Innovative Cryo Enterprises L.L.C.

EMBRYO VITRIFICATION PROTOCOL

Use for vitrification of human cleavage-stage (2-cell to 8-cell) embryos or zygotes only. Do not freeze blastocysts.

Embryo Vitrification	**Zygotes & 2cells-10cells**
1) V1-Embryo	5 min at 23-26°C
2) V2-Embryo	2.5 min at 23-26°C
3) V3-Embryo	2-5 min at 23-26°C

Use a 270-300um pipette to move cells.

Transfer embryo(s) into V3-Embryo with minimal carry-over, rinse embryo(s) by pipetting to ensure complete coverage by V3. Be sure to give the embryos at least 2min in the V3 prior to vitrification.

4) Load embryo(s) in straw between columns of V3-Embryo. Aspirate the first column of V3 to the cotton plug, as this will allow the columns to remain intact.

Can also use a micro-volume device.

5) Heat seal both ends of the straw. *You must heat-seal. Periodically check seal under microscope to ensure sealer is working properly.*

6) Plunge and store in LN_2.

Notes:

Push the cotton plug in about 2 cm to have room for heat-sealing.

Label the straw directly or use a flattened 0.5cc straw as a label that can be heat-sealed onto the 0.25cc straw.

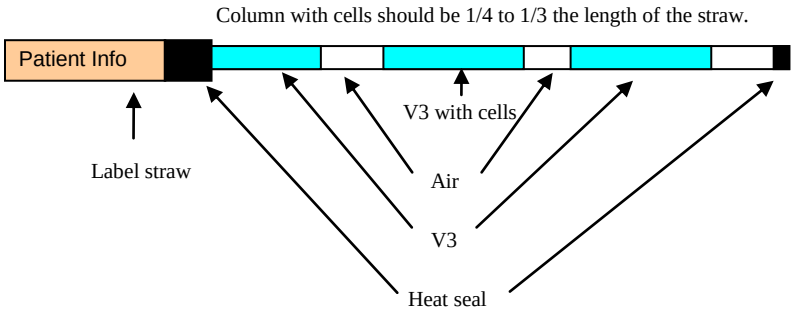
Use a 60 mm dish to freeze in. Use large drops (75-100 µl) with no oil overlay.

Use a new dish (new medium) for each set of cells frozen.

Because the time allowed in V3 can be up to 5 min, there is time to prepare multiple straws for vitrification.

Aspirate the first column of V3 to the cotton plug, as this will allow the columns to remain intact.

Store media at 5°C.



Jstachecki@gmail.com 973-632-8635

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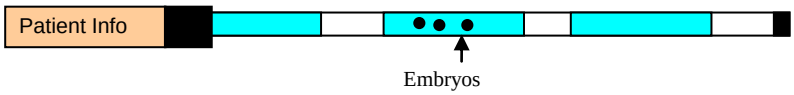
EMBRYO THAWING PROTOCOL

Please Note: Embryos are vitrified in a conventional 0.25cc straw or 0.3cc CBS straw, but other devices can be used. The cells are located in the middle column. See diagram below.



Embryo Thawing Protocol:

1) Thaw 3 sec in room temperature air and then 7 sec in 37°C water.

   Pull straw out of tank slowly and hold in air. Move straw around in the water bath, make sure straw is completely submerged.

2) Quickly wipe straw and cut off top heat-sealed end of straw, insert syringe and cut off other sealed end.
3) Empty contents of straw into 300ul of T1-Embryo in a 60mm dish.
4) Shake dish on workbench for 30 sec to mix contents.

   This helps dilute out the cryoprotectants and allows the embryo(s) to settle to the bottom of the drop. Be careful not to let the media touch the sides of the dish as this will make locating the cell(s) more difficult. Start timer once embryo(s) are expelled.

Transfer embryo(s) to (50ul-75ul drops microdrops under oil):

5) T1-Embryo	3 min at 23-26°C
6) T2-Embryo	3 min at 23-26°C
7) T3-Embryo	3 min at 23-26°C
8) T4-Embryo	3 min at 23-26°C
9) T5-Embryo	3 min on 37°C place on warmer
10) Equilibrated, warmed Culture medium.	37°C incubator

For optimal performance:

For best results use a new dish (new medium) for each straw thawed.

Store media at 5°C.

Culture thawed cells in media containing 15-20% HSA or a similar protein for the first day after thawing.

We suggest culturing the thawed embryos overnight before transfer. This will allow verification of cleavage and further development post-thaw.

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