Performing Cell Growth Curve Experiments with different Substances on the Eppendorf epMotion® 5075 LH

René Thierbach, Babette Wagenhaus and Pablo Steinberg, Stiftung Tierärztliche Hochschule Hannover, Hannover, Germany
Renate Fröndt, Eppendorf AG, Hamburg, Germany

Abstract
In the present Application Note we make use of the liquid handling system epMotion® 5075 LH to analyze simultaneously the influence of seven different concentrations of three substances dissolved in three different solvents on the growth of the fibroblast cell line NIH/3T3. Three independent experiments were performed and each experiment is divided into 5 steps: Cell seeding, Cell treatment, Culture media change, Fixation and Sulforhodamin B staining and measurement.

Introduction
The procedure to obtain a cell growth curve is quite simple. In a first step a low number of cells are seeded in several different cell culture dishes. At different time points the cell number or a parameter directly proportional to cell number (e.g. protein content) is determined by making use of a hemocytometer, a Coulter counter or by protein staining. By means of a graphical analysis of the data the cell growth curve is obtained. The linear part of the growth curve can be used to determine the doubling time of the cell line.

However, in practice there are several experimental conditions, which are considered to be crucial when wanting to obtain high quality data. First, it is very important to seed nearly identical numbers of cells into the different cell culture dishes. Second, in most cases cell culture medium change must be performed every two to three days, thereby getting a break in the curve. Third, if the cells are directly counted, it is necessary that the same person trypsinizes and counts the cells at each time point.

It was shown previously [1] that a continuous contamination-free handling of mammalian cell cultures for more than one week is possible with the liquid handling system epMotion 5075 LH. In the present Application Note a simple and highly reproducible protocol to obtain a cell growth curve, which combines the protein staining method in 96-well microplates described by Skehan et al. [2] with the liquid handling system epMotion 5075 LH and which circumvents all the experimental difficulties described above, is presented.
Materials and Methods

Eppendorf epMotion 5075 LH equipped with:
- Dispensing tools TS 1000 and TM 1000-8
- Reservoir Rack
- Seeding cylinder with magnetic stirrer
  (available from general Lab suppliers)

Eppendorf Consumables
- epTIPS Motion Filtertips 1,000 µl
- 30 ml Reservoir
- Eppendorf Deepwell plate 96/2,000 µl

Consumables and reagents from other vendors:
- NIH/3T3 cells
- Cell culture medium: Dulbecco’s modified Eagle’s medium supplemented with 10 % v/v fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin
- 96-well cell culture plates

RNA Selection

Cell seeding
A cell seeding protocol has been described in detail [3], but in order to optimize it the dispensing step has been modified. Briefly, a custom-built seeding cylinder with the magnetic stirrer was placed in a source position (C4). A magnetic mixer was positioned under the epMotion 5075 LH and under the stirrer in position C4. The cells were trypsinized and the obtained cell suspension was filled into the seeding cylinder. Six destination positions were equipped with 96-well microtiter plates, each on a 85 mm height adapter. In three steps the plates were loaded. Four lines at a time received 180 µl of the cell suspension per well (350 cells/well, 1x pre-wetting, multidispense) after discarding the first 180 µl of cell suspension. The cells plated in this way were incubated for 24 h at 37 °C, 95 % humidity and 5 % CO₂ to ensure attachment to the bottom of the wells.

Cell culture treatment and cell culture media change
The epMotion 5075 LH was first loaded with the three cell culture media, each of them including the final solvent concentration, and the three cell treatment media, each of them containing the highest substance concentration and the final solvent concentration. It has previously been shown that the concentration range selected for the three compounds does not lead to acute cytotoxicity (technical details in [4]). In a 96-well deepwell plate on a 55 mm height adapter the different conditioned media were prepared by mixing the loaded media [5]. Thereafter, 90 µl of the old culture medium present in each well of the culture plates (Figure 1) were replaced by 90 µl of the fresh medium (8-channel tool, 1,000 µl filter tips, multiaspirate and multidispense). In the case of the cell treatment protocol the concentration of each compound in the fresh medium was doubled in order to reach the correct final concentration of each compound in the corresponding well.

Fixation
From day 2 to day 7 one plate per day was fixed (Figure 2). In order to do so, 45 µl trichloroacetic acid (3 M) per well were added. After 45 minutes at 4 °C the plate was carefully washed with demineralized water and dried.

Sulforhodamin B staining and measurement
At the end of the experiment all fixed plates were stained with sulforhodamin B and washed with acetic acid. Sulforhodamin B staining is used to quantify the cellular protein level and the amount of protein-bound sulforhodamin B is directly proportional to the cell number in the well [2]. The protein-bound dye was solubilized with Tris base and the optical density was measured with a spectrophotometer at 565 nm.
Results

Three experiments were carried out. As an example the effect of sulforaphane on the growth behavior of the cells is shown in Figure 3. During the lag-phase the cells attach to the bottom of the well and adapt to the culture conditions. One day after beginning the treatment of the cells with sulforaphane the cells go over into the exponential growth phase. At this stage cells duplicate at a maximal rate and the curve is nearly a quadratic function. After day 5 cell growth slows down due to the fact that there is almost no more free space available for the cells - the stationary phase begins.

Figure 3 shows that sulforaphane inhibits cell growth in a concentration-dependent manner, whereas vitamin C and menadione do not (see Figure 4). For a quantitative comparison the growth curve for each substance concentration in every experiment was converted (y-axis as doubling number) and the linear growth phase was used to calculate the doubling time.

Figure 4 illustrates that the doubling time is increased after treatment of the cells with sulforaphane. Whereas control cells need 13.2 h for one doubling, the cells treated with 2 µM sulforaphane need 22.3 h. Vitamin C and menadione do not affect cell growth at the concentrations tested.

Conclusion

In the present Application Note it has been shown that the epMotion 5075 LH is a powerful tool to obtain cell growth curves in 96-well multiplates and to analyze the effect of different compounds on a given cell line. In order to do so, the cells were seeded onto the plates by using the seeding cylinder in combination with a magnetic stirrer. Daily partial medium change was carried out and at 6 time points cells were fixed. The SRB staining can be used to easily determine the relative cell number in each well. The advantages of using the epMotion 5075 LH are:

1. more accurate loading of each well than with a manual approach (see also [3]),
2. high accuracy when preparing diluting concentrations of compounds in the same or in different solvents at a time,
3. high economy of time in steps like cell culture media change.

Fig. 3: Example of growth curves.
Increasing concentrations of sulforaphane were added to the cells as indicated.


References


Ordering Information Eppendorf

<table>
<thead>
<tr>
<th>Product</th>
<th>Order no. international</th>
<th>Order no. North America</th>
</tr>
</thead>
<tbody>
<tr>
<td>epMotion® 5075 LH</td>
<td>5075 000.008</td>
<td>960020006</td>
</tr>
<tr>
<td>Dispensing tool TM 1000-8</td>
<td>5280 000.258</td>
<td>960001061</td>
</tr>
<tr>
<td>Dispensing tool TS 1000</td>
<td>5280 000.053</td>
<td>960001036</td>
</tr>
<tr>
<td>Reservoir rack</td>
<td>5075 754.002</td>
<td>960002148</td>
</tr>
<tr>
<td>epMotion Reservoir 30 ml</td>
<td>0030 126.505</td>
<td>960051009</td>
</tr>
<tr>
<td>Eppendorf Deepwell Plate 96/2,000 µl</td>
<td>0030 501.306</td>
<td>951033405</td>
</tr>
<tr>
<td>epTIPS Motion 1000 µl, Filter</td>
<td>0030 003.993</td>
<td>960050100</td>
</tr>
</tbody>
</table>