## 1. PRODUCT DESCRIPTION

Collagenase MA GMP Grade (MA) is an aseptically filled, lyophilized mixture of 60% (w/w) purified Class I (C1) and 40% (w/w) purified Class II (C2) collagenases from *Clostridium histolyticum*. The lyophilized cake/powder consists of the blended mixture in the presence of low concentration of biological buffer salts sealed under vacuum in an amber glass vial.

## 2. APPLICATION

MA is formulated to contain a sufficient amount of collagen degradation activity (CDA) units for the isolation of hepatocytes from human liver[1]. MA is a highly purified collagenase product and contains negligible quantities other proteolytic activities. The product must therefore be supplemented with sufficient neutral protease to successfully release islets from the extracellular matrix.

## 3. STORAGE & STABILITY

This product is stable for at least four years from date of manufacture if stored unopened as a lyophilized material at -20±5°C. Internal studies have shown the reconstituted enzyme is stable as a frozen solution at -20±5°C for at least one year as long as no other protease enzymes had been added to the solution. Additional studies have shown the reconstituted collagenase was successfully frozen and thawed three times as a concentrated or dilute solution without apparent loss of potency as assessed by the CDA assay. The product is shipped on Enviro ice packs to keep the product cold and minimize the potential for high temperature excursions during shipment.

## 4. PRODUCT USE

### 4.1. Enzyme Reconstitution

Reconstitute the lyophilized enzyme with 5 mL of water or buffer (recommend HBSS or similar non-phosphate buffer) and allow enzyme to rehydrate for 30 minutes. Occasionally invert the vial to aid in the dissolution process. The enzyme solution should not be vortexed or swirled excessively as enzyme denaturation may occur. Failure to allow the enzyme to completely rehydrate will affect the enzyme potency and could negatively impact the success of the tissue dissociation procedure. The enzyme is lyophilized in a buffer containing calcium so the initial reconstitution has sufficient calcium for enzyme stability. However, for optimal stability the final working buffer for tissue dissociation should have at least 0.1 mM $Ca^{2+}$.

### 4.2. Digestion Solution Preparation

Once completely in solution, MA must be combined with a neutral protease and diluted to the appropriate volume for use in a specific tissue dissociation procedure. The collagenase may be degraded by neutral protease. To minimize this problem, the enzymes should be mixed just prior to beginning the digestion. At most, the mixture can be stored for two hours between 2°C and 8°C prior to use. This enzyme solution can be sterile filtered through 0.2 µm cellulose acetate or PES filter membranes without compromising enzyme potency. Surfactant free cellulose acetate (SFCA) and PES filters from several major vendors were tested and no measurable loss of CDA was observed. The exact concentration of collagenase and protease is dependent on the specific application. Guidance for common cell targets is available at www.vitacyte.com.

### 4.3. Human Liver Preparation & Digestion Protocol

MA contains approximately 2.5 million CDA units. This vial is sufficient to perform two hepatocyte isolations on surgical resection tissue specimens up to 150 g or one hepatocyte isolation from a deceased donor organ specimen up to 300 g. For deceased donor organ specimens > 300 g additional collagenase is recommended.

MA should be mixed with BP Protease which contains approximately 1.1 million neutral protease units. This vial is sufficient to perform one hepatocyte isolation on surgical resection tissue specimens up to 150 g. Additional vials are required to perform isolation on deceased donor organs. We recommend using two vials on deceased donor organs up to 300 g and 2 ½ vials on organs > 300 g.

This protocol is based on the method of Nakazawa[2], et al. using the following buffers:

- P1 1x HBSS without $Ca^{2+}$/$Mg^{2+}$ (MediaTech 20-021-CV), 25 mM HEPES, 0.5 mM EGTA
- P2 1x HBSS without $Ca^{2+}$/$Mg^{2+}$ (MediaTech 20-021-CV), 25 mM HEPES
- P3 Eagles-modified Minimal Essential Medium (EMEM with EBSS and 25 mM HEPES and without L-glutamine; Lonza 12-136Q), supplemented with Collagenase MA and BP Protease as described above

| Step | Procedure |
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| 4.3.1. | Both warm and cold ischemia times should be kept to a minimum for best results, but cold ischemia times up to 36 hours have provided acceptable yields of healthy hepatocytes. |
| 4.3.2. | Warm 500 mL of P1, 500 mL P2, and 500 mL of the unsupplemented EMEM in a 37°C water bath. Volumes for all three buffer solutions should be doubled for organ masses of > 300 g. The amount of enzyme recommended is detailed in the Reagent Preparation section above |
| 4.3.3. | Prepare the collagenase and neutral protease per instructions in the Reagent Preparation section above. |
| 4.3.4. | Place catheters made from silicone tubing in branches of the hepatic and portal veins and secure with purse-string sutures. If isolating hepatocytes from a surgical resection tissue sample, tie off unused vessels and seal the cut surface with medical glue to create a closed circuit in the organ. |
| 4.3.5. | When the MA and BP Protease are completely in solution, add the recommended amounts to the warmed EMEM, which will complete the P3 buffer. This collagenase-neutral protease mixture (P3 buffer) should be used within one hour of making it. |
| 4.3.6. | Place the liver in a sterile bag and connect tubing to a peristaltic pump that delivers the perfusate at a rate of 45 mL/minute. Pull through 500 mL of P1 without recirculation. |
| 4.3.7. | Discard the P1 perfusate by aspiration and immediately follow with 500 mL of P2, under the same conditions. |
| 4.3.8. | Discard the P2 perfusate by aspiration. Initiate the enzymatic dissociation of the liver by perfusing 500 mL of the P3 buffer. The P3 buffer should be recirculated through the liver. |
| 4.3.9. | Digestion will be quenched with ice-cold EMEM when cells begin to release beneath Glisson's capsule (usually 15-25 minutes). We recommend using visual clues to determine the digestion endpoint and not using a predetermined set time. |

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| 4.3.10. | Cells should be washed, purified and cultured per user preferred protocols. |

### 4.4. Rodent Digestion Recommendations

**Rat** – Use 2,500 CDA U/mL of MA and 550 NP U/mL of BP Protease enzyme concentration in the digestion solution.

**Mouse** – Use 2,000 CDA U/mL of MA and 500 NP U/mL of BP Protease enzyme concentration in the digestion solution.

### 4.5. Digestion Optimization

The recommendations made in this product insert represent the best guidance available based on experiences from product development activities and observations shared by users. Individual results may vary, and some optimization may be required to achieve the desired outcome. Moderate adjustments to the enzyme concentration can be made with the goal of improving stem cell yield or minimize cell damage leading to low viability. Other factors that can be adjusted include digestion time and the mechanical contribution by gentle agitation during digestion. Contact VitaCyte to discuss specific problems or optimization strategies.

## 5. TROUBLESHOOTING

**5.1.** Many factors contribute to the successful isolation of cells from tissue and inadvertent oversight to any of these conditions may drastically reduce the yield and viability of target cell population. While far from a complete list, the guidance below may help identify commonly encountered problems. Contact VitaCyte if this guidance does not help resolve specific issues.

**5.2.** Prolonged or Incomplete Digestion may be caused by:

- Loss of enzyme potency (activity)
- Incomplete enzyme rehydration during reconstitution
- Inappropriate enzyme dilution
- Presence of enzyme inhibitors
- Low incubation temperature
- Inefficient digestion solution perfusion

**5.3.** Low Yield and/or Cell Viability

- Prolonged organ warm ischemia time
- Aggressive mechanical disruption
- Extended incubation time
- Elevated incubation temperature
- Inappropriate enzyme dilution

## 6. ADDITIONAL INFORMATION

### 6.1. Intended Use & Regulatory

MA is for ex-vivo use only to recover cells from tissue. Guidance for use of reagents in clinical cell transplantation procedures is governed by local Institutional Review Boards and regional Health Authorities. MA is manufactured in accordance with the principles for clinical trial material outlined in Guidance Document Q7 Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients published by the FDA and ICH in September 2016. This product is labeled as 'GMP Grade' to indicate the quality system and manufacturing facility are consistent with requirements set forth in the above document. Guidance for the qualification and acceptance of reagents used in cell therapy applications can be found in the USP General Chapter <1043> Ancillary Materials for Cell, Gene, and Tissue-Engineered Products. Further details can be found on the VitaCyte Commitment to Quality document available upon request.

### 6.2. Animal Origin

No bovine derived animal products are used in any step of manufacturing of MA. This product is purified from culture supernatants of *C. histolyticum* that contain porcine gelatin and pancreatic enzymes derived from US and Canadian sources.

### 6.3. Manufacturing Summary

Enzymes are purified from the culture supernatants results from the fermentation of native organisms. The purification processes use standard protein column chromatography and tangential flow filtration concentration and diafiltration techniques. The purification processes have been optimized to yield the highest purity attainable for each enzyme while minimizing undefined and contaminating protease activities. After characterization, the purified collagenases are sterile filtered in a qualified biosafety cabinet and aseptically dispensed into amber vials on activity units, lyophilized, sealed under vacuum then secured and labeled. The final lyophilized product is then further characterized to confirm each batch meets established specification ranges.

### 6.4. Activity Assessment

VitaCyte relies on several biochemical tools to characterize and ensure the consistency of MA. The Pz-peptide substrate (Wünsch Assay) has historically been used to characterize collagenase activity[4]. While this assay has advantages in terms of reproducibility and historical precedence, it also has several limitations. The Wünsch Assay is strongly biased towards C2 and is not sensitive to the different molecular forms of C1. In addition, the substrate assesses the catalytic activity of the enzyme and does not assess the ability of collagenases to degrade native collagen. Degraded collagenases lacking a collagen binding domain are able to cleave the Pz-peptide substrate but are not functional in degrading native collagen. The Pz-peptide activity provides potentially misleading information about the ability of collagenase to isolate islets. The limitations of the Wünsch assay led to the development of a fluorescent microplate CDA using fluorecein isothiocyanate labeled calf skin collagen fibrils as substrate[5]. The intact molecular form of purified C1 with two collagen binding domains (~116kDa) has approximately 10-fold higher CDA when compared the CDA found with same amount of purified C1 containing only one collagen binding domain (~100kDa) or intact C2 (~114kDa).

Collagenase MA is manufactured with both 116kDa and 100kDa molecular forms of C1 to provide the optimal collagenase activity for the isolation of hepatocytes.

### 6.5. Additional Considerations

In addition to the quality of the dissociation enzymes, additional factors impact the outcome of success of cell isolations including: the quality of the organ/tissue and experience of the cell isolation team. The team needs to assess many variables that affect islet recovery. These include but are not limited to the characteristics of the donor, transport of the organ/tissue, the cell isolation procedure, and subsequent cell culture.

### 6.6. Resources & Support

Further details on manufacturing, quality control testing and use of products are available at www.vitacyte.com or technical support at 317-917-3457.

### 6.7. References

1. Gramignoli R, Green ML, et al. (2012) Development and application of purified tissue dissociation enzyme mixtures for human hepatocyte isolation. *Cell Transplant.* 21(6), 1245-60.

2 Nakazawa F, Cai H, Miki T, Dorko K, Abdelmeguid A, Walldorf J, Lehmann T, and Strom SC (2002) Human hepatocyte isolation from cadaver donor liver., pp. 147-158. Kluwer Academic Publishers

3 U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). (2016) Q7 Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients, Guidance for Industry. www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm073497.pdf

4 Wünsch E and Heidrich H-G. (1963) Zur quantitativen bestimmung der kollagenase. *Hoppe-Seyler's Zeitschrift Physiologische Chemie* 333, 149-151.

5 McCarthy R.C., Spurlin B., et al. (2008) Development and characterization of a collagen degradation assay to assess purified collagenase used in islet isolation. *Transplantation Proceedings* 40, 339-42.