



THERMOGENESIS CORP.
3146 Gold Camp Drive
Rancho Cordova, CA 95670-6022
(916) 858-5100 Tel
(916) 858-5199 Fax
(800) 337-9431 Sales
(800) 783-8357 Customer Service
www.thermogenesis.com

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January 20, 2000

Via UPS Next Day

Dockets Management Branch (HFA-305)
Food and Drug Administration
12420 Parklawn Drive, Room 1-23
Rockville, MD 20857

Re: Docket Number 97N-0068

THERMOGENESIS CORP. hereby submits the following comments and data reflecting our concern that cryopreserved hematopoietic stem and progenitor cells sourced from placental/cord blood (PCB) may be damaged as a result of certain thermal events that may occur during routine processing, storage and shipping of PCB grafts. One particular thermal event has been shown to result in a substantial loss (> 50%) of CFCs within the PCB unit.

We have named these thermal events "transient warming events (TWE)" because they occur during transfers of frozen samples from the controlled rate freezer to the quarantine dewar, the storage dewar, the cryo shipper and finally into the storage dewar at the transplant center as shown in Figure 1. The rising temperature of the cells that results from exposing the cryogenically frozen sample to the warm ambient air is followed by a sudden lowering of temperature when the sample is returned to the permanent storage temperature (-196°C in liquid nitrogen or other validated environment).

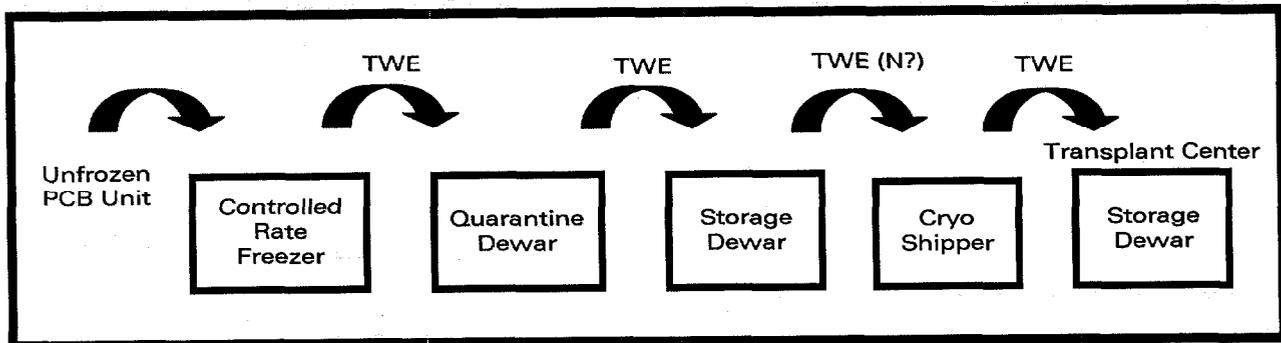


Figure 1

The damage to the cells that we have observed likely results from the sudden drop in temperature that occurs when frozen PCB units warmed through exposure to the ambient air are transferred into environments maintained at -196°C and thereby undergo an uncontrolled rate drop to the -196°C storage temperature. Cryobiologists are in agreement that injuries to cells occur during freezing as the result of either osmotic cell dehydration or of intracellular ice formation - in both cases resulting in damage to the

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cell membranes¹. Cryoprotectants such as DMSO are effective in significantly diminishing cell damage by reducing the freezable water converting to ice thus limiting cell dehydration. Nevertheless reduced cell viabilities have been observed as a result of rapid freezing² at certain temperatures. The temperature range which is of the greatest concern during freezing is:

- 0°C to -30°C (the eutectic point of water to the lowest eutectic point of the blood proteins in solution)

Although much less examined, another temperature range in which the cells may come under duress due to a change of state in water from crystalline to glass is:

- -115°C to -120°C (glass transition temperature range of water-based cell solutions)

TWEs are especially rapid when the frozen PCB specimens are of a small mass which occurs when units of placental blood are volume-reduced to 25 ml as is now becoming the international standard³. Rapid warming of these cryogenically frozen PCB units occurs not only because of their absorption of heat from the warmer ambient air by radiation and convection, but also because water vapor within the air condenses and freezes on the cold surfaces of the PCB containers. This frost is proof that a thermodynamic event in which the latent heat of vaporization (580 cal/gm) and latent heat of fusion (80 cal/gm) of water moisture in the air is transferred to the frozen specimen. This heat invasion is exacerbated in laboratories where nitrogen dewars are stored because higher than normal air-exchanges within the room are required to evacuate the evaporating nitrogen from the dewars. This rapid air exchange brings more warm, moist air in contact with the frozen PCB units.

TWEs are visited upon multiple units at one time when a rack of PCB units is removed from the storage dewar in order to retrieve a single unit. This heat exposure may occur many times over the years in which the PCB units are archived. Further, the duration of these TWEs is often longer than it may seem to the laboratory technician, who is concentrating on the mechanics of the transfer and thus can easily lose track of the elapsed time. (In addition, since the PCB unit remains frozen during the transit, it is easy to assume the unit remains closer to -196°C than the actual fact.)

¹ Meryman HT. *Principles of Cryopreservation and the Current Role of Frozen Red Blood Cells in Blood Banking and Clinical Medicine*. Cryopreservation of Tissue and Solid Organs for Transplantation; 1-12 (1983)

² Douay L et al. *A Technical Bias: Differences in Cooling Rates Prevent Ampoules from Being a Reliable Index of Stem Cell Cryopreservation in Large Volumes*. *Cryobiology*; 23; 296-301 (1986)

³ Rubinstein et al. *Processing and Cryopreservation of Placental/Umbilical Cord Blood for Unrelated Bone Marrow Reconstitution*. *PNAS*; 10119-22 (1995).

Whether a PCB unit incurs a single TWE or many, this thermal history for each exposed specimen during processing and prolonged storage, is not typically documented nor are the possible consequences to cell viability well understood.

In order to investigate whether these TWEs could result in a loss of recovery of viable CFCs, we proceeded to:

- Rely upon the protocol for controlled rate freezing PCB (Appendix A) and the protocol for thawing PCB (Appendix B) developed by the New York Blood Center to establish the baseline for CFC recoveries obtainable from 25 ml PCB units.
- Measure the elapsed time required to warm PCB units from -196°C to 0°C when exposed to the ambient air at temperatures between 20°C and 30°C and humidities between 30% and 40% utilizing the following equipment:

Recording Instrument: Keithley Metrabyte Das-TC Data Logger
16 Channel, 16 Bit, A/D Converter

Sensor Type: Type K Thermocouple, .010 Diameter, Rapid Response

Set Up Data: Dastc Log File Date 01-05-2000
Number Type – Integer
NMR Frequency – 60 Hz
Logging Interval – 6 seconds

- Freeze the 25 ml PCB units at controlled-rates faithful to the New York Blood Center protocol down to specific target temperatures followed by transfers to -196°C to determine if the recovery of viable CFCs are reduced over those reported in the New York Blood Center protocols. The target temperatures were -30°C , -35°C , -40°C and -45°C .

Hypothesis: If the recovery of viable CFCs is reduced when a transferred PCB unit attains a certain temperature and is then transferred to the -196°C liquid nitrogen environment, then the critical question becomes how long does it take for the warming PCB unit to rise to that critical temperature.

Rate of Warming

The two most commonly used freezing bags for volume-reduced PCB (25 ml) are the Baxter 4R9953 Cryocyte and the Medsep 791-05 as shown in Figure 2 in both frontal and cross section views.

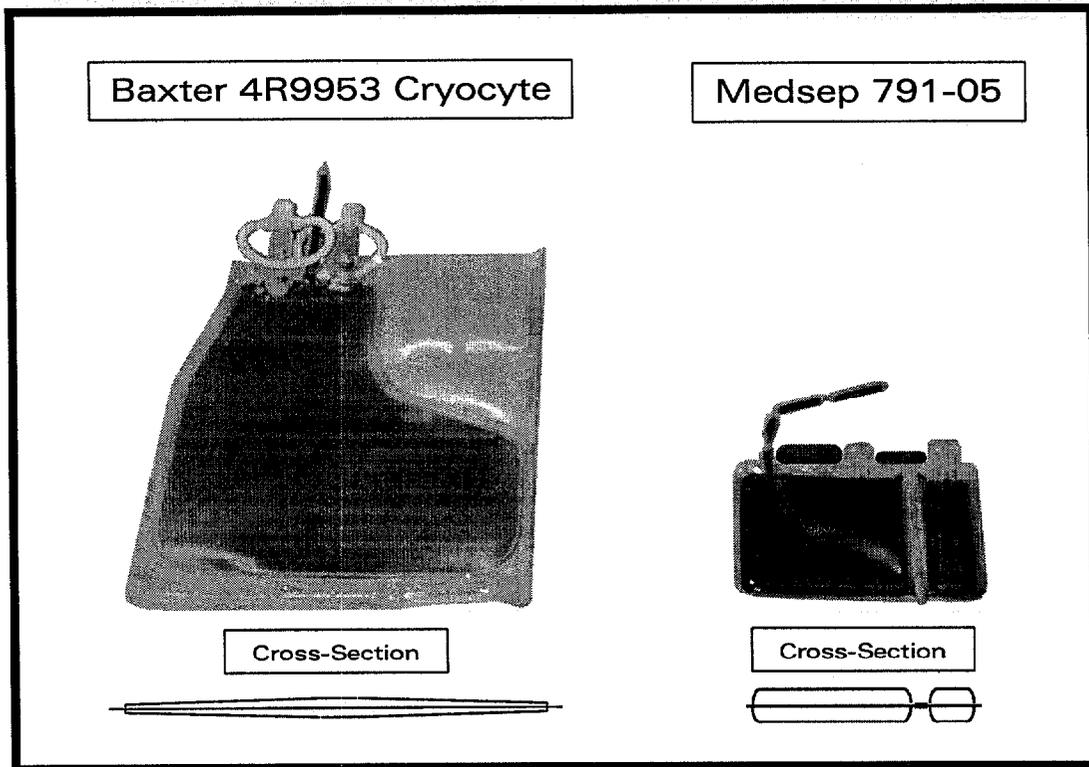
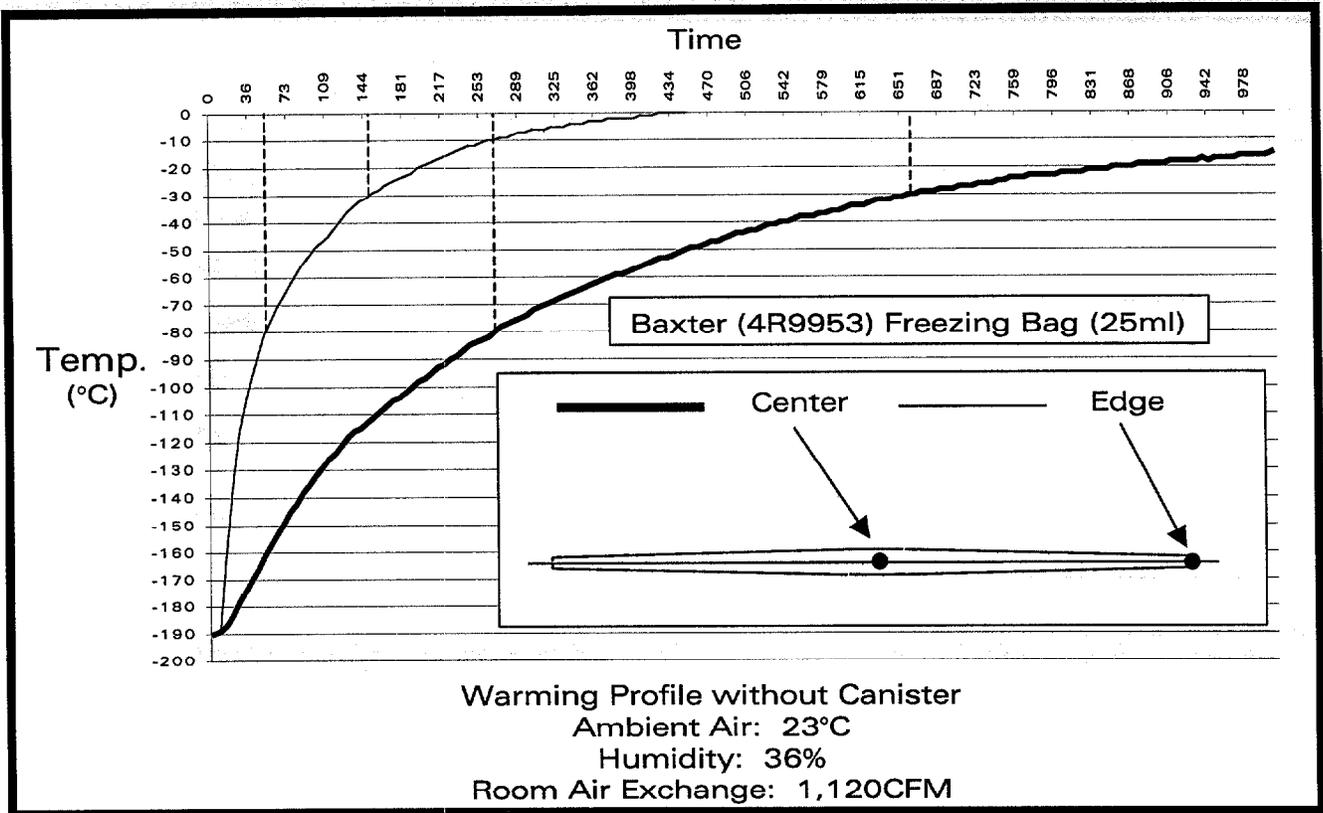
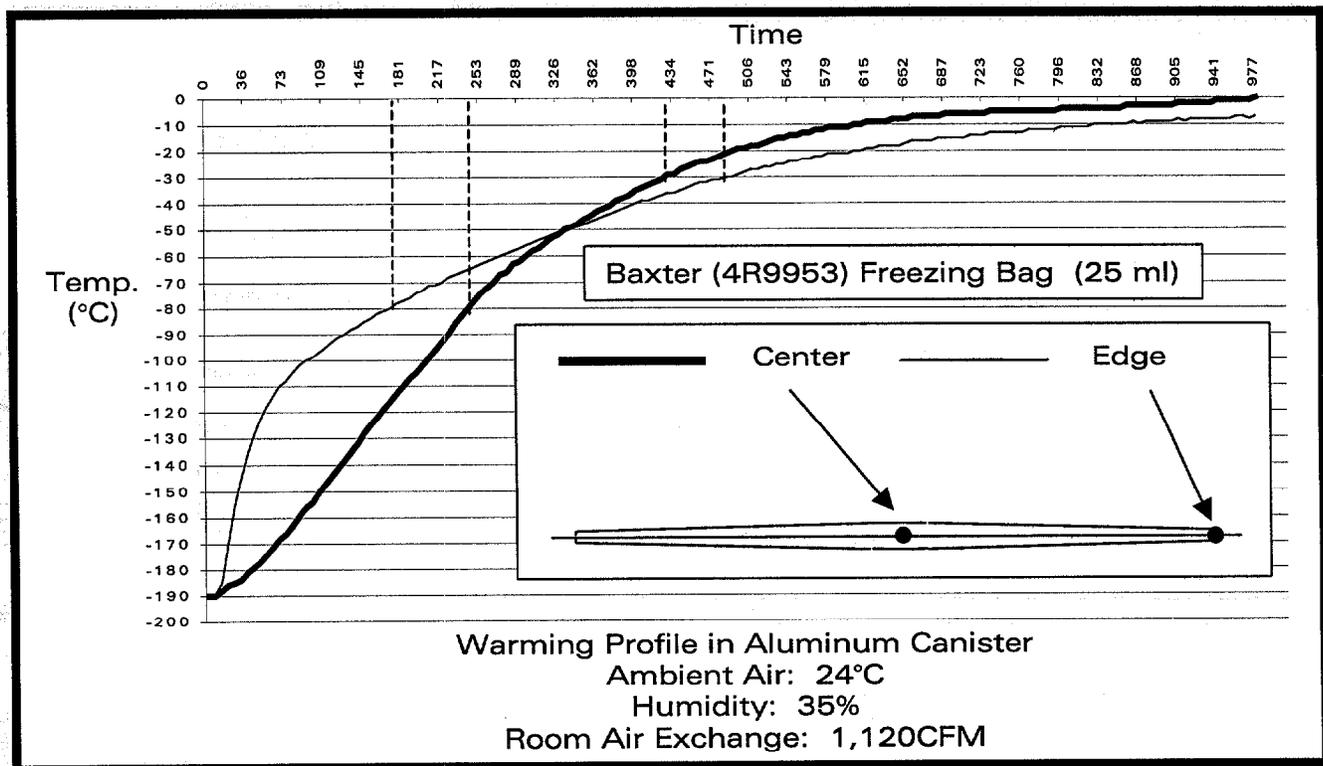


Figure 2

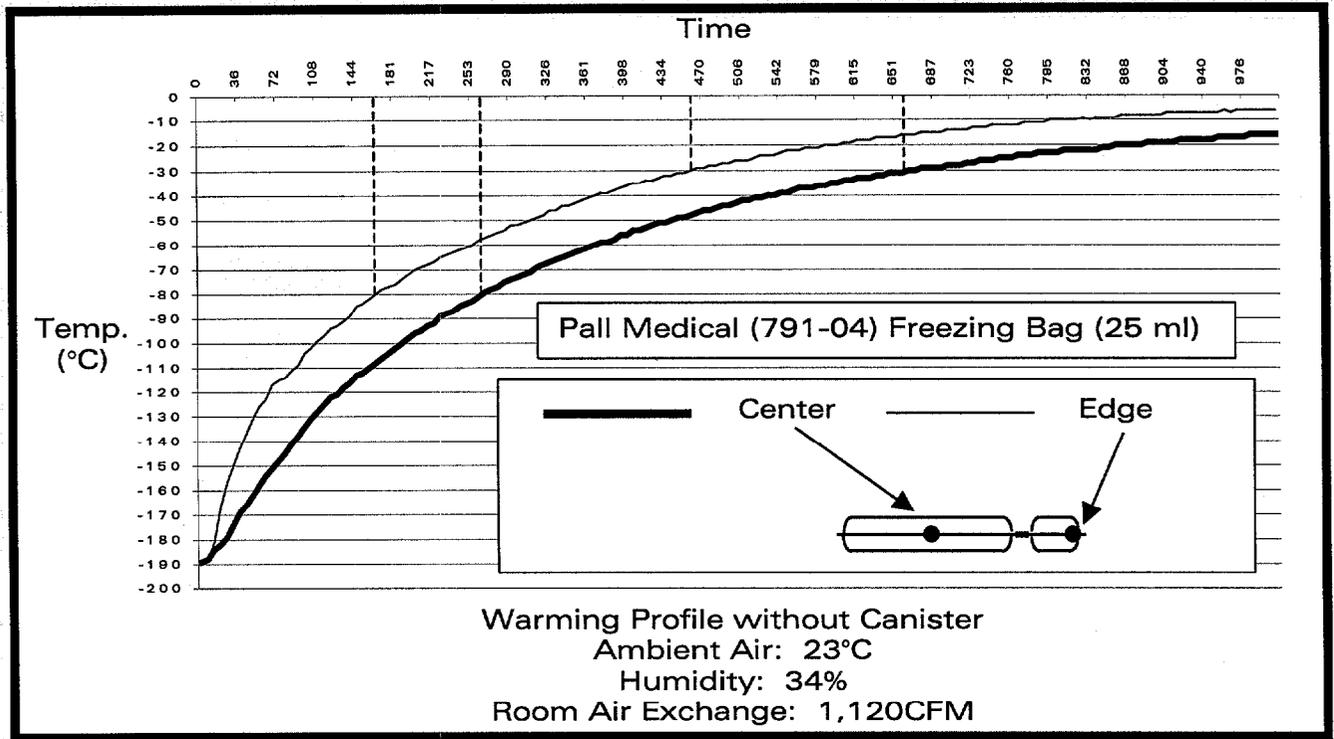
The rate of warming of cryopreserved PCB in these two bags from -196°C to 0°C under specified ambient conditions is illustrated in Graphs 1 – 5 as shown below. Thermocouples were placed within the bags in order to detect the earliest moment at which any portion of the PCB unit rises to the measured temperature T (edge) and the moment when 100% of the PCB unit is at the measured temperature or higher T (center).



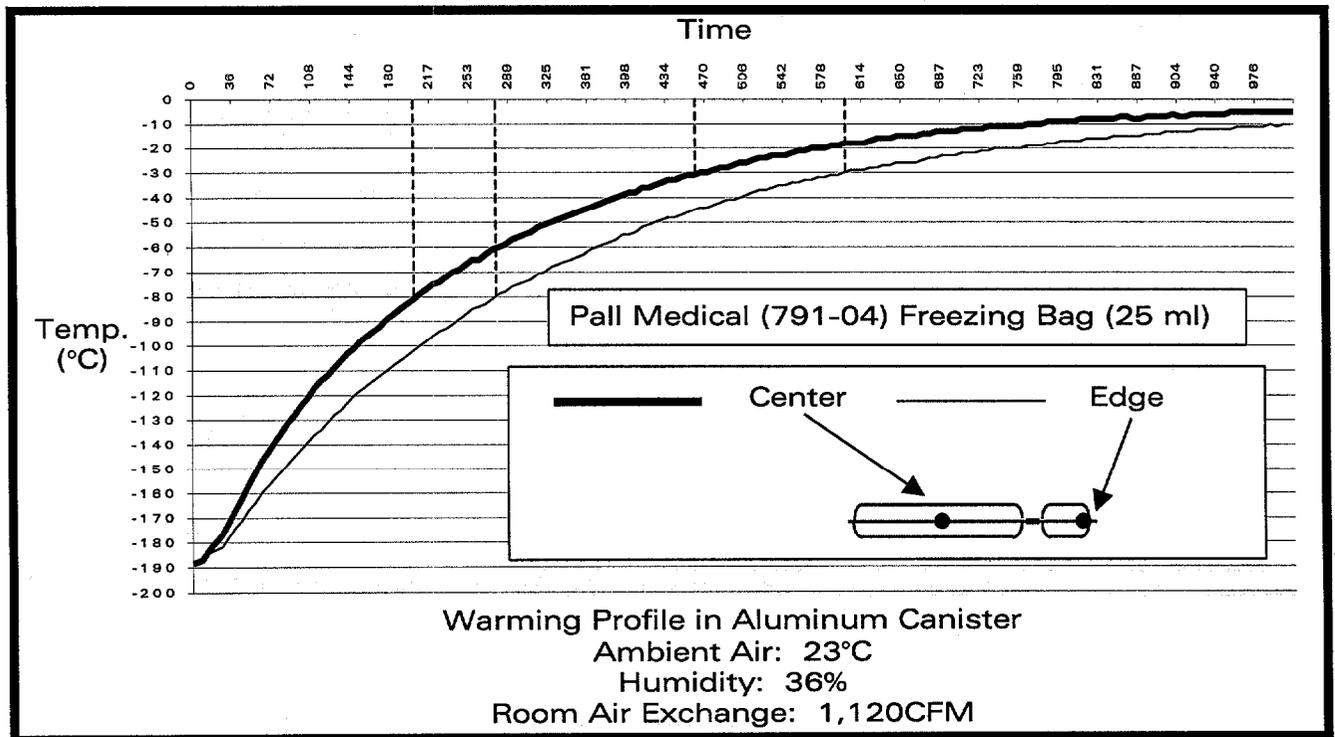
Graph 1



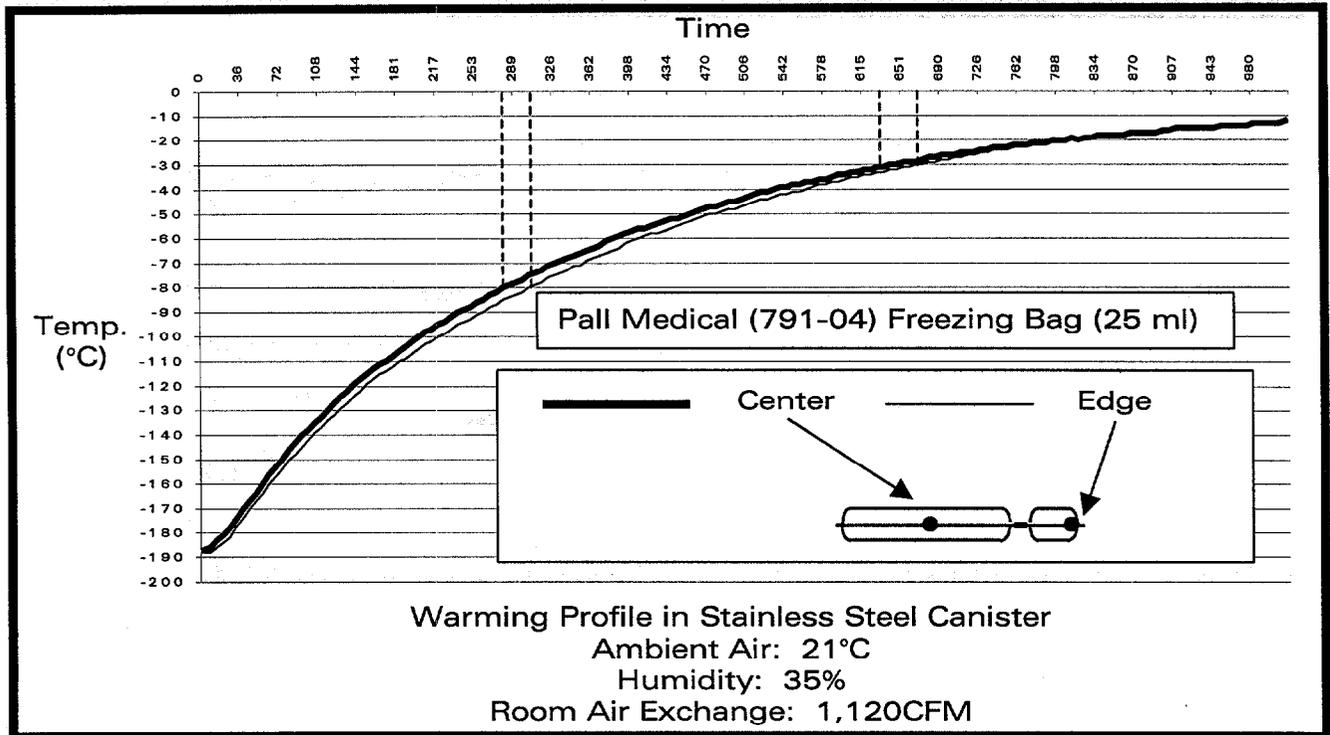
Graph 2



Graph 3



Graph 4



Graph 5

Table 1 below summarizes the elapsed times of the warming of the frozen PCB units. Column 1 details the seconds required for the freezing bag to rise to the glass transition temperature (-120°C). Column 2 details the seconds required to rise to the lowest eutectic temperature of a blood protein (-30°C) and Column 3 details the seconds required to rise from a typical CRF final temperature (-80°C) to the lowest blood protein eutectic temperature (-30°C).

Freezing Bag	Temp. Probe	Elapsed Time (sec) from:		
		-196°C to -120°C	-196°C to -30°C	-80°C to -30°C
Baxter Cryocyte	T Edge	26	138	95
	T Center	108	644	391
Baxter Cryocyte in aluminum canister	T Edge	36	469	313
	T Center	144	409	181
Pall Medical (791-041)	T Edge	61	453	296
	T Center	121	598	284
Pall Medical (791-041) in aluminum canister	T Edge	138	580	325
	T Center	96	458	265
Pall Medical (791-041) in stainless steel canister	T Edge	145	658	362
	T Center	133	633	362

Table 1

CFC Recoveries

To test the hypothesis that allowing a frozen unit (at -196°C or -80°C) to reach the “critical” temperature, we first determined the CFC recovery after; (a) allowing the unit to chill down to a target, “end” temperature, and (b) then plunging the unit directly into liquid nitrogen.

After removing aliquots for establishing the initial CFC counts, three PCB units were tested at each of the four target temperatures (-50°C, -40°C, -35°C and -30°C). The freezing rate of each unit followed the New York Blood Center protocol until the target temperature was achieved, then the unit was directly lowered into the -196°C environment.

The following day, the PCB units were retrieved from the nitrogen, thawed, and prepared for colony forming according to New York Blood Center protocols.

The CFC recoveries for each of the 12 units are detailed in Figure 3.

RESULTS			
End Temp.	# Colonies (initial total)	# Colonies (thawed total)	% Recovery from initial
-50	420000	512500	122
-40	516666	568750	110
-35	530000	656250	123
-30	466666	125000	27
-50	355000	354062	100
-40	365000	390625	107
-35	363333	354062	97
-30	490000	187500	38
-50	510000	631125	123
-40	495000	525000	106
-35	370000	459375	124
-30	423333	124875	29

Figure 3

The reciprocal experiments, in which the units are; (a) frozen down to -196°C or -80°C under the optimal protocol, (b) then allowed to warm up to these "critical" temperatures and re-cooled down to -196°C a specified number of times, and (c) finally, thawing the unit in the regular way, are now in progress.

Discussion

This brief examination of the effect of TWEs upon CFC recoveries in cryopreserved PCB units indicates that substantial losses ($> 60\%$) may occur if a PCB unit is warmed on only one occasion to -30°C and is then returned to -196°C environment. We are now investigating whether warming a previously frozen (-196°C) unit to -30°C or even lower critical temperatures has a similar, larger or smaller deleterious effect. This temperature (-30°C) was reached in the center core of the PCB unit in all our tests in less than 11 minutes (660 seconds) from -196°C and, in one case, in less than 7 minutes (420 seconds). The elapsed time to reach -30°C from -80°C , a typical final CRF temperature, ranged from about 3 minutes (180 seconds) to six minutes (360 seconds). The rate of warming will be accelerated for frozen PCB units if the ambient temperatures, humidities and air exchanges at which they tested are elevated above those tested.

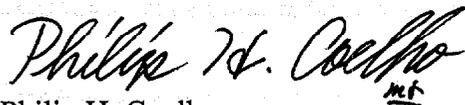
It is also interesting to note that, unlike the stainless steel canisters, the aluminum canisters did not inhibit rapid warming of the frozen PCB units.

A further question, unanswered by this brief exploration of TWEs, is what effect on CFC recovery would occur as a result of multiple TWEs in which the PCB unit rose to a less elevated temperature, i.e. -50°C , -80°C or -120°C ?

Clearly, more exploration of this phenomenon is required before definitive directions to licensed PCB bankers can be made. However, it may be prudent to require PCB banks to document the thermal histories, including TWEs, in order to provide the transplant physicians with this information to better inform their choice between two or more PCB units with similar HLA-typing, and pre-freezing cell counts.

Sincerely,

THERMOGENESIS CORP.



Philip H. Coelho
Chairman/CEO

PHC/mr
Attachment

Appendix A

Validation Report

Viability of Placental/Cord Blood (PCB) Stem Cells Controlled-Rate Frozen and Stored in the BioArchive™ System

Introduction

Freezing in liquid nitrogen has been used for cryopreservation of cell suspensions from blood and other biological tissues for several decades. Storage of cells at cryogenic temperatures stops or significantly reduces their metabolism and thus preserves them for prolonged periods of time. During freezing, however, formation of large ice crystals tends to occur inside the cells, which would irreversibly damage them and must be avoided. Use of cryoprotective agents such as glycerol or dimethyl sulfoxide (DMSO), along with careful control of the rate of freezing, helps retard or prevent the formation of ice crystals and thus protects the cells from injury during freezing.

In the BioArchive, the computerized user interface allows definition and selection of an appropriate freezing profile by entering the freezing and ending temperatures of the freeze process and entering the cooling rates that are to be maintained during each of the three freezing phases e.g. pre-freeze, freezing, post-freeze.¹

Purpose

The aim of this study was to validate the method for controlled rate freezing using the BioArchive System. The experiments were performed at the New York Blood Center's Placental Blood program under the direction of Ludy Dobrila, Ph.D., Associate Director, Placental Blood Program.

Materials and Methods

Processing of Placental/Cord Blood

Placental blood was collected in disposable collection bags containing anticoagulant (25.0 ml CPD). Multiple units were matched for ABO and pooled to total in excess of 10^9 total nucleated cells and then aliquoted into five samples. The red blood cells were sedimented using hydroxyethyl starch (final concentration: 1% w/v) followed by gentle centrifugation (50 g at the expected red blood cell-plasma interface for 6 min). The leukocyte-rich plasma was then transferred to a processing bag set. The cells were concentrated to a volume of 20 ml by a second centrifugation step (400G 13

¹ See BioArchive Operator's manual for further information.

minutes) and removal of the excess supernatant plasma. Five ml of the cryoprotective solution (50% w/v DMSO / 5% w/v Dextran40), was then slowly added to the concentrated cells, and the mixture transferred to a THERMOGENESIS CORP.-approved two-compartment freezing bag for programmed freezing and storage in the BioArchive System.^{2, 3}

The 25 ml units were kept frozen for at least 48 h. After storage, the units were retrieved from the BioArchive, kept in the vapor phase of liquid nitrogen for 5 min before being immersed in a 37°C waterbath for thawing. The units were subsequently diluted with 25 ml of a 2.5% albumin and 5% Dextran40 solution and mixed gently for a few minutes to allow complete equilibration and then further diluted with 50 ml of the same 2.5% albumin and 5% Dextran40 and centrifuged at 400 g for 20 min. The excess supernatant was carefully removed and the sedimented cells were suspended in albumin/Dextran40 solution.

Samples for viability testing were taken before freezing and after thawing of the unit.

Assessment of viability by hematopoietic colony formation

Cord blood was diluted 1:500 in culture tubes containing Iscove's modified Dulbecco's culture medium. The following human recombinant growth factors were added: 50 ng erythropoietin, 25 ng granulocyte/macrophage colony-stimulating factor, 250 ng stem cell factor, 250 ng granulocyte colony-stimulating factor and 25 units of IL3. Triplicate samples were cultured in Petri dishes for 2 weeks at 37°C in humidified controlled atmosphere (5% O₂/ 5% CO₂ / 90 % air). Colonies were identified and enumerated with a phase contrast microscope³.

Freezing profiles

Six parameters which define the freezing curve can be varied by the operator: Pre-freeze rate (1°C/min to 10°C/min), start freeze temperature (0°C to -15°C), fan power (10 % to 100%), end freeze temperature (-3°C to -30°C), post freeze rate (1°C/min to 10°C/min), and end temperature (-20°C to -55°C). Numerous combinations of the parameters were tried and the best recoveries were obtained when the pre-freeze rate was set at 10°C/min, the start freeze at -3°C, fan speed during fusion at 100% and the post freeze rate at 2°C/min.

² See Inservice guide-lines 'New York Blood Center Protocol for Reduction of Excess Red Cells and Plasma from Placental Blood Followed by Cryoprotection using the Medsep Processing bag Sets and the THERMOGENESIS CORP. Auto-volume Expressor', THERMOGENESIS CORP.

³ Rubinstein et al. *Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution*. PNAS 1995;10119-22

In order to define the zone in which optimum recoveries will occur, the following freeze profiles were tested:

Pre-freeze rate °C/min	Start freeze temp. °C	Fan power %	End freeze temp. °C	Post freeze rate °C/min	End temp. °C
10	-3	100	-10	2	-50
10	-3	100	-12	2	-50
10	-3	100	-15	2	-50

Five samples were evaluated for each profile.

Result

Each table shows the freezing time and the number of colony forming units (CFU) in the units before freezing and after thawing. The recovery was calculated as the percentage of the number of CFU after thawing compared to the number of CFU before freezing.

End freeze temperature -10°C:

Id no	Time min	CFU, initial number	CFU, thawed	Recovery from initial number %	Recovery from mean %
100129P	19	430000	515625	120	110
100130P	24	450000	628125	139	134
100131P	19	426666	562500	131	120
100132P	24	473333	512499	108	109
100133P	25	563333	562500	100	120
Mean	22 min			119 %	119
± sd	3			16	10

Freezing profiles are shown in graphs 1 – 5.

End freeze temperature -12°C:

Id no	Time min	CFU, initial number	CFU, thawed	Recovery from initial number %	Recovery from mean %
100105P	23	720000	712500	99	108
100106P	24	643333	693750	107	105
100107P	17	655000	787500	120	120
100108P	22	645000	656250	101	100
100109P	21	610000	662500	108	101
Mean	21 min			107 %	107
± sd	3			8	8

Freezing profiles are shown in graphs 6 – 10.

End freeze temperature -15°C:

Id no	Time min	CFU, initial number	CFU, thawed	Recovery from initial number %	Recovery from mean %
100110P	22	716666	743750	103	103
100111P	15	690000	806250	116	111
100112P	17	725000	740625	102	102
100113P	15	753333	759375	100	105
100114P	22	721500	705000	98	98
Mean	18 min			104 %	104
± sd	4			7	5

Freezing profiles are shown in graphs 11 – 15.

Conclusion

No difference in the number of CFUs was found when comparing samples taken pre-freezing with samples taken post-thawing.

The results show that a freezing profile with a pre-freeze rate of 10°C/min, fan power during fusion of 100%, post-freeze rate of 2°C/min and an end freeze temperature set between -10 and -15 °C, will consistently give post-thaw cell viability comparable to pre-freezing values.

Appendices

Raw data, Table 1.

Freezing profiles for each unit, graphs 1 - 15

Table 1- Cont.

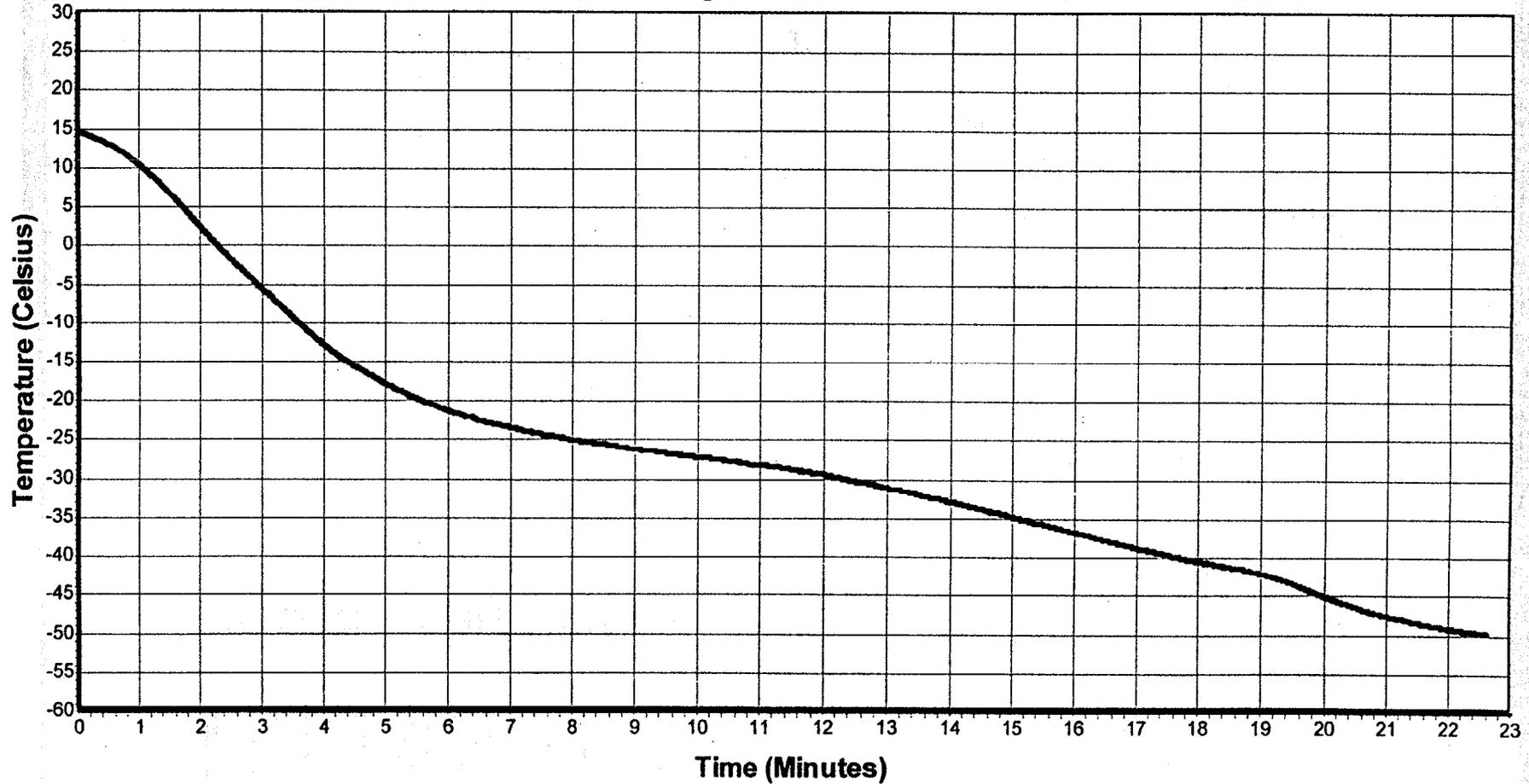
PROFILE													
Exp. #	Unit ID#	Date	Pre freeze oC/min	Start freeze temp.	Fan power %	End freeze temp.	Post freeze oC/min	End temp.	Time min.	# Colonies initial total	# Colonies thawed total	%Recov. from initial	%Recov. from mean
VII	100105P	11/9/98	10	-3	100	-12	2	-50	23	720000	712500	99	108
	100106P	11/9/98	10	-3	100	-12	2	-50	24	643333	693750	107	105
	100107P	11/9/98	10	-3	100	-12	2	-50	17	655000	787500	120	120
	100108P	11/9/98	10	-3	100	-12	2	-50	22	645000	656250	101	100
	100109P	11/9/98	10	-3	100	-12	2	-50	21	610000	662500	108	101
VIII	100110P	11/9/98	10	-3	100	-15	2	-50	22	716666	743750	103	103
	100111P	11/9/98	10	-3	100	-15	2	-50	15	690000	806250	116	111
	100112P	11/9/98	10	-3	100	-15	2	-50	17	725000	740625	102	102
	100113P	11/9/98	10	-3	100	-15	2	-50	15	753333	759375	100	105
	100114P	11/9/98	10	-3	100	-15	2	-50	22	721500	705000	98	98
XIII	100129P	11/20/98	10	-3	100	-10	2	-50	19	430000	515625	120	110
	100130P	11/20/98	10	-3	100	-10	2	-50	24	450000	628125	139	134
	100131P	11/20/98	10	-3	100	-10	2	-50	19	426666	562500	131	120
	100132P	11/20/98	10	-3	100	-10	2	-50	24	473333	512499	108	109
	100133P	11/20/98	10	-3	100	-10	2	-50	25	563333	562500	100	120

ThermoGenesis Corp.

3146 Gold Camp Drive
Rancho Cordova, CA 95670
(916) 858-5100

Sample: 100105P

Storage Date: 11/9/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 5

Profile Used: Endfr12

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -12C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1008

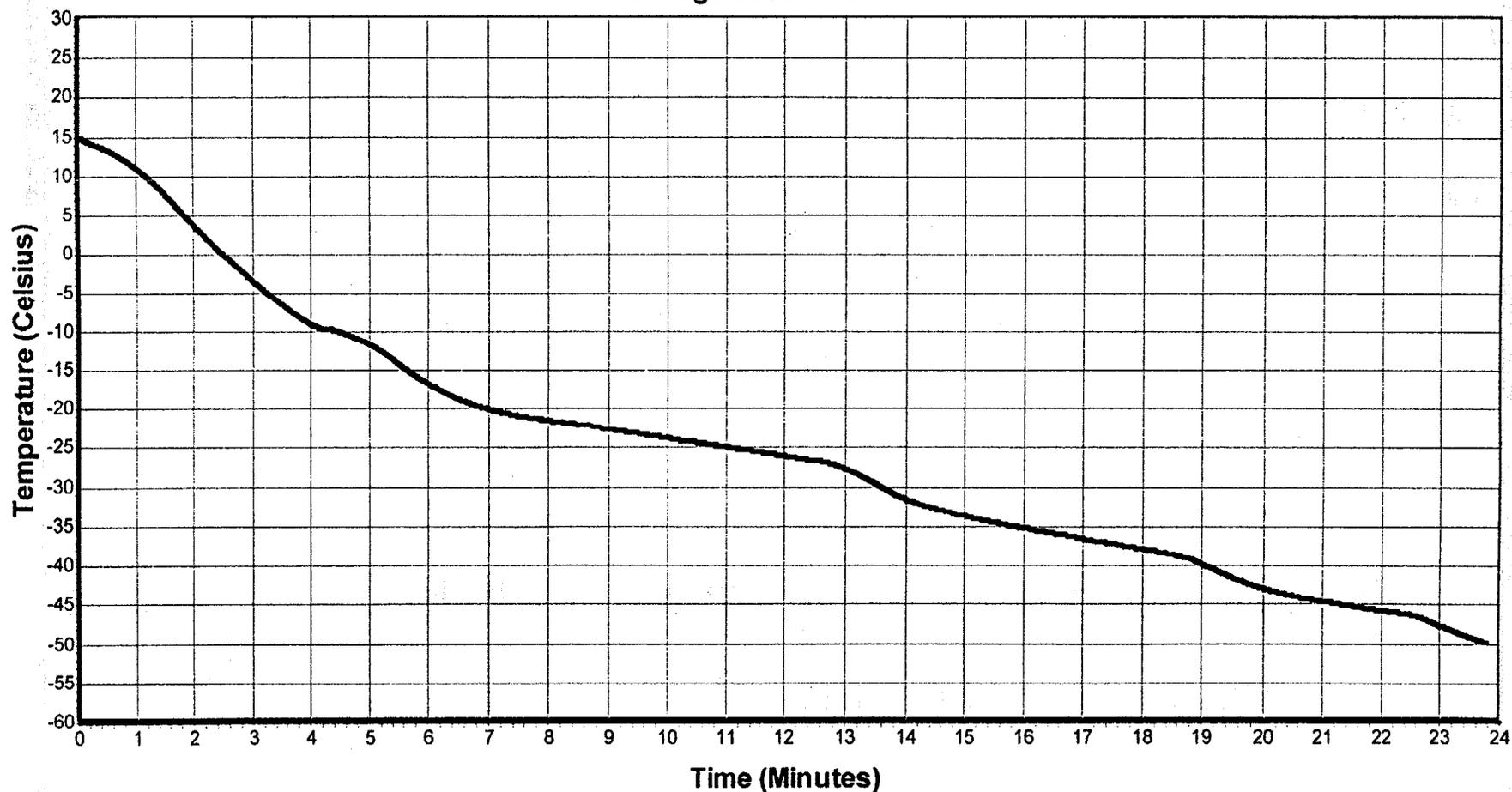
CRF Version: 1.

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(916) 858-5100

Sample: 100106P

Storage Date: 11/9/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 6

Profile Used: Endfr12

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -12C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1034

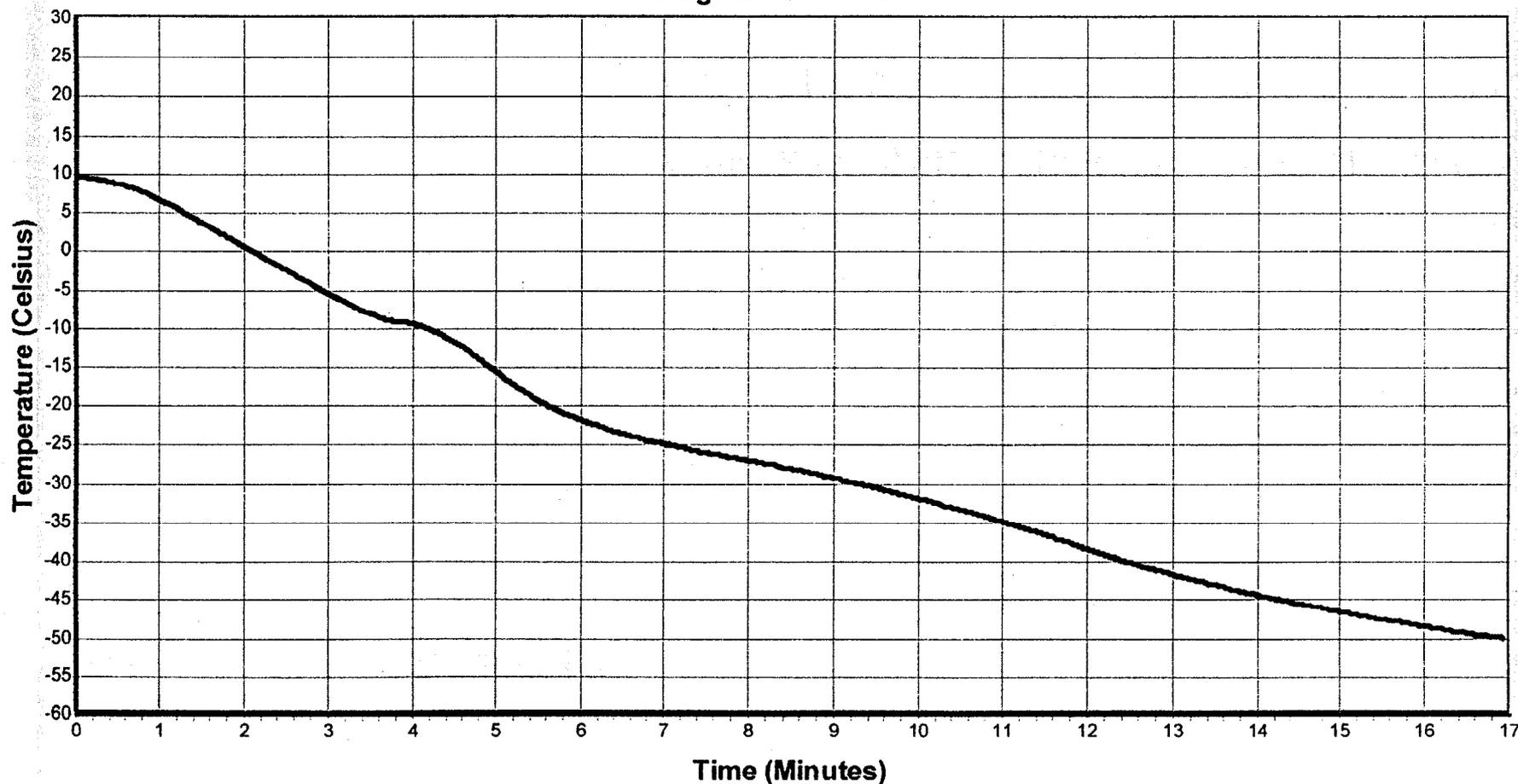
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Sample: 100107P

Storage Date: 11/9/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 7

Profile Used: Endfr12

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -12C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1010

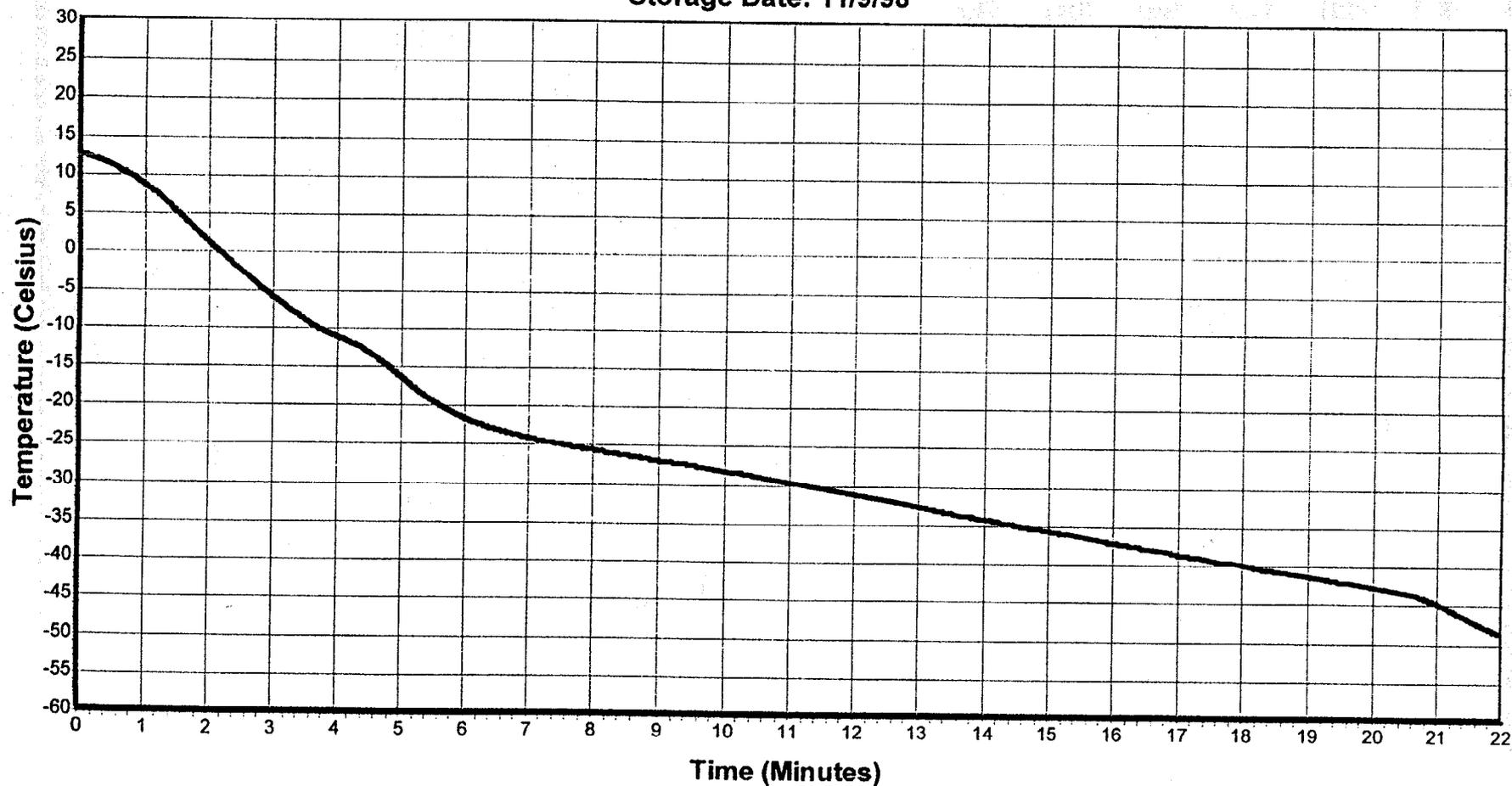
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Sample: 100108P

Storage Date: 11/9/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 8

Profile Used: Endfr12

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -12C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1011

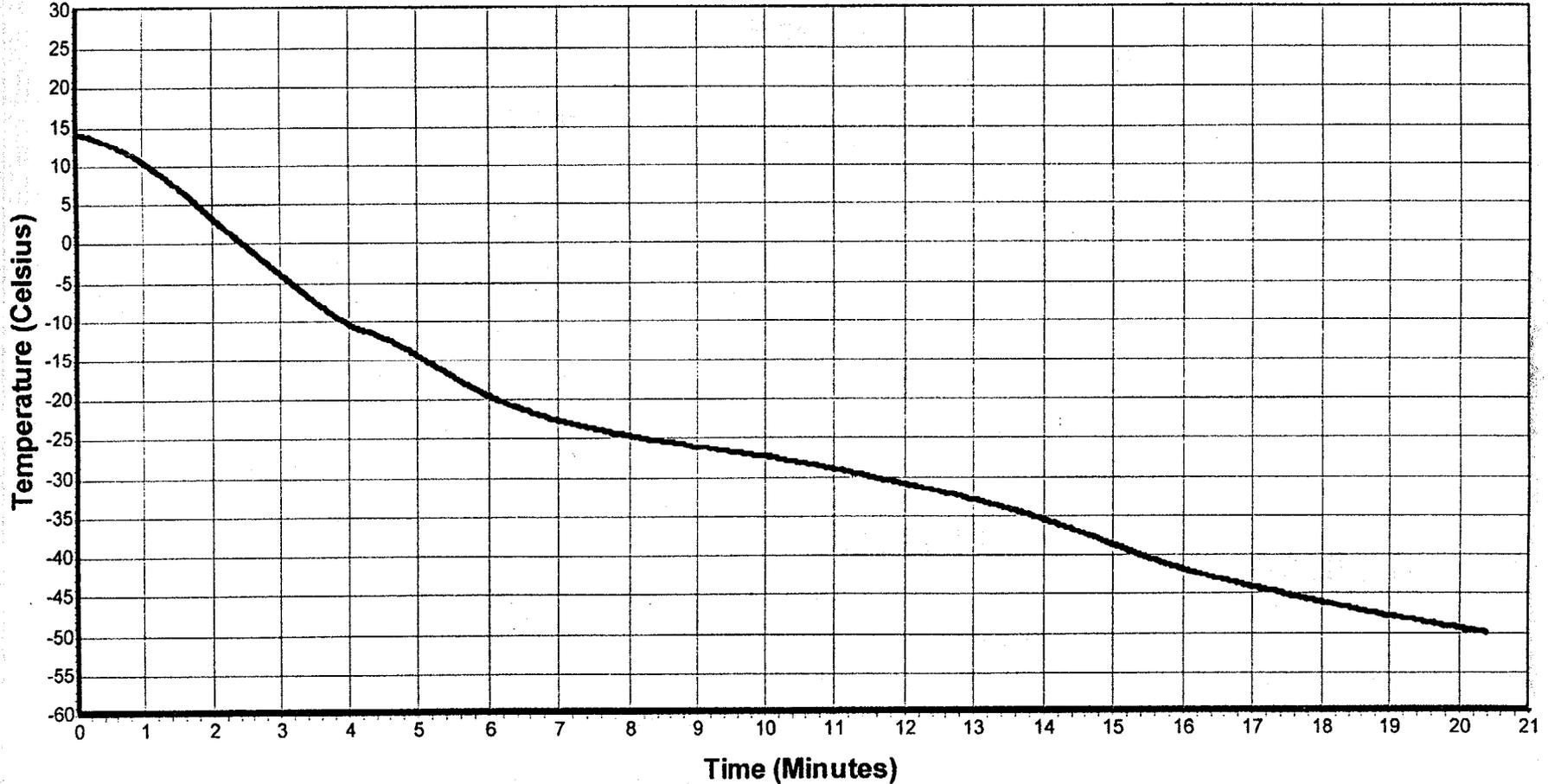
CRF Version: 1.

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Sample: 100109P

Storage Date: 11/9/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 9

Profile Used: Endfr12

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -12C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1008

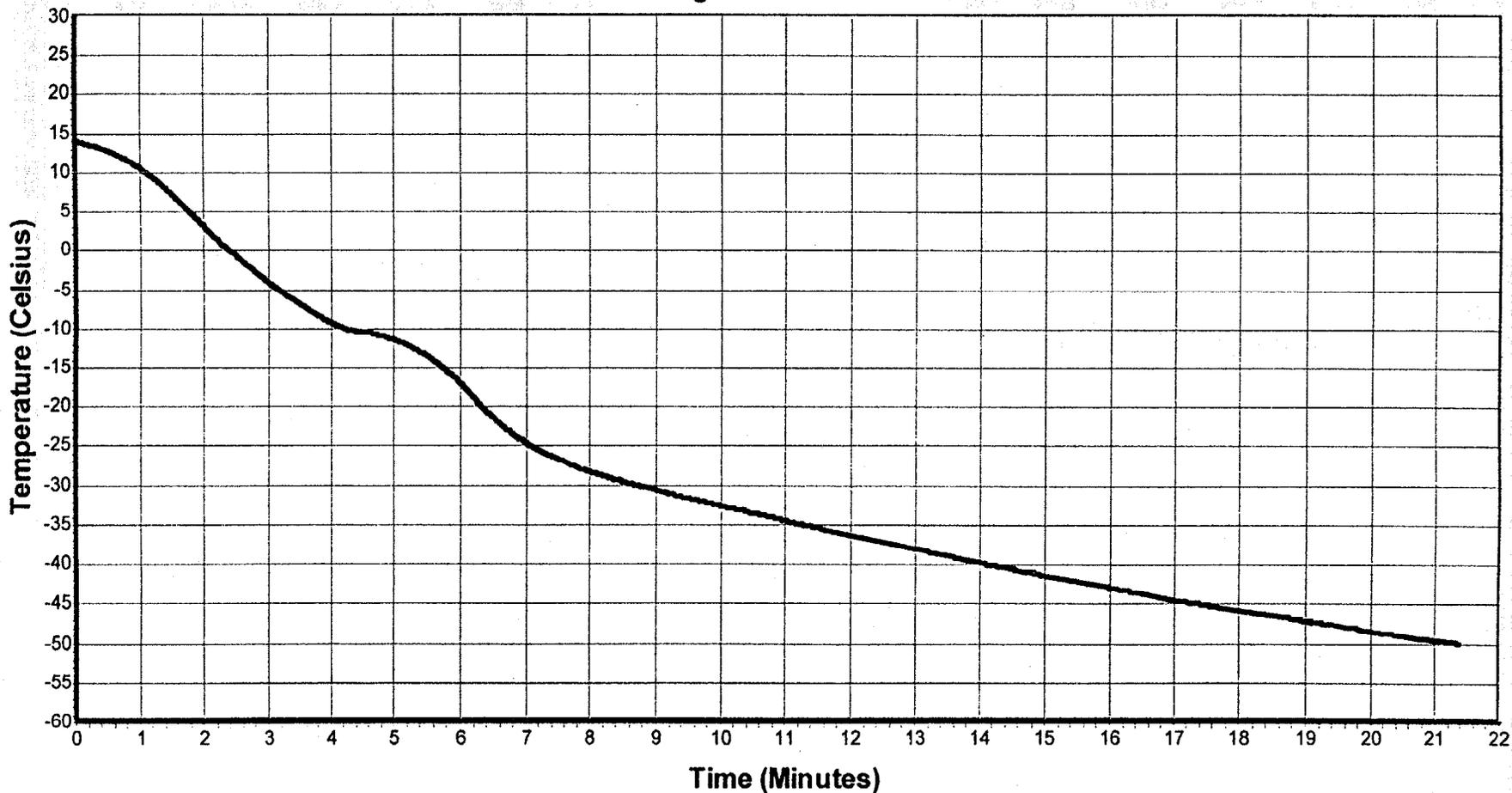
CRF Version: 1.

ThermoGenesis Corp.

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(916) 858-5100

Sample: 100110P

Storage Date: 11/9/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 10

Profile Used: Post2

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -15C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1034

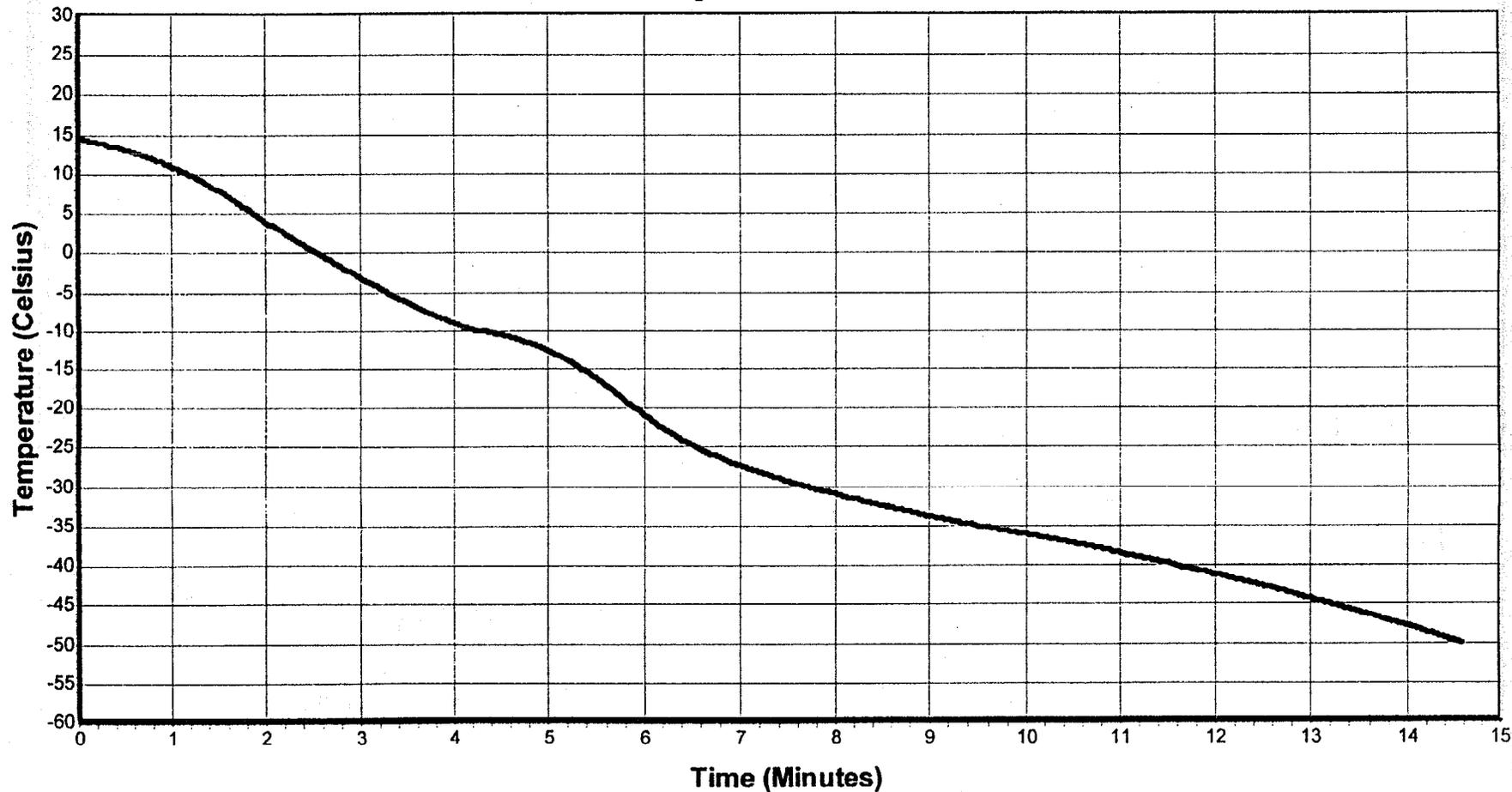
CRF Version: 1.

ThermoGenesis Corp.

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Rancho Cordova, CA 95670
(916) 858-5100

Sample: 100111P

Storage Date: 11/9/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 11

Profile Used: Post2

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -15C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1011

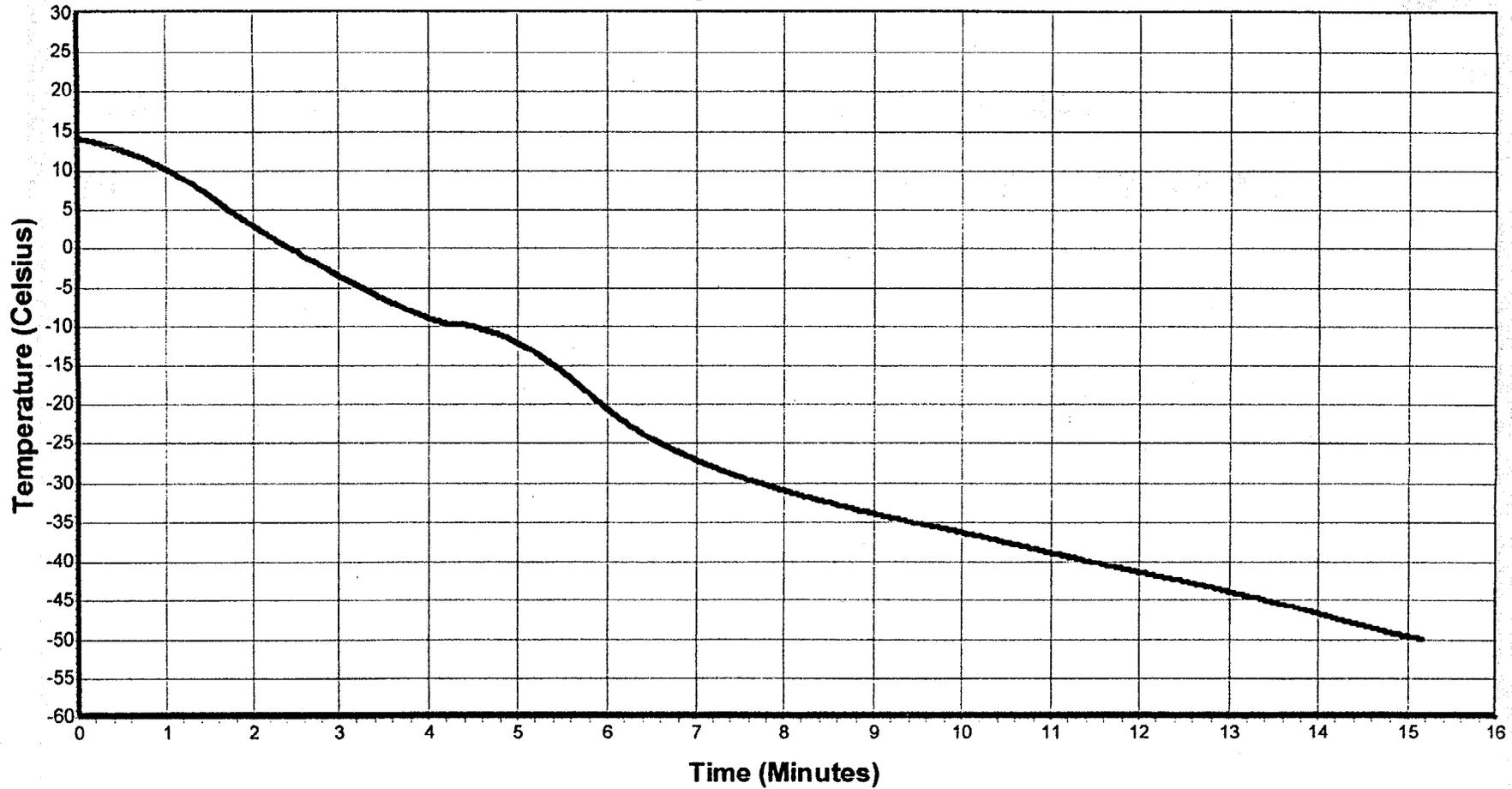
CRF Version: 1.

ThermoGenesis Corp.

3146 Gold Camp Drive
Rancho Cordova, CA 95670
(916) 858-5100

Sample: 100113P

Storage Date: 11/9/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 13

Profile Used: Post2

Pre-Freeze: 10C/Min.
Start Freeze: -3C
Fan Power: 100%

End Freeze: -15C
Post Freeze: 2C/Min.
End Temp: -50C

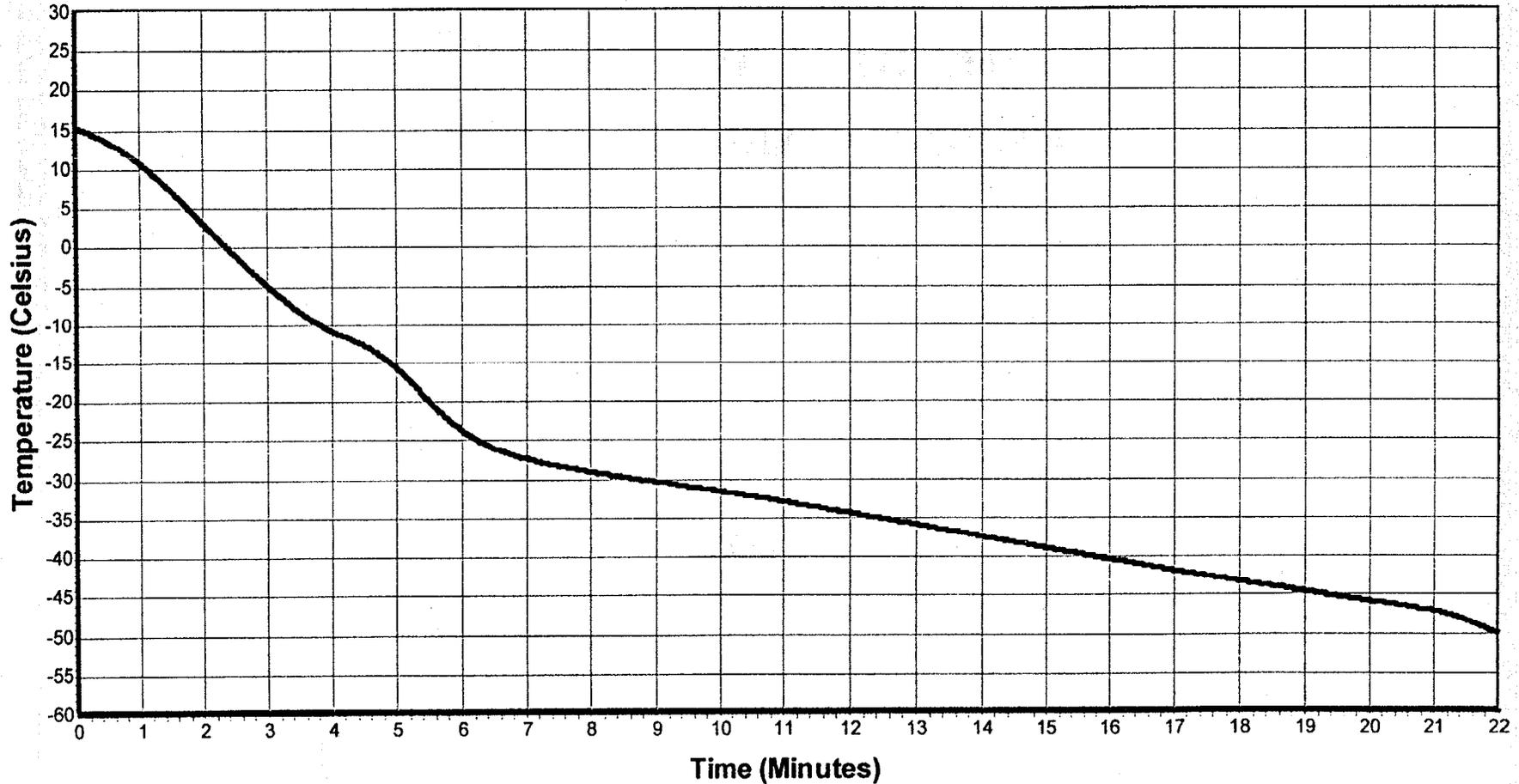
CRF Serial Number: 1034
CRF Version: 1.

ThermoGenesis Corp.

3146 Gold Camp Drive
Rancho Cordova, CA 95670
(916) 858-5100

Sample: 100114P

Storage Date: 11/9/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 14

Profile Used: Post2

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -15C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1008

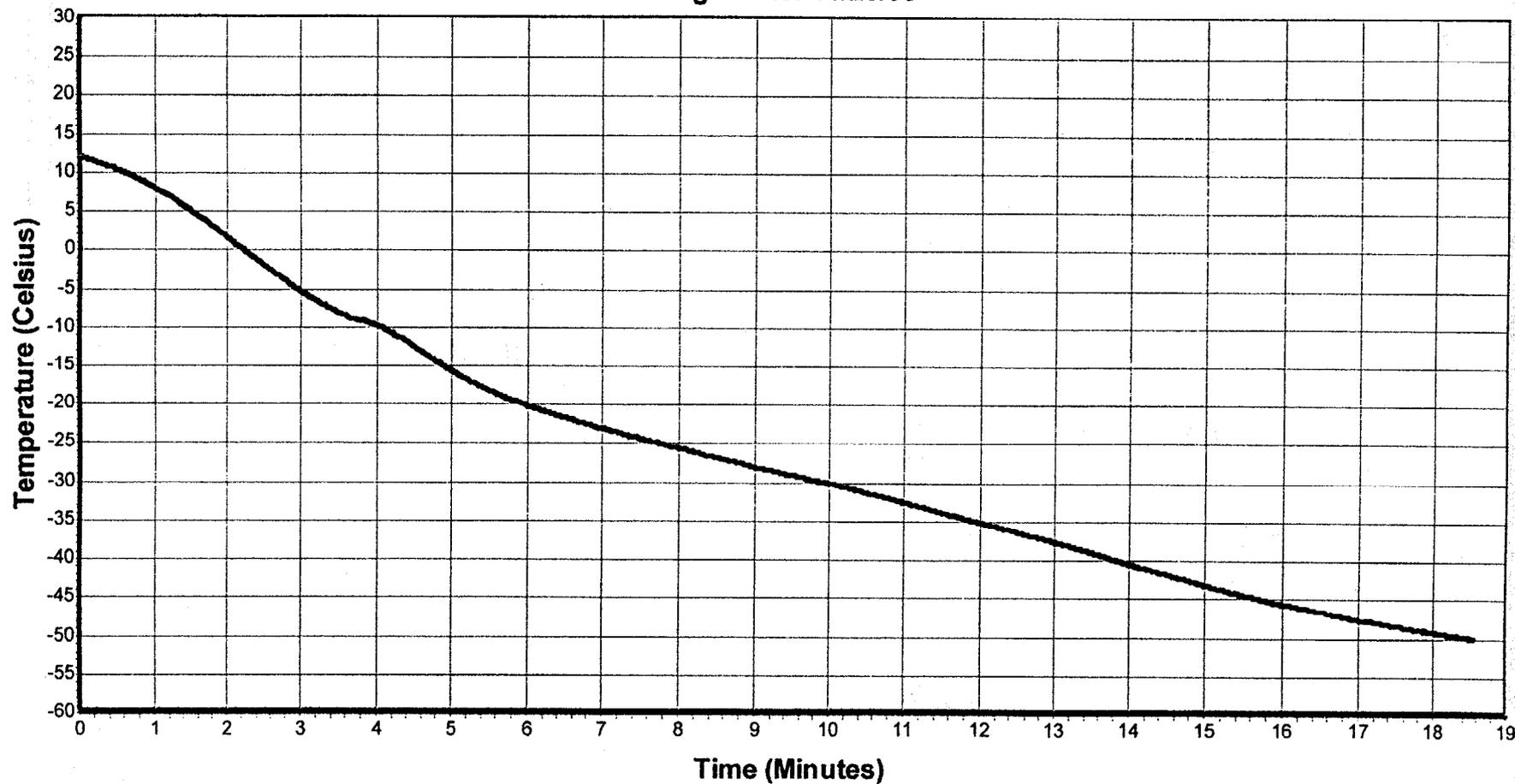
CRF Version: 1.

ThermoGenesis Corp.

3146 Gold Camp Drive
Rancho Cordova, CA 95670
(916) 858-5100

Sample: 100129P

Storage Date: 11/20/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 6

Profile Used: Endfr10

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -10C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1034

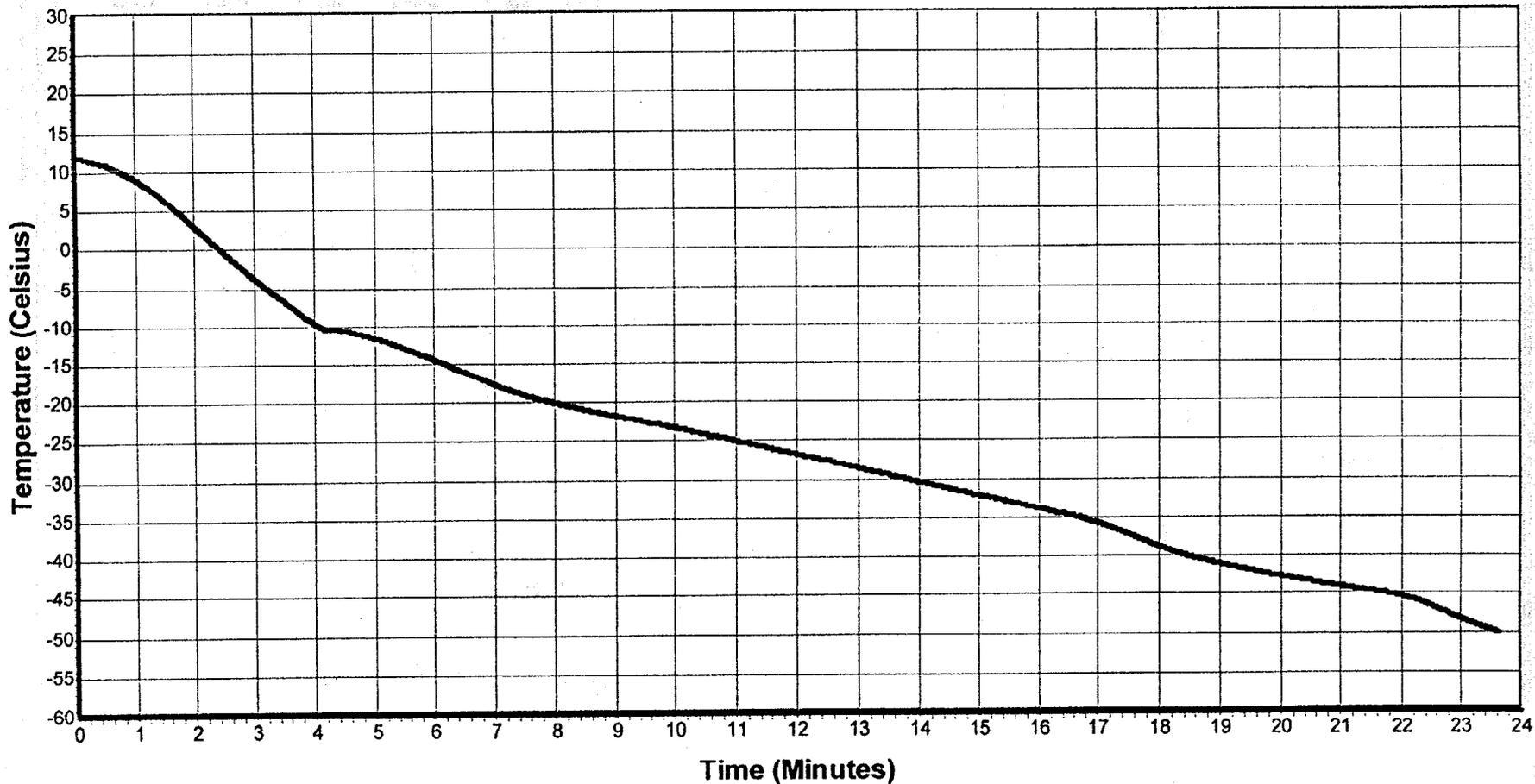
CRF Version: 1.

ThermoGenesis Corp.

3146 Gold Camp Drive
Rancho Cordova, CA 95670
(916) 858-5100

Sample: 100130P

Storage Date: 11/20/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 7

Profile Used: Endfr10

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -10C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1008

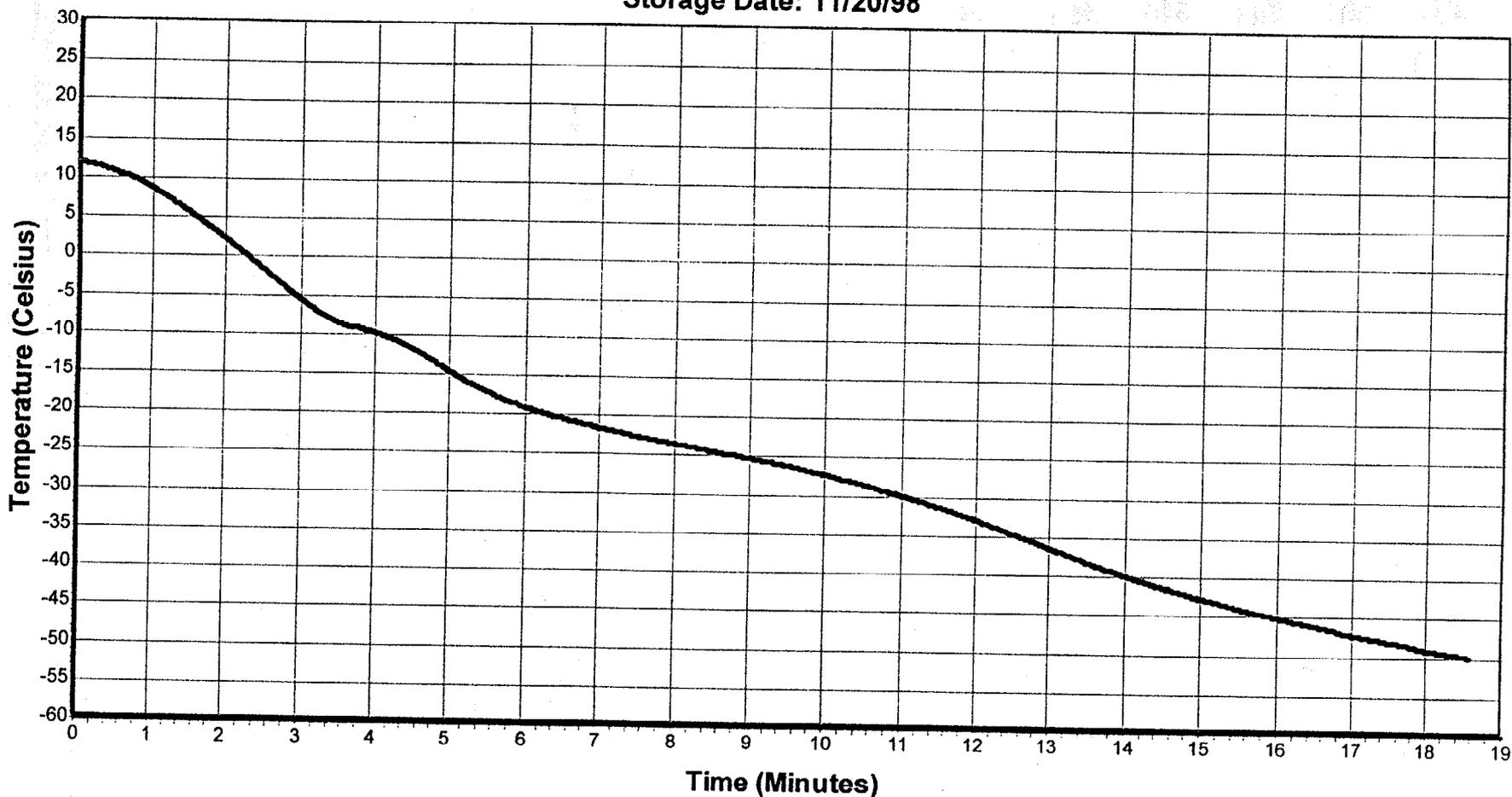
CRF Version: 1.

ThermoGenesis Corp.

3146 Gold Camp Drive
Rancho Cordova, CA 95670
(916) 858-5100

Sample: 100131P

Storage Date: 11/20/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 8

Profile Used: Endfr10

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -10C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1011

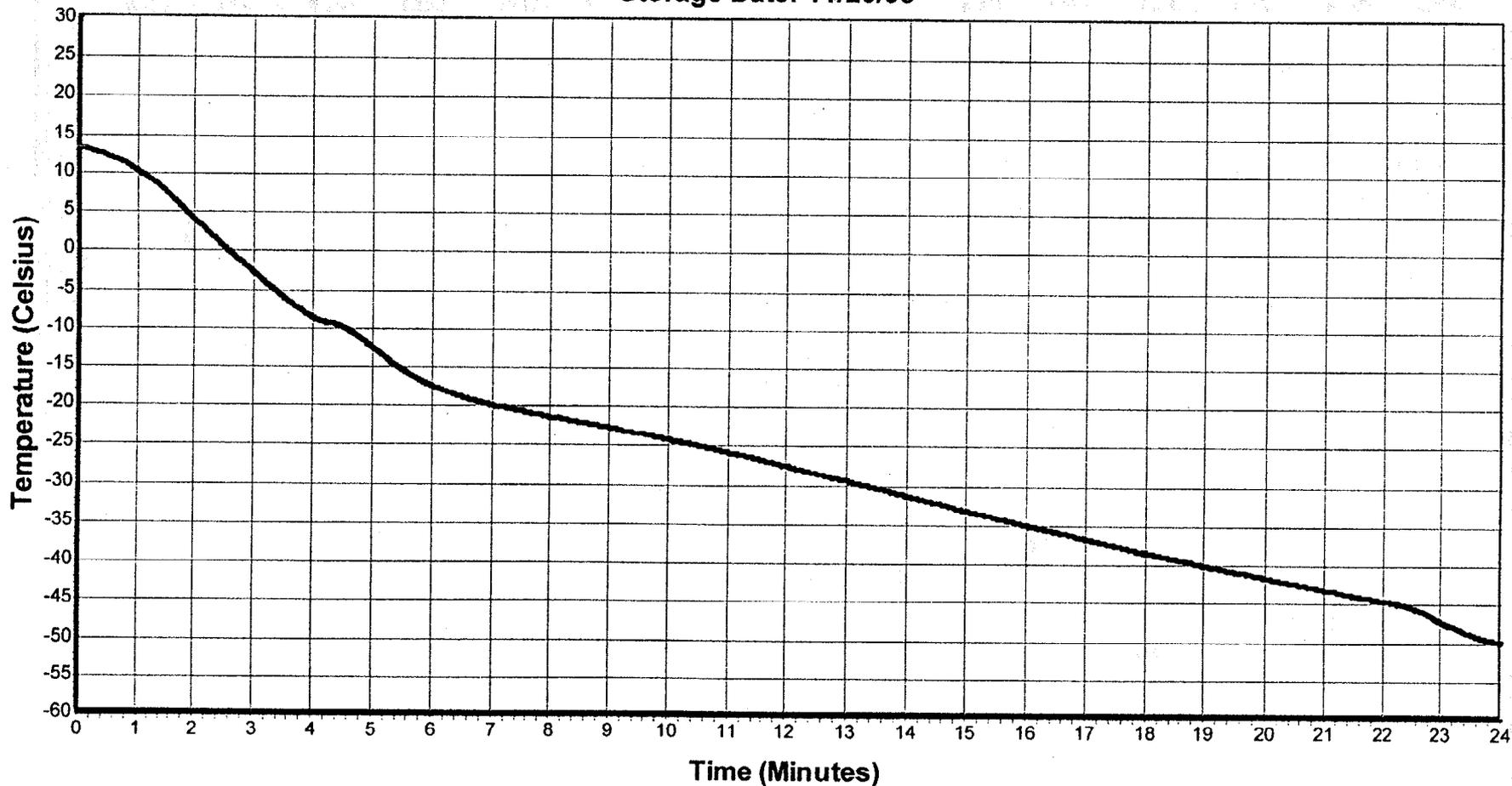
CRF Version: 1.

ThermoGenesis Corp.

3146 Gold Camp Drive
Rancho Cordova, CA 95670
(916) 858-5100

Sample: 100132P

Storage Date: 11/20/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 9

Profile Used: Endfr10

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -10C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1010

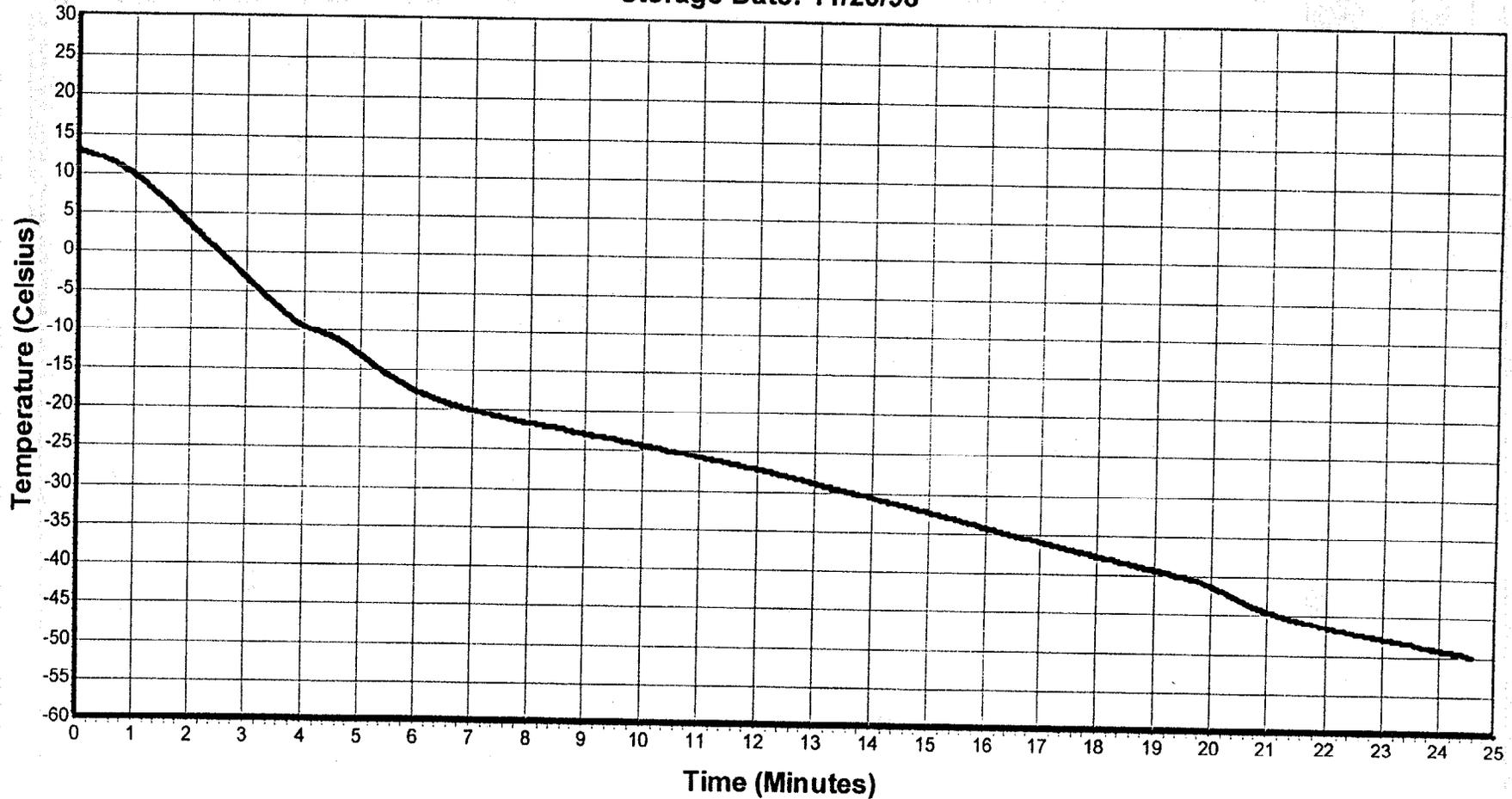
CRF Version: 1.

ThermoGenesis Corp.

3146 Gold Camp Drive
Rancho Cordova, CA 95670
(916) 858-5100

Sample: 100133P

Storage Date: 11/20/98



Operator ID: LUDY

Location: Ring: 2

Rack: 1

Slot: 10

Profile Used: Endfr10

Pre-Freeze: 10C/Min.

Start Freeze: -3C

Fan Power: 100%

End Freeze: -10C

Post Freeze: 2C/Min.

End Temp: -50C

CRF Serial Number: 1008

CRF Version: 1.

Appendix B

IN-SERVICE GUIDELINES

New York Blood Center

Protocol for Preparing Frozen Placental Cord Blood Units for Transfusion

Draft 11/15/1999

BACKGROUND

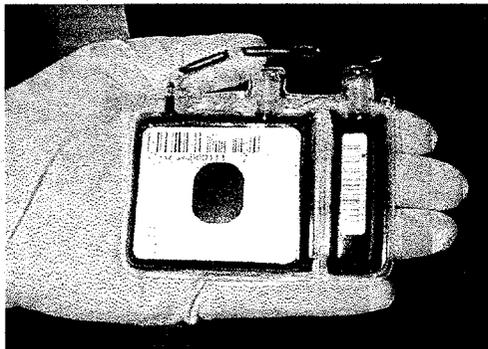
Until recently, it was the customary practice to thaw frozen stem cell containing bone marrow, peripheral blood cells and placental blood units directly from storage in liquid nitrogen (LN₂) and administer them to the patient with all its DMSO content, as quickly as possible afterwards.

This practice should be replaced by the new procedure described below as:

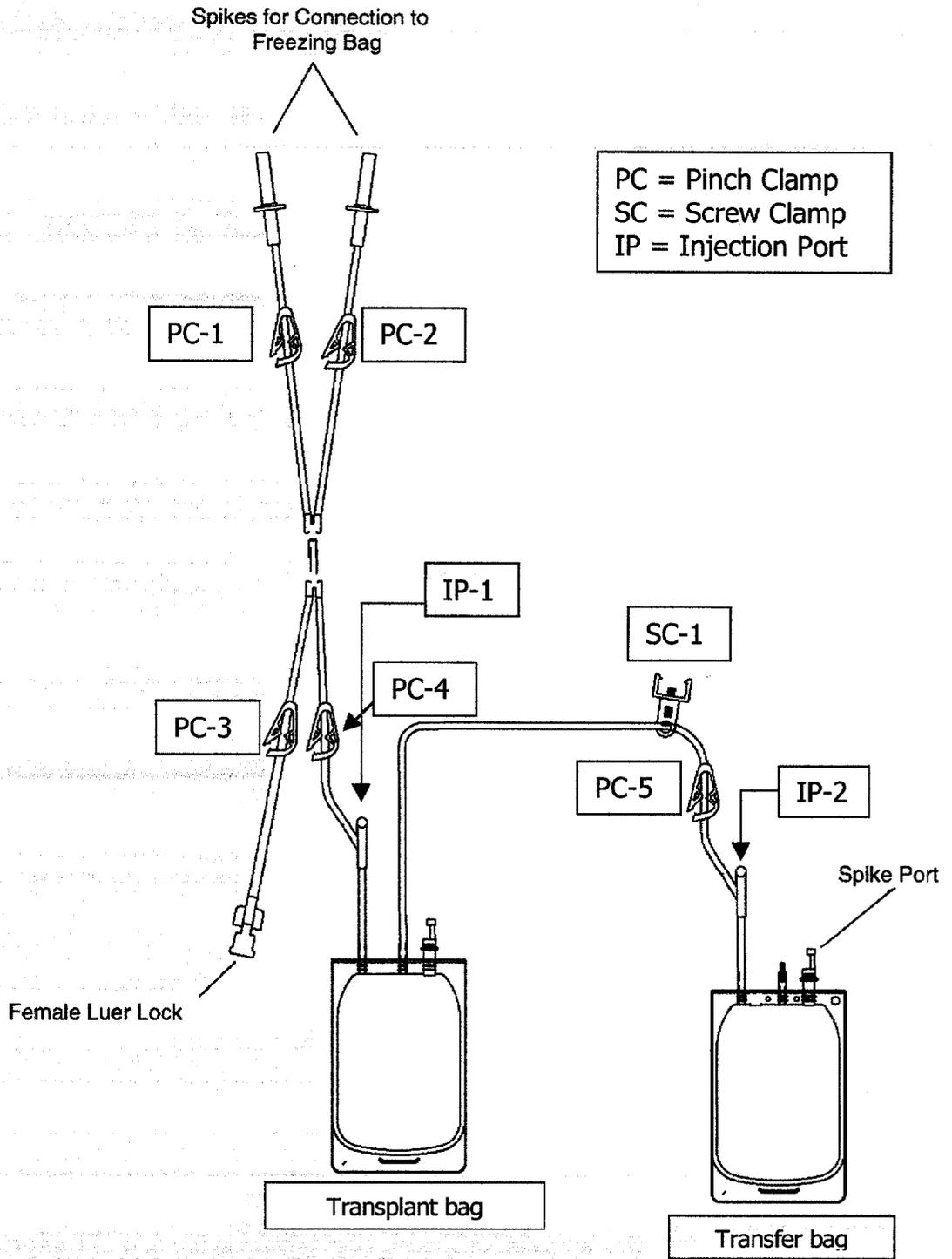
1. Data from the New York Blood Center (PNAS 92:10119-10122, 1995) indicates that recovery of viable cells can be significantly improved by restoring the osmolarity of the blood cell suspension and removing the supernatant with DMSO.
2. DMSO causes adverse effects in vivo after reinfusion, including blood pressure instability, fever, chills and nausea.

I. NEW PROCEDURE

The placental cord blood (PCB) unit is stored in a new, more compact freezing bag (Pall Medsep re-order code 791-02 or 791-05).



The procedure to restore the osmolarity of the blood cell suspension and remove the supernatant with DMSO is assisted by a new transplant bag set, included with this shipment (Pall Medsep re-order code 791-03), designed especially for this freezing bag. See page 3.



**Pall Medsep (791-03)
Transplant Set**

II. MATERIALS

II.1 PCB unit

Frozen PCB unit (identified as described in section III) in a foam canister sleeve maintained at LN₂ temperature (-196° C), either inside the container used for shipping (Dry-Shipper) or other LN₂-cooled storage device.

II.2 Equipment

- Laminar flow hood
- Refrigerated blood bank centrifuge
- Plasma expessor (Baxter 4R4414)
- Digital balance
- Tube sealer compatible for PVC plastic
- Automated cell counter
- Microscope and chamber for counting of cells (optional)
- Waterbath (4 liters or more at 38°C)
- THERMOGENESIS CORP. (TG) canister opener

II.3 Reagents

- Albumin (5% human UPS)
- 10% Gentran 40 (Dextran 40) and 0.9% NaCl, USP (both can be purchased from Baxter)
- Diluted acetic (2%) acid for lysing red blood cells
- Vital Stain for staining of cells, if desired
- Bacterial culture bottles

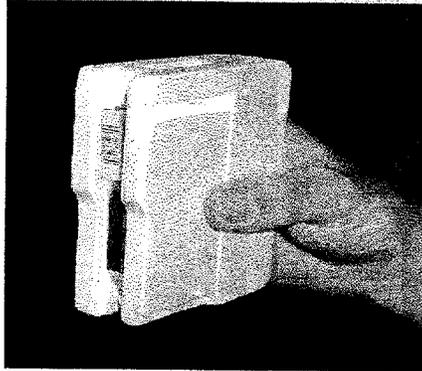
II.4 Supplies

- Transplant Processing Two-Bag Set (Pall Medical 791-03)
- Sterile Disposable Syringes: 1 ml, 50 ml and 60 ml (Becton-Dickinson)
- 18 gauge injection needles
- Sterile gloves
- Hemostats (optional)
- Sterile small plastic zipper-lock bags
- Alcohol prep pads
- Iodine swab sticks
- Tubes for cell counts, progenitor assays (optional)
- Protective freezer gloves.
- PCB thawing form procedure.

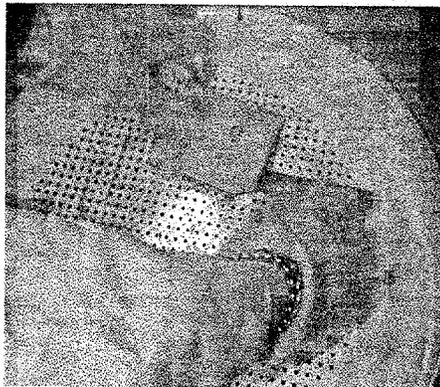
III. VERIFICATION OF UNIT'S IDENTITY

Wear protective freezer gloves.

- III.1 The PCB unit is in a canister which is contained in a foam insulated sleeve. The bar coded ID of the PCB unit is attached to the canister and visible through the open side of the sleeve. Check to confirm its identity with the ID of the expected unit as soon as it is received.



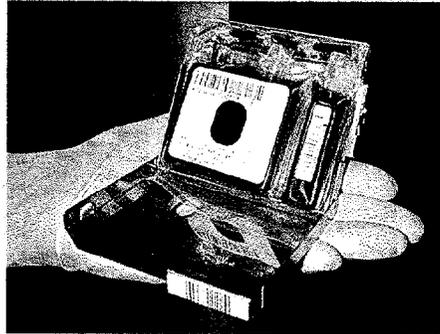
- III.2 Transfer the PCB unit from the Dry-Shipper to the vapor phase of an LN₂ storage tank. Utilize the TG canister opener to pry open from top and bottom, as shown below. Work carefully to avoid damaging the frozen plastic PCB bag.



- III.3 Check the bar coded label on the unit against your records to verify that the bar coded and human-readable printed number absolutely conform to:

III.3.1 the information previously provided.

III.3.2 the documentation included with the unit.



NOTE: If there is any error or ambiguity, close the canister and keep the unit at LN₂ temperature. Immediately advise the Placental Blood Program and the transplant physician. Do not proceed until the problem is resolved. If your LN₂ storage tanks have no space to store the unit in its canister and insulated sleeve, add LN₂ to the dry-shipper to maintain the unit frozen until a completely satisfactory determination is made.

IV. METHOD

IV.1 Preparation of thawing solutions

IV.1.1 Fit a 18 gauge needle to a 50 ml syringe. Draw 12.5 ml of 10% Dextran40 and 12.5 ml of 5% human albumin into the syringe. This syringe is for dilution of cells after thawing.

IV.1.2 Fit 18 gauge needles to four 60 ml syringes. Draw 30 ml of 10% Dextran40 and 30 ml of 5% human albumin into each syringe.

Alternatively, prepare the solution in a 300-ml transfer bag by adding 150 ml 10% Dextran40 and 150 ml 5% albumin.

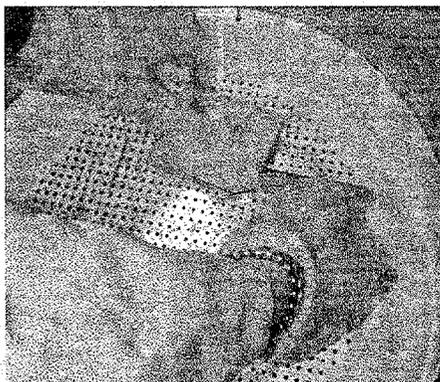
IV.2 Thawing of unit

Wear protective freezing gloves.

IV.2.1 Remove the canister with the unit from the LN₂ container. Place the canister in the vapor phase of the LN₂. Leave the canister in the vapor phase for 5-10 min. before proceeding.

NOTE: If two units are stored in the LN₂ container at the same time, open canister with the TG canister opening tool as described in III.2. Carefully check the ID number on the labels attached to the canister and the unit, respectively. Close canister and leave it in the vapor phase for 5-10 min. before proceeding.

IV.2.2 Open canister with the TG canister opening tool as described in III.2. Work carefully to avoid damaging the frozen plastic PCB bag.



Remember that plastic at this temperature is very brittle and breaks easily.

IV.2.3 Remove the unit from the canister.

CAUTION: Do not handle plastic bags at liquid nitrogen temperature with the thongs intended for metal canisters!! Do not allow unit to bend or it will crack!!

IV.2.4 Place the unit inside a zipper-locked plastic bag, let the air out and close the bag. Put the unit in a water bath at 38° C. Take care not to break the plastic tube segment, if still attached.

IV.2.5 To accelerate thawing, carefully move the unit in the water and knead its contents gently but assiduously.

NOTE: *Inspect for leaks.*

Plastic bags for frozen blood may have pin-holes, other defects or develop leaks. It is most important to prevent serious blood losses or bacterial contamination caused by cracks or leaks.

If blood leaks out into the zipper-locked bag, find the site of the break in the freezing bag and position the unit so as to prevent further escape of blood. While maintaining that position, thaw the unit. Proceed to IV.2.6.

IV.2.6 As soon as the blood has thawed, remove the bag from the water bath.

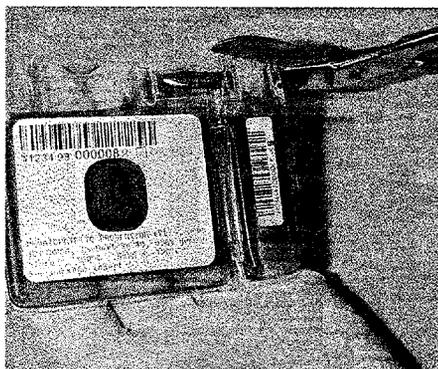
IV.3 Joining the Freezing Bag to the Transplant Set

Work under laminar flow.

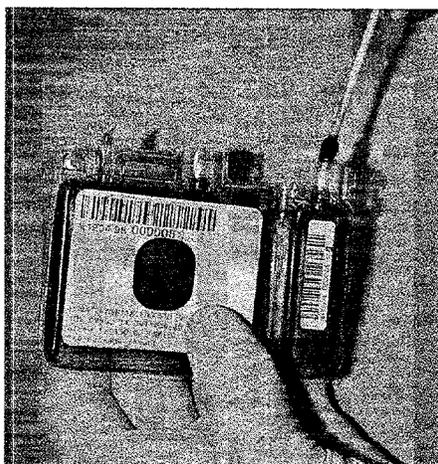
IV.3.1 Close all clamps on the Transplant Set.

IV.3.2 Remove the unit from the zipper-locked bag. Using an iodine swab stick, disinfect the covers of both ports of the freezing bag.

IV.3.3 With scissors, cut off both of the hermetically sealed covers to the spike ports on the freezing bag. Disinfect the scissors before using.



IV.3.4 Using an iodine swab stick, disinfect the cut surfaces of the spike port area of the freezing bag.



IV.3.5 Insert the spikes of the transplant set in the ports of the freezing bag.

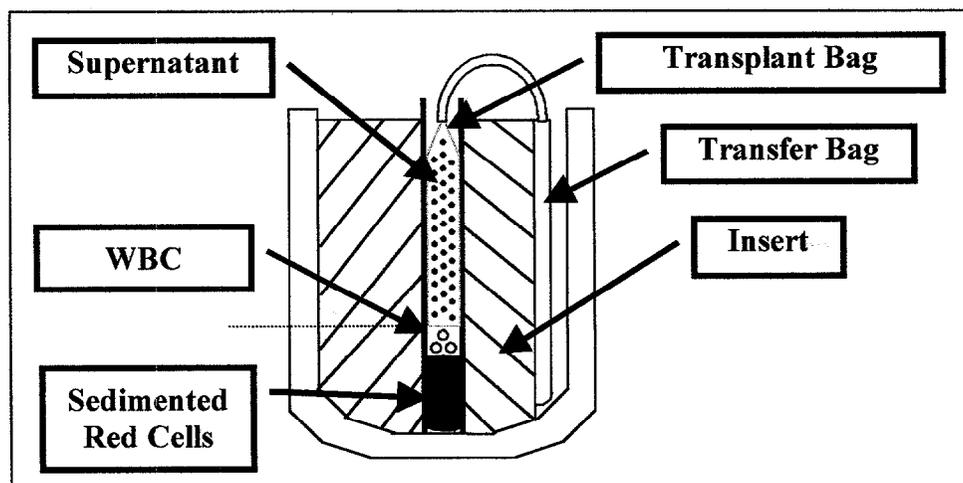
NOTE: Label the transplant bag with the unit number of the PCB and the name of the recipient, or according to local standard practice!

IV.4 Washing of the PCB

- IV.4.1 Attach the 50 ml syringe with thawing solution to female luer lock. Open PC-1, PC-2 & PC-3 and then slowly introduce the 25 ml of dextran-albumin solution to the 25 ml PCB in the freezing bag. Mix the fluids during transfer. Close PC-3.
- IV.4.2 Allow 5 minutes for equilibration.
- IV.4.3 Open PC-4. Transfer the diluted PCB from the freezing bag to the transplant bag.
- IV.4.4 Pass the diluted PCB back & forth between the transplant bag and the freezing bag in order to more completely wash all cells out of the freezing bag and into the transplant bag. Close PC-1 and PC-2.
- IV.4.5 Attach a 60 ml syringe with dextran-albumin solution to the luer lock. Open PC-3. Transfer the 60 ml solution to the diluted PCB in the transplant bag. Mix during transfer.

Repeat with a second 60 ml syringe. The final volume is now approximately 170 ml (50 ml diluted PCB and 120 ml dextran40-albumin solution).

- IV.4.6 Close PC-3 and open PC-1 and PC-2. Pass the diluted PCB back & forth between the transplant bag and the freezing bag in order to more completely wash all cells out of the freezing bag and into the transplant bag. Close PC-4.
- IV.4.7 Seal tubing between PC-4 and IP-1 and disconnect. Discard the spikes, luer lock and connecting tubing.
- IV.4.8 Place the transplant bag and the transfer bag in a centrifuge cup. Fully support the transplant bag with inserts to prevent formation of creases during centrifugation as show in IV.4.9. Make sure PC-5 and SC-1 are closed.
- IV.4.9 Centrifuge at 400 x G, for 20 minutes, at 10°C.



You may now call the Transplant Unit and advise them that the transplant will be ready in about 30 minutes!!

- IV.4.10 Place the centrifuged transplant bag in the plasma extractor. Open PC-5. Use SC-1 to adjust the flow and very slowly transfer most of the supernatant to the transfer bag. Leave approximately 10-15 ml supernatant (Supernatant-1) with the cells. Empty the tubing between the bags by transferring air from the transfer bag to the transplant bag. Close PC-5.

NOTE: If you detect passage of cells to the transfer bag, return the contents to the transplant bag, re-suspend the cells and repeat the centrifugation.

IV.4.11 Inspect the supernatant for escaped cells, even if there is no appearance of escape.

IV.4.11.1 Express 10 ml from the Supernatant-1 bag into a conical centrifuge tube (accurate volume will help the accuracy of estimations).

IV.4.11.2 Centrifuge at 600G x 10 minutes.

IV.4.11.3 Carefully aspirate 9.5 ml of supernatant without disturbing the (possible) cell button in the tip of the tube.

IV.4.11.4 Resuspend the cell button thoroughly in the 0.5 ml of supernatant and load into a cell-counting chamber. Count the nucleated cells per μL and calculate the total number of cells in the remaining volume of Supernatant-1.

IV.4.11.5 Determine the number of nucleated cells per Kg of patient's weight.

NOTE: A decision can then be made by the Transplant Physician on whether these cells should be added to the main Sediment-1 cells, the graft, to increase the cell dose. This addition may not be necessary when the graft, Sediment-1, already contains a high cell-dose. If the Sediment-1 cell dose is low, or borderline, a meaningful addition to the cell dose would be useful and desirable. If so, proceed to IV.4.11.6.

IV.4.11.6 Centrifuge the Supernatant-1 bag at 400G x 20 minutes.

IV.4.11.7 Remove the new supernatant (Supernatant-2) to a new 300 ml Transfer Bag, with care not to disturb the sedimented cells (Sediment-2).

IV.4.11.8 Weigh Supernatant-2 and calculate the volume left with Sediment-2.

IV.4.11.9 Resuspend Sediment-2.

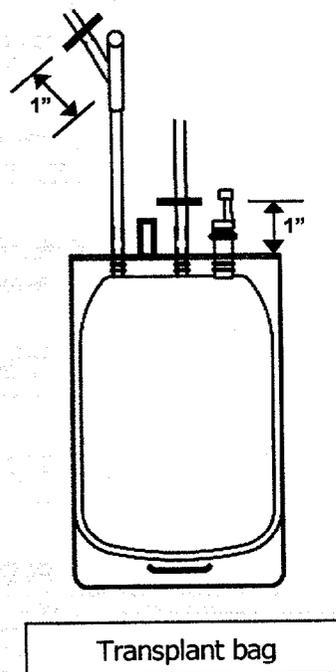
IV.4.11.9.1 In an equal volume of dextran-albumin solution, if it is to be infused to the patient, or

IV.4.11.9.2 In cryopreservative solution, if it is to be transferred to vials and frozen for reference.

IV.4.12 Seal the tubing between the bags close to the transplant bag.
Disconnect the transfer bag with the supernatant.

IV.4.13 Re-suspend the cell pellet by slowly adding 25-50 ml of the dextran-albumin solution in a 60 ml syringe through the IP-1. Mix cells and solution thoroughly during transfer.

IV.4.14 The weight of the transplant bag if cut and sealed as shown below is 21.6 gm. Calculate the weight of the cells by subtracting the weight of the empty transplant bag.



IV.4.15 Remove a small volume for cell count and viability determination.
Samples for bacteriology can be taken from the supernatant.

IV.4.16 If cells are recovered from the first post-thaw supernatant, add 10 ml of dextran-albumin solution to the bag through IP-2. Re-suspend the cells carefully.

NOTE: The volumes injected in step IV.4.13 may be modified if the Transplant Physician prefers injecting the patient with a smaller total volume. In this case, re-suspend the cell pellet to the desired volume by injecting the desired volume of dextran-albumin solution.

IV.4.17 Bring the transplant bag to the Transplant Unit even if the second is being prepared; the second can be infused separately afterwards.

V. ADMINISTRATIVE REQUIREMENTS

V.1 Prepare a report on the procedure. Note the condition of the bag and whether leaks or cracks were detected.

Include in the report:

- Unit number
- date of receipt of the Unit
- storage conditions in your facility
- date of thawing
- cell count
- viability of the cells recovered

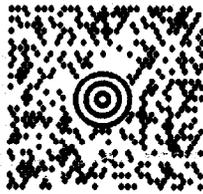
Submit the report to the Transplant Unit, mail a copy (preferably fax it first) to the placental/cord blood bank from which you have received this unit. Keep a copy for your Processing team records.

V.2 Return the metal canister (and the dry shipper if it is still at your site) to the New York Blood Center Placental Blood Program.

THERMOGENESIS
(916)638-8357
3146 GOLD CAMP
RANCHO CORDOVA CA 95670-6022

1 LBS

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FOOD AND DRUG ADMINISTRATION
(916)858-5105
12420 PARKLAWN DRIVE, ROOM 1-23
ROCKVILLE MD 20857

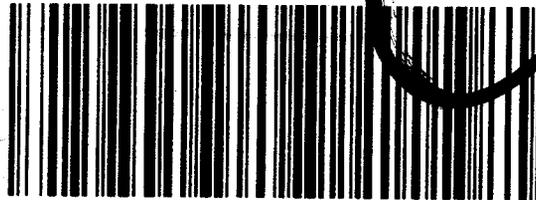


MD 2070-06



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DEPT #: PC1688

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