Automated Off-Line 2-D LC: A Powerful Tool for the Separation of Complex Samples with the UltiMate 3000 Proteomics MDLC System

INTRODUCTION

Analytical laboratories have experienced an increase in the number of analytes that have to be measured in a single experiment. For example, samples from proteomics experiments can contain thousands of species, a number that is well beyond the peak capacity of a one-dimensional (1-D) separation. However, separation of these species is necessary to accurately characterize the sample. One approach to achieving this is to use multidimensional LC (MDLC), one of the strongest and most flexible ways to increase the peak capacity of a separation. The peak capacity of an MDLC method can be calculated by multiplying the peak capacities of the applied dimensions (provided that they are orthogonal).

MDLC methods can be performed in either on- and off-line modes. Typically, on-line MDLC methods have the advantage of full automation, but lack flexibility. Off-line MDLC methods offer virtually unlimited flexibility, but are difficult to automate.

A technique that overcomes the limitations of both approaches and offers unique possibilities for the MDLC analysis of complex samples is automated off-line MDLC.

This technical note describes a unique automated off-line MDLC solution, based on the microfraction collection (µFC) option of the WPS 3000PL autosampler. The µFC option allows injection, fractionation, and reinjection on a single LC system. Complemented by smart software tools, the automated off-line MDLC solutions offers the flexibility of an off-line strategy with the ease-of-use of an on-line approach, and allows laboratories to easily resolve a large number of analytes.

AUTOMATED OFF-LINE MDLC

System and Related Advantages

Automated off-line MDLC can be applied to the separation of both peptides and proteins, making it an ideal tool for proteomics research. The UltiMate® 3000 Proteomics MDLC system with the microfraction collection (µFC) option is the basis for all automated off-line MDLC solutions. The unique benefits offered by each system feature are summarized in Table 1.

Table 1. Benefits of an Automated Off-Line MDLC System	
System Feature	**Benefit**
Microfractionation	• Multiple analysis of fractions • Ability to use different column diameters • Ability to use different column chemistries • Intermediate sample handling, e.g. derivatization, digestion. • Temperature control (cooling/heating)
Dual gradient pump	• Small footprint • Two independent ternary gradients
Flow manager	• Column switching valves to switch between 1st and 2nd dimension • UltiFlow eluent delivery system providing nano flow rates • Optimal retention time reproducibility by precise column thermostatting
UV detection	• Online separation monitoring
Chromeleon software	• Fractionation tracking • Automated 2-D sequence generation • 2-D Data representation for comprehensive overview • System wellness monitoring

Injection and Fractionation Using the µFC Option

The 8-port valve of the µFC option allows for sample injection and fractionation of the column effluent. Figure 1 shows the valve in the load (top) and inject (bottom) position. In the load position, the syringe is used to draw the sample into the loop. Upon switching, the sample is loaded onto the column and separated. The column effluent returns to the valve (port 7) and to the needle (port 6). By moving the needle from the drain position to empty vials or a well plate, the eluting sample can be fractionated.

The injection of the fractions occurs in a similar way to the injection of the original sample. The switching valves in the FLM direct the reinjected sample to the second dimension column as shown in Figure 2.

Workflow for Automated Off-line Two Dimensional LC

Figure 2 shows a complete setup with an 8-port valve on the WPS and two 10-port switching valves in the FLM. The right 10-port valve is used to switch between the first and second dimensions.

The DGP-3600 pump delivers two independent ternary gradients to both dimensions. The left pump is typically used for the first dimension gradient (A_1B_1) and second dimension sample loading (C_1). The right pump is typically used for the second dimension gradient (A_2B_2).

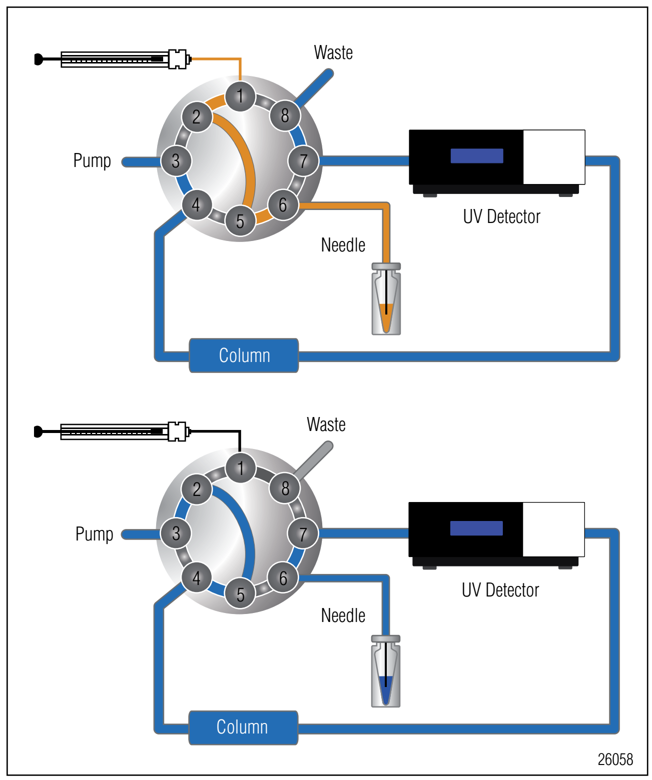

Figure 1. Fluidic setup on the 8-port valve. Top: Load position; column effluent is directed to waste. Bottom: Inject position; effluent is redirected to the injection needle.

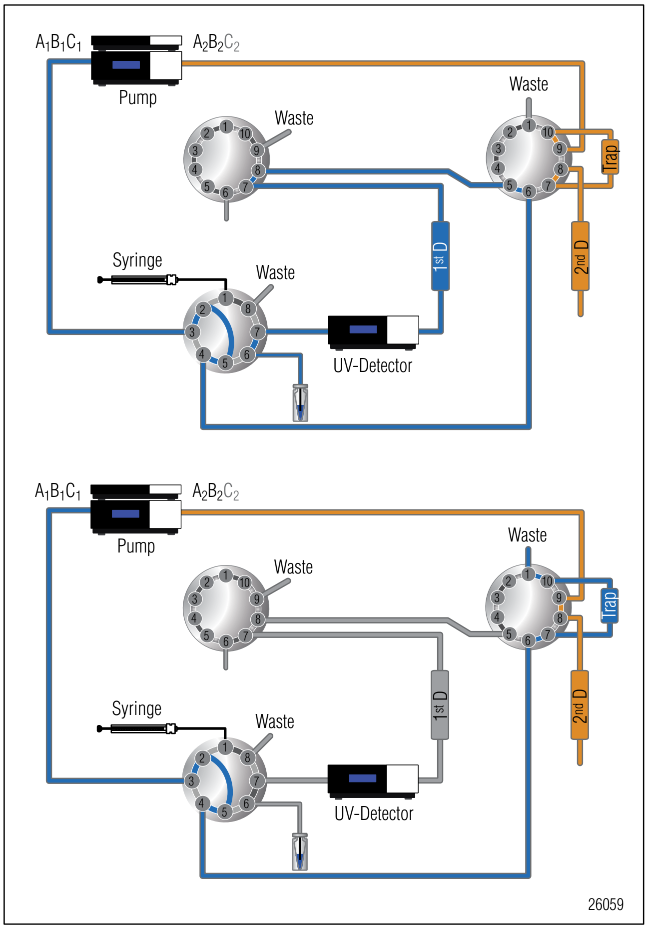

Figure 2. Automated off-line two-dimensional configuration; Top: the injected sample is separated in the first dimension and fractionated by the WPS. Bottom: The reinjected fraction is loaded onto the trap column for second-dimension analysis.

Figure 2 (top) shows the separation on the first-dimension column and the fractionation of the column effluent. In Figure 2 (bottom) the reinjected fractions are loaded onto the trap column after which they are analyzed according to a standard preconcentration method. The second dimension can be combined with a second UV detector or interfaced directly with an MS instrument.

Table 2 shows the details of the applied solvents and the positions of the valves during the analysis.

The configuration shown in Figure 2 is the basic setup; there are many variations possible (e.g. dual fractionation), which are discussed in more detail in dedicated application notes.

SOFTWARE

Running off-line MDLC separations is often considered a complex process with many factors to take into account. Chromeleon® Chromatography Data System software includes dedicated tools to simplify this process. The Chromeleon is used to control the UltiMate 3000 LC system and provides software tools for maximum ease-of-use in the automated off-line application.

Table 2. Details on the Applied Solvents and Valve Positions at the Different Steps of the 2-D Analysis

	Pump Solvent		Valve positions		
Step	**Left**	**Right**	**WPS**	**FLM Left**	**FLM Right**
1. Injection of sample	A_1	Off	1_2	1_2	1_2
2. Separation on 1st D column and fractionation	A_1B_1	Off	8_1	1_2	1_2
3. Reinjection of fractions	C_1	A_2	1_2	10_1	10_1
4. Separation on 2nd D column	C_1	A_2B_2	8_1	10_1	1_2

Automated Sequence Generation

The Chromeleon can generate a new sequence automatically for the collected fractions. During analysis Chromeleon records where and how many fractions are collected. Next, the post-acquisition steps functionality uses this information to generate the second-dimension sequence for follow-up analysis (Figure 3).

2-D Retention Map

The MDLC data consists of multiple, unique chromatograms of the same original sample. It is necessary to view all these chromatograms together to get the maximum amount of information about the sample. This is supported in Chromeleon through the 2-D retention map tool.

The tool displays the UV data of the second dimension in a 2-D retention map. The correlation between the separate fractions becomes immediately clear and a comprehensive view of the sample is given. With UV traces in both dimensions, it is possible to actively link the fractions from the first dimension to the corresponding second dimension chromatograms (Fig.2)

APPLICATIONS

The strength of the automated off-line MDLC solution is further demonstrated by two applications that can be readily applied to proteomics analysis. The first is a bottom-up separation and the second is a top-down analysis in which the autosampler performs three roles; injection, fractionation, and digestion of the samples.

Bottom-up Analysis of Complex Peptide Samples

The most common 2-D LC analysis for peptides in proteomics is the combination of SCX and RP chromatography. These are typically used in well-known online approaches such as 2-D salt plugs and MudPit, and with MS detection.

The fractionation step in an off-line workflow allows intermediate sample handling. In the workflow shown below, Heptafluorobutyric acid (HFBA) was added to the fractions to enhance the trapping efficiency during the loading and desalting phase. In this bottom-up example, a 300 µm ID SCX column was used in the first dimension, and a PepSwift monolithic column in the second dimension. The fluidic setup is a derivative of Figure 2.

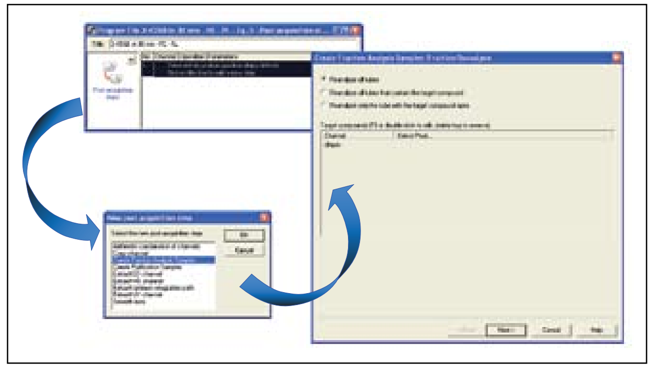

Figure 3. A wizard assists in setting up the post-acquisition steps.

Retention time reproducibility is important to verify experimental results and is especially desired in biomarker validation. UltiFlow™ technology is designed to deliver reproducible flow and gradients, but in a 2-D LC analysis the complete system should also work reproducibly. A complex tryptic digest was injected three times to evaluate the reproducibility of the entire bottom-up analysis.

Figure 5 shows the MS and MS/MS data of the same fraction of the three independent 2D analyses. As demonstrated by the retention times, the MS and the

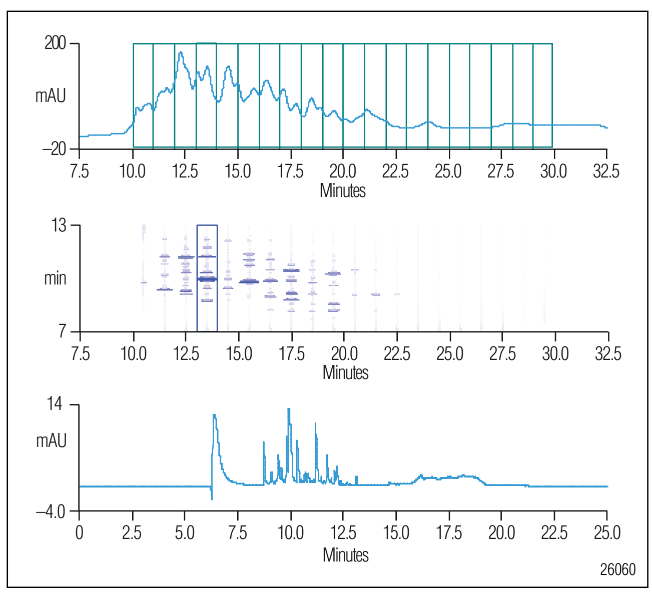

Figure 4. The 2-D retention map with 1st dimension and 2nd dimension chromatogram and fractions.

MS/MS spectra are identical, even though the spectra were acquired at 12 hour intervals.

These results demonstrate that the automated off-line 2-D LC analysis was able to inject, separate, fractionate, reinject, and separate the 2nd dimension with excellent reproducibility.

A detailed description, including experimental conditions, is given in Technical Note 78.

Gel Free Top-Down Workflow

The automated off-line MDLC solution is not restricted to one sample type. By choosing different columns, an entire protein workflow can be followed.

Figure 6 shows a gel-free top-down workflow consisting of a 2-D protein separation and dual fractionation. The intermediate sample handling in this example involved protein digestion. The autosampler was used to add trypsin to each fraction and perform an in-well digestion at 30 °C for 3 h. This was performed overnight and allowed peptide analysis of selected fractions immediately the next morning.

The 2-D retention map can be used to evaluate the protein separation and select fractions of interest for follow-up analysis. This is another example how an off-line approach can be more efficient; no analysis time is wasted on empty fractions.

The analysis was performed using intact proteins from an *E.Coli* cell lysate and the 2-D retention map of the protein separation is given in Figure 7. The identified and externally verified proteins are shown for each of the selected fractions.

A detailed description, including experimental conditions, is given in Technical Note 79.

Other Applications

The flexibility of the automated off-line MDLC solution allows many different column chemistries to be combined. Exciting applications that show good orthogonality include RP (basic)-RP (acidic), or HILIC-RP. Another possibility would include post-fractionation derivatization with the autosampler.

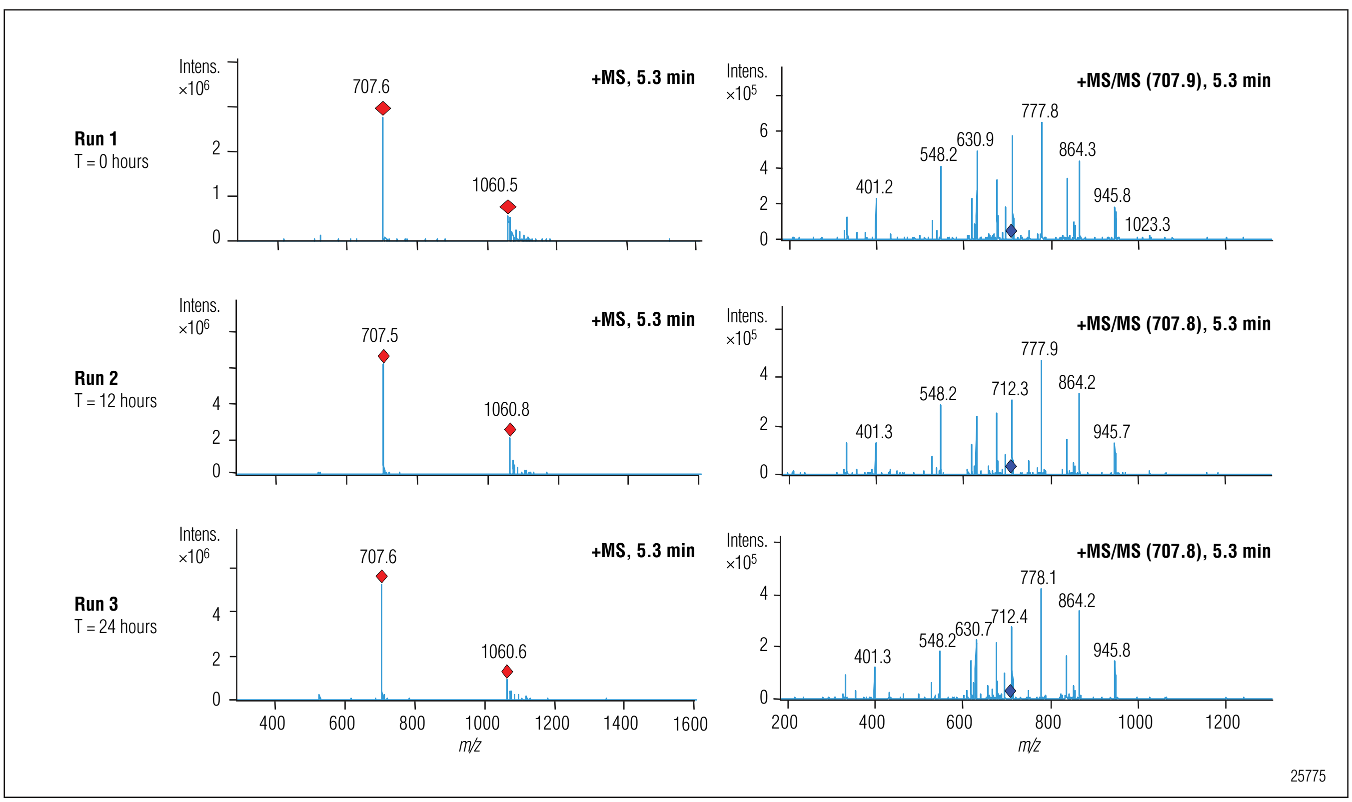

Figure 5. MS and MS/MS spectra of fraction 13 of three replicate 2-D experiments. Each set of MS and MS/MS spectrum was recorded at 12 h intervals.

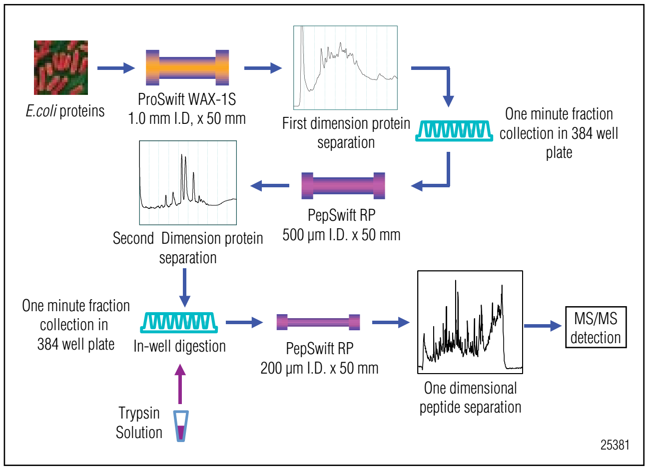

Figure 6. Schematic layout of the protein workflow, including digestion and peptide MS analysis.

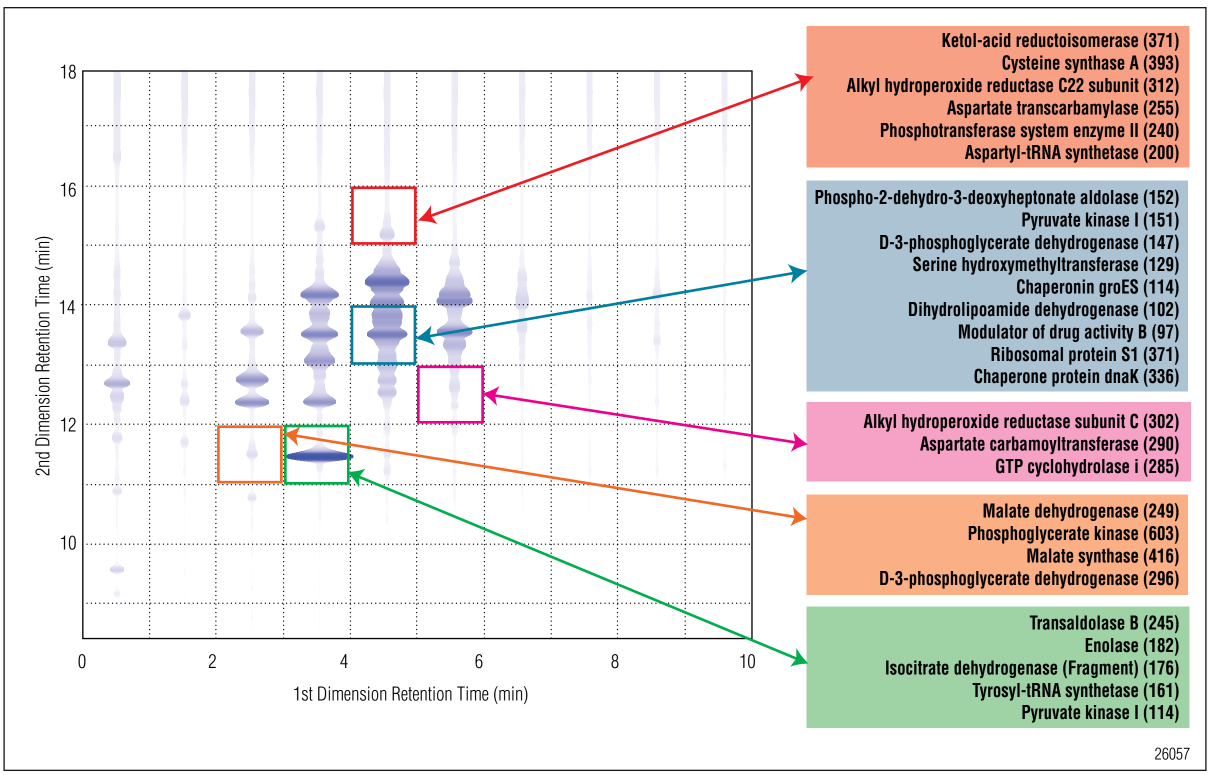

Figure 7. 2-D retention map of the 2-D E. Coli *separation. The fractions selected for follow up analysis are indicated in red outline*

CONCLUSIONS

- Off-line MDLC provides superior separation performance over most on-line techniques, due to the true gradient elution. This is critical for proteomics analyses, as better separations lead to identification of more species.
- The microfractionation ability of the autosampler allows immediate and unattended reinjection of collected fractions. The UltiMate 3000 Proteomics MDLC and Chromeleon Chromatography Data System ensure the flexibility and ease-of-use of the system.
- Excellent performance was demonstrated for peptides and proteins analysis in terms of robustness, reproducibility, and flexibility. Both of the examples shown here are supported by dedicated application kits.

MATERIALS

Standard System

UltiMate 3000 Proteomics MDLC (contact local Dionex representative for P/N)

Microfraction Collection Option, WPS-3000PL (P/N 6820.0051)

Off-line 2-D LC kit for Peptides, Polysulfoethyl ASP 300 µm i.d./PepSwift™ 200 µm i.d. (P/N 6720.0100)

Off-line 2-D LC kit for Peptides, Polysulfoethyl ASP 1.0 mm i.d./PepMap™100 C18 75 µm i.d. (P/N 6720.0101)

Off-line 2-D LC kit for Proteins, ProSwift® WAX 1.0 mm i.d./PepSwift 200 µm i.d. (P/N 6720.0102)

Off-line 2-D LC kit for Proteins, ProSwift WAX 1.0 mm i.d./PepSwift 500 µm i.d. (P/N 6720.0103)

Biocompatible System

UltiMate 3000 Proteomics MDLC, biocompatible (contact local Dionex representative for P/N)

Micro Fraction Collection Option, WPS-3000PL, biocompatible (P/N 6821.0051)

Off-line 2-D LC kit for Peptides, Polysulfoethyl ASP 300 µm i.d./PepSwift 200 µm i.d., Biocompatible (P/N 6721.0100)

Off-line 2-D LC kit for Peptides, Polysulfoethyl ASP 1.0 mm i.d./PepMap100 C18 75 µm i.d., Biocompatible (P/N 6721.0101)

Off-line 2-D LC kit for Proteins, ProSwift WAX 1.0 mm i.d./PepSwift 200 µm i.d., Biocompatible (P/N 6721.0102)

Off-line 2-D LC kit for Proteins, ProSwift WAX 1.0 mm i.d./PepSwift 500 µm i.d., Biocompatible (P/N 6721.0103)

Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
U.S./Canada (847) 295-7500

South America
Brazil (55) 11 3731 5140

Europe
Austria (43) 1 616 51 25 Benelux (31) 20 683 9768 (32) 3 353 4294
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0
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Switzerland (41) 62 205 9966 United Kingdom (44) 1276 691722

Asia Pacific
Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190
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