An automated system for a stable and reproducible long-term cultivation of epithelial cells at the air-liquid interface

The CULTEX® LTC module

Cultex® Laboratories
The Airborne Exposure Experts

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Introduction

In vitro approaches, especially cell culture techniques, have gained more and more importance in toxicology during the last decade, due to the ongoing progress in the development of novel technologies and the increasing call for alternatives to animal testing. One major requirement for the reliability of toxicological data is their reproducibility. For that purpose, identical cell cultures are required for repeatable experiments. However, the handling of cell cultures is laborious and vulnerable to person-specific variations as well as pipetting errors. Thus, automated cell cultivation systems are highly desirable for in vitro toxicology. There are various automated systems that are specifically designed to fulfill particular requirements, ranging from different bioreactors up to sophisticated modular working stations.

Today, cell cultures that reflect the in vivo conditions in the best possible manner are often used for toxicological studies. The lung, skin and intestinal epithelium are target organs of many drugs and pollutants and are thus the subject of most toxicological studies. The differentiation of epithelial cells in vitro is achieved by cultivating them at the air-liquid interface (ALI).

The CULTEX® **L**ong-**T**erm **C**ultivation (LTC) system was specifically designed for the computer-controlled cultivation of cells grown on commercial cell culture inserts under ALI conditions. The system allows a continuous as well as an intermittent medium supply and the medium level is controlled by an ultrasonic sensor. The results demonstrate that the CULTEX® LTC module enables the long-term cultivation of epithelial cells under stable and reproducible conditions and can thus highly enhance the reliability of toxicological studies.

Materials and Methods

CULTEX® LTC unit:

The LTC equipment is specifically designed to provide ALI cell cultures with culture medium over a longer time period (e.g. 3 – 6 weeks). The medium change interval and volume of medium renewal as well as the frequency and intensity of medium circulation can be varied.

Functional components of the LTC incubator module:

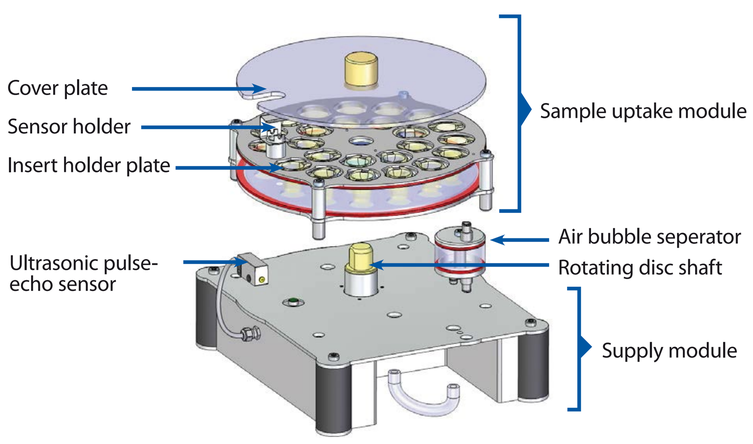


Figure 1: Overview on the CULTEX® Long Term Cultivation Continuous (LTC-C) unit for cultivating cell cultures growing at the air-liquid interface (ALI). The incubator module consists of the cover plate, sample uptake module, air bubble precipitator and supply module. All components except the supply module are autoclavable. 24 cell culture inserts (12-well format; e.g. Costar® or Falcon®) can be handled at the same time. The CULTEX® LTC-C is connected to a control unit (Figure 3), regulating the time intervals and volume of medium renewal as well as the frequency of medium circulation. Before entering the sample uptake module, the fresh culture medium passes the air bubble precipitator in order to eliminate air bubbles. Control of the medium level is enabled by an ultrasonic pulse-echo sensor.

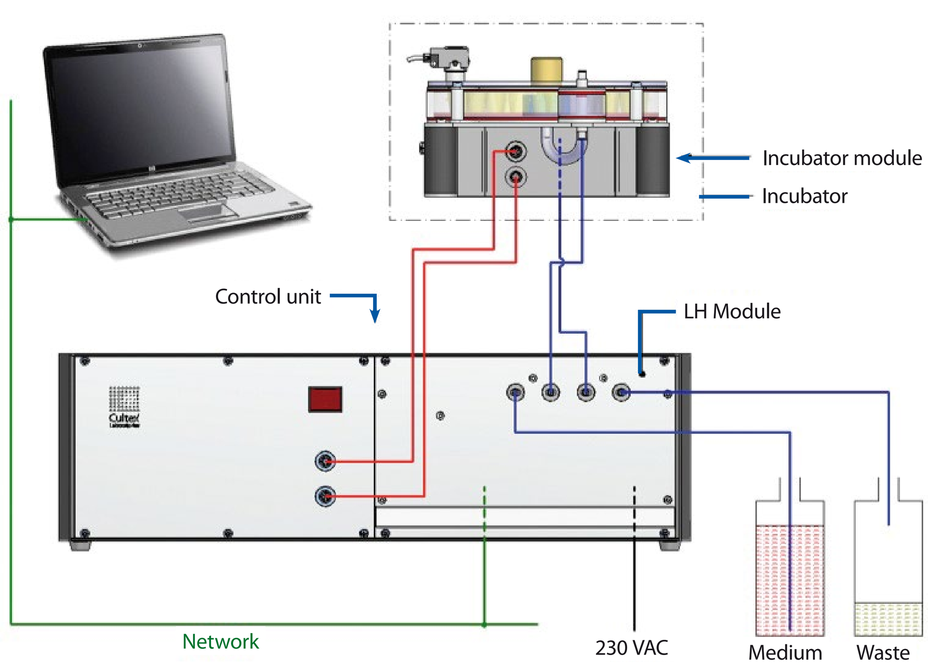


Figure 2: The control unit which is located outside the incubator includes a programmable logic controller (PLC) and pumps for the supply and removal of medium. The control unit is further equipped with an integrated web server. Visualization of the procedures and measurement data is carried out by a web browser, e.g. via a laptop computer. The pumps are located inside the control unit in a sliding cassette, the **L**iquid **H**andling (LH) module (Figure 3), where they are easily accessible for maintenance and sterilization.

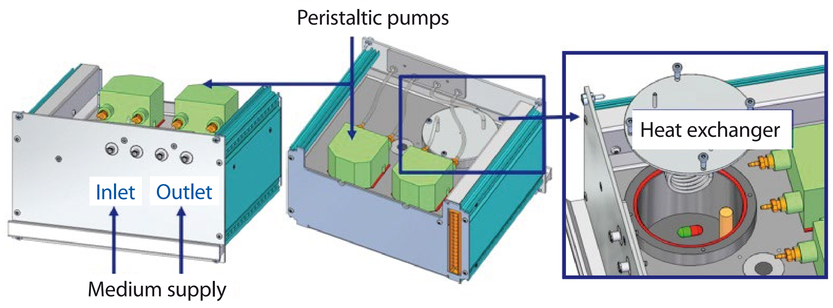


Figure 3: The main components of the LH module are two peristaltic pumps (supply and removal pump), the Heat exchanger, and the leakage sensor. The leakage sensor recognizes the leaking fluid in the case of an error and triggers a relevant error message. The Heat exchanger preheats the culture medium to about 37 °C before it is conducted to the incubator module. The right figure shows the heat exchanger (with opened top) which is filled with water. During operation, the heating cartridge first heats the water to 37 °C and then transfers the heat to the medium, which is conducted through the immersed spiral tubing. A magnetic stirrer circulates the water and this ensures optimal heat transport between the heating cartridge, the spiral tubing and the temperature measurement points on the Heat exchanger housing. The heat energy transferred to the medium is balanced continuously by reheating the water. All parts contacting the medium can be removed individually and are autoclavable at 121 °C.

Preparation of cell cultures:

The A549 cells were expanded in 75 cm^2 cell culture flasks with DMEM (including L-glutamine, sodium pyruvate) supplemented with 10 % FBS and 0.05 % gentamicin. After passaging, the cells were seeded on cell culture inserts (12-well format) with a density of approximately $2 \times 10^5/cm^2$ and cultivated under submerged conditions with their respective growth medium (DMEM) until reaching 100 % confluency. Afterwards, the air-liquid interface was established by removing the apical medium from the insert housing and replacing the growth medium in the basal chamber by the differentiation medium [BEBM (Lonza)/DMEM (1:1) supplemented with SingleQuots™ (Lonza; without retinoic acid), 100 nM retinoic acid and 2 % Ultroser G (Pall Corporation)]. The A549 cultures were immediately placed into the CULTEX® LTC-C and cultivated for varying culture periods.

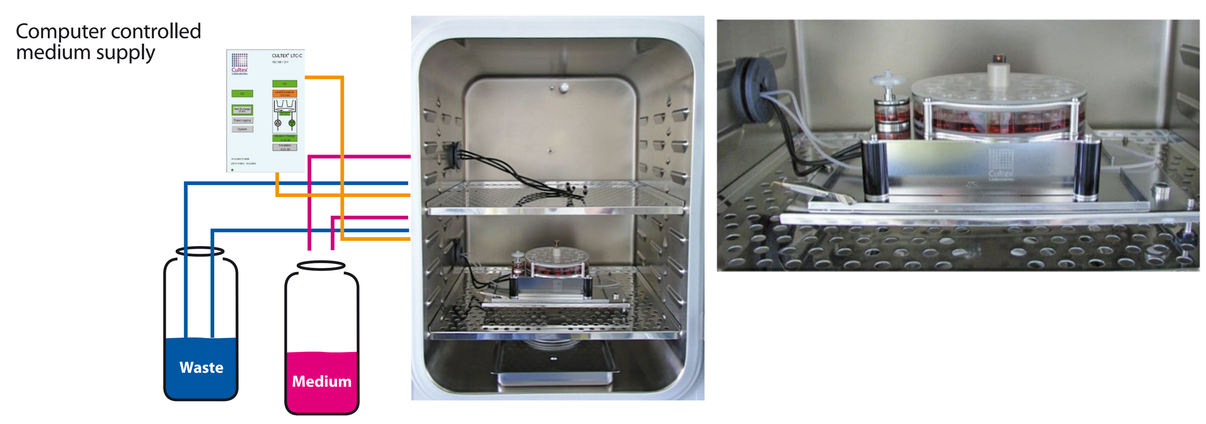


Figure 4: The CULTEX® LTC-C in operation. The incubator module is placed into the incubator, allowing the cultivation under physiological conditions. The control unit as well as the vessels for culture medium and waste are located outside the incubator.

NHBE cells (passage 2) were cultivated in BEGM (Lonza). The cell culture inserts were inoculated with 1.5×10^5 cells per cm^2 and the cultures were cultivated under submerged conditions with BEGM. When reaching 100 % confluency, the ALI cultivation was started (same culture conditions as for A549 cultures). After 10 days under ALI conditions, the NHBE cultures were placed into the CULTEX® LTC-C and cultivated for varying culture periods.

The experimental conditions are given in the results section. For evaluation of the cultures, the cell cultures were fixed with 10 % formaldehyde and the inserts membranes were embedded in paraffin. Histological sections of 5 – 7 µm were produced and stained with hematoxylin and eosin (HE).

Results

Functionality of the long-term cultivation device & procedure was analyzed by using A549 cells.

Cell growth was studied dependent on (1) cultivation time, (2) position in the support plate (Figure 5), (3) influence of the sensor and (4) reproducibility (3 independent experiments).

Experimental conditions: Medium renewal: 36 hours, circulation interval: 30 minutes, circulation frequency: 2 every 30 minutes

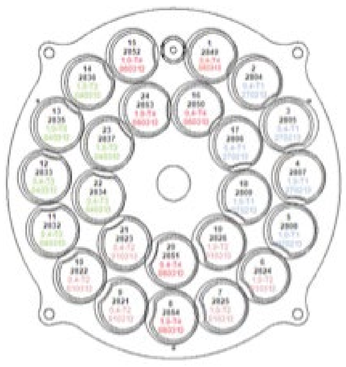


Figure 5: Distribution scheme for the inserts within the support plate (supply uptake module).

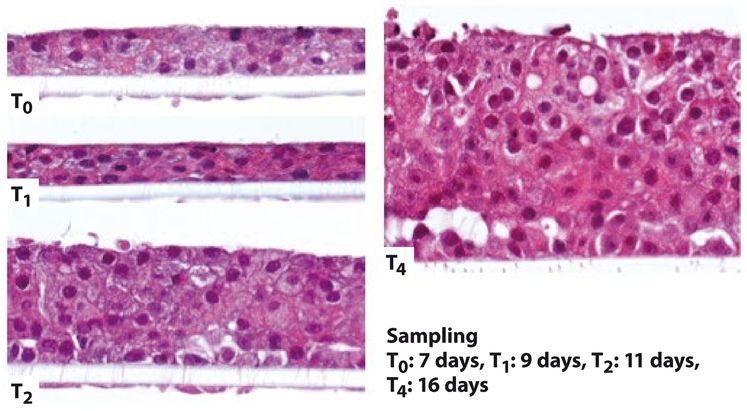
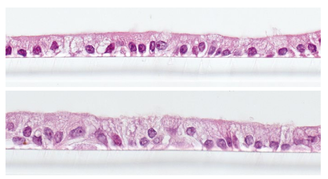


Figure 6: Growth of A549 cells on cell culture inserts with a pore size of 1.0 µm in the LTC-C module dependent on the cultivation time. Samples were taken at different time points from the inner and outer circle.

T_0: Starting point corresponds to the incubator control, 7 day culture. T_1: 9 day culture; T_2: 11 day culture; T_4: 16 day culture.



Initial investigations on the usefulness of the LTC-C for NHBE cultivation revealed that the cells showed a normal growth and developed natural differentiation characteristics, like cilia, during the automated cultivation.

Experimental conditions: Medium change: 36 hours; circulation interval: 30 minutes; circulation frequency: 2 every 30 minutes

Figure 7: NHBE cells cultivated in the LTC-C for 18 days.

Conclusions and Outlook

The investigations demonstrated

- Cells grown under long term conditions in the CULTEX® LTC-C exhibited a homogenous growth independent on the insert position within the support plate.
- In comparison to the control cultures which were grown under conventional culture conditions (manual medium exchange every 36 hours), it became obvious that the circulation of the medium induced a considerable proliferation activity in A549 cells, reflecting their cancerous origin.
- The cell growth of ALI cultures can be influenced by the frequency of the medium renewal and circulation of the medium.

The advantages of such an experimental approach can be summarized as follows:

- A desired number of ALI cultures can be grown under comparable and reproducible culture conditions – independent on interferences by manual handling procedures.
- The provision of comparable cultures for exposure experiments resulting in stable and reproducible data can be ensured.
- The influencing factors for growth and differentiation can be defined in order to select the relevant exposure conditions with regard to the susceptibility of the cells.

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