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Rapid cryopreservation of five mammalian and one mosquito cell line at -80°C while attached to flasks in a serum free cryopreservative

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ABSTRACT

Cell culturing, and the requisite storage of cell lines at ultra-low temperatures, is used in most laboratories studying or using eukaryotic proteomics, genomics, microarray, and RNA technologies. In this study we have observed that A72(dog), CRFK(cat), NB324K(human), MCF7(human), WI38(human), and C636(mosquito) cells were effectively cryopreserved at -80°C while attached to the substratum of 25cm^2 tissue culture flasks. This was accomplished using a serum free crypreservative recently developed by Corsini and co-workers. The technique allows for significant savings of time and money in laboratories that rapidly process numerous cell lines.

INTRODUCTION

Culturing of eukaryotic cells is required in most laboratories that study or utilize proteomics, genomics, microarray, and RNA-based technologies, and long term cryostorage of these cells is a necessary aspect of the cell culturing techniques. The ability to omit lengthy procedures and expensive cryopreservatives would be both beneficial and economical, especially in laboratories that use and process large numbers of cultured cells. Previous work with multi-well plates has shown that a variety of cell lines can remain viable during storage while attached to substratum of multi-well plates (1-3), thus eliminating time consuming steps. In addition, our recent experiments with serum-free cryopreservatives have shown that a phosphate-buffered saline (PBS)/10% dimethyl sulfoxide (DMSO) solution performs nearly as well as a serum-containing preservative (4). Together, these findings suggested the possibility of cryostoring cells with a serum-free preservative while attached to a 25cm^2 tissue culture flask. Such a procedure would, at times, represent a significant saving in time and money.

With this in mind, we have extended our initial studies (1, 4) in three ways. First, we show that cells stored in a frozen state while attached to substratum of 25 cm^2 culture flasks remain viable for 30-180 days at -80°C. The ability to store cells while attached in flasks is an improvement over the multi-well plates because there is no need to seal the flask, as one must with multiwell plates, in plastic to prevent air exchange. Second, we have employed a serum free cryopreservative recently developed by Corsini *et al*. (4), and established that this preservative is effective during procedures that preserve cells while attached to substratum of a tissue culture flask. Third, we have extended the observation (that various cells can be cryopreserved while attached to substratum of culture vessel) to an insect and to a non-transformed 'primary' human cell line, WI38.

MATERIALS AND METHODS

The experiments proceeded as follows: No antibiotics were used in any of the growth media. All mammalian cells were seeded into 25cm^2 Falcon flasks (Becton

Dickinson Labware, Franklin Lakes, NJ, USA). These flasks were of the type with solid lids that allow no exchange of air when tightened. Flask lids should seal down and prevent air exchange, which in turn prevents oxidation and sublimation of cryopreservative. Cells were typically grown in 5 mls of RPMI 1640 + 10% FBS (Sigma, St. Louis, MO, USA) in a 37°C and 5% CO_2 incubator with lids slightly open to allow free exchange of air from the incubator. In order to prevent dehydration of the culture medium, high humidity in the CO_2 incubator was maintained by filling an 18x12x8 inch rubber pan with water and placing it in the bottom of the incubator. *Aedes albopictus* C6/36 cells (5) were grown at 28°C in L-15 media (Sigma) supplemented with 10% FBS, in the absence of CO2. As cells approached confluency, the growth medium was aspirated from the cells and 2-4 mls of freezing solution were applied. The solution consisted of PBS + 10% DMSO (Sigma). The flasks were then placed directly into the ultra low freezer (-80°C) for 30-180 days. Cell viability (survival) after this process was assessed by removing flasks from the freezer and then placing them in 37°C incubator for 15-25 minutes until the cryopreservative thawed. The cryopreserving solution was then aspirated from the flasks and new growth medium RPMI 1640 + 10% FBS (Sigma) overlaid onto the cells. The flasks, with lids loosened to allow exchange of CO_2, were then placed in a 37°C and 5% CO_2 incubator (or 28°C and no CO_2 for the mosquito line) and growth was observed for 5-7 days. The mosquito cells were incubated with lids tightened in the 28°C incubator, so dehydration was not a concern.

RESULTS AND DISCUSSION

NB324K: Two experiments with NB324K human kidney cells (6) were conducted. In experiment 1, flasks were frozen at 90% confluency, and the cells were 40% confluent upon thawing 6 months later; they grew to confluency within 3 days. In experiment 2 the cells were frozen at ca. 5% confluency and were less than 1% confluent after two months of cryostorage, and within 7 days they grew into many healthy colonies that occupied 20-30% of the substratum.

CRFK: Two experiments were conducted with CRFK feline kidney cells (7). In experiment 1 the flask was frozen at close to 100% confluency and after thawing, the cells remained 70% confluent and grew to complete confluency again within 1-2 days. The experiment 2 cells were also near 100% confluent when frozen, and after being thawed they were still 60% confluent. They grew to confluency again in 3 days.

A72: A canine fibroma cell line called A72 (8) was also tested. In the first experiment the flask was grown to 100% confluency and then frozen for 2 months. Less than 1% of the cells remained attached immediately after thawing; these increased to 1-2% within 48 hours, and confluency soon thereafter. 10-20% of the cells remained viable in the cryopreservative and attached within two days after seeding into fresh flasks. This phenomenon of cell detachment was previously observed with A72 in previous experiments where 80-90 % of A72 cells detached from substratum during storage on multi-well plates (4). In the second experiment, A72 cells were grown to 95% confluency and then stored for 2 months at –80°C. Upon thawing, they were 0.5% confluent, and within a week they expanded to confluency. Many of the cells had detached in the cryopreservative; these floating cells, upon seeding in fresh flasks, also quickly grew to confluency.

MCF-7: This is a human breast cancer cell line (9). In our experience, these cells grew slowly, forming tight groups that we term "patches." For the first experiment, the flasks were frozen at about 90% confluency. Five months later, thawing and seeding resulted in less than 1% attached cells, which grew into many healthy colonies within a week. In the second experiment, MCF-7 cells were frozen at about 95% confluency. Upon thawing the flasks, two months later, they where less than 1% confluent, and the cells grew to many healthy colonies within 5 days. Also upon thawing, MCF-7 flasks contained viable cells that had detached into the cryopreservative and could be rescued by centrifugation and seeding into fresh flasks.

WI38: This is a human diploid fibroblast line with a finite life of 50+/-10 divisions and normal karyotype that has been utilized in production of poliovirus vaccine (10, 11). In two experiments these cells were frozen at 75-80% confluency. After two (experiment 1) or three (experiment 2) months at -80°C, both flasks were ca. 1-2% confluent upon thawing. These surviving cells grew into healthy colonies within a week. Very few viable cells were rescued out of the cryopreservative.

C6/36: An *Aedes albopictus* mosquito line, C636 (5), was also tested. The two experiments yielded very similar results. In both experiments live cells attached to the substratum and recovered in supernant showed about 1% survival rate when thawed after one month of storage. The flasks grew to confluency within 7-8 days. Many healthy cells also detached into the cryopreservative; upon rescue by centrifugation and seeding into fresh flasks, these floating cells also grew to confluency within 7-8 days.

These experiments indicate that frozen storage of a variety of cell lines, while attached to the substratum of a tissue culture flask, is possible. As mentioned in the results, we have noticed that some cell lines tend to detach from the substratum, but they are easily rescued by centrifugation of the cryopreservative collected from the flask. If testing this protocol on a cell line for the first time, it will be prudent to centrifuge the cryopreservative from the flask to collect these cells. There also appears to be some flexibility with the cryopreservative. While we have not rigorously examined the possibility of using standard growth medium with 10% DMSO in this process with flasks, we have successfully stored and rescued A72 from standard growth medium plus 10% DMSO on two occasions. This, combined with the fact that previous experiments successfully stored and retrieved six different cell lines (including A72, CRFK, MCF7, and NB324K) grown and frozen while attached to the sub-stratum of multi-well plates (1), suggests that our PBS/DMSO cryopreservative is interchangeable with a standard formulation containing growth medium plus FBS in place of the PBS. We also note that effective recovery occurs at either high or low seeding densities, but we recommend that high seeding densities be used to reduce the possibility of selecting unusual mutants that 'misbehave' during subsequent experimental work.

Because the non-transformed and the insect lines were successfully recovered after the process, this technique promises to be widely applicable. There are many situations that will find this technique helpful. For example, experiments designed to isolate useful clones of genetically altered (transfected or transduced) cells often generate thousands or even millions of possible clonal isolates growing on the substratum of numerous flasks. With our technique, many of the flasks can be stored temporarily as they await analysis. This technique will also be useful in situations that involve the screening of large numbers of cell lines for virus susceptibility, drug susceptibility, or any other property of interest. As cell lines come into the lab (sometimes dozens of lines per week), each line can be stored frozen in its flask until use, saving a significant amount of time. Those lines that prove useful can then be processed and placed in vials for long-term storage in liquid nitrogen. In summary, our experiments with the rapid cryopreservation of five mammalian and one insect cell lines indicate that storing cultured cells while attached to the substratum of a tissue culture flask is possible. In laboratories that process large numbers of cell lines, this procedure represents a significant saving of both time and effort because time-consuming detachment and centrifugation steps can be eliminated. It also represents a cash savings through reduction of centrifuge tube and serum use (due to the serum-free cryopreservative).

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PROTOCOLS

Storage

1. Grow cells to 50-90% confluency in a 25cm^2 flask.
2. Remove all of the culture medium from the cells.
3. Immediately add 2mls of phosphate-buffered saline containing 10% DMSO (this solution should be filter-sterilized before use). Ensure that the solution has spread over the bottom of the flask, covering the cells, by rocking back and forth several times.
4. Tighten lid and place the flask directly into -70°C to -80°C until use. These lids should be of a style that prevents air exchange when tightened.

Recovery

1. Remove the flask containing cells from the freezer and place in cell culture incubator until cryopreservative has thawed (15-20 minutes).
2. Remove cryopreservative to a sterile, conical centrifuge tube and immediately add 4-5 mls of growth medium to the flask. Place flask into incubator and monitor growth through the following week.
3. If the cell line tends to detach from the flask during the process, spin the supernatant (transferred to a conical centrifuge tube instep 2) at 200g for 5 minutes at room temperature.

Remove supernatant, re-suspend the cells in 5mls of fresh growth medium (warm or cold), and transfer to a 25cm^2 flask. Place the flask in incubator and monitor growth.