PepSets™REPLi

A library of FRET tripeptides that accelerates protease drug development

Mimotopes' jointly developed rapid endopeptidase profiling library (REPLi) is a "small but smart" generic screening tool that overcomes known hurdles to identifying the cleavage site requirements for protease substrates.[1]

Narrowing the search for specific inhibitors and possible *in vivo* substrates

Proteases are involved in the regulation of a wide variety of essential physiological processes, and their dysregulation has been implicated in a number of disorders including cardiovascular disease, rheumatoid arthritis, Alzheimer's disease and cancer, to name only a few. Hence, proteases and their substrates are increasingly viewed as valuable drug targets in disease treatment. However given the large number of proteases in the human genome (>560), the task of characterizing the biological function *in vivo* of all proteases, including the identification of all protease substrates, is daunting.
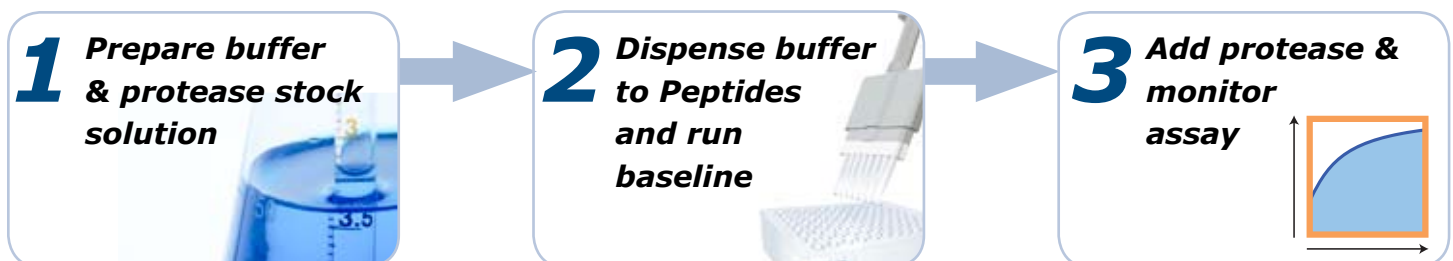
Information relating to the specific residue requirements spanning the protease cleavage site in peptide substrates is invaluable in assisting the development of specific inhibitors and in identifying possible *in vivo* protein substrates. In addition, specificity information can be used to design highly sensitive and specific synthetic fluorogenic substrates and these in turn enable screening of compounds to identify small molecule inhibitors. Hence, there is an urgent need for more rapid techniques of substrate discovery on a system-wide basis, with the capability of identifying new protease substrates directly from cells, tissues and body fluids, and quantitating differences in substrate processing of low-abundant proteins as disease markers.
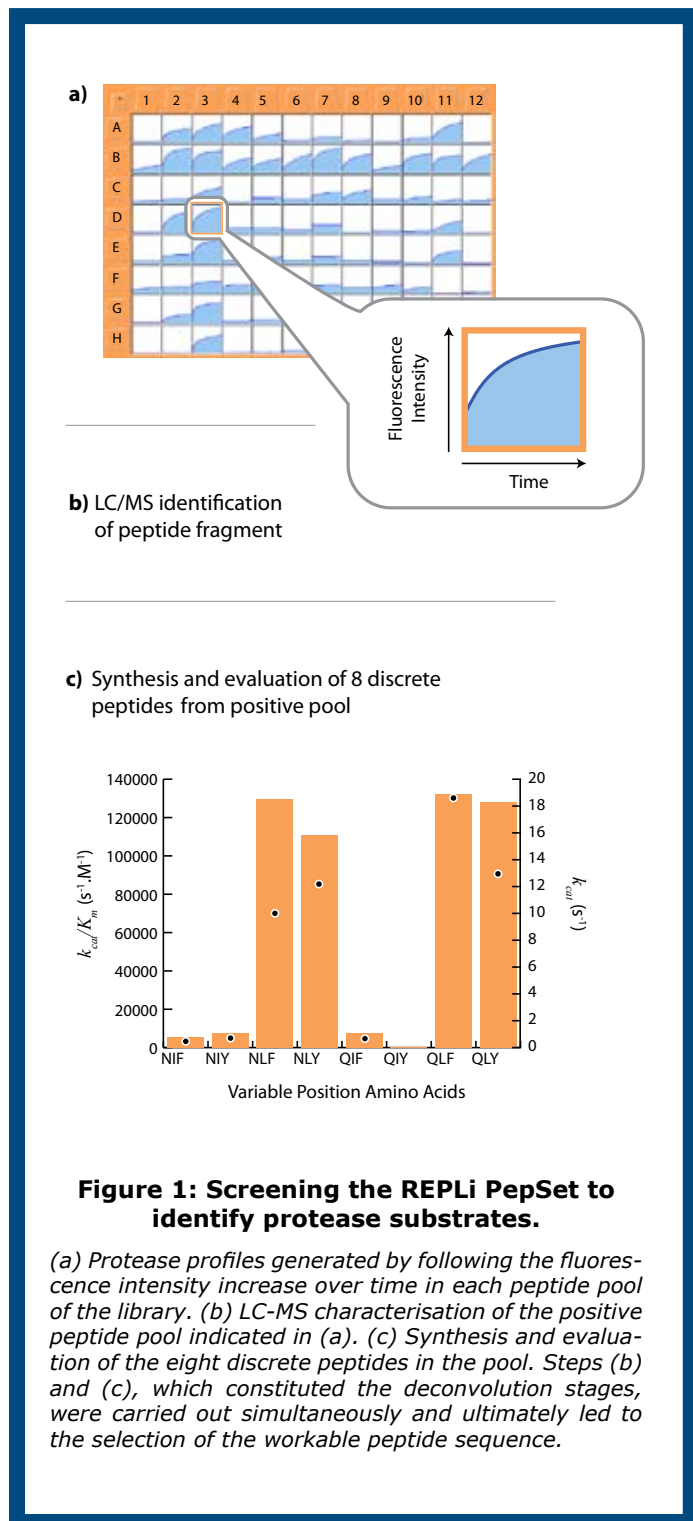
Features of the REPLi PepSet

- **Identifies new protease substrates for drug discovery and assay development**

- **Condenses a massive search to a manageable 512-pool experiment**

- **Continuous monitoring of proteolytic activity**

- **Determines protease specificity on both sides of the scissile bond**

- **Fully validated in the four mechanistic protease classes**

- **Economically presynthesized; ready to ship**

- **Available in two sizes: Pilot (5 nmol) and Research (50 nmol)**

Finding new protease substrates is pure simplicity

1 *Prepare buffer & protease stock solution*

2 *Dispense buffer to Peptides and run baseline*

3 *Add protease & monitor assay*

Figure 1: Screening the REPLi PepSet to identify protease substrates.

(a) Protease profiles generated by following the fluorescence intensity increase over time in each peptide pool of the library. (b) LC-MS characterisation of the positive peptide pool indicated in (a). (c) Synthesis and evaluation of the eight discrete peptides in the pool. Steps (b) and (c), which constituted the deconvolution stages, were carried out simultaneously and ultimately led to the selection of the workable peptide sequence.

Protease substrate identification

Various methods have been employed to characterize protease specificity, generally consisting of the screening of chemically or biologically produced peptide libraries. The early mixture-based peptide libraries provided both general applicability and speed, with digestion of the library mixtures followed by N-terminal sequencing giving specificity information C-terminal to the scissile bond (P' sites, see Figure 2). However, such methods preclude the continuous monitoring of proteolytic activity and do not provide information on the protease specificity N-terminal to the scissile bond (P sites). Libraries generated using solid phase synthesis where peptides are tagged with a fluorogenic or chromogenic group that fluoresces/absorbs light after cleavage, allows the proteolytic activity to be monitored in real time. However, the lack of information on the primed subsite specificity (P' sites) hinders its application to endoproteases that recognise residues C-terminal to the scissile bond.

Development of the REPLi PepSet

To overcome issues such as feasibility and deconvolution, associated with large peptide libraries, Mimotopes has developed and launched in collaboration with GlaxoSmithKline (Stevenage, UK) and the University of Leeds (Leeds, UK) a 'small but smart' generic fluorescence resonance energy transfer (FRET) rapid endopeptidase profiling library (REPLi) as a tool for rapidly identifying protease substrates.

To keep the number of peptides relatively small while still representing the residue requirements for the largest number of proteases, similar amino acids are paired within a tripeptide core giving rise to a relatively small library of 3375 peptides divided into 512 distinct pools each containing only 8 peptides. The variable central core is flanked with multiple Gly residues and an additional two Lys residues are added at the C-terminus to confer adequate solubility to peptides bearing hydrophobic variable sequences (see Figure 2).

Potentially problematic amino acids that have been removed from the selection set include Cys (potential for introducing disulphide bonds), His (not generally observed within substrates at sites of protease cleavage), Met (due to both its hydrophobicity and bulky nature being shared by Leu and Ile, and its propensity to being oxidised) and Trp (interference with fluorescence signal due to abs.λ280nm/em. λ320nm). Gly is also omitted since functionalised amino acids furnish comparatively more information and because cleavage around flanking Gly residues can be detected. The remaining 15 amino acids are grouped in matching pairs (Ala + Val, Arg + Lys, Asp + Glu, Asn + Gln, Leu + Ile, Ser + Thr, Phe + Tyr), while Pro is left as a single residue to allow access to any potential conformational information.

The judicious choice of amino acid partners ensures that maximum SAR information can be derived from an initial

result. This 'matching pair' design greatly simplifies the deconvolution steps. The complete peptide library is synthesised using Mimotopes' PepSets™ and SynPhase™ Lantern technology and is provided for direct usage in 96- well plate format.

## Validation: Identification of substrates for calpain-1 and -2.

To validate the library, scientists at GSK and the University of Leeds profiled the REPLi library against representative members from each of the four mechanistic protease classes (trypsin, pepsin, matrix metalloprotease (MMP)-12 (macrophage elastase), MMP-13 (collagenase-3), calpain-1 (μ-calpain) and calpain- 2 (m-calpain).

The substrate specificities of calpains-1 and -2 are almost identical and few peptide substrates for either enzyme have been reported. Thus, it was of particular interest that the REPLi returned several substrates that were preferentially cleaved by either one or other enzyme.

The variable core sequence of the peptide pool that was specifically cleaved by calpain-1 was -Pro-[Ile/Leu]-[Phe/Tyr]-, while that which was specifically cleaved by calpain-2 contained -[Ile/Leu]-[Asn/Gln]-[Phe/Tyr]- in the variable core. The peptide pool comprising the eight peptides with the core motif [Asn/Gln]-[Ile/Leu]-[Phe/Tyr] that returned the highest signal:background ratio with calpain-2 was subjected to full LC-MS characterization (see Figure 2). This type of analysis enables the identification of the cleavage site within the peptide (MS measurement) and aids the determination of the preferred substrate sequence within the pool (LC data).

To identify the actual peptide sequences in the pool cleaved by calpain-2, all eight peptides were synthesised as discrete candidates and monitored in real-time assays. These allowed the concomitant determination of the nature of the optimal residues for each of the three variable positions and also provided kinetic data for each substrate (Fig 1). As predicted, the kinetic characterisation of single peptides was in complete agreement with LC-MS data obtained for the pool and showed that the activity was confined to specific sequences. Utilizing this information obtained from the REPLi, a highly sensitive and efficient fluorogenic substrate for calpain-2 was developed from a minimum number of experimental iterations.

The thorough validation of this 'small but smart' peptide library with representatives from each of the four mechanistic protease classes indicates that the REPLi PepSet has genuine potential applications in high-throuput screening of substrates for multiple proteases.
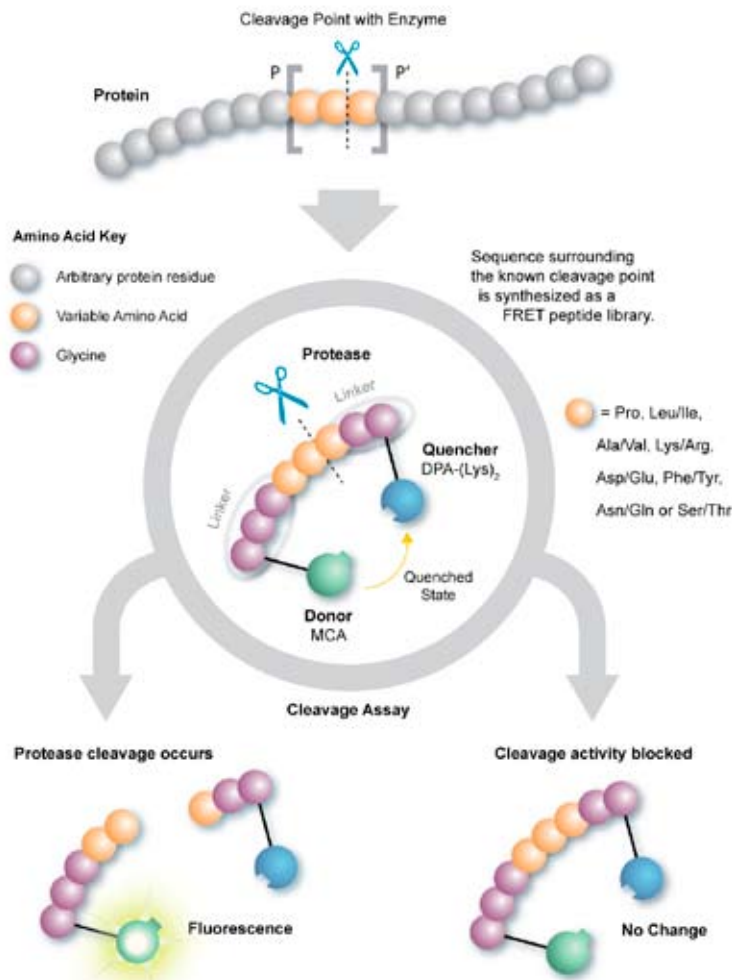
Figure 2: Principle of the REPLi PepSet FRET assay.

The peptide library was constructed around a central tri-variable peptide (orange spheres) flanked by Gly residues (mauve spheres) that linked the FRET pair. Two Lys residues were included at the C terminus to aid solubility. The DPA acceptor quenches the MCA donor; excitation at 320 nm produces only a background emission at 400 nm. Upon proteolytic cleavage, the acceptor and donor moieties move apart allowing the MCA to emit light.

Specifications and ordering information

Feature / Specification	Value
Total number of peptides: (In 6 x 96-well tube racks)	3375
Total number of wells: (8 peptides per well)	512
Analysis of control peptides by:	HPLC, MS, AAA

PepSets REPLi is available in two scales, letting you verify this powerful screening tool before commiting to use in research.

Scale >	Pilot	Research
Quantity of peptide per well:	5 nmol	50 nmol
Catalog code:	PSREPLI005	PSREPLI050

Other PepSet formats

Mimotopes' peptide libraries (PepSets) are created from a choice of formats, each designed to solve a particular screening application with utmost efficiency. Multiple formats can be combined in the same PepSet, provided quantities are of the same scale. Whichever format you chose, Mimotopes can help with designing the best peptide library for your project.

References

1. Daniel A. Thomas, Peter Francis, Carla Smith, Steven Ratcliffe, Nicholas J. Ede, Corinne Kay, Gareth Wayne, Steve L. Martin, Keith Moore, Augustin Amour and Nigel M. Hooper, A broad-spectrum fluorescence-based peptide library for the rapid identification of protease substrates., Proteomics, **6**, (2006), 2112–2120.

PepSets for specific applications

Screening Application		Recommended PepSets Format
General purpose: epitope mapping, SAR and binding studies	STD	Standard cleaved PepSet for general applications
Cytotoxic T-cell epitope mapping	CTL	Truncation peptide sequences
Protease-inhibitor interactions	FRET	(Fluorescence resonance energy transfer) labeled peptide analogs
Protease substrate profiling	REPLi	Rapid endopeptidase profiling library of FRET tripeptide analogs
Kinase mapping and cell signal transduction studies	PHOS	Phosphorylated peptide analogs
Cell penetrating peptide studies	CELL	Cell penetrating peptides
High sensitivity antibody epitope mapping	NCP	Non-cleavable PepSets
Self cleavable PepSet for T-cell epitope mapping	DKP	Diketopiperazine forming PepSets (available cleaved or uncleaved)
Antibody / B-cell eptiope mapping	BIO	Biotinylated peptides for Avidin/Streptavidin binding
Studies requiring conformation restraint	CYC	Cyclized mapping sequences or analogs
Immune-ready presented epitope mapping	CNG	Protein conjugated peptide sequences

Visit www.mimotopes.com to learn more about PepSets REPLi, or contact your nearest Mimotopes consultant

PepSets™ Peptide Libraries