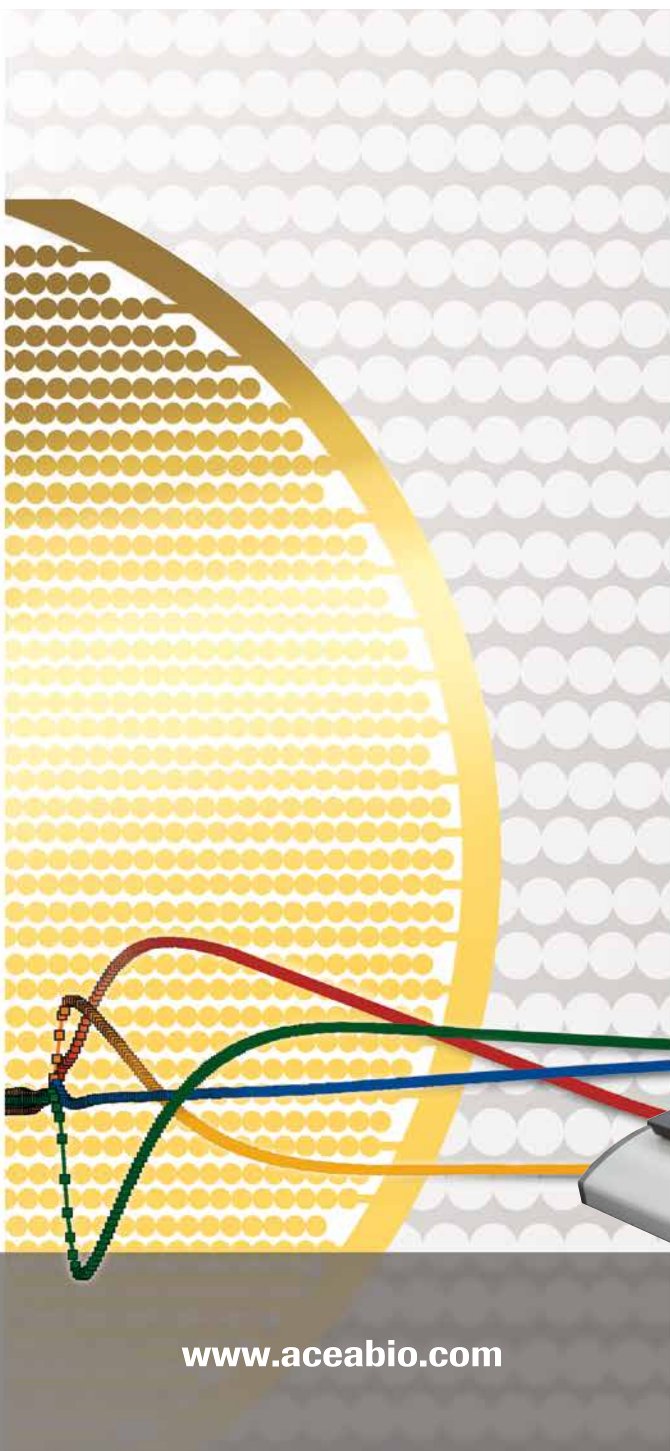
xCELLigence System

Application Note No. 5 / January 2013



Label-Free Assay for NK Cell-Mediated Cytolysis

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Label-Free Assay for NK Cell-Mediated Cytolysis

Introduction

Nature Killer (NK) cells are bone-marrow-derived lymphocytes originally identified by their large granular morphology. The NK cell lineage has been considered for cancer eradication owing to its ability to kill a wide variety of tumor cells spontaneously while sparing normal cells. Importantly, while T cells must be educated by antigen-presenting cells before they recognize tumors, NK cells spontaneously lyse certain tumor targets *in vivo* and *in vitro* without requiring immunization or pre-activation. Several *in vivo* and *in vitro* studies have shown that, in addition to extravasation and the ability to infiltrate tumor tissues, NK cells have promising anti-tumor effects. Mice with compromised NK cell function are more susceptible to carcinogen-induced cancers. In addition, individuals lacking NK cells suffer from persistent viral infections and as a consequence die prematurely. The potency of uncontrolled or inappropriate NK cell responses is evident in disease conditions such as allograft rejection, graft vs. host disease, diabetes, various autoimmune and neurological diseases, and aplastic anemia/neutropenia. Therefore, NK cells play a prominent role in various physiological and disease states and the assessment of their cytolytic activity is not only important for monitoring of immunocompetence in cancer, infectious diseases, and autoimmune diseases, but also in determining the proteins which mediate the cytolytic effect.

The standard methods for measuring NK cell cytolytic activity are radioactive label release assays[1,2] using:

- Chromium (^{51}Cr), or
- Indium (^{111}In)

In these assays, the target cells are radioactively labeled and then mixed with effector cells. The release of the radioactive isotope, which correlates with NK cell-mediated cytolysis, is then measured at a given time point (less than 4 hours). Several non-radioactive labeling assays are also available, including flow cytometry, ELISA-based granzyme measurement, and morphometric analysis by microscopy[3].

ACEA Biosciences developed a label-free assay format that allows dynamic monitoring of NK cell-mediated cytolysis. The assay uses impedance-based technology (RT-CES®)[4,5]. This system is the predecessor of the new xCELLigence System. The attachment and interaction of adherent cells with wells on a 96X E-Plate leads to impedance changes, which correlate with cell number, size and shape. In contrast, addition of suspended cells to the wells results in undetectable changes due to non-existent or weak interaction with the electrodes. Therefore, NK cell-mediated cytolytic effects on the cancer cell monolayer can be quantitatively and dynamically monitored on the sensor plate without labeling the target cells.

In this application note a series of experiments is described to determine whether this new impedance-based system is suitable for monitoring NK cell-mediated cytolysis.

Materials and Methods

Cells

The NK92, the NIH 3T3 cell line, and all the cancer cell lines used in these experiments were obtained from ATCC. The mouse NK cell line (mNK) was provided by Dr. Hui Shao of University of Louisville. All the cell lines were maintained in a 37°C incubator with 5% CO_2. The NK92 and mNK lines were maintained in Alpha MEM with 2 mM L-glutamine, 1.5 g/L Sodium bicarbonate, supplemented with 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 12.5% horse serum, 12.5% FBS, and 100-200 U/ml recombinant IL-2. Other cancer cell lines were maintained in RPMI media containing 5% FBS, 1% penicillin and 1% streptomycin (GIBCO). The NIH 3T3 cells were maintained in DMEM media containing 10% FBS, 1% penicillin and 1% streptomycin.

Materials and Methods continued

Cytolytic analysis

Target cells were seeded into the wells of 96X E-Plates in 100 µl of media. Cell growth was dynamically monitored with the impedance-based RT-CES® system until they reached log growth phase and formed a monolayer (24-34 hours, depending on the experiment). Effector cells at different concentrations were then directly added to individual wells containing the target cells. For background control, effector cells were added to a well without target cells. After addition of the effector cells the system continued to take measurements every 15 minutes for up to 20 hours.

Cell morphology analysis by microscopy

The effect of NK cell-mediated cytolysis on target cells was examined using a Nikon upright microscope. When the Cell Index dropped to 50% (relative to the control) upon addition of effector cells, cells were removed from the system, fixed in 80% methanol for 5 minutes and stained with Giemsa blue.

The morphology of the cells was examined by microscopy and photographed using an accompanying CCD camera.

Experiment data analysis

The integrated system software is able to display the entire history of the experiment from seeding the cells to the end of cytolysis. The time- and effector-to-target-ratio (E/T) -dependent curves can be displayed in real time, so NK cell activity can be monitored dynamically. The assay system expresses impedance in arbitrary Cell Index (CI) units. The CI at each time point is defined as $(R_n\text{-}R_b)/15$; where R_n is the cell-electrode impedance of the well when it contains cells and R_b is the background impedance of the well with the media alone.

To quantify the lysis at specific time points, the data was exported to Microsoft® Excel and percentage cytolysis at specific E/T ratios was determined by comparison to the control.

Results and Discussion

Dynamic monitoring of NK cell-mediated cytolysis

To assess NK cell-mediated cytolytic activity, a murine NK cell line (mNK line), and a target cell line, NIH 3T3 cells were used. The target NIH 3T3 cells were seeded in the wells of 96X E-Plates at 5,000 cells per well and the impedance-based system dynamically monitored the cell growth every 60 minutes until the cells reached growth phase, 34 hours later. The effector murine NK cells were then directly added to the well at different E/T ratio, and the NK cell-mediated cytolysis was dynamically monitored on the system. As shown in Figure 1A, a significant decline of the Cell Index (relative to the control) was seen in NIH 3T3 cells after the addition of mNK cells at the E/T ratio of 15 to 1. Furthermore, no significant decline of the Cell Index was seen in the effector control wells using YAC cells, a T lymphocyte line, without cytolysis effect. This indicates the decrease in the Cell Index due to addition of the mNK cells is specific and is most likely mediated by cytolysis. A time-dependent cytolysis of the NIH 3T3 cells was also seen in the presence of mNK cells but not in the presence of YAC cells (Figure 1B). To further confirm the cytolysis effect, target cells were stained when the cytolysis was approximately 50% (8 hours after addition of the mNK cells), and then examined under a microscope. As shown in Figure 1C, in the presence of mNK cells, the target cells were effectively cleared away by the cytolytic action of the mNK while control YAC cells did not affect the target cells.

In summary, the impedance-based system is the only currently available assay format that can directly monitor NK cell-mediated cytolysis without labeling the target cells and without using any chemical reporters. In addition, the entire history of the cytolysis can be dynamically monitored on the system, a feature that would be difficult to replicate with any label-based, endpoint assay format. Analysis of the kinetics of cytolysis indicates that a slow cytolysis was detected with the mNK cells. The cytolysis is detected within 2 hours of mNK cell addition. Since 4 hours is the standard incubation time for radioisotope-based assays, it is interesting to note that this assay shows less than 30% of the cytolytic activity remaining after 4 hours. Data obtained with the impedance-based system clearly shows that cytolytic activity can reach up to 70% within 12 hours after adding the mNK cells. Since the maximum cytolytic activity occurs after the standard incubation time of existing label-based assays, they could easily underestimate this activity. Therefore, this new system not only offers label-free detection, but since it can dynamically monitor the entire history of cytolysis, the system provides a more accurate assessment of cytolytic activity.

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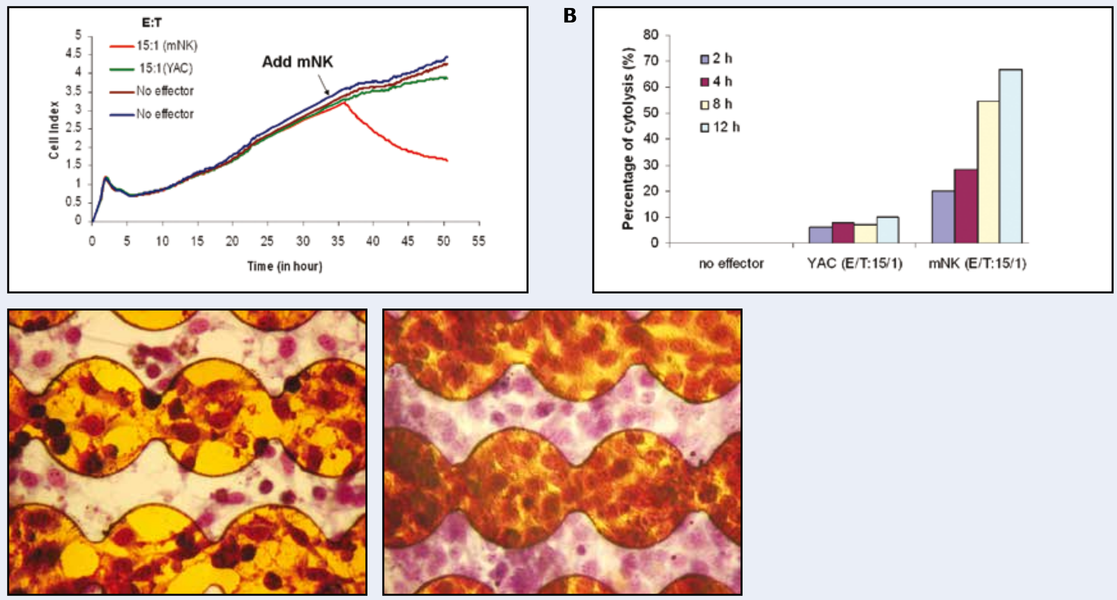

mNK (E/T = 15:1)
YAC (E/T = 15:1)

Figure 1: Dynamic monitoring of NK cell-mediated cytolysis
(A) Dynamic monitoring of NK cell-mediated cytolysis of NIH 3T3 cells. The NIH 3T3 cells were seeded in the wells of the 96X E-Plate at 5000 cells/well. Cell attachment, spreading, and proliferation were monitored in real time. 34 hours after seeding of the cells, Cell Index values reached 3, which is equivalent to approximately 10,000 cells/well. 150,000 mNK cells or YAC (negative control) cells were added to each well in triplicate. The mNK cell-mediated cytolysis was then dynamically monitored. **(B)** Time-dependent cytolytic activity of mNK cells. The cytolytic activity at a given time point was calculated and presented as the percentage of cytolysis {% of cytolysis = $(CI_{no\ effector} - CI_{effector})/CI_{no\ effector}$ X 100}. **(C)** The morphological examination of cytolysis by mNK cells. The specific cytolysis of NIH 3T3 cells by mNK cells was examined by microscopy after the cells were stained with Giemsa blue.

Quantitative measurement of NK cell-mediated cytolysis

The Cell Index is correlated with cell number[5], and has been used to quantitatively monitor cytotoxicity induced by chemical compounds such as anticancer drugs. To test whether mNK cell activity can also be quantitatively assayed, cytolysis was monitored at different ratios of effector/target. Both murine and human NK cell lines (mNK and NK92) were used as effectors; the NIH 3T3 line and the MCF7 line (human breast cancer cells) were used as the targets for each of the effectors, respectively. As described above, the target cells were first seeded to the 96X E-Plate at 5,000 cells/well, and the cell growth was monitored with the impedance-based system. When the target cells reached growth phase, the NK cells were directly added to wells at different concentrations. The NK cell-mediated cytolysis at different E/T ratios were then monitored in real time on the system.

As shown in Figure 2, subsequent to the addition of mNK or NK92 cells to its target cells, the Cell Indices declined relative to the "no effector" control. The decline in the Cell Index values is E/T-ratio dependent; it is caused by a decrease in cell/electrode interaction that occurs during cytolysis. For either case, the higher the E/T ratio, the lower the Cell Index value obtained. This strongly indicates that the impedance-based system permits specific and quantitative measurement of NK cell-mediated cytolytic activity. Moreover, the dynamic monitoring of the cytolysis may provide more insights into the underlying mechanisms of NK cell-mediated killing. For example, analysis of the dynamics of cytolysis indicates that the NK92 cells are much more potent effectors than mNK cells. At an E/T ratio of 4:1 or higher, the MCF7 cells are more than 90% cytolyzed within 4 hours after addition of NK92 cells, whereas for mNK cells only 30% cytolysis occurs within that time. The difference in cytolytic kinetics of NK cells indicates that the nature of the interaction between effector and target is cell-specific, and may involve such factors as expression of NK receptors and ligands, or different mechanisms of NK cell-mediated cytolysis.

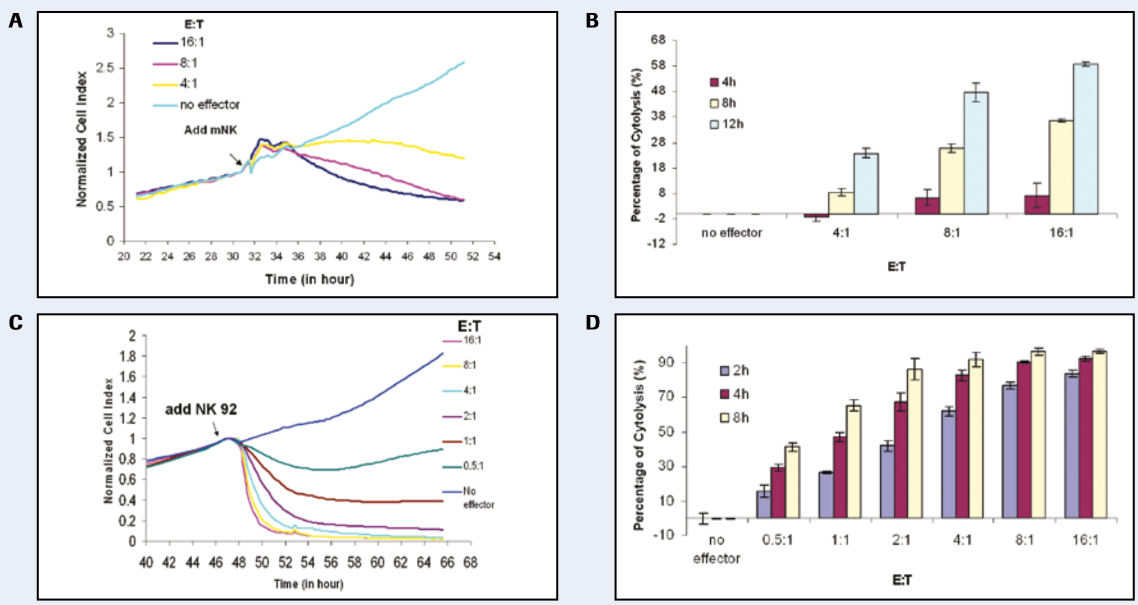

Figure 2: Label-free and quantitative measurement of cytolytic activity of mNK cells and NK92 cells. (A). Quantitative measurement of cytolytic activity of mNK cells. The NIH 3T3 cells were seeded to the 96X E-Plate. Cell growth was monitored in real time on the RT-CES® system until the CI values reached 3, equivalent to 10,000 cells/well. The mNK cells were added to target cells at different cell concentrations to generate a series of E/T ratios. The cytolysis of the target cells at different E/T ratios was dynamically monitored on the system. A normalized Cell Index was used, in which the Cell Index values obtained after addition of mNK cells were normalized against the Cell Index value from the same well before the addition of mNK cells. **(B)**. Time-dependent cytolytic activity of mNK cells at different E/T ratios. The percentage of cytolysis of the NIH 3T3 cells by mNK cells was calculated as described in Figure 1. The time-dependent cytolytic activities are indicated. **(C)**. Quantitative measurement of cytolytic activity of NK92 cells. The MCF7 target cells were seeded and the cell growth was monitored on the system as described above. The NK92 cells were then added to each well at different concentration to generate the series of E/T ratios indicated. The cytolytic activities of NK92 cells on MCF7 cells at different E/T ratios were dynamically monitored on the system. **(D)**. Time-dependent cytolytic activity of NK92 cells at different E/T ratios. The percentage of cytolysis of the MCF7 cells by NK92 cells was calculated as described in Figure 1.

Label-free assessment of NK cell cytolytic activity in a variety of target cell lines

Cytolytic activities of mNK and NK92 were tested using 9 cell lines, which include 8 different human cancer cell lines and the NIH 3T3 cell line. The susceptibility of different target cells lines to mNK- or NK92-mediated cytolysis is summarized in Tables 1 and 2. NK92 shows a broad spectrum of cytolytic activity on cancer cell lines. The cytolysis mediated by NK92 occurs quickly and reaches the maximum killing activity less than 8 hours after addition of NK92 cells. A comparison of susceptibility of 7 cancer cell lines to NK92 cells is shown in Figure 3A. Over 90% cytolysis was achieved with 4 out of 7 target cell lines tested, including H460, HepG2, MCF7 and MDA-MB231. In contrast, mNK cell-mediated cytolysis appears to be more selective than NK92 (Figure 3B). For 4 of 9 target cell lines tested, over 30% cytolysis was observed after 12 hours of incubation with mNK cells, including NIH 3T3, A549, Hela, and MDA-MB-231. No cytolysis (0%) or weak cytolysis (10%) was found in 5 target lines, including OVCAR4, HT1080, HepG2, H460 and MCF7. In addition, the cytolysis mediated by mNK was much slower than that mediated by NK92, reaching the maximum about 12 hours after mNK cells were added.

In summary, these experiments demonstrate that an impedance-based system can be used for label-free assessment of NK cell-mediated cytolytic activity. Both human and murine NK cell lines were tested for their cytolytic activities on 9 different target cell lines, including human cancer cell lines commonly used in the field. The quantitative, dynamic measurement of NK cell-mediated cytolysis was achieved on the system without any labeling steps or reagents. Moreover, this new technology offers fully automated measurement of cytolysis in real time, which could make possible a large-scale screening of chemical compounds or genes responsible for the regulation of NK cell-mediated cytolytic activity.

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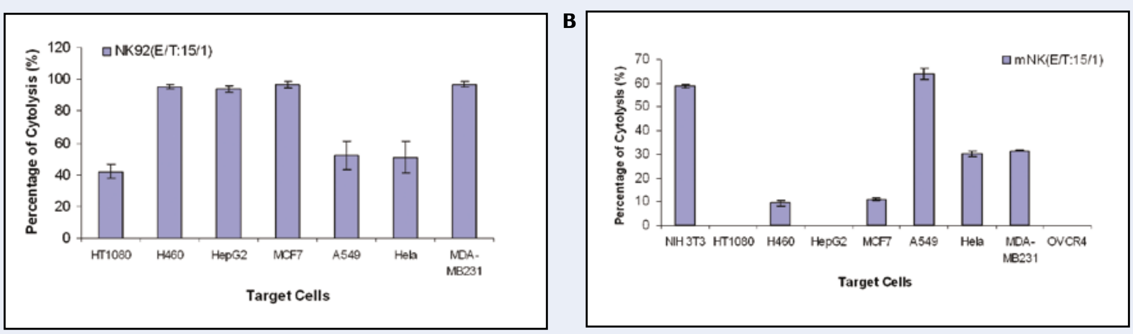

Figure 3: Label-free assessment of NK cell-mediated cytolysis using a variety of cell lines.
(A) The NK92-mediated cytolysis of 7 different cancer lines. The percentage of cytolysis indicated for each line is calculated based on the Cell Index value of individual wells 8 hours after NK92 cells were added; cytolysis reached a maximum at that time. **(B)** The mNK-mediated cytolysis of 9 different cell lines. The percentage of cytolysis indicated for each line is calculated based on the Cell Index values of individual wells 12 hours after mNK cells were added; cytolysis reached a maximum at that time.

Cell Name	Cell Type	Species	Maximum Cytolysis (%) at 12 h
NIH 3T3	Fibroblast	Murine	58.8
HT1080	Fibrosarcoma	Human	0.1
H460	Non-small cell lung cancer	Human	9.5
HepG2	Hepatoma	Human	0
MCF7	Breast cancer	Human	11.0
A549	Non-small cell lung cancer	Human	64.0
HeLa	Cervix cancer	Human	30.3
OVCAR4	Ovarian cancer	Human	0
MDA-MB-231	Breast cancer	Human	31.5

Table 1: mNK cell-mediated cytolysis of 9 cell lines.

Results and Discussion continued

Cell Name	Cell Type	Species	Maximum Cytolysis (%) at 12 h
HT1080	Fibrosarcoma	Human	42.2
H460	Non-small cell lung cancer	Human	95.4
HepG2	Hepatoma	Human	94.1
MCF7	Breast cancer	Human	96.5
A549	Non-small cell lung cancer	Human	52.2
HeLa	Cervix cancer	Human	51.0
MDA-MB-231	Breast cancer	Human	97.0

Table 2: NK92 cell-mediated cytolysis of 7 cell lines.

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The RT-CES® system, which was used to perform all the experiments described in this application note, will soon be replaced by the xCELLigence System. While retaining the advantages of impedance-based technology described in this publication, the xCELLigence System will have improved functionality over the RT-CES® system.

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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