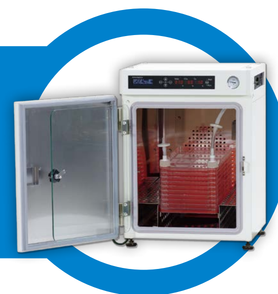


Application data sheet #06

Active Gas Ventilation CO_2/Multi-gas incubator *Prescyto* MG-71C/M-A

Effects of active gas ventilation in a mass culture using a multilayer culture plate

Visualizing the differences in natural and active gas ventilation for a multilayer culture plate, while comparing culture results

About active gas ventilation (AGV)

Gas exchanges in a culture plate by using active gas ventilation (AGV*) is a method in which we have a patent for equipment structure that is key to precise gas branching. We have shown that with the Custom BioShaker CO_2-BR series, AGV can improve cultural efficiency and matter production numbers of microorganisms and mammalian cells. Our CO_2 incubator, the Prescyto MG-71 series, also has a model supporting AGV. This sheet describes a case involving the mass culture of mammalian cells with our AGV CO_2 incubator, the Prescyto MG-71C-A, by using a multilayer culture plate.
The MG-71C-A can be applied to an AGV culture in an Erlenmeyer flask with a shaker and in a spinner flask with a low-speed stirrer. However, both of methods are in a suspension culture accompanied by shaking or propeller agitation. Also, many researchers are more or less satisfied with the results achieved by using normal gas ventilation (NGV*). On the contrary, a multilayer culture plate is an adherent static culture. As the name suggests, it has a structure with stacked culture trays, meaning that it is questionable if efficient gas exchanges are taking place at every layer with NGV. (In actual fact, upon learning that results were not good with a multilayer culture plate in the field of pharmaceutical R&D and production, we learned that NGV was being used in many cases.) There is another question as to whether any shortages of gas exchange negatively affect a culture. Therefore, we decided to use a non-destructive oxygen sensor to monitor the gas exchange efficiency in a multilayer culture plate with NGV and AGV, together with the results of comparisons between the two by actually cultivating cells.

＊There are a number of equivalent phrases for active gas ventilation, but our company uses this expression (in addition to its counterpart, natural gas ventilation as employed in reference document 1).

Monitoring gas exchange status in a multilayer culture plate

Off-the-shelf multilayer culture plates are for NGV or AGV. (The former is attached with a vent cap, while the latter has a connector for a gas supply hose.) There are no generally established methods to directly and easily monitor how internal gas phases change (gas exchanges) from atmospheric composition when a multilayer culture plate is fed with 5% CO_2, etc. by active gas ventilation, and when it is statically placed in the atmosphere with natural gas ventilation. The only methods available for determining the status of ventilation or its needs were the color changes of a culture medium by the changes in gas phases as identified by a pH indicator or whether the culture results were good or bad. However, cell culture with a multilayer culture plate is extremely expensive, and it is better to have knowledge about internal gas exchanges. As we can take advantage of non-destructive oxygen sensors to monitor internal gas exchange situations, we will introduce the results and color changes of pH indicators. Consequently, this allowed us to confirm the superiority of AGV's gas exchange efficiency.

(1) Monitoring method and results: NGV

We installed a non-destructive oxygen sensor chip (on 5th layer from the top) in a manner without affecting the airtightness onto an NGV type multilayer culture plate (ten layers), which was then statically placed in our multi-gas incubator, the Prescyto MG-71M, and set to 1% O_2. Signals from the non-destructive oxygen sensor chip are read from outside the multilayer culture plate (Figure 1). We also put a trisbuffer containing phenol red, which is a pH indicator often added to a cell culture medium, in another multilayer culture plate of the same model and filled each layer (2000 mL). It was statically placed in our multi-gas incubator, the Prescyto MG-71C, and set to 5% CO_2. We then took video of the color changes of the trisbuffer (Figure 2.) With gas exchanges with NGV, O_2 concentration did not reach that in the incubator after 24 hours had elapsed following static placement. Regarding the color changes of the trisbuffer, changes to yellow started 20 minutes later, but changes after that were gradual and did not turn to a perfect yellow when 60 minutes had passed.

(2) Monitoring method and results: AGV

We installed a non-destructive oxygen sensor chip (on the 1st, 5th, and 10th layers from the top) in a manner without affecting airtightness onto an AGV type multilayer culture plate (ten layers), which was then connected to our nitrogen gas generator, the NS GENESIS 200, via a flowmeter. Nitrogen gas at a flow rate of 500 mL/min. was fed using AGV. Signals from the non-destructive oxygen sensor chip were read from outside the multilayer culture plate (Figure 3). We also put a trisbuffer containing phenol red, as was the case with the above (1) in another multilayer culture plate of the same model. The plate was then put into our AGV CO_2 incubator, the Prescyto MG-71C-A, and set to 5% CO_2·500 mL/min. We then took video of the color changes of the trisbuffer (Figure 4). With gas exchanges with AGV, in two to four hours after starting ventilation, it quickly became low in oxygen. (Slight differences among layers were likely to be caused by structural factors of the multilayer culture plate being used.) As for the color changes of the trisbuffer, changes to yellow started ten minutes later and turned a perfect yellow when 60 minutes had passed.

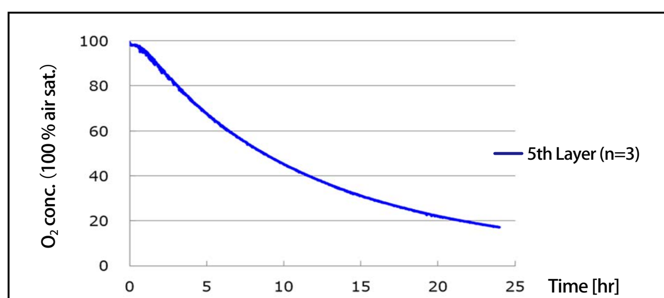


Fig 1. Changes of O_2 concentration in a multilayer culture plate by NGV

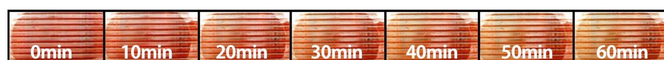


Fig2. Color changes by NGV (video captured image, saturation adjusted)

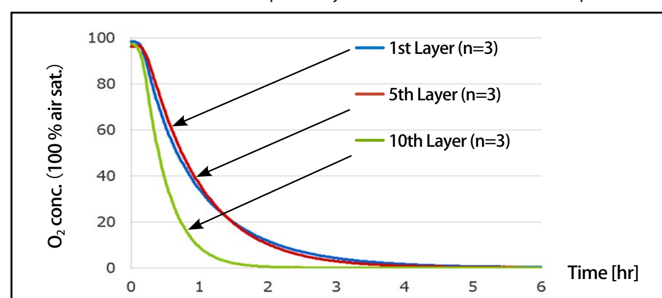


Fig 3. Changes of O_2 concentration in a multilayer culture plate by AGV

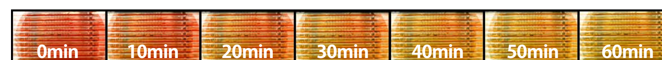


Fig4. Color changes by AGV (video captured image, saturation adjusted)

Confirming AGV's effects in a cell culture by using a multilayer culture plate

●Materials and method

Cells: Vero cells (kidney epithelial cells extracted from the African green monkey, adherent cells); culture medium: EMEM with 10% FBS and antibiotics, 1.5 L
Container: Cell Factory (for NVG) and Cell Factory with Active Gassing (for AGV), both ten layers, manufactured by Thermo Fisher Scientific
Cell density at the start: 4.0 x 103 cells/cm2, culture condition: 5% CO2, +37℃
Culture time: About 90 hours; evaluation items: cell density, cell viability
Collection method of cells: After collecting the culture medium, the inside of the container was cleansed with PBS (-), then trypsin-EDTA was added for 20 minutes of incubation at +37° C
Comparison: Conventional culture (NGV) was performed on a cell culture dish of φ6 cm, and the two are compared

●Results and discussion

In the culture of Vero cells in a multilayer culture plate (ten layers), cell density with AGV was about 3.7 times as much as that with NGV and close to that in a cell culture dish (φ6 cm) to which they were compared (Table 1). It turned out that AGV was effective to achieve an efficiency comparable to normal dishes in a mass culture using a multilayer culture plate. Needless to say, it was reported that the result was 1.44 times with iPS cells1), so it is apparent that depending on cell types, an increase of cell density will change, but in consideration of the above-mentioned monitoring results, it is highly likely that AGV works effectively. Regarding the cell viability, there was no difference between NGV and AGV, and both were similar to that of a cell culture dish. In the above-mentioned AGV culture of iPS cells, it was reported that the cell viability was not different from that with NGV.
For reference, in this experiment, during trypsin treatment, Vero cells in the multilayer culture plate did not easily come off. Even after a long period of incubation for the trypsin treatment of about 20 minutes, the cells remained massive and was difficult to break up, with some of the cells adhered near the outlet hole and unable to be collected. Due to this loss, the difference of cell density from that of a cell culture dish was likely to be caused. In a different experiment where we cultivated HeLa cells in a multilayer culture plate (ten layers), the cell density with AGV was closer to the cell density in a cell culture dish with about ten minutes of trypsin treatment when compared to this experiment (Table 2.) To minimize the loss at the time of collection, the optimization of trypsin treatment may be necessary such as increasing the times of cleansing with PBS (-) or trypsin concentration and shaking at the time of incubation, depending on the cell types. It is added that as multilayer culture plates need more time for supplying the culture medium, cell seeding, and collection when compared with dishes and flasks, this may consequently affect culture efficiency in general.

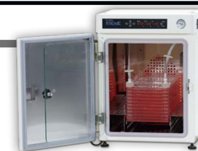
Table 1. Results of this experiment (comparison of NGV and AGV in Vero cell culture using a multilayer culture plate)

Culture Vessel	Multilayer Culture Plate/NGV	Multilayer Culture Plate/AGV	Culture Dish/NGV
Incubator	MG-71C	MG-71C-A	MG-71C
Surface Area	10-layer, 6320 cm^2		φ6 cm, 21 cm^2
Culture Volume	1500 mL		5 mL
Cell	Vero		
Growth Medium	EMEM with 10% FBS and antibiotics		
Seeding	**4.0×10^3 cells/cm^2**		
Time of Culture	96 hr		
Condition	+37℃、5% CO_2 in air	+37℃、5% CO_2 in air、500 mL/min	+37℃、5% CO_2 in air
Yeild	**1.2×10^4 cells/cm^2**	**4.4×10^4 cells/cm^2**	**5.0×10^4 cells/cm^2**
Viability	**97%**		

Table 2. Reference data (Comparison of cultivating HeLa cells in a multilayer culture plate and a normal cell culture dish)

Culture Vessel		Multilayer Culture Plate/AGV	Culture Dish/NGV
Incubator		MG-71C-A	Competitor's Conventional CO_2 Incubator
Surface Area		10-layer, 6320 cm^2	φ6 cm, 21 cm^2
Culture Volume		2000 mL	6.5 mL
Cell		HeLa	
Growth Medium		DMEM with 10 % FBS and antibiotics	
Seeding		**6.0×10^3 cells/cm^2**	
Time of Culture		72 hr	
Condition		+37℃、5% CO_2 in air、500 mL/min	+37℃、5% CO_2 in air
Yeild		**4.9×10^4 cells/cm^2**	**5.3×10^4 cells/cm^2**
Viability		**98%**	**94%**

Now on sale
●AGV CO_2 incubator *Prescyto* MG-71C-A
●AGV multi-gas incubator *Prescyto* MG-71M-A
●CO_2 incubator *Prescyto* MG-71C
●Multi-gas incubator *Prescyto* MG-71M
●Nitrogen gas generator N_2 GENESIS 200



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Reference

1) Tohyama *et al.*, Efficient Large-Scale 2D Culture System for Human Induced Pluripotent Stem Cells and Differentiated Cardiomyocytes. *Stem Cell Reports* (2017) **Vol. 9**, 1-9.

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