1. PRODUCT DESCRIPTION

CIzyme AS is an aseptically filled, lyophilized mixture of 60% purified class I (C1) and 40% purified class II (C2) collagenase from *Clostridium histolyticum* and neutral protease from *Bacillus polymyxa*. The lyophilized cake/powder consists of the blended mixture of enzymes in the presence of an inert protein stabilizing excipient in a low concentration of biological buffer salts sealed under vacuum in an amber glass vial.

2. APPLICATION

CIzyme AS is formulated to contain an optimal amount of collagen degrading activity (CDA) units and neutral protease activity (NP) units for the isolation of the stromal vascular fraction from human lipoaspirate[1] using the procedure described by Dubois, et al.[2] Specific details are presented in Section 4.

3. STORAGE & STABILITY

This product is stable for at least two years from date of manufacture if stored unopened between 2-8°C. Internal studies have shown the reconstituted enzyme is stable as a frozen solution between -15 to -25°C for at least 3 months.

4. PRODUCT USE

4.1. Enzyme Reconstitution

Reconstitute the lyophilized enzyme with 2 mL of water or buffer (recommend HBSS or similar non-phosphate buffer) and allow enzyme to rehydrate for 15 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, dilute the reconstituted enzyme into PBS or KRB. Use 1 vial of CIzyme AS per 100 grams of human lipospirate. At most, the rehydrated enzyme can be stored for 2 hours between 2°C and 8°C prior to use or freezing. Enzymes 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.

4.3. Aspirate Preparation & Digestion Protocol

Step	Procedure
4.3.1.	Maintain lipoaspirate at +4°C and process within 24 hours of procurement from patient.
4.3.2.	Warm 2-3, 500 mL bottles of PBS or KRB to room temperature.
4.3.3.	Reconstitute CIzyme AS per the instructions in Step 4.1.
4.3.4.	Once enzyme is reconstituted, further diluted per Step 4.2 and warm enzyme solution to 37°C.

4.3.5.	Place 100 grams (~100 mL) of lipoaspirate in a 250 mL bottle.
4.3.6.	Add an equal volume of PBS or KRB.
4.3.7.	Agitate 15 to 30 seconds to wash the adipose tissue and then allow phases to separate, 2-4 minutes.
4.3.8.	Aspirate away the infranatant solution (lower, liquid phase).
4.3.9.	Repeat steps 6-8 at least two additional times (or more) to remove contaminating erythrocytes and white blood cells, achieving a straw colored infranatant solution.
4.3.10.	Add the digestion solution from Step 4.3.4 to each centrifuge bottle. Add one part collagenase solution to one part adipose tissue (i.e. 100 mL of collagenase solution per 100 mL adipose tissue).
4.3.11.	Place the tubes in a 37°C shaking water bath at 100 rpm for 30 to 60 minutes until the tissue becomes visibly broken down. If a shaking water bath is not available, gently swirl by hand for 15 seconds every 5 minutes until the tissue is visibly broken down.
4.3.12.	Balance the centrifuge tubes, if needed, with buffer and centrifuge at 1,200 rpm at room temperature for 5 minutes. The centrifugal force should be approximately 300xg.
4.3.13.	Carefully aspirate off top layer of fat, the primary adipocytes (yellow layer of floating cells), and the collagenase solution. Leave behind approximately 10ml of the layer above the pellet so that the stromal-vascular fraction (dark red cells on bottom) is not disturbed.
4.3.14.	Add 10-20 ml of PBS or KRB to each centrifuge tube and resuspend the cells. Pool the cells into one tube and centrifuge again.
4.3.15.	Aspirate the supernatant and then resuspend the cells in 10 ml/100ml tissue with Stromal Medium.
4.3.16.	Plate the cells at a density equivalent to approximately 0.16 ml of lipoaspirate per cm^2 of surface area. For example, in a T225 flask, you would plate the stromal vascular cells isolated from the equivalent of approximately 35 ml of tissue in a volume of 35- 40 ml of medium.
4.3.17.	Divide the cells equally accordingly to the appropriate number of flasks, making sure the flasks are level so that the stromal cells are evenly distributed throughout the flask.
4.3.18.	Incubate at 37°C in 5% CO_2.
4.3.19.	Aspirate the medium from the flask 48 hours after plating. Wash with PBS or HBSS and aspirate, and then add fresh media.
4.3.20.	Grow cells to 70-80% confluence.

4.4. 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 islets from rodents and inadvertent oversight to any of these conditions may drastically reduce the yield and viability of cells. While far from a complete list, the recommendations 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

5.3. Low Yield and/or Cell Viability

- Prolonged organ warm ischemia time
- Aggressive mechanical disruption
- Extended incubation time
- Incubation above 37°C
- Inappropriate enzyme dilution

6. ADDITIONAL INFORMATION

6.1. Intended Use & Regulatory

CIzyme AS is for research use only. Guidance for use of reagents in clinical cell transplantation procedures is governed by local Institutional Review Boards and regional Health Authorities. This product is manufactured in accordance with the principles for clinical trial material outlined in ICH Q7a[3]. The document control system in place is in alignment with FDA guidance for Phase I material. Document controls are in place to minimize the chances of cross-contamination.

6.2. Animal Origin

No bovine derived animal products are used in any step of manufacturing of CIzyme AS. 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 a thorough characterization of the purified collagenases and proteases, the individual components are blended based on activity to prepare a specific formulation. This formulation is dispensed into amber vials, lyophilized and then sealed under vacuum. The final

lyophilized product is then further characterized to confirm each batch meets established specification ranges.

6.4. Activity Assessment

CIzyme AS is assessed by several biochemical tools to characterize and ensure the consistency of components used and the final blended and lyophilized product. Collagenase activity is characterized using both the traditional Wünsch (Pz-peptide)[4] substrate and a more recently developed fluorescent microplate functional collagen degrading activity (CDA) assays[5]. The CDA assay relies on fluorecein isothiocyanate (FITC) labeled calf skin collagen fibrils as substrate. The B. polymyxa neutral protease activity (NPA) is measured using a FITC labeled BSA substrate[6]. Focus on manufacture of products with consistent collagenase and neutral protease activity improves control over the cell isolation process with the goal of more consistent results.

6.5. Additional Considerations

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

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. Aronowitz JA, Lockhart RA, et al. (2015) Clinical Safety of Stromal Vascular Fraction Separation at the Point of Care. *Annals of Plastic Surgery* 75(6), 666-71.

2 Dubois SG, Floyd EZ, et al. (2008) Isolation of human adipose-derived stem cells from biopsies and liposuction specimens. *Methods Mol Biol* 449, 69-79.

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.

6 Breite AG, Dwulet FE, McCarthy RC. (2010) Tissue Dissociation Enzyme Neutral Protease Assessment. Transplant Proceedings 42(6), 2052-4.