

Application Note:

**Harvesting of Adherent Cells
from Corning RoboFlask Vessels
with Tecan Freedom EVO**

1 Introduction

This application note serves as a guide to adapt and establish harvesting protocols for various adherent cell lines growing in Corning® RoboFlask™ robotic-friendly cell culture flasks using Tecans' Freedom EVO® robotic liquid handling platform equipped with a flask handling device (flask flipper).

The application note describes and discusses the relevant parameters such as protease incubation time, its incubation temperature and number of flask knocks. Examples of established protocols for three cell lines with different characteristics are provided. These were chosen either for their low aggregate rate (*mouse fibroblast*), their strong culture flask attachment (*human osteogenic sarcoma; SAOS-2*) or their high aggregate rate (*human breast adenocarcinoma; MCF-7*).

Finally, a Standard Operation Procedure (SOP) is provided, which outlines the consecutive adaptation steps to be taken and points out areas of attention.

2 Summary

Protocols for three different cell lines (mouse fibroblast, SAOS-2 and MCF-7) were successfully developed to allow automated cell detachment on Tecan's Freedom EVO robotic platform equipped with a flask handling device. No significant differences in cell number, cell viability and aggregation rate were detected between the automated and the manual (benchmark) harvest (Figure 2-1 for harvested cell numbers per flask).

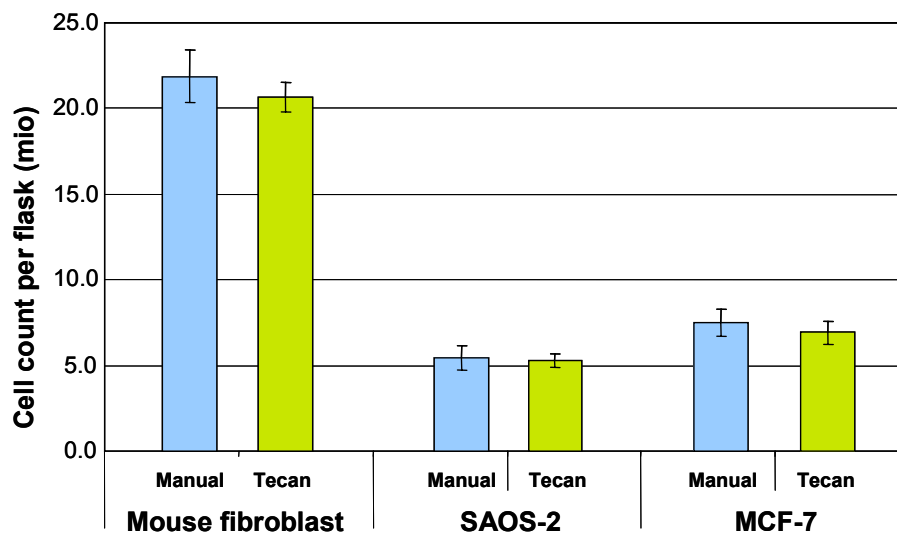


Figure 2-1: Cell number comparison of mouse fibroblast, SAOS-2 and MCF-7 cells between the automated and the manual harvest process. No significant differences were visible.

To achieve complete cell removal from a tissue culture treated flask only three parameters had to be optimized: These were the incubation time and temperature of the dissociation reagent and the number of flask knocks. These experiments effectively demonstrated how to establish further protocols for different cell lines for automated cell handling with Tecans' Freedom EVO – based robotic solutions such as Cellerity™.

3 Material and Methods

Automated cell detachment: A Freedom EVO 150 equipped with a liquid handling arm (LiHa), a robotic manipulator arm (RoMa) and a flask flipper were used for all robotic actions and liquid handling steps.

The RoMa was used to load RoboFlask vessels onto the flask flipper and into and out of a small incubator placed on the worktable for proteinase incubation.

The liquid handling arm was used to add and remove media into and out of the RoboFlask. Four channels were connected to a peristaltic pump (Masterflex L/S) which assisted the removal of liquids from the tissue culture flasks into waste. Four channels equipped with 2.5ml syringes were used to add media or dissociation reagent to the flasks. All channels were equipped with Teflon® (DuPont, Wilmington) coated steel tips which allowed repeatable piercings of the flask septa. Preliminary experiments (results not shown) demonstrated that flasks can be pierced hundreds of times without losing septum integrity.

The flask flipper (Figure 3-1) was used to hold up to four microplate sized cell culture flasks. It was used to flip the flasks into an upright position to allow the LiHa to pierce the septa and access the flask for adding or removing liquids. The flask flipper was also used as a shaker to evenly distribute liquids over the entire growth surface and also to knock cells off the surface.

The Freedom EVO and all accessories and options were controlled by the Freedom EVOware® robotics control software.



Figure 3-1: Cellerity flask flipper with LiHa tips piercing Corning RoboFlask vessels

The flow chart of the manual as well as automated harvesting process is shown in Fig. 3-2.

Media: Phosphate-buffered saline (PBS), fetal calf serum (FCS) and high glucose Dulbecco's modified eagle's medium (DMEM) were purchased from Gibco® BRL (Grand Island, NY). As cell dissociation reagent the recombinant enzyme TrypLE™ Express (Gibco; 12605-028) was used. Proliferation media for mouse fibroblast and SAOS-2 cells was DMEM with 10% fetal calf serum (FCS) and 1% antibiotics (penicillin, streptomycin 100 mg ml⁻¹ / 100 IE ml⁻¹), for MCF-7 cells it was minimum essential Eagle medium (ATCC; 30-2003) with 10% FCS.

Cell culture: For detachment experiments mouse fibroblasts, bone tumor cells (SAOS-2) and human breast adenocarcinoma (MCF-7) cells, were used. The cells were cultivated with media at 37°C and 5% CO₂ in tissue culture treated RoboFlask vessels (Corning Life Sciences; 3070). The media was completely exchanged every two to three days. After the cells reached approximately 75% confluency they were rinsed with 6 ml PBS and detached with 2.4 ml TrypLE either manually or with the automated process.

Analytical methods: Cell number, viability and aggregation rate were determined by trypan blue staining with the Cedex AA20 (Innovatis, Bielefeld).

Results were statistically analyzed (F-test and student t-test). Standard deviation and coefficient of variation (CV%) were calculated and experiments with a CV% above 13% were discarded and repeated again.

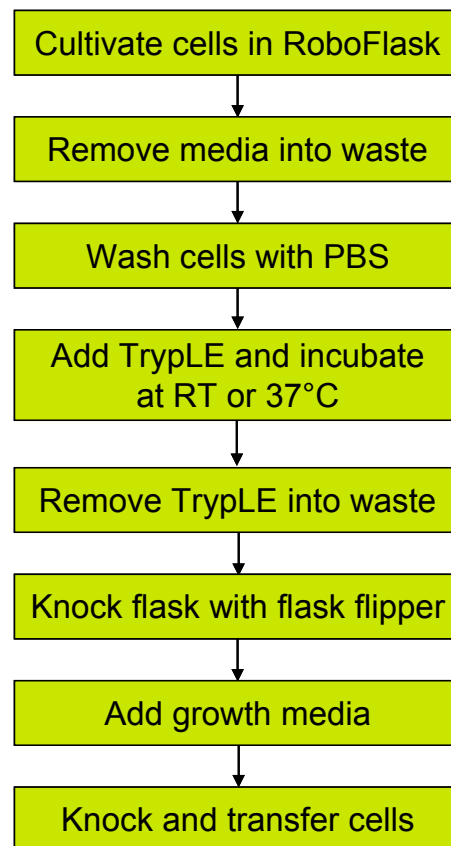


Figure 3-2: Flow diagram of harvest process

4 Automated cell detachment experiments

4.1 Mouse Fibroblasts

Mouse fibroblasts were used in this experiment since they exhibit average cell aggregation and their adherence to culture flask is moderately strong. Mouse fibroblast cells were used as a model for typical cell lines.

After cultivation¹ the cells were almost confluent (Figure 4-1). They were automatically detached according to the process described in Figure 3-2 with the parameters given in Table 4-1. TrypLE was incubated for 6 minutes at room temperature and removed thereafter. By that time 1.5% of the cells were already detached from the surface and consequently discarded together with the TrypLE solution.

After cells were detached (by knocking the flasks using the flask flipper) the cells were collected in 10 ml media (6 ml for cell harvest and 4 ml to rinse the flask after collection). The manual process was conducted identically.

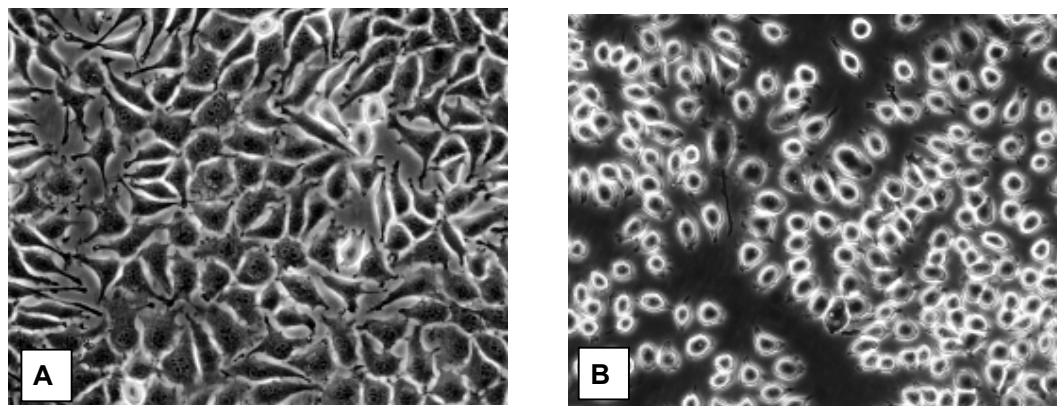


Figure 4-1: Microscopic picture of a nearly confluent mouse fibroblast cell culture growing in RoboFlask vessels (resolution 1:200).

A: Before incubation with TrypLE

B: After 6 min. of TrypLE incubation, right before protease was removed and cells detached.

The protease incubation time had to be observed carefully because at 4.5 min of incubation time, merely 70% of the cells could be detached and with 7.5 min of incubation already one third of the cells were discarded with TrypLE removal.

¹ According to the protocols outlined in chapter three (Material and Methods)

Cell number, viability and aggregate rate were measured in automatically and manually harvested samples (Figure 4-2). The data of both processes were comparable and no statistical significant deviations were detected (student t-test).

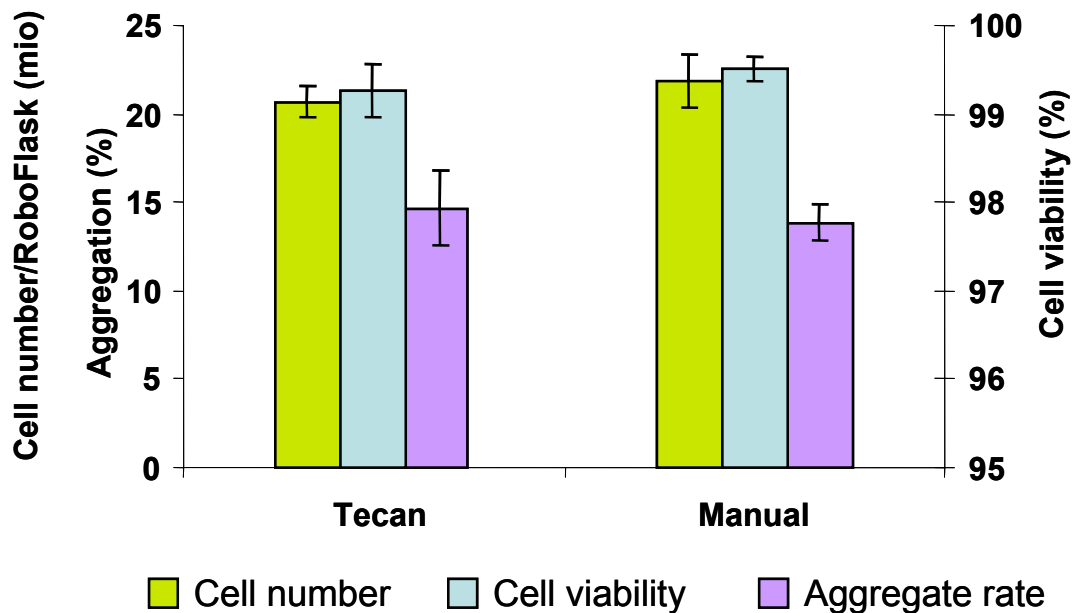


Figure 4-2: Comparison of cell number, cell viability and cell aggregation after harvesting manually or using a Tecan Freedom EVO liquid handling robot. No significant differences between the two harvesting approaches were detected (n=5; coefficient of variation (CV) < 8%).

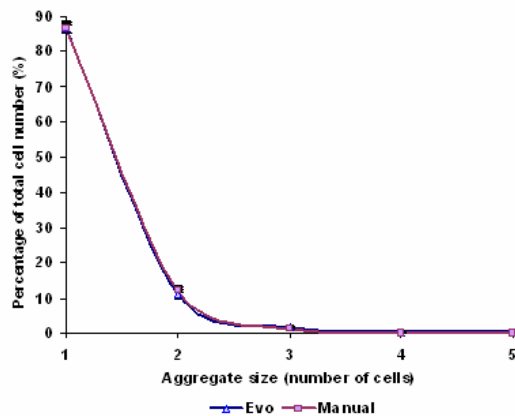


Figure 4-3: Aggregate distribution of mouse fibroblast cells. 86% were not aggregated, more than 11% of the cells were of an aggregate size of two, less than 1.5% of an aggregate size of three and only 0.4% of the cells were aggregating in clusters of four or more cells.

Table 4-1: Parameters determined for successful cell detachment of mouse fibroblast cultures from RoboFlask vessels using Freedom EVO and a flask flipper.

Variables	
Volume TrypLE	2.4 ml
Incubation Time	6 min
Temperature	RT
Flipper knocks	15
Flipper knocks with media	5

Aggregate formation of mouse fibroblasts was quite low (Figure 4-3). In average over 86% of the cells did not aggregate at all and only 1.8% of the cells were congregated in an aggregate with three or more cells.

Compared to the manual process which was used as a bench mark 95% of the cells could be harvested with the automated process. This difference is derived from residual media in the flask as approximately 300 μ l remained in the flask even though the flask base was slightly tilted to allow as much recovery as possible.

4.2 Human Osteogenic Sarcoma Cells (SAOS-2)

Human SAOS-2 cells were used for this experiment since the cell adhesion to the culture flask is fairly strong. This cell line was used to test the flipper for its capability to remove strongly adherent cells from culture vessels.

After cultivation² the cell cultures were nearly confluent (Fig 4-5). They were automatically detached using the parameters specified in Table 4-2.

To accelerate cell detachment (at room temperature TrypLE incubation was more than 10 minutes) cells together with the protease solution were incubated at 37°C in a heatable RoMa-accessible hotel (Fig. 4-4). At 37°C TrypLE was incubated for only 7 minutes before it was removed. It was found that 0.5% of the cells were already detached and consequently discarded together with the protease solution. After detachment the cells were collected in 10 ml media. The manual process was performed identically.

Incubation time had to be observed carefully since 10 min incubation at 37°C led to removal of 17% of the cells which were discharged whereas at 6 min incubation time only 60% of the cells could be detached. The number of necessary flipper knocks was not investigated in detail. Some experiments indicated that a significantly lower cell number could be retrieved with 5 knocks compared to 10 knocks (data not shown).



Figure 4-4: RoMa arm loading a robotic-compatible 37°C incubator

² According to the protocols outlined in chapter three (Material and Methods)

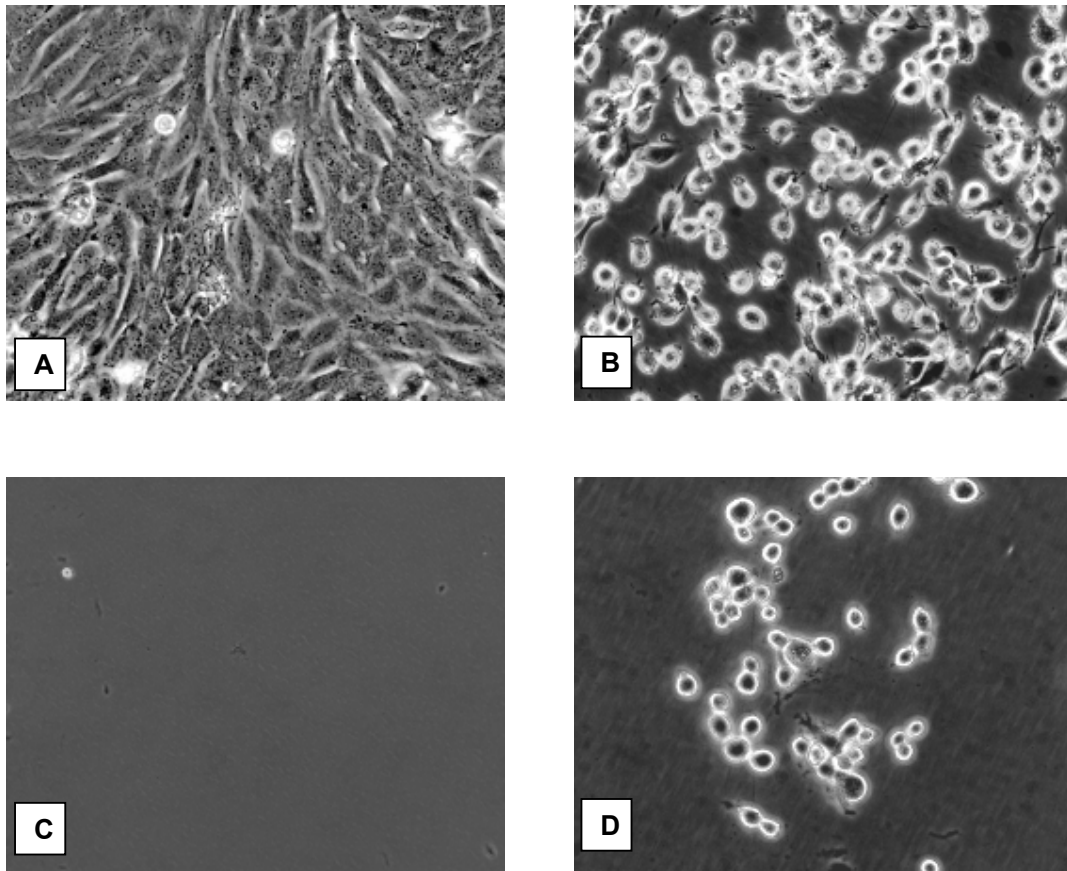


Figure 4-5: Nearly confluent SAOS-2 cell culture growing in Corning RoboFlask vessel (resolution 1:200)

A: Before incubation with TrypLE

B: After 7 min. of TrypLE incubation at 37°C before protease was removed and cells detached

C: Culture flask after cell collection. Very few cells remained in the flask (1:100)

D: Residual volume in culture flask where most of the non collected cells remained (1:200).

Compared to the manual process which was taken as the bench mark 97% of the cells could be harvested with the automated process (Figure 4-6). The cell number harvested manually or robotically is comparable and did not deviate significantly (student t-test).

The viability of cells harvested automatically was significantly higher (with a probability p of 0.93) than for cells harvested manually (Fig. 4-6).

Aggregate formation of SAOS-2 was high. On average over 35% of the cells were aggregated and over 11% of the cells formed aggregates of three or more cells (Fig. 4-7). However, cell aggregation was somewhat dependent on the detachment process. It was significantly (p : 0.97) lower for cell cultures harvested by Freedom EVO®.

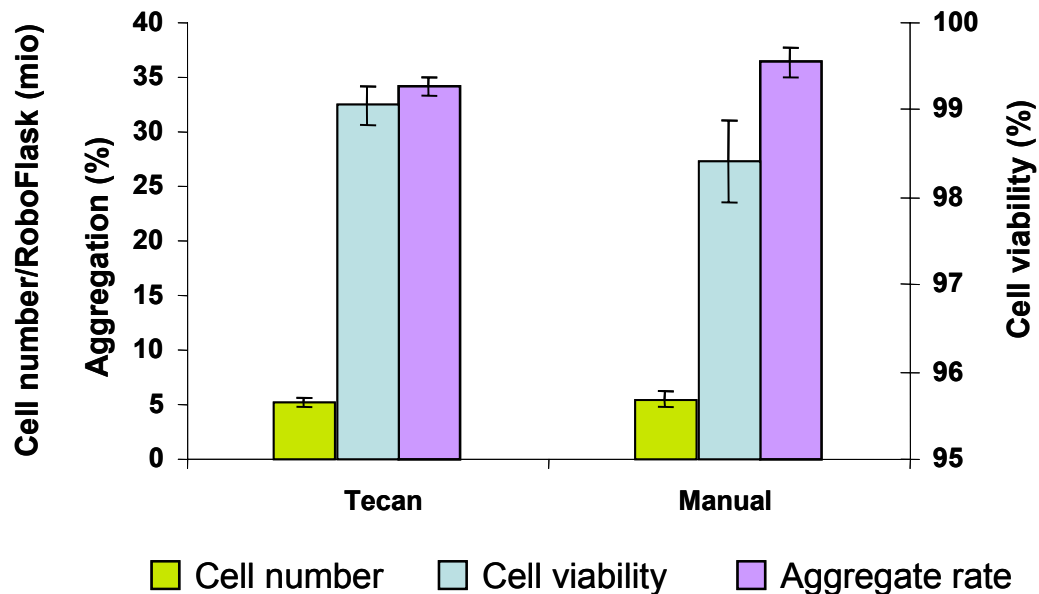


Figure 4-6: Comparison of manually and robotically harvested SAOS-2 cell cultures. Number of SAOS-2 cells did not vary significantly when cells were detached automatically with Freedom EVO or manually. However with the Tecan instrument a significantly higher cell viability and a lower aggregate rate was detected (n=4; CV% < 13%).

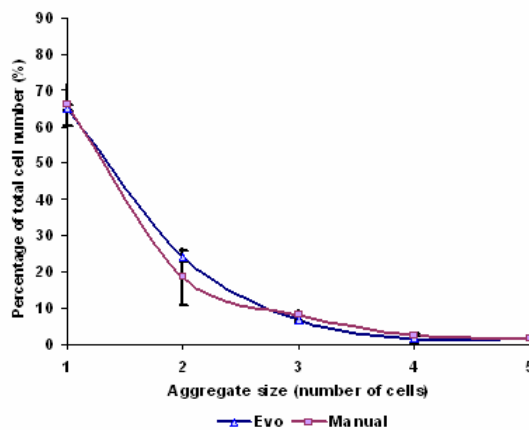


Figure 4-7: Aggregate distribution of human SAOS-2 cells. 65% of the cells were not aggregated, 21% of the cells were of an aggregate size of two, 8% of an aggregate size of three and the remainder were 4 or more cells aggregating.

Table 4-2: Specification of cell detachment for human SAOS-2 cells.

Variables	
Volume TrypLE	2.4 ml
Incubation Time	7 min
Temperature	37 °C
Flipper knocks	15
Flipper knocks with media	5

4.3 Human Breast Adenocarcinoma (MCF-7)

Human breast adenocarcinoma cells (MCF-7) were used for this experiment since their adhesion to the culture flask is fairly weak and their cell aggregation is quite high.

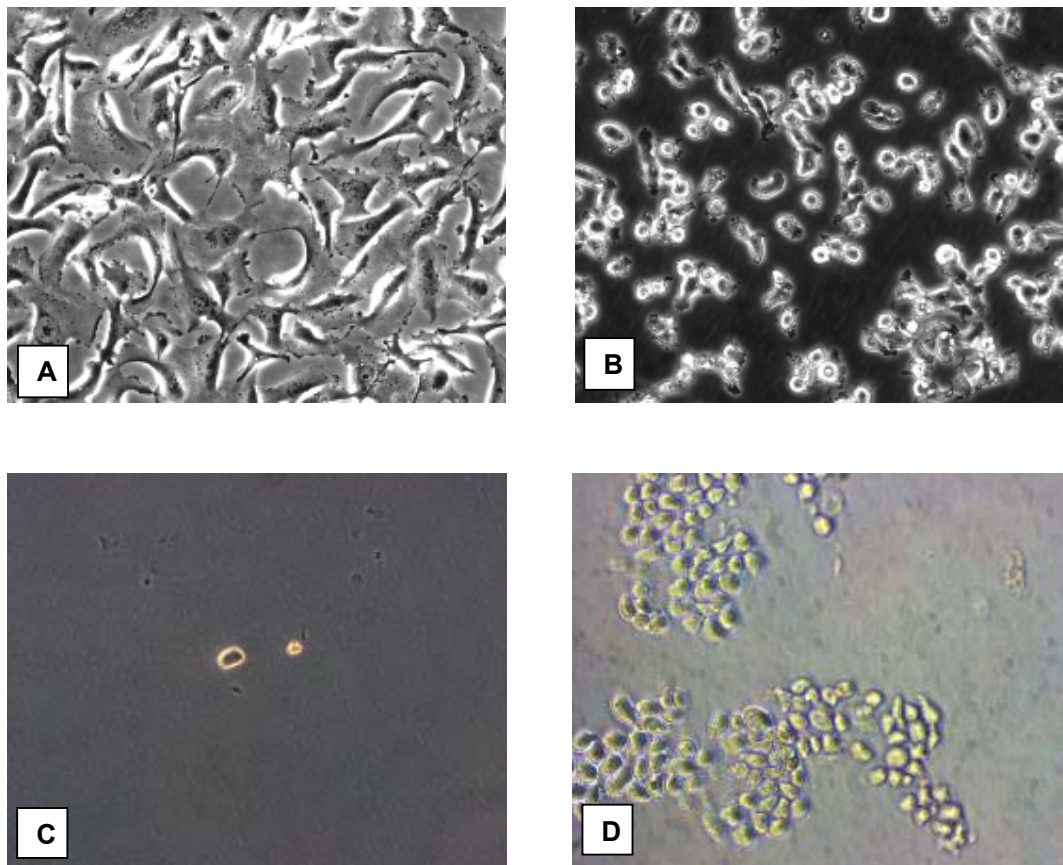


Figure 4-8: Nearly confluent MCF-7 cell culture (resolution 1:200).

A: Before incubation

B: After 1 min. of TrypLE incubation, immediately before protease was removed and cells detached

C: culture flask after cells were collected

D: Cell aggregates in residual volume after cells were collected.

After cultivation³ nearly confluent cell cultures (Figure 4-8) were automatically detached using the parameters specified in Table 4-3. After 1 min, the trypsin-like enzyme was removed. Roughly 0.8% of the cells were already detached and removed with the protease.

After cells were detached they were collected in 10 ml media. The manual process was performed identically. Incubation time was observed carefully since 2 min of incubation time removed more than 17% of the cells which were subsequently discharged with the

³ According to the protocols outlined in chapter three (Material and Methods)

protease. Flipper knocks should not be reduced below ten, since with five flipper knocks only about 60% of the cells could be detached compared to 10 knocks (data not shown).

As seen in Figure 4-9 the cell number as well as viability and aggregate rate are comparable for both approaches and did not deviate significantly (t-test).

Compared to the manual process which was used as a bench mark, 93% of the cells were harvested with the automated process and no significant deviation was detected. Also the cell viability of the automatically and manually harvested cell cultures was comparable.

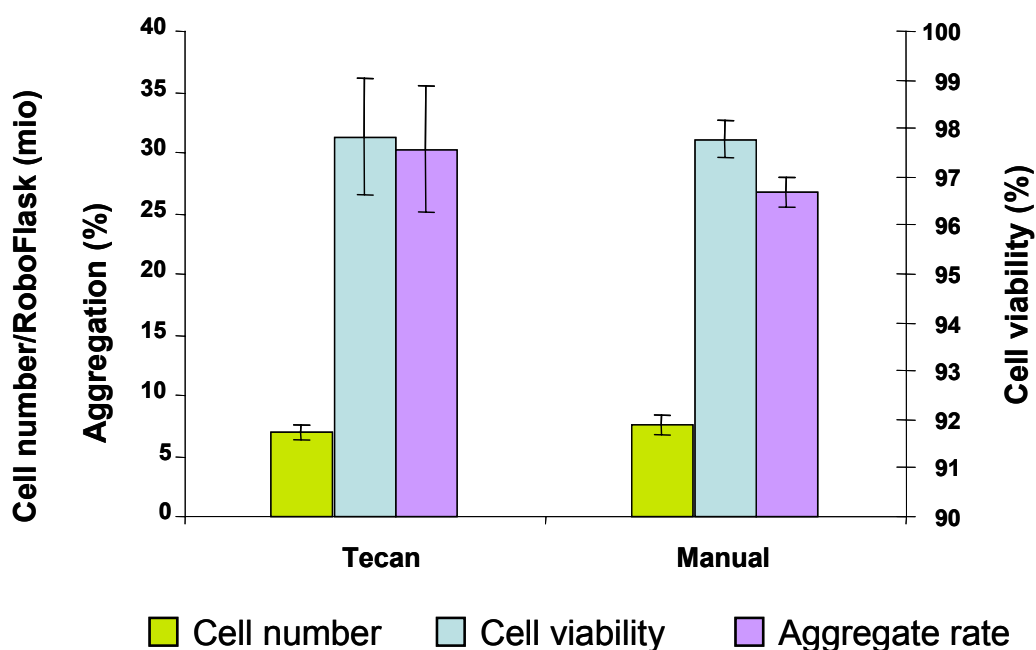


Figure 4-9: Comparison of automatically and manually harvested MCF-7 cell cultures. Cell number, aggregate rate and viability are not significantly different when cells were detached with the Freedom EVO liquid handler or manually (n=4; CV% <11%).

Aggregate formation of MCF-7 was high and not dependent on the detachment process (automatically or manually). In average over 29% of the cells were aggregated and 9% of the cells were aggregates of three or more cells (Fig. 4-10).

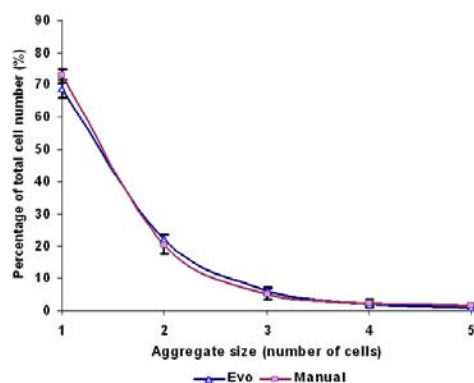


Figure 4-10: Aggregate distribution of human MCF-7 cells. 71% of the cells were not aggregated, whereas more than 21% of the cells were of an aggregate size of two, less than 6% of an aggregate size of three and the remainder were aggregates of 4 or more cells.

Table 4-3: Specification of cell detachment for human MCF-7 cells.

Variables	
Volume TrypLE	2.4 ml
Incubation Time	1 min
Temperature	RT
Flipper knocks	15
Flipper knocks with media	5

4.4 Conclusions

These exemplary cell culture experiments demonstrated the successful automated cell detachment with Freedom EVO liquid handling robot equipped with a flask flipper from Corning RoboFlask cell culture vessels. No significant differences in cell number, cell viability and cell aggregation were detected as compared to the manual process. In general, experiments were more repeatable if performed automatically.

If automated and manual cell harvesting experiments are to be compared, then both processes must be performed in identical vessels since cell adherence can deviate considerably to other flask types (for example in manual tissue culture flasks from another vendor cells adhered only half as strong as on the Corning RoboFlask vessel (data not shown).

To ensure coverage of the complete culturing surface, protease volume must not be reduced below 2.4 ml. Incubation time and temperature should be observed carefully, since they have a large influence on cell detachment and harvest yield.

Fifteen flask knocks were sufficient in all cases to remove the cells from the surface. Without undue negative influence on the harvest yield, flask knocks can be reduced to about 5 knocks in most cases. The cells ought to be collected with a minimum of 6 ml media, in order to avoid cell loss due to residual volumes (approximately 0.3 ml media remains in the flask). The whole automated detachment process takes about 15 min operation time (without incubation), which is slightly longer than with the manual process.

5 How to develop a cell harvesting process on Freedom EVO[®] flask flipper

The following standing operation procedure (SOP) describes the adaption of cell detachment protocols of a specific cell line to an automated process on Freedom EVO flask flipper. For a graphical depiction of the process see Fig. 3-2. This SOP builds on the manual detachment process in which the protease solution is removed right before cell detachment⁴. The only variables that have to be adjusted are protease incubation time, incubation temperature, and number of flipper-knocks.

1. The cells are seeded into RoboFlask vessels and cultivated with the respective media at 37°C and 5% CO₂ until they are almost confluent (It is not advisable to use flasks from a different manufacturer or brand, since the cell adhesion may deviate considerably).
2. Media is removed and the flask is rinsed with the desired volume of PBS. After removing PBS, the desired volume of recombinant TrypLE Express is added (the minimal volume to ensure full flask coverage is 2.4 ml).
3. The flask is then placed under an inverse microscope at room temperature (RT) and detachment of the cells, without knocking or vigorously shaking the flask is observed. The majority of the cells should be rounded but still attached to the flask (even when flask is slightly shaken (Figs. 4-1, 4-5 and 4-8)).
4. At this point the TrypLE will be completely removed from the flask. As a control the cells in the removed protease solution should be counted (in which not more than 2% of the total cell number should be present). The time when the protease is removed is noted and used as the basis for automation. If the incubation time exceeds 10 min, the flask can alternatively be placed in an incubator at 37 °C (respectively heatable hotel) which should reduce the needed incubation time significantly.
5. The flask is then placed manually on the flipper and the cells are detached by the adapted program. Usually 15 knocks are sufficient and in most cases they can be reduced (harvested cell number however should not decrease significantly).
6. Cell detachment is stopped by the addition of media (at least 6 ml culture media to minimize cell loss due to approximately 0.3 ml of residual media in the flask). The cells are subsequently transferred out of the flask. The cell number and viability are determined and are compared to the already established manual detachment process. No significant difference in cell number and viability should be visible, otherwise the incubation time or amount of knocks have to be adjusted.

⁴ In other laboratories this process is sometimes performed differently (e.g. protease remains in the culture media and is quenched by the addition of serum-containing media or cells are centrifuged and protease is removed). If appropriate such a process might also be adapted on Freedom EVO. The proposed process offers the advantage to remove protease which might damage cells and it avoids centrifugation which is stressful to cells and more expensive to automate.