

35 minute presentation

Cryopreservation: trouble shooting

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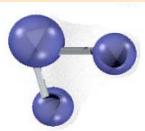


Clinical context for cryopreservation

Cryopreservation is typically used for a variety of cell types and can be combined with liquid storage.

Cryopreservation permits

- Coordination with patent care regimes
- Completion of safety testing and quality control testing.
- Genetic diversity of cells available
- Transportation to/from collection/administration site.



Liquid storage/
prefreeze processing

Introduction of solution

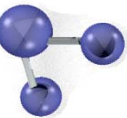
Cooling protocol

Storage conditions

Warming protocol

Post thaw assessment

Components of a Cryopreservation Protocol



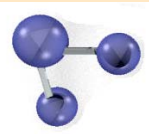
Categories of problems

Problems obvious to operator

- Failure during controlled rate freezing
- Delayed latent heat

Problems not obvious to operator

- Poor post thaw viability



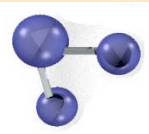
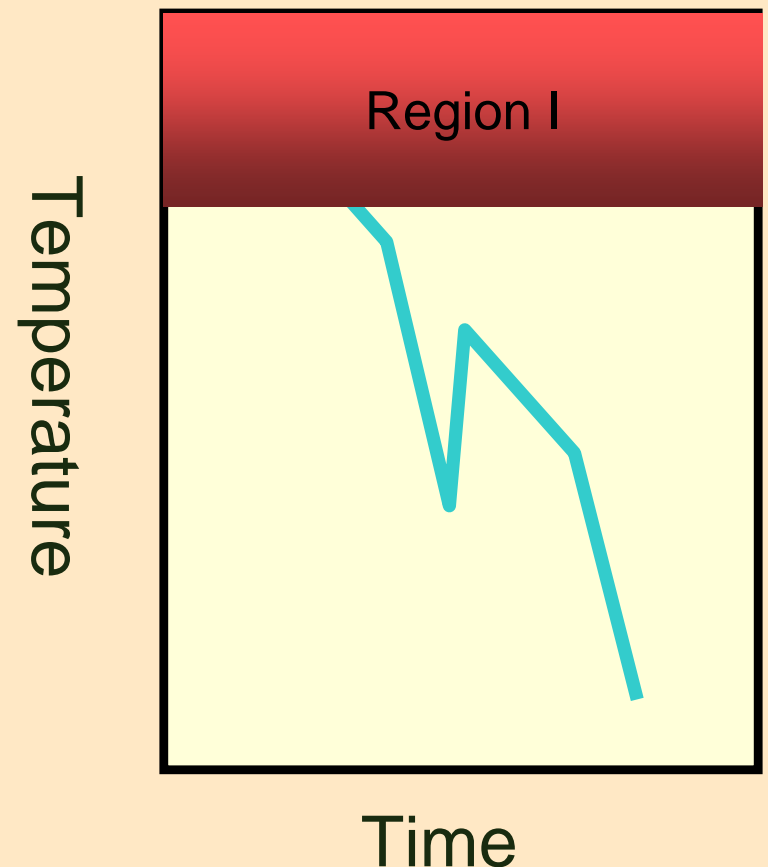
Failure during CRF

Mechanisms of failure

- Running out of LN2
- Failure of solenoid

Region I:

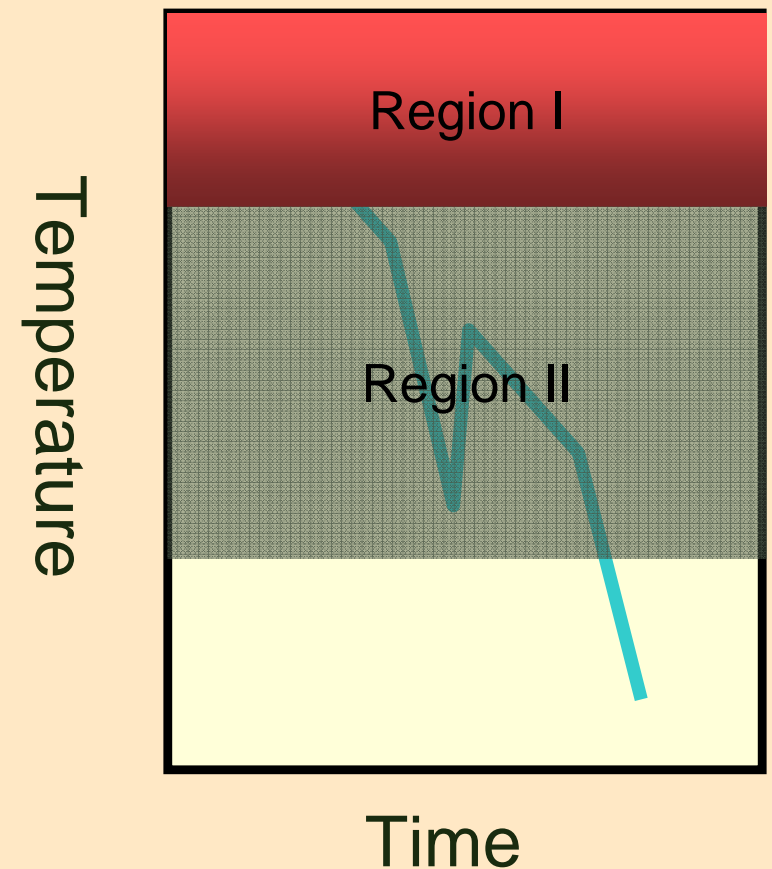
- Remove from CRF and place in back up freezer
- Viability of sample should be unaffected



Failure during CRF, cont.

Region II (T_{nuc} to -40 C)

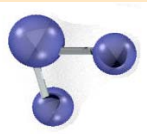
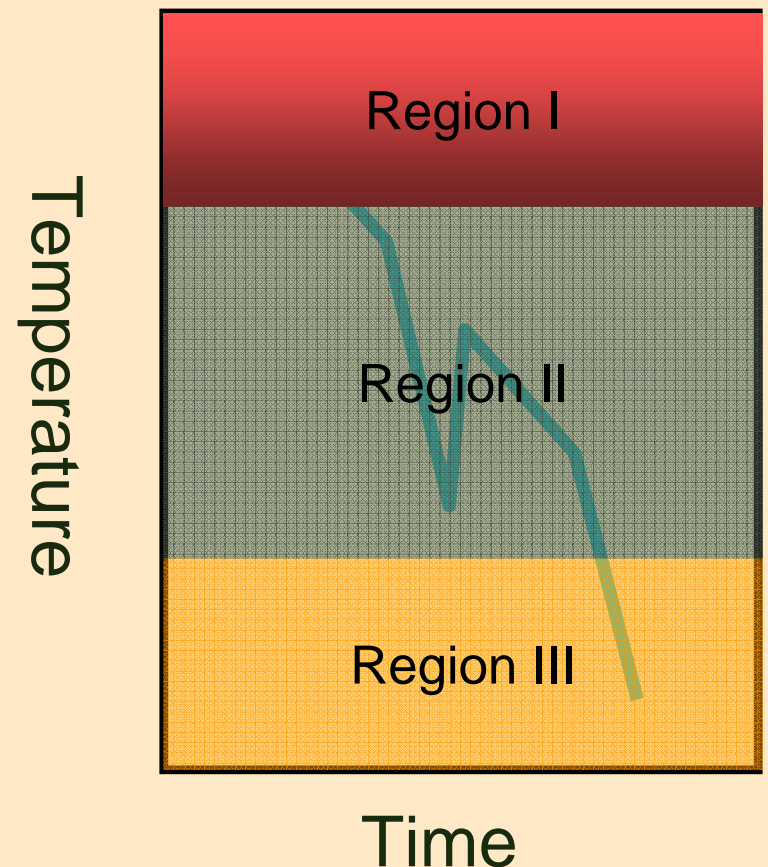
- Remove sample and place in -80 C freezer
- High probability that sample has been damaged
- Region of greatest activity
 - Solidification
 - Biological



Failure during CRF, cont.

Region III (-40 C to T_{end})

- Place in LN2 or -80 C freezer
- Minimize warming during transfer
- Sample unlikely to be affected



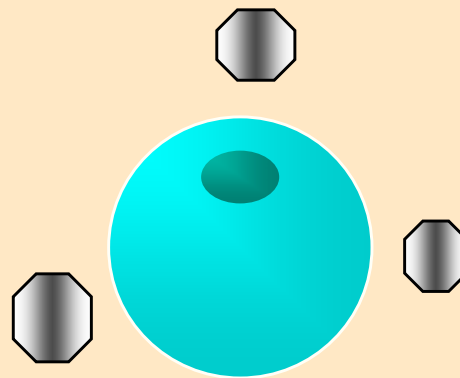
“Delayed” Latent heat

Nucleation and growth of ice releases the latent heat of fusion.

The temperature at which this occurs, T_{nuc} is important.

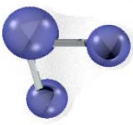
Material Science

- Concentration of extracellular solution
- Ice crystal growth characteristics



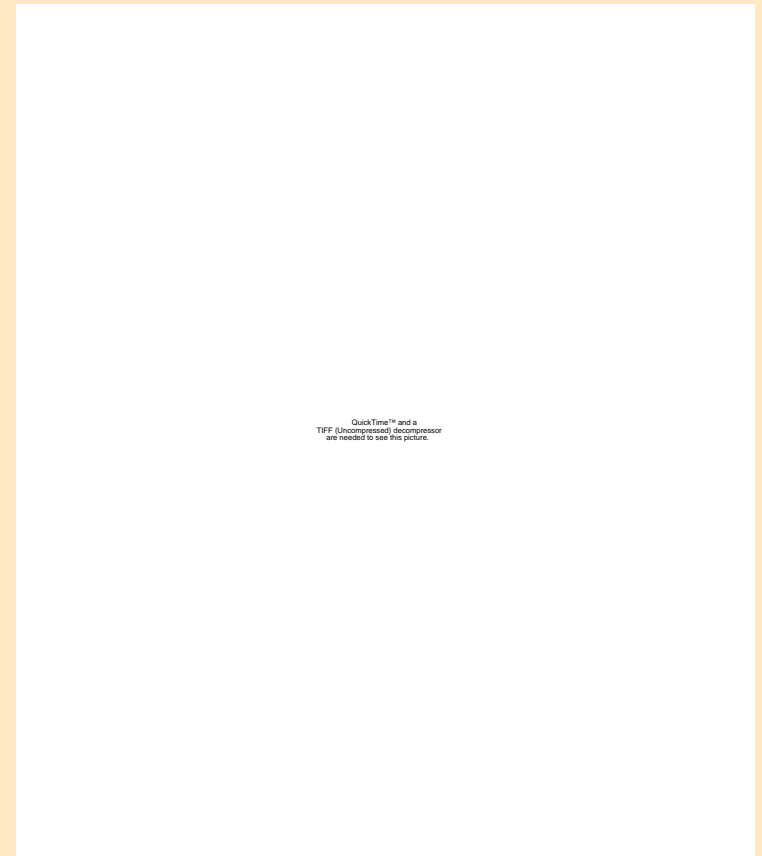
Biology:

- Permeability of cell to water
- Membrane phase characteristics

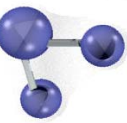


Relationship between T_{nuc} and IIF

- IIF is an accepted mechanism of damage.
- Cooling rate, T_{nuc} and cell type influence fraction of cells with IIF.

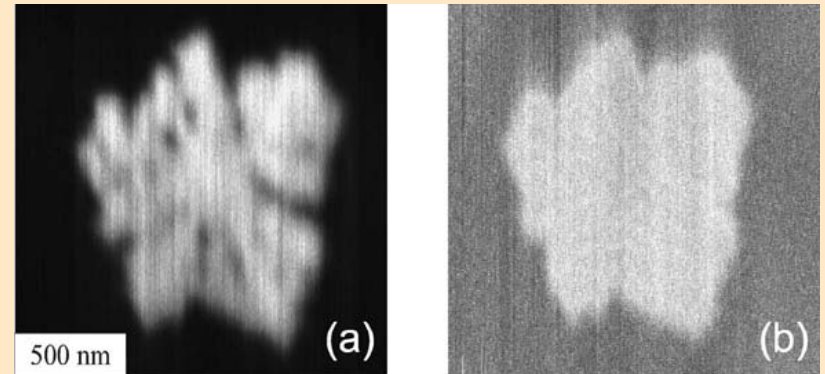


Toner, 1992

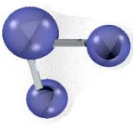


Methods of seeding sample

- “Automatic seeding”
 - Clinical protocols.
- Manual seeding
 - IVF protocols.
- Uncontrolled seeding.
 - Both clinical and laboratory settings.
- None of the methods really controls T_{nuc} for all samples being frozen
- Temperature history for all samples is not known



Bottom line: a solution for the problem does not yet exist

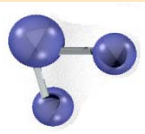


Technology in development

- CRF with controlled nucleation for each vial
- Wireless temperature measurement for each vial

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

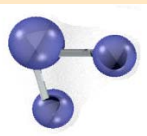
(Petersen, Cryobiology, 2006)



Poor post thaw viability

Caveat: Previous studies have established feasibility of freezing cells

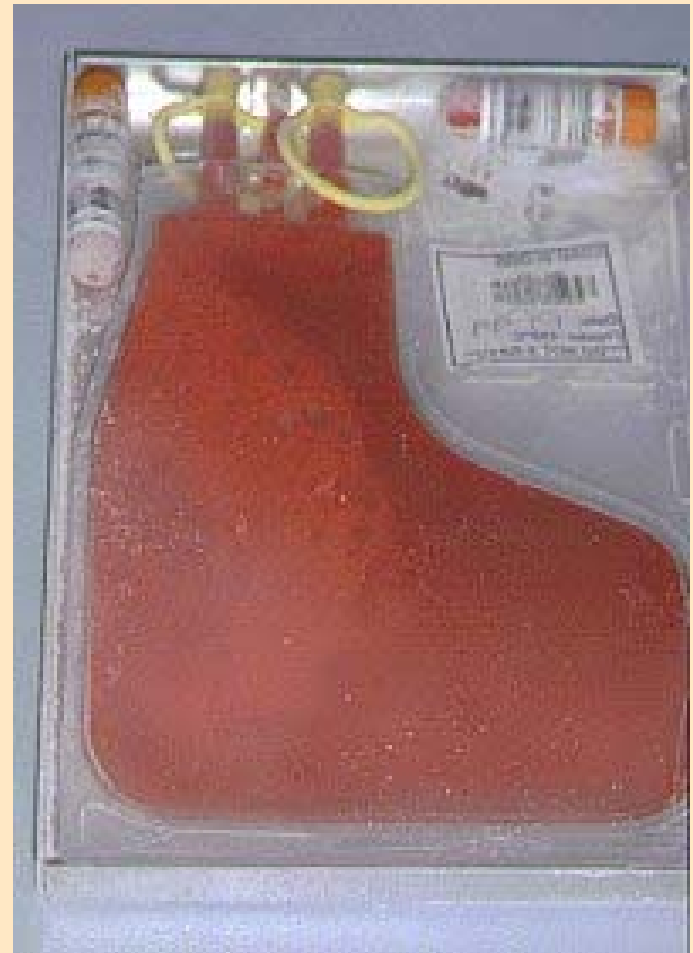
Presumption: flaws in implementation of the protocol



'Debugging' a protocol

Starting point:

- Each protocol element
 - Prefreeze processing
 - Cryopreservation solution introduction
 - Segments of CRF protocol
 - Storage
 - Warming
 - Post thaw assessment

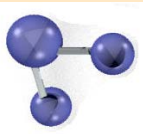


Prefreeze processing

Protocols should minimize stress, oxygen/nutrient deprivation, etc.

Measure: Screen for early markers of apoptosis, biochemical markers of stress, etc.

Modify: reduce duration of liquid storage, develop storage solution, etc.

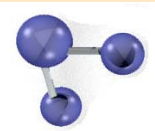
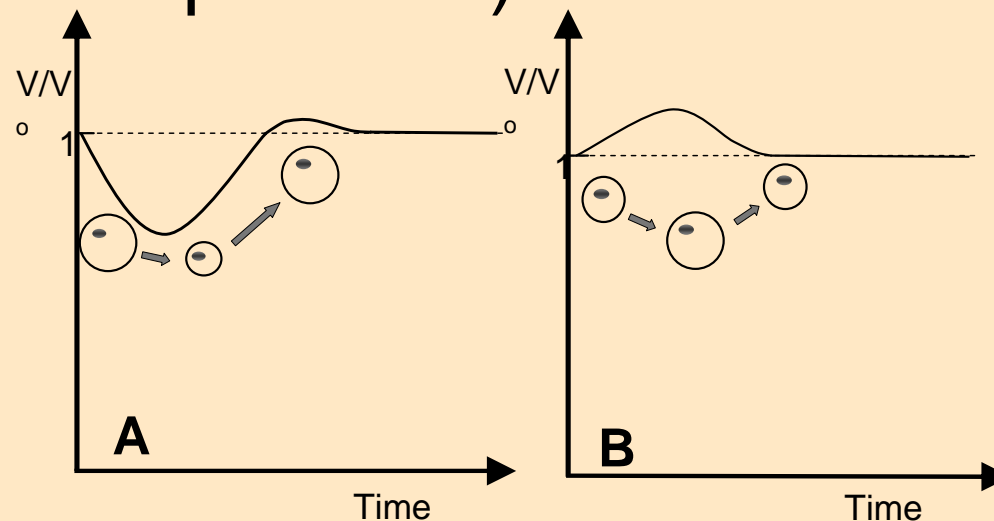


Cell processing for preservation

- Preservation requires the use of specialized solutions.
- Introduction and removal of the solutions can result in cell death.

Measure: cell losses after introduction and removal of solution (without freezing)

Modify: introduction protocol (multistep, gradual, reduced temperature)

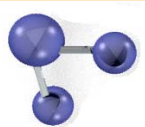
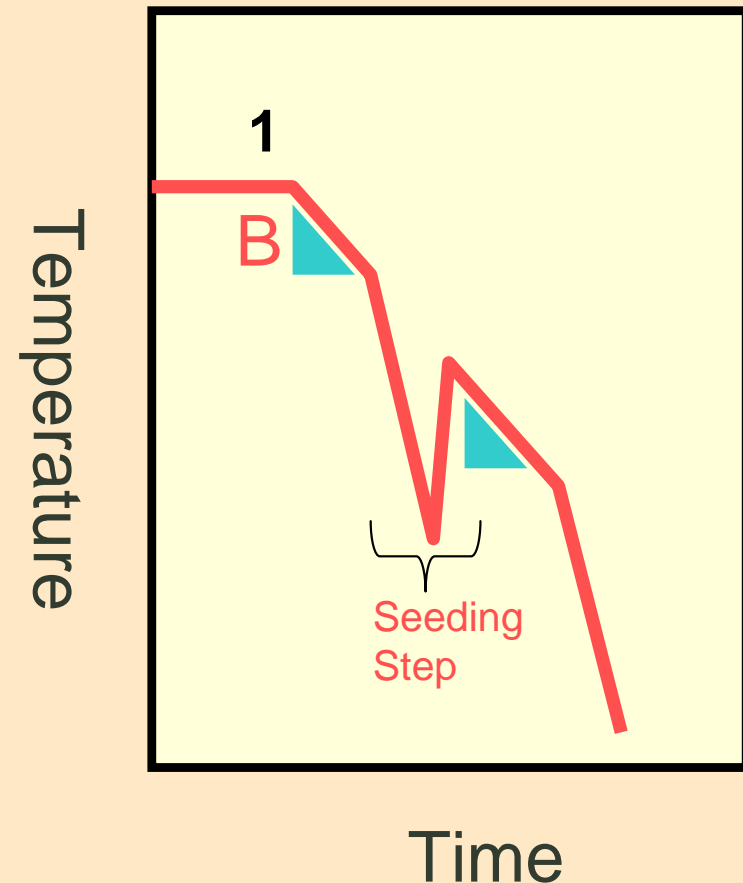


Controlled rate freezing

Segment One:

Measure: temperature in sample and compare to chamber temperature.

Modify: hold time to permit sample temperature to track chamber

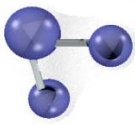
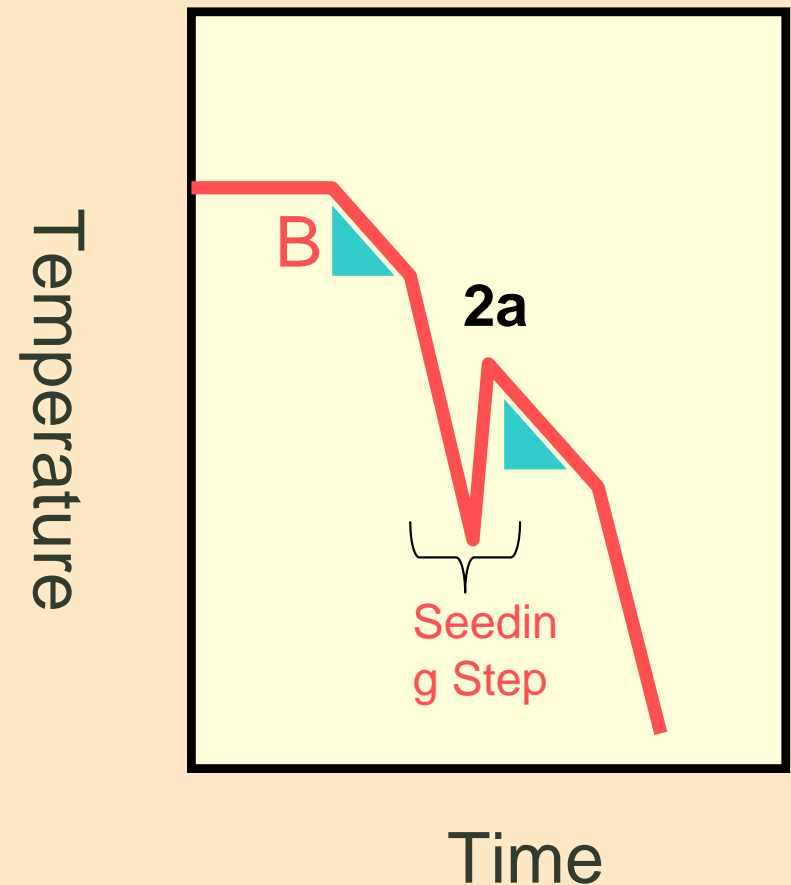


Controlled rate freezing, cont.

Segment 2a: seeding step

Measure: temperature at which sample seeds; viability of cells

Modify: seeding protocol to increase seeding temperature

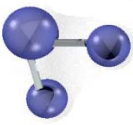
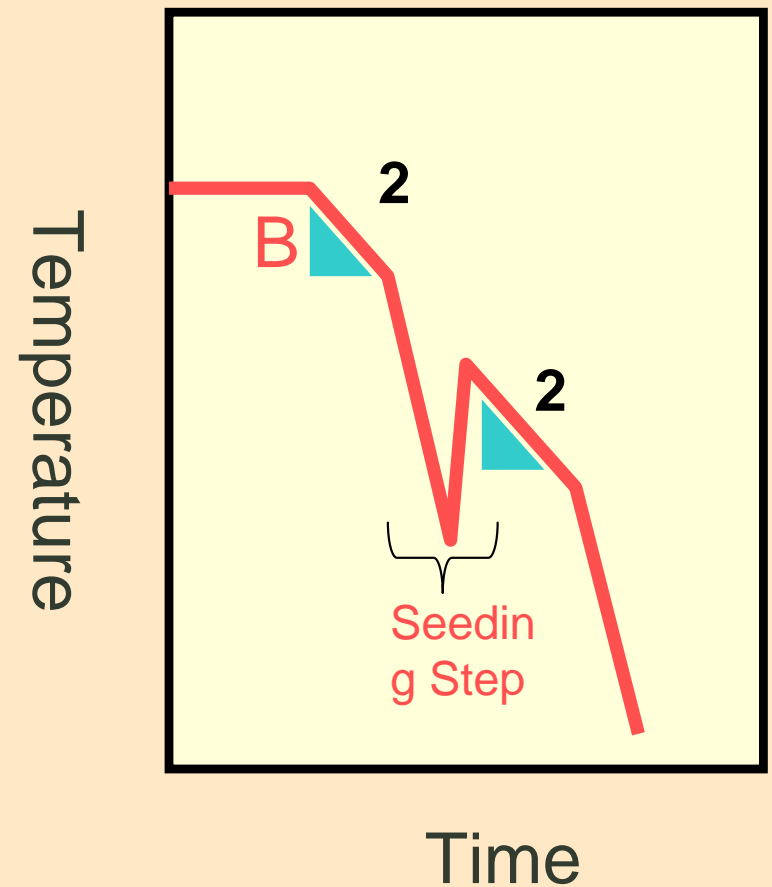


Controlled rate freezing, cont.

Segment two: cooling rate

Measure: cooling rate over high subzero temperatures

Modify: cooling rate to achieve improved survival

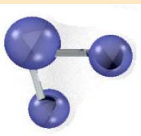


Storage

Measure: Viability as a function of storage temperature

Monitor: temperature fluctuations in storage unit near sample.

Modify: reduce storage temperature and protocol for accessing repository



Warming

Measure: warming
rate for sample.

Objective: $B > 200$
C/min

Modify thawing
protocol (increase
agitation or bath
temperature)



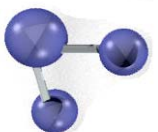
Post thaw assessment

It is easy to perform post thaw assessment badly

Kansas Stem Cell Program Sued

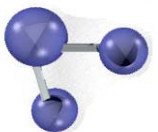
October 2006

A group of patients has sued a Kansas City stem cell transplant program alleging that it used shortcut methods to prepare the cells, resulting in the deaths of one-fourth of 40 patients treated between 1998 and 1999. Those patients died within 100 days of the treatments from complications including hemorrhages, infections and the return of their cancer. In two years, half of the patients died -- a much higher death rate than other centers.



Post thaw assessment

Assessing the viability of a frozen and thawed cell is not the same as assessing the viability of a cell



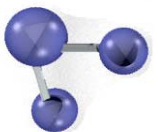
Post thaw assessment

Freezing and thawing produces:

- Changes in membrane integrity
- Metabolic function
- Tendency toward apoptosis

Implying that:

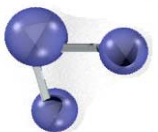
- Membrane integrity tests \neq viability
- Viability may change with time post thaw



Post thaw assessment, cont.

Helpful hints

- Do not use trypan blue
- Perform post measures at the same time point post thaw
- Prevent measurement bias (discussed in upcoming slides)
- Use multiple measures of post thaw assessment



Post thaw assessment: measures

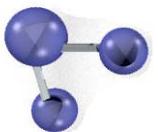
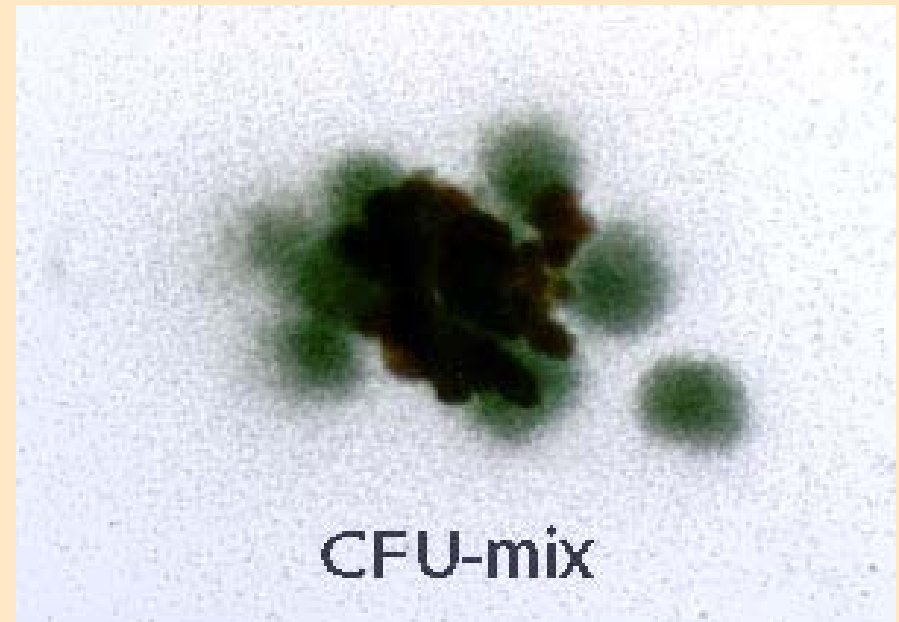
- Physical integrity (fluorescent dyes).
- Metabolic activity
- Mechanical activity (attachment, contraction)
- Mitotic activity (proliferation assay)
- Transplantation potential.

Method of assessment should depend upon the system (cell/tissue) of interest. It is common to need more than one assay.



Assays for HSCs

- CFU
- CD34+ enumeration via flow cytometry
- These assays are performed on intact cells
- 'Recovery' $> 100\%$ frequently reported



Measurement Bias

Prefreeze Assay

Total number of cells = 100

Number of target cells = 6

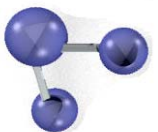
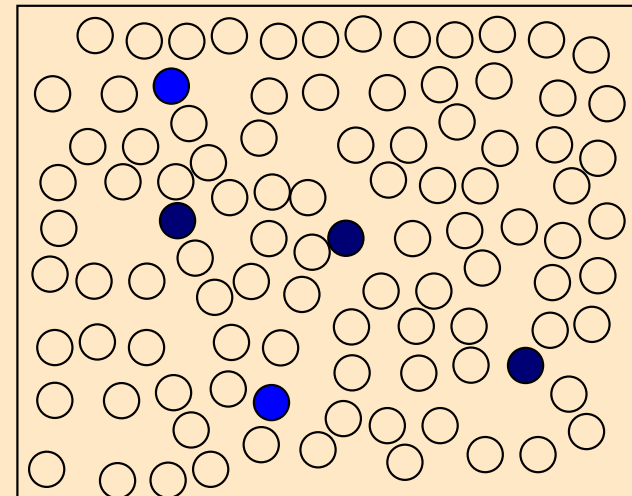
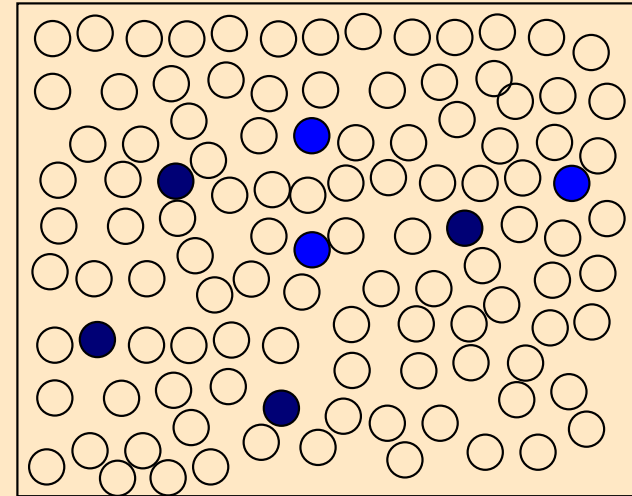
Frequency of target cells = $6/100 = 6\%$

Post thaw Assay

Total number of cells = 71

Number of target cells = 5

Frequency of target cells = $5/71 = 7\%$



Measurement Bias, cont.

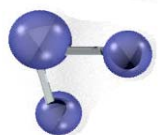
Current method of accounting

Recovery of target cell = frequency of target cell
post thaw / frequency of target cell prefreeze =
 $0.07/0.07 = 100\%!!!$

⇒ Measurement Bias

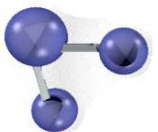
You have not accounted for the cells that were
lost

Recovery of target cell = total number of target
cells post thaw / total number of target cells
prefreeze

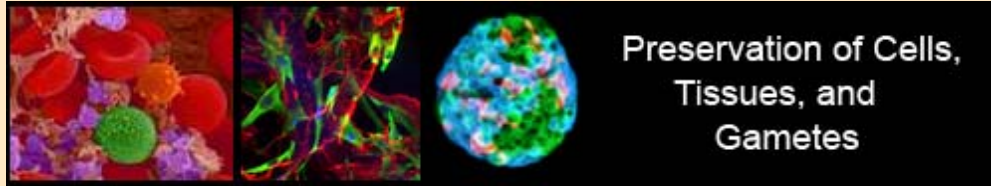


Summary

- Problems with cryopreservation can be both apparent and hidden from the operator
- Various strategies can be used to ‘debug’ problems with the protocol
- Post thaw assessment critical
- Properties of thawed cells vary from non-frozen cells
- Improper interpretation common
- Measurement bias is common



Professional short course



Topics covered:

- Liquid storage of cells and tissues.
- Fundamentals of cryopreservation
- Protocol development
- Quality systems
- Clinical cell cryopreservation
- Repository design
- Gamete preservation
- Tissue preservation
- Regulatory issues for cell/tissues

Lecturer

- Allison Hubel, University of Minnesota
- Charles Lee, University of North Carolina, Charlotte
- Ian Pope, CoreCryolab
- Ken Roberts, University of Minnesota
- Marilyn Waxberg, RCRI
- Alptekin Aksan, University of Minnesota
- David McKenna, University of Minnesota
- Diane Kadidlo, University of Minnesota

