Serum-free Culturing of Mammalian Cells – Adaptation to and Cryopreservation in Fully Defined Media

Yanela González Hernández and René W. Fischer
Laboratory of Organic Chemistry, Swiss Federal Institute of Technology, Zurich, Switzerland

Summary
Long term storage of living cells is a central issue in cell biology and medicine. In addition to the cryoprotectant dimethyl sulphoxide (DMSO), foetal bovine serum (FBS) is often added to the freezing medium for the cryoconservation of serum dependent cell lines. FBS, with its high protein content, protects cells against shear forces and gives the medium a desirable osmotic environment with a physiological viscosity. However, the harvesting of FBS is painful for the foetus and should be avoided for ethical reasons. In this work we describe the adaptation of several commonly used cell lines to serum- and protein-free media; however, such cell lines should not be frozen in a conservation medium containing serum. We tested the synthetic surfactant "Pluronic F68™", known to protect mammalian cells grown in serum-free bioreactors (Papoutsakis, 1991), as an active cryoprotectant. In samples containing 0.1 to 1% Pluronic F68, we found a significant increase in viable cells after thawing. Values up to 115% of starting cell number indicate that the cells proliferate within the first 24 hours after thawing, a property which was not observed in cryoconservation media without Pluronic F68.

Zusammenfassung: Serumfreie Kultivierung von Säugerzellen – Gewöhnung an und Kryokonservierung in chemisch definiertem Medium
Die Langzeitlagerung von lebenden Zellen ist eine zentrale Notwendigkeit in der Zellbiologie und der Medizin. Für die Kryokonservierung wird neben dem Gefrierschutzreagens Dimethylsulfoxid (DMSO) bei serumabhängigen Zelllinien häufig fötales Kälberserum (FBS) eingesetzt. FBS schützt die Zellen durch seinen hohen Proteingehalt gegen Scherkräfte und verleiht dem Medium die geeignete Osmolarität sowie eine physiologische Viskosität. Die Gewinnung von FBS ist für den Fötus schmerzhaft und sollte schon aus ethischen Gründen vermieden werden. In dieser Arbeit beschreiben wir die Gewöhnung verschiedener häufig eingesetzter Zelllinien an serum- und proteinfreies Medium. Serumfrei wachsende Zellen sollten jedoch nicht mit Serum eingefroren und gelagert werden. Wir testeten das synthetische Tensid "Pluronic F68™" als Zusatz in chemisch definierten Kryomedien. Pluronic F68™ wird bereits zum Schutz von serumfreien Zellkulturen in Bioreaktoren eingesetzt (Papoutsakis, 1991). Wir fanden, dass Pluronic F68™ in Konzentrationen von 0,1 bis 1% die Ausbeute an lebenden Zellen nach dem Auftauen signifikant erhöhte. Werte von 115% der eingesetzten Zellen deuten darauf hin, dass die frisch aufgetauten Zellen schon in den ersten 24 Stunden proliferieren. Dies geschah nicht, wenn den Kryokonservierungsmedien kein Pluronic zugesetzt wurde.

Keywords: cryopreservation, cryoprotectant, serum- and protein-free, animal component free, chemically fully defined

1 Introduction

Since the beginning of the 20th century, when the first attempts were made to maintain eukaryotic cells *in vitro*, until today, animal sera have played an important role in cell culture. In the early days, animal sera were known as "physiological nutrition" and general "cell protecting" solutions. Today, foetal bovine serum (FBS) is widely used as a growth promoting additive in cell culture media. However, due to the efforts of animal protection groups, it became known that the harvesting of FBS is cruel and painful to the foetuses. Consequently, some scientists are trying to find alternatives to FBS (Van der Valk et al., 2004). The search for alternatives to FBS is further driven by the fact that the purification of cell culture products obtained from cells raised in FBS-containing medium is costly and their registration with governmental authorities is very difficult. However, the majority of cell lines used today in cell biology and molecular biology require serum for growth. For such cells FBS is typically used at a concentration of 5 to 20% in the cultivation medium. In the present paper, we describe how we successfully adapted nine frequently used cell lines (both adherent and in suspension) to chemically defined medium not supplemented with any animal derived components. These cell lines

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are available to the scientific community through the European Collection of Cell Cultures "ECACC". An updated overview of serum-free cell lines and serum-free media can be found in the database supported by the 3R Research Foundation Switzerland at www.sefrec.com

Apart from keeping cells growing in culture, FBS is also widely used for their long term storage, i.e. for the cryopreservation of cell lines. Cryopreservation of mammalian cell lines has been and still is an absolute requirement for cell culturists. The freezing and thawing processes are complex and very stressful for cells. A substantial portion of the cryopreserved cells does not survive this treatment (Han and Bischof, 2004). In addition, the surviving cells may, due to the harsh treatment, exhibit a lag phase that delays growth after thawing.

The freezing process of a cell suspension can be divided into several stages. As the temperature is decreased below 0°C, ice starts to form in the extracellular medium, whereas the cytosol remains unfrozen. Depending on the cooling rate, the cytosol can even become supercooled and stay liquid at temperatures as low as -15°C (Mazur, 1970). When the water of the extracellular medium begins to form ice crystals, the solutes become more concentrated, a phenomenon also referred to as the "solution effect". As a consequence, the concentrated medium equilibrates with the intracellular liquid, resulting in cell shrinkage. At lower temperatures, ice formation also takes place inside the cell, and the same solution effect is observed in the cytosol with disadvantageous consequences, such as changes in pH or cytotoxic solute concentrations. A further decrease in temperature lets the solute concentration rise to a point which makes crystallisation impossible. At a certain temperature, the highly concentrated liquid solidifies in a process called "eutectic freezing". In addition, ice crystals, occurring intra- or extracellularly may disrupt cell membranes, resulting in poor cell recovery after thawing. Recrystallisation of ice crystals during thawing is thought to be another factor that is detrimental for cell survival.

The recovery of living cells after storage is thus greatly affected by the freezing protocol, the cooling rate, and the compounds present in the freezing medium, such as DMSO, which is used as an anti-freeze agent that prevents ice crystal formation. We believe that the non-ionic surfactant Pluronic F68™ is able to act as a cryoprotectant by stabilising the cell membrane. In this work, we compare existing cryo-media formulae, evaluate the optimal cooling rate and demonstrate the positive influence of Pluronic F68™ in the newly developed freezing medium "FILOCETH" on the survival rate of cryopreserved cells after thawing.

2 Material and methods

2.1 Reagents and cell lines

Cell lines:
All cell lines were obtained from the ECACC. After successful adaptation to serum-free medium, the cells were sent back to ECACC for quality control and proper cell banking as indicated below.

Cat.# 85011420 P3X63Ag8.653 Mouse BALB/c myeloma
Cat.# 03092502 P3X63Ag8.653-TurboDoma adapted to grow in chemically defined protein- and peptide-free medium. Suitable for use as a fusion parent for serum-free generation of hybridomas.

Cat.# 88031701 COS-1 Monkey African green kidney, SV40 transformed
Cat.# 04092902 COS-1-InVitrus Monkey African green kidney, SV40 transformed, adapted to grow in chemically defined protein- and peptide-free medium. This cell line is substrate dependent, i.e. adherent. COS-1 was derived from CV-1 simian cells transformed by an origin-defective mutant of SV40, which codes for wildtype T Ag. Possible host for propagation of pure populations of recombinant SV40 virus.

Cat.# 87021302 COS-7 Monkey African green kidney, SV40 transformed
Cat.# 05063001 COS-7-InVitrus Monkey African green kidney, SV40 transformed, adapted to grow in chemically defined protein- and peptide-free medium. This cell line is substrate dependent, i.e. adherent. COS-7 was derived from CV-1 simian cells transformed by an origin-defective mutant of SV40.

Cat.# 84113001 Vero African Green Monkey kidney
Cat.# 03092503 Vero-Hektor African Green Monkey kidney cells, adapted to grow in chemically defined protein- and peptide-free medium. This cell line is substrate dependent, i.e. adherent, and may be suitable for the replication of viral particles.

Cat.# 85120602 293 Human Embryo Kidney
Cat.# 05030204 293-Hektor Human Embryo Kidney, adapted to grow in chemically defined protein- and peptide-free medium. Cells grow in suspension and tend to form aggregates in static culture. The cells grow as finely dispersed single cell suspensions under optimally agitated culture conditions.

Cat.# 5072401 SP2/0-Ag14 Mouse x Mouse hybridoma, non-producing.
Cat.# 03092501 Sp2/0-Ag14-TurboDoma Mouse x Mouse hybridoma, non-producing, adapted to grow in chemically defined protein- and peptide-free medium. Suitable for use as a fusion parent for serum-free generation of hybridomas as well as a host for the production of recombinant proteins.

Cat.# 85110503 NS0 Mouse myeloma
Cat.# 06020202 NS0 Mouse myeloma, adapted to grow in chemically defined protein- and peptide-free medium. Cholesterol is not required as a supplement for this culture. This cell line is suitable for use as a fusion parent for serum-free generation of hybridomas.

Cat.# 85011433 BHK 21 (clone 13) Hamster Syrian kidney
Cat.# 05062302 BHK21-InVitrus Hamster Syrian kidney, adapted to grow in chemically defined protein- and peptide-free medium.

"HEP-1" a home-made antibody secreting mouse-hybridoma cell line.

Cell culture reagents: Foetal bovine serum (FBS), Iscove's modified Dulbecco's medium (IMDM), glutamine and

gentamicin were purchased from Sigma-Fluka Buchs, Switzerland.
Chemically defined, serum-free media: TurboDoma, Hektor and InVitrus were obtained from Cell Culture Technologies, Gravesano, Switzerland.

Conditioned medium (CM): Conditioned medium was harvested from confluent cell culture by centrifugation of the cell suspension (5 min/3300xg). The cell free medium is partially depleted of nutrients, but enriched with cytokines and growth factors originating from the cells.

Unless otherwise indicated all other chemicals were either "cell culture tested" or of highest available purity from Sigma-Fluka Buchs, Switzerland.
Pluronic F68™ Cat.# P-1300 Sigma-Fluka Buchs, Switzerland.

Cryo-media: Hypothermosol (HTS) was prepared essentially as previously described (Lakey, 2001), applying the values listed in Table 1.

Methylcellulose (MC) Fluka #64632 and polyvinylpyrrolidone (PVP) Fluka # 81385 were dissolved in PBS (phosphate buffered saline) at concentrations of 1% and 10% respectively, and then sterilised by autoclaving for 20 min at 121°C as described (Merten, 1995). The pH was controlled and adjusted prior to autoclaving.

The FILOCETH freezing medium developed in this work has the following composition:
89% chemically defined, serum-free medium (TurboDoma)
10% dimethyl sulphoxide
1% Pluronic F68™

FILOCETH with an improved composition, named FILOCETHplus is commercially available: http://www.procryotect.com.

2.2 Adaptation of cell lines to chemically defined medium

Cell lines were adapted to chemically defined medium by lowering the content of the original, serum-containing medium in a stepwise fashion, keeping cell density above 4x10E7 cells/ml for cells growing in suspension. Adherent cell lines were kept at a confluence of >50%. Detachment of the cells was achieved by incubating the medium-depleted and PBS-washed cell culture vessel with the non-enzymatic cell dissociation solution (SIGMA #C5914 prepared in PBS w/o calcium and magnesium).

Insulin was added in a final concentration of 10 mg/l to the media used for the cell lines Vero, COS-1, COS-7, BHK and HEK-293 (Insulin recombinantly expressed, Sigma I 9278).

A typical adaptation scheme is outlined in Table 2.

2.3 Determination of the doubling time

Adherent cells: The "Cell Titer 96 Aqueous One Solution Cell Proliferation Assay" (Promega Corporation, Madison, WI, USA) was performed following the manufacturer's instructions. In brief, the cell concentration was adjusted to a density of 2.5x10E4 cells/ml, and cells were

Table 1: Modified composition of the freezing medium "Hypothermosol" (Lakey, 2001)

Compound	Molecular weight	g/L	Osmolarity mOsm calculated
KH_2PO_4	136.1	1.361	30
D-Glucose	180	0.901	5
KCl	74.55	0.559	14
$MgCl_2$ x 2 H_2O	203.31	1.016	12
Sucrose	342.30	6.846	20
$CaCl_2$ x 2 H_2O	147.02	0.007	-
D-Mannitol	182.18	3.644	19
Dextran (40'000)	162	60.0	1.5
Lactobionic Acid	358.3	35.83	200
Glutathione red.	307.3	0.922	3
Adenosine	267.25	0.534	2
HEPES	238.3	5.957	37
$KHCO_3$	100.12	0.5	10
			353 (Measured osmolarity: 350 mOsm)

Table 2: A typical adaptation scheme.

Step	Original medium / New Medium (%)	Time in Culture
1	100/0	>10 days
2	80/20	>10 days
3	60/40	>10 days
4	40/60	>10 days
5	20/80	>10 days
6	10/90	>10 days
7	5/95	>14 days
8	2.5/97.5	>14 days
9	1.25/98.75	>14 days
10	0/100	>14 days

Stepwise adaptation from serum-containing medium to fully defined medium. Occasionally, an extra step had to be introduced to asymptotically achieve serum free conditions.

seeded on a 96-well plate (Techno Plastic Products, Trasadingen, Switzerland). At intervals of 24, 48, 54, 72, 77, 96 and 120 hours after seeding, MTS was added and incubated. The appearance of formazan, the coloured compound formed from MTS by living cells, was monitored by measuring the optical density at 490 nm in an ELISA plate reader (Spectra MAX 250, Molecular Devices, Sunnyvale, CA, USA) after 1 and 2 hours of MTS incubation.

Suspension cells: The cell concentration was adjusted to 5x10E4cells/ml and the cultures were started in 6 ml of pre-warmed fresh medium. At intervals of 24, 48, 54, 72, 77, 96 and 120 hours after inoculation, samples were counted as described below.

Doubling times were derived from exponential fitting of the measured OD values.

2.4 Cell counting

Cells were counted using the CASY 1® cell counting system (Schärfe System GmbH, Reutlingen, Germany), which classifies the cells as living or dead based on their size or membrane permeability.

2.5 Freezing procedure

Cells were kept in culture until they reached a confluence of between 80-90%. Cell suspensions with viabilities of ≥75% were aliquoted in portions of 2 x 10E6 living cells. Cell number was checked again and adjusted if needed. After centrifugation at 350xg the cell pellets were re-suspended in 1 ml of ice cold freezing media and transferred to Nunc cryo-vials (Fisher Scientific, Wohlen, Switzerland). Samples were kept on ice for 10 min. The actual freezing process from 0°C to -80°C was performed at controlled cooling rates of 0.5, 1, 1.5 or 5°C/min. The temperature control unit was made by the Institute of Electron Microscopy ETH Zürich, kindly provided by Martin Müller. After ≥3 h samples were transferred to the liquid nitrogen tank where they were kept for at least 24 h prior to thawing.

2.6 Thawing procedure

The cryo-vials were placed in a 37°C water bath for exactly 2 min (a little ice was still present in the tube). The cell suspension was diluted into 10 ml of fresh culture medium and centrifuged at 350xg for 5 min. The cell pellet was re-suspended in 6 ml pre-warmed culture medium and plated into a 6-well plate. 24 h after thawing, cell recovery was deter-

Table 3: Adapted cell lines with the corresponding original and adaptation medium.

Adapted cell lines		Original Medium	Adaptation Medium
1	VERO	DMEM 10% FBS	A
	Cos-1		
	Cos-7		
	Hek 293	EMEM 10% FBS	
2	BHK-21C13-P	GMEM 10% FBS	
	SP2/0	RPMI 10% FBS	B
	NS0		
	P3X63Ag8.653	IM10% FBS	

1: Adherent cell lines,
2: Suspension cell lines,
A: In Vitrus + Insulin (10µg/mL) or HEKTOR + Insulin (10µg/mL),
B: Turbodoma (HP-1),

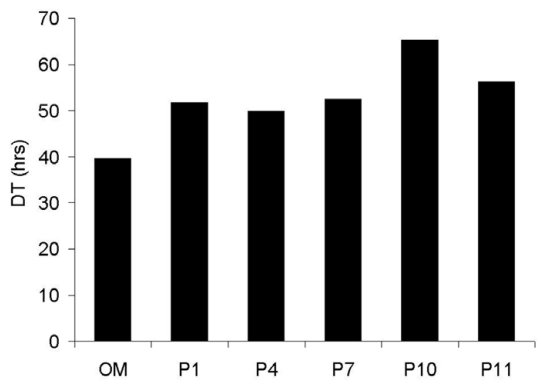

Fig. 1a: Adaptation of the fast responding cell line COS-7.
Doubling times (DT) of a fast adapting cell line COS-7 during adaptation to fully defined medium are plotted for selected passage times. The doubling time increased from 40 h in original medium (OM) to about 55 h after the first passage (P1). Subsequent passages had little effect on the DT.

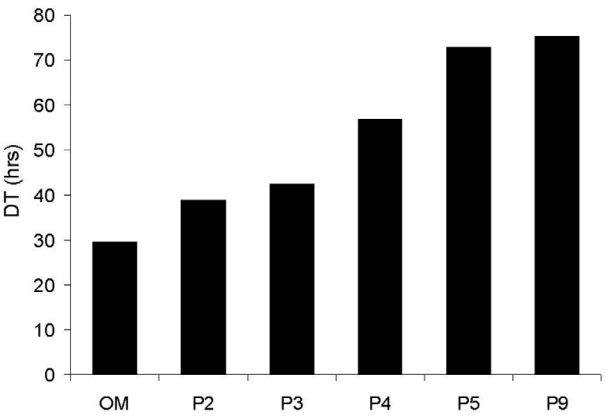

Fig.1b: Adaptation of the slow adapting VERO cell line.
Values of the doubling time (DT) for VERO cells reached a plateau after five passages (P5). The fully adapted cells exhibit a doubling time 2.5 times slower than those in original, serum-containing medium (OM).

mined by cell counting with the CASY 1® cell counting system. Doubling time was determined as described in section 2.3, starting at least 24 h after thawing.

3 Results

3.1 Adaptation of cell lines to fully defined media

As summarised in Table 3 the type of fully defined culture medium was chosen according to the origin of the cell line, i.e. TurboDoma for mouse cells (mouse BALB/c myeloma, NS0 mouse myeloma and mouse hybridoma), and InVitrus or Hektor for kidney cells of monkey, human or hamster origin. The media for Vero, COS-1, COS-7, BHK and HEK-293 cells were supplemented with insulin.

All cell lines examined could be adapted to fully defined medium, usually according to the protocol described in Material and methods, although sometimes extra adaptation steps, e.g. 0.5/99.5 (0.5% original medium + 99.5% new medium) had to be introduced to approach the serum free condition asymptotically.

All cell lines exhibited a slower proliferation rate after adaptation to serum-free media. In Figure 1a and 1b we show the doubling times of two typical cell lines adapted to defined media in relation to the number of cell culture passages in the defined medium.

3.2 Cooling rate

The survival rates with two different freezing media, "90/10" (90% FCS + 10% DMSO) and "45/45/10" (45% conditioned medium + 45% fresh medium + 10% DMSO) at different cooling rates are shown in Figure 2. A cooling rate of 5°C/min resulted in the lowest recovery of living cells 24 h after thawing, whereas 0.5, 1.0 and 1.5°C/min exhibited similar recovery rates. The pattern obtained with the serum-containing and the fully defined freezing media appeared similar. However, in the latter, fewer than half the cells survived as compared to the classic FBS/DMSO cryo-medium.

3.3 Comparison of different freezing media

The recovery rates of living cells (Mouse BALB/c myeloma cells P3X63Ag8.653) after freezing in differently composed freezing media are summarised in Figure 3. From top to bottom, the first six samples represent freezing media with simple composition, culture medium, or PBS plus DMSO at different concentrations as cryoprotectant. Fresh medium and PBS with 10% DMSO performed rather poorly, and only 20% of the initial cells could be recovered after thawing. Combination of fresh and conditioned medium with varying amounts of DMSO improved the recovery rate up to 50%. The same values could be found for the complex medium Hypothermosol with DMSO concentrations between 2.5 and 10%. Altering the viscosity by addition of either polyvinylpyrrolidone or methylcellulose within the recommended concentrations of 0.1 to 3% (Merten, 1995) had no effect on the survival rate. All these formulations did not match the performance of the "gold standard", FBS + 10% DMSO, which exhibited a recovery rate of 80%, as displayed at the bottom of the graph. FILOCETH however, with Pluronic F68 as active protection agent, performed significantly better, resulting in about 120% living cells.

Similar results were obtained with the Hybridoma cell line HEP-1.

3.4 Optimal Pluronic concentration

Figure 4 shows the effect of the Pluronic concentration on the survival rate of mouse BALB/c myeloma P3X63Ag8.653 cells after low temperature freezing. The tested concentration range was between 0.1 and 10% Pluronic added to fresh medium. The data describe a bell-shaped curve, with maximal efficiency occurring at concentrations between 1 and 5% Pluronic.

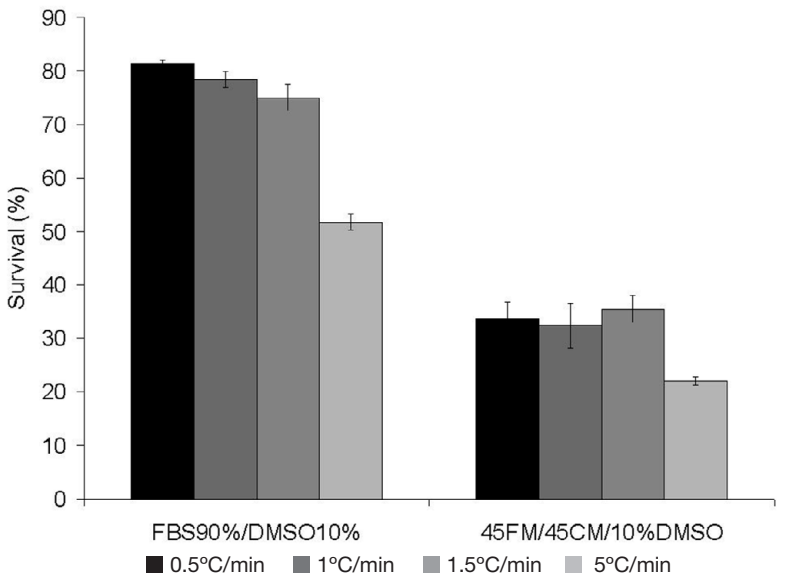

Fig. 2: Survial rates in relation to the cooling rate.
Survival rate of HEP-1 cells 24 h after thawing are shown as a function of different cooling rates. Two different combinations of freezing media were used: Foetal Bovine Serum (FBS) plus 10% DMSO and 45% fresh medium (FM) plus 45% conditioned medium (CM) plus 10% DMSO.

4 Discussion

The replacement of FBS in cell cultures is, for ethical and practical reasons, a longstanding goal that has not yet been achieved to the satisfaction of the cell culturist. Many formulations of serum free culture media are commercially available. Currently, we distinguish three main types of culture media: serum-containing medium, serum-free medium and serum- and protein-free medium (often fulfilling the requirements to be called: "free of animal derived components"). The terms "chemically fully defined" or

simply "fully defined" clearly exclude the existence of any unidentified component in the culture medium. In the present project we show that many cell lines can be successfully adapted to fully defined medium. However, the adaptation may cause changes in the phenotype of the cell, e.g. cell lines originally growing adherently may lose this ability after adaptation and begin to grow in suspension. All cell lines described in this report exhibited a significant increase in their doubling time during growth in fully defined media. A stable proliferation rate was therefore one criterion for successful completion of the adaptation process. The stabilisation of the doubling time required several passages in fully defined medium after weaning the cells off serum.

The genotype of the commonly used cell lines was compared with its pre-adapted state by the ECACC prior to deposition in the cell bank for further distribution among scientific institutions. A functional test was introduced for hybridoma cells to examine their antibody production.

The weaning process *per se* was cell line specific. Certain cell lines, mostly hybridoma, often could be adapted in a harsh way, i.e. by resuspending cell pellets obtained from cultures in original serum-containing medium directly in the defined medium. However, the majority of the treated cell lines required a very slow adaptation phase to approach the serum-free state.

The availability of serum-independent cell lines used for production of recombinant proteins opens new horizons for biotechnology. Transfection of serum-independent cell lines will allow scientists to circumvent a tedious weaning process with uncertain outcome in order to register the cell product with the authorities.

The low temperature conservation of serum independent cells was achieved with the newly developed fully defined freezing media FILOCETH (patent pending). The active ingredients are dimethyl sulphoxide (DMSO) and Pluronic F68™. DMSO and other cryo-

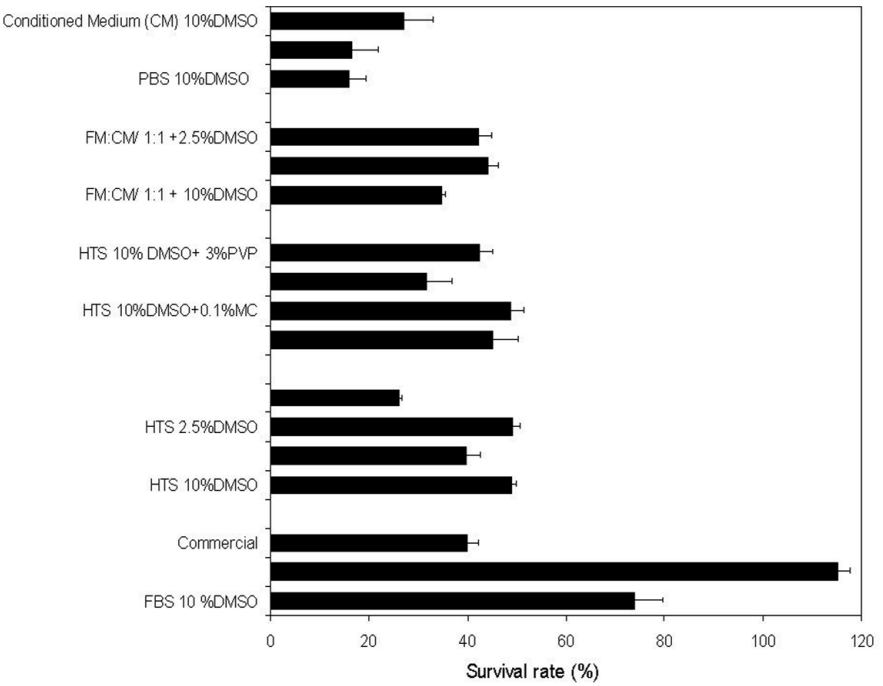

Figure 3. Survival rates of different serum-free freezing media compared to FBS/DMSO.

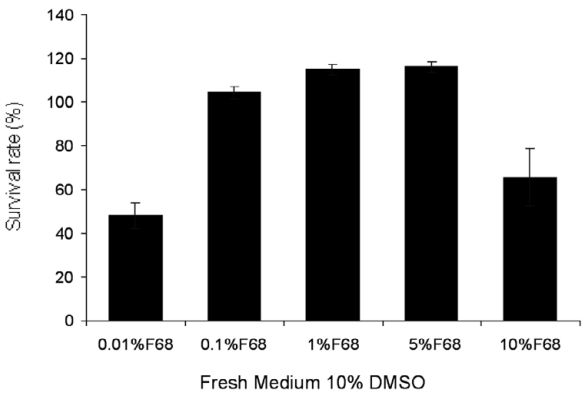

Fig. 4: Effect of Pluronic F68 concentration on cell recovery after freezing.
The survival of mouse BALB/c myeloma P3X63Ag8.653 cells 24 h after thawing, as a function of different concentrations of Pluronic F68.
All the samples contained fresh medium plus 10% DMSO.

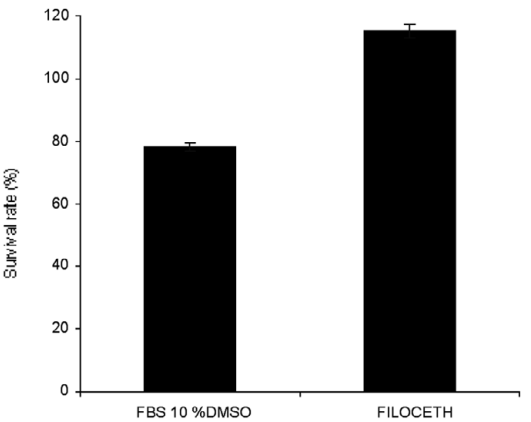

Fig. 5: A comparison of the "gold standard", FBS+DMSO, and the new FILOCETH.
Survival of mouse BALB/c myeloma P3X63Ag8.653 cells 24 h after thawing. The "gold standard", FBS + 10% DMSO, is compared directly the new fully defined freezing medium FILOCETH.

protectants, e.g. glycerol, play an important role in cryo-conservation. They prevent the disruption of cell membranes by intra- and extracellular ice crystal formation. DMSO was effective at concentrations between 5 and 10%. However, DMSO is cytotoxic at room temperature and higher. Prior to freezing, the cells should be incubated with this reagent for about 10 min at 4°C in order to allow the DMSO to penetrate the cell membrane and become effective intracellularly. After thawing it is important to quickly remove the DMSO to minimise its cytotoxic effects at elevated temperatures.

The cooling rate, thought to be a crucial step in cryopreservation, did not prove to be particularly sensitive: cell survival was relatively insensitive to cooling rates between 0.5°C/min and 1.5°C/min. This finding permits the researcher to use a simple and cheap freezing device, which consists mainly of a polystyrene block with holes for the cryo-vials. The cooling rate of samples in a polystyrene block placed in a -80°C freezer was found to be between 1.4°C/min and 1.6°C/min, except during the transition phase at which the latent heat of fusion is released (between -6°C to -10°C) where it drops to 0.3°C/min.

The addition of Pluronic F68™ to a simple freezing medium caused a dramatic increase in surviving cells after thawing as compared to any other known cryo-medium. Immediately after thawing, cells are in a shrunken state and need time to re-hydrate and to regain a normal morphology prior to determination of cell number and viability. In this work, the recovery time was standardised to 24 h. With the classic freezing media, no cell proliferation was observed during the first 24 h. With FILOCETH, however, cell survival rates >100% indicate significant cell proliferation during the first 24 h after thawing.

Pluronic F68™, a non-ionic polyoxyethylene (POE)-polyoxypropylene (POP) surfactant with the general formula $(POE)_{76}$-$(POP)_{30}$-$(POE)_{76}$ is a well known block copolymer used in biotechnology for many years. Its primary application is in plant- and animal cell culture systems, to protect cells against fluid-mechanical damage in agitated cultures (Handa-Corrigan, 1989). It has been proposed that Pluronic F68™ protects the cell by adsorbing to the cell membrane (Wu, 1997). The interaction of surface active polymers may alter the hydrophobicity of the cell surface and thus affect its ability to adhere to other cells and/or air bubbles. Seeman (1966) demonstrated an increased resistance of erythrocytes to osmotic lysis after adsorption of surfactants to the cell membrane. These mechanisms may explain the positive effect of Pluronic F68™ on the survival rate of eukaryotic cells after low temperature preservation. In Fig. 5, the direct comparison of the classic FBS/DMSO freezing medium with the fully defined FILOCETH clearly illustrates the dramatic improvement we achieved in cryopreservation.

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Abbreviations

ECACC: European Collection of Cell Cultures, Health Protection Agency, Porter Down, Wiltshire, SP4 0JG, UK
MTS: (Owen's reagent) (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)--2-(4-sulfophenyl)-2H-tetrazolium, inner salt.
DMSO: Dimethyl sulphoxide

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Correspondence to

René W. Fischer
Laboratory of Organic Chemistry
Swiss Federal Institute of Technology
CH-8093 Zurich
Switzerland
e-mail: rene.fischer@org.chem.ethz.ch