1. PRODUCT DESCRIPTION

PD Collagenase 100 and 800 products are equivalent to the DE Collagenase 100 and 800 products sold earlier by VitaCyte. PD 100 and 800 are aseptically filled, lyophilized preparations of > 95% pure *Clostridium histolyticum* collagenases and purified neutral protease from *Paenibacillus polymyxa*. The composition of each product is shown below:

Components	1g PD Collagenase 100	1g PD Collagenase 800
C. histolyticum collagenase	55 mg	440 mg
BP Protease (Dispase™ equivalent enzyme)	18 mg	18 mg
Approximate mg polypeptide excipient	927 mg	542 mg

The minimally hygroscopic polypeptide excipient preserves enzyme stability during storage and adds convenience because users can weigh the precise amount of product needed immediately before use. These lyophilized products showed no loss of enzyme activity after stressing the lyophilized products by incubation at higher temperatures. These products have a five-year shelf life at 4°C.

The PD 100 product is formulated to have enzyme activities like those found in Worthington Type 1 collagenase, the first crude collagenase product used for cell isolation. The PD 800 product contains an eight-fold increase in collagenase mass. This formulation was defined by reverse engineering "good lots" of crude collagenase used for human islet isolation. The high collagenase-low protease activity mixture works well for many other cell types. This product contains the same amount of BP Protease found in the PD 100 product.

2. APPLICATION

The PD Collagenase 100 and 800 alone or mixed in different ratios enable you to develop a continuum of collagenase to protease ratios for application-specific formulations. If the DE Collagenase product line was used in the past, see Appendix 1 to determine how to use the PD 100 and 800 products to prepare the enzyme formulations equivalent to DE 200, 400, or 600 products.

Alternatively, these products can also be used to prepare purified enzyme mixtures to replace your existing product or to prepare an enzyme formulation to isolate a specific cell type. Here, the collagenase and protease enzyme activities must be translated into VitaCyte's collagen degradation activity (CDA) and neutral protease activity (NPA) units (U). Appendix 2 describes the processes to prepare a purified collagenase-protease enzyme mixture that will approximate the enzyme activities in your current collagenase product.

3. STORAGE & STABILITY

PD Collagenase is stable for at least five years from the date of manufacture if stored as a lyophilized powder at ≤ 2-8°C. The product is shipped ambient but should be stored ≤ 2-8°C.

4. PRODUCT USE – Enzyme Reconstitution

While preparing for tissue digestion, equilibrate PD Collagenase to room temperature. PD Collagenase is supplied as a lyophilized powder. This powder may appear as a solid cake or clumps when first received.

Vigorous shaking of the bottle or mechanical disruption with a laboratory spatula should quickly convert the material into a partially flowing powder. Weigh out the required amount of enzyme powder. The remaining enzyme may be resealed in the bottle and returned to storage at 2-8°C.

PD Collagenase may be reconstituted in a small volume of buffer or water and further diluted into the working buffer (suggest HBSS or a similar non-phosphate buffer) or added directly to the desired volume of working buffer. Allow the powder to rehydrate for a minimum of 15 minutes to ensure complete dissolution of the enzyme. Occasionally, invert the bottle to aid in the dissolution process. Enzyme denaturation may occur if the enzyme solution is vortexed or swirled excessively. 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+} and contain no cation-chelating agents. The 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 collagenase's collagen degradation activity was observed. See details for preparing a mixture of the PD Collagenase 100 and 800 products in Appendix 1.

5. ADDITIONAL INFORMATION

5.1. Intended Use & Regulatory

PD Collagenases are for research use only. Local Institutional Review Boards and Regional Health Authorities govern guidance for the use of reagents in clinical cell transplantation procedures. This product is manufactured following the principles for clinical trial material outlined in ICH Q7a. The document control system aligns with the FDA guidance for Phase I material. Document controls are in place to minimize the chances of cross-contamination.

5.2. Animal Origin

No bovine-derived animal products are used in any step of manufacturing PD Collagenases. Collagenase is purified from culture supernatants of *C. histolyticum* that contain porcine gelatin and pancreatic enzymes. Before lyophilization, a non-mammalian peptide excipient is added to the solution containing the purified enzyme mixture.

5.3. Manufacturing Summary

The purification processes use standard protein column chromatography, tangential flow filtration concentration, and diafiltration techniques. After characterization, the purified collagenases are sterile filtered in a qualified biosafety cabinet and aseptically dispensed by volume into amber bottles to contain 1 g dry weight of protein product. The final lyophilized product is then further characterized to confirm each batch meets established specification ranges.

5.4. Activity Assessment

Each lot of product is characterized for collagenase activity using the FALGPA peptide substrate[1] and neutral protease activity using succinyl casein substrate[2]. The clostripain and trypsin-like activities are determined on the specific lot of collagenase used to prepare PD Collagenase products[3].

5.5. 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.

5.6. References

1. Van Wart HE and Steinbrink DR. A continuous spectrophotometric assay for *Clostridium histolyticum* collagenase. *Analytical Biochemistry* 113 (1981); 356-65.
2. Hatakeyma T, Kohzaki H, and Yamasaki N. A micro assay for proteases using succinylcasein as a substrate. *Analytical Biochemistry* 204 (1992); 181-184.
3. Mitchell WM and Harrington WF. Clostripain. *Methods in Enzymology* 19 (1970) 635-642.

Appendix 1

Refer to the table below to prepare products equivalent to the former DE Collagenase 200, 400, or 600 using PD Collagenase 100 & 800 products.

Enzyme Formulation in reference to PD 100 product (Collagenase/Protease activity ratio)	Directions to prepare 1 mg equivalents of PD 200, PD 400, or PD 600 using PD 100 and PD-800	
	PD-Collagenase 100 (cat # 011-1010)	PD Collagenase 800 (cat # 011-1050)
DE 200 (2.6)	85.7% = 6 parts PD 100	14.3% = 1 part PD 800
DE 400 (5.2)	57.1% = 4 parts PD 100	42.9% = 3 parts PD 800
DE 600 (7.8)	28.6% = 2 parts PD 100	71.4% = 5 parts PD 800

The illustrative table shows how to prepare 100 mg of DE 200, DE 400, or DE 600

To check your calculations, add the mg of the PD 100 and PD 800. The answer should be the amount of DE 200, DE 400, or DE 600 products required for your cell isolation procedure.

Prepare the enzyme mixtures by weighing the appropriate mass of each product shortly before use—reconstitute and use within two hours. DO NOT STORE POWDERED MIXTURES OF THE TWO PRODUCTS FOR RE-USE SINCE THE HOMOGENEITY OF THE NEW MIXTURE IS NOT KNOWN.

	100 mg
DE 200	100 x 0.857 = 86 mg PD 100 + 100 x 0.143 = 14 mg PD 800
DE 400	100 x 0.571 = 57 mg PD 100 + 100 x 0.429 = 43 mg PD 800
DE 600	100 x 0.286 = 29 mg PD 100 + 100 x 0.714 = 71 mg PD 800

APPENDIX 2: Preparing a purified defined enzyme mixture by matching collagenase and protease activities in your current collagenase product or for a specific application.

The steps outlined below describe the process. If you have any questions, contact VitaCyte technical support.

1. Begin by determining if the "Reference Material" (i.e., the collagenase product you want to match or a cell isolation application you want to replicate with VitaCyte enzymes) has an acceptable collagen degradation activity (CDA)/neutral protease activity (NPA) ratio by translating the Reference Material's enzyme activities into VitaCyte's CDA and NPA U. The table below estimates the collagenase/protease ratios of Worthington and Liberase research products in VitaCyte's enzyme units. The values for the Sigma or Nordmark products are not included because of the variability in specific CDA due to variable proteolytic degradation of collagenase. Contact VitaCyte technical support if you want to prepare a PD enzyme mixture to match a Sigma or Nordmark product.

SUPPLIER	VITACYTE'S TRANSLATED COLLAGENASE CDA U/NEUTRAL PROTEASE NPA U RATIO			
WORTHINGTON	Type 1 = 1.2	Type 2 = 1.0	Type 3 = 3.2	Type 4 = 1.6
ROCHE*	Liberase TL = 28.8	Liberase TM = 3.1	Liberase TH = 1.5	Liberase DH = ?

- See https://www.vitacyte.com/news/replace-liberase-with-vitacyte/ for estimated protease mass. Thermolysin activity is determined by multiplying the Thermolysin mass by 260,000 (Specific activity of purified Thermolysin, NP U/mg protein, at VitaCyte)
- Yellow highlights indicate TL enzyme formulation outside the range of PD collagenase mixtures. Dispase activity in DH products is unknown

2. If this ratio is close to or within the 1.3 to 10.4 CDA U/NPA U ratios, enter the desired collagenase/protease ratio (y) and solve for x using the algebraic equations below. The first set of equations below determines the appropriate percentages of the PD 100 and PD 800 products needed to generate an enzyme mixture with a CDA U/NPA U activity ratio similar to the Reference Material.

   1st equation to determine x, the amount of PD Collagenase 100 used to prepare the desired collagenase CDA/Neutral Protease activity ratio (y). The desired ratio (y) must be between 1.3 to 10.4. The amount of PD Collagenase 800 used in the mixture will be 1-x. The second equation will determine the amount of each product you must weigh to prepare a sufficient quantity of collagenase & protease for your application.

   1st equation to determine the ratio of each product

   $$x = 10.47 - y/9.16$$

An illustrative calculation to generate a collagenase-protease mixture to replace Liberase TM

1st Step: prepare a collagenase-protease enzyme mixture equivalent to Liberase TM

The collagenase/protease ratio = y = 3.1 for Liberase TM

Substituting 3.1 for y in the equation below:

x = 10.47 – 3.1/9.16

x = 0.805 amount of PD Collagenase 100 in the mixture

1-x = 0.195 amount of PD Collagenase 800 in the mixture

2nd Step is to determine the appropriate amounts of PD 100 and PD 800 to prepare an enzyme mixture similar to Liberase TM*

PD 100 contains 3,300 CDA U/mg dry weight (wt)

PD 800 contains 26,400 CDA U/mg dry wt

PD 100 or 800 contains 0.018 mg BP Protease/mg dry weight = 2520 NP U/mg dry wt

For PD 100: 3300 CDA U/mg x 0.805 = 2656.5 CDA U/mg of mixture

For PD 800 26,400 CDA U/mg x 0.195 = 5148 CDA U/mg of mixture

Total CDA = **7804 CDA U/mg of mixture**

To prepare a Liberase TM equivalent product that has 600,000 CDA U, then

600,000/7804 = 76 mg of the above enzyme mixture

This mixture is prepared by adding

76 x 0.805 = 61.2 mg of PD 100 to

76 x .195 = 17.8 mg of PD 800

This 76 mg will contain 191,520 NP U (76 x 2,520 NP U) since the NP U are the same in PD 100 & 800

To confirm the collagenase/NP ratio in the PD Collagenase mixture ≈ Liberase TM

600,000 CDA U/ 191,520 NP U = **3.13**

*CDA and BP protease-specific activities: collagenase 60,000 CDA U/mg, BP Protease 140,000 NP U/mg

Applying the calculation above to prepare a collagenase-protease mixture by application or by product

Species & cell type *Volume solution*	Enzyme U/mL solution	CDA/NP Ratio	Final volume *Required U*	The final mass of the enzyme using the following amounts PD 100 & 800	Calculated CDA & NP Activity in the final solution (Round up to 1 decimal)
Rat hepatocytes *300 mL*	2500 CDA U 1000 NP U	2.5	300 mL *750,000 CDA U* *300,000 NP U*	119 mg mixture by adding 103.5 mg PD 100 + 15.5 mg PD 800	750,750 CDA U 299,800 NP U CDA/NP ratio = 2.5
Rat or mouse islets *40 mL*	8333 CDA U 1667 NP U	5	40 mL *333,320 CDA U* *66,680 NP U*	26.6 mg 16 mg PD 100 + 10.6 mg PD 800	332,640 CDA U 67,032 NP U CDA/NP ratio = 5.0

Recommendations to use PD Collagenases to replace other collagenase products

Product *Required mass*	Enzyme U/bottle	CDA/NP Ratio	Mass to prepare an equivalent mixture	The final mass of enzyme using the following amounts PD 100 & 800	Calculated CDA & NP Activity in the final solution
Liberase TM *10 mg*	600k CDA U 192.4k NP U	3.1	76 mg	61.2 mg PD 100 + 14.8 mg PD 800	592,680 CDA U 191,520 NP U CDA/NP ratio = 3.1
Liberase TH *10 mg*	600k CDA U 390k NP U	1.5	159.5 mg	156.3 mg PD 100 3.2 mg PD 800	600,270 CDA U 401,940 NP U CDA/NP ratio = 1.5

There may be cases where you develop a purified defined collagenase-protease mixture that does not provide cell yields similar to those obtained with the reference material. In those cases, consider supplementing the purified-defined collagenase-protease enzyme mixture with clostripain. Clostripain is one of many enzymes secreted by *C. histolyticum* into the culture supernatants. It is a common contaminant of enriched or purified *C. histolyticum* collagenase products. The instructions for use can be found in the Clostripain package insert.

Contact VitaCyte for technical guidance on any questions about the above method for

- estimating the CDA U/NP U ratio for Sigma or Nordmark products.
- matching an enzyme mixture where the CDA U/NP U ratio is outside the 1.3 to 10.4 CDA U/NP U ratios