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WORKING WITH PEPTIDES

Synthetic custom peptides offer an increasingly affordable approach for exploring protein-protein interactions and more complex phenomena such as immune responses directed against specific epitopes. To get the best from peptides in your experiments, you need to consider all relevant factors when designing and using peptides. This guide provides expert advice on the storage and handling of custom peptides, to enable you to get optimal results. It will also cover the implications of the presence of certain amino acids in the peptide sequence and suggest design considerations to help achieve your desired outcomes.

CONTENTS

BUILDING BLOCKS

Sometimes you need a particular peptide sequence for your work,
but have you considered how that peptide will need to be handled?

The amino acid composition of your peptide will affect its
physiochemistry - meaning you need to think carefully
about synthesis, solubilization and storage.

Peptide Design

There are a number of elements to consider when designing individual peptides,
specifically amino acid composition, length, solubility and the application in which the
peptides are to be used. As the length of the peptide increases, since the coupling
efficiency of adding each individual amino acid is less that 100%, so the proportion of
full-length peptide obtained from the synthesis will decrease.

The amino acid content strongly influences the purification of a peptide and the
resulting solubility. The ratio of charged amino acids to uncharged and hydrophobic
residues is critical in determining the ease of solubility of a peptide, and therefore its
usefulness in the downstream application. A high content of hydrophobic residues will
reduce the solubility of a peptide in aqueous solution, and it may not be possible to
use the peptide in experiments such as cellular assays that don't accommodate the
concentrated organic solvents that will dissolve a hydrophobic peptide. Further, a
hydrophobic peptide is technically more demanding to make, so it may be difficult to
obtain large quantities or high purity.

A design with at least one charged residue every five amino acids is recommended,
otherwise it is recommended to replace hydrophobic amino acids with charged or
polar residues where possible. If determination of peptide concentration is critical,
the addition of a Tyrosine residue at the N- at C-terminus of the peptide is
recommended if the peptide sequence does not already contain Tyrosine or
Tryptophan (see *Measuring Concentration*, page 12).

Certain amino acids can cause other problems in synthesis, solubilization and storage
of peptides:

N-terminal Asparagine

The protecting group for Asparagine can be difficult to remove when at the N-
terminus. To avoid problems remove Asparagine or substitute.

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BUILDING BLOCKS

Cysteine, Methionine and Tryptophan

These residues are prone to oxidation and their presence in the sequence can cause problems with cleavage and subsequent purification of peptides. To eliminate this issue, use Serine to replace Cysteine and use Norleucine to replace Methionine. If multiple Cysteine residues are present, disulfide links may form in the presence of oxygen. To minimize this, use a buffer containing a reducing agent such as DTT, or replace Cysteine with Serine.

N-terminal Glutamine

Glutamine will cyclize to form Pyroglutamate when exposed to the acidic conditions of cleavage; to avoid this either acetylate the N-terminus, synthesize with Pyroglutamate instead of Glutamine to stabilize the peptide, or remove or substitute the Glutamine.

Aspartic Acid

Peptides containing Aspartic acid (Aspartate) can undergo hydrolysis. The peptide chain may be cleaved under acidic conditions when particular amino acid pairs are present. Avoid Aspartate-Glycine, Aspartate-Proline and Aspartate-Serine pairs if possible.

Multiple Serine or Proline

Avoid adjacent Serine residues as synthesis frequently results in a product that is low in purity and that also may contain many deletions. Proline may undergo cis/trans isomerization in solution and subsequently show low purity.

Secondary Structure

The presence of beta-sheet can lead to deletion sequences in the final peptide. Multiple Glutamine, Isoleucine, Leucine, Phenylalanine, Threonine, Tyrosine or Valine residues can lead to beta-sheet formation. If possible, break up these stretches of amino acids by making replacements, such as Asparagine for Glutamine or Serine for Threonine, or add Proline or Glycine every third residue.

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AMINO ACID PROPERTIES

Amino Acid Name	3-Letter Code	1-Letter Code	Side Chain Polarity	Side Chain Acidity / Basicity	Hydropathy Index*	Molecular Weight (Da)	Isoelectric Point**
Alanine	Ala	A	Nonpolar (hydrophobic)	Neutral	1.8	89.09	6.00
Arginine	Arg	R	Polar (soluble)	Basic (strongly)	-4.5	174.20	11.15
Asparagine	Asn	N	Polar	Neutral	-3.5	132.12	5.41
Aspartic acid	Asp	D	Polar	Acidic	-3.5	133.10	2.77
Cysteine	Cys	C	Polar	Neutral	2.5	121.16	5.02
Glutamic acid	Glu	E	Polar	Acidic	-3.5	147.13	3.22
Glutamine	Gln	Q	Polar	Neutral	-3.5	146.15	5.65
Glycine	Gly	G	Nonpolar	Neutral	-0.4	75.07	5.97
Histidine	His	H	Polar	Basic (weakly)	-3.2	155.16	7.47
Isoleucine	Ile	I	Nonpolar	Neutral	4.5	131.18	5.94
Leucine	Leu	L	Nonpolar	Neutral	3.8	131.18	5.98
Lysine	Lys	K	Polar	Basic	-3.9	146.19	9.59
Methionine	Met	M	Nonpolar	Neutral	1.9	149.21	5.74
Phenylalanine	Phe	F	Nonpolar, aromatic	Neutral	2.8	165.19	5.48
Proline	Pro	P	Nonpolar	Neutral	-1.6	115.13	6.30
Serine	Ser	S	Polar	Neutral	-0.8	105.09	5.68
Threonine	Thr	T	Polar	Neutral	-0.7	119.12	5.64
Tryptophan	Trp	W	Nonpolar, aromatic	Neutral	-0.9	204.23	5.89
Tyrosine	Tyr	Y	Polar, aromatic	Neutral	-1.3	181.19	5.66
Valine	Val	V	Nonpolar	Neutral	4.2	117.15	5.96

*Hydropathy Index: a number representing the hydrophobic or hydrophilic properties of the side-chain of an amino acid (Kyte and Doolittle, 1982: A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105-132.)

**Isoelectric Point: the pH at which the net charge of the amino acid is equal to zero.

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PEPTIDE PURITY

When planning your experiment, it is important to bear in mind the effect that peptide impurities could have - they could give you a false result, or even be toxic to the cells you are using. However, highly purified peptides are very expensive, and might not be required for all experiments. So how do you choose which purity to order?

Peptide purity, measured by analytical reversed phase HPLC, is the percentage of full-length peptide compared to impurities that absorb at 210-220 nm (peptide bond absorption wavelength). Care should be taken regarding the LC method used. A crude gradient can suggest high purity results when the actual purity may be much lower. Impurities consist of deletion sequences, incompletely deprotected sequences, truncated sequences and side products of the synthesis process. Residual salt and water that do not absorb at this wavelength are not quantified. In experimental planning it is important to consider that some peptide or non-peptide impurities may be toxic to cells. This can generally be avoided by purifying the peptide, which will result in only trace amounts of toxic impurities.

Choose the best purity for your application:

95% to >98%	Peptides for use in quantitative studies or assays e.g., receptor-ligand interaction, enzyme-substrate studies, blocking and competition assays, ELISA and RIA NMR, Mass Spectrometry and crystallography In vivo studies and in vitro bioassays Monoclonal antibody production
>80%	Non-quantitative use in western blotting, immunocytochemistry or enzyme-substrate studies Phosphorylation reactions Antibody affinity purification Coating of tissue culture plates for cell attachment
>70%	For use as a standard in an ELISA For use in ELISpot assays Polyclonal antibody production

Peptide Content

Lyophilized peptides contain impurities in the form of counterions such as acetate or trifluoracetate, and residual water. Net peptide content, measured by amino acid analysis, is the percentage of all peptides relative to these non-peptide impurities. It varies according to the purification and lyophilization procedures, and is affected by the amino acid composition, particularly the presence of hydrophilic amino acids in the sequence.
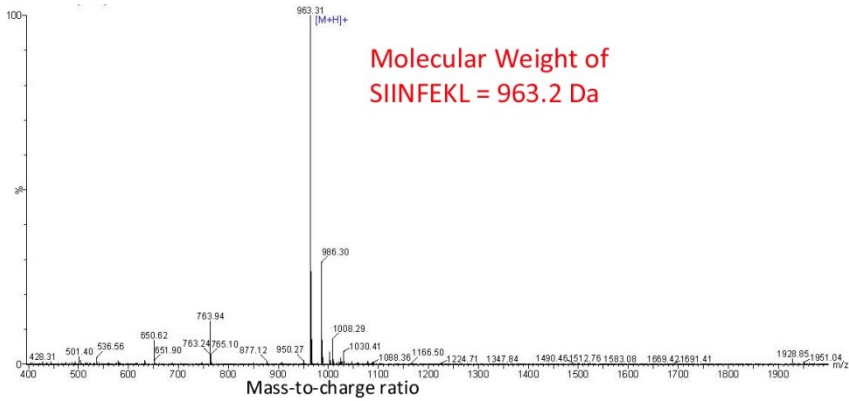
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PEPTIDE PURITY

What do all the datasheets provided with your peptide mean?

You should receive two traces with your peptide, a mass spectrometry (MS) trace and an HPLC trace.

MS traces

MS traces are used to confirm that the molecules with the correct mass are present as a major species. On your trace, the largest peak corresponds to the major ion species created from your peptide in the mass spectrometer. Use this trace to make sure that the mass of your desired peptide corresponds to the mass shown for the major peak. The value assigned to it should be close (within 1Da) to the calculated molecular weight for your peptide.

HPLC traces

HPLC traces are used to confirm the purity of your peptide.

The area under the major peak corresponds to the percentage purity. In the example shown on the right, for the same SIINFEKL peptide described by the MS trace above, SIINFEKL accounts for 96.88 of the peptide species in the sample. In other words, the peptide is almost 97% pure.
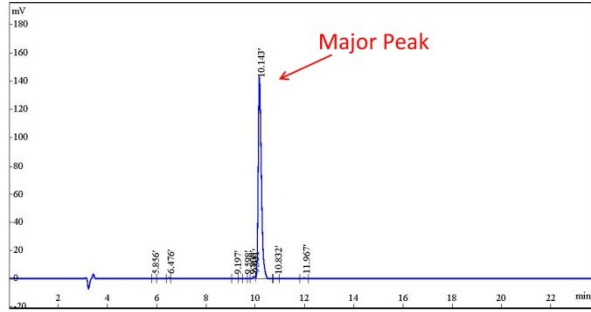
Rank	Time	Conc.	Area
1	5.856	0.09785	1292
2	6.476	0.3677	4855
3	9.197	0.3144	4151
4	9.598	0.08868	1171
5	9.808	0.12	1584
6	9.931	1.269	16758
7	10.143	96.88	1279175
8	10.832	0.1091	1440
9	11.967	0.7475	9869
Total		100	1320295

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AVOID A PEPTIDE DISASTER!

Aliquotting and weighing are by no means the most exciting things you'll do with your peptides, but it is important to get them right.

Weighing Out Lyophilized Peptides

Allow the peptide to warm to room temperature before opening the vial, to prevent condensation of atmospheric moisture onto the peptide. This ensures that it is easier to handle and weigh. Highly hydrophobic peptides will be particularly hard to work with in the presence of moisture.

Check that there is temperature and humidity control in the laboratory. Make sure there is no draft blowing over the workspace, e.g., from an air conditioning unit. Set up sensitive weighing scales; the use of a marble slab between the scales and bench top minimizes vibrations and improves accuracy of weighing.

To avoid the effects of static electricity, which could result in your peptide sticking to the spatula or weighing boat, use a Teflon coated spatula (e.g., Sigma Z51333-4, Fisher FB65115) and weigh small amounts into an antistatic weighing boat (e.g., Fisher FB50343).

Dissolve only the required amount of peptide into a buffer. The remainder of the stock should be stored dry at -20 C or -80 C. Ideally, lyophilized stock should be stored under inert gas – Nitrogen or Argon – but usually this is not a practical option. For more on storing peptides, see page 14.

Custom peptides are valuable, making it imperative that one gets as many experiments out of a sample as possible. Calculating ahead of time to determine how much peptide will be needed for the specific experiments planned and only solubilizing that amount is highly recommended.

Peptides containing Cysteine, Tryptophan or Methionine are susceptible to oxidation. Once dissolved, these peptides have limited stability and long-term storage in solution is not recommended. It is advisable to blanket the dry, stock peptide with argon or nitrogen before the vial is resealed.

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AVOID A PEPTIDE DISASTER!

Hygroscopic peptides containing several charged residues (Arginine, Aspartic Acid, Glutamic Acid, Histidine, Lysine) may take up water when exposed to air. To prevent this, reseal the vial of remaining dry, stock peptide under Argon or Nitrogen. If inert gases are unavailable, then storage in a desiccator is a viable alternative. Before use, bring the sample to room temperature in a desiccator.

Aliquotting Dissolved Peptides

Peptides in solution are only stable for up to one week when stored at +4 C. If peptides are to be used frequently, solubilize and aliquot, then store frozen at -20 C (-30 C if in DMSO; note that DMSO is hygyroscopic so will not freeze well once it has absorbed water) or lower, and avoid freeze-thaw cycles. Typically the peptide will remain stable for several months if handled this way.

Estimate the number of aliquots needed for your series of experiments; if necessary an aliquot can itself be aliquoted again. Follow the recommendations for solubilization on page 10 of this guide. Aliquot the peptide into high quality polypropylene tubes. The tubes should have a screw cap with an O-ring to ensure a tight seal. Do not fill the tubes completely, as the solution is likely to expand on freezing. Avoid exposure to light for peptides with amino acids containing aromatic rings (Phenylalanine, Tryptophan, Tyrosine).

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SOLUBILIZATION

Choose an appropriate solvent, compatible with your experimental procedures and that does not react with or degrade the peptide. The solvent should be dry (if it is non-aqueous) and degassed. Since most peptide work is small-scale, it is most practical to buy small volumes of dry solvent, rather than attempting to dry it yourself.

Determine if the peptide is acidic, basic or neutral (this depends on the ratio of different types of amino acid present in the sequence, and can be calculated using a range of free online tools, or the table in this guide).

Proceed with solubilization using a small amount of peptide. Most acidic and basic peptides are more soluble at neutral pH. Peptides are acidic when the manufacturing process is complete, due to the presence of trifluoroacetate (TFA) as the counter ion (a result of the cleavage or purification process). Check whether the peptide is supplied as a TFA salt before you start the solubilization process.

Short non-hydrophobic peptides (< 5 amino acids) and peptides containing >25% non-clustered, charged residues and <25% hydrophobic residues will typically dissolve in aqueous solutions.

Simply adding water may dissolve **basic** peptides. If it does not, first try a drop (10-20 µl) of glacial acetic acid and vortex gently. For problematic sequences, increase up to 30% acetic acid by volume.

Acidic peptides with net charge of -1 or lower should be dissolved in a small amount of basic solvent such as 0.1% ammonium hydroxide or ammonium bicarbonate and diluted to the required stock concentration with water. The exception is peptides containing Cysteine, as disulfide bonds may form at alkaline pH.

Neutral or hydrophobic peptides with a net charge of zero can sometimes be brought into solution by addition of base, but often a gel forms. Generally this gel will only respond to dilution with higher amounts of distilled, deionized water, along with gentle vortexing.

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SOLUBILIZATION

Peptides that are comprised of **>50% hydrophobic** amino acids may be difficult to dissolve in water alone and should be dissolved in a small amount of organic solvent, e.g., acetonitrile, methanol, isopropanol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF). This should be added drop wise, followed by sonication and vortexing after every drop until the peptide dissolves. The drop wise addition of the organic solvent can also be used for peptides that do not respond to pH adjustment.

Peptides that are **>75% hydrophobic** are unlikely to dissolve in aqueous solution alone and may require solubilization in a stronger solvent such as TFA or formic acid, and at high concentration. The peptide may precipitate out when aqueous buffer is added. These conditions may not be compatible with some cell culture based experiments.

Organic solvents at certain concentrations are incompatible with some biochemical assays. A small amount of DMSO should be compatible with most immunological assays, but avoid DMSO if the peptide contains Methionine, Cysteine or Tryptophan due to sulfoxide or disulfide formation. These peptides should be prepared using 1,2-ethanediol or dithiothreitol (DTT) in order to prevent oxidation. Oxygen-free water or buffers, or DTT are recommended for solubilizaton.

Note, if DMSO solvent is at all wet it will not freeze at –20ºC, so freezing at –30ºC is recommended. DMSO is hygroscopic and will adsorb water if subjected to repeated freeze-thaw cycles. To minimize these issues, buy small volumes of dry DMSO for solubilization purposes (e.g., Sigma Aldrich 15,493-8).

Peptides that are prone to aggregation may require **strong denaturants** (e.g., >6 M urea or guanidine hydrochloride), which may then be able to be diluted.If peptides are to be used in cellular assays, start by making a concentrated stock solution e.g., 5-10 mM, or 5-10 mg/ml. Dilute the peptides into physiological buffer for use, such that the original solvent is present at no more than 0.1% in the final working solution.

Diluted peptide solutions may also be **filter-sterilized** (0.22 µm) if they are to be added to sterile cultures.

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MEASURING CONCENTRATION

Measurement Of Concentration

Establishing the actual peptide concentration based on the weight of the lyophilized peptide is inaccurate because there may be a significant quantity (10-70%) of bound water, and salts or counter ions. The type of counter ion and the amount is dependent on the peptide sequence and the solvents that have been used in the manufacturing and purification process. The peptide concentration can be estimated based on whether there are Tryptophan or Tyrosine resides present in the sequence.

If your Peptide contains Tryptophan or Tyrosine[1,2]

To measure concentration, measure the absorbance of your peptide at 280nm using UV spectrophotometry, and applying the following calculation:

$$[\text{Peptide concentration}] \, \text{mg/ml} = (A_{280} \times DF \times MW) / \varepsilon$$

A_{280} is the absorbance of the peptide solution at 280 nm in a 1 cm cell

DF is the dilution factor

MW is the molecular weight of the peptide

ε is the molar extinction coefficient of Tryptophan or Tyrosine at 280 nm.

Tryptophan $\quad \varepsilon \, (\text{Trp}) = 5690 \, M^{-1}cm^{-1}$ $\qquad$ Tyrosine $\qquad \varepsilon \, (\text{Tyr}) = 1280 \, M^{-1}cm^{-1}$

If the peptide contains more than one Tryptophan or Tyrosine, or a mixture, then the extinction coefficients are added, e.g., if there are two Tryptophan and one Tyrosine, $\varepsilon = (2 \times 5690) + (1 \times 1280)$.

(Note that this calculation assumes that the peptide will be in an extended unfolded conformation. For longer peptides, folding may obscure some Trp or Tyr residues and affect the A_{280} value. The most accurate measurement will be obtained if the peptide is measured in a denaturing buffer such as 6M Guanidine hydrochloride, pH 6.5, 0.02 M phosphate buffer.)

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MEASURING CONCENTRATION

If your peptide does not contain Tryptophan or Tyrosine[3]

It is possible to determine the concentration of peptides that do not contain aromatic amino acids by measuring absorbance at 205 nm. However this measurement is far more prone to external influence, since many solvents and other chemicals will absorb at this wavelength. A high quality, dual-beam spectrophotometer is required in order to reduce the effects of non-specific absorption and to measure low concentrations. For a high level of reproducibility, a combination of absorption measurements at a range of wavelengths is recommended.

If routine analysis is required, then HPLC analysis with measurement at 205 nm would be a far superior option because of its ability to resolve the peptide from the buffer constituents, thus allowing for more accurate concentration determination. Integration of the peak of interest will allow for accurate determination of peptide quantity. Using control peptide solutions to determine the absorbance at 205 nm that corresponds to a fixed concentration of peptide is recommended.

References

1) Edelhoch, H., 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry* 6:1948-1954.

2) Gill, S.C., von Hippel, P.H., 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* 182:319-326.

3) Scopes, R.K., 1974. Measurement of protein by spectrophotometry at 205 nm. *Anal. Biochem.* 59:277-282.

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PEPTIDE STORAGE

Lyophilized Peptide

Most commercial manufacturers of custom peptides ship the product as a lyophilized powder. Store lyophilized peptides in the freezer upon receipt. For short-term storage (up to 2 months), store at -20 C; for long-term storage -80 C is recommended. The stability of a stored peptide will be highly sequence-dependent, but stored dry and cold like this, the majority of peptides will be stable for 6 months and may for much longer. Ideally, vials for storage should be high-density, polypropylene tubes, airtight, well sealed with a screw cap with an O-ring. Glass vials could be considered for storage of peptides. However, they are more expensive and fragile, and may crack if frozen at very low temperature. Avoid storing or making up peptide solutions in polystyrene containers as they adsorb peptide material to their surface. Some peptides are provided in an inert atmosphere, e.g., under Argon or Nitrogen. Do not open and transfer to another vial for storage purposes, as the inert gas will be released.

Solubilized Peptide

Peptides in solution are only stable for up to one week when stored at +4 C. If peptides are to be used frequently, solubilize and aliquot, then store frozen at -20 C (-30 C if in DMSO) or lower, and avoid freeze-thaw cycles. Typically the peptide will remain stable for several months if handled this way.

Estimate the number of aliquots needed for your series of experiments; if necessary an aliquot can itself be aliquoted again. Follow the recommendations for solubilization on pages 10-11. Aliquot the peptide into high quality polypropylene tubes. The tubes should have a screw cap with an O-ring to ensure a tight seal. Do not fill the tubes completely, as the solution is likely to expand on freezing. Avoid exposure to light for peptides with amino acids containing aromatic rings (Phenylalanine, Tryptophan, Tyrosine).

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