VitaCyte®

Unravelling Cell Isolation

webinar

Certificates of Analysis for Tissue Dissociation Enzymes: Practical Utility

Thursday, May 11, 2023

Bob McCarthy

President, VitaCyte LLC

rcmccarthy@vitacyte.com | 317-269-7142

CAN HUMAN ISLET ISOLATIONS BE CONTROLLED?

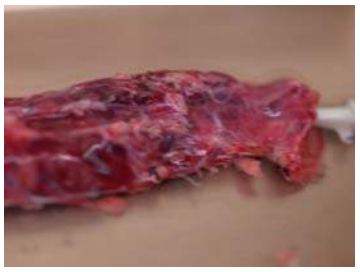

Acceptable Organ/ Tissue
Experienced Cell Isolators
Successful Isolation
Optimized Collagenase-Protease Mixture

Acceptable Pancreata

Normal

< 32 YO

> 32 YO



Diseased

Experienced Islet Isolators

- Islet isolation is more art than science
- Tacit Knowledge: Knowledge is difficult to transfer (not explicit): “We know more than we can tell”
- Explicit Knowledge: Knowledge that can be written down, verbally communicated, transferred to others

Optimized Collagenase-Protease Mixture

- All agree the enzyme mixture is a critical factor for successful islet isolation
  but still searching for best enzyme formulation
- Applying QBD terminology: the collagenase-protease enzyme mixture used for isolation is a critical process parameter (CPP) since it impacts many critical quality attributes (CQAs)
- If you agree that enzymes are CPPs, then we must know more about the Certificate of Analyses (CoAs), which is the focus of this webinar
- Enzymes are the only variable that can and should be controlled in islet isolation process

Outline of Presentation

- Liberase HI Certificates of Analyses (CoAs): Past vs Present
- Description of analyses and enzymatic activity assays: relationship to *Clostridium histolyticum* collagenase structure-function
- Application of analyses and enzyme activity assays to troubleshoot enzyme problems from Liberase HI to now
- Model for collagenase-mediated tissue dissociation
- Neutral protease assays and other factors to consider
- Summary, useful tips, VitaCyte's recommendations

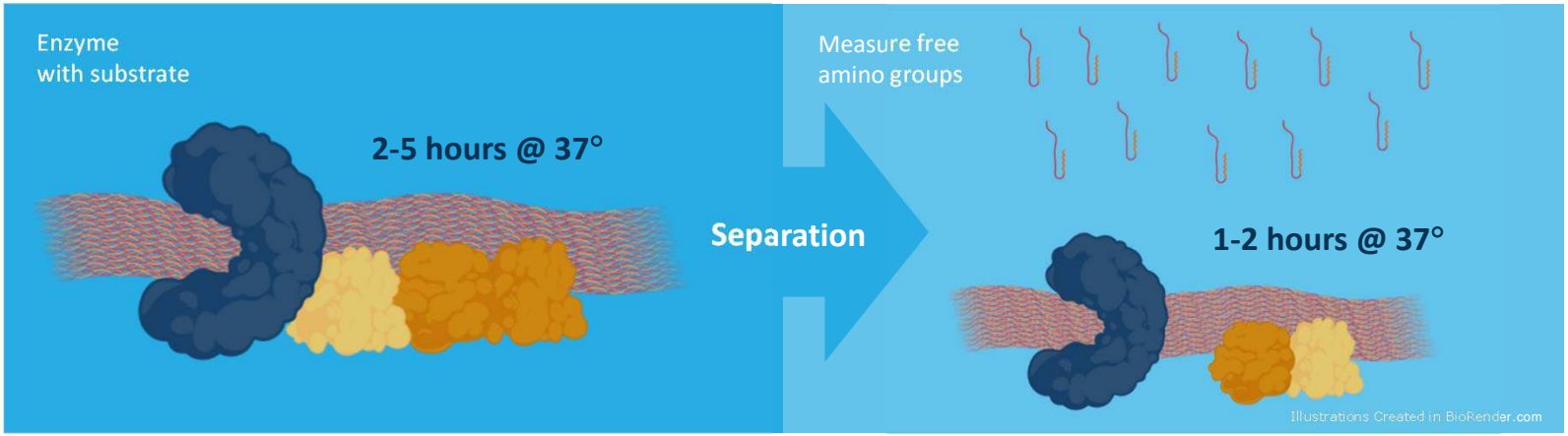
LIBERASE HI CERTIFICATES OF ANALYSIS: PAST VS PRESENT

Assay Results Reported on Liberase HI COAs

Assay	Steps	Analyte
Wünsch Assay: Peptidase Activity	Incubate > Quench> Extract > Dry > Read A_{320} (Typical run 14 tubes)	Class II Collagenase Activity
Anion Exchange Analytical Chromatography	Sample Prep > Passage over Mono-Q column > Analyze results	Detection of Intact C1 and Truncated C1 Forms
Endotoxin	Limulus or Limulus equivalent assay	Endotoxin EU/A280
Neutral Protease Activity (FITC Casein)	Incubate > Precipitate > Read fluorescence emission	Neutral Protease Activities
BAEE Assay Peptidase Activity	Untreated or reduced sample pipetted into quartz cuvette containing BAEE peptide > Read A_{253} over 2 min	Trypsin-like and Clostripain Activities

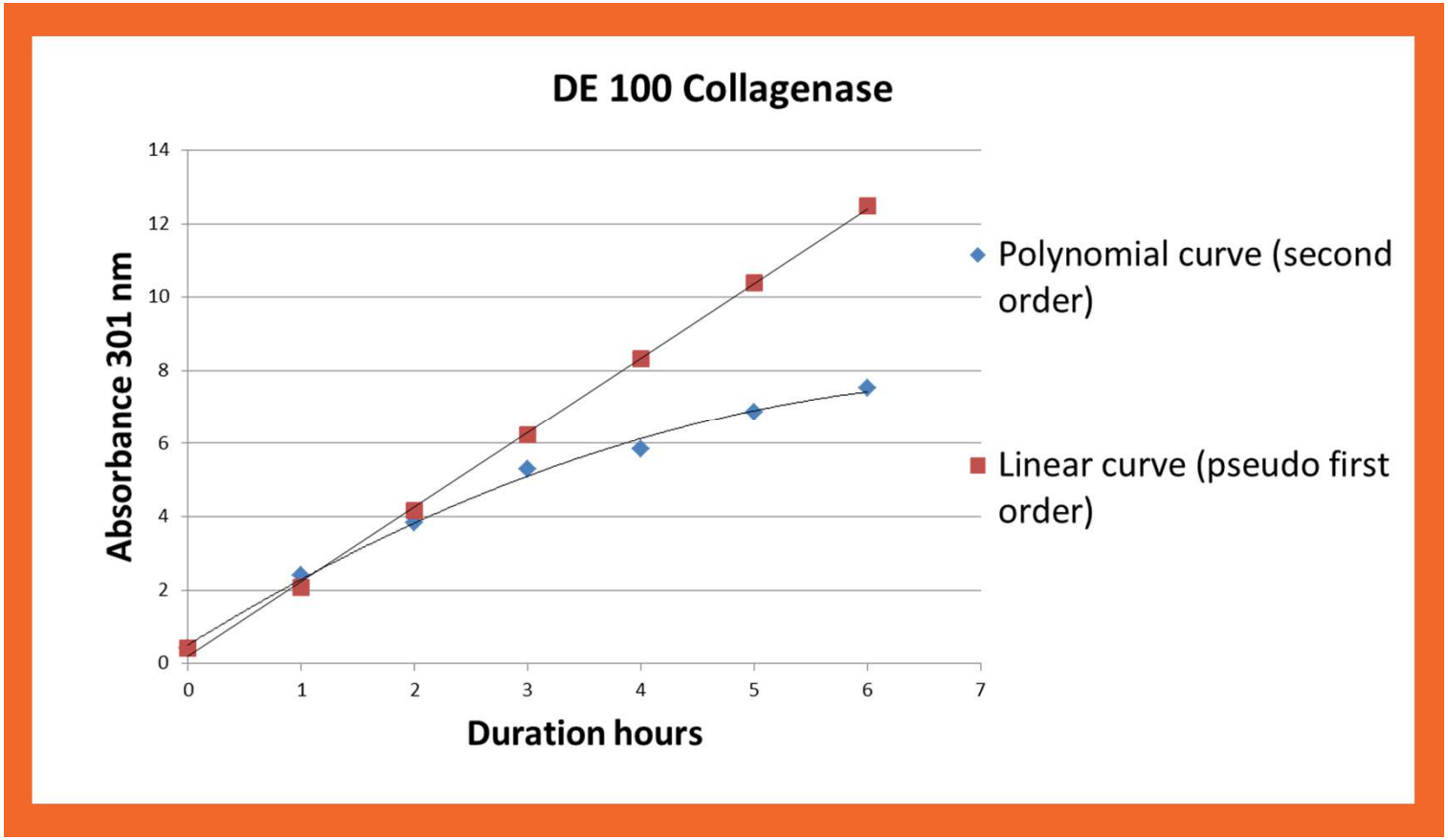
Evaluated and Dropped use of Mandl Assay for Collagenase Activity

[1953] Mandl CDA Assay: *Heterogeneous, Endpoint Assay*



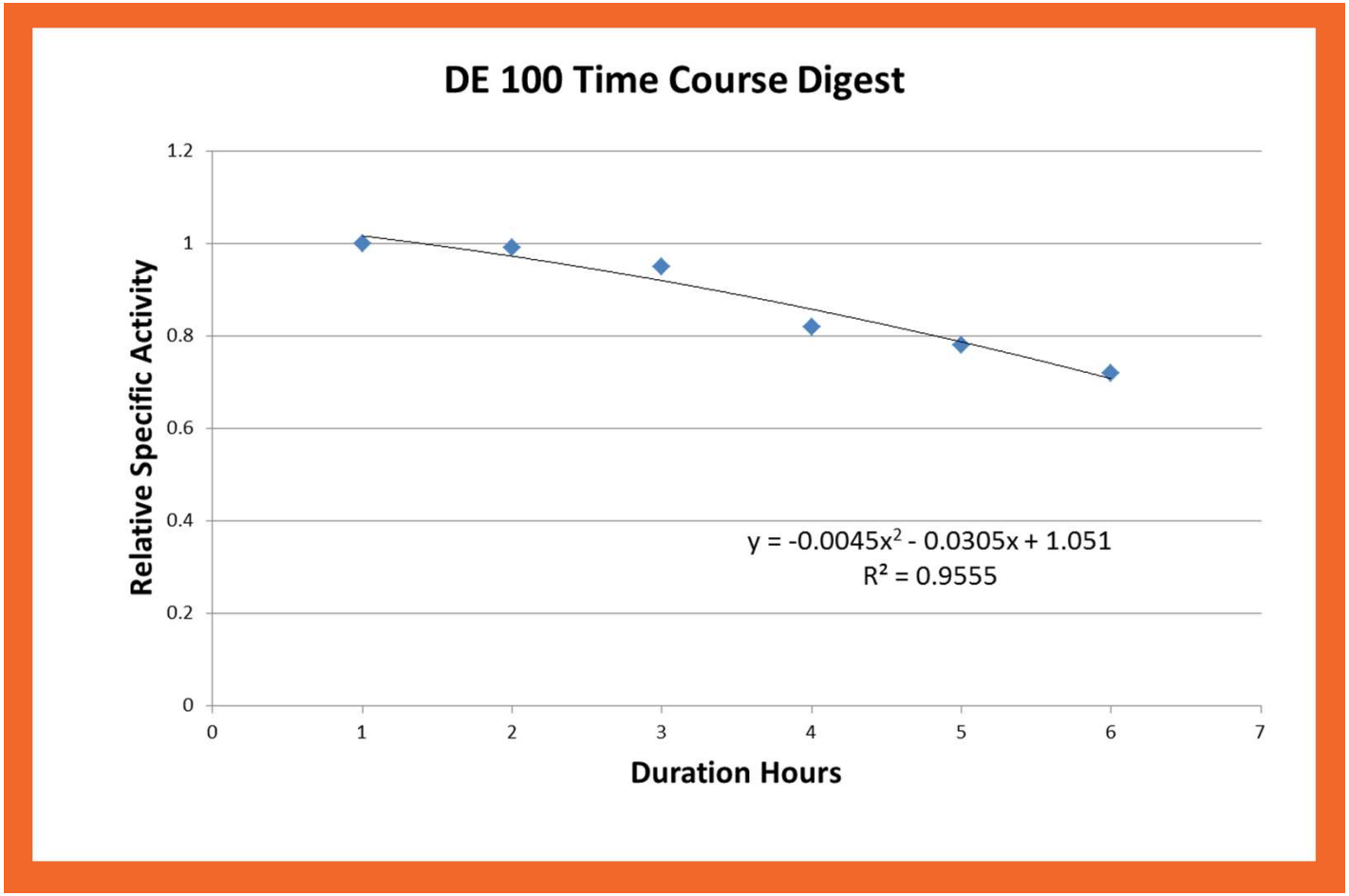
- Collagen fiber substrate (Achilles tendon)
- Appropriate dilution purified collagenase
- On completion, separate undigested fiber from supernatant
- Transfer supernatant to another test tube for detection free amine groups
- Mix fixed volume supernatant with reagent to detect free amine groups
- On completion, stop reaction
- Read absorbance at 310 nm
- Calculate specific activity usually in µM leucine equivalents released per min or mg collagenase

Kinetic Analysis of Collagen Fiber Degradation

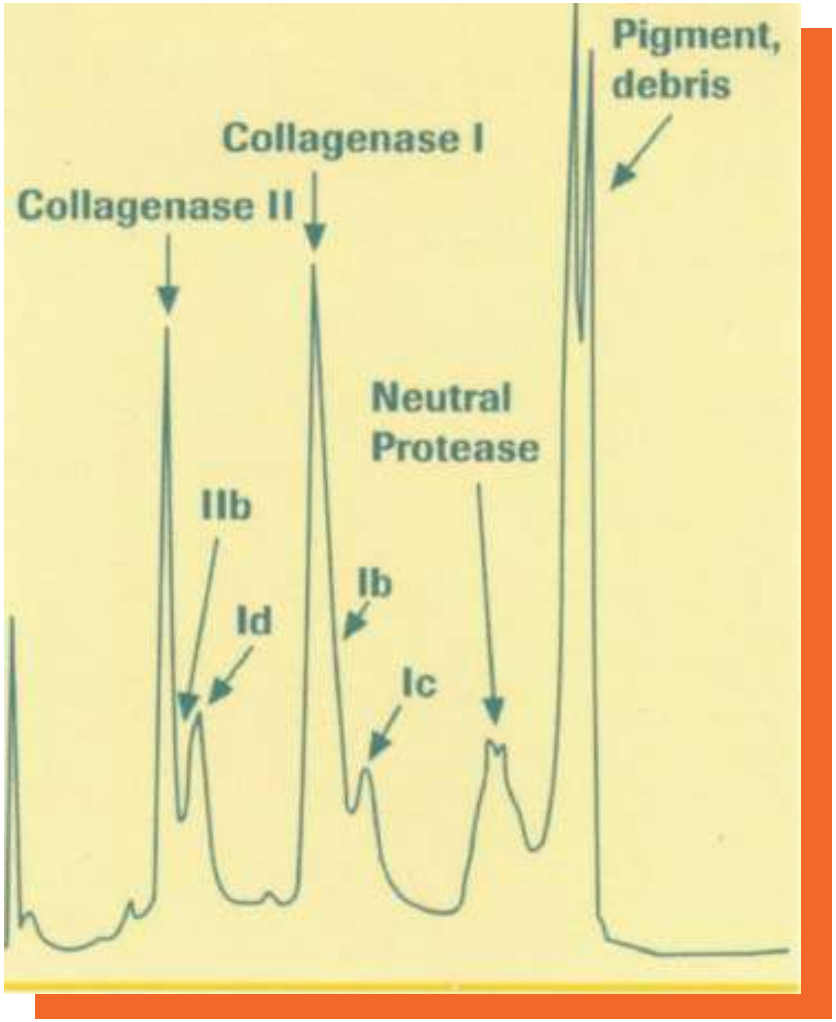


See https://www.vitacyte.com/collagen-degradation-by-clostridial-histolyticum-collagenases/

Assay Conditions Effect CDA Specific Activities

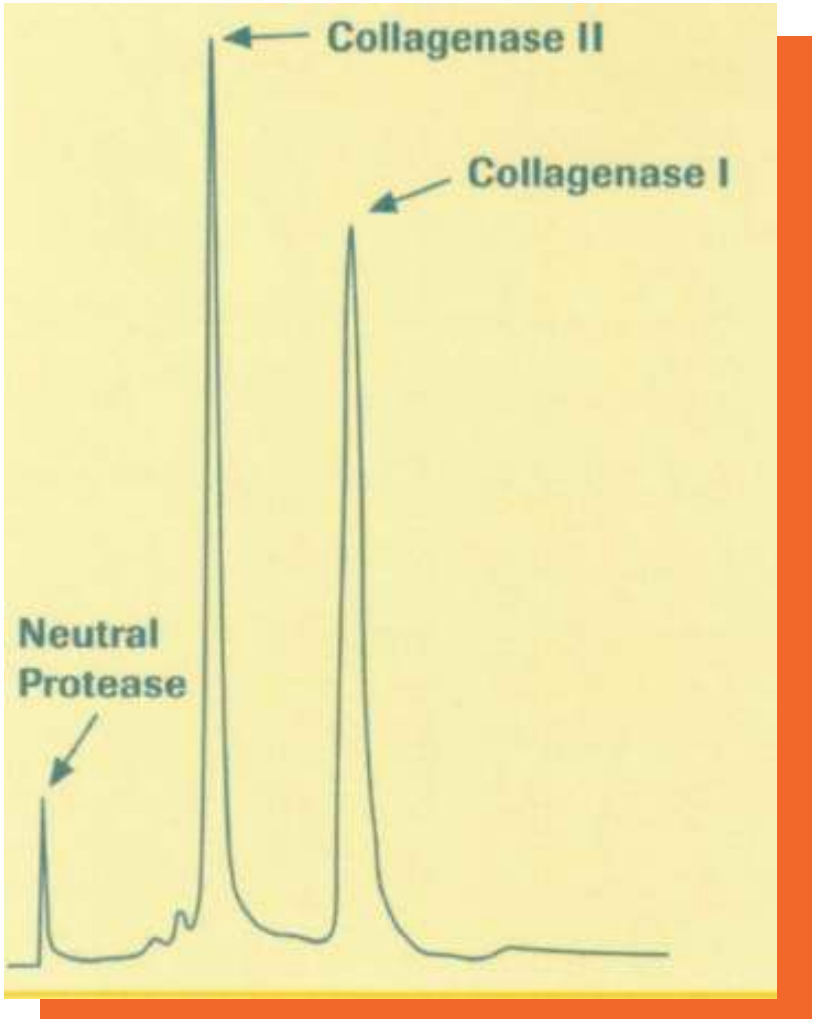


Collagenase P



- Uncontrolled processing
- Lot qualification before use
- About 30% lots effective
- Shelf life < 18 months

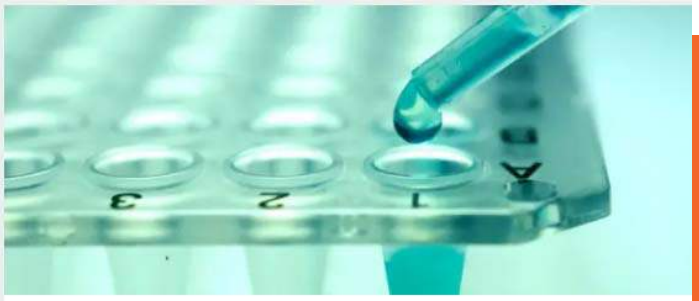
Liberase™ HI



- Identify key enzymes: class I & II collagenase & protease
- Purification improves product consistency: 60:40 C1:C2 mixture
- Supplement with thermolysin

USP Monographs for *C. histolyticum* Collagenase

- Monographs <89.1> class I (C1) collagenase & <89.2> class II (C2) collagenase introduced in 2016 describe
  - Peptidase activity assay using (Wünsch assay)
  - Anion exchange chromatography analyses



Reference Standards

Buy direct from USP:

- Collagenase I
- Collagenase II
- Trypsin Recombinant Porcine
- Fetal Bovine Serum
- Protein A
- rProtein A
- rProtein A, C-Cys

SEE ALL BIOLOGICS REFERENCE STANDARDS

Documentary Standards

For associated documentary standards, please visit *USP-NF Online* (note: you will need a log-in to access specific standards)

- <89.1> Collagenase I
- <89.2> Collagenase II
- <89> Enzymes used as Ancillary Materials in Pharmaceutical Manufacturing
- <90> Fetal Bovine Serum Quality Attributes and Functionality Tests
- <130> Protein A Quality Attributes

ACCESS *USP-NF ONLINE*

Content on Today's Certificates of Analyses

Parameter	Nordmark	Roche	VitaCyte
Collagenase			
Wűnsch activity	√	√	√
FALGPA activity			
Anion exchange chromotography		√	√*
Collagen degradation activity (CDA)			√**
Trypsin Like activity	√	√	√
Clostripain activity	√	√	√
Endotoxin	√	√	√
Expiration date	√	√	√
Neutral protease			
Neutral protease activity***	√	√	√
Endotoxin	√	√	√
Expiration date	√	√	√

* Perform analyses but do not put on the CoA

** CDA is fluorescent kinetic microtiter plate assay not the Mandl assay used by Sigma and Worthington

*** Each supplier uses a different neutral protease assay Nordmark DMC casein, Roche FITC casein, VitaCyte FITC HSA format same as CDA

DESCRIPTION OF ANALYSES & ENZYMATIC ASSAYS: RELATIONSHIP TO COLLAGENASE STRUCTURE-FUNCTION

Wünsch assay

Advantages vs Disadvantages

Advantages	Disadvantages

• Precise: between run CV < 10%
• Similar values between manufacturers
• Common practice is to dose collagenase for islet isolation: Wünsch U (WU)/g tissue
	• Technically challenging
	• Collagenase class II specific activity > 50x higher than class I
	• Wünsch activity ≠ collagen degradation activity
	  • Peptidase activity assay assesses ability of **catalytic domain** to cut the peptide
	• Wünsch activity ≠ collagenase mass:
	  • Mass calculated by using Wünsch specific activity and collagenase class I:class II ratio

Wünsch Activity ≠ CDA in Following Reports

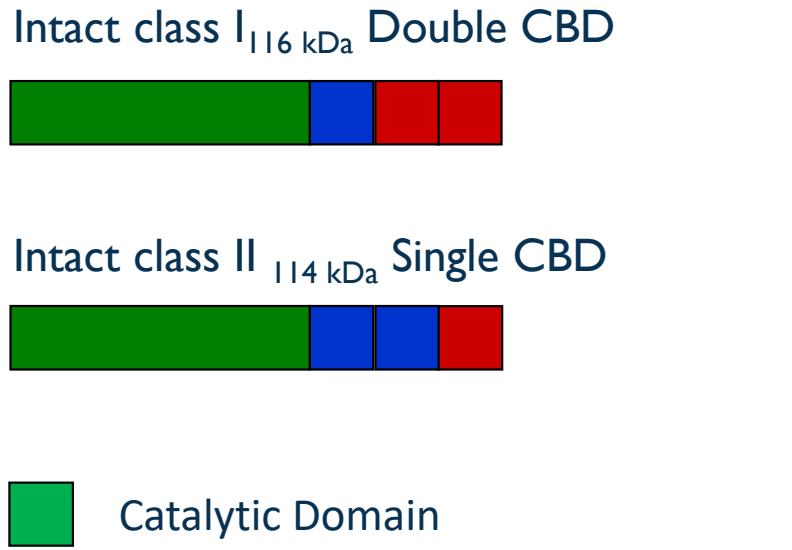
- Digestion of C1 or C2 with chymotrypsin
  - > 84% and > 95% loss of CDA for C1 and C2 respectively
  - 7.1 % loss of C2 Wünsch activity
- Lakey found decrease in the ability of Collagenase P to recover porcine or canine islets 6 to 16 months after date of manufacture
  - Unlikely Wünsch activity affected by storage
  - Loss of activity likely due to clostripain degradation of C1
- Kin, et al. reported on 251 human islet isolations and found that Wünsch activity was not important as neutral protease activity to predict islet isolation success
  - Noted the limitations of Wünsch activity to detect C2

explanation of the Wünsch results

Collagenase Structure-Function > CDA

- *C. histolyticum* collagenase multi-domain structure
- "Functional" collagenase has catalytic and collagen binding domain (CBD), degrades native collagen
- "Non-functional" collagenase cannot degrade native collagen, has catalytic domain but no CBDs

Collagenase Domain Structure



Correlation of Physico-Chemical Analyses with CDA

Intact C2 $_{114\ kDa}$ Single CBD

9500 CDA U/mg

Intact C1 $_{116\ kDa}$ Double CBD

100,000 CDA U/mg

C1b Single CBD C1 $_{100\ kDa}$

9500 CDA U/mg

C1c Single CBD C1 $_{100\ kDa}$

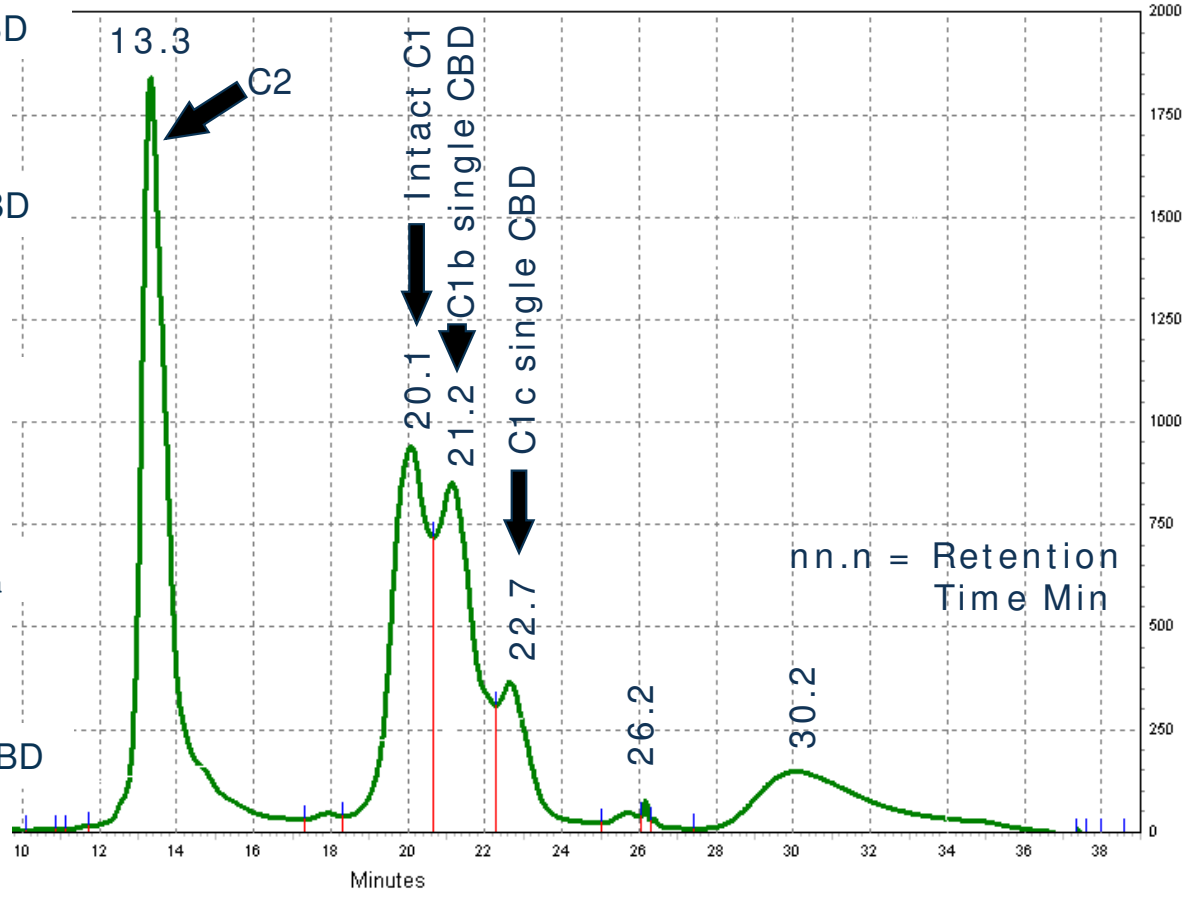
9500 CDA U/mg

Degraded C1 $_{<84\ kDa}$ NO CBD

0 CDA U/mg

Degraded C2 $_{<100\ kDa}$ NO CBD

0 CDA U/mg



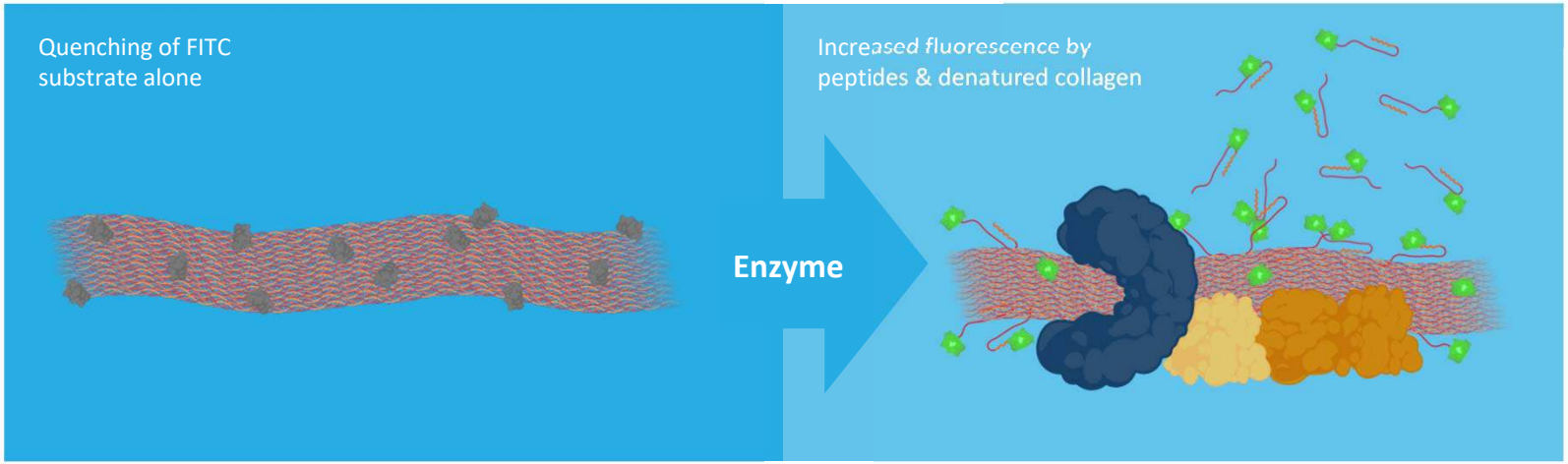
Catalytic Domain

PKD Domain

Collagen Binding Domain

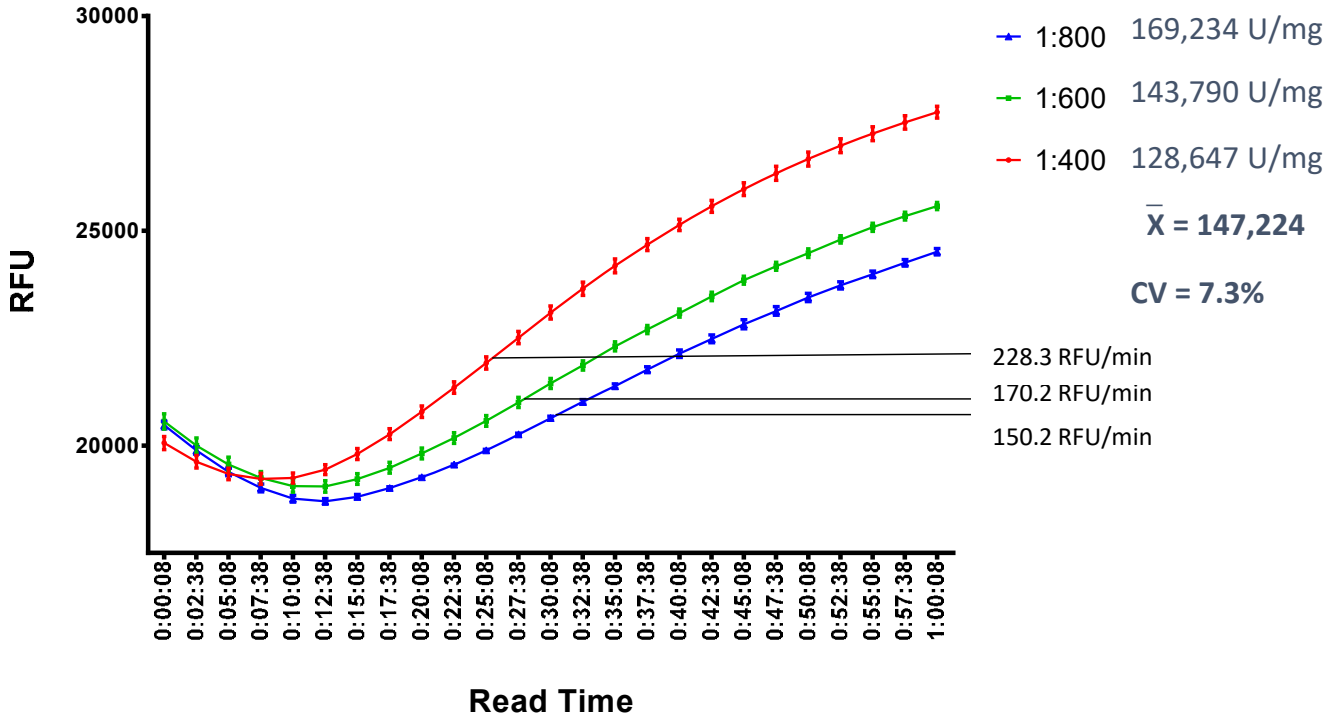
[2008] Fluorescent, Kinetic Microtiter Plate CDA Assay:

Homogeneous, Kinetic Assay



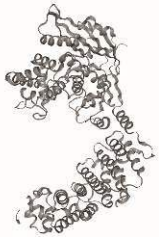
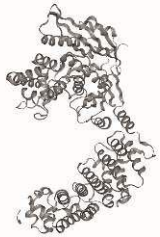
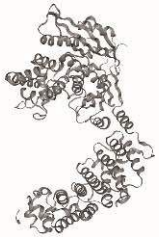
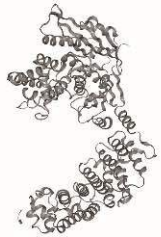
- Appropriate amount of fluorescein isothiocyanate-collagen fibrils (FITC-fibrils) from bovine skin- (Type 1) in 50 µL
- 50 µL of an appropriate dilution purified collagenase
- Run each sample at 2 or 3 dilutions, in duplicate
- Measure release of fluorescence in relative fluorescence U (RFUs) by exciting FITC fluorescence at 485/20 nm and detecting fluorescence at 528/20 nm
- Determine Vmax using mean from 7 fluorescent readings, pseudo first order kinetics
- Calculate specific activity per mg protein

Fluorescent Kinetic Microtiter Plate (FL-MTP) Assay



Assay in duplicate, 7 points to calculate mean rate fluorescent units (RFU/min)

Correlation of Collagenase Structure to Activity

	Functional Collagenase			Non-Functional Collagenase	
	Intact Class I 116 kDa	Intact Class II 114 kDa	Truncated Class I 100 kDa	Degraded Class II 100 kDa	Degraded Class I 85 kDa
Catalytic Domain					
Linking Domain					
Collagen Binding Domain					
Collagen Degrading Activity	++++	++	++		
Gelatinase Activity	++++	++	++++	++	++++
Peptidase Activity (PZ peptide, FALGPA)	+	++++	+	++++	+

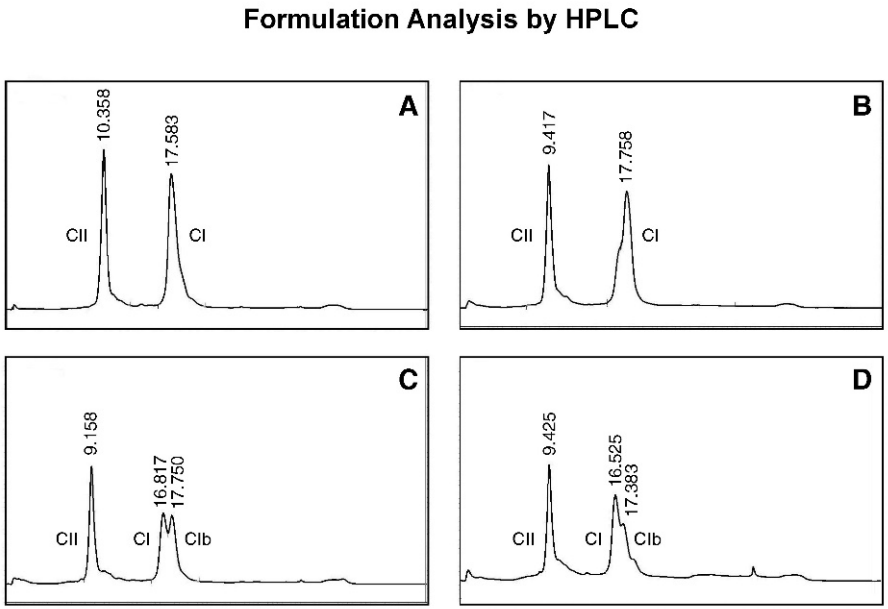
Doesn't degrade collagen in ECM!

Functional and non-functional forms of C. histolyticum collagenase and enzyme activity profile

The 3-dimensional structures above are for illustration and are individual domains of the *C. histolyticum* C1 collagenase obtained from the Protein Database[4-6]. Three different assays can be used to assess collagenase activity, however only the collagen degrading activity assay measures functional collagenase activity which is required to degrade native collagen leading to the release of cells from tissue.

APPLICATION OF ANALYSES & ASSAYS TO TROUBLESHOOT ENZYME PROBLEMS

Changes in Liberase™ HI Collagenase Affected Human Islet Yields



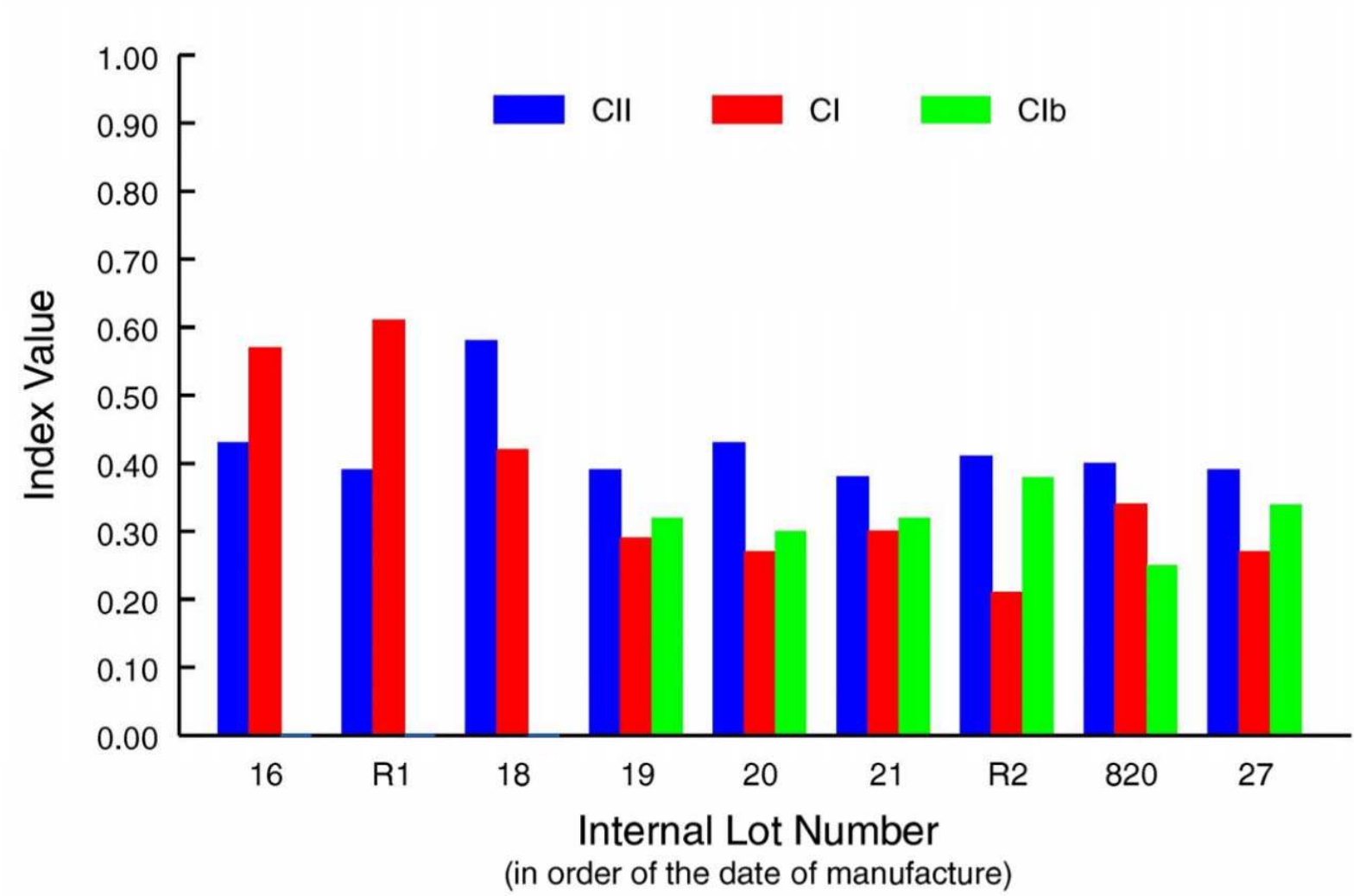
Isolation based on CI gelatinase enzyme activity

	Unsuccessful N=5		Successful N=5	
	CI Activity CDU/ug	IE Yield	CI Activity CDU/ug	IE Yield
Mean	1.64†	177,764 IE	2.98†	488,075 IE
SEM	+/-0.2	+/-73,687 IE	+/-1.1	+/-122,769 IE
t-test	*p= 0.0447*† one-tailed, paired: p<0.05			

- Lakey's lab in Edmonton found a temporal change in quality of Liberase HI
- Correlated change with loss of C1 gelatinase activity and suboptimal human islet yields where success is recovery of >300,000 IEQ/organ
- Change unlikely to be detected by Wunsch activity

From Barnett, et al. *Transplantation* (2005) 80: 723-28 & JRT Lakey islet workshop presentation July 2005

Chronology of Changes in Liberase HI Collagenase Quality



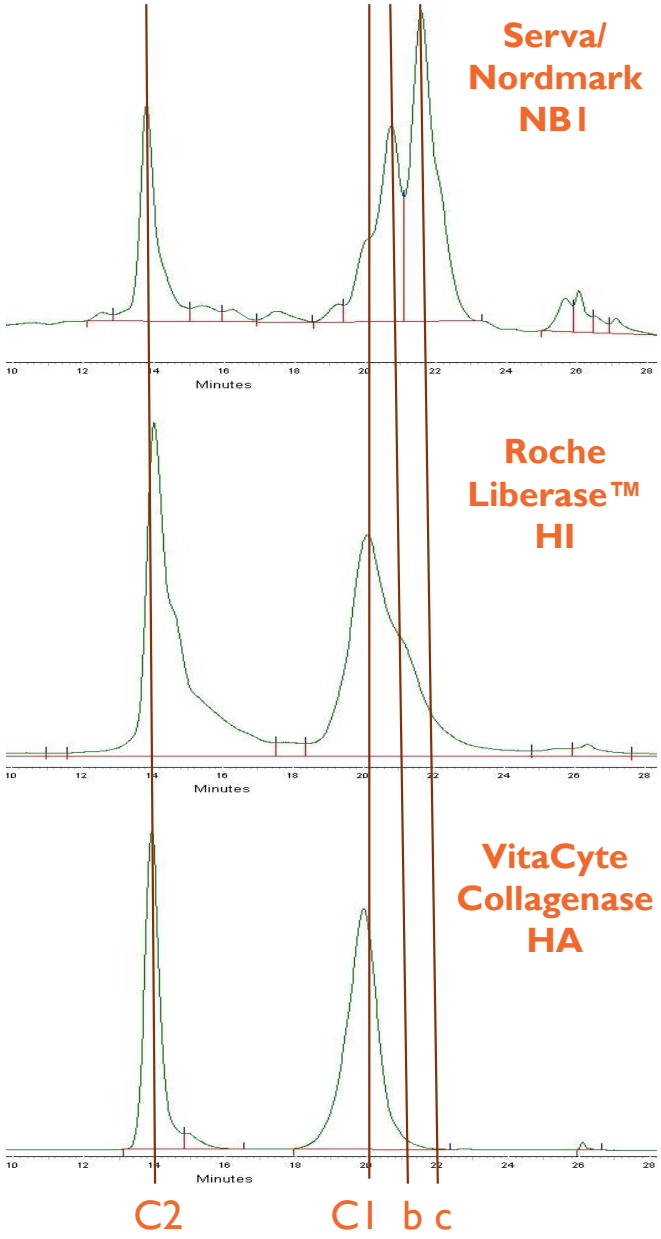
similar case

Switch From Liberase HI to Nordmark NB-1 Collagenase in CITC Trial

- 2007: NIH dropped use of Liberase HI in CITC clinical trial because of risk for TSE transmission, led to switch to GMP Grade Nordmark enzymes
- At the time, limited knowledge of key characteristics of good collagenase
  - Wünsch activity measured C2 collagenase activity, no assay for C1 activity
  - No reproducible collagen degradation activity assay
  - No knowledge of how collagenase and neutral protease released cells from tissue

Problems with Supplier Switch

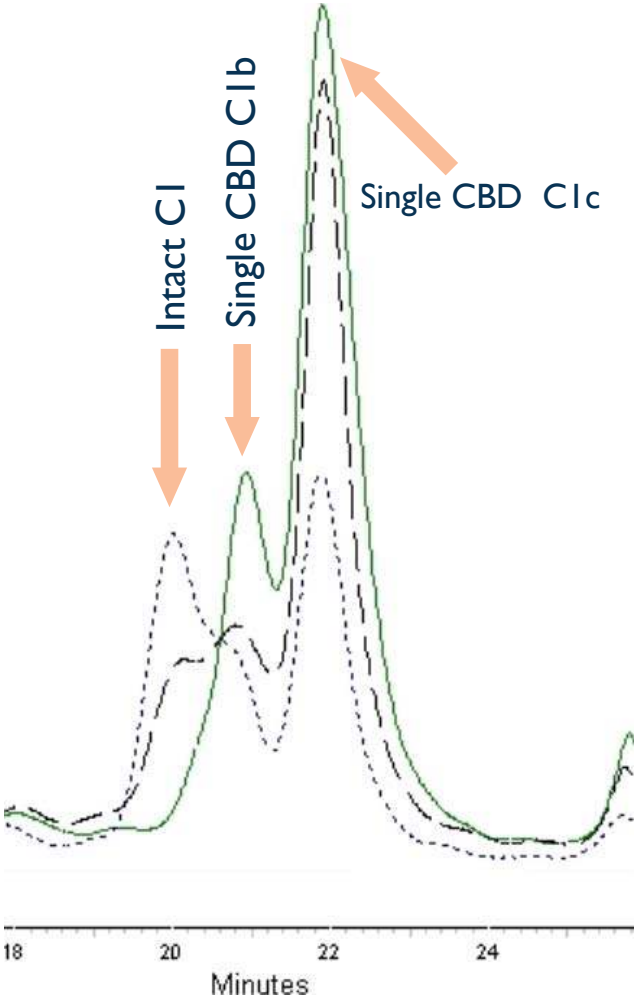
- The centers who successfully reproduced the Edmonton Protocol in the ITN trial were unable to recover sufficient numbers of islets using Nordmark's enzymes
- Collagenase from different suppliers had different biochemical characteristics
- Validated Barnett et al. prior observation



Difference in Collagenase Supply

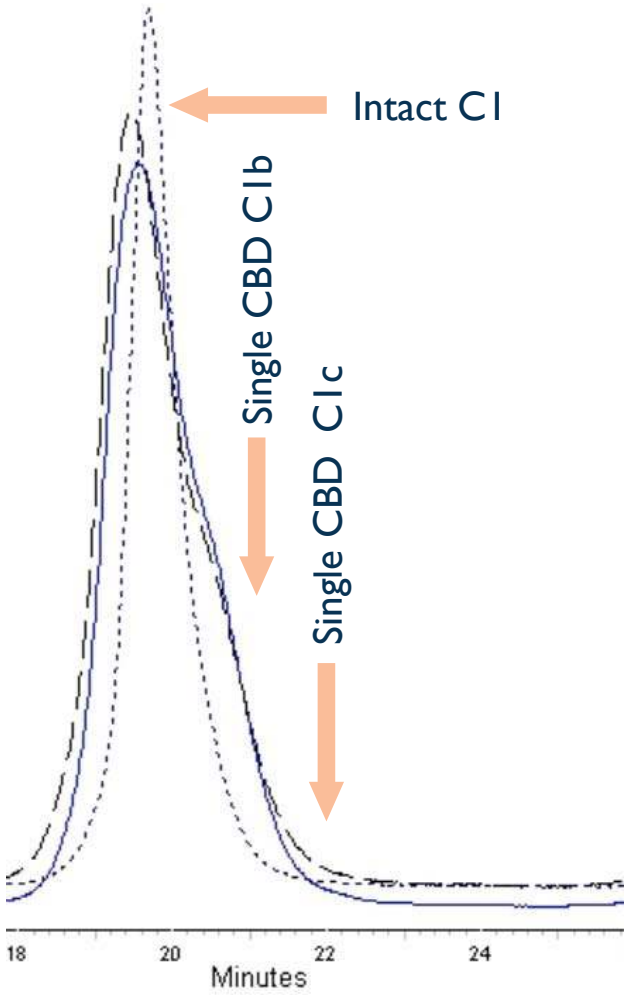
Nordmark NB-1 Collagenase

CDA/mg = 12710 ± 1646



VitaCyte Collagenase HA

CDA/mg = 37854 ± 4791



From Balamurugan AN et al. *Transplantation* (2010) 89:954-961

Importance of Intact C1 for Increasing Human Islet Yields

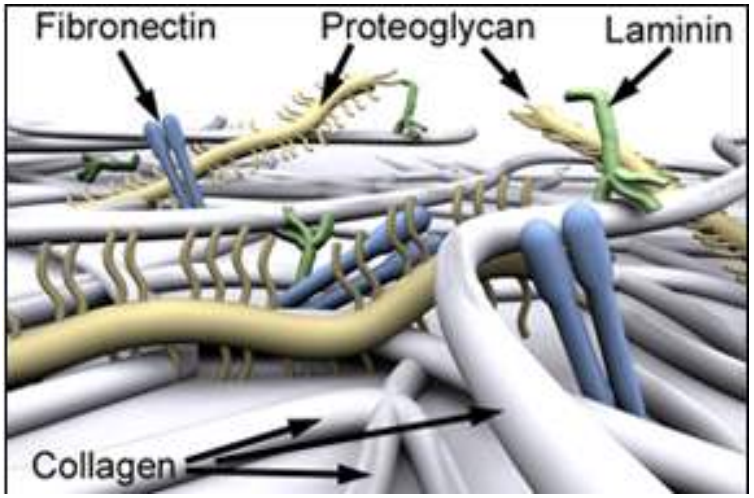
Parameter	VitaCyte n=14	Serva/Nordmark n=27	P Value
Trimmed Pancreas Weight	106.4 ± 26.6	106.6 ± 33.6	0.98
Digest Time	20.1 ± 4.1	23.7 ± 5.5	0.03
Percent Digested	81.1 ± 9.5	78.5 ± 13.0	0.52
Post-Purification IEQ (x 10^3)	419.1 ± 150.9	217.5 ± 152.4	0.001
Post-Purifications IEQ/g	4147 ± 1759	2134 ± 1524	0.002
Percent Recovery After Purification	86.1 ± 22.0	67.1 ± 34.3	0.07
Oxygen Consumption Rate	122.3 ± 20	123.2 ± 41.4	NS
Stimulation Indes (GSIR)	3.7 ± 1.1	3.1 ± 2.0	NS
Islet Viability (FDA/PI %)	94.4 ± 2.3	95.5 ± 2.5	NS

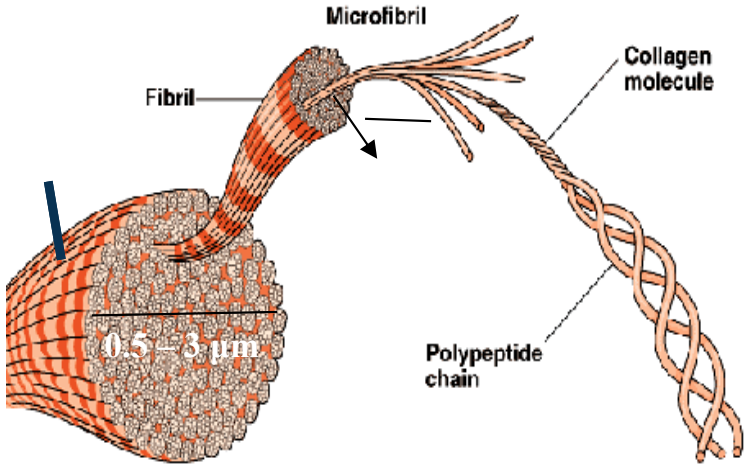
From Balamurugan AN et al. *Transplantation* (2010) 89:954-961

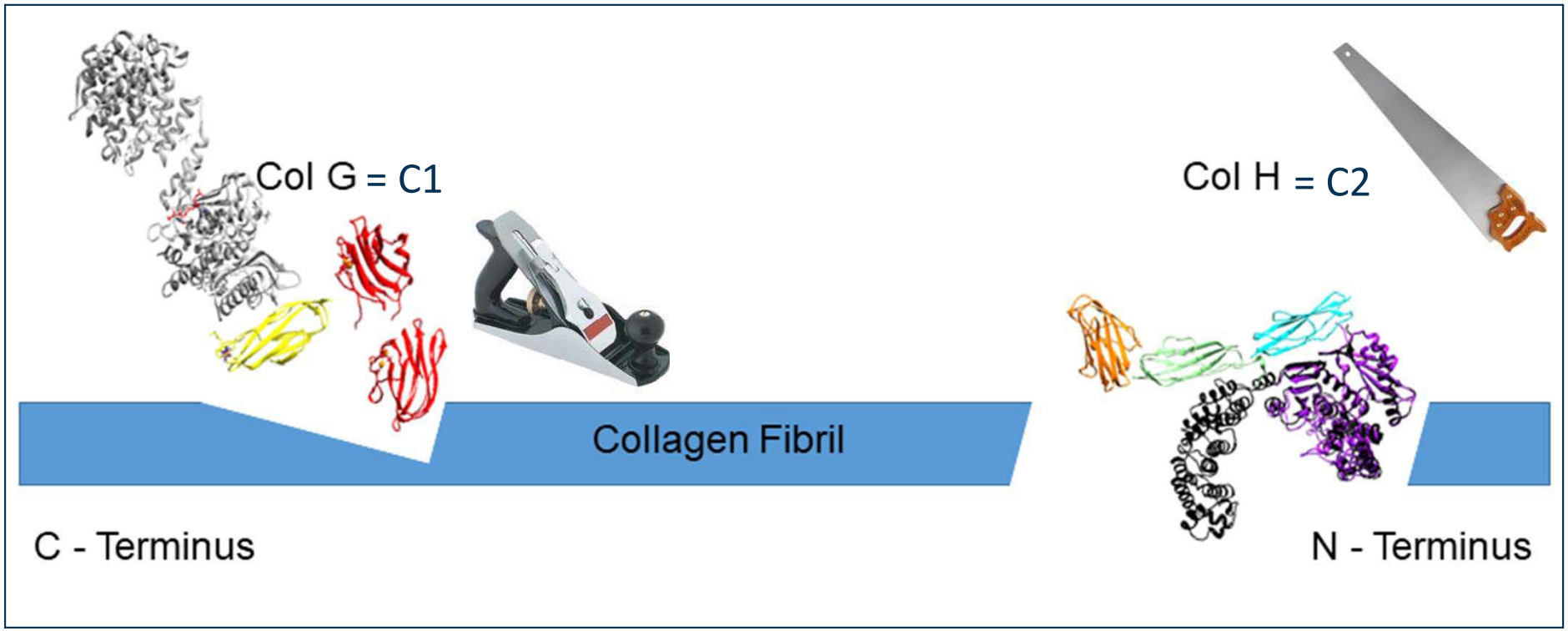
MODEL FOR COLLAGENASE MEDIATED TISSUE DISSOCIATION

ECM Designed to Resist Proteolysis

- Many different proteins in the extracellular matrix (ECM)
- Collagen protects the structural integrity of the ECM, resistant to proteolytic damage
- Cell anchoring proteins directly or indirectly attached to ECM hold cells in tissue
- *C. histolyticum* collagenase used for tissue dissociation because of its ability to degrade all types of collagen



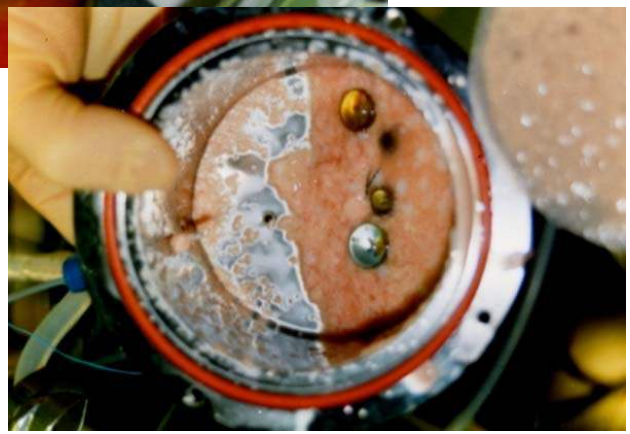




Slides provided by K Tanaka & J Sakon, University of Arkansas

Model for Tissue Dissociation

- As collagen is degraded, matrix loosens, exposing protease sensitive sites on ECM proteins
- ECM proteins cut by proteases, further loosening the tissue
- If a sufficient number of "anchoring proteins" that hold cells to tissue are broken, then cells released from tissue
- 2 critical factors to control
  - Is the CDA activity in excess?
  - Is the neutral protease activity in control
- Excess purified collagenase unlikley to damage cells but too much neutral protease will damage cells, impact CQAs



NEUTRAL PROTEASE ASSAYS & OTHER FACTORS TO CONSIDER

Neutral Protease Activity Assays

Most of the assays described below were developed > 30 years ago and measured activities associated with the enzyme without knowledge of their structure or function

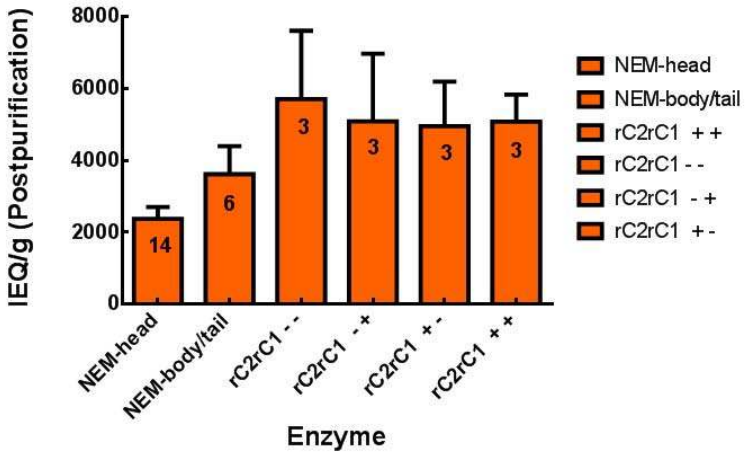
Vendor	Separation Step	Assay/Readout	Characteristics
Nordmark	N/A	Dimethyl casein/measure appearance of amino groups with trinitrobenzene sulfonic acid	Endpoint, low throughput
Roche Liberase HI	TCA	FITC-casein/ FITC fluorescence	Endpoint, low throughput
Roche Liberase MTF	TCA	Casein/absorbance release equivalent to tyrosine	Endpoint, low throughput
VitaCyte	N/A	FITC-human serum albumin/measure increase in fluorescence	Kinetic, high throughput

Conversion factors Nordmark or Roche NPs to VitaCyte NPA U:

- Nordmark: DMC U to VitaCyte NPA U: multiply DMC U by 13,400
- Roche Thermolysin: (mg Thermolysin/1.6) x 260,000

Other Factors to Consider

- Class I:class II recombinant collagenase ratios from 27:73, 38:62, 43:57, & 55:45, does not impact ability of collagenase to degrade pancreatic collagen
- Must know exogeneous sources of neutral protease activities since increased dose of collagenase protease mixture may result in damage to islets
  - Presence of trypsin like activity impacts human islet yields in high dose NEM at UMN



+ C1 = 200K CDA U/g tissue
- C1 = 100K CDA U/g tissue
+ C2 = 20 WU/g tissue
- C2 = 12 WU/g tissue

All information above applicable to Ricordi method as performed today

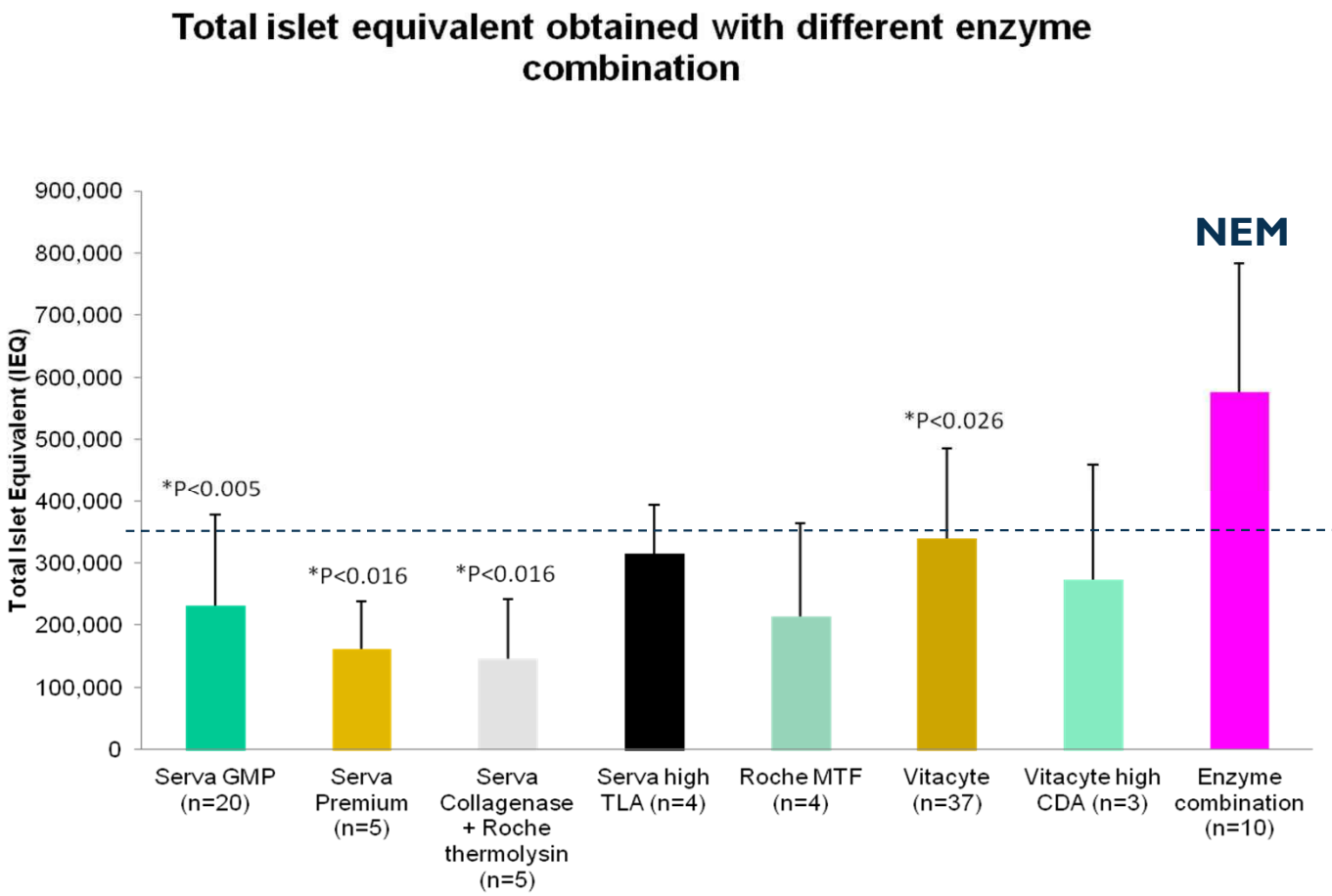
If perform a static digestion, must reassess impact of these factors (NHP islet isolation Ricordi vs static digestion method)

SUMMARY

Issues Covered Up Until Now

- Review key assays presented on CoA and addressed limitations of each
- Shown how these assays were used to troubleshoot problems with inconsistent Liberase HI products and when switch made to Nordmark NB-1 Collagenase and NB Protease products
- Focus on two issues
  - Do you have excess collagen degradation activity?
  - Are you using an appropriate dose of neutral protease enzyme
- All information above restricted to a Ricordi islet isolation method

Intact C1 & Protease Critical for Successful Human Islet Isolation



Serva = Nordmark enzymes

From Balamurugan AN et al. *Transplantation* (2012) 93:693-702

Tissue Dissociation Enzymes for Isolating Human Islets for Transplantation: Factors to Consider in Setting Enzyme Acceptance Criteria

Robert C. McCarthy, Andrew G. Breite, Michael L. Green, and Francis E. Dwulet

Tissue dissociation enzymes are critical reagents that affect the yield and quality of human pancreatic islets required for islet transplantation. The United States Food and Drug Administration's oversight of this procedure recommends laboratories to set acceptance criteria for enzymes used in the manufacture of islet products for transplantation. Currently, many laboratories base this selection on personal experience because biochemical analysis is not predictive of success of the islet isolation procedure. This review identifies the challenges of correlating results from enzyme biochemical analysis to their effectiveness in human islet isolation and suggests a path forward to address these challenges to improve control of the islet manufacturing process.

Keywords: Tissue dissociation, Collagenase, Islet isolation, Protease, Biochemical characterization, Type 1 diabetes.

(*Transplantation* 2011;91: 137–145)

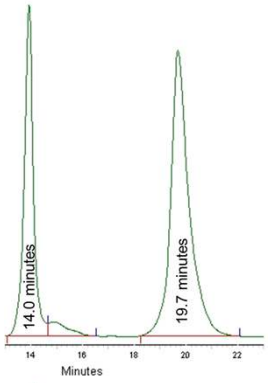
- Collagenase
  - Wünsch assay activity
  - Collagen degradation activity
  - Anion exchange chromatography
- Neutral Protease
  - Neutral protease activity
  - TLA & clostripain if use *C. histolyticum* neutral protease
- Other Parameters
  - Endotoxin
  - Expiration date: important to document assays used to assess stability

Which Assay is the Canary in the Coal Mine When Selecting Collagenase Products?

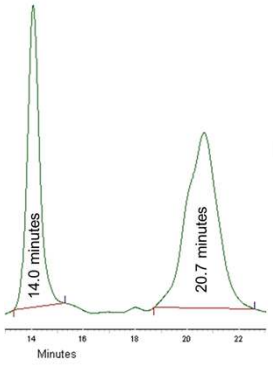
- Most insightful assay to begin evaluation of new lot of collagenase is to review anion exchange profile
- Clostripain very effective in degrading class I collagenase (e.g., Collagenase P) leading to loss of islet recovery
- ⬆ C1b or C1c peaks indicate degradation of collagenase and if all C1b, may impact C2
- Best example is Lakey's lab's work discovering change in manufacturing Liberase HI

VitaCyte's Focus on Selling Purified-Defined Collagenase & Proteases

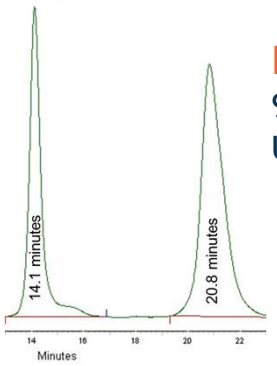
- VitaCyte primary focus is to provide consistent enzymes using 60:40 C1:C2 ratio
- First products identified as Collagenase HA (high), MA (moderate) and LA (low) collagen degradation activity
- Each product has the same specific Wunsch activity but different specific CDAs
- Current specific activities last 10 lots
  - HA # 001-1000 59,695 ± 6,306 (CV=10.6%) CDA U/mg
  - MA # 001-2030 25,398 ± 2,007 (CV=7.9%) CDA U/mg
  - LA: dropped, no market interest



HA
35209 CDA U/mg



MA
18873 CDA U/mg



LA
9024 CDA U/mg

Guiding Principle

If I had to reduce my message for management to just a few words, I'd say it all had to do with reducing variation

- W. Edwards Deming