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Liberase T-Flex Research Grade

 **Version 03**

Content version: April 2011

Partially blended purified enzymes for tissue dissociation

	Cat. No.	Content
Liberase	05 989 132 001	1 × 500 mg Collagenase blend
T-Flex Research Grade		2 × 15 mg Thermolysin

Store Liberase Research Grade products at –15 to –25°C

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1. What this Product Does

Product Characteristics Liberase T-Flex kit contains highly purified collagenase blend and neutral protease (Thermolysin) enzyme in separate vials. The kit contains 2 vials of Thermolysin to allow flexible adjustment of Thermolysin in the proteolytic enzyme mixture. Use a mixture of this two components for efficient, gentle, and reproducible dissociation of tissue. The purified collagenase enzymes are isoforms I and II, as specified by the nomenclature of Bond and Van Wart (3). The target substrates for these enzyme blends are the collagen and non-collagen proteins that comprise the intercellular matrix.

Formulation Liberase Research Grade products are white lyophilizates consisting of aseptically filled, blended enzymes, and a small quantity of buffer salts.

Target Activities Each vial of Liberase T-Flex Research Grade purified enzyme is filled by total protein mass. Combined collagenolytic activity of the Collagenase I and II isoforms is measured by the method of Wunsch (1). Neutral protease activity is measured by a non-fluorescent Caseine assay (2). The kit components allow to make individual blends of Collagenase and Thermolysin ratios.

Product	Target Enzyme Mix (mg)	Target Collagenase Activity (Wunsch units/vial)	Target Neutral Protease Amount	Enzyme Mixture Aggressiveness	Main Application
Collagenase I/II Blend Research Grade	500	2600			Very gentle isolation of islets from pancreas
Thermolysin Research Grade	15		Low	+(+)	
Collagenase I/II Blend Research Grade	500	2600			Isolation of islets from rodent pancreas
Thermolysin Research Grade	24		Low	++	
Collagenase I/II Blend Research Grade	500	2600			More harsh isolation of islets from pancreas
Thermolysin Research Grade	30		Low	++ (+)	

+ = (lowest neutral protease activity/mg protein mixture)

+++++ = (highest neutral protease activity/mg protein mixture)

Storage and Stability

- The lyophilized enzyme is stable at -15 to -25°C until the expiration date printed on the label of the product.
- Liberase Research Grade products are shipped on dry ice.

1.1 Enzyme Characteristics

Temperature and pH

pH Optimum	Temperature Optimum
<ul style="list-style-type: none"> In general, the optimal pH for tissue dissociation is the one which is physiologically appropriate for the cells to be isolated (pH 7.4). 	<ul style="list-style-type: none"> For general tissue dissociation, use a temperature range of +35 to +37°C. Although lower temperatures may be used, enzyme activity (and the rate of tissue dissociation) will be reduced.
<p>Ⓞ Liberase Research Grade products are mixtures of enzymes that act differently upon different substrates. Plots of <i>in vitro</i> enzyme activity vs. pH or temperature (measured with artificial substrates) cannot predict the effects of pH and temperature on tissue dissociation.</p>	


Modifying Factors

Purified collagenase contains approximately 1 mole of Zn⁺ and 2 to 7 moles of Ca²⁺ per mole of enzyme (3). Exposure of the enzyme to divalent cation chelators removes Zn⁺ and Ca²⁺, thus rendering the enzyme inactive (4).

Modification	Factor
Inhibitor	0.1 M EDTA (5) Cysteine (4) Mercaptoethanol (4) Protease inhibitors Serum Albumin
Stabilizer	Ca ²⁺ (3)
Cofactor	Zn ⁺ , Ca ²⁺ (3)

2. How To Use this Product

2.1 Reconstitution and Storage

 Do not use bacteriostatic water for injection. This type of water contains preservatives that inhibit collagenase enzyme activity.

- 1 Reconstitute the lyophilized enzyme with tissue-dissociation buffer or phosphate buffered saline (PBS). Do not add serum or other components that may influence enzyme activity (*e.g.*, albumin or protease inhibitors) to the dissociation buffer. Enzyme stability is reduced at higher concentrations and warmer temperatures ($> 4^{\circ}\text{C}$). Therefore, avoid both conditions for any duration of time. Reconstitute the entire vial. Do not weigh individual aliquots of the lyophilizate. The introduction of moisture into the vial results in a decline in enzymatic activity.

Vial	Collagenase	Thermolysin
Reconstitution volume	20 ml	15 ml
Collageanse Wünsch Units/ml	130	
Enzyme Concentration [mg/ml]	25	1.00

- 2 Place vials on ice to rehydrate the lyophilized enzyme.
- 3 Gently agitate the vials at $+2$ to $+8^{\circ}\text{C}$ until enzyme is completely dissolved (several min to max. 30 min).
- 4 Remove an aliquot of the stock solutions to prepare the working solution (see section 1 and section 2.2).
- 5 Store unused stock solutions in single-use aliquots at -15 to -25°C for up to one month.

 **Strictly avoid repeated freezing and thawing.**

2.2 Application

Liberase T-Flex Research Grade Composition

The Liberase T-Flex Research Grade kit enables to make individual blends of collagenase and Thermolysin ratios. As a guideline please refer to the ratios described in the section “Target Activities” (chapter 1). Mix different ratio of neutral protease activity, relative to the collagenase activity to get an enzyme blend appropriate for your application.

**Factors Affecting
Liberase
Selection**

Prior to choosing a Liberase Research Grade for your application, you must be familiar with the factors that influence enzyme requirements. Enzyme requirements for tissue dissociation are determined by:

- Tissue type
- Species
- Dissociation protocol
- Desired outcome of tissue dissociation

**Select a Liberase
Research Grade**

If ...	Then ...
you have previously used collagenase for this application	refer to section 2.3, "Select a Liberase Research Grade to replace traditional collagenase".
you have previously used a 1st generation Liberase Enzyme for this application	refer to section 2.4, "Select a Liberase Research Grade to replace a 1st generation Liberase Enzyme blend".
you are dissociating a tissue with which you have no previous experience	refer to section 2.5, "Select a Liberase Research Grade for a new procedure".
you have not previously isolated cells from tissue through enzymatic digestion	refer to a basic cell culture text (6, 7, 8) for background information, then refer to section 2.6, "Liberase Research Grade Enzyme Working Concentration".
<p>Ⓢ Liberase Enzymes are formulated for use with most calcium-containing buffers. However, protease inhibitors, serum, and BSA will inhibit Liberase Enzyme performance. Therefore, they must be excluded from the dissociation.</p>	

2.3 Select a Liberase Research Grade to Replace Traditional Collagenase**Select Liberase
Research Grade
based on
Application**

Visit our Liberase Enzyme website at www.collagenase.com to learn if we have identified a Liberase Research Grade for your specific application. If your application is not included in our current database, please go to the next step, "Select Liberase Research Grade based on current collagenase."

**Select Liberase
Research Grade
based on Current
Collagenase**

In most cases, you can select a Liberase Research Grade based upon your previous experience with traditional collagenase.

**Determine
Liberase
Research Grade
Working
Concentration**

All Liberase Enzymes have substantially higher specific activities than traditional collagenases. This means that identical working concentrations of Liberase Enzymes and traditional collagenase, expressed in mg/ml, yield very different effective enzyme concentrations. To estimate a working concentration of Liberase Enzymes, go to section 2.6, "Liberase Research Grade Enzyme working concentration."

Optimize Protocol After selecting a Liberase Research Grade and corresponding starting concentration, apply it to your procedure. Optimize your protocol by reviewing the optimization table (section 2.7).

2.4 Select a Liberase Research Grade to Replace a 1st Generation Liberase Enzyme Blend

You can select a Liberase Research Grade based upon your previous experience with Liberase Enzymes and Liberase Blendzymes of the 1st generation. Visit our Liberase Enzyme website at www.collagenase.com for information how to replace your current collagenase:

- Type of Liberase Enzyme used previously
 - Previously used working concentration (mg/ml or Wünsch units/ml)
- ③ The mg/vial amounts indicated for the Liberase Enzymes and Liberase Blendzymes of the 1. generation referred to whole protein amounts (Collagenase I + Collagenase II + neutral protease). The mg/vial indications of second generation Liberase Research Grade Purified Enzyme Blends refer to the total collagenase amount without considering the protein amount of neutral protease. Therefore, it is essential to express collagenase concentration of the first generation product in Wünsch units/ml, instead of mg/ml. Wünsch units/ml of 1. and 2. generation blends are equivalent.
- ③ Please note that the second generation Liberase Products show considerably higher purity of the Collagenase I + II compared to the former Liberase Products. Therefore, the incubation time has to be adapted to each application specifically. In most of the cases, a reduction of the incubation time of 10 – 30% gave optimal results.

2.5 Select a Liberase Research Grade for a New Procedure

Select a Liberase Research Grade based on Application Visit our Liberase Enzyme website at www.collagenase.com to learn if we have identified a Liberase Research Grade for your specific application. If your application is not included in our current database, please go to the next section, “Liberase TM Research Grade.”

Liberase TM Research Grade Liberase TM Research Grade blended with a medium content of Thermolysin has the greatest overall range of applicability and is a good starting point for the dissociation of many tissues. The Liberase Research Grade panel offers a continuous range of “Enzyme Mixture Aggressiveness”. Liberase DL Research Grade is the most gentle (lowest protease activity per mg of protein), and Liberase TH Research Grade is the most aggressive (highest neutral protease activity per mg protein). For more information, go to the section 5 “Supplementary Information” / “Ordering Information” and refer to “Enzyme Mixture Aggressiveness” (chapter 1).

Liberase Research Grade Enzyme Working Concentration

All Liberase Enzymes have substantially higher specific activities than traditional collagenases. This means that the working concentration of Liberase Research Grade Purified Enzymes, expressed in mg/ml, will be much lower than that of traditional collagenase.

if . . .	then . . .
your application is included in our list of applications	use the Liberase Research Grade concentration recommended in that application
your application is NOT included in our list of applications	use Liberase TM Research Grade at a concentration of 0.08 – 0.28 Wunsch units/ml

Optimization

After selecting a Liberase Research Grade blend and concentration, apply it to your procedure. Optimize your protocol by reviewing the optimization table (section 2.7).

2.6 Liberase Research Grade Enzyme Working Concentration

Background Information

The goal of this section is to estimate the best starting concentration of Liberase Research Grade to use. This is only a first step, due to differences in procedure and lot-to-lot differences in traditional collagenase. After working with this starting concentration, consult the optimization table (section 2.7) to find the best enzyme concentration, based upon your experimental needs.

Collagenase Specific Activity

Collagenase is traditionally diluted to a concentration expressed in mg/ml. Significant lot-to-lot differences in traditional collagenase specific activity require that you establish a new working concentration each time you change lots. This is not the case with Liberase Research Grade Purified Enzymes. Each Liberase Research Grade product is blended from highly purified enzymes. It is essential to express collagenase concentration in Wunsch units/ml, instead of mg/ml.

- ☉ For consistency in your protocol, always express collagenase concentration in terms of enzyme units per ml.

Convert Collagenase Specific Activity to Wunsch units/mg

Use the following table to convert the collagenase enzyme activity of your current collagenase to Wunsch (collagenase) units/mg. This table calculates Wunsch units/mg from either FALGPA units/mg, or collagen degrading units/mg (Mandl units; CDU).

- ☉ These conversions are a reasonable approximation, based upon the expected precision of the different collagenase assays.

To Convert from	To	Divide	Example
FALGPA units/mg	Wunsch units/mg	FALGPA units/mg by 3.9	3.5 FALGPA units/mg ÷ 3.9 = 0.9 Wunsch units/mg
CDU/mg or Mandl units/mg	Wunsch units/mg	CDU/mg by 1000	200 CDU/mg ÷ 1000 = 0.2 Wunsch units/mg

Collagenase Working Concentration

Multiply your previous collagenase working concentration (mg/ml) by its specific activity (Wünsch units/mg, [as determined above]), to obtain Wünsch units/ml. To determine how much Liberase Research Grade to use, first multiply your collagenase working concentration (in Wünsch units/ml) times the total volume of your working enzyme solution to obtain the total collagenase activity needed (Wünsch units). Divide the total collagenase activity required by the Liberase Research Grade stock concentration (see step 1 in “Reconstitution and Storage”). This will tell you how many milliliters of Liberase Research Grade stock solution to use in your working enzyme solution.

Optimization

After selecting a Liberase Research Grade and concentration, apply it to your procedure. Optimize your protocol by reviewing the optimization table (section 2.7).

2.7 Optimize Your Tissue Dissociation Procedure**Introduction**

This section will help you interpret your tissue dissociation results, and find opportunities to improve your cell yield, viability, and/or functionality. Before continuing, refer to the points in section 2.2 regarding enzyme requirements for tissue dissociation, as well as the points below:

- Liberase T-Flex Research Grade enzymes contain only collagenase and neutral protease.
- Collagenase enzymes digest the intercellular matrix.
- Neutral proteases act synergistically with collagenase.
- Given sufficient time and concentration, neutral proteases damage cell surface proteins.
- Time of dissociation, enzyme ratios, and enzyme concentration all affect the tissue-dissociation outcome.
- Use Liberase T-Flex Research Grade enzymes without modifying factors, such as serum, BSA, or protease inhibitors.

Optimization Table

Use the following table in the sequence provided. Note whether the yield, viability, or functionality of your cells isolated with Liberase enzyme is less than optimal. Find the probable cause, then act on the recommendation.

Refer to enzyme mixture aggressiveness described in “Target Activities” (chapter 1) for information on neutral protease specific activity increasing within the Liberase Research Grade panel.

Observation 1	Observation 2	Possible Cause	Recommendation
Low cell viability	Dissociation very rapid	Enzyme concentration too high	Reduce enzyme concentration by 50%.
		Enzyme mixture aggressiveness too high	Select a Liberase Research Grade Purified Enzyme mixture containing lower amounts of neutral protease.
	Dissociation very slow	Enzyme concentration too low	Increase enzyme concentration by 50%.
		Enzyme mixture aggressiveness too low	Select a Liberase Research Grade Purified Enzyme mixture containing higher amounts of neutral protease.
Impaired cell function	Cell viability > 80%, cell yield is reasonable	Enzyme concentration too high	Reduce enzyme concentration by 25%.
		Enzyme mixture aggressiveness too high	Select a Liberase Research Grade Purified Enzyme mixture containing lower amounts of neutral protease.
Low cell yield	Cell viability > 80%	Enzyme concentration too low	Increase enzyme concentration by 25 – 50%.
		Enzyme mixture aggressiveness too low	Select a Liberase Research Grade Purified Enzyme mixture containing higher amounts of neutral protease.
	Cell viability < 80%	Enzyme concentration too high	Reduce enzyme concentration by 50%.
		Enzyme mixture aggressiveness too high	Select a Liberase Research Grade Purified Enzyme mixture containing lower amounts of neutral protease.
	Mechanical (shear) force is excessive	Reduce shear force in all aspects of dissociation. Treat tissue gently.	
Released cells clump in gelatinous stringy form	Cell yield and viability are acceptable	DNA release, subsequent to cell lysis, causes clumping	More prevalent in some tissues. If cell viability is acceptable, add DNase to dissociation mixture.
	Cell yield or viability are reduced	Mechanical (shear) force is excessive	Reduce shear force in all aspects of dissociation. Treat tissue gently.

3. Troubleshooting

If you are unable to optimize a Liberase Research Grade enzyme mixture for your procedure, or if you encounter difficulties in effective cell isolation, refer to the following table:

	Possible Cause	Recommendation
Prolonged dissociation time or incomplete dissociation	Enzyme decay	Follow appropriate storage conditions (chapter 1).
	Inappropriate Enzyme reconstitution time	Follow appropriate reconstitution conditions (chapter 2.1).
	Inappropriate Enzyme dilution	Verify dilution.
	Enzyme inhibition or tissue exposed to enzyme inhibitors	Check for presence of inhibitors in all buffers (chapter 1).
	Incubation temperature too low	Verify +37°C is incubation temperature.
Low cell viability and yield	Tissue stored at elevated temperature prior to dissociation	Reduce time and temperature of ischemia.
	Prolonged tissue ischemia time	Reduce time of tissue ischemia.
	Incubation time too long	Reduce incubation time.
	Inappropriate Liberase Research Grade dilution	Verify dilution.
	Incubation temperature too high	Verify +37°C is incubation temperature.
Decreased cell viability or <i>in vitro</i> survival	Endotoxin exposure	Check all tissue dissociation reagents for endotoxin contamination.
Liberase Research Grade does not go into solution within 30 minutes		Increase volume of reconstitution buffer two-fold.

4. Additional Information on this Product

How This Product is Purified and Blended Liberase T-Flex Research Grade kit contains enzymes of purified collagenase isoforms I and II (3), and the neutral protease Thermolysin. The collagenase isoforms are purified from the fermentation of *Clostridium histolyticum*. Thermolysin is purified from the fermentation of *Bacillus thermoproteolyticus*. The methods for purification and blending of these components are described in one or more of the following patent numbers: US 5,753,485 and US 5,830,741.

References

- Target Activities**
- 1 Wünsch, E. & Heidrich, H.G. (1963) Zur quantitativen Bestimmung der Kollagenase. *Z. Physiol. Chem.* **333**, 149.
 - 2 Matsubara, H. (1970) Purification and assay of thermolysin. *Methods in Enzymology* **19**, 642-651.
- Modifying Factors**
- 3 Bond, M.D. & Van Wart, H.E. (1984) Characterization of the individual collagenases from *Clostridium histolyticum*. *Biochemistry* **23**, 3085-3091.
 - 4 Seifter, S. & Gallop, P.M. (1960) Collagenase from *Clostridium histolyticum*. *Methods in Enzymology* **5**, 659-665.
 - 5 McShane, P., et al. (1989) Protease activity in pancreatic islet isolation by enzymatic degradation. *Diabetes* **38** (Suppl. 1), 126-128.
- Basic Tissue Culture**
- 6 Pollard, J.W., & Walker, J.M. (1997) *Basic Cell Culture Protocols*, Humana Press.
 - 7 Jones, G.E. (1996) *Human Cell Culture Protocols*, Humana Press.
 - 8 Freshney, R.I. (2005) *Culture of Animal Cells: A Manual of Basic Technique*, Wiley & Sons.

Quality Control

Several tests are performed on Liberase Research Grade Purified Enzyme Blends prior to release for sale. In the following table acceptance ranges are shown.

Product	Pack size	Collagenase content (mg/vial) ¹	Collagenase Ib, c part of Collagenase I ¹	Endotoxin (EU/mg) ²
Collagenase 500 mg I/II Blend Research Grade	500 mg	430 – 644	≤ 10% area	≤ 10
Thermolysin Research Grade	15 mg	12.0 – 18.0		≤ 50

¹ Measured by High Performance Liquid Chromatography (HPLC) analysis.

² Measured by a standard Limulus-Amebocyte Lysate (LAL) assay.

The actual activities determined for each lot are available in the respective Certificates of Analysis. They can be retrieved from the Roche Applied Science Homepage (<https://www.roche-applied-science.com/techresources>).

In the following, the measurement of Collagenase I and Collagenase II integrity by HPLC is described. Almost no fragmentation of both collagenase enzymes can be detected in Liberase™ Research grade blends proving the purity of this enzyme mixture (Figure 1).

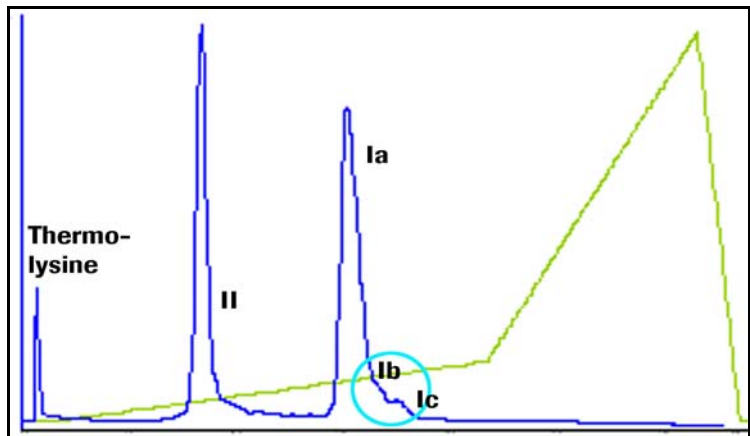


Fig. 1: HPLC analysis of Liberase™ Research Grade

5. Supplementary Information

5.1 Conventions

5.1.1 Text Conventions

To make information consistent and easier to read, the following text conventions are used in this Instruction Manual:

Text Convention	Usage
Numbered stages labeled ①, ② etc.	Stages in a process that usually occur in the order listed.
Numbered instructions labeled ①, ② etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Applied Science.

5.1.2 Symbols

In this Instruction Manual, the following symbols are used to highlight important information:

Symbol	Description
④	Information Note: Additional information about the current topic or procedure.
⚠	Important Note: Information critical to the success of the procedure or use of the product.

5.2 Changes to Previous Version

- Editorial changes
- License Disclaimer information deleted
- Addresses updated

5.3 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, www.roche-applied-science.com.

Product	Pack Size	Cat. No.
Liberase DL Research Grade	10 mg	05 401 160 001
Liberase DL Research Grade	100 mg	05 466 202 001
Liberase TL Research Grade	10 mg	05 401 020 001
Liberase DH Research Grade	10 mg	05 401 054 001
Liberase DH Research Grade	100 mg	05 401 089 001
Liberase TM Research Grade	10 mg	05 401 119 001
Liberase TM Research Grade	100 mg	05 401 127 001
Liberase TH Research Grade	10 mg	05 401 135 001
Liberase TH Research Grade	100 mg	05 401 151 001
Liberase T-Flex Research Grade (Thermolysin in separate vial)	500 mg	05 989 132 001
Liberase Selection Kit Research Grade	5 mg each	05 401 046 001

5.4 Trademarks

LIBERASE is a trademark of Roche.

5.5 Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

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