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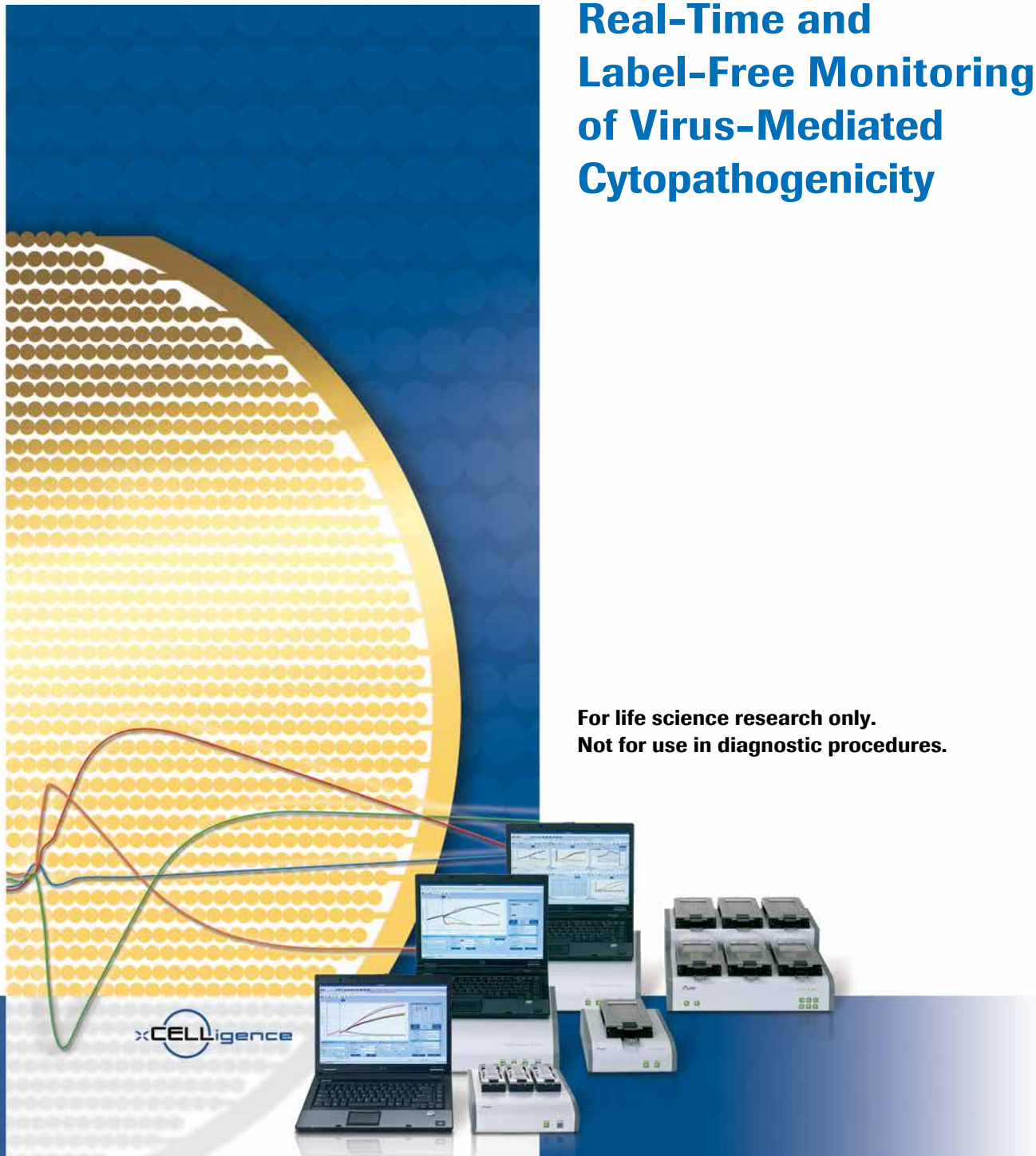
xCELLigence System

Application Note No. 9/January 2013

Real-Time and Label-Free Monitoring of Virus-Mediated Cytopathogenicity



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Real-Time and Label-Free Monitoring of Virus-Mediated Cytopathogenicity

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Introduction

To understand virus infectivity, progression, and disease-onset, it is important to perform a detailed examination of cytopathic effects. Depending on the type of virus and cells infected, different cytopathic effects will be observed dependent on the time point of infection (*e.g.,* during the growth or stationary phase), and the amount of virus involved.

One of the most common methods for measuring lysogenic/lytic activity of viruses is the plaque test (Knipe and Howley, 2007). In this assay, culture plates with confluent cells that are sensitive towards the specific virus type/strain are used. Lytic activity of virus is detected by macroscopic analysis of plaques in a confluent cell layer after staining with, for example, crystal violet. This technique is an endpoint assay and well suited for determining viral titers. However, it provides no information about the onset of cytopathic effects and the kinetics of the viral replication. Moreover, infection with a number of viruses such as attenuated mutants leads to the formation of small turbid plaques which are difficult to detect, or produce no plaques although the virus replicates successfully.

The xCELLigence System, using microelectronic biosensor technology allows dynamic, real-time label-free and non-invasive analysis of cellular events (Solly *et al.,* 2004), including viral cytopathogenicity. The system measures electronic impedance using an array of microelectrodes located in the bottom of each culture well. When adherent cells attach to the sensor surface of electrode arrays, the changes in impedance are detected and recorded. The microelectrodes are integrated in special cell culture plates, called E-Plates 96, allowing experiments in the standard 96-well format.

By this means, the xCELLigence System can monitor any cell events, including cell number change, cell adhesion, cell viability, cell morphology, and cell motility. These Real-Time Cell Analyzer (RTCA) Instruments can be used in a broad spectrum of research fields, including drug development, toxicology, oncology, medical microbiology, and virology (Solly *et al.,* 2004). This technology has already been used for proliferation and cytotoxicity assays (Solly *et al.,* 2004), cell adhesion and spreading (Atienza *et al.,* 2005), cell culture quality control (Kirstein *et al.,* 2006), receptor tyrosine kinase activation (Atienza *et al.,* 2006), mast cell activation (Abassi *et al.,* 2004), and G protein-coupled receptor (GPCR) activation (Yu *et al.,* 2006).

The xCELLigence System comprises three components,

1. **the RTCA Single Plate (SP) Station,**
2. **the RTCA Analyzer, and**
3. **the RTCA Control Unit with integrated software.**

The RTCA SP Station with E-Plate easily fits inside a standard cell culture incubator, ensuring a temperature-, humidity-, and CO_2-controlled environment. The RTCA SP Station can accommodate one E-Plate 96. The bottom of each well of an E-Plate 96 contains the sensor electrode arrays required to record cell impedance changes in the overlying cells. Measured electrode impedance is expressed as a dimensionless Cell Index (CI) and graphically represented using the software to show the phenotypic changes of a cell population over time.

Introduction continued

This application note describes the experimental setup for studying virus-mediated cytopathogenicity using the Real-Time Cell Analyzer (RTCA) Instruments. This assay overcomes many of the limitations of the single endpoint plaque test, providing evidence that RTCA Instruments are a simple, easy way to identify and characterize virus-mediated cytopathic effects comprehensively.

Materials and Methods

Cells. Cells were cultured in a standard humidified incubator at +37°C with 5% CO_2 saturation. Vero E6, obtained from the ATCC, is an African green monkey kidney-derived cell line with deficiency of the type I interferon genes. HEK 293, obtained from Microbix Biosystems, is a human embryonic kidney cell line with intact interferon system. Both adherent cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, 2 mM glutamine and 1% penicillin/streptomycin.

Virus. The vesicular stomatitis virus (VSV), Serotype Indiana, was grown and titrated on Vero E6 cells at +37°C with 5% CO_2.

Cell Proliferation Assays. For real-time cell analysis, 100 µl of growth media was added to each well of the E-Plate 96 to obtain background readings. For each cell type, a sequential 1:1 dilution series with 7 different cell numbers ranging from 50,000 to 781 cells/well were resuspended in 100 µl of media and then seeded into the E-Plate 96. The E-Plates 96 containing cells were incubated for 30 minutes at room temperature, and placed on the RTCA SP Station located in the cell culture incubator. Cell attachment, spreading, and proliferation were monitored every 30 minutes using the RTCA SP Instrument. Measured impedance recordings from cells in each individual well on the E-Plate 96 were automatically converted to Cell Index (CI) values by the RTCA Software.

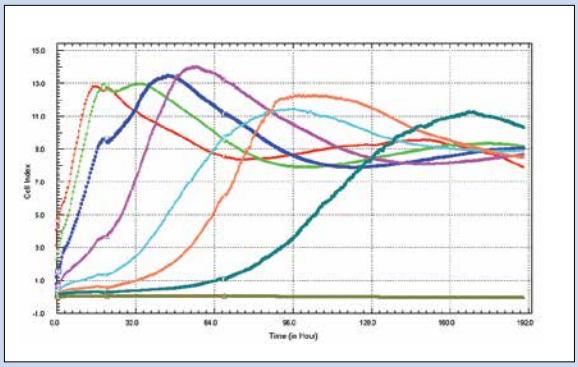
Assessment of virus-mediated cytopathogenicity. For viral studies, 25,000 cells/well and 12,500 cells/well of each cell line were seeded into each well of an E-Plate 96. After 20.5 hours (Vero E6 cells) and 68.5 hours (HEK 293 cells), when the cells had reached either confluency (25,000 cells/well) or were still in the growth phase (12,500 cells/well), cells were infected with the vesicular stomatitis virus (VSV). The E-Plate 96 was removed from the RTCA SP Station, and 800,000 ("high MOI") or 80,000 ("low MOI") PFU VSV, resuspended in 10 µl growth media, were added to the wells. As the control, eight wells were mock-infected by adding 10 µl growth media only. The E-Plate 96 was then placed back immediately into the RTCA SP Station in the incubator, and the CI values were measured every 15 minutes for up to 190 hours.

Results and Discussion

Dynamic Monitoring of Cell Proliferation

To identify the optimal time point for viral infection, a cell proliferation analysis was performed with Vero E6 and HEK 293 cells. Suitable time points for virus infection were defined at 20.5 hours for Vero E6 cells and 68.5 hours for HEK 293 cells (see Figure 1 A & B). At these time points, cells were either in the growth phase when 12,500 cells had been used for seeding or in the early stationary phase when 25,000 cells had been used for seeding. Therefore, viral cytopathogenicity was monitored in either the growth phase or the early stationary phase.

A: Proliferation Curve of Vero E6 Cells



B: Proliferation Curve of HEK 293 Cells

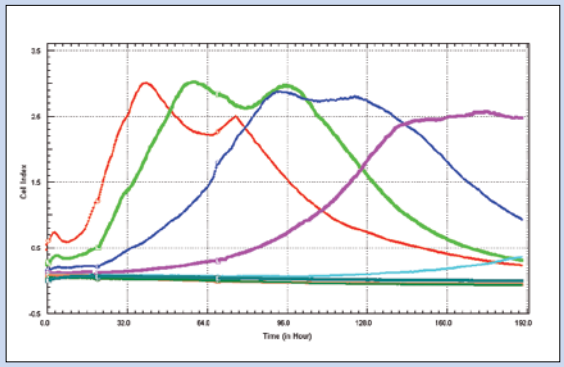
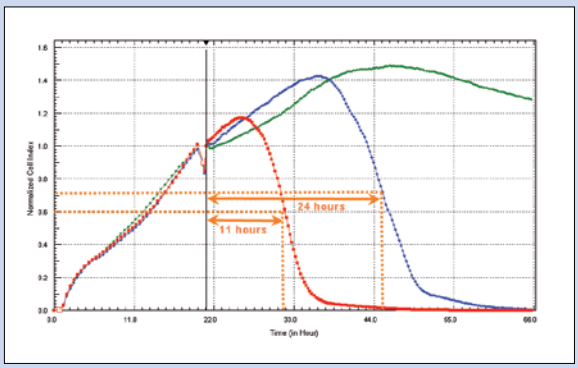


Figure 1: Dynamic monitoring of cell proliferation.
Cells were seeded in the E-Plate 96 and continuously monitored by measuring CI to identify a suitable time point for addition of virus (growth or early stationary phase). The adhesion, spreading, and proliferation of **(A)** Vero E6 cells and **(B)** HEK 293 cells were dynamically monitored every 30 minutes using the RTCA SP Instrument. Colored curves indicate the different cell numbers seeded per well in an E-Plate 96 (from left to right): **red, 50,000; green, 25,000; blue, 12,500; magenta, 6,250; cyan, 3,125; coral, 1,562; dark green, 781; olive green, medium control (without cells).**

VSV Cytopathogenicity profile using Vero E6 cells

Based on the dynamic monitoring of cell proliferation, at 20.5 hours after seeding, Vero E6 cells either in growth phase or in early stationary phase were infected with VSV using two different MOIs.

A: VSV Cytopathogenicity profile on Vero E6 Cells (12,500 Cells/well)



B: VSV Cytopathogenicity profile on Vero E6 Cells (25,000 Cells/well)

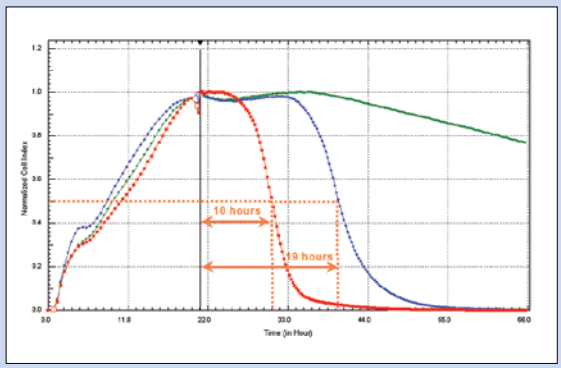


Figure 2: Dynamic monitoring of Vero E6 cells during VSV infection. (A) Normalized Cell Index values of growing cells, **(B)** Normalized Cell Index values of confluent cells. The virus-mediated effect on adhesion, spreading, and proliferation of the cells was monitored by measuring cell impedance every 15 minutes using the RTCA SP Instrument. Time of addition of virus at 20.5 hours is indicated by the black vertical line. The time point when the Cell Index (CI) value had decreased to 50% of the maximum (CI_{50}) value is indicated by the dotted orange lines.
Green curves: control (no viral infection); blue curves: 80,000 PFU VSV; red curves: 800,000 PFU VSV.

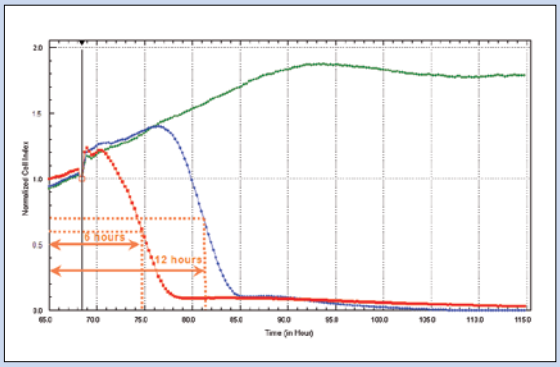
When Vero E6 cells were infected with VSV during the growth phase, there was a clear correlation between the amount of virus used for infection and the onset of the virus-mediated cytopathic effect (see Figure 2). After infection with a low MOI (80,000 PFU VSV), the cells continued to grow for 15 hours (see Figure 2A, blue curve) similar to mock-infected cells (see Figure 2A, green curve). Thereafter, CI values decreased, indicating that the cells were dying as a consequence of VSV replication. In contrast, mock-infected cells continued to grow. At 24 hours after infection, the CI values had decreased to 50% of the maximum value (CI_{50}) and then continued to decline to zero, indicating complete cell death in the infected culture. In contrast, the CI of Vero E6 cells infected with a high MOI (800,000 PFU VSV) started to decline already at 4 hours post infection (see Figure 2A, red curve), and the CI_{50} was already reached after 11 hours.

Very similar results were obtained when confluent Vero E6 cells were infected (see Figure 2B). The CI_{50} was reached at 10 hours post infection (high MOI, see Figure 2B, red curve), and 19 hours post infection (low MOI, see Figure 2B, blue curve) respectively. Again, complete death of the infected cultures was observed, as indicated by the decrease of CI values to zero.

VSV Cytopathogenicity profile using HEK 293 Cells

Based on the dynamic monitoring of cell proliferation, HEK 293 cells in the growth phase and in early stationary phase were infected with VSV 68.5 hours after seeding (see Figure 3 A & B).

A: VSV Cytopathogenicity profile on HEK 293 Cells (12,500 Cells/well)



B: VSV Cytopathogenicity profile on HEK 293 Cells (25,000 Cells/well)

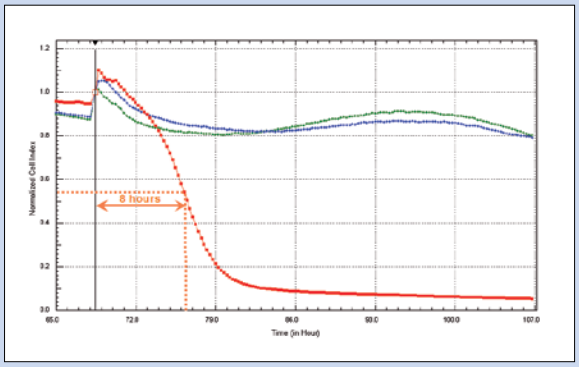


Figure 3: Dynamic monitoring of HEK 293 cells during viral infection. (A) Normalized Cell Index values of growing cells, (B) Normalized Cell Index values of confluent cells. The virus-mediated effect on adhesion, spreading, and proliferation of the cells was dynamically monitored every 15 minutes using the RTCA SP Instrument. Time of addition of virus at 68.5 hours is indicated by the black vertical line, the time point when the Cell Index had decreased to 50% of the maximum value (CI_{50}) is indicated by the dotted orange lines. **Green curves show the control (no viral infection); blue curves: 80,000 PFU VSV; red curves: 800,000 PFU VSV.**

HEK 293 cells showed a different response compared to Vero E6 cells when infected with VSV. HEK 293 cells in the growing phase were much more sensitive to VSV infection, as indicated by the drop in CI values to the CI_{50} value by 6 hours post infection when a high MOI was used (see Figure 3A, red curve). Cells infected with a low MOI reached the CI_{50} by 12 hours post infection (see Figure 3A, blue curve). Interestingly, a completely different result was obtained when confluent HEK 293 cells were infected (see Figure 3B). While confluent cells infected with a high MOI exhibited a drop in CI values similar to growing cells (see Figure 3B, red curve), cells infected with a low MOI appear to be completely resistant to VSV infection, exhibiting CI values virtually identical to mock-infected cells (see Figure 3B, blue and green curves).
With respect to the different responses to the VSV infection, the main difference between Vero E6 and HEK 293 cells is the ability of producing type I interferons. Vero E6 cells are devoid of the interferon genes (Emeny and Morgan 1979).

Results and Discussion continued

As a consequence, they cannot upregulate the expression of interferon-induced antiviral active proteins such as MxA and OAS/RNaseL in response to viral infections.

In contrast, HEK 293 cells possess an intact interferon system. Upon viral infection, they produce interferons which activate the JAK/STAT signaling pathway in an autocrine and paracrine manner. As a consequence, the expression of antiviral active proteins is initiated and an antiviral state is established. It is tempting to speculate that the observed resistance of confluent HEK 293 cells to VSV infection with a low MOI is due to the antiviral response mounted by their interferon system.

Additionally, confluent cells may represent a suboptimal environment for VSV replication, because it is well known that confluent cells have reduced metabolic activity compared to growing cells. In line with this hypothesis is the observation that growing HEK 293 cells are much more sensitive to VSV, independent of the MOI used for infection.

On the other hand, it should be noted that the VSV-M protein is known to counteract the interferon system by inhibiting host RNA and protein synthesis which contributes to the shutoff of host-directed gene expression (Ferran and Lucas-Lenard 1997). Therefore, the observed differences in response to VSV infection in growing or confluent Vero E6 and HEK 293 cells and the dependency of the outcome on cell number (and VSV MOI), most likely reflects the interplay of cellular antiviral mechanisms and viral countermeasures.

In contrast to conventional endpoint assays, real-time cell analysis using the xCELLigence System now offers the possibility of continuously monitoring virus-host interactions to better define the responses in which the viral or the cellular activities are more dominant.

References

Abassi YA, Jackson JA, Zhu J, O'Connell J, Wang X, and Xu X (2004). Label-free, real-time monitoring of IgE-mediated mast cell activation on microelectronic cell sensor arrays. *J Immunol Methods 292,* 195-205.

Atienza JM, Yu N, Wang X, Xu X, and Abassi Y (2006). Label-free and real-time cell-based kinase assay for screening selective and potent receptor tyrosine kinase inhibitors using microelectronic sensor array. *J Biomol Screen 11,* 634-643.

Atienza JM, Zhu J, Wang X, Xu X, and Abassi Y (2005). Dynamic monitoring of cell adhesion and spreading on microelectronic sensor arrays. *J Biomol Screen 10,* 795-805.

Emeny, J. M. and M. J. Morgan (1979). Regulation of the interferon system: evidence that Vero cells have a genetic defect in interferon production. *J Gen Virol 43(1):* 247-252.

Ferran, M. C. and J. M. Lucas-Lenard (1997). The vesicular stomatitis virus matrix protein inhibits transcription from the human beta interferon promoter. *J Virol. 71(1):* 371-377.

Kirstein SL, Atienza JM, Xi B, Zhu J, Yu N, Wang X, Xu X, and Abassi YA (2006). Live cell quality control and utility of real-time cell electronic sensing for assay development. *Assay Drug Dev Technol 4,* 545-553.

Knipe, D. M. and Howley, P. M., Eds. (2007). *Fields Virology,* 5th edition. Lipincott Williams & Wilkins: 37-39.

Yu N, Atienza JM, Bernard J, Blanc S, Zhu J, Wang X, Xu X, and Abassi YA (2006). Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: An approach to study G protein-coupled receptors. *Anal. Chem. 78,* 35-43.

Solly K, Wang X, Xu X, Strulovici B, and Zheng W (2004). Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. *Assay Drug Dev Technol 2,* 363-372.

Ordering Information

Product	Cat. No.	Pack Size
xCELLigence RTCA DP Instrument	**00380601050**	**1 Bundled Package**
RTCA DP Analyzer	05469759001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
xCELLigence RTCA SP Instrument	**00380601030**	**1 Bundled Package**
RTCA Analyzer	05228972001	1 Instrument
RTCA SP Station	05229057001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
xCELLigence RTCA MP Instrument	**00380601040**	**1 Bundled Package**
RTCA Analyzer	05228972001	1 Instrument
RTCA MP Station	05331625001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
E-Plate 16	05469830001	6 Plates
	05469813001	6 x 6 Plates
E-Plate VIEW 16	06324738001	6 Plates
	06324746001	6 x 6 Plates
E-Plate Insert 16	06465382001	1 x 6 Devices (6 16-Well Inserts)
CIM-Plate 16	05665817001	6 Plates
	05665825001	6 x 6 Plates
E-Plate 96	05232368001	6 Plates
	05232376001	6 x 6 Plates
E-Plate VIEW 96	06472451001	6 Plates
	06472460001	6 x 6 Plates
E-Plate Insert 96	06465412001	1 x 6 Devices (36 16-Well Inserts)
E-Plate Insert 96 Accessories	06465455001	6 Units (6 Receiver Plates + 6 Lids)

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