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Protocol Development for Vitrification of Tissue-Engineered Cartilage

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The success of tissue-engineered cartilage constructs (TECCs) as treatment options for healing cartilage defects can only be achieved if suitable preservation methods are found that can maintain their viability and function. Simply lowering the temperature of cells and tissues to below their freezing point invariably destroys them due to ice crystals that form in the water-laden cells and tissues. In addition, high salt concentrations that result from removal of water due to ice formation create a toxic imbalance. If the formation of ice crystals can be minimized while still halting metabolic activity of cells at low temperatures, then the viability and functionality of the preserved tissue may be maintained.

Several important variables have been studied that affect cryopreservation of cells and tissues to maximize cell survival and tissue functionality: cryoprotectant agents, cryoprotectant concentrations, methods of introduction and elution, cooling and warming rates, and storage temperatures.[1–5] In 1949, Polge *et al.*[6] discovered a significant breakthrough in the role of glycerol as an intracellular cryoprotectant, which permeates cells to minimize intracellular ice formation. Later on, other intracellular cryoprotectants were discovered such as dimethylsulfoxide (Me₂SO)[7] and extracellular cryoprotectants such as starches and sucrose, which protect cell membranes or act as osmotic buffers.[2,8] Formamide, acetamide and other such chemicals reduce the toxicity of cryoprotectants used in high concentration. Amides, in general, are weak cryoprotectants but improve cell viability when combined with cryoprotectants.[9] Combinations of intracellular and extracellular cryoprotectants can have additive or synergistic effects on cell viability upon rewarming of cryopreserved samples.[1]

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¹Sigmacoat™ is a trademark of Sigma-Aldrich, Inc.

²alamarBlue® is a registered trademark of Invitrogen Corp.

There are two main approaches to cryopreservation for cells, tissues, and organs. The first is the conventional freezing technique where up to 30% of cell water is substituted by a cryoprotective compound, usually Me₂SO, permitting the storage of many types of cells in vitrified channels within ice.[1] Conventional freezing relies on slow cooling and warming rates. In contrast, vitrification uses high concentrations of cryoprotectants resulting in greater than 50% replacement of water in the cell or tissue. When combined with fast cooling and rewarming rates, both intracellular and extracellular ice formation is avoided. This method minimizes or prevents formation of ice, creating a glass-like material, while still halting metabolic activity and maintaining an osmotic balance between cells and their environment. The major limitation of vitrification, however, is the potential cytotoxicity of the high cryoprotectant concentrations employed.[1]

Representative live-dead staining, original magnification 10x, post-rewarming across the thickness of a 28 day TECC vitrified with VS70 in four steps in the PCC bioreactor. The surface (top) shows a predominance of live (green) cells. Live cells were also found in the interior of the construct. Dead cells and cell fragments fluoresced red. This TECC had 50.1% live cells.

Vitrification has been shown to provide effective preservation for a number of tissues including oocytes, early embryos, cartilage, skin, blood vessels (natural and engineered), and heart valves.[10–19] Furthermore, vitrified arterial blood vessels and tissue-engineered vascular grafts have been shown to maintain viscoelastic properties similar to fresh vascular tissues while demonstrating superior biomechanical performance when compared with frozen cryopreserved specimens.[15–17] Song *et al.* achieved significantly better viability by employing vitrification (80%) with a 55% (w/v) vitrification solution (VS), known as VS55, than with conventional freezing (12.8%) employing 0.6 mm thick rabbit articular cartilage.[20] Guan *et al.* demonstrated that 51% viability occurred when bovine cartilage was vitrified, and only 5% viability when bovine cartilage was preserved without cryoprotectants.[21] Subsequently, we found that VS55 was inadequate for preservation of thicker, large mammal cartilage and that better preservation was obtained using a more concentrated 83% vitrification formulation.[22,23] Kuleshova *et al.* demonstrated the need to consider different approaches in the preservation of engineered tissues due to potential interactions between the scaffold material and tissue matrix.[24] There are three main variables affecting cell viability during cryopreservation by vitrification (cooling/warming rates, effective tissue permeation, and cytotoxicity) and protocols that work for one tissue type do not necessarily apply for other tissues or, in the case of cartilage, even the same tissue type from different species.

The investigation of long-term storage is an important field in tissue engineering. The main reason for this study was to determine whether vitrification protocols developed for native articular cartilage were effective for tissue-engineered cartilage constructs. TECCs were harvested from a bioreactor, vitrified employing different protocols, and cell viability was assessed after rewarming and cryoprotectant removal. The end goal of this study was to employ a bioreactor, the perfusion concentric cylinder bioreactor, to aid in the cryopreservation of tissue-engineered cartilage.

Materials and Methods

Reagents and Culture Supplies

Unless otherwise stated, reagents were obtained from Sigma (St. Louis, Missouri) and tissue culture supplies from VWR Scientific (West Chester, Pennsylvania).

Cartilage Source and Isolation

Articular chondrocytes were isolated aseptically from the femoral-patella groove and femoral condyles of the knee joint from freshly slaughtered 2–14 day old male calves (Research 87, Inc., Boylston, Massachusetts).[25] Cartilage digestion, chondrocyte isolation, and cell culture was performed, as previously described.[26]

Polymer Scaffolds

Poly-L-lactic acid (Polysciences, Warrington, Pennsylvania) with a molecular weight of 50,000 was used to prepare 90% porous scaffolds. The scaffolds were made using salt-polymer casting and particulate leaching method, as previously described.[27] The sodium chloride crystals ranged in size from 106–150 μm . Cylindrical scaffolds, 10 mm in diameter and approximately 1.87 mm thick, were used for all experiments. Scaffolds were sterilized by triplicate washes with 70% ethanol followed by triplicate washes with deionized water, and finally UV light exposure.

Bioreactor Design and Operation: The Perfusion Concentric Cylinder Bioreactor

The bioreactor (Figure 1) consists of an immobile hollow polycarbonate inner bob (McMaster Carr, Atlanta, Georgia) with 16 construct arms (hollow cylindrical tubes) radiating from the central hub containing embedded scaffolds affixed via tension. The 16 scaffolds were arranged in two rows near the bottom of the bob. The inner bob was placed in an outer Pyrex glass cup (Wilma Glass, Vineland, New Jersey). The outer cup rotated at a constant speed of 38 rpm, changing direction every twelve hours to produce a homogeneous laminar flow regime within the annulus. Numerical simulations have determined that the center of the construct surface experienced uniform hydrodynamic loading with the average surface shear stress of 0.4 dynes/cm². Prior to experimentation, the inner bob and outer cup were treated with Sigmacoat™ to prevent cell adhesion. Except for the inner bob, all bioreactor components were steam sterilized for 20 min at 121°C and 2.2 atm. The bioreactor inner bob was sterilized with ethylene oxide.

The bioreactor was assembled aseptically in a laminar flow hood. The scaffolds embedded in the bioreactors were prewetted in culture media for 48 hours prior to seeding. At time zero, approximately 1.5×10^8 cells were suspended in 350 mL of culture medium in the bioreactor. The bioreactor was mounted on a rotating base and placed in an incubator at 37°C with 5% CO₂, and the caps covering the bioreactors were raised slightly to allow gas exchange. These same incubator conditions were used over a four day period for scaffold cell seeding. On the fourth day, the medium was replaced with 400 mL of fresh medium and every four days thereafter until the end of each experiment. A peristaltic pump moved media

from the annulus region into the central hub to radiate through the construct arms, perfuse through the constructs, and then exit into the annulus region again.

After the four-day seeding period, perfusion was initiated at a flow rate of 0.6 mL/min/construct. At the wall, a level of shear stress between 0.13 and 0.18 dynes/cm² was maintained. The seeded scaffolds were mechanically stimulated in the bioreactor for 7 or 28 days to induce chondrogenesis and extracellular matrix growth. At the end of the culture period, the constructs were harvested for cryopreservation studies.

Cryopreservation

Vitrification solutions used in the cryopreservation studies consisted of Me₂SO, formamide, and 1,2-propanediol in EuroCollins (EC) solution (0.97 M dextrose, 0.07 M potassium phosphate monobasic [KH₂PO₄], 0.21 M potassium phosphate dibasic [K₂HPO₄], 0.08 M potassium chloride [KCl], and 0.05 M sodium bicarbonate [NaHCO₃]) and 0.01 M HEPES buffer with a pH between 7.9 – 8.1. The three vitrification solutions used in this study (VS55, VS70, and VS83) varied in cryoprotectant concentration, and their formulations are listed in Table 1.

The cell viability levels of harvested TECCs were quantified both before and after the cryopreservation process. The full-strength mixture of each vitrification solution (both the addition and removal solutions) was introduced or eluted into or out of the TECCs at 4°C on an orbital shaker (approximately 100 rpm) in a step-wise method consisting of six and seven discrete addition and removal steps for 15 minutes each.[20] Some studies used only four steps for addition and removal. After the last solution was removed, culture medium (4°C) was added for 15 minutes. Finally, the culture medium was exchanged for fresh culture medium and the samples were incubated under physiological conditions in a tissue culture incubator for a one-hour recovery period before the viability levels were retested.

In the step-wise introduction/elution method, TECCs were exposed to 5 mL of each addition solution in 6-well dishes on ice, and an orbital shaker was employed to facilitate diffusion. The addition solutions, ranging from 0% (A1), 18.7% (A2), 25% (A3), 50% (A4), 75% (A5), and finally 100% (A6), were each added in EC solution. The TECCs were then placed in scintillation vials filled with 5 mL of the A6 (full-strength) solution and 0.8 mL of isopentane (EMD Chemicals Inc., Darmstadt, Germany) to limit direct air contact with the vitrification solution.[20] Samples filled with VS55 (including a dummy vial without tissue) were loaded into a tube rack, placed in a pre-cooled isopentane bath and then stored in a –150°C mechanical storage freezer (or a small-scale benchtop system [53 × 34 × 30 cm³] containing approximately 7 L of liquid nitrogen). A temperature-monitoring thermocouple was also placed in with the samples. When sample temperatures reached –100°C, they were removed from the isopentane bath and placed on a shelf in the mechanical freezer (or in the vapor phase of the liquid nitrogen) to slowly lower the temperature from –100°C to –135°C. After samples reached –135°C, they were stored overnight in the freezer or, in the case of the bench-top method, removed immediately to begin the rewarming process.

First, sample vials were removed from the freezer and allowed to rewarm slowly from –135°C to –100°C. Then the vials were placed in a 37°C water bath where they warmed from –100°C to 4°C at a faster rate of approximately 40°C. TECCs were unvialled and placed in a 6-well dish (filled with 5 mL of the first removal solution) on an orbital shaker at 4°C. The removal solutions were then introduced in a stepwise manner to elute the cryoprotectants. Removal solution strengths were: 100% (R1), 75% (R2), 50% (R3), 25% (R4), 12.5% (R5), 0% (R6), and 0% (R7 [mannitol-free]), each in EuroCollins solution with 200 mM mannitol added to all but R7.

Gradient additions of cryoprotectants to the bioreactor are shown in Figure 2. While the step-wise method on the orbital shaker introduced and eluted the cryoprotectants in discrete steps, the bioreactor method introduced and eluted the cryoprotectants gradually. Three reservoirs filled with 150 mL cryoprotectant solutions (of increasing concentration) were connected by tubing, and solutions were pumped into the central hub of the bioreactor with TECCs at 4°C. The TECCs were harvested, tested for initial viability levels, and then eight TECCs were returned to the bioreactor (placed in the bottom row of the inner bob).

The outer cup was filled with 150 mL of the first solution (A1) which permeated the constructs resulting from a pressure gradient between the inner hub and outer cup region. After 15 minutes, the second solution (A3) was pumped at a perfusion rate of 1.25 mL/min/construct into the bioreactor while the first solution was pumped out to a waste beaker at 10 mL/min. After another 15 minutes, the next solution (A5) was pumped in as A3 was pumped out. Then 15 minutes later, the final solution (A6) was pumped in as A5 was pumped out.

Ten minutes after the A6 solution pumped in, the TECCs were removed from the bioreactor and placed in scintillation vials containing A6 solution with isopentane (as mentioned earlier). Samples were then vitrified and rewarmed, as described earlier. For cryoprotectant removal, the TECCs were placed back in the bioreactor, removal solutions were pumped in using the reservoir method (four reservoirs containing 150 mL of R4, R6, R7, or culture medium at 4°C) as shown in Figure 2. The TECCs were harvested after the last of the medium had been perfused through the constructs and placed in 6-well dishes containing culture medium.

Viability Assessments

The cell viability levels of TECCs treated in the cryopreservation studies were quantitatively determined using an alamarBlue® assay (Invitrogen, Carlsbad, California), to measure the metabolic activity of cells before and after treatment. Once the last cryoprotectant elution solution was removed, culture media (0°C) was added for 15 minutes. Finally, the media was exchanged with fresh culture media and the samples were incubated under physiological conditions in a tissue culture incubator for a one-hour recovery period before the viability levels were retested. Each TECC was incubated with 2.5 mL of medium and 250 µL of alamarBlue for three hours at 37°C and 5% CO₂. 100 µL aliquots of the medium containing alamarBlue were measured in 96-well plates on a SpectraMax Plus fluorescence plate reader (Molecular Devices, Sunnyvale, California) at an excitation of 544 nm and emission of 590 nm. Because the dye is non-cytotoxic, the assay could be performed on the TECCs before and after vitrification to determine the viability of cells within each TECC.

After viability assessment, the TECCs were washed, lyophilized, and digested overnight with papain (Worthington Biochemical, Lakewood, New Jersey) at 60°C for DNA quantification. Construct cell number was determined using a Picogreen DNA quantification kit (Invitrogen). Chondrocyte cell number was calculated assuming 7.7 pg of DNA per chondrocyte.[28]

Statistical Analysis

For each data set, values are presented as either mean or mean ± standard error mean (SEM) for n constructs or N experimental trials. Statistical analyses were performed using Student's t-test and p-values < 0.05 considered statistically significant.

Results

Cytotoxicity of Vitrification Solutions

Four experimental trials were conducted employing 7-day TECCs where one set of TECCs was treated with VS55 in 6/7 steps and the other with VS70 in 6/7 steps but not subjected to vitrification cooling and rewarming. Instead, TECCs were kept at 0°C in the full-strength mixture of VS55 or VS70 for approximately 30 minutes. The non-vitrified samples treated with VS70 had a $36.0 \pm 11.3\%$ decrease in cell viability levels compared to VS55 ($p < 0.05$, $N = 4$). The highest viability level observed with VS55 was 46% of pre-treatment values.

The Effect of Cryopreservation Formulations and Cooling Rates on Cell Viability

Experiments comparing cryoprotectant formulations are summarized in Figure 3. The total time to introduce cryoprotectants in 6/7 steps versus 4/4 steps was 195 and 120 minutes, respectively. A comparison of all results for solutions employing either VS55 or VS70 revealed that VS70 resulted in significantly better cell viability ($p < 0.05$). VS70 normalized to VS55 showed a 2.3-fold (± 0.2) increase in cell viability levels ($N = 5$, $p < 0.05$). VS83 was tested only once and produced the lowest viability values observed in our studies. The 4/4 step cryoprotectant addition and removal method with VS70 was compared with 6/7 steps. The 4/4 step method only showed a 1.2-fold (± 0.2) increase in cell viability that was not statistically significant ($N = 4$, $p = 0.53$). Visible ice formation was observed at cooling rates $< -10^\circ\text{C}/\text{min}$.

Introduction of Cryoprotectants as a Gradient in the Bioreactor

Finally, based upon the results of the previous studies, VS70 was used in 4/4 steps for comparison of step-wise additions employing an orbital shaker and gradient additions of cryoprotectant employing the bioreactor (see Figure 2 for setup). In these studies, the four addition solutions were A1, A4, A5, and A6, and the removal solutions were R1, R4, R6, and R7. Three trials were conducted in which one set of TECC was treated with VS70 in 4/4 steps on the orbital shaker, and the other with VS70 using the bioreactor. The bioreactor results were normalized to those obtained using the orbital shaker. The results were very similar (Figure 4), demonstrating a 1.0-fold (± 0.1) increase in cell viability levels after cryopreservation ($p = 0.97$). The highest cell viability level reached for vitrified 7-day constructs containing 2.6 million cells (± 0.2) per construct using the orbital shaker was $56.0\% (\pm 3.3)$ ($n = 4$), while $47.8\% (\pm 5.6)$ ($n = 4$, $p = 0.23$) was observed in the bioreactor. There was no discernable difference between the two treatment methods.

Discussion

Long-term storage that maintains cartilage viability and function can provide an “off-the-shelf” availability for patients without the need to wait for cells to grow and make TECCs. To date, the majority of research performed in cartilage cryopreservation has been on native tissues and cells. The interaction between newly formed tissue and the scaffold matrix during vitrification is not well understood and as such, few studies have been carried out on preservation of TECCs. The focus of this study was to employ the vitrification methods currently used for native articular cartilage to investigate the post-preservation cell viability of tissue engineered cartilage. We have characterized the bioreactor-derived cartilage constructs in a companion manuscript submitted for publication in 2010 by Farooque *et al.* Briefly, over the course of 28 days, cells were propagated and an extracellular matrix was deposited preferentially on the construct face that experienced surface shear. Immunohistochemical staining showed the presence of collagen type II indicating cells expressing an articular cartilage phenotype.

VS70 yielded higher chondrocyte viability than either VS55 or VS83. VS70 was more cytotoxic than VS55 but preserved twice as many cells as VS55. The more concentrated VS70 solution provided the best balance of cryoprotectants to preserve cells when compared to VS55. Fahy *et al.*[3] hypothesized that solutions with higher hydrogen bonding energy could vitrify solutions with high water concentrations, but were also more toxic than solutions with low hydrogen bonding energy between water and the cryoprotectants' hydrogen bonding groups. They suggested that there existed a concentration threshold (CT) in which cryoprotectants below the CT were not cytotoxic.[3] We reduced the number of steps during addition and removal of the cryoprotectants from 6/7 to 4/4 but there was no significant difference ($p = 0.53$). Further variations in formulation or conditions of exposure (*e.g.*, variation of incubation time, step number, and temperature of exposure) may increase cell viability by reducing cryoprotectant toxicity.

The highest chondrocyte viability observed in TECC vitrification experiments was 56%. In comparison, Song *et al.*[20] was able to preserve up to 80% viability of vitrified rabbit articular cartilage with VS55 in 6/7 steps. It is likely that these differences in outcome are due to cryoprotectant toxicity. Minimizing the number of steps reduced cryoprotectant exposure time from 195 minutes (6/7 steps) to 120 minutes (4/4 steps). Though reduction of steps increased the concentration gradient, and thus, osmotic imbalances, our results show that less steps produced no negative effects upon cell viability levels. Pegg *et al.*[29] found that chondrocytes can withstand osmotic imbalances far better than other cells. The higher concentration gradient in the 4/4 step method, however, may have underexposed the TECCs to the cryoprotectants. Only 15 minutes was allotted for cryoprotectant diffusion to prevent reduced cell viability levels. Longer incubation times, possibly at lower temperatures, might result in better cell viability.

The final studies utilized the 4/4 step method which was adapted as a continuous gradient employing the bioreactor. The hypothesis was that increasing mass transport of cryoprotectants through the TECCs would improve cell viability levels. The highest cell viability level reached using VS70 in the bioreactor was 47.8% while VS70 in 4/4 steps using an orbital shaker to introduce and elute the cryoprotectants maintained 56%. There was no significant difference between the three experiments (Figure 4). This result was surprising when mass transfer estimations were made. Diffusion of Me_2SO , formamide and 1,2-propanediol was the main limitation to mass transfer. The diffusivity measured by Mukherjee *et al.*[30] for Me_2SO , formamide, and 1,2-propanediol into cartilage tissue at room temperature was $4.63 \times 10^{-10} \text{ m}^2/\text{s}$, $3.47 \times 10^{-10} \text{ m}^2/\text{s}$, and $6.67 \times 10^{-10} \text{ m}^2/\text{s}$, respectively. Based on Fick's second law of diffusion, the time it took for VS55 to permeate native cartilage and equilibrate with the surrounding bulk VS55 solution was approximately 2–3 hours.[30] If these diffusivity constants were applied to TECCs, then the 15-minute exposures to vitrification solutions using the orbital shaker method may not have been adequate for complete cryoprotectant permeation. Therefore, we hypothesized that using convective flow (direct perfusion) in the bioreactor to aid in cryoprotectant permeation through the TECCs would overcome these transport limitations and improve cell viability, but clearly this was not the case. However, it offers the opportunity to streamline the bioprocess scheme from construction to preservation as a more continuous process, reducing sample handling and potential contamination risks. Nevertheless, the introduction of cryoprotectants using the bioreactor was not detrimental to cell viability in comparison to the orbital shaker method.

The difference in cell viability levels achieved for TECCs and native rabbit cartilage indicates that TECCs require different vitrification protocols than those for native tissues. Tissue thickness may be important. The rabbit cartilage previously vitrified was only approximately 0.6 mm thick[20] while in contrast, the tissue-engineered constructs in this

study were approximately 1.8 mm thick. However, most cells were superficially placed near the outer surface of the construct and should have been readily accessible to the cryoprotectants. This may have introduced an unavoidable cytotoxicity bias because most cells in the constructs were located nearer to the cryoprotectants. The interaction between engineered tissue and the scaffold matrix may also play an as yet unknown role in the success of vitrification.

In Conclusion

The VS70 formulation resulted in the best post-vitrification cell viability levels compared with VS55 and VS83. Step reductions for formulation addition and removal had no significant effect upon cell viability. The addition of convective flow employing the bioreactor and a gradient to add and remove cryoprotectants also had no effect upon cell viability levels. The highest experimental chondrocyte viability level observed post-vitrification was only 56%; however, cryoprotectant exposure without vitrification caused similar losses of viability due to cytotoxicity. Until novel cryoprotectant formulations are developed, the goal of lowering cytotoxic effects may be achieved by limiting the temperature and duration that tissues are exposed to cryoprotectants.

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Figure 1.

Schematic representation of the perfusion concentric cylinder bioreactor. The bioreactor consists of a stationary inner bob with 16 spokes (arranged in two rows) which contains embedded constructs radiating from a central hub. The gap between the construct holders and outer cup is 0.14 mm. The peristaltic pump flows media from the outer cup region to the interior of the central hub to exit radially through the spokes at an average set flow rate of 0.6 mL/min/construct. Inset represents a close-up of construct arm. The diagram is not strictly to scale, but the dimensions are correct.

Figure 2.

Bioreactor introduction/elution diagram for the addition and removal of vitrification solutions. TECCs are embedded into the bottom row of the bioreactor where the solution is pumped through the thickness of the constructs. Reservoirs consist of A3 (25%), A5 (75%), A6 (100%) of VS addition formulation and R4 (25%), R6 (0% with 200 mM mannitol), R7 (0% with 0 M mannitol) of VS removal formulation, and culture media. Waste: the solutions pumped out of the bioreactor.

Figure 3.

Effects of cryopreservation formulation and cooling rate upon cell viability. Ten experiments, each represented by a line, were performed comparing three vitrification formulations: VS55 to VS83 (squares), VS55 to VS70 (triangles), and VS70 in 6/7 steps to VS70 in 4/4 steps (circles) at cooling rates ranging from -5°C to $-46^{\circ}\text{C}/\text{min}$ during TECC cooling from 0°C to -100°C . Fast cooling rates ($>-25^{\circ}\text{C}/\text{min}$) are represented by the dashed lined while slow cooling rates are represented by the solid line. The data points represent the mean of four samples except for VS83 comparison where there were three samples. VS83 was only tested once because it had the lowest viability value observed. VS70 normalized to VS55 showed a 2.3-fold (± 0.2) increase in cell viability levels ($N = 5$, $p < 0.05$), while VS70 in 4/4 steps normalized to VS70 in 6/7 steps showed a 1.2-fold (± 0.2) increase in cell viability levels ($N = 4$, $p = 0.53$).

Figure 4.

VS70 in the PCC bioreactor compared with VS70 using the orbital shaker, both added and removed in 4/4 steps. VS70 in the PCC bioreactor steps maintained equivalent amounts of viable cells compared to VS70 using the orbital shaker (mean $\pm$ 1 standard error of three experiments, $p = 0.97$). TECCs were cultured for seven days before cryopreservation.

TABLE 1

Vitrification solution formulations.

Vitrification Solution	Me ₂ SO	Formamide	1,2-propanediol	Total
VS55	3.10 M	3.10 M	2.21 M	8.40 M
VS70	3.97 M	3.97 M	2.83 M	10.80 M
VS83	4.65 M	4.65 M	3.30 M	12.60 M