No.2

BULLETIN

Evaporation from Cell Culture Plates

When cells are cultured in plates with loose-fitting lids, the medium will always lose water to the plate surroundings by evaporation, i.e. by water vapour escape.

This may cause culture problems since the medium components, especially the salts, may reach concentrations which are harmful to the cells.

The evaporation is due to the fact that the air just above the surface of the medium is always saturated with water vapour, whereas the surroundings under realistic circumstances will never be completely saturated. Thus, water molecules will constantly escape along a concentration gradient from the surface of the medium to the surroundings, by diffusion.

In the following text, the various parameters influencing evaporation are discussed.

Nunc Plate	Evaporation index cm/cm^2	Culture area/well cm^2	Nunc Lab uses	
			Medium vol. well/ml	Medium depth mm
Petri Dish 35	1.2	9	3	3.4
Petri Dish 60	0.8	21	5	2.4
Petri Dish 100	0.5	57	12.5	2.2
Petri Dish 140	0.3	146	35	2.4
Square Dish 235	0.2	520	135	2.6
Multidish 4 rectang.	0.6	16	5	3.1
Multidish 8 rectang.	0.6	8.5	3	3.5
Multidish 4 SonicSeal	3.4	1.5	1	6.7
Multidish 4*	2.1	2	1	5.0
Multidish 6*	0.7	9.5	3	3.2
Multidish 12*	1.0	3.5	2	5.7
Multidish 24*	0.9	2	1	5.0
Multidish 48	0.8	1	0.5	5.0
MicroWell 96F	0.7	0.33	0.2	6.0

*Plates having a reservoir for additional liquid.

Table 1
Evaporation indices and other figures for Nunc cell culture plates with loose-fitting lids. The indices, which are the circumferences of the plates divided by the respective surface areas of medium, can be regarded as the relative speeds by which the water losses become »critical« provided that the opening-slit widths and the depths of medium are equal for all the plates. Thus, to obtain actual, relative figures, the indices should be multiplied by the respective actual slit widths and divided by the actual medium depths. See text for further explanation.

Slit Area

The rate of evaporation may be assumed to be proportional to the area of the slit between the lid and the plate edge separating the plate lumen from the surroundings. Hence, if the slit width is constant, the speed of water loss is directly proportional to the plate's total edge length, i.e. its circumference.

Further, the time it takes before the water loss becomes critical is proportional to the volume of medium in the plate. If the depth of medium is constant, the volume of medium is in turn proportional to the surface area of the medium in the plate.
Therefore, the various Nunc cell culture plates with loose-fitting lids can be characterized as more or less

prone to critical water loss by evaporation according to their specific circumference/medium surface area ratio, which may be denoted »evaporation index« (Table 1).

In principle, the larger the index is for a product, the more prone it is to critical water loss by evaporation.
Due to this principle, the tendency should be to use greater depths of medium (i.e. larger volumes of medium per square cm^2), the larger the ratios are.
Also, a critical water loss from evaporation can be postponed by adding liquid to the reservoir between the wells, if present (see Table 1).
Because of the relatively high evaporation index for MicroWell Plates, the MicroWell Plate Lid has been equipped with an edge-rib for narrowing the slit width and thus diminishing the total slit area. For the same reason it has been given a ringshaped rib above each well, as any narrowing in the vapour path from the medium to the surroundings will add to the evaporation delay.
Fig. 1 shows the results obtained from a comparison of the evaporation from MicroWell 96F Plates with new lids versus old lids, i.e. lids with edge rib and rings versus lids without edge rib and rings.
As seen, water loss has been reduced by approx. 50% due to the edge rib and rings.

Edge Effect

In the multiwell plates, where all the wells are not positioned identically in relation to the edge of the plate, i.e. Multidish 6, Multidish 24, and MicroWell Plates, a larger water loss is seen in the peripheral wells than in the central ones. This is especially so in the corner wells because they are more exposed to the surroundings.
In MicroWell Plates, the evaporation from the four corner wells has been shown to account for fully 15% of the total water loss regardless of the kind of lid used. In comparative studies, this figure was also found for corresponding »low-evaporation« plates of another brand.

Surrounding Humidity

To avoid significant evaporaton from culture plates with loose-fitting lids, the plates are placed in an incubator with humidity control aimed at 100% relative humidity. However, the highest obtainable humidity in practice would normally be about 95%. A small water vapour gradient is therefore still operating causing a water loss which may become critical during long term culture. One reason for the inability to keep the humidity at 100% is that the

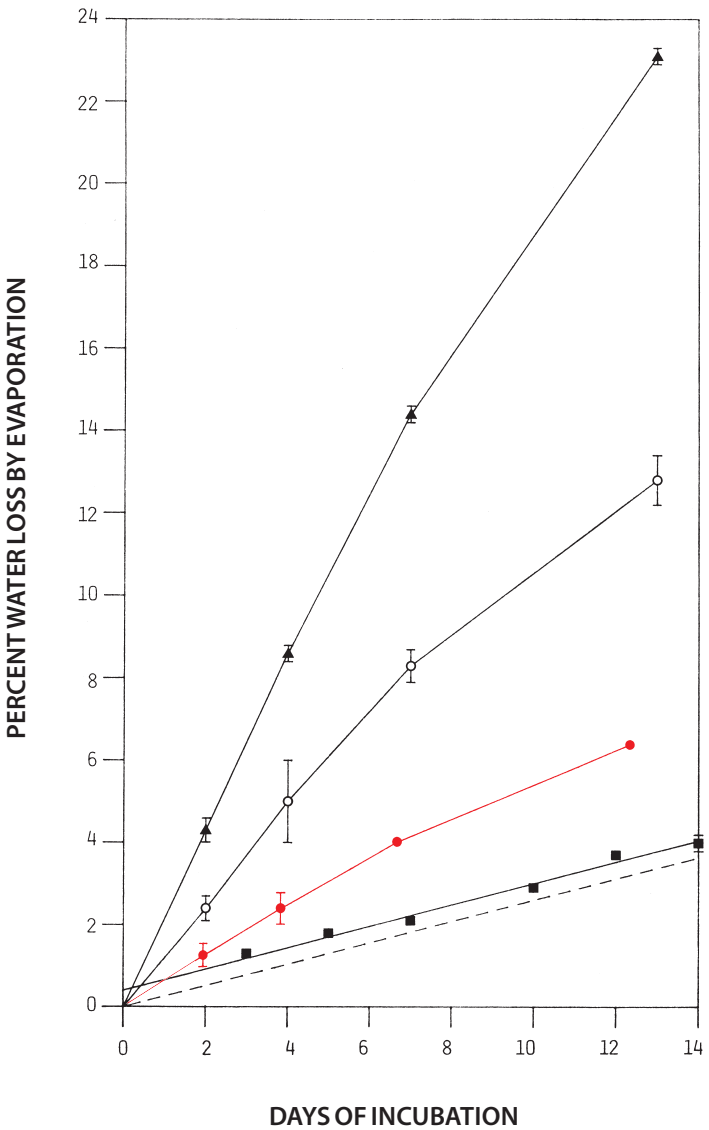

Fig. 1
Evaporation curves for MicroWell Plates incubated at 37°C with 200 µl growth medium per well.
Closed circles (red curve): *Incubation at 95% relative humidity, lids with edge rib and rings. This curve represents the most realistic evaporation from a Nunc MicroWell Plate.*
Open circles: *Incubation at 95% relative humidity, lids without edge rib and rings (out of production).*
Triangles: *Incubation at 80% relative humidity, lids with edge rib and rings.*
Each of these three curves represents the average evaporation from four plates, which were removed at intervals, weighed, and returned to the incubator.
Squares: *Incubation at 95% relative humidity, lids with edge rib and rings.*
As opposed to the method described above, these data points represent the average evaporation from separate pairs of plates which were removed at intervals from an incubated series, weighed, and discarded.
A regression line is fitted to these data points.
The axis-intercept represents the evaporation that occurs between removal and weighing of the plates.
The dotted line is a parallel displacement of the regression line through the zero point representing the (theoretical) evaporation curve when plates are left undisturbed in the incubator.
See text for discussion.

incubator is normally opened frequently during incubation.
Fig. 1 shows the results from a comparison between evaporation from MicroWell Plates incubated at 95% and at 80% relative humidity. As seen, evaporation is almost four times higher at 80% humidity than at 95%.
A critical factor affecting evaporation seems to be the frequency with which plates are removed from the incubator for microscopic examination of the cells.
The effect of this was investigated by monitoring the evaporaton (by weighing) from plates which were removed at intervals from the incubator and comparing the results with those obtained from plates which had been incubated undisturbed for the same length of time.
As shown in Fig. 1, evaporation is almost halved if the plates are left undisturbed in the incubator.
Thus, it seems that the measured evaporation is to a large extent due to the plates' »outside trips«. This is also indicated by the levelling off tendency of the evaporation curves for plates that are returned to the incubator (see Fig. 1): the longer the interval is between the measurements, the less steep the evaporation curve is. This can be explained by the occurence of a relatively large additional evaporation every time the plates have been out of the incubator. Therefore, the number of outside inspections should be kept at a minimum.

Temperature

Animal cell cultures are normally incubated at 37°C, at which temperature the pressure of saturated water vapour is about 2.5 times higher than at 20°C (room temperature). In addition, the surrounding humidity is far lower outside the incubator than inside. Therefore, the evaporation gradient is greatly increased when the plates are removed from the incubator.
However, the evaporation will decrease with the falling temperature, and because air can contain about 2.5 times more water at 37°C than at 20°C, some of the water vapor will be trapped by condensation. This is observed by drop formation on the inside of the lids soon after removal of the plates from the incubator.
Obviously, plates should not be outside the incubator for longer than absolutely necessary.

Air Circulation

If the plates are exposed to »draught«, the evaporation will be more rapid than if only pure diffusion forces are operating.
There is always some draught even inside a closed incubator due to its air circulating system, but when the incubator is opened, or when the plates are moved outside the incubator, a significant draught may be experienced. Therefore, the incubator should only be opened when absolutely necessary, and plates should be moved slowly and only taken the shortest possible distance from the incubator.

Summary

Generally, evaporation from cell culture plates only causes problems during long term culture, e.g. two weeks or more. However, peripheral wells, especially in MicroWell Plates, may lose »critical« amounts of water long before then, but the evaporation very much depends upon the actual culture conditions. The following precautions would help to postpone evaporation problems:

1. Use an optimally performing humidity control.
2. Limit the number of outside inspections.
3. Keep the »outdoor« periods as short as possible.
4. Take the plates the shortest possible distance from the incubator.
5. Move the plates slowly.
6. Do not open the incubator unnecessarily.
7. If possible, add water or medium to the spaces between the wells.
8. Use larger volumes of medium in corner wells, or in all peripheral wells.
9. Use greater depths of medium in the plates which have a high evaporation index (see Table 1).

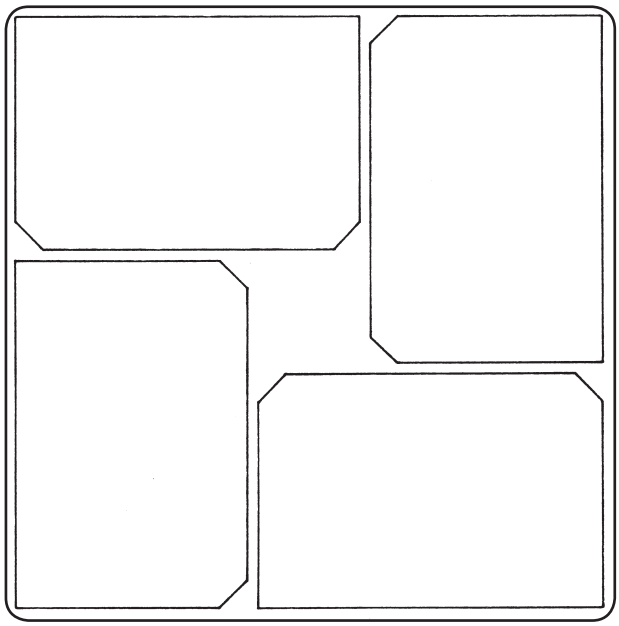

Fig. 2.
Placement pattern of four MicroWell Plates in a Nunc Square Dish.

These precautions can to a large extent be substituted by keeping the plates in a humidity chamber during incubation and transport. The Nunc Square Dish equipped with a wet filter paper in the bottom is highly suitable for this purpose. As illustrated in Fig. 2, the Nunc Square Dish will hold four MicroWell Plates.

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Vibration Patterns in Cell Culture Vessels

Do you observe similar patterns in your cultures?

During the years, Nunc has collected items with »strange« cell density patterns.
At first sight, one may find no logical explanation of the patterns, especially as they are very seldom formed and seem to arise spontaneously. However, on closer examination one very often finds a high degree of regularity or symmetry. In most cases, this indicates that one should look for physical forces affecting cell attachment and/or growth.
A frequent problem with incubators is vibrations transmitted from the fan to the shelves.
Depending on the frequency of the vibration, standing waves may be formed in the medium, i.e. when resonance is attained.
A typical example of standing waves in a petri dish is shown in Fig. 3a. As can be seen, the cells grow in concentric circles. When comparing the distance between the circles with the diameter of the dish, one sees that the diameter corresponds to 17 half wavelengths.
The standing waves have nodes at the rim and at every »cell circle« (see Fig. 3b).

The reason why the cells are growing only at the nodes is that in nodes, the growth medium is motionless. Between the nodes, the medium is moving up and down preventing the cells from attaching and moving them towards the nodes.
A similar effect in a flask is shown in Fig. 4. In this case, the standing waves have been formed across the flask.
A more complex pattern due to incubator vibrations is shown in Fig. 5.
In this case, the cells grow in a spotlike manner, as a result of 2 standing waves, one across and one lengthwise in the flask.
In Fig. 6, the cells are growing at the centerline of the flask because of very slow vibrations. In this case, the vibrations are not coming from the incubator but from carrying the flask in a horizontal position with slow and gentle shaking (walking).

How to eliminate the problem?

The only effective way is to stop the vibration, but this is not always possible, as the vibrations may be due to the construction of the fan or the fanmotor. As the problem is due to resonance, one can try to alter the frequency by placing something heavy in one corner of the incubator, but one should be aware that by changing the frequency, one may hit the resonance frequency of another item. The frequency also changes with the load of the incubator.

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Fig. 3b
Standing waves in a petri dish:

_____ *wave at* $t = 0$

- - - - - - - *wave at* $t = \frac{T}{2}$
(half period later).

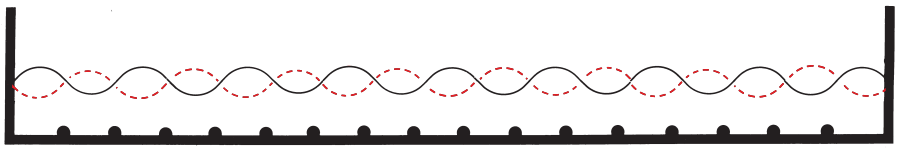

Fig. 3a

Fig. 4

Fig. 5

Fig. 6

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