRP Batch 3.

Peak Integration Parameters. In order to take full advantage of the software's ability to analyze data as it is acquired, a few parameters needed to be set in the 32 Karat software.

The integration parameters optimized for the analysis of EPO BRP3 was set as follows:

#		Event	Start Time	Stop Time	Value
1	<input checked="" type="checkbox"/>	Width	0.000	0.000	0.3
2	<input checked="" type="checkbox"/>	Threshold	0.000	0.000	25
3	<input checked="" type="checkbox"/>	Percentile Point	0.000	0.000	25

Figure 5: Peak integration parameters for analysis of EPO BRP3.

Named Peaks		Groups					
#		Name	ID	Mig. Time	MT Window	Ref. ID #	ISTD. ID #
1	<input checked="" type="checkbox"/>	System	1	48.2667	10	1	0
2	<input checked="" type="checkbox"/>	Iso 1	2	51.1	2.555	1	0
3	<input checked="" type="checkbox"/>	Iso 2	3	52.3667	2.61833	1	0
4	<input checked="" type="checkbox"/>	Iso 3	4	53.6667	2.68333	1	0
5	<input checked="" type="checkbox"/>	Iso 4	5	55	2.75	1	0
6	<input checked="" type="checkbox"/>	Iso 5	6	56.4	2.82	1	0
7	<input checked="" type="checkbox"/>	Iso 6	7	57.8333	2.89167	1	0
8	<input checked="" type="checkbox"/>	Iso 7	8	59	3	1	0
9	<input checked="" type="checkbox"/>	Iso 8	9	60	3	1	0

Figure 6: The peak IS table was set as shown above so that the software can call the peak names properly.

Note that one must set-up integration parameters and a peak ID table in order to be able to set up system suitability.

According to European Pharmacopoeia, the system suitability must be run in triplicate and the parameters are set as follows on 32 Karat:

Step 1. Largest peak is 50 times greater than baseline noise.

Step 2. Resolution of peaks 5 and 6 is not less than 1.

Step 3. Percent RSD for migration time of isoform 2 is 2%.

The figure displays three screenshots of the 32 Karat software interface, each showing the 'Compound:' selection list and a table of system suitability parameters. In all three, 'Iso 5' is selected in the list.

Screenshot 1 (Top): The 'Compound:' list shows 'Iso 5' selected. The parameter table is:

#	Parameter	Min	Max	%RSD
1	S/N (ASTM)	50		
2				

Screenshot 2 (Middle): The 'Compound:' list shows 'Iso 6' selected. The parameter table is:

#	Parameter	Min	Max	%RSD
1	Resolution (USP)	1		
2				

Screenshot 3 (Bottom): The 'Compound:' list shows 'Iso 2' selected. The parameter table is:

#	Parameter	Min	Max	%RSD
1	Migration Time			2
2	Corrected Migration Time			2
3				

Figure 7: Set-up on 32 Karat software to run system suitability in triplicate.

Run #	Run Type	Reps	Sample Inject Duration	Sample ID	Method	Filename
1	Sys Suit Begin	1		EPO BRP 3 V3	suit 2.met	epo brp 3 v3-004.dat
2	Sys Suit Std.	1		EPO BRP 3 V3	suit 2.met	epo brp 3 v3-005.dat
3	Sys Suit End	1		EPO BRP 3 V3	suit 2.met	epo brp 3 v3-006.dat

Figure 8: A sequence table can be created to run triplicates of the system suitability standard as shown in Figure 7.

For a system passing the suitability parameters, a sequence suitability report is automatically generated by 32 Karat.

System Suitability Report Page 1 of 1

Sequence : C:\32Karat\Projects\Default\Sequence\epo.seq
User : Proteom elab
Printed : 11/06/2008 11:11:08 AM

System is Suitable

UV - 214nm	Compound	Parameter	Min	Max	%RSD
	Iso 2	mt			2
		correctmig			2
	Iso 5	snastm	50		
	Iso 6	resusp	1		

Sample ID	Compound	Parameter	Average	Low	High	%RSD	Status
	Iso 2	mt	49.589	49.267	49.900	0.639	
EPO BRP 3 V3			49.900				Passed
EPO BRP 3 V3			49.600				Passed
EPO BRP 3 V3			49.267				Passed
		correctmig	52.29775119	52.28310328	52.30943076	0.026	
EPO BRP 3 V3			52.28310328				Passed
EPO BRP 3 V3			52.30943076				Passed
EPO BRP 3 V3			52.30071953				Passed
	Iso 5	snastm	348.151193	225.296212	433.467512	31.321	
EPO BRP 3 V3			385.689854				Passed
EPO BRP 3 V3			433.467512				Passed
EPO BRP 3 V3			225.296212				Passed
	Iso 6	resusp	2.00092	1.94620	2.04229	2.470	
EPO BRP 3 V3			2.01426				Passed
EPO BRP 3 V3			1.94620				Passed
EPO BRP 3 V3			2.04229				Passed

Figure 9: Sample 32 Karat sequence suitability report.

Summary

Proper characterization of EPO destined for therapeutic use is critical for patient safety. Capillary Electrophoresis technology can be readily applied to this process and can easily be implemented to achieve high resolution isoform separation of this inherently complex glycoprotein. Using the European Pharmacopeia CZE method and applying it to the PA 800 Protein Characterization System, we were able to separate EPO isoforms from one another in a qualitative and quantitative manner. This work illustrates the utility of the PA 800 system for analysis of a complex glycoprotein.

Troubleshooting Aid

Severe Shift in Migration Time or Unresolved EPO Peaks.

This behavior indicates the capillary

may not have been properly equilibrated. The equilibration of the capillary is quite long but necessary to stabilize both the UV baseline and the capillary surface for reproducible migration time, improvement of peak shape and thus resolution. Figure 1 shows 4 runs of an 11 run sequence, where the capillary was voltage equilibrated for only 5 min. Note the severe drift on the baseline for run no. 1 and only a small disturbance on the baseline indicative of the presence of EPO. As the sequence gradually progresses the baseline starts to stabilize but the EPO peaks show a very poor resolution, even after 10 runs.

Peak Shape and Resolution. Desalting and filtration of the sample is quite important for a successful separation⁴. Figure 13 shows an electropherogram of EPO BRP3 which was not filtered or desalted. Note that the 8 isoforms are not visible.

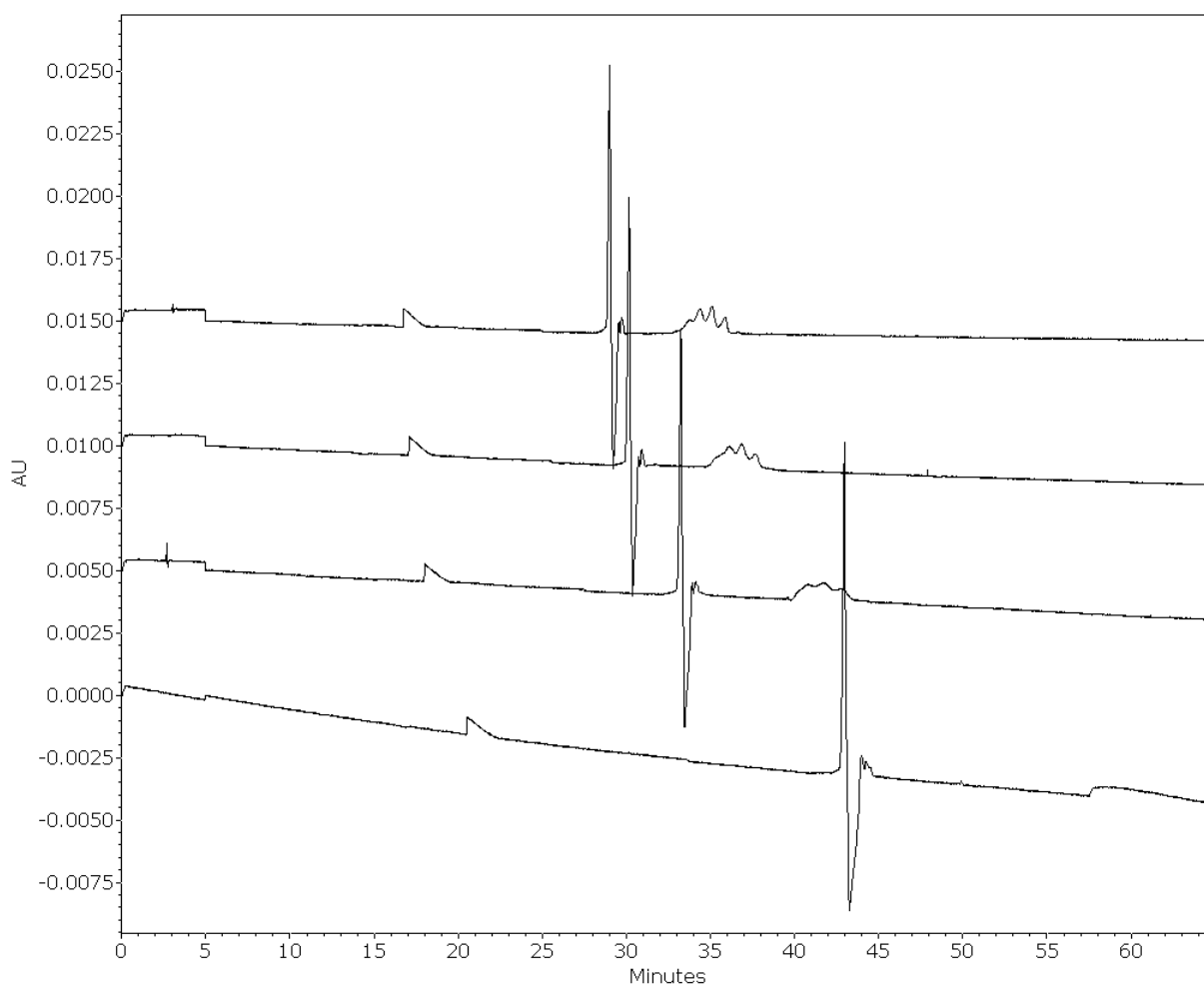


Figure 10: Peaks poorly resolved following improper capillary equilibration.

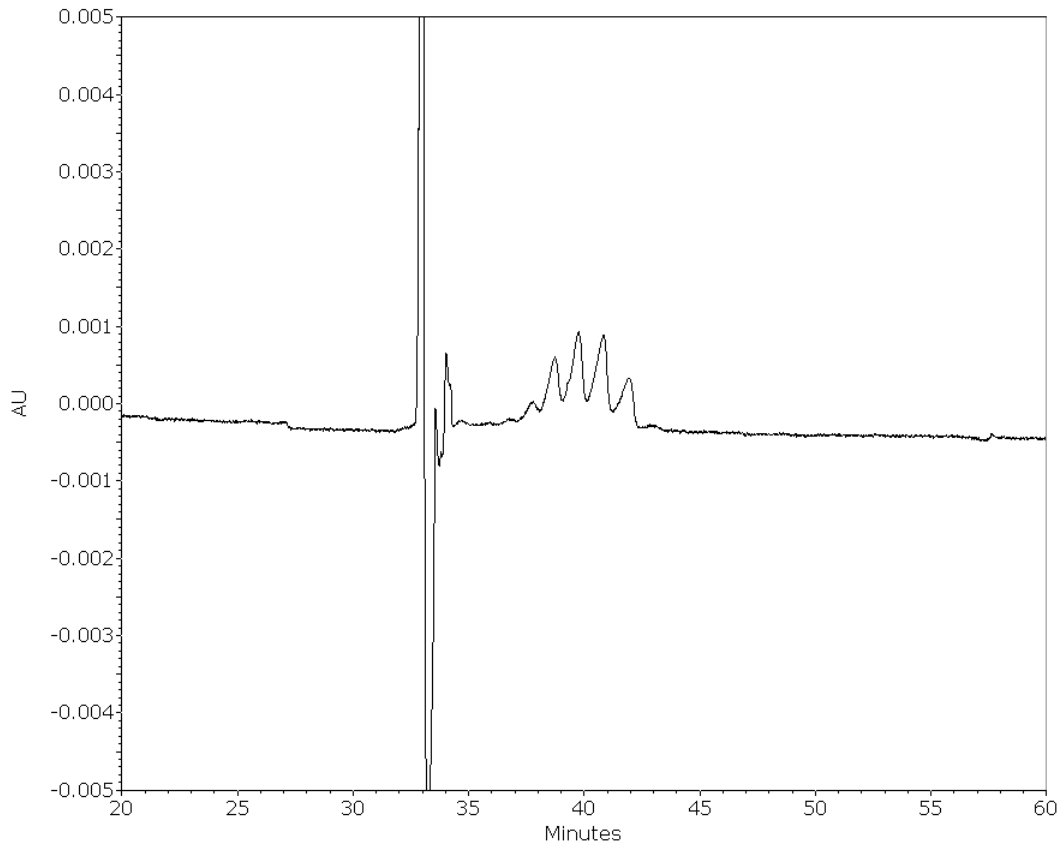


Figure 11: Example of unfiltered and not-desalted EPO CE separation.

References

1. M. Wadman, *Nature* 404(2000)532.
2. <http://www.ich.org/cache/compo/363-272-1.html>
3. A. Bristow and E. Charton, *Pharmeuropa*, 11-2(1999)290.
4. V. S. Nebot, F. Benavente, A. Valverde, N. Guzman and J. Barbosa, *Anal. Chem.* 75 (2003) 5220.

The DU 800 and the ProteomeLab PA 800 Protein Characterization System are for laboratory use only; not for use in diagnostic procedures.

Beckman Coulter, the stylized logo and DU are registered trademarks of Beckman Coulter, Inc. ProteomeLab and 32 Karat are trademarks of Beckman Coulter, Inc. Nalgene is a registered trademark of Thermo Fisher Scientific. Microcon is a registered trademark of Millipore.

Australia, Gladesville (61) 2 9844-6000 Canada, Mississauga (1) 905 819 1234 China, Beijing (86) 10 6515 6028
Czech Republic, Prague (420) 272 01 73 32 Eastern Europe, Middle East, North Africa, South West Asia: Switzerland, Nyon (41) 22 365 3707
France, Villepinte (33) 1 49 90 90 00 Germany, Krefeld (49) 2151 33 35 Hong Kong (852) 2814 7431 India, Mumbai (91) 22 3080 5000
Italy, Cassina de' Pecchi, Milan (39) 02 953921 Japan, Tokyo (81) 3 5530 8500 Korea, Seoul (82) 2 404 2146 Latin America (1) (305) 380 4709
Mexico, Mexico City (001) 52 55 9183 2800 Netherlands, Woerden (31) 348 462462 Puerto Rico (787) 747 3335 Singapore (65) 6339 3633
South Africa/Sub-Saharan Africa, Johannesburg (27) 11 564 3203 Spain, Madrid (34) 91 3836080 Sweden, Bromma (46) 8 564 85 900
Switzerland, Nyon (41) 0800 850 810 Taiwan, Taipei (886) 2 2378 3456 Turkey, Istanbul (90) 216 570 17 17 UK, High Wycombe (44) 01494 441181
USA, Fullerton, CA (1) 800 742 2345

