High-Throughput Kit 96x 10 µmol

Manual

Catalog number: 180484100

(reductively cleavable PEC-Linker RC+)

V1.0 – DRAFT 31.03.2020

Introduction

Belyntic's Peptide Easy Clean (PEC) technology breaks barriers in peptide purification.

Key for a universal application is our novel purification linker, the PEC-Linker.

The target peptides are chemically retained on our activated filter material via robust oxime ligation. Our innovative chemistry allows traceless release of the purified peptides.

This manual provides step-by-step instructions for the PEC purification using our kits, alongside with helpful information and notes to assure a successful use.

Please watch our product video on YouTube (https://youtu.be/gihpfHr1bfI) for step-by-step visual guidance. Please also refer to the FAQ on our website belyntic.com.

Now, let's get ready to re-think peptide purification!

Contents

Product Description

Kit Content

quantity	label / description	amount
1	**PEC filter plate with activated filter material** / 50% suspension of Agarose100 provided in 96-well filter plate 400 µL/well, closed with top- and bottom-sealing-mat	38.4 mL
1	**PEC-Linker** / RC+	3.2 g
1	**Buffer salt** / mixture of citric acid-sodium carbonate	5.6 g
1	**Blocking agent** / L-cysteine	1.0 g
1	**Reducing agent** / Dithiothreitol	5.0 g
1	**TFA collection plate** / deep-well plate to collect crude peptides, perform ether precipitation and centrifugation closed with top-sealing-mat	
1	**Peptide collection plate** / perforated deep-well plate to collect purified peptides and perform freeze-drying in the plate	

Safety Information

Please read the Safety Data Sheets, available at belyntic.com before use.

Storage Conditions

Upon receipt, store the kit components in the fridge at 2-8°C. The agarose beads should not be frozen or dried under reduced pressure. Buffers (cf. page 8) can be stored for 2 weeks in the fridge at 2-8°C.

Product Use

For Research Use Only. The use in or for clinical or diagnostic products, applications, or services is excluded.

Preparations

Purification Considerations

- PEC-Linker RC+ will be installed at the *N*-terminus of the peptide
- reductive conditions will be applied in the purification process
- azide and disulfide groups in the peptide might be reduced

Precautions During Peptide Synthesis

- **acetylation after each amino acid coupling (capping) step is key to ensure a successful PEC purification**

  efficient conditions for blocking unreacted amino groups are 2 M acetic anhydride (20 vol%) with either 2 M pyridine (18 vol%) or 2 M 2,6-lutidine (21 vol%) in DMF for 5 min[1]

- **special attention must be paid to possible side reactions during SPPS**
  - minimize by-products within the fullength chain (e.g. aspartimide)
  - minimize by-products during TFA cleavage[1], ideally use Fmoc-Lys(Trt)-OH to prevent *t*Bu on Lysine[2] and Fmoc-Cys(StBu)-OH to prevent alkylation on -SH during TFA cleavage
  - use Ramage- or Sieber-amide resins for C-terminal peptide amides and chlorotrityl resin for C-terminal peptide acids (avoid Wang-linker)
  - N-terminal glutamine bearing sequences may show 10-20% of pyroglutamate after purification, contact us for support if you want to purify N-terminal glutamine peptides
- **avoid aldehydes and/or ketones in ether and solvents**

  the free oxy-amine on PEC-linker will readily react with traces and contaminations of ketones and/or aldehydes that will deactivate it

[1] O. Reimann, O. Seitz, D. Sarma, R. Zitterbart, *J. Pept. Sci.* **2019**, *25*, e3136.
[2] J. Pawlas, U. Törnvall, *Proceedings of the 33rd European Peptide Symposium* **2014**, 108-109.

Other Chemicals & Consumables

Reagents

- ethyl-2-cyano-2-(hydroxyimino) acetate (Oxyma) or 1-hydroxybenzotriazol (HOBt) as coupling reagent
- scavengers for TFA cleavage cocktail, e.g. TIS, EDT, thioanisol, phenol
- guanidinium chloride (GdmCl) - in case of technical grade, filter the solution prior to use
- sodium chloride (NaCl)

Acids / Bases

- *N,N*-diisopropylethylamine (DIEA)
- trifluoroacetic acid (TFA)
- sodium hydrogen carbonate ($NaHCO_3$)

Solvents

- *N,N*-dimethylformamide (DMF), ≥ 99.5% (peptide synthesis grade)
- dimethyl sulfoxide (DMSO), ≥ 99.5% (synthesis grade)
- dichloromethylene (CH_2Cl_2), ≥ 99.5% (synthesis grade)
- acetonitrile (MeCN), ≥ 99.5% (synthesis grade)
- deionized water
- ethanol (EtOH), denatured
- suitable ether for peptide precipitation (Et_2O and n-heptane 3:1), ≥ 99.5% (synthesis grade, acetone-free)
- 1,1,1,3,3,3-hexafluoro-propan-2-ol (HFIP), if using synthesis grade (≤99%) please reflux 1 h with 1 w% L-cysteine to remove trifluoroacetone, distill then to get pure HFIP

Preparation of Buffers

- **Buffer#1**

  dissolve 5.6 g of the supplied buffer salt slowly in 200 mL water (final pH = 4.5, caution: strong CO_2 evolution); To prepare more Buffer#1 see below[3]

- **Buffer#2 (7 M GdmCl in Buffer#1)**

  dissolve 13.4 g of GdmCl in 20 mL Buffer#1

- **Wash A: DMSO with 0.9 M GdmCl**

  dissolve 13 g of GdmCl in 150 mL of DMSO

- **Wash B: EtOH/H_2O (7:3) with 0.1 M NaCl**

  dissolve 1168 mg of NaCl in 60 mL water **first**, then add 140 mL EtOH

- **$NaHCO_3$ pH 8:**

  dissolve 2.5 g of $NaHCO_3$ in 50 mL water, to give a pH of 8

Equipment & Processing

please don't hesitate to contact us, if in doubt about suitable equipment

- shaking device for deep-well plates, ideally with 800 rpm
- centrifuge with rotor for deep-well plates
- Belyntic's Acrylic Vacuum Manifold, catalog number: 180484150. Other manifolds cannot be recommended since they may produce cross-contaminations
- 30-300 µL multichannel pipette (8- or 12-channel) or dispenser with 8- or 12-channel manifold exit

[3]Buffer#1: dissolve 19.2 g Citric Acid (anhydrous, or 21.0 g of monohydrate) together with 8.75 g Na_2CO_3 (anhydrous) slowly in 1 L water.

Step 1: PEC-Linker Coupling

Note: The following procedure describes manual coupling of PEC-Linker RC+ after peptide assembly in a synthetic filter plate. The PEC-Linker can also be coupled to the peptides on a synthesizer; higher volumes might be necessary due to synthesizer dead volume. The base (DIEA or *N*-methylmorpholine) must be added to the pre-mixed solution of the PEC-Linker with Oxyma (or HOBt) before coupling.

- ☐ swell synthetic resin in DMF for 15 min and remove DMF before PEC-Linker addition
- ☐ weigh in the amount of PEC-Linker and Oxyma or HOBt (Table 1) and combine in a suitable vessel

Table 1: PEC-Linker coupling for 96x 10 µmol peptide

PEC-Linker RC+ (>4 eq.)	3200 mg	4255 µmol
Oxyma/ HOBt (>6 eq.)	907/862 mg	6383 µmol
add DMF to have ~13.8 mL	10630 µL	0.3 M PEC-Linker
DIEA (>6 eq.)	1116 µL	6383 µmol

- ☐ add DMF to dissolve PEC-Linker and Oxyma or HOBt (Table 1)
- ☐ then add DIEA (Table 1, on a synthesizer DIEA or *N*-methylmorpholine should be added directly in a pre-activation vessel before coupling)
- ☐ quickly add 150 µL of the yellow solution to each well

Note: Other common solvents can be used (e.g. CH_2Cl_2, DMSO or NMP)).

- ☐ couple for ≥ 3 h (shaking is not necessary; overnight is possible)
- ☐ wash synthesis resin with 3x ≥ 200 µL/well DMF and 3x ≥ 200 µL/well CH_2Cl_2

Note: You may pause here and store the linker-modified peptide on synthesis resin below 8°C.

Step 2: TFA Cleavage

- ☐ choose and prepare proper cleavage cocktail for your peptides (500 µL/well = 48 mL)

> *By-products caused by re-addition of protecting groups cannot be removed with PEC, please use proper scavengers: Reagent Bel TFA/H_2O/PhSMe/EDT/TIS (83:5:5:5:2) or a non-odorous cocktail of TFA/H_2O/DTT/TIS (88:4:6:2) are recommended.*

- ☐ remove the sealing-mat, put the TFA collection plate in a vacuum block from your synthesizer and place the synthesis plate on the manifold
- ☐ in a fume hood, add TFA-cleavage cocktail to each well of the synthesis plate and carry out cleavage for 2 h (no shaking); use reduced pressure at the end to collect cleavage solution
- ☐ take the TFA collection plate that is containing the peptides in TFA cocktail out of the manifold and precipitate the peptides by addition of 1.2 mL **cold** (≤ -20 °C) Et_2O/n-Heptane (3:1) to each well

> *Aldehyde- and ketone-free ether is crucial during this step, in doubt use THF/n-Heptane (1:1) as an alternative.*

- ☐ close the plate with the sealing-mat (**make sure that it is closed properly**), shake for 30 seconds and put it in the freezer (≤ -20 °C) for ≥ 10 min
- ☐ centrifuge the plate for ≥ 4 min at ≥ 4000 rpm and discard the ether supernatants by carefully turning the plate upside down. The precipitates will stay in the TFA collection plate.
- ☐ put the TFA collection plate back in the vacuum manifold
- ☐ place the synthesis plate on the manifold and elute each well with 2x 200 µL TFA/H_2O (95:5), use reduced pressure to decrease elution time
- ☐ take the TFA collection plate out of the manifold

If you have methionine containing peptides, please add 7 µL TMSBr and 8 µL EDT to each well containing such peptides and shake the plate gently (300 rpm) for 15 min.

- ☐ precipitate the peptides again by addition of 1.2 mL **cold** (≤ -20 °C) Et_2O/n-Heptane (3:1) to each well
- ☐ close the plate with the sealing-mat (**make sure that it is closed properly**), shake for 30 seconds and put it in the freezer (≤ -20 °C) for ≥ 10 min
- ☐ centrifuge the plate and discard the ether supernatant as described above
- ☐ let the ether precipitates dry by putting the plate uncovered in a fume hood for ≥ 30 min
- ☐ cover the TFA collection plate with a paper tissue fixed with rubber bands and dry the precipitates carefully in an exicator with a membrane pump (**after** drying in a fume hood)
- ☐ clean the sealing-mat with water

Note: You may pause here and store the crude linker-modified peptides as a dried solid below 8 °C.

If crude analysis is performed, check delta mass list in Table 2 (cf. page 16). On some analytical LC systems, the linker-modified peptide cannot be detected or shows lower purities. Dilute your sample with a 50 mg/mL solution of methoxyamine hydrochloride (CAS: 593-56-6), that will prevent analytical artifacts due to aldehydes or ketones. However, if you still don't see linker-modified peptide, please continue the purification process described in this manual as the desired peptide will be visible again after PEC-Linker cleavage.

Step 3: Dissolution & Bead Conditioning

- ☐ first add 450 µL/well DMSO and close the plate with the sealing-mat

Note: For very hydrophobic peptides HFIP can be used (make sure it is free of trifluoroacetone, solvent section page 7). Other solvents, such as DMF or MeCN up to 90 vol% in Buffer#1, may be used. Ketone- or aldehyde-containing solvents are not tolerated.

- ☐ shake heavily for ≥ 1 h (overnight possible)

Please make sure to use a shaker that is suitable for deep-well plates and use a shaking speed of 800 rpm.

Before removing sealing-mats, centrifuge the plates for 1 min at ≥ 4000 rpm to prevent cross-contaminations

If you have methionine containing peptides and you want to use DMSO, please use DMSO purged with inert gas (N_2 or Ar) and try not to exceed dissolution time of 1 h and immobilization time of 4 h.

- ☐ if all peptides are dissolved (inspection with flashlight), please add 50 µL/well Buffer#2; **don't add Buffer#2 when using HFIP as solvent**
- ☐ take the PEC filter plate that is prefilled with agarose suspension provided in your High-Throughput Kit, remove top- and bottom-sealing-mat, place it in the vacuum manifold and remove the supernatants
- ☐ wash 3x 300 µL/well with water and 3x 300 µL/well with Buffer#1 (2.4 mL/8-wells, 3.6 mL/12-wells, 90 mL in total each)
- ☐ wash once with 300 µL/well DMSO **or again with 300 µL/well water when using HFIP as peptide solvent**
- ☐ close the PEC filter plate with the bottom-sealing-mat and add 200 µL/well of the solvent used for peptide dissolution

Step 4: Immobilization (Catch)

- ☐ add the dissolved peptides to their respective wells
- ☐ apply the top-sealing-mat and immobilize peptides by shaking for ≥ 4 h (overnight possible, when not dealing with methionine containing sequences)
- ☐ remove the sealing-mats from the PEC filter plate and drain the immobilization supernatants by usage of the vacuum manifold
- ☐ wash 3x 300 µL/well with DMSO (2.4 mL/8-wells, 3.6 mL/12-wells)

Note: You may monitor immobilization efficiency by LC or MS analysis of the supernatant of single wells prior to addition of Blocking Buffer.

- ☐ prepare Blocking Buffer (2 w% solution of L-cysteine): dissolve all L-cysteine (1 g) provided in your High-Throughput Kit in 50 mL Buffer#1
- ☐ close the PEC filter plate with the bottom-sealing-mat and add 500 µL/well of Blocking Buffer
- ☐ apply top-sealing-mat and shake for 30 min to block unreacted aldehyde groups and to liberate imines
- ☐ remove the sealing-mats, place the PEC filter plate on the vacuum manifold and remove the Blocking Buffer

Step 5: Washing

Each wash is 3x 300 µL/well (2.4 mL/8-wells, 3.6 mL/12-wells, 90 ml in total)

- ☐ **wash A**: DMSO with 0.9 M GdmCl
- ☐ **wash B**: EtOH/H_2O (7:3) with 0.1 M NaCl

Note: You may pause here by storing the immobilized peptides after one additional H_2O wash in the fridge at 2-8 °C.

Step 6: Cleavage & Work-up (Release)

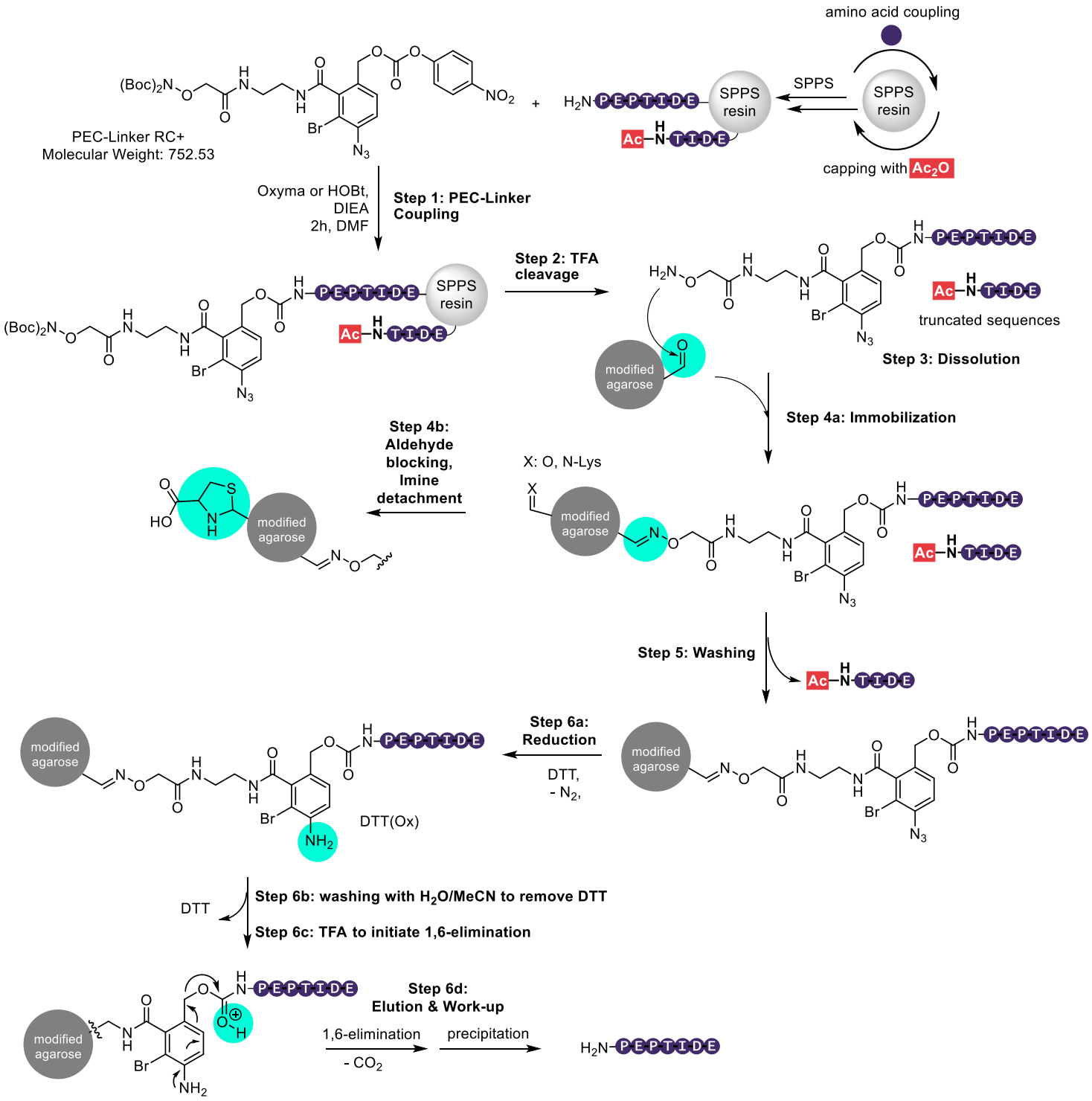
- ☐ prepare Reducing Agent: dissolve 5.0 g DTT in 50 mL 5 w% aq. $NaHCO_3$ (0.6 M; pH 8)
- ☐ close the PEC filter plate with the bottom-sealing-mat and add 500 µL/well MeCN
- ☐ add 500 µL/well of the Reducing Agent, apply top-sealing-mat and shake for 30 min, shake for 90 min when Cys(StBu) has been used
- ☐ wash 3x 300 µL/well with water, 3x 300 µL/well with MeCN and discard to remove excess DTT

> *From this point on, please collect all filtrates in the perforated peptide collection plate, since they hold your product.*

- ☐ put the peptide collection plate in the vacuum manifold and place the PEC filter plate on the manifold
- ☐ add 200 µL/well H_2O/MeCN/TFA (90:8:2)
- ☐ let the plate stand in the vacuum manifold for ≥ 4 h (overnight possible)
- ☐ collect the peptides by reduced pressure and further elute peptides:
  1st: 600 µL/well H_2O/MeCN (7:3) + 0.1% TFA
  2nd: 500 µL/well H_2O/MeCN (3:7) + 0.1% TFA
  (use reduced pressure to decrease elution times)
- ☐ take the peptide collection plate out of the vacuum manifold and take your analytical samples
- ☐ cover the plate with a paper tissue fixed with rubber bands, freeze in liquid nitrogen **for ≥ 30 min** and lyophilize the peptides

Appendix

Reaction overview



Belyntic's PEC Delta Masses

Table 2: Relevant delta masses during the purification process.

+412.03/414.03 (MH^{2+}: +206.52, MH^{3+}: +137.67)	PEC-Linker RC+ on peptide
+ 40.05 (MH^{2+}: +20.03, MH^{3+}: +13.33)	acetone oxime of peptide-linker conjugate, +453.08 Da with PEC-Linker RC+
+ 26.03 (MH^{2+}: +13.02, MH^{3+}: +8.68)	acetaldehyde oxime of peptide-linker conjugate, +439.06 Da with PEC-Linker RC+
+ 15.99 (MH^{2+}: +8.00, MH^{3+}: +5.33)	oxidation of methionine residues
+ 12.01 (MH^{2+}: +6.01, MH^{3+}: +4.00)	formaldehyde oxime of peptide-linker conjugate, +425.04 Da with PEC-Linker RC+
+56.07 (MH^{2+}: +28.04, MH^{3+}: +18.69)	reattached *t*Bu; thiols (e.g. EDT) should be used in the TFA cleavage cocktail (make sure they are not oxidized)
+106.05 (MH^{2+}: +53.03, MH^{3+}: +35.35)	para-hydroxybenzyl cation, generated during TFA cleavage; use RAM-linker or add EDT and 1,3,-Dimethoxybenzene during cleavage
+252.06 (MH^{2+}: +126.03, MH^{3+}: +84.02)	non-cleaved or reattached Pbf; treat purified peptide with Reagent K or another thioanisol-containing TFA cocktail for 3-4 h.

Abbreviations

AcOH	acetic acid
Buffer#1	immobilization buffer
Buffer#2	7 M guanidinium chloride in immobilization buffer
CAS	Chemical Abstracts Service
DIEA	*N*,*N*-Diisopropylethylamine
DMF	*N*,*N*-Dimethylformamide
DTE	dithioerythritol (Cleland's reagent)
DTT	dithiothreitol (Cleland's reagent)
EDT	ethanedithiol
Et_2O	diethyl ether
EtOH	ethanol
GdmCl	guanidinium chloride
HFIP	1,1,1,3,3,3-hexafluoro-propan-2-ol
HOBt	1-hydroxybenzotriazole
LC	liquid chromatography
MeCN	acetonitrile
MS	mass spectrometry
MTBE	t-butyl methyl ether
NMP	N-methyl pyrrolidone
Oxyma	ethyl-2-cyano-2-(hydroxyimino) acetate
Pbf	pentamethyldihydrobenzofuran-5-sulfonyl
PEC	Peptide Easy Clean
PhSMe	Thioanisol
RAM	modified Rink-Amide
SDS	safety data sheet
SPPS	solid phase peptide synthesis
tBu	t-butyl
TFA	trifluoro acetic acid
THF	tetrahydrofuran
TIS	triisopropylsilane
TMSBr	trimethylsilyl bromide

Technical Support

Please watch our product video on YouTube for visual step-by-step guidance (https://youtu.be/gihpfHr1bfI). Please also refer to the FAQ on our website http://belyntic.com.

For further technical support, submit your questions directly via mail to **support@belyntic.com** or call +49 30 8104-1113.

Safety Data Sheets (SDS)

Safety Data Sheets (SDS) are available on our website http://belyntic.com.

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Your Notes

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