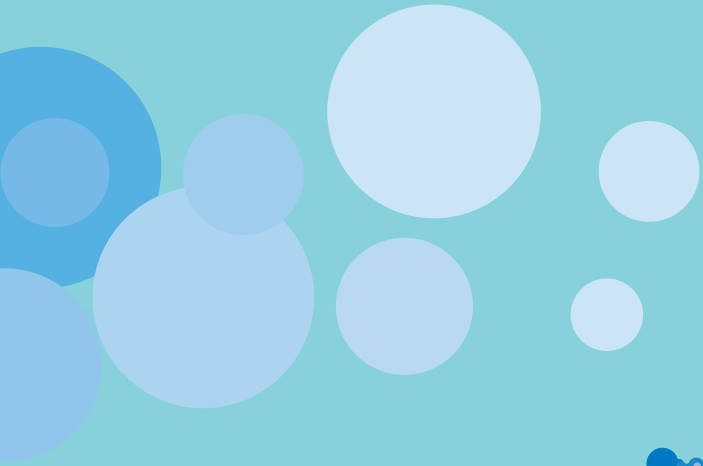
LavaDigest – protease monitoring kit



fluorotechnics
naturally fluorescent

Ordering

LP-031020 **LavaDigest** protease monitoring kit for up to 2000 assays

Order from *http://www.fluorotechnics.com*

LavaDigest Protocol August 2007

Content

LavaDigest - protease monitoring kit

LavaDigest™ is a simple assay that provides real-time monitoring of proteolysis. Monitoring proteolysis is important in many areas ranging from digestibility studies in the food industry, to ensuring complete protein digestion prior to MS-analysis in proteomics, to the removal of tags from recombinant proteins. Other methods for monitoring proteolysis such as HPLC[1,2], circular dichroism[3], mass spectrometry[4] and SDS-PAGE[5] are slow, may require expensive instrumentation, and are unsuitable for real-time monitoring.

Quick Facts

Storage

Store Part A at –15 °C to – 30 °C in the original brown bottle to protect from light.
Store Part B at room temperature.
Store Part C at 3-6 °C.

Disposal

LavaDigest is an environmentally safe solution and requires no special disposal procedures.

Detection

Optimum excitation wavelengths: 390, 520 nm. Suitable light sources include green (e.g. 543, 532 nm) blue (e.g. 488 nm); violet (e.g. 405 nm) or UVA.

Emission wavelength: Maximum emission is at 610 nm, irrespective of the excitation source. Suitable filters include the 610 nm band pass or 560 long pass. For fluorescence spectra, see *www.fluorotechnics.com/content/documents/Spectral Characteristics of Epicocconone ec.pdf*

Ordering

LP-031020 LavaDigest is sufficient for 2000 x 200 µL assays.
Order from *http://www.fluorotechnics.com*

Features

LavaDigest Kit:

- provides a simple, convenient and robust approach to the real-time monitoring of the progress of protease digestion
- is suitable for all proteins and most proteases tested
- does not interfere with proteolytic activity
- is fully compatible with downstream proteomic analyses
- replaces expensive and time-consuming gel electrophoresis for validation of proteolytic digestion
- can be used to derive kinetic parameters of proteolytic activity

Safe Handling and Disposal

All chemicals should be considered potentially hazardous. This product should only be handled by people trained in laboratory techniques, and used in accordance with the principles of good laboratory practice. Wear suitable protective clothing including laboratory overalls, safety glasses and gloves. **LavaDigest** kit contains three parts. Part A is a natural organic fluorophore (epicocconone) supplied as a lyophilized power. Part B is a solvent mixture and Part C is a bicine buffer (pH 8.5) suitable for tryptic digestion. The diluted working solution is non-flammable. The complete properties of the dye component have not been fully investigated. Part C contains bicine buffer which can cause irritation to eyes and skin and may be harmful if inhaled, ingested or absorbed through the skin.

Tips and Troubleshooting

- Ensure that you follow the protocol outlined below.
- **LavaDigest** reacts with primary amines and these should not be used in your samples or buffers.
- Bicine buffer has been provided to replace the ammonia based buffer that is routinely used with tryptic digestions.

- Use high-grade chemicals and freshly prepare reagents that are unstable.

- **LavaDigest** will work with proteases within the pH range (pH 7.7 to 8.5), however **LavaDigest** is not suitable for proteases (such as pepsin) that are assayed at low pH values.

- With **LavaDigest** the fluorescence of the sample falls during digestion. The settings on your fluorescence plate reader should be set at maximum fluorescence at the start of the assay.

- Freshly prepared fluorophore solution should be used.

Reagents and Equipment

LavaDigest Kit Components

Part A is a fluorophore used to monitor protease digestion. Two vials of lyophilized fluorophore (0.25 mg/vial) are provided.

Part B consists of a solvent mixture of acetonitrile and DMSO (1.5 mL/vial) for reconstituting Part A.

Part C comprises a 10× concentrate (100 mL) bicine buffer (1 M, pH 8.5).

Reagents and Equipment not Provided

- Protease e.g. trypsin
- Dithiothreitol (DTT)
- Iodoacetamide
- Sodium dodecyl sulfate, (SDS)
- Microcentrifuge tubes 1.5 mL
- 96-well plate (black, flat-bottom)
- Fluorescence plate reader, with an excitation filter (540±10 nm) and an emission filter (630±10 nm)

Working Solutions

1. **LavaDigest** concentrate – Reconstitute one vial of Part A (0.25 mg) in 0.5 mL of Part B.
2. **Bicine buffer 1x** - Dilute Part C 1:10 in high purity water, e.g. 1 mL of Part C and 9 mL of water.
3. **LavaDigest** working solution - Dilute 1 part of **LavaDigest** concentrate above (1) in 199 parts of 1× bicine buffer (2) (see "Monitoring Protease Digestion" in the Protocol). Vortex to mix and the solution becomes pink.
4. **SDS solution** - Prepare 10% SDS in high purity water.
5. **DTT solution** - Prepare a 200 mM solution of dithiothreitol (DTT) in 1× bicine buffer (2).
6. **Iodoacetamide solution** - Prepare a 1 M solution of iodoacetamide in 1× bicine buffer (2).

Storage/Stability

Upon receiving the kit, store Part A at -20 °C. Part A is stable for 1 year if stored unopened at -20 °C. **LavaDigest** concentrate (1) is stable at 4 °C for 1 month.The working solution of bicine buffer (2) can be prepared in advance. If stored in the refrigerator (4 °C) the bicine buffer will be stable for up to 6 months. **LavaDigest** working solution (3), DTT and iodoacetamide solutions should be freshly prepared.

Protocol

Preparation of Protein Sample

Prepare protein sample as a 10 mg/mL solution in 1x bicine buffer (1). If your protein is already dissolved, dilute appropriately using the 10x bicine buffer (Part C) and water to achieve approximately 10 mg/ml protein in 1x bicine buffer. Accurate quantification of your protein can be achieved using FluoroProfile™ see *www.fluorotechnics.com/protein.php*

Reduction and Alkylation: To 100 µL of protein sample in a micro-centrifuge tube, add 1µL of SDS solution (4) and 5 µL of DTT solution (5). Vortex mix then centrifuge (5 seconds, 6,000 rpm) to bring the solution to the bottom of the tube. Incubate at 70 °C for 10 min then add 4 µL of iodoacetamide solution (6). Vortex mix, centrifuge and incubate at room temperature in the dark for 45 min – 1 hr. Add 20 µL of DTT solution (5), vortex mix, centrifuge and incubate at room temperature for 45 min – 1hr.

To 1 part reduced and alkylated protein, add 9 parts 1x bicine buffer (1). Your sample is now ready for protease digestion.

Monitoring Protease Digestion

- Add 100 µL of your protein to two wells of a black, flat-bottom, 96-well microtitre plate that is suitable for your fluorescence plate reader. One sample will act as a reference.
- Prepare fresh solution of **LavaDigest** working solution as previously described (see **Working Solutions**) and add 100 µL of **1× LavaDigest** working solution (3), to both samples.
- Insert the plate into the fluorescence plate reader and pre-incubate to allow the fluorescence to develop for 50 minutes.
- Obtain the gain setting of your fluorescence plate reader by measuring the fluorescence of your samples using appropriate filters to provide an excitation wavelength of 480-540 nm (e.g. λ_{Ex}540 +/-10nm) and emission wavelength of 580-630 nm (e.g. λ_{Em}630 +/-10nm).
- For a protein digestion with trypsin add 2.0 µL of your protease to one protein sample and 2.0 µL of the protease buffer to the reference (reference solution first). For example, for a tryptic digestion, first add 2 µL of 1 mM HCl to the reference (no trypsin). Then add 2.0 uL of trypsin (1 µg/µL in 1 mM HCl) to the sample to be digested.
- Incubate in the fluorescence plate reader at a temperature optimal for your protease.
- Immediately start measuring the fluorescence in each well at appropriate intervals. This is every 1 – 10 minutes depending on the rate of digestion. Tryptic digestions typically take between 30 – 600 minutes to complete.
- The digestion is complete when the fluorescence reaches 10 x the half-life (see section on **Determination of Rate Constants** below). As a rough guide the digestion is complete when the fluorescence of a protein sample (with protease) is stable.
- If the fluorescence readings of the sample (with protease) are similar to those of the reference, the digestion has failed.

Determination of Rate Constants

LavaDigest is ideal for determining rates of protease digestion, e.g. the rate constants and half life. To determine reaction kinetics, and the rate constant for fluorophore degradation, fit the progress curve for the reference

sample (with no trypsin) to a one-phase exponential decay (Y=span*exp (-kX)+bottom). Note the value of the rate constant (k). Next fit a two phase exponential decay (Y=span1*exp(-k1X)+span2*exp(-k2X)+bottom) to the progress curve of the protein with protease. Fix one of the rate constants to the value determined for the sample without protease, and use non-linear regression to determine the rate constant for proteolytic digestion and the half-life (0.69/k). Suitable software packages such as Prism™, Origin™ and Excel™ can be used.

Mass Spectrometry

Protein samples used for monitoring proteolytic digestion using **LavaDigest** can be analysed directly by mass spectrometry without any pre-treatment.

Limit of Detection and Interfering Compounds

At least 1.5 µM (10 µg/well) of a protein sample is required for **LavaDigest**. The maximum acceptable limits of interfering compounds are listed below.

Chemicals	Recommended limit of chemicals in the LavaDigest assay
SDS	0.02%
Triton X100	0.001% v/v
Tween 20	0.002% v/v
guanidine-HCl	500 µM
CHAPS	0.02%
NaOH	10 mM
dithiothreitol	4 mM
iodoacetamide	3 mM
urea	1 M
calcium chloride	5 mM
tris-HCl	2.5 mM

An updated list of interfering compounds can be found at www.fluorotechnics.com/content/documents/lavadigest_inhibitory_260607.pdf

LavaDigest has been found to be suitable for in-solution but not in-gel digestions.

Related Products

Fluorotechnics offers a family of compatible products including total protein gel and blot stains, a protein quantification kit, a peptide quantification kit, and a live cell imaging reagent. For details of all of our products visit *http://www.fluorotechnics.com/*

References

1. Kohse, K.P. and Wisser, H. (1987). Quantitative monitoring by high-performance liquid chromatography of the dissociation of human serum cholinesterase by limited proteolysis. *J. Chromatogr.* **420**:25-33.

2. Noda, Y., Fujiwara, K., Yamamoto, K., Fukuno, T. and Segawa, S. (1994). Specificity of trypsin digestion and conformational flexibility at different sites of unfolded lysozyme. *Biopolymers* **34**:217-26.

3. Caballero, R., Fernandez, B.A., Moran, F. and Montero, F. (1985). Kinetics of conformational changes in the globular head of calf thymus H1 upon tryptic digestion. *Biochem. Int*. **11**:765-72.

4. Bothner, B., Chavez, R., Wei, J., Strupp, C., Phung, Q., Schneemann, A. and Siuzdak, G (2000). Monitoring enzyme catalysis with mass spectrometry. *J. Biol. Chem.* **276**:13455-13459.

5. Thomas,K,et al (2004). A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assesing the safety of novel proteins. *Regal.Toxicol.Pharma*. **39:**87-98

Legal

Trademarks