### ONPRC Module 4B Saturday Academy
**Cryopreservation & Endangered Species**

<table>
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<tr>
<th>Main Question</th>
<th>Laboratory Questions</th>
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<td>What is &quot;cryopreservation&quot; and how can we use it to preserve fertility in endangered species?</td>
<td>• What are the main causes for cell or tissue damages cryopreservation?</td>
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<td>• What are the 2 main techniques used in cryopreservation to minimize freezing-induced damages?</td>
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<td>• What are cryopreservation agents and how do they work?</td>
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<td>• How can we preserve fertility in endangered species using cryopreservation?</td>
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### What is cryopreservation?

Cryopreservation is a process where cells or whole tissues are preserved by cooling to low sub-zero temperatures (usually the temperature of liquid nitrogen, -196°C). At these low temperatures, any biological activity, including the biochemical reactions, that would lead to cell death is effectively stopped.

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Cryopreservation Vocabulary

**Crystalline structure:**
A solid composed of molecules or atoms arranged in a very specific and orderly way that displays perfect symmetry.

**Amorphous state (glass transition):**
A solid in which there is no specific order of the atoms. Usually resulted from rapid cooling (before the molecule has enough time to organize into an orderly structure (crystal)).

http://upload.wikimedia.org/wikipedia/commons/e/e9/Sodium-chloride-3D-ionic.png

**Viscous:** Thickness of a certain fluid. Water is "thin", having a lower viscosity, while honey is "thick", having a higher viscosity.

**Cryoprotective agents (CPAs):** A substance that is used to protect biological tissue from freezing damage (damage due to ice formation). CPAs are usually very thick and have high viscosity.

**Dehydration:** The removal of water from a cell, tissue, or organism.

**Liquid Nitrogen:** Nitrogen in a liquid state at a very low temperature (-196°C). It can cause rapid freezing on contact with living tissue, which may lead to frostbite (use with caution! Always use goggles and thick gloves).

**Vitrification:** The transition of a substance into a glass (amorphous state). Cells in a solution of water and CPAs can be vitrified by rapid freezing in liquid nitrogen.

**Devitrification:** The growth of ice crystals during rewarming of a vitrified solution.

**Supercooled liquid:** Liquid at normal freezing temperature without ice formation.

**Slow rate freeze:** Freezing of biological samples using programmable steps at a very slow declined rate of temperature.

**Seeding:** Seeding is the process of inducing ice crystal formation outside the cell during slow rate freeze.

http://www.biopoliticaltimes.org/img/original/egg%20freezing.jpg
Cryopreserved Endangered Species

Cheetah
http://nationalzoo.si.edu/SCBI/EndangeredSpecies/Cheetah/

Cryobiology helped Zoo scientists breed black-footed ferrets, once thought to be extinct. (Mehgan Murphy/NZP)
http://nationalzoo.si.edu/publications/zoogoer/2010/1/FrozenAssets.cfm
Cryopreservation of sperm and embryos is being used to preserve endangered species. They are stored in liquid nitrogen – “a zoo in a freezer”. Sperm can be thawed and used for mating with a female. Embryos can be thawed and transferred to a female for live birth.

Drawing: Joel Ito, Medical Illustrator ONPRC

Timu - baby lowland gorilla, Photo Courtesy of Cincinnati Zoo

Baby Panda Bao Bao  Photo: Abby Wood, Smithsonian’s National Zoo
http://nationalzoo.si.edu/Animals/GiantPandas/PandaUpdates/
Cryopreservation Laboratory Experiments

Vitrification Experiment

• Label 1 round bottom plastic tube with “0%.”

• Label 4 round bottom glass tubes with “20%, 40%, 60%, 80%.” Place all labeled tubes in a rack.

• Add 3ml water to a round bottom plastic tube labeled with “0%” glycerol.

• Make up 20, 40, 60, and 80% glycerol in water for a total volume of 3ml (First do the calculation (table below) and try to figure out how much glycerol and water you will need to make up each solution.

<table>
<thead>
<tr>
<th>Glycerol (Gly) (ml)</th>
<th>0%</th>
<th>20%</th>
<th>40%</th>
<th>60%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (ml)</td>
<td>3.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Volume (ml)</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

• Mix each solution thoroughly with a transfer pipette (pipette the solution up and down 20 times).

• Set the timer for 30 seconds. Wear safety goggles and thick gloves and hold one tube (starting with the lowest %) with forceps. Submerge the tube into liquid nitrogen for 30 seconds. Make sure that all the solution is submerged below the surface of liquid nitrogen.

• At 30 seconds, carefully take the tube out of liquid nitrogen and observe the solution. Ask yourself, “is the solution vitrified?” How are you tell? Record your results in the table below.

• Repeat the last 2 steps for all your tubes.

Results

<table>
<thead>
<tr>
<th>Glycerol</th>
<th>0%</th>
<th>20%</th>
<th>40%</th>
<th>60%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evidence of Ice? Yes or No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitrified? Yes or No</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Students Notes or Questions:
________________________________________
________________________________________
________________________________________
Exercise

Question 1
How can you tell that a solution is vitrified successfully?

Question 2
What is the percentage of glycerol (CPA) needed for successful vitrification?

Question 3
Give an example of cryopreservation in nature.

Question 4
How can cryopreservation be used to help endangered species?

Question 5
Why is cryopreservation important for preservation of endangered species?