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September 2009

HUMAN HEPATOCYTE ISOLATION PROTOCOL

Purpose:

This document describes the recommended protocol for isolating human hepatocytes from surgical resection tissues and deceased donor whole organs using the VitaCyte Clzyme™ purified enzyme formulations. The protocol used here is based on the Seglen procedure widely used in leading hepatocyte isolation laboratories³.

Products:

The VitaCyte Clzyme™ Collagenase MA and BP Proteases are used in this procedure in place of the traditional crude or enriched collagenase products. Clzyme™ MA is an aseptically filled, lyophilized protein mixture containing 60% purified class I (C1) and 40% purified class II (C2) collagenase from *Clostridium histolyticum*. This material is purified from *C. histolyticum* culture supernatants that contain porcine gelatin or porcine pancreatic enzyme derived from US or Canadian sources. No bovine derived animal products are used in any step used to prepare the purified collagenase enzymes.

This product is not manufactured under U.S. Good Manufacturing Procedures nor are individual lots tested for sterility. However, the product is manufactured under document control. The product is shipped on dry ice to provide the most stable shipping conditions. The product is stable for two years from date of manufacture if stored between -15 to -25°C. VitaCyte guarantees the stability of this product during this time period.

This product is not intended for use in diagnostic or therapeutic procedures; it is intended for research use only. Advances in cell transplantation procedures may lead to the use of this product to prepare cells for clinical transplantation. The guidance for use of research reagents in these procedures is governed by existing laws and regulations. This includes approval of human experimental protocols by a local Institutional Review Board. Responsibility for applying this product in any clinical procedure lies solely with the Principal Investigator and Institution supervising this activity. If you plan to use this product in any clinical transplant procedure, please consult the policies of your institution or the appropriate regulatory authorities.

Reagent Preparation:

The VitaCyte Clzyme™ Collagenase MA (**Cat# 001-2030**) contains approximately 2.5 million CDA units¹. This vial is sufficient to perform **two** hepatocyte isolation 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.

The product is supplied as a lyophilized cake in vacuum sealed amber bottles. Reconstitute the lyophilized enzyme with 5 mL of cold water on ice for a minimum of 30 minutes to ensure complete dissolution of the enzyme. Occasionally invert the vial to aid in the dissolution process. The enzyme solution should not be vortexed or swirled excessively. Failure to allow the enzyme to completely redissolve will affect the enzyme potency and could negatively impact the success of the tissue dissociation procedure. In our hands, the reconstituted enzyme is stable frozen at < -20°C for 1 year as long as no other protease enzymes had been added to the solution. In internal studies, the reconstituted collagenase was successfully frozen and thawed three times as a concentrated or dilute solution without apparent loss of potency.

The VitaCyte Clzyme™ BP-Protease (**Cat# 003-1000**) contains approximately 1.1 million neutral protease units. This vial is sufficient to perform **one** hepatocyte isolation on surgical resection tissue specimens up

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to 150 g. Additional vials are required to perform an 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. Reconstitute the enzyme as described above for the Collagenase MA, except using 2 mL of cold water. Reconstituted BP Protease is only stable for about 6 months once reconstituted and should only be subjected to one freeze-thaw cycle and should be aliquoted if a remainder is to be saved.

This protocol was developed using the following buffers:

P1 1x HBSS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (MediaTech 20-021-CV), 25 mM HEPES, 0.5 mM EGTA

P2 1x HBSS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (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 Clzyme™ BP Protease and Clzyme™ Collagenase MA (VitaCyte; Indianapolis, IN).

Protocol (see Nakazawa et al²):

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.
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.
3. Prepare the collagenase and neutral protease per instructions in the Reagent Preparation section above.
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 close circuit in the organ.
5. When the Collagenase 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 1 hour of making it. If desired, this enzyme solution can be sterile filtered through sterile cellulose acetate or PES filter membranes without compromising enzyme potency. The surfactant free cellulose acetate (SFCA) and PES filters from several major vendors were tested and no measurable loss of collagen degradation activity (CDA) or neutral protease (NP) was observed.
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.
7. Discard the P1 perfusate by aspiration and immediately follow with 500ml of P2, under the same conditions.
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.
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.
10. Cells should be washed, purified and cultured using user preferred protocols.

Resources:

VitaCyte has details on the significance of enzyme activities and the assays used in the manufacturing and quality control of products on the website, www.vitacyte.com. Additional details about enzyme activity or questions regarding your specific isolation are available by contacting VitaCyte directly. Additional reagents can be order by sending an e-mail to orders@vitacyte.com or by phone at (317) 917-3457.

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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. Seglen P.O. (1976) Preparation of isolated rat liver cells. *Methods in Cell Biology* 13, 29-83.