

# VivaThermic Ultra-300 and VivaThermic Super-150

YingYing Zhou, Biological Handling Division, Vivakor  
Tannin Fuja\*, Vivakor

**Abstract:** Freezing injury, occurred during routine cryopreservation practices, has a significant impact on the cell survival rate and viability. It often causes problems for biological materials in maintaining their long desired efficacy and integrity, and consequently leads to the failure of the biological materials. Existing products for cryopreservation cannot provide accurate temperature control or achieve fast cooling/thawing rates, resulting in less than optimal cell cryosurvival. Vivakor is actively developing the technologies required for the successful cryopreservation of diverse biological samples with improved recovery of viable cells post-cryopreservation. In this update, we introduce two new products developed at Vivakor: (a) VivaThermic ultra-300 and (b) VivaThermic super-150, and present the comparison studies with current market leading cryovials. Results show that VivaThermic ultra-300 and VivaThermic super-150 are superior to the existing cryovials. VivaThermic ultra-300 is 300% better in temperature control and can achieve 3 times faster cooling/thawing rates whereas VivaThermic super-150 is 150% better in temperature and can achieve 1.5 times faster cooling/thawing rates. In addition, existing cryovials are not centrifugable. To process biomaterials after cryopreservation, biomaterials have to be transferred into a centrifuge tube. This is time-consuming process, wastes bioconsumables, and may cause cross-contamination and is time-consuming. Contrary to the existing cryovials, VivaThermic ultra-300 and VivaThermic Super-150 are designed to fit into standard centrifuge motors, and can withstand the centrifuge force up to 20,000 times gravity. Biomaterials cryopreserved in VivaThermic ultra-300 and VivaThermic Super-150 can be directly centrifuged after thawing.

Nearly \$1.5 billion are spent annually in the cryopreservation of biological materials for clinical and research applications. Cryopreservation techniques are routinely used in regenerative and reproductive medicine, pharmacology, basic scientific research, transgenics, animal breeding and agriculture applications. This process provides a therapeutic option in treatment of infertility and an alternative in the breeding and preservation of domestic and wild animals, where embryos, oocytes and sperm have all been successfully cryopreserved, stored and recovered [1, 2, 11, 12, 13].

Cryopreservation of eukaryotic cells is complicated by the fragility of the cell membrane composed of a phospholipid bilayer embedded with numerous proteins, channels and biochemical moieties. Mammalian oocytes and embryos, while

possessing unique membrane protectant features, are particularly sensitive to cryoinjuries. This has been attributed to their increased total lipid content and varied developmental stage. Most cryoinjuries are the result of intracellular ice crystal formation during freezing and thawing processes [11]. To eliminate deleterious ice crystal formation, ice-free solidification, which is achieved by rapid cooling using concentrated cryoprotectants, has been applied to the cryopreservation of mammalian reproductive cells. Successful cryopreservation has been reported at cooling rates approximately 200 °C/min with the aid of highly concentrated cryoprotectants [9]. To minimize the potential toxic effects of cryoprotectants, current ice-free solidification methods achieve ultra rapid cooling (over 10<sup>4</sup> °C/min) by reducing the total sample volume and requiring direct sample contact with liquid nitrogen (LN<sub>2</sub>).

In spite of its great potential in cryopreservation, ice-free solidification is not widely used in clinical applications and market use is primarily restricted to experimental laboratories. There are various reasons for this. First, direct contact of medium solution with LN<sub>2</sub> raises the concern of cross-contamination. Secondly, the small volume approach usually lacks the capability of direct transfer of samples from different cryopreservation phases. It requires special tools and complex procedures to aid the sample transfer from the freezing stage to the storage vessel and on to sample reloading. Thirdly, small samples (<1 µL) may not tolerate more than a fraction of a second in accidental warming. This further increases the difficulty in the sample handling and imposes a potential risk for long-term storage. Lastly, various biological samples such as hepatocytes and human embryo stem cells cannot be vitrified in very small volumes.

To minimize the risk of cross-contamination, many recently introduced fast-cooling techniques separate the cooling and storage phases to reduce the direct contact with LN<sub>2</sub>, allowing this small amount of LN<sub>2</sub> to be filtered or UV sterilized [10]. Another technology takes the hybrid approach, (e.g. the straw-in-straw method) by wrapping the OPS straws into the 0.5 mL protective straws before cooling. The rate of the cooling diminished to 400°C/min or even 200 °C/min (10 times less than the rates achievable in sealed 0.25 mL common insemination straws) [5, 6, 8, 9]. Although it has been reported that cooling at a rate of 400°C/min was appropriate for human immature oocytes and blastocytes, and a moderately low cooling rate of 120°C/min was effective for fast-cooling of human embryos [9],

\*Corresponding author

compromises in cooling and thawing rates may jeopardize biological samples, especially those sensitive to cryo-injuries.

To overcome the inadequate heat transfer/cooling rates experienced with existing devices, cryobiologists current takes the approach by over-compensating with excessive heating or cooling. This current strategy leads to variation in cooling and warming rates and subsequently results in less than optimal cell cryosurvival. If one can precisely control the temperature of the specimen that resides within the walls of the cryovial, one should be able to reduce the risk of intercellular ice crystal formation by optimizing cell dehydration, cryoprotectant permeation and ultimately improve cell cryosurvival.

### TESTING AND COMPARISON STUDY

VivaThermic Ultra-300 and VivaThermic Super-150 are patent-pending disposable vials that can be used to process and store biological specimens at cryogenic temperature. Figure 1 shows the VivaThermic Ultra-300 and Figure 2 shows the VivaThermic Super-150. They are designed to fit into standard centrifuge motors, and thus can be used for bio-materials' separation. Unique cap designs allow the seal without using o-rings. Special design and material selection provide the entire surface for writing.



Figure 1. VivaThermic Ultra-300



Figure 2. VivaThermic Super-150

In this comparison study, we perform the following tests as shown in Table I.

Table – Tests Performed

1	Thermal Cycle Test
2	Seal/Leakage Test
3	Write-ability Test
4	Autoclave test
5	MCF Measurement
6	Cooling/Thawing Rate Measurement

Table II – Vials Tested

Manufacturer/Distributor	Vials
Vivakor	VivaThermic Ultra-300
Vivakor	VivaThermic Super-150
Fisher Scientific (033377P)	Market Existing Vial-F
VWR International (T308-2A)	Market Existing Vial-V

Vials being tested are shown in Table II. In addition to VivaThermic Ultra-300 and VivaThermic Super-100, we select 2 different vials from 2 market leading distributors. They are: (1) 033377P from Fisher Scientific, and (2) T308-2A from VWR International.

### 1. Thermal Cycle Test

Thermal cycle test is performed on three samples from each type of vials listed in Table II. Samples are filled with 1.0 ml of 20% dimethyl sulfoxide (DMSO) solution with no cells. The following protocol is used for the testing:

- Immerse the vials in liquid nitrogen for 5 minutes
- Take the vials out of liquid nitrogen
- Immerse the vials in 37 °C warm water for 5 minutes
- Take the vials out of warm water
- Air-dry the vials
- Examine the vials for cracks, surface defects or any other deformation
- Repeat the process for 5 times

All of the vials perform well. No cracks or any other defects have been observed.

### 2. Seal/Leakage Test

During the thermal cycle test, examination is also performed to detect the seal/leakage issue. It is found that two out of three Market Existing Vial-F from Fisher Scientific have loose caps. The amount of the DMSO solution in one of the two vials was less than the original. This may cause a severe cross-contamination in the cryopreservation.

Unique cap designs of VivaThermic Ultra-300 and VivaThermic Super-150 seal the vials very well. All caps are tight and no leakage has been found in any of VivaThermic Ultra-300 and VivaThermic Super-150 vials.

### 3. Write-ability Test

Write-ability test is performed with two types of markers: (1) VWR lab marker, and (2) Stratpie marker. Three tests are performed, following the protocol below:

First test:

- Write with both markers
- Wipe with Kimwipes wipers
- Check the readability

Second test

- Write with both markers
- Spray 70% ethonal
- Wipe with Kimwipes wipers
- Check the readability

### Third test

- Write with both markers
- Immerse the vials in liquid nitrogen
- Thaw the vials with warm water
- Air-dry the vials
- Spray 70% ethonal
- Wipe with Kimwipes wipers
- Check the readability

Each test is conducted on three samples from each type of vials. Based on the observation, it can be concluded that

#### For Market Existing Vial-F from Fisher Scientific

- Marks can be easily wiped off no matter which markers are used with or without 70% ethonal spray

#### For Market Existing Vial-V from VWR International

- Marks can be made only on the writable area, which is a small portion of the entire vial surface
- Marks can withstand 70% spray only when it was written by VWR lab marker
- Marks can be completely wiped off if Stratprie marker is used with 70% ethonal spray

#### For VivaThermic Ultra-300

- Entire vial surface is writable
- Marks can withstand 70% spray only when it was written by VWR lab marker
- Marks are still readable when Stratprie marker is used and after 70% ethonal spray and wiping

#### For VivaThermic Super-150

- Similar to VivaThermic Ultra-300

## 4. Autoclave Test

Omega ST Autoclave is used to test the autoclave-ability. Ten samples from each type of vials are placed into 250 ml VWR 250 ml glass bottles. The caps of the glass bottles are set loose. Program 4 is used and samples are autoclaved at 121°C for 30 mins.

Samples are examined after autoclaving. No defect or deformation is observed.

## 5. MCF Measurement

Eppendorf centrifuge 5810R is used to determine the maximum centrifuge force (MCF) the vials can withstand. Since Market Existing Vial-F from Fisher Scientific and Market Existing Vial-V from VWR International cannot fit into the centrifuge motor, it is impossible to determine the MCF these two vials can sustain.

To determine the MCF, a serial of 5 tests is performed, starting from a lower force (10,000 × g) and gradually increasing to

20,000 × g. Each test is performed on 2 vials filled with 1 ml of 20% DMSO solution. The testing protocol is the follows:

- Test at 10,000 × g for 5 mins, and examine the vials
- Test at 12,500 × g for 5 mins, and examine the vials
- Test at 15,000 × g for 5 mins, and examine the vials
- Test at 17,500 × g for 5 mins, and examine the vials
- Test at 20,000 × g for 5 mins, and examine the vials

It is found that both VivaThermic Ultra-300 and VivaThermic-150 can sustain centrifuge force up to 20,000 × g. Since 20,000 × g is the maximum force that Eppendorf centrifuge 5810R can provide, we are unable to test the vials beyond this force.

To confirm the result, 4 more VivaThermic Ultra-300 and 4 more VivaThermic-150 with 1 ml of 20% DMSO solution are centrifuged at 20,000 × g for 5 mins. All vials are examined after centrifugation, and no defects or deformation is observed.

Furthermore, 4 VivaThermic Ultra-300 and 4 VivaThermic-150 with 1 ml of 20% DMSO solution are centrifuged at 20,000 × g for 5 mins after being freezing and thawing crypreservation cycle to determine the impact of freezing/thawing. All vials are examined after centrifugation, and no defects or deformation is observed.

## 6. Cooling/Thawing Rate Measurement

Cooling and thawing is measured by an apparatus designed and built in-house. Thermocouples and a data acquisition system are used to record the temperature continuously. Tested vials are filled with 1.2 ml of 20% DMSO solution with no cells. Liquid nitrogen is used as the cooling medium whereas 37 °C warm water for thawing.

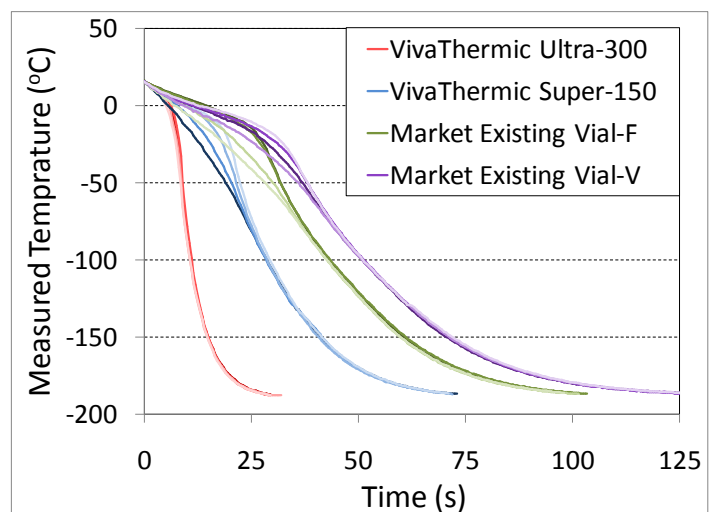


Figure 3. Temperature measurement (cooling)

Temperature measurement is performed on randomly chosen samples, five from VivaThermic Ultra-300, five from

VivaThermic Super-150, four from Market Existing Vial-F from Fisher Scientific and four from Market Existing Vial-V from VWR International. The measured temperature vs. time at the stage of cooling is shown in Figure 3.

The cooling rate can be deduced from the continuously measured temperature by taking the derivatives. Shown in Figure 4 are the average cooling rates at temperature range of 15 to -15 °C and at temperature range of -15 to -130 °C. We choose these two ranges for comparison purpose because they are critical for cryopreservation. It is known that ice-crystallization typically occurs around temperature of -15 °C depending on the usage of cryo-protectants and biological reaction stops at -130 °C.

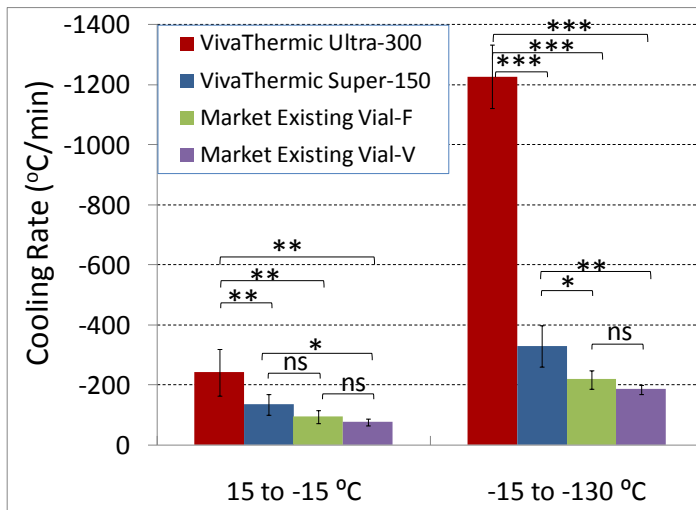


Figure 4. Comparison of cooling rates

t-test is used to compare the cooling rates. It assesses whether the mean value of the cooling rate achieved by one type of vials is statistically different from the mean value achieved by another. The statistical significance shown in Figure 4 follows the convention as indicated in Table III.

Table III – Statistic significance

P value	Wording	Symbol
>0.05	Not significant	ns
0.01 to 0.05	Significant	*
0.001 to 0.01	Very significant	**
< 0.001	Extremely significant	***

Temperature measurement and similar analysis are performed on the thawing stage, and results are shown in Figure 5. It shows the average thawing rates at three temperature ranges for the tested vials: (a) from -130 to -50 °C, (b) from -50 to -20 °C, and (c) -20 to 15 °C. Statistic significance is also shown in Figure 5 following the same convention as in Table III.

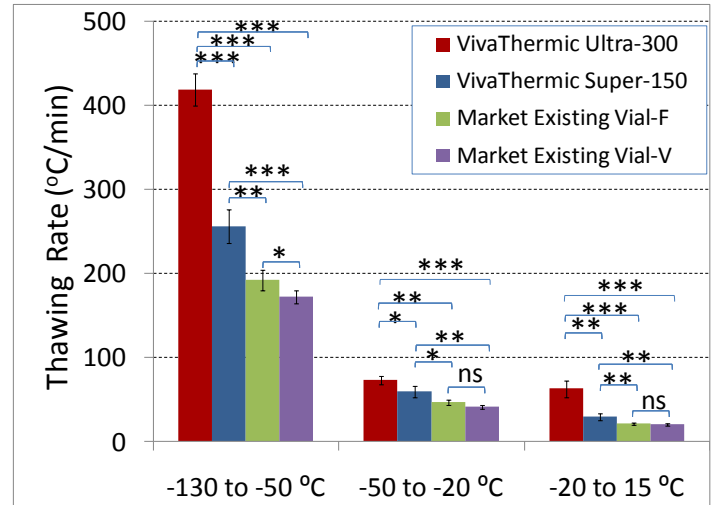


Figure 5. Comparison of thawing rates

Further analysis reveals that thawing biocontent frozen in VivaThermic Ultra-300 and Super-150 takes much shorter time. As shown in Figure 6, thawing 1.2 ml 20% DMSO from -20 °C to 4 °C takes approximately 30 s and 50 s for Vivathermic Ultra-300 and Super-150 respectively, comparing to 69 s and 71 s for Market Existing Vial-F and Vial-V. They are statistically very different.

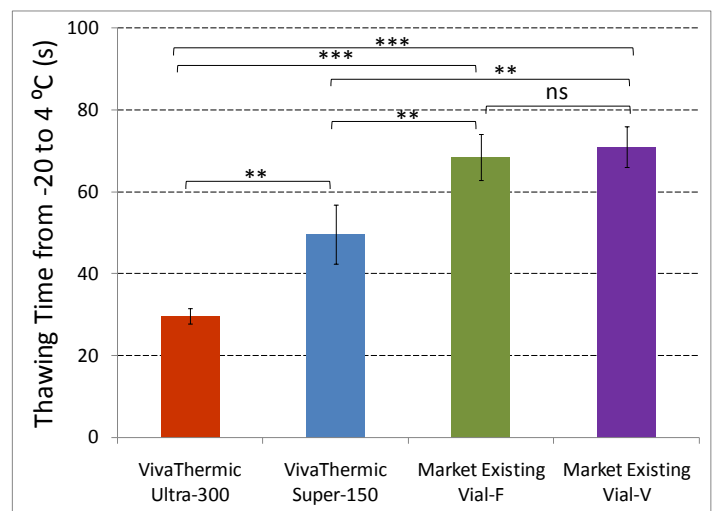


Figure 6. Comparison of Thawing Time from -20 to 4 °C

Based on the testing results and the statistic significance analysis, it can be concluded that in terms of temperature control and cooling/thawing rates,

- Market Existing Vial-F from Fisher Scientific is not statistically different from Market Existing Vial-V from VWR International
- VivaThermic Super-150 is different from both Market Existing Vial-F and Market Existing Vial-V. The

difference is statistically significant or statistically very significant

- Cooling/thawing rates achieved by VivaThermic Super-150 is approximately 1.5 times higher than those achieved by either Market Existing Vial-F or Market Existing Vial-V
- VivaThermic Ultra-300 is different from both Market Existing Vial-F and Market Existing Vial-V. Overall, the difference is statistically extremely significant
- Cooling/thawing rates achieved by VivaThermic Ultra-300 is approximately 3 times higher than those achieved by either Market Existing Vial-F or Market Existing Vial-V
- Thawing biocontent frozen in VivaThermic Ultra-300 and Super 150 takes much shorter time, approximately 40% and 70% of the time for thawing biocontent frozen in the market existing vials tested

At the beginning of the product development, a measurement is also performed to compare the prototype of VivaThermic Ultra-300 with the existing stainless steel microvial from BioSpec. The measured temperature and its derived cooling rate vs. concurrent temperature are shown in Figure 6 and Figure 7. Plotted also in the Figure 6 is the temperature measured by directly inserting a thermocouple into the LN<sub>2</sub> for the evaluation of thermocouple sensitivity and response time. This temperature measurement also indicates the fastest cooling rate (average of approximately -1600 °C/min) which can be measured by this setup without tangible error.

As shown in Figure 7 and 8, the cooling rate achieved by the prototype of VivaThermic Ultra-300 is the highest among the three tested vials. It quickly reaches -500 °C/min or higher, and maintains at this elevated level until the temperature of the tested solution gets to -130 °C. It is two and half times higher than those by Fisher conventional cryovial and Biospec stainless steel vial, which are approximately at -200 °C/min.

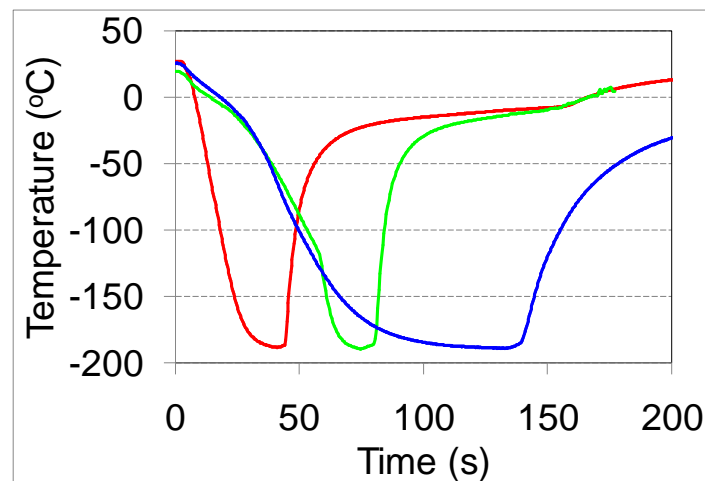


Figure 7. Temperature measurement: VivaThermic Ultra-300 prototype (red), Stainless steel vial (green), Market Existing-F (blue)

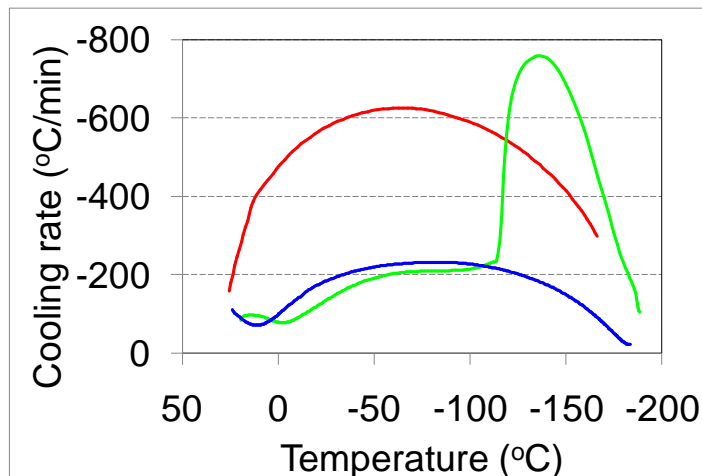


Figure 8. Comparison of cooling rates: VivaThermic Ultra-300 prototype (red), Stainless steel vial (green), Market Existing-F (blue)

It is necessary to emphasize that the cooling/thawing rates achieved by VivaThermic Ultra-300 are achieved with 1.2 ml solution, which is several orders of magnitude higher than the small volumes applied in existing ultra-fast cooling technologies.

## RESULTS AND DISCUSSION

We have demonstrated that VivaThermic Ultra-300 and VivaThermic Super-150, with thoughtful material selection and unique designs, are superior to market existing products. To summarize, we highlight the major advantages below:

- 2-in-1 design allows VivaThermic Ultra-300 and VivaThermic-150 to be used as conventional cryovials and as centrifuge tubes. As a result, it saves time, reduces the bioconsumables, and decreases or eliminates the risk of cross-contaminations
- VivaThermic Ultra-300 and VivaThermic Super-150 can achieve 3 or 1.5 times faster cooling/thawing rates than those achieved by market existing vials, respectively
- It takes much shorter time to thaw biocontent frozen in VivaThermic Ultra-300 and Super-150, approximately 40% and 70% of the time for thawing biocontent frozen in the market existing vials tested
- VivaThermic Ultra-300 and VivaThermic Super-150 can provide 300% and 150% better temperature control than market existing vials, respectively
- VivaThermic Ultra-300 and VivaThermic Super-150 are centrifugable, with MCF up to 20,000 × g
- Unique design allows the sealing without the usage of o-rings

- VivaThermic Ultra-300 and VivaThermic Super-150 are autoclavable
- Entire surface of VivaThermic Ultra-300 and VivaThermic Super-150 is writable

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