

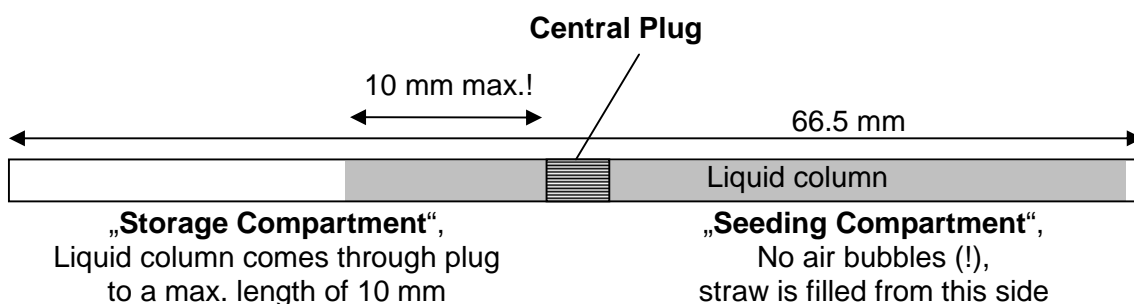
Quick guide: using CTE security straws for cryopreservation of pronuclear stages, embryos and sperm

1. How to prepare straws for pronuclear stages and embryos (use straws with plug, 66.5 mm)

Straws are filled bubble-free with the last cryopreservation solution **before** loading any cells or embryos. This is different from most other straw types. Use sterile, mouse embryo tested syringes with sterile, long (60 mm) cannula to fill straws.

Thoroughly mix all cryopreservation solutions straight before using them! This is essential for successful cryopreservation.

- a) Attach the cannula to the syringe, then fill syringe with the last cryopreservation solution.
- b) To remove any air from the cannula, release a droplet of medium from its tip before filling any straw.
- c) Hold the straw horizontally and insert the cannula into one of its ends, almost reaching the plug. Slowly fill half of the straw with cryopreservation solution, simultaneously retracting the cannula. This half of the straw is dedicated to automatic seeding (“seeding compartment”) and completely filled. **Avoid any major air bubbles here as this will impair ice growth in the seeding compartment. Do not move the plug by touching it with the tip of the cannula!**
- d) Turn the straw to a vertical position to make the solution enter the “storage compartment” (other half of the straw) through the plug. The storage compartment is not filled actively. The liquid column in the storage compartment should come to a **maximum length of 10 mm**. If too long, simply invert the straw to reduce the liquid volume in the storage compartment.
- e) When the liquid column in the storage compartment has reached adequate length, orient the straw horizontally again. Then refill the seeding compartment, leaving the last 2 mm empty for proper sealing. Heat seal the opening of the seeding compartment without locking in major air bubbles.
- f) Handle the straws carefully and store in a sterile Petri dish until loading the cells.



2. Loading straws with pronuclear stages or embryos

For loading pronuclear (PN) stages or embryos into the straws, use the same capillaries you are normally using for handling and transferring cells and embryos (e.g., unopettes or stripper tips). Capillaries must be long enough to almost reach the central plug of the straw.

- a) Switch your stereo microscope to low magnification, then aspirate as many PN stages / embryos with your capillary as you want to load into one straw. Take one of the pre-filled straws and hold both the straw and the capillary horizontally in the focal plane of the stereo microscope. Insert the capillary into the straw and place the PN stages / embryos in the liquid column of the storage compartment. Control the whole loading process through the eyepieces of your stereo microscope.
- b) Make sure that there are no air bubbles left in the liquid column of the storage compartment.
- c) Place the PN stages / embryos close to the central plug of the straw. However, avoid touching the plug with the capillary!
- d) After loading the straw with cells, heat seal the opening of the storage compartment. Carefully load the straws into the straw holder of the freezer.

3. Labelling of straws for PN stages / embryos

Wrap self-adhesive, cryo-proof labels around the lower part of the straw (corresponding to the seeding compartment). This will keep the storage compartment accessible for visual control. There is no need to label the reference straw covering the temperature sensor.

4. How to prepare straws for ejaculate (use straws without plug, 66.5 mm)

Straws are heat sealed on one end before filling them with a mixture of ejaculate and cryoprotectant.

- a) Carefully aspirate the mixture (ejaculate + cryoprotectant) into a sterile, non-toxic syringe.
- b) Attach a sterile, long cannula (min. 60 mm) to the syringe.
- c) Hold the straw vertically, then insert the cannula to the bottom of the straw. Be careful not to penetrate the straw walls. Slowly fill the straw, avoiding any air bubbles. Gradually retract the cannula while filling the straw. Leave the upper 15-20 mm of the straw empty for pressure compensation! The inner straw walls of this area should be kept as dry as possible.
- d) Heat seal the upper end of the straw.

Please note:

You may use straws with plug for highly sensitive sperm samples (e.g., OAT). When doing so, fill the seeding compartment of the straws first, using pure sperm cryoprotectant. The storage compartment, however, should only be filled by 1 mm above the plug. Heat seal the seeding compartment as usual (see 1.) and fill the storage compartment with a mixture of ejaculate and cryoprotectant. Keep half of the storage compartment empty for pressure compensation!

Validate this procedure by using highly diluted normal semen and evaluate sperm concentration and survival rate before cryopreserving critical OAT samples!

5. How to handle the heat sealer

- a) Inspect the jaws of the welding tongs in regular intervals. The jaws of the heat sealer can be changed after loosening the small hexagon sockets. Before using the welding tongs of the heat sealer, make sure that the welding jaws are in parallel orientation (i.e., they must not be wedged) and the hexagon sockets are tightened. **The welding jaws must close without gap when pressed against each other!** Otherwise the welding tongs might not be able to properly seal straws.
- b) Inspect the silicon protection of the welding jaws
The silicon protection must protrude the welding jaws by approx. 1 mm! Otherwise the welding tongs might burn holes into the straws or the straws and the welding jaws might stick together. Keep the silicon covers clean by removing media remnants with a paper towel moistened with 70 % alcohol or water immediately after sealing. The silicon covers have to be changed from time to time.
- c) Sealing temperature should be set to 185 – 190°C to seal CTE straws reliably. To change sealing temperature, press the “↓” button once until the “o” symbol in the display starts blinking, then change temperature by using “+” and “-“ buttons.
- d) Sealing straws
Hold the welding tongs horizontally and place the end of a straw centrally on one of the welding jaws. Then press the welding tongs and hold for 3-5 sec (count in your head). The welding seams should be approx. 2 mm long.
- e) Inspect the welding seams
Practice on straws without cells but filled with liquid before switching to routine sealing! Appropriate welding seams should be smooth and transparent and lack any inclusions or channels. Check under the stereo microscope!
- f) The welding seams of all straws should be similar in length to maintain equal cooling conditions for all cells.

6. Recommended freezing program for PN stages and embryos

Once a month and after changing the Pt100 temperature sensor, an auto-zero calibration has to be performed to ensure reliable operation of your CTE freezer (see manual).

Program:

Ramp	Cooling rate (°C/min)	Final temperature (°C)
1	- 5.0	4
2	- 1.0	- 1
3	- 0.3	- 34
4	- 50	- 190

(Total duration of this temperature profile: 121 min)

Please note: when watching ice crystal formation inside the straws, do not mistake frost formation on the outer surface of the straws for ice growth! Frost formation might mimic fast ice growth or even subsequent thawing whereas real ice formation will progress slowly and continuously. If necessary, use a magnifying glass to identify real ice growth.

Reference straw and reference straw holder

Always use a sperm straw (66.5 mm, no plug) as reference straws. Seal this straw on one end, then fill with cryopreservation solution using a cannula. Avoid any air bubbles inside the reference straw. Carefully (!) insert the cone of the temperature sensor into the upper end of the reference straw. **Avoid deflecting the temperature sensor from its longitudinal axis as it is coated by glass and sensitive towards breakage!** Spilling cryopreservation solution has to be removed from the outside of the reference straw (paper towel) as it might disturb temperature measurements. Use a new reference straw for each cryopreservation procedure.



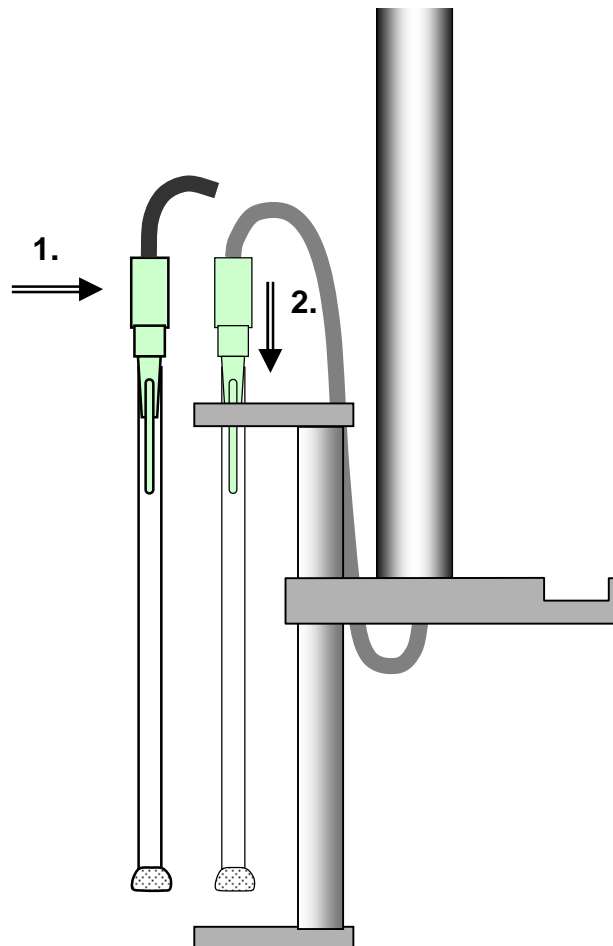
Caution: *The platinum sensing resistor is coated with glass (diameter 0.9 mm) and is highly sensitive towards any mechanical impact!*

PLEASE NOTE: for cryopreservation of ejaculate in the respective straws (no plug), the reference straw is left empty (no filling with cryopreservation solution)!

Hold the temperature sensor and attached reference straw vertically. The cone of the temperature sensor must be above the reference straw holder which is vertically oriented. The welding seam of the reference straw must be correctly oriented to fit the slot of the lower part of the reference straw holder (see figure). Insert the reference straw into the upper part of the straw holder from the side, then carefully move downwards and plug the cone of the temperature sensor into the drill hole of the holder. Simultaneously, the welding seam of the reference straw must fit its slot. **Do not try to latch the cone of the temperature sensor from the side!**



Do not try to latch the cone of the temperature sensor from the side. Danger of breakage!



Inserting the reference straw: two steps!

The reference straw holder can be turned by 90° as both auto-zero calibration and seeding point determination (see manual) have to be performed with special straws (EC-02; rectangular shape with “tail”). **To turn the reference straw holder, do not loosen the screw of the swivel joint!** It is sufficient to just pull the holder by approx. 1 mm and turn it by 90°. A spring mechanism will arrest it in the new position. Do not turn the reference straw holder when the temperature sensor is attached!

When rectangular straws and horizontal holder position are used, the straws are attached to the holder in the same way as shown above. Make sure that the “tail” of the straw is vertically oriented.

7. Handling and storage of straws

Make sure that the straws are never warmed to temperatures above -130°C after completing a freezing program. Use the CTE handling unit to pack the straws without warming. We recommend to use multi purpose cassettes for packing the straws of an individual patient. Cassettes can be stored in conventional canisters of liquid nitrogen storage tanks.

8. How to remove PN stages / embryos from straws

Use sterile scissors and a micro pipettor (see figure) to open and flush straws containing PN stages or embryos. For flushing, a sterile syringe filled with air and a piece of silicon tubing fitting the straw diameter may also be used.

- a) After performing the common thawing steps, hold the straw horizontally. Carefully cut both ends with sterile scissors.
- b) Briefly turn the straw to a vertical position, storage compartment down, to make the liquid column move downwards, approx. 2 mm away from the open end of the seeding compartment. This will help to keep the flushing device clean.
- c) Turn the straw back to horizontal orientation and carefully attach the micro pipettor or other flushing device to the open end of the seeding compartment. The flushing device should be tightly connected to the straw.
- d) Hold the open end of the storage compartment above the dish or well you want to place the cells in.
- e) Carefully (!) empty the straw by slowly pressing air from your flushing device into the seeding compartment of the straw. The cryopreservation solution from the seeding compartment will flush the plug, making sure that no cell is left in the straw.
- f) After all liquid has left, foam will be generated by the air passing through the plug. Do not flush the foam into the dish or well as it will considerably impair your sight!
- g) Check under the stereo microscope whether the full number of PN stages / embryos has been regained from the straw.

To flush the straw for missing cells, have a sterile, non-toxic syringe with long (60 mm) cannula ready. Before thawing the straws, fill this syringe with 0.5 ml thawing solution without air bubbles.

In case a PN stage or embryo is missing, refill the seeding compartment of the straw with thawing solution (see 1.). Let part of the liquid percolate through the plug by holding the straw vertically, then attach the air-filled flushing device again to flush the straw a second time.



Micro pipettor for flushing straws

8. Auto-zero calibration of the Pt 100 sensor

Please note: For auto-zero calibration of the Pt 100 sensor, special straws with 90° “tail” have to be used! For this purpose, the reference straw holder has to be turned by 90° (to horizontal position).

In the new security straws, ice crystal formation is accurately controlled. Consequently, the heat of crystallization will rapidly dissipate, there will be no heat peak which is used by the machine to determine the zero point.

Please note:

MTG Medical Technology Vertriebs-GmbH and CTE Cryo Technik Erlangen GmbH have established the procedures described above in all conscience. Nevertheless, we strongly recommend to practice all steps extensively on spare cells before integrating them into clinical routine.

We strongly advise you to run tests on both the new freezer and associated procedures before cryopreserving valuable PN stages, embryos or semen. For doing tests, immature or non-fertilized oocytes as well as sperm from diagnostic samples can be used.

According to individual lab setups or personal preferences, slight modifications to the procedures described above might be beneficial. MTG and CTE assume no liability with regard to any adverse effects or damage related to the use of this quick guide.