Cleavage, Deprotection, and Isolation of Peptides after Fmoc Synthesis

Cleavage and deprotection is one of the most crucial steps in peptide synthesis. The treatment of a peptidyl-resin with a cleavage cocktail is not one simple reaction, but a series of competing reactions. Unless suitable reagents and reaction conditions are selected, the peptide can be irreversibly modified or damaged.

The goal of cleavage/deprotection is to separate the peptide from the support while removing the protecting groups from the side-chains. This should be done as quickly as possible to minimize the exposure of the peptide to the cleavage reagent. The peptide is then recovered from the reaction mixture and analyzed. This technical bulletin describes procedures for cleavage of peptides from solid supports assembled *via* Fmoc/*t*Bu-based strategies.[1]

There are numerous procedures in the literature which describe a variety of cleavage and deprotection methods for peptides synthesized with an Fmoc/*t*Bu strategy.[2-4]. Most of these techniques are TFA-based, and they differ primarily in the final concentration of TFA, types of scavengers used, and reaction times. Some of the variablity in methods reflects the preferences of individual labs, but it is mainly dictated by the amino acid composition of the peptide.

Some amino acids have potentially reactive side-chains which generate carbonium ions and other reactive species during TFA cleavage of the peptide from the support. Therefore, the appropriate scavengers and reaction conditions must be chosen to minimize modification or destruction of the sensitive amino acids.

Since prolonged treatment with the cleavage acid is needed to remove some protecting groups, scavengers must be used to protect the reactive sites of the peptide during extended reaction times. The 21 naturally-occurring Fmoc amino acids and their side-chain protecting groups are listed in Table 1.

Abbreviations: Acm: acetamidomethyl, **ACN:** acetonitrile, **Al:** allyl, **Ala:** alanine, **Aloc:** allyloxycarbonyl , **Arg:** arginine, **Asn**: asparagine, **Asp**: aspartic acid, **Boc:** *t*-butyloxycarbonyl, **Cys**: cysteine, **DCM:** dichloromethane, **DMF:** *N,N*-dimethylformamide, **DTT:** dithiothreitol, **EDT**: 1,2-ethanedithiol, **Fmoc:** 9-fluorenylmethoxycarbonyl, **Gln**: glutamine, **Glu:** glutamic acid, **Gly**: glycine, **His**: histidine, **HPLC:** high performance liquid chromatography, **Ile**: isoleucine, **Leu:** leucine, **Lys**: lysine, **Mbh**: 4,4-dimethyloxybenzhydryl, **MeOH:** methanol, **Met:** methionine, **Mtr:** methoxytrimethylbenzene sulfonyl, **OAl**: allyl ester, **Orn:** ornithine, **O*t*Bu:** *t*-butyl ester, **Pbf:** 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl, **pGlu:** pyroglutamic acid or pyrrolidone glutamic acid, **Phe:** phenylalanine, **Pmc**: 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl chloride, **Pro**: proline, **SDS:** sodium dodecyl sulfate, **Ser**: serine, ***t*Bu**: *t*-butyl ether, **TES:** triethylsilane, **TFA:** trifluoroacetic acid, **Thr**: threonine, **TIPS**: triisopropylsilane, **Tmob**: 2,4,6-trimethoxybenzyl, **Trp:** tryptophan, **Trt**: trityl or triphenylmethyl, **Tyr:** tyrosine, **Val**: valine

Potential Problems

Certain amino acids can cause problems during TFA cleavage and deprotection. These fall into three broad categories:

1. Amino acids whose protecting groups are easily removed, but whose deprotected side-chains are especially labile in acid conditions (e.g., **Met, Cys, His, Trp**).
2. Amino acids which need more than the normal two hours for complete removal of the side-chain protecting groups (e.g., **Arg(Pmc/Mtr)**, **Asn/Gln(Mbh)**).
3. Amino acids whose side-chain protecting groups, once removed from the side-chain, are extremely reactive and must be scavenged to prevent reattachment or modification of the deprotected side-chains (e.g., **Arg(Pmc/Mtr), Asn/Gln(Tmob)**).

Table 1: Side-chain Protecting Groups for Fmoc Amino Acids

Amino acid	Protecting Group (bold is recommended)	Functionality Protected
Ala (A)	none	
Arg (R)	**Pbf**, Mtr, Pmc	guanidino N
Asn (N)	**Trt** , Mbh, Tmob	Amide
Asp (D)	**O*t*Bu**, OAl°,	Carboxyl
Cys (C)	**Trt,** Acm°, *t*Bu°, *St*Bu	Sulfhydryl
Gln (Q)	**Trt**, Mbh, Tmob,	Amide
Glu (E)	**O*t*Bu,** OAl°	Carboxyl
Gly (G)	none	
His (H)	**Trt**, Boc	Imidazole
Ile (I)	none	
Leu (L)	none	
Lys (K)	**Boc**, Aloc°, Fmoc°	Amino
Met (M)	none	
Orn (O)	**Boc**	Amino
Phe (F)	none	
Pro (P)	none	
Ser (S)	***t*Bu**	Hydroxyl
Thr (T)	**tBu**	Hydroxyl
Trp (W)	**Boc**	Indole
Tyr (Y)	***t*Bu**	Phenol
Val (V)	none	

° Indicates protecting groups not removed by TFA

Methionine

Peptides containing methionine are easily oxidized to methionine sulfoxide, which can be reduced back to methionine by treatment with dithiothreitol (DTT) or *N*-mercaptoacetamide[2,3]. The conversion of methionine sulfoxide back to methionine can be monitored by reverse-phase HPLC, since the sulfoxide is more polar, eluting slightly earlier. Short deprotection times will greatly reduce the amount of oxidation that occurs.

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Cysteine
Peptides containing cysteine are prone to oxidative formation of disulfide bonds, which can form intramolecularly, forming a cyclic peptide, or intermolecularly, resulting in oligomerization[2]. If more than one cysteine is present in a molecule, intramolecular disulfides will cause aggregation of the peptide molecules[5]. Disulfide bonds can be reduced using DTT. However, some aggregates are very difficult to reduce. Since oxidation occurs most frequently after exposure of the deprotected peptide to air, care should be taken to keep the peptide as anaerobic as possible when multiple cysteines are present. When multiple cysteines are present, and the user wishes to direct disulfide bond formation to specific sites, it is recommended that different protecting groups, which are removed in different manners, be utilized (refer to Table 1).

Formation of an undesired impurity (+51 Da by positive-ion mode MALDI-TOF) has been seen during the synthesis of *C*-terminal cysteine-containing peptides[6]. The amount of this impurity is dependent on the polymeric support, the side-chain protecting group, the linker incorporated to anchor the cysteine to the resin, and the state of the N^{α}-amino group during chain elongation. The reaction is minimized by using Trt-protection and PEG-PS supports.

Tryptophan
The indole ring of tryptophan is susceptible to both oxidation and alkylation by unscavenged protecting groups released by other side-chains[2-4]. Oxidation products of tryptophan generally cannot be reversed by reduction. Use of Boc-protected tryptophan, and minimal exposure to the cleavage cocktail, will reduce the chances of indole ring damage[7]. *C*-terminal tryptophan-containing peptides are also known to undergo reattachment to the resin[1,8]. This undesired back-alkylation can be prevented by the use of Fmoc-XAL-PEG-PS for the construction of *C*-terminal amides[9].

Histidine
The imidazole ring in histidine can be acylated[8]. Short deprotection times will reduce the chances of damage.

Tyrosine
Although unlikely, the side-chain of tyrosine can be alkylated by unscavenged protecting groups released by other side-chains[3]. Peptides containing *C*-terminal tyrosine may undergo reattachment to the resin[1].

Asparagine
Dehydration of unprotected asparagine can occur during coupling[3]. Therefore, a protecting group is recommended. The Mbh group may require longer deprotection times, especially when multiple ones are present, and the Tmob group is difficult to scavenge. However, the Trt group is readily removed and easily scavenged. *N*-terminal Asn(Trt) may need extended cleavage times[4,10]. Use of the faster deprotecting cyclopropyldimethylcarbinyl (Cpd)-group for *N*-terminal Asn has proven useful[11].

Glutamine
Like asparagine, dehydration of unprotected glutamine can occur during coupling[3]. Therefore, a protecting group is recommended. The Mbh group may require longer deprotection times, especially when multiple ones are present, and the Tmob group is difficult to scavenge. However, the Trt group is readily removed and easily scavenged[4]. *N*-terminal Gln(Trt) may need extended cleavage times. However, *N*-terminal glutamine can also undergo a cyclization to form pyroglutamic acid[2]. While it can occur slowly at basic or neutral pH, it is most commonly seen under highly acidic conditions (as in cleavage/ deprotection). Deprotection times under 4 hours will greatly reduce the chances of this occurring. Like Asn, Gln(Cpd) has proven useful for *N*-terminal glutamine[11]

Arginine
Mtr-protected arginine requires long deprotection times[3,12,13]. When multiple Arg(Mtr) are present in the peptide, deprotection times of up to 12 hours may be required! Pmc-protection has greatly reduced this time[13], but it can still take more than 4 hours when multiple Arg(Pmc) are present. Pmc is also difficult to scavenge and has a tendency to reattach or alkylate sensitive residues. The use of Pbf-protection for arginine is therefore strongly recommended, since deprotection times, even with multiple Arg(Pbf) present, are under 4 hours (2 hours is usual), and the Pbf group is easily scavenged[14].

Isolation of the Peptide from the Solid Support

Various cleavage cocktails have been reported in the literature and are typically applied to peptides based upon their amino acid composition. Unfortunately, no single cleavage and deprotection procedure is optimal for all peptides. This technical bulletin is meant as a guide to simplify the selection of cleavage cocktails based upon both the nature of the linker and the amino acid composition of the peptide.

The overall scheme for a post-synthetic work-up is shown in Figure 1. This figure outlines a general strategy for isolation of a peptide from a polymeric support. More detailed schemes for selecting the procedures that are appropriate for the amino acid sequence of the synthesized peptide are shown in Figures 2-5.

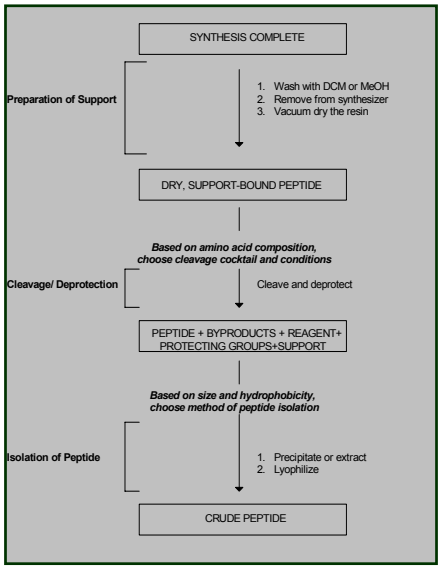


***Figure 1**: Post-Synthetic Work-up Scheme*

NOTE: Since cleavage/deprotection is a chemical reaction in which bonds are irreversibly broken, it is recommended that an initial trial cleavage using a small amount of the resin (50-100 mg) be performed to verify the proper conditions.

Linkers

There is a wide range of commercially available polymeric supports with linkers (anchors) attached to the resin. The linker provides a point of attachment for the *C*-terminal amino acid, and the structure of the linker determines the method of cleavage and the functionality of the final product. The nature of the cleavage cocktail selected is dependent on the linker[15].

Low concentrations of TFA
Peptides synthesized on supports with a XAL ("Seiber") or HAL linker are cleaved in low percentages of TFA to yield fully-protected peptide amides or peptide acids respectively.

High Concentrations of TFA
Peptides synthesized on supports with a PAC ("Wang"), PAL, AM ("Rink Amide"), or BAL linker are cleaved in high percentages of TFA to yield fully deprotected peptides. There are numerous cleavage cocktails cited in the literature for cleavage of peptides from these type of supports.

Other Linkers
Linkers have been developed that release peptides from the solid support upon treatment with reagents other than TFA. These include Linker B (or KB) , which is base-labile, Hycram (an allyl linker), "Safety-catch", and photolabile linkers. These are not commonly used, and are, therefore, out of the scope of this technical bulletin. For cleavage of peptides from these types of supports, please contact your vendor.

Choosing the Cleavage Cocktail

The selection of the cleavage cocktail depends on the nature of the cleavable linker attaching the peptide to the support, the nature of the protecting groups and the reactive properties of the unprotected side-chain.

Low concentrations of TFA
Peptides synthesized on supports with a XAL or HAL linker are cleaved in low concentrations of TFA, and are typically used to prepare protected fragments or sequences containing acid-sensitive residues, such as *N*-methyl amino acids (e.g. cyclosporin).

For protected peptides and fragments, cleave from the resin using TFA/DCM (1:99) and allow to react using one of the following methods:

- 1 x 1 hour
- 6 x 5 minutes

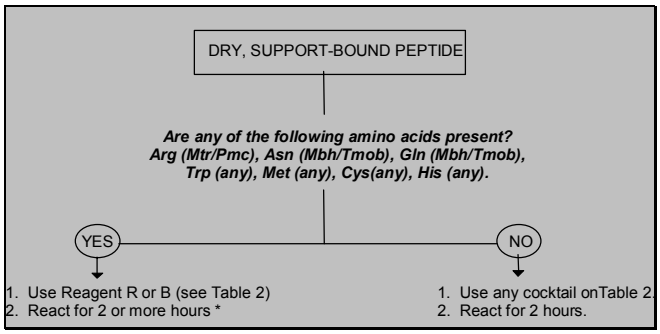
For fully deprotected and acid-sensitive peptides, use one of the following methods:

- TFA/DCM (1:19) for one hour, followed by TFA/DCM (1:1) for 1 hour. Add scavengers as appropriate. Scavengers are necessary only for the removal of Pmc, Pbf, and/or Tmob groups.
- High concentration of TFA (see Table 2) for 2 hours.

High concentrations of TFA
The most commonly used supports for peptide synthesis are those cleaved with a high concentration of TFA. The most common cleavage cocktails are listed in Table 2.

All cleavage cocktails must be prepared fresh prior to use. Scavengers should also be fresh. Buy them in small quantities, and discard any unused scavenger after a few months.

Reagents R and B, which are compatible with most sequences, are highly recommended for those peptides containing **Trp, His, Met, Cys, Arg(Mtr/Pmc), Asn(Mbh/Tmob), Gln(Mbh/Tmob),** as well as for peptides constructed on a PAL or Rink Amide resin (See Figure 2). While Reagent R may give higher cleavage yields, it is highly noxious and may not be preferable to handle. Reagent R or B are also recommended when extended cleavage times (those in excess of 2 hours) are needed, since these cocktails contain a mixture of additives, some of which accelerate cleavage from the resin and scavenge reactive carbocations produced during cleavage/deprotection.



*Reaction times:
Arg(Mtr): one=4 hours
two or more=8-12 hours.
Do not use with Trp.
Asn(Mbh/Tmob): 4 hours.
Gln(Mbh/Tmob): 4 hours.
Trp (any): 2-4 hours maximum.
Unprotected Trp: Protect from light.
Blanket with N_2.
Cys (any), His (any), Met (any): 2 hours.
Blanket with N_2.

***Figure 2**. Choice of Cleavage/Deprotection Cocktail (High conc. of TFA) and Reaction Times.*

Table 2: Fmoc Cleavage Cocktails: High Concentrations of TFA.

	Recipe	Hrs	Comments
B	TFA/phenol/water/ TIPS (88/5/5/2)	1-4	All peptides[1].
K	TFA/phenol/water/ thioanisole/EDT (82.5/5/5/5/2.5)	1-4	All peptides[1].
K'	TFA/phenol/water/ thioanisole/ 1-dodecanethiol (82.5/5/5/5/2.5)	1-4	All peptides[1].
L	TFA/DTT/Water/ TIPS (88/5/5/2)	1-4	All peptides[1]
P	TFA/phenol (95/5)	1-4	*t*Bu group. Do not use with Trp, Met or Cys.
P+	TFA/phenol/ Methanesulfonic acid (95/2.5/2.5)	15 min	All peptides[1,2].
R	TFA/thioanisole/ EDT/anisole (90/5/3/2)	1-8	All peptides[1].
T	TFA/TES (95/5)	1-4	Boc, *t*Bu,Trt. Do not use with Arg or Trp.
	TFA/water (95/5)	1-4	Boc, *t*Bu, Trt, Pbf[3]. Do not use with Trp, Met or Cys.
	TFA/DCM/indole (70/28/2)	1-4	Do not use with Arg[1].

1. Possible alkylation of unprotected tryptophan residues.
2. Possible deamidation of Asn and Gln residues.
3. May be used if one Arg(Pbf) is present. Moderate success if more than one Arg(Pbf) is present.

Amount of Cleavage Cocktail to Use

The amount of cleavage cocktail used depends on both the amount of the resin and its properties.

Polyethylene glycol-grafted polystyrene (PEG-PS) supports and polystyrene (PS) supports swell differently. PS supports float in the TFA. Enough cocktail solution should be used to saturate and swell the resin, with about 2-3 mm of clear solution below the floating beads. PEG-PS supports swell in TFA, and do not float. Use enough cocktail solution to swell the resin. This is approximately 5 mL of cleavage cocktail for 0.5 g of resin.

Choosing the Reaction Time

With most linkers, the peptide-resin anchoring is fully severed following a two-hour treatment with TFA. However, longer times may be necessary for the complete removal of some of the side-chain protecting groups.

A guide to reaction times is provided in Figure 2. Optimal conditions should be determined using an initial trial cleavage. Using HPLC to monitor the efficiency of the removal of the side-chain protecting groups over time can be a useful tool. Since protected peptides will elute differently from unprotected peptides, and may also absorb at different wavelengths, it may be possible to monitor the disappearance of the protected peptide over the course of the cleavage reaction. It is important to realize that due to differences in the extinction coefficients, there is no direct correlation between peak heights/areas of protected peptides and their actual percentage in the peptide mixture. For example, a peptide containing a small percentage of protected Arg(Mtr) may show two peaks of equal height.

NOTE: Met, Cys and unprotected Trp may be modified during prolonged acid treatment, even in the presence of scavengers. Take care to exclude oxygen and light. Do not let cleavage times exceed 4 hours. One to two hours is optimal.

Cleavage Cocktail Preparation

Cleavage cocktails must be prepared fresh using high quality TFA and fresh scavengers. Depending on the amino acid composition of the peptide (see Figure 2 and Table 2), select a cleavage cocktail and prepare the appropriate amount for the reaction scale.

CAUTION:
Preparation of cleavage cocktails and the cleavage/deprotection reaction must be done in a well-vented fume hood.

For most applications, 5-10 mL of cocktail is required. It is convenient to dedicate a set of glassware to the handling of cleavage cocktails.

Thiol scavengers are especially noxious. It is recommended that a large beaker containing a 50% aqueous bleach solution be kept in the fume hood, and all glassware and disposables, such as pipette tips and gloves, be allowed to soak in this solution for several days before cleaning or disposal.

Requisite Chemicals and Equipment

- Gloves, goggles and protective clothing
- TFA (sequencing grade)
- Scavengers [thioanisole, EDT, anisole, phenol, TIPS, etc.] (reagent grade)
- Peroxide-free anhydrous diethyl ether, cooled in an explosion-proof freezer or in a dry ice/acetone bath.
- Household bleach
- Nitrogen or argon (pre-purified), for blanketing the reaction mixture
- Scintillation vials with polypropylene cap inserts, for the cleavage reaction
- Pasteur pipettes and glass wool, or polypropylene syringes with polypropylene frits and rubber septa (see Figure 3)
- Glacial acetic acid
- Dry ice
- Benchtop centrifuge (for 50 mL tubes)
- 50 mL centrifuge tubes
- Lyophilizer
- 10 mL and 25 mL graduated cylinders
- Automatic pipettors
- Miscellaneous glassware: flasks, beakers
- Rotary evaporator (optional)
- Magnetic stirrer and stirbars (optional)

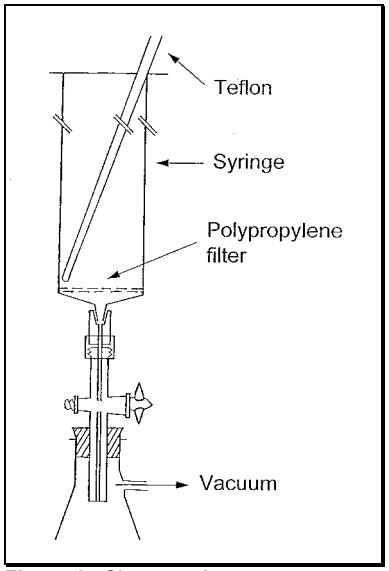


Figure 3. Cleavage Apparatus

Reagent Preparation

Below is a typical procedure for the preparation of a cleavage cocktail, and it is applicable to any cocktail solution.

Reagent R:
To prepare 10 mL of Reagent R, use the following (by volume):

TFA:	9.0 mL (90%)
Thioanisole:	0.5 mL (5%)
EDT:	0.3 mL (3%)
Anisole:	0.2 mL (2%)

1. Add the scavengers to a graduated cylinder (10 or 25 mL) with an automatic pipettor.
2. Dilute with the TFA.
3. Mix vigorously for 30 seconds *via* shaking or nitrogen bubbling (recommended for sequences containing Cys or Met).

For those cocktails containing phenol, use phenol crystals, not liquified phenol. The crystals may either be weighed out (w/v) or be melted and the subsequent liquid delivered to the graduated cylinder (v/v).

The Cleavage/Deprotection Reaction

Reagents R and B are compatible with most sequences (see Figure 2 and Table 2), and are *strongly recommended* for sequences containing Trp, His, Met, Cys, Arg (Mtr/Pmc), Gln (Mbh/Tmob), or Asn(Mbh/Tmob), as well as for peptides constructed on a PAL or Rink Amide resin.

NOTE: It is strongly recommended that an initial trial cleavage using a small amount of the resin (50-100 mg) be performed to verify the proper conditions.

1. Wash the resin with DMF.
2. Wash the resin with DCM.
3. Add the appropriate cleavage cocktail to the peptidyl-resin.
4. Mix the solution periodically or bubble nitrogen through the solution.

If the peptide contains Trt-protected amino acids (Cys, His, Asn, Gln) the resin may turn deep yellow during the cleavage reaction. This is due to the trityl carbonium ion chromophore, which is produced when the protecting group is removed under acidic conditions.

If cleavage is performed on a PAL or Rink amide resin with a cocktail other than Reagent R or B, the resin may turn from pink to red, depending on the amount of scavengers in the cleavage cocktail. This should not effect the peptide quality.

Separating the Cleaved Peptide from the Support

To induce peptide precipitation, add the cleavage mixture dropwise to cold (-20ºC) ether. The non-peptide products remain in the ether solution.

Until the peptide is analyzed, it is recommended that neither the ether solution nor the resin be discarded, and that they be stored under nitrogen or argon at 0ºC.

NOTE: Some small (less than 6 amino acids) or hydrophobic peptides may not precipitate when added to the ether and must be isolated by alternative methods. In this event, dry down the ether, dissolve the peptide in 10% aqueous acetic acid, and extract with chloroform. Other possible causes for no precipitate and hints for peptide recovery are provided at the end of this section.

The separation of the peptide solution from the support can be performed using a variety of techniques provided that the addition of the solution to the ether is dropwise. The following procedure is the simplest:

1. Prepare a filter to retain the resin particles either by placing a small plug of glass wool into the neck of a 9” pasteur pipette or by placing a polypropylene frit into a disposable polypropylene syringe (see Figure 3).

2. Clamp the filter over a container containing at least a 10-fold excess of cold (-20 ºC) diethyl ether.

3. Add the cleavage mixture dropwise to the filter. Minimize the amount of support transferred to prevent clogging of the filter.

   A white precipitate should appear in the ether as the drops hit the surface. The greater the amount of peptide material per drop, the greater the probability of precipitation; therefore, maintain a cleavage volume as low as possible.

   If a precipitate has not formed following the addition of 10 drops of the cleavage solution to the cold ether, stop adding the cleavage solution and

observe the ether solution. If no visible precipitate appears after 10 minutes, use the chloroform extraction procedure given later in this section.

4. Rinse the support with a small volume of cleavage cocktail or TFA.

5. Once the peptide has been precipitated and the resin washed, chill the ether solution in the freezer or in a dry ice/acetone bath. For maximum recovery, incubate the ether-peptide mixture at 4ºC overnight.

6. Collect the precipitated material by filtration or centrifugation.

7. Wash the precipitate at least three times with cold ether to remove any residual scavengers.

8. Dissolve the peptide in glacial acetic acid, shell freeze and lyophilize.

NOTE: **The precipitate dried directly from the ether gives a TFA salt. TFA salts may affect the pH of peptide solutions or the viability of cells in culture. Lyophilization from acetic acid replaces the TFA salt with an acetate salt, which allows easier handling of the peptide and removes trace amounts of scavengers.**

NOTE: **Do not discard the support or the ether until the peptide is analyzed. Blanket it with nitrogen or argon and store in the cold.**

No Formation of Precipitate upon Ether Treatment

The following are several reasons that a precipitate may not form:

1. Peptides which are less than 6 amino acids and/or very hydrophobic may be difficult to precipitate. An alternative work-up is to evaporate the ether, dissolve the sample in 10% aqueous acetic acid, and extract the organic scavengers and byproducts with chloroform.

2. The peptide may be cleaved, but binds to the support. Additional washing of the resin with TFA may be needed to remove the peptide from the support.

3. The peptide may still be covalently attached to the support; cleavage from the support did not occur or was very inefficient due to the following:

    i. DMF was not replaced by a volatile solvent before the peptide was dried, contaminating the resin and interfering with acidolysis.

    ii. The support was washed with DCM but not thoroughly dried. A support containing DCM reduces the TFA concentration in the cleavage cocktail, resulting in a less efficient cleavage reaction and low yields. Wash the support again with DCM, thoroughly dry under vacuum, and repeat the cleavage reaction.

4. Prolonged acid treatment may break sensitive amide bonds, such as those between Asp and Pro, producing fragments of the peptide.

5. The peptide reattached to the resin-bound cations *via* electron-rich side-chains during the cleavage reaction. This has been reported in peptides containing *C*-terminal Trp, Tyr and Met, and may result in drastically decreased yields. *This is an undesirable side-reaction that may be minimized by choosing the appropriate scavengers in the presence of susceptible amino acids.* If Reagent R or B is used and a low yield is obtained, suspect incorrect preparation, or poor quality scavengers If this happens, you must repeat the synthesis.

6. Poor quality TFA or scavengers may result in any of the above problems. Recovery may not be possible, but try repeating the cleavage reaction after washing and drying the support.

Chloroform Extraction

If the peptide did not precipitate from the ether, it may be possible to extract the undesired by-products into chloroform using the following procedure:

1. Dry down the TFA-peptide mixture under high vacuum (equipped with a proper trap), or rotary evaporate. If the cleavage cocktail contained thiols, this mixture will be highly noxious.

2. Dissolve the residue in 10-20% aqueous acetic acid (1-5 mL).

3. Transfer into a separatory funnel and add a two-fold volume of chloroform.

4. Mix thoroughly by shaking (periodically relieve the pressure by removing the stopper) and allow the layers to separate.

NOTE: The separation of the layers may take several hours. Gradual swirling will accelerate the process. Separation of the layers is considered complete when the two layers are *entirely clear*.

5. Remove the organic layer (the bottom one) which contains the protecting groups and non-volatile by-products.

6. Repeat the extraction of the aqueous layer (steps 3-5) twice.

NOTE: The time for the two layers to separate decreases with each subsequent extraction.

7. Dry the aqueous layer under high vacuum.

8. Dissolve the residue in glacial acetic acid, shell-freeze, and lyophilize.

Analysis and Purification of the Crude Peptide

Hydrophobic Peptides

Amino acids are frequently grouped according to the polarity of the side-chains.

Non-Polar (Hydrophobic):
small: **Cys, Pro, Ala, Thr**
large: **Val, Ile, Leu, Met, Phe**

Intermediate polarity:
large: **Trp, Tyr, His**

Polar (Hydrophilic):
small: **Ser, Gly, Asp, Asn**
large: **Glu, Gln, Lys, Arg**

The hydrophobicity values of amino acid side-chains are listed in Table 3, and their corresponding plots are shown in Figure 5.
These values may be used as a guide when trying to determine if a given peptide is likely to precipitate from the ether after the cleavage reaction, as well as to approximate chromatographic behavior.

The first column of Table 3 contains the Kyte and Doolittle hydropathy scale, which is often used to predict hydrophobic regions in proteins and peptides (the "grease plot"). The numerical values represent a consensus from studies of partitioning the individual amino acids between organic solvent and water, and the positions of the amino acids within a folded protein as determined from crystal data. The most hydrophobic peptides possess a value of +4.5; the least hydrophobic -4.5.

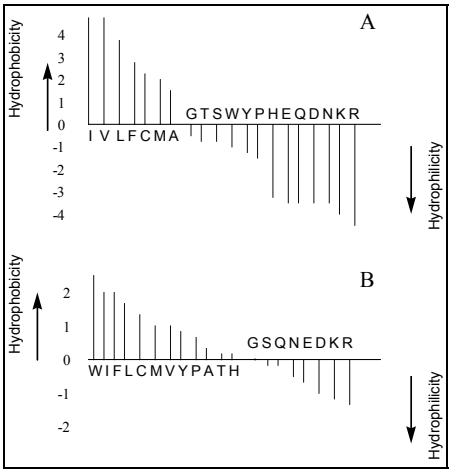
Kyte and Doolittle scales are based partially on crystal data, and reflect the inside-outside positions of *folded* proteins, presumably in their native state. These values may not always correlate to the denatured state of synthetic peptides. Partition data of amino acid side-chains between water and octanol, referred to as Fauchere and Pliska values, are listed in the second column of Table 3. The alpha amino and carboxyl groups of the individual amino acids were masked, and partition coefficients for the various side-chains were compared to Gly (side-chain = H). Although values for some amino acids vary between the two scales, the hydrophobic residues **Ile, Val, Leu, Phe, Cys**, and **Met,** and the hydrophilic residues **Arg, Lys, Asp, Glu, Asn, and Gln** are closely grouped. It should be noted that both the Kyte and Doolittle and Fauchere and Pliska scales are only estimates of the hydrophobic properties of a peptide. The actual solubility characteristics of a peptide may differ from their calculated values.

Table 3. Hydrophobicity Scales for Amino Acid Side-chains

Amino Acid	Kyte & Doolittle	Fauchere & Pliska
Ala (A)	1.8	0.31
Arg (R)	-4.5	-1.01
Asn (N)	-3.5	-0.60
Asp (D)	-3.5	-0.77
Cys (C)	2.5	1.54
Gln (Q)	-3.5	-0.22
Glu (E)	-3.5	-0.64
Gly (G)	-0.4	0.00
His (H)	-3.2	0.13
Ile (I)	4.5	1.80
Leu (L)	3.8	1.70
Lys (K)	-3.9	-0.99
Met (M)	1.9	1.23
Phe (F)	2.8	1.79
Pro (P)	-1.6	0.72
Ser (S)	-0.8	-0.04
Thr (T)	-0.7	0.26
Trp (W)	-0.9	2.25
Tyr (Y)	-1.3	0.96
Val (V)	4.2	1.22

Souces:
J. Kyte and R.F. Doolittle 1982. *J. Mol. Bio*. **157**:105. Consensus scale from partition experiments and amino acid distributions in proteins from crystal data. Scale: most hydrophobic: +4.5 to least hydrophobic: -4.5.

J.L. Fauchere and V. Pliska 1983. *Eur. J. Med. Chem*. **10**:369. Derived from partition measurements of amino acids between water and octanol, relative to Gly. Scale: most hydrophobic: +2.25 to least hydrophobic: -1.01.



A: Kyte and Doolittle values, based on consensus of partition and crystal data.
B: Fauchere and Pliska scale, reflecting partition data only

Figure 5. Hydrophobicity Plots of Amino Acid Side-chains

The Solubility Problem

Many crude synthetic peptides, especially those that were precipitated and dried several times, may be notoriously insoluble in standard HPLC solvents, due to the following reasons:

1. A high content of hydrophobic residues; these peptides tend to "oil-out" and form compact clusters to minimize contact with water and maximize hydrophobic interactions.

2. Non-specific aggregation.

3. Disulfide formation in Cys-containing peptides.

4. Secondary structure formation, especially in longer sequences.

5. Specific interactions of reactive side-chains, including various types of hydrogen-bonding, salt-bridges, etc.

The following procedures may be used to solubilize peptides (disulfides are covalent interactions and must be reduced chemically):

1. For highly hydrophobic peptides with aromatic or hydrocarbon side-chains (Val, Leu, Ile, Met, Phe, Tyr, Ala), add DMF (up to 50%); or dissolve the peptide in a minimal amount of DMF and then dilute with HPLC buffer. Upon reversed phase chromatography, DMF will elute with the buffer front. The DMF peak may be large, but most peptides are retained longer, and the DMF does not interfere with the analysis. If the peptide is not retained on the column upon injection in DMF, the gradient must be adjusted to decrease the initial concentration of buffer B.

2. If there are many *basic* amino acids, dissolve the peptide in aqueous acetic acid (1-10%).

3. If *acidic* amino acids are present, dissolve the peptide in a volatile basic buffer (up to pH 8) such as *N*-ethyl morpholine acetate or bicarbonate. (If the peptide is dissolved in a basic solution, the pH may have to be adjusted before chromatography, unless a small amount is injected onto the column.)

4. Longer sequences may dissolve in salts. High concentrations of chaotropic salts, such as guanidine HCl or urea, assist in dissolving the peptide by breaking up the secondary structure.

5. Propanol may be used to help dissolve medium-sized peptides (the amount of propanol injected should be minimized to reduce the effect on retention time.)

6. Small amounts of TFA may help to dissolve aggregates. This technique is useful, but may damage C18 columns during reverse-phase HPLC.

7. Detergents (such as SDS) may be added. Detergents that contain polyethers may form peroxides and harm some peptides. Peroxide-free detergents are available from Pierce.

Hints on Storage and Handling of Synthetic Peptides

Both natural and synthetic peptides may irreversibly attach to glass and plastics (e.g., polystyrene and nitrocellulose). Silylation of glassware prior to use prevents peptides from binding to the surface of the glass. Pure silylating reagents must be used to avoid the introduction of contaminants. Polypropylene containers appear to have less of a tendency to adsorb peptides and are, therefore, suggested.

It is recommended to leave the peptide attached to the solid support for storage and to cleave it from the support in small quantities. Once the side-chain protecting groups are removed from the peptide, the amino acids may react inter- and intramolecularly, as well as with other contaminants. Peptides and proteins may retain a significant amount of solvent even after lyophilization; care should be taken to replace the potentially harmful solvents with inert ones. For example, it the peptide was lyophilized from acid, residual amounts may cause unwanted reactions, including slow hydrolysis of acid-labile peptide bonds such as Asp-Pro, deamidation of Gln and Asn, and cyclization of *N*-terminal Gln to pGlu. Thus, suspend/redissolve the peptide in water, re-dry, and repeat until all the residual acid is removed. This procedure should be performed if the peptide is to be stored for any length of time without purification.

HPLC Analysis and Purification

HPLC is one of the most powerful and rapid tools to analyze and purify peptides. A detailed description of HPLC is beyond the scope of this technical bulletin, and many books and articles are available on this subject. The following guidelines are suggested:

1. If the elution pattern of the peptide is unknown, use a C-18 reversed phase column for small-to-medium-sized peptides (up to 20-30 amino acids) that are at least moderately hydrophilic. Begin with a low percentage mobile organic phase (%B, see below), use a shallow gradient and moderate flow rates. For more hydrophobic peptides, a weak, polymeric reversed phase column may perform better.

   Use the following protocol for a standard analytical column (4 mm x 20 cm):

Buffer A:	0.1% TFA in water
Buffer B:	0.1% TFA in ACN
Flowrate:	1-1.5 mL/min
Gradient:	0-90% B in 90 min

   When the approximate elution time is known, considerable time can be saved by only running the relevant portion of the gradient. For example, if the peptide elutes at 25% B, start the run at 10% B and stop the gradient at 40% B.

2. For "medium-sized" peptides (20-40 amino acids), we recommend a C-8, C-4 or a polymeric reversed phase column. These peptides can be expected to elute at a higher percentage of ACN, and it is usually safe to start the gradient at 10-20% B.

3. Long peptides, or those having large amounts of similarly charged groups, may best be separated on either polymeric reversed phase or aqueous ion exchange columns.

4. Some protecting groups, such a Mtr or Trt, absorb very strongly in the UV: even a small percentage of Mtr or Trt still remaining on the peptide will give disproportionately large peaks. Peptides which have Mtr or Trt attached will elute later than completely deprotected peptides.

5. Peptides containing Tyr, Phe, or Trp can be monitored at 240-260 nm, due to the strong absorbance of the aromatic ring; otherwise, monitor at wavelengths closer to the absorbance of the peptide (amide) bond (210-214 nm).

6. Running reagent and gradient blanks is strongly recommended, especially if the chromatogram seems to show constant contaminant peaks.

7. If DMF is used to dissolve the peptide, expect a large peak at the beginning of the gradient. The size of the peak depends on the amount of DMF injected. If the peptide is very small and elutes early, initial % B may be lowered to separate the peptide from the DMF peak.

Reduction of Disulfides and Methionine Sulfoxide

Cys- and **Met**-containing peptides may air-oxidize. For Cys-containing peptides, this is especially prevalent at a near-neutral pH, resulting in formation of disulfide bridges. If only one Cys is present, oligomerization may occur, giving extra peaks *via* HPLC analysis. If more than one Cys is present in the peptide, all possible conformers can be produced in varying ratios producing a number of peaks, complicating the interpretation of the chromatogram. Therefore, it is common practice to reduce any disulfides or sulfoxides before chromtography.

Method 1: Reducing Disulfides and Sulfoxides using DTT.

1. Prepare 0.05 M *N*-ethylmorpholine acetate or bicarbonate, pH 8.3. Bubble nitrogen through the buffer for about 30 seconds.

2. To 100 µl of the buffer, add 100-300 µg of the peptide (peptide concentration 1-3 mg/mL). Blanket the solution with nitrogen.

3. Add DTT to give a final concentration of 0.05 M.

4. Blanket with nitrogen and react at room temperature for 4 hours, or at 55°C for 2 hours.

5. Acidify with dilute TFA to lower the pH to approximately 3.0, and analyze 10 µl by HPLC.

Note: *N-ethylmorpholine and DTT should be of the highest grade available (Aldrich reagents are recommended).*
N-ethylmorpholine should be stored under argon or nitrogen, refrigerated, and protected from light. Do not use if either reagent is discolored.

Method 2: Reducing Methionine Sulfoxide using *N*-methylmercaptoacetamide (MMA)

1. Dissolve the peptide to a concentration of 1-5 mg/mL in 10% HOAc.
2. Add MMA to give a 10% solution.

3. Blanket with nitrogen and react at 37ºC for 24-36 hours.

4. Analyze by HPLC.

Deprotecting Cys(Acm) and Cys(*t*Bu)

Although the Trt protecting group is readily removed upon treatment with a high concentration of TFA, the Acm and *t*Bu groups are stable to these conditions and must be removed using alternative methods.

Method 1: Mercury (II) Acetate

1. Dissolve 0.1 mmol of peptide in 10 mL HPLC-grade, deoxygenated water. If solubility is a problem, add a minimum amount of DMF. Adjust the pH to 4.0 with dilute acetic acid or ammonia. Blanket with nitrogen or argon.

2. Dissolve 100 mg mercuric acetate for each Acm or *t*Bu group in 10 mL HPLC-grade deoxygenated water. Blanket with nitrogen or argon.

3. Mix solutions from steps 1 and 2, blanket with nitrogen or argon, and react at room temperature for 90 minutes.

4. Add 0.5 mL of β–mercaptoethanol. Heat at 45-50°C for 60 minutes. A precipitate forms as the reaction nears completion. If no precipitate is visible, incubate for another hour. Deprotection of Cys(*t*Bu) usually takes longer than Cys(Acm).

5. Vacuum filter the mixture through a medium-porosity sintered glass funnel containing Celite® rinsed with deoxygenated water.

6. Lyophilize or rotary-evaporate the filtrate.

7. Dissolve or suspend the residue in HPLC-grade water, and repeat the drydown to remove traces of β–mercaptoethanol.

8. Analyze by HPLC. If two or more major peaks elute, disulfides have formed and the peptide must be reduced as above.

Method 2: Thallium (III) Trifluoroacetate

NOTE: Acid sensitive amino acids, such as Trp and Met should be protected (Met as its sulfoxide) if using this method.

1. Dissolve the peptide in TFA.
2. Add 1-2 equivalents of thallium (III) trifluoroacetate and 2 equivalents of anisole.
3. Place the reaction mixture in a ice bath (0ºC) and allow to react for 1-2 hours.
4. Evaporate the TFA, resuspend the mixture in 10% aqueous HOAc, and extract into chloroform.
5. Analyze the peptide by HPLC.

NOTE: Thallium is a toxic metal and should only be used on a small scale.

Thallium oxidation may also be performed on the resin prior to cleavage, allowing disulfide bond formation to be performed on solid-phase:

1. Swell the resin in DMF.
2. Dissolve 1-2 equivalents of thallium (III) trifluoroacetate in DMF to a concentration of 6 mM.
3. Add this to the resin and allow to react for 1-2 hours at 0ºC.
4. Wash the resin three times with DMF.
5. Proceed with the disulfide bond formation, or wash the resin three times with DCM and dry *in vacuo*.

Determination of the Correct Product by Mass Spectroscopy

The most popular technique for the mass spectroscopy analysis of synthetic peptides is matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF). In MALDI-TOF mass spectroscopy, the peptide is surrounded by crystals of a low molecular weight organic molecule, known as the matrix. When the matrix is bombarded by the laser, energy is absorbed and transferred to the peptide. The peptide becomes ionized and travels down the flight tube when a voltage is applied. Peptides of different masses are separated since they travel down the tube at different velocities. Depending on the matrix, the concentration of the peptide, and the power of the laser, peptide masses may be determined to an accuracy of $\leq$0.05% (a few Daltons for most peptides). While this is a very powerful tool, it should be noted that this technique should be used in conjunction with other tools, such as HPLC, for it is difficult to quantify the impurities. It is also known that if the laser strength is too high, sensitive peptide bonds, such as Asp-Pro, can be broken and the peptide fragmented.

It is recommended that mass spectra be obtained for both the crude and the purified peptide.

We recommend the following guidelines for the MALDI-TOF analysis of peptides:

Peptide: Dissolve to 1 pmol/µLl in 0.1% TFA (aqueous). If the peptide is hydrophobic, use a volatile organic solvent such as ACN or methanol.

Matrix: Use a solution of α-cyano-4-hydroxycinnamic acid in water/ACN (1:1) containing 0.1% TFA (5 mg/mL).

Mix 1 µL of peptide with 1 µL of matrix and allow to dry slowly for proper crystal formation.

It is recommended that a mass standard be used to calibrate the mass spectrometer. The standard may be performed on another position of the sample plate, mixed with the peptide sample (an internal standard), or chosen from a list of standards from the instrument. Internal standards are the most accurate, but can interfere with the spectrum of the peptide if not chosen properly.

Synthetic peptides have a tendency to form salt adducts on the MALDI-TOF plate. The most common salt adducts are sodium (Na+ = 22 Da) and potassium (K+ = 35 Da). Multiple salt adducts, such as +Na+K (+57 Da) may also occur. These do not affect the quality of the peptide, but may affect the mass accuracy as the length of the peptide increases. Desalting the peptide, by dialysis or by a size-exclusion membrane, will assist in the anaylsis.

Table 4: Masses of Amino Acid Residues

Amino Acid	Residue Composition	Average Mass
Ala (A)	C_3H_5NO	71.0788
Arg (R)	$C_6H_{12}N_4O$	156.1876
Asn (N)	$C_4H_6N_2O_2$	114.1039
Asp (D)	$C_4H_5NO_3$	115.0886
Cys (C)	C_3H_5NOS	103.1448
Glu (E)	$C_5H_7NO_3$	129.1155
Gln (Q)	$C_5H_8N_2O_2$	128.1308
Gly (G)	C_2H_3NO	57.0520
His (H)	$C_6H_7N_3O$	137.1412
Ile (I)	$C_6H_{11}NO$	113.1595
Leu (L)	$C_6H_{11}NO$	113.1595
Lys (K)	$C_6H_{12}N_2O$	128.1742
Met (M)	C_5H_9NOS	131.1986
Phe (F)	C_9H_9NO	147.1766
Pro (P)	C_5H_7NO	97.1167
Ser (S)	$C_3H_5NO_2$	87.0782
Thr (T)	$C_4H_7NO_2$	101.1051
Trp (W)	$C_{11}H_{10}N_2O$	186.2133
Tyr (Y)	$C_9H_9NO_2$	163.1760
Val (V)	C_5H_9NO	99.1326

Table 5: Masses of Common Side-Chain Groups

Protecting Group	Mass
Acetyl	42
Acm	71
Allyl	40
Aloc	84
Boc	100
Fmoc	222
Mtr	212
*t*Bu	56
Tetramethylguanidinium*	101
Tmob	180
Trt	242
Pbf	252
Pmc	266

*See Technical note entitled "**Guanidinium Formation during *in situ* Activation of Amino Acids by Uronium-Salts**." PerSeptive Biosystems (1997).

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