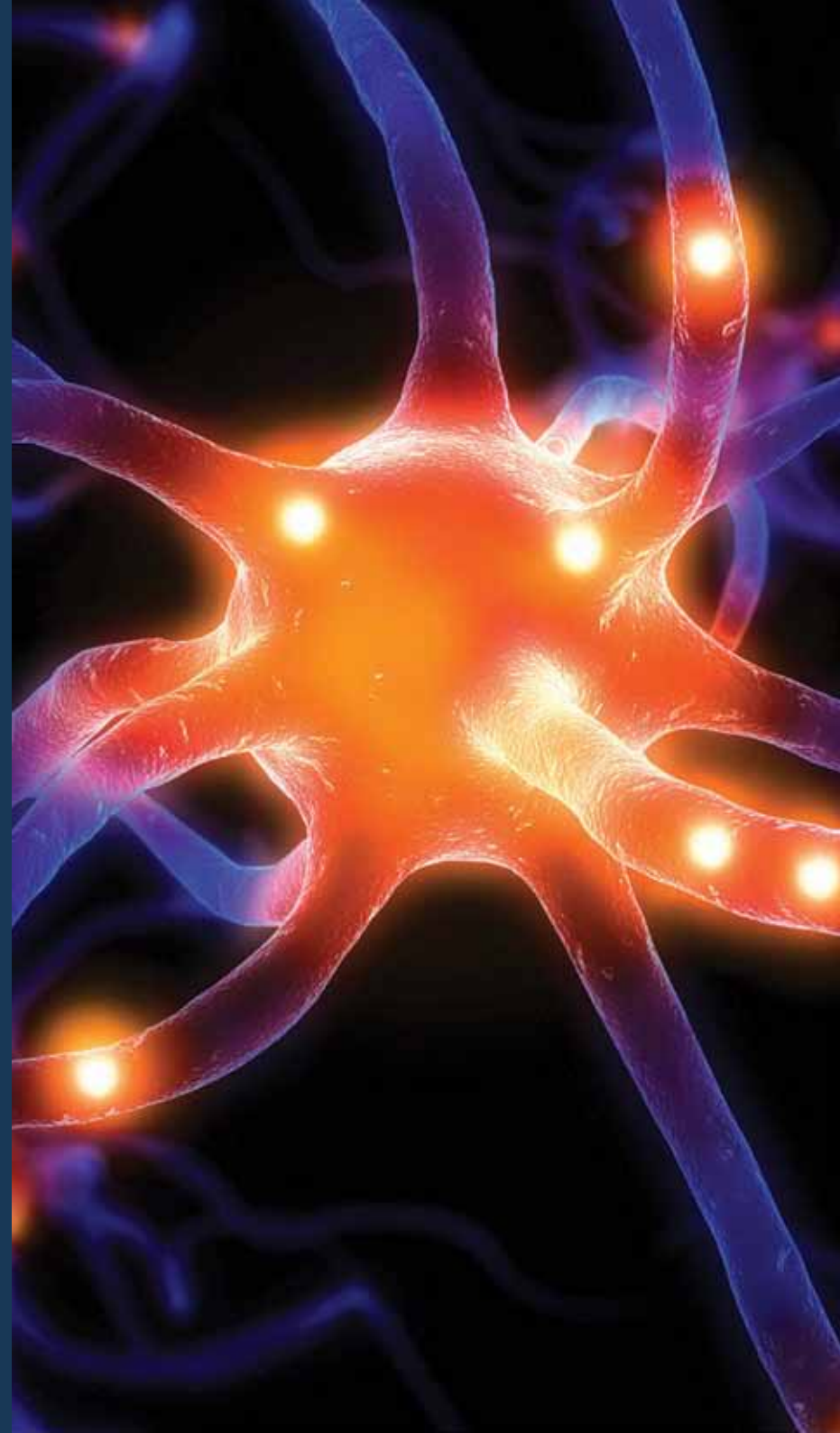


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3.6 Custom peptides

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Peptide Manufacturing Process

Eurogentec and AnaSpec have combined their deep knowledge and yearly experience to further satisfy your needs in custom peptide synthesis, both in terms of Peptide length, Modifications and Synthesis scale (up to kg peptides).

Sequence analysis

Our skilled chemists make feasibility evaluations based on software and our own internal database on all peptide sequences prior to synthesis. Should any of these parameters be too low, you will be informed prior to the acceptance of your order:

- Difficult to synthesize sequences
- Hydrophobicity/ hydrophilicity plot: Hoop-Woods and Kyte-Doolittle algorithms.
- Antigenic index (Jameson-Wolf) and surface probability evaluations (in case of peptide for immunization)

Peptide synthesis

The automatic peptide synthesis starts from a special resin on which the whole peptide sequence is synthesized sequentially by stepwise addition of the different amino acids. The growing peptide chain stays fixed to the resin until the end of the synthesis. Each single amino acid addition consists of 3 individual reaction steps: deprotection, activation and coupling.

Deprotection

Raw material consists of commercially available protected amino acids in which the alpha amino group is protected by the so-called Fmoc group (9-Fluorenylmethyloxycarbonyl), while the reactive side chain is also protected to avoid secondary reactions.

The decisive advantage of using Fmoc is that it can be cleaved under relatively mild basic conditions using Piperidine.

During the deprotection phase, Piperidine is used to remove the Fmoc protection of the last amino acid of the elonging chain, leading to a free and reactive amino group that can react with the next amino acid to be added to the chain.

Activation

The next amino acid to be added, fully protected, is then introduced in large excess. In order to allow for a quick reaction, its carboxyl group is to be activated. The activator transforms the carboxylic acid to a so-called active ester.

Coupling

This active ester in turn reacts on the free amino group of the elonging chain, resulting in the formation of a new amide bond and thus to the addition of one amino acid to the chain. The whole process is subsequently repeated with the next amino acid.

Completion of synthesis

After addition of all amino acids to the chain, the peptide is present in protected form and still linked to the resin. Piperidine is then used to remove the remaining Fmoc protection at the end of the chain and TFA is applied both to remove side chain protections and to free the peptide from the resin.

The figure beside summarizes the peptide synthesis process.

Specifications

Length	From 6 to 100 a.a.
Synthesis scale	From µg to Kg
Modifications	More than 100 !
Purification	5 purity levels from crude to 98 % pure peptides
Quality control	HPLC and MALDI-TOF MS
Format	Dried
Documentation	Technical Data Sheet
Shipping	Room temperature

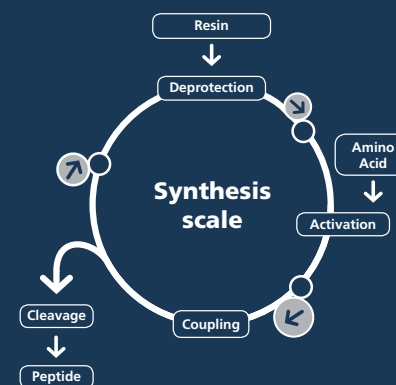


Figure 1 : Peptide synthesis process

Purification and QC

The Identity of all peptides is analysed by MALDI Mass Spectrometry. Comparison of the theoretical molecular weight with the experimentally determined molecular weight confirms the absence of amino acid deletions or double couplings.

The Purity of all non-crude peptides is analysed by Reverse-Phase HPLC (RP-HPLC). Precipitated peptides are analysed on a Waters Alliance, RP-HPLC instrument, equipped with an auto-sampler for continuous analysis. Other tests such as sequencing and Amino acid analysis are available on demand.

Delivery

In order to ensure short delivery time, all peptides are shipped by express courier. 70 % pure peptides are shipped lyophilised within 10–15 working days of receipt of the order. For purified peptides (> 85 % and > 95 % pure), 5 additional days are required.

After purification and corresponding quality control, the peptide is lyophilised, weighted and shipped. If ever we were unable to obtain the ordered quantity, a resynthesis is started immediately and the additional quantity delivered afterwards.

The peptide is always shipped together with:

- The HPLC analysis profile
- The mass spectrum
- A data sheet containing useful information (molecular weight, isoelectric point,...)
- Information about solubilization and storage

Eurogentec products are guaranteed to meet or exceed the specifications listed. This warranty is limited to the replacement of the product, no other warranty, expressed or implied is provided by Eurogentec. Our products are sold for research and laboratory use only and are not to be administered to humans or used for medical diagnostics.

Working with peptides

The first choice solvent for most peptides is ultra-pure water. If the peptide does not dissolve easily, sonication may help. Dilute acetic acid or ammonium hydroxide may be necessary to dissolve basic or acidic peptides, respectively. For peptides which are not dissolved by these methods, guanidinium chloride or acetonitrile may be necessary. The use of these compounds may have a detrimental effect on some experiments, so we recommend that care be taken when designing the peptide. Residues such as Ala, Cys, Ile, Leu, Met, Phe and Val increase the chance that the peptide will have solubility problems.

Most peptides are stable for years at -20 °C, especially if they have been lyophilized and stored in a desiccator. Allow lyophilized peptides to come to room temperature before exposing them to air. This will minimize moisture-related effects. When lyophilization is not possible, the next best method of storage is small, working-size aliquots. For peptides which contain Cys, Met or Trp, deoxygenated buffers are a must for solubilization because the peptides will readily oxidize by air. Nitrogen or Argon passed slowly over the peptide before closing the vial will also decrease oxidation. Peptides containing Gln or Asn are also easily degraded. All these peptides have a limited lifetime in comparison to those that do not contain these problem residues.

Custom Peptides modifications

We are able to provide the following modifications to your custom peptide. For quotation or advices about your project please contact: info@eurogentec.com

Conjugations-Labeling

- ▣ BSA, KLH,OVA, THY conjugated peptides for antibody production
- ▣ Drug-peptide conjugates
- ▣ FRET Substrates
 - ↳ 5-FAM/QXL-520
 - ↳ HiLyte Fluor™ 488/QXL-520
 - ↳ TAMRA/QXL-570
 - ↳ EDANS/DABCYL
 - ↳ Other FRET pairs
- ▣ Glycopeptides
- ▣ Lipopeptides
 - ↳ Prenylated
 - ↳ Farnesyl
 - ↳ Geranyl
- ▣ Pegylated peptides
- ▣ TR-FRET Substrates
 - ↳ Europium/QXL-610
 - ↳ Europium/HiLyte Fluor 647

Terminal Modifications

- ▣ C-terminal modifications
 - ↳ Aldehydes
 - ↳ Alcohols
 - ↳ Chloromethyl Ketones
 - ↳ Cysteamide
 - ↳ Ester
 - ↳ N-Alkyl Amides
 - ↳ Hydrazides
 - ↳ Thio ester
- ▣ Dye-labeled peptides
 - ↳ C-terminal
 - AFC, AMC, Dap(Dnp), Lys(Dye), pNA, Rh110
 - ↳ N-terminal
 - HiLyte Fluor™ dyes (405, 488, 555, 647, 680, 750)
 - QXL™ quenchers
 - FAM, FITC, MCA, Sulforhodamine 101, TAMRA
- ▣ N-terminal Modifications
 - ↳ Acetylated
 - ↳ Biotinylated (also for internal lysine)
 - ↳ Bromoacetylated
 - ↳ DOTA
 - ↳ Formylated
 - ↳ Myristoylated
 - ↳ Succinylated

Structural Modifications

- ▣ Branched peptides
- ▣ Click Chemistry peptides
- ▣ Cyclic peptides
 - ↳ N -> C
 - ↳ Disulfide (S-S bond formation)
- ▣ Dityrosine Cross linked peptides
- ▣ Hydrocarbon Stapled Peptides
- ▣ Lactam peptides
- ▣ Multiple Antigen Peptides (MAP) for immunization
- ▣ Peptide bond modifications
 - ↳ Ester (Depsipeptides)
 - ↳ Amine (reduced amide bond)
- ▣ Thiolactone peptide

Amino acid Modifications

- ▣ Dehydro peptides
 - ↳ dehydroalanine; dehydroleucine, dehydroproline
- ▣ Heavy Isotope Labeled Peptides (ClearPoint™ peptides labeled with D, C13, N15)
- ▣ Methylated peptides
 - ↳ Lys(Me), Lys(Me)₂, Lys(Me)₃, Arg(Me)₂ symmetrical, Arg(Me)₂ asymmetrical
 - ↳ N α -Methylated
 - ↳ C α -Methylated
- ▣ Phosphopeptides
 - ↳ Phosphoserine, Phosphothreonine, Phosphotyrosine
- ▣ Sulfated Tyrosine or Serine
- ▣ Peptidomimetics

Peptide purity levels & applications

We propose five purity levels depending on your application.

- ▣ Crude Peptides are useful for first pass screening applications.
- ▣ > 70 % pure peptide is usually sufficient for generating or testing antibodies.
- ▣ Peptides that are > 85 %, > 95 % or > 98 % pure are usually required in enzymology or biological activity studies.

The quality of these peptides is checked by HPLC using a photodiode array detector and by Mass Spectrometry. These data are included in the peptide delivery sheet.

NOTE : > 98 % pure peptides are available as special request

Crude peptides / SePOP purified

These are useful for first pass screening applications where large numbers of peptides must be tested. Once the initial “good hits” have been identified a second pass with a small number of more highly purified peptides is recommended. Average purity is 65 %.

Common applications involving crude peptides

- ▣ Epitope mapping
- ▣ Alanine walking
- ▣ Mutation screening
- ▣ Sequence screening

Crude peptides

Minimum quantity	Length	Included in price	Reference
1-3 mg	6-18 aa	Mass spectrometry analysis for correct molecular weight	AS-PECR-02XX where XX is the number of aa

Immunograde (> 70 %)

A > 70 % pure peptide is sufficient to raise sequence specific polyclonal antibodies. As the amount of contaminating peptide species is very low, the antigenic response to these will be small. Sequence selection will play a greater role in antibody specificity than the cross-reactivity from contaminating peptides purified at immunograde level. Nevertheless, the presence of organic impurities inherent to the synthesis process can be the source of adverse side effects like inflammatory or even toxic effects during the antibody production procedure. Therefore, our immunograde peptides always undergo a special treatment for the complete extraction of organic impurities and, where necessary, undergo one standard purification step.

Common applications involving immunograde (> 70 %) peptides

- ▣ Immunization to raise Polyclonal antibodies
- ▣ ELISA tests: antibody titre measurements do not require higher purity than > 70 % pure peptides.
- ▣ Affinity purification of polyclonal antibodies

Immunograde purity (> 70 %)

Minimum amounts	Length	Included in price	Reference
5 to 500 mg	6-30 aa	HPLC analysis and Mass Spectrometry analysis	AS-PE70-XXYY where XX = number of mg of peptide in aa (06-30)

Biochemistry grade (> 85 %)

- ▣ Semi-quantitative enzyme-substrate studies
- ▣ Phosphorylation studies
- ▣ Peptide blocking studies by Western blotting
- ▣ *In vitro* bioassays
- ▣ Cell attachment studies

Biochemistry purity (> 85 %)

Minimum amounts	Length	Included in price	Reference
2 to 100 mg	6-30 aa	HPLC analysis and Mass Spectrometry analysis	AS-PE85-XXYY where XX = number of mg YY = Length of peptide in amino acids (06-30)

Very high purity (> 95 %)

- ▣ Generating standard curves
- ▣ Quantitative receptor-ligand interaction studies
- ▣ *In vitro* bioassays
- ▣ *In vivo* studies
- ▣ Quantitative blocking and competitive inhibition assays
- ▣ Quantitative phosphorylation studies
- ▣ Quantitative proteolysis studies
- ▣ NMR studies
- ▣ Physical properties standards

Highly purified peptides (> 95 %)

Minimum amounts	Length	Included in price	Reference
2 to 100 mg	6-30 aa	HPLC analysis and Mass Spectrometry analysis	AS-PE95-XXYY where XX = number of mg YY = Length of peptide in amino acids (06-30)

FRET peptides

General overview

Numerous methods are used in the analysis of proteases present in solutions, cells or tissues; however, spectrophotometric method has been favored due to its high speed, better accuracy and ease of use. FRET (Fluorescence Resonance Energy Transfer) is a non-radioactive based detection system. This method has been predominantly used in high throughput screening (HTS) of protease activities and inhibitors. The spectral and enzymatic properties of chromogenic and fluorogenic substrates play a critical role in the successful use of spectrophotometric methods for analyzing proteases. In general, fluorogenic substrates are several orders of magnitude more sensitive than chromogenic substrates, they have a wide linear dynamic range and offer good reproducibility. In recent years, FRET-based assays have been used extensively in the detection of different proteases, which made the continuous assay of protease activity and HTS of protease inhibitors faster and easier.

Applications

- ▣ Proteolysis inhibitor discovery
- ▣ Proteolysis selectivity profiling
- ▣ Can also be used in phosphatase/kinase screens

FRET principle

FRET is a transfer of the excited state energy from the initially excited donor to a nearby acceptor molecule. A fluorophore absorbs light energy and is promoted to an excited state. In the absence of a quencher, the fluorophore falls back to the ground state and the excess of energy is released as fluorescence. In the presence of a quencher, the

quencher accepts the energy from the fluorophore and no fluorescence is detected from the first acceptor molecule.

The transferred energy is either released from the quencher as fluorescence at a higher wavelength, or as heat (dark quencher). To obtain optimal quenching, the absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor. The efficiency of the process is dependent on the fluorophore-quencher distance. Donor and acceptor molecules must be in close proximity (typically 10–100 Å or 8-10 amino acids) for quenching to take place.

FRET peptides as substrates for proteases, contain a fluorophore and a quencher, each on one side of the cleavage site. Quenching will be perturbed upon cleavage of the peptide by the presence of a protease, thus separating fluorophore and quencher and resulting in a new fluorescent emission (Figure 1).

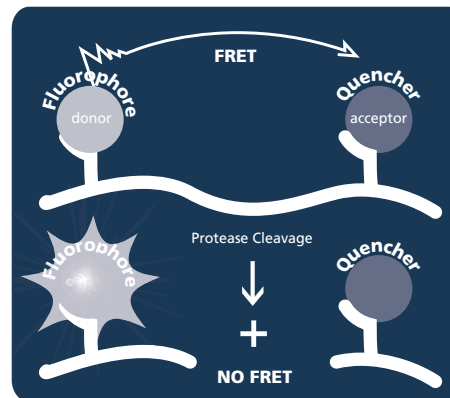
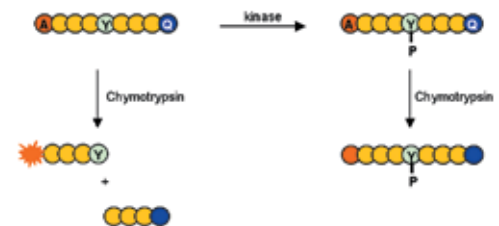


Figure 1: Principle of FRET peptides - Quenching is eliminated upon peptide cleavage resulting in a fluorescent signal.

Kinase / phosphatase detection with FRET peptides

Over 500 kinases and phosphatases are encoded in the human genome. The phosphorylation and de-phosphorylation process is one of the major regulatory processes *in vivo* and aberrant activity of these enzymes has been associated with numerous pathologies. FRET based technologies are also useful for the detection of phosphorylation / de-phosphorylation events.



Quenching process is lost due to cleavage of the peptide and a signal is detected.

The peptide is not a substrate for chymotrypsin activity when the site of cleavage is phosphorylated. The FRET peptide is intact and the quenching process is maintained.

Figure 2: Detection of Tyrosine Phosphorylation

Guidelines in Designing FRET Peptide Substrates

Choose a donor/acceptor pair where the absorption spectrum of the quencher overlaps with the emission spectrum of the donor. We generally use a fluorescent donor and a non-fluorescent acceptor (quencher) to build protease peptide substrates (QXL™ 520 has been proven to be an efficient quencher for FAM and HiLyte Fluor™ 488).

Within the same peptide sequence, the donor and acceptor molecules must be in close proximity (typically 8-10 aa) in order to get good quenching. The time-dependent increase in fluorescence intensity is related to the extent of substrate hydrolysis.

Beside using the native sequence, sequences containing unnatural amino acid or modified bonds other than a regular amide bond can be used to increase efficiency of cleavage, to protect the peptide from degradation or to increase solubility.

Most fluorophores are amino reactive, which means they can be conjugated to the α -amino group or the ϵ -amino group of Lysine.

Thiol reactive dyes can be used to conjugate to Cys-containing peptides. This is an economical way to utilize the dyes since the peptides can be HPLC purified first before reacting with the dyes.

For hydrophobic sequences, Lysines or Arginines may be added to increase solubility. These amino acids must be added at the appropriate positions without adversely affecting the protease recognition site.



See also our SensoLyte™ Assay kits catalogue containing hundreds of validated protease and kinase assays.

See also page 246.

ClearPoint™ peptides

ClearPoint™ peptides are heavy isotope labeled peptides that can be used as internal mass spectrometry for the absolute quantitation of proteins.

Custom **ClearPoint™** peptides offer a choice of H², C¹³, or N¹⁵-isotope labels in single, multiple or universal positions. with triple-stage mass spectrometry and HPLC analysis, **ClearPoint™** peptides are rigorously validated for optimum consistency and quality.

- ▣ Clear Point™ β -amyloid peptides
- ▣ Clear Point™ Angiotensin peptides
- ▣ Clear Point™ BSA, Mass spectrometry standard

Custom peptide libraries

Using a new modification of standard Fmoc chemistry for peptide synthesis, Eurogentec now offers the synthesis of custom peptide libraries in 96-well format for high-throughput screening purposes.

Peptide libraries include

- 1 200 – 500 µg of each peptide
- 2 96-well format
- 3 Unbound, free crude peptides
- 4 Lengths between 5-22 amino acids
- 5 Amino N-terminus, CONH₂ C-terminus
- 6 MALDI-TOF QC on 10 % of peptides

Applications

- ▣ Epitope mapping
- ▣ Alanine walking
- ▣ Single amino acid mutation screening
- ▣ Protein-protein interaction studies
- ▣ Kinase motif discovery
- ▣ Protease motif discovery

Requirements

Excel format list of peptides to synthesize

Epitope Mapping

Epitopes recognized by antibodies are commonly 6 amino acids in length. By generating overlapping 10-mer peptides, each shifted by 5 amino acids, one can unequivocally determine which amino acids make up the epitope.

Schematically the approach looks as follows based on the following simplified “protein” example:

KNCSHIQPWETDCLSCLPERQDEYDPKGPVSDG
Peptide library for the identification of the interacting epitope would be generated as follows:

```

1  KNCSHIQPWE
2      HIQPWETDCL
3          WETDCLSCLP
4              CLSCLPERQD
5                  LPERQDEYDP
6                      QDEYDPKGPV
7                          DPKGPVSDG
  
```

Alanine Scan

By systematically replacing every amino acid position in a peptide with Alanine, one can associate an activity with a particular amino acid. Schematically the approach looks as follows based on the following peptide example:

```

KNCSHIQPWETDCLSCLP
ANCSHIQPWETDCLSCLP
KACSHIQPWETDCLSCLP
KNASHIQPWETDCLSCLP
KNCAHIQPWETDCLSCLP
KNCSAIQPWETDCLSCLP
KNCSHAQPWETDCLSCLP
KNCSHIAPWETDCLSCLP
KNCSHIQAWETDCLSCLP
: : :
: : :
  
```

Amino Acid Optimization

By systematically replacing every amino acid position in a peptide with each possible amino acid, one can optimize the activity of a particular peptide sequence.

Schematically the approach looks as follows based on the following peptide example:

```

KNCSHIQPWETDCLSCLP
ANCSHIQPWETDCLSCLP
CNCSHIQPWETDCLSCLP
DNCSHIQPWETDCLSCLP
ENC SHIQPWETDCLSCLP
: : :
: : :
KACSHIQPWETDCLSCLP
KCCSHIQPWETDCLSCLP
KDCSHIQPWETDCLSCLP
KEC SHIQPWETDCLSCLP
: : :
: : :
  
```

High-throughput screening peptide libraries

Description	Reference
Peptide synthesis < 1 mg crude peptide, min 24 peptides	AS-HTPP-CRYY
Biotin labelling	AS-HTPP-NBIOT
EDANS-DABCYL labelling	AS-HTPP-EDDA
Additional QC by MALDI	AS-HTPP-ADDMS
Addition of 1 phosphate or 1 peptide	AS-HTPP-PHOS

YY = length of peptide (5-22 aa)