Chapter 13

Screening One-Bead-One-Compound Peptide Libraries for Optimal Kinase Substrates

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Abstract

Protein kinases phosphorylate specific serine, threonine, and/or tyrosine residues in their target proteins, resulting in functional changes of the target proteins such as enzymatic activity, cellular location, or association with other proteins. For many kinases, their in vivo substrate specificity is at least partially defined by the amino acid sequence surrounding the phosphorylatable residue (or sequence specificity). We report here a robust, high-throughput method for profiling the sequence specificity of protein kinases. Up to 10^7 different peptides are rapidly synthesized on PEGA beads in the one-bead-one-compound format and subjected to kinase reaction in the presence of [γ-S]ATP. Positive beads displaying the optimal kinase substrates are identified by covalently labeling the thiophosphorylated peptides with a fluorescent dye via a disulfide exchange reaction. Finally, the most active hit(s) is identified by the partial Edman degradation-mass spectrometry (PED-MS) method. The ability of this method to provide individual sequences of the preferred substrates permits the identification of sequence contextual effects and non-permissive residues. This method is applicable to protein serine, threonine, and tyrosine kinases.

Key words Protein kinase, Substrate specificity, Sequence specificity, Peptide library, One-bead-one-compound library

1 Introduction

Approximately 30 % of human proteins are phosphorylated by 518 putative protein kinases on >100,000 serine, threonine, and tyrosine residues [1, 2]. In general, the phosphorylation events are highly specific with respect to both the kinase and the substrate protein. It is now established that protein kinases utilize a combination of several mechanisms to achieve exquisite substrate specificity in vivo, including temporal expression of the kinase and/or substrate, localization of the kinase and/or substrate to subcellular structures, protein-protein interaction through the use of recruiting domains/surfaces or scaffolding proteins, and interactions between the kinase active site and the linear sequence motif surrounding the phosphorylatable residue (or the intrinsic

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sequence specificity of the kinase domain) [3, 4]. For some protein kinases (e.g., protein kinase A), the intrinsic sequence specificity of the kinase active site is the major determinant of their in vivo substrate specificity, whereas other kinases show little sequence selectivity [3, 4].

One of the first tasks toward understanding the biological functions of a protein kinase is to identify its specific protein substrates. For kinases that recognize specific sequence motifs, a productive approach to substrate identification involves first defining the sequence specificity of the kinase domain using peptide substrates followed by searching the proteome against the consensus motif(s) [5–7]. Optimal peptide substrates also provide robust in vitro assays for the kinase as well as useful guide in designing specific inhibitors against the kinase. Several methods have previously been reported to determine the sequence specificity of protein kinases (reviewed in ref. [8]). However, the previous methods generally suffer from one or more drawbacks, e.g., inability to provide individual sequences and therefore detect any sequence contextual effect. Here we report a simple, robust, and general method for on-bead screening of one-bead-one-compound (OBOC) peptide libraries against a protein serine, threonine, or tyrosine kinase to determine its optimal peptide substrates.

For a protein kinase of unknown specificity, we recommend that one starts with a generic kinase substrate library in the form of X_5ZX_5NNBBRM-resin (library I), where B is β-alanine, Z is Ser, Thr, or Tyr, and X is any of the 19 proteinogenic amino acids except for methionine [replaced by L-norleucine (Nle or M)] and cysteine. The inclusion of a fixed Ser, Thr, or Tyr ensures that each peptide contains at least one phosphorylatable residue, although phosphorylation may also take place at any of the randomized positions. The linker sequence, NNBBRM, permits selective peptide release (cleavage after Met by CNBr) and facilitates peptide sequencing by partial Edman degradation-mass spectrometry (PED-MS; Arg provides a fixed positive charge and improves aqueous solubility) [9]. This library has a theoretical diversity of 19^9 or 2.6×10^{11} and should be synthesized on amino polyethylene glycol polyacrylamide (PEGA) resin (300–500 μm in water, ~1 million beads/g) (*see* **Note 1**). It is convenient to synthesize the library on ≤5 g of PEGA resin in a research lab setting (up to ~5 million different peptide sequences). Thus, the number of peptide sequences actually synthesized represents only a small percentage of the theoretical diversity. However, we have previously shown that the specificity profile of a protein can be unambiguously determined by sampling just a small fraction of the entire sequence space [8, 10–12]. If necessary, one may also synthesize and screen a secondary, biased library by fixing some of the random positions with preferred amino acids identified from the primary screening, in order to define the selectivity at less critical positions [8].

A major challenge associated with on-bead screening of enzymatic substrates is how to differentiate the reaction product(s) (typically <1 %) from a large excess of unreacted substrates. The key innovation of our method is a simple, robust assay for the kinase products (Fig. 1a). Briefly, the peptide library is treated for a limited amount of time with a kinase of interest in the presence of adenosine 5′-O-(3-thio)triphosphate ([γ-S]ATP) (instead of ATP), so that only beads carrying the optimal kinase substrates undergo a small amount of reaction (usually <1 %). The use of [γ-S]ATP results in the addition of a thiophosphoryl group to the positive beads, which are subsequently labeled with a fluorescent group (e.g., tetramethylrhodamine) through a disulfide exchange reaction. The fluorescent beads (Fig. 1b) can be manually isolated from the library with a micropipette under a fluorescence microscope and individually sequenced by PED-MS [9]. Substitution of [γ-S]ATP

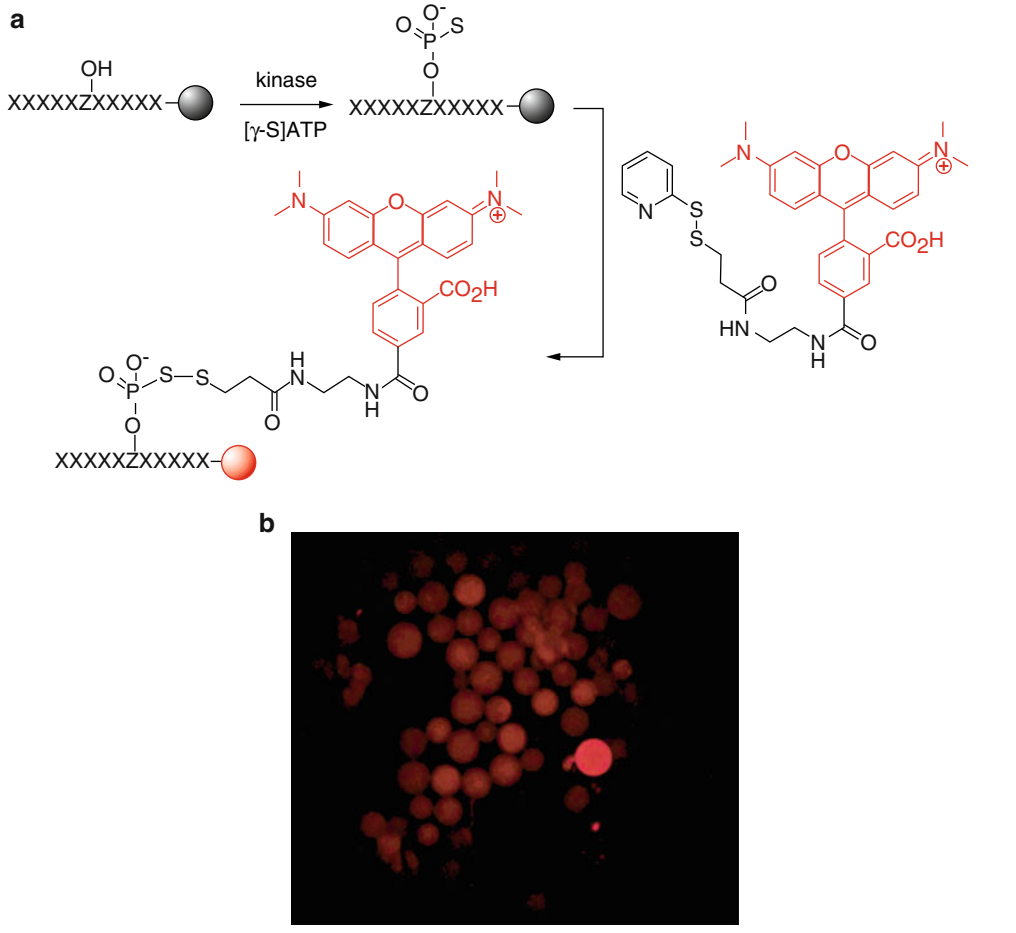

Fig. 1 Strategy for screening OBOC peptide libraries against protein kinases. (*a*) Reactions involved in the kinase screening strategy. X, random residues; Z, Ser, Thr, or Tyr. (*b*) A portion of the peptide library beads after the screening reactions (viewed under a fluorescence microscope)

for ATP decreases the catalytic activity of kinases by 15–30-fold but does not alter the sequence specificity [13, 14]. This screening method has a wide dynamic range and was able to profile the specificity of kinases that have catalytic efficiency (k_{cat}/K_M) ranging from 0.1 to 10^6 M^{-1} s^{-1} [8]. It provides individual peptide sequences, thus permitting the identification of not only amino acids that contribute positively to the kinase-substrate interaction (permissive residues), but also amino acids that negatively impact the kinase function (non-permissive residues), as well as any sequence covariance.

2 Materials

2.1 Library Synthesis

1. 0.2 mmol/g PEGA resin, 300–500 μm (Varian).
2. Fmoc-protected L-amino acids, 3-Fmoc-aminopropanoic acid (Fmoc-β-Ala-OH).
3. O-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU).
4. 1-Hydroxybenzotriazole hydrate (HOBt).
5. *N*-methylmorpholine (NMM).
6. 20 % Piperidine in DMF.
7. *N*,*N*-dimethylformamide (DMF).
8. Dichloromethane (DCM).
9. *N*-methylpyrrolidone (NMP).
10. CD_3CO_2D (Sigma-Aldrich).
11. $CH_3CD_2CO_2D$ (Sigma-Aldrich).
12. Trifluoroacetic acid (TFA).
13. Allyloxycarbonyl *N*-hydroxysuccinimide ester (Alloc-OSu).
14. Phenol, liquefied.
15. Double-distilled water (ddH_2O).
16. Thioanisole.
17. Ethanedithiol (EDT).
18. Triisopropylsilane (TIPS).
19. Anisole.
20. Kaiser test solution 1: 5 % (w/v) Ninhydrin in ethanol.
21. Kaiser test solution 2: 80 % (w/v) Phenol in ethanol.
22. Kaiser test solution 3: 2 % (v/v) of a 1 mM aqueous solution potassium cyanide, in pyridine.
23. Modified reagent K: 7.5 % phenol, 5 % water, 5 % thioanisole, 2.5 % EDT, 2.5 % TIPS, and 1.25 % anisole in TFA.

24. Rotary shaker.
25. Peptide synthesis vessels.
26. Spin columns, 1.2 mL bed volume (Bio-Rad).
27. Vacuum manifold with Luer connections.
28. Vacuum source.
29. Vortex mixer.

2.2 Synthesis of Labeling Reagent and Kinase Screening

1. 5-(and 6)-carboxytetramethylrhodamine, succinimidyl ester (TMR-NHS; Pierce).
2. Dimethyl sulfoxide, anhydrous (DMSO).
3. *N*-Boc-ethylenediamine.
4. *N*-Succinimidyl 3-[2-pyridyldithio]-propionate (SPDP).
5. Kinase buffer 1: 60 mM Tris, 150 mM NaCl, 5 mM $MnCl_2$, 2 mM dithiothreitol (DTT), 0.02 % Tween-20, pH 7.4.
6. Kinase buffer 2: 30 mM HEPES, 30 mM NaCl, 20 mM $MgCl_2$, 2 mM DTT, 0.02 % Tween 20, pH 7.4.
7. Adenosine 5′-O-(3-Thiotriphosphate), Tetralithium Salt ([γ-S]ATP; EMD Millipore).
8. 4 M Guanidine hydrochloride solution: Dissolve guanidine hydrochloride in water. Adjust pH to 7.4 with 6 M NaOH.
9. Labeling solution: 1:1 (v/v) NMP:50 mM HEPES, pH 7.4.
10. Selection solution: 50 mM NaCl, 0.02 % Tween-20.
11. Tris (2-carboxyethyl)phosphine hydrochloride (TCEP-HCl).
12. 50 mM TCEP solution: 28.6 mg TCEP-HCl in 2 mL selection solution.
13. Spin columns, 0.8 mL bed volume (Bio-Rad).
14. 35 mm petri dish.
15. Parafilm.
16. Orbital shaker.
17. Fluorescence microscope (Olympus).

2.3 Partial Edman Degradation and Mass Spectrometry for Sequencing Resin-Bound Peptides

1. Custom-designed reaction vessel (12 mm diameter, 20 mm height, a 10–20 μm glass frit, and 1 mm luer tip at the bottom (PED) vessel).
2. Pyridine.
3. TFA.
4. DCM.
5. Phenylisothiocyanate, liquid (PITC; Sigma-Aldrich).
6. *N*-(9-Fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu).

7. Triethylamine (TEA).
8. Dimethyl sulfide.
9. Ammonium iodide.
10. Cyanogen bromide, 40 mg/mL solution in 70 % TFA in water.
11. Acetonitrile.
12. 4-Hydroxy-α-cyanocinnamic acid (α-CCA).
13. α-CCA solution: 10 mg/mL α-CCA in 50:50:0.1 (v/v) acetonitrile/water/TFA.
14. Tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$].
15. Triphenylphosphine (PPh_3).
16. Tetrahydrofuran, anhydrous (THF).
17. *N*-methylaniline.
18. Sodium dimethyldithiocarbamate (SDDC).
19. Dissection microscope (10× to 40× magnification).
20. SpeedVac concentrator (Thermo Scientific).
21. Matrix-assisted laser desorption ionization (MALDI) sample plate (Bruker).
22. MALDI-TOF system (Bruker).

3 Methods

3.1 Synthesis of Solid-Phase Peptide Library

3.1.1 Synthesis of Linker Region: NNBBRM-Resin

1. Weigh about 2 g of PEGA resin (~20 g wet resin) into a 30 mL glass or polypropylene reaction vessel, equipped with a filter. The diameter of the reaction vessel should be sufficient so that the height of the resin bed is less than 2 in. PEGA resin typically comes as soaked in ethanol at 10 % concentration.
2. Wash the resin five times with DMF. This is typically done by covering the resin completely with DMF, and then drain.
3. Swell the resin by incubating in DMF for 20 min on a rotary shaker.
4. Drain the solvent. The resin is now ready for library synthesis.
5. Dissolve 4 eq. of Fmoc-Met-OH (594.2 mg), 4 eq. of HBTU (606.8 mg), and 4 eq. of HOBt (244.8 mg) in 20 mL of DMF. Add 8 eq. of NMM (352 μL), mix well for 10 s and add to resin. Incubate the mixture on a rotary shaker for 90 min.
6. Wash the resin with DMF (3 times), and then with DCM (3 times).
7. Prepare Kaiser test mixture by adding 40 μL of each Kaiser test solution (1, 2, and 3) into a glass test tube. A control tube should also be prepared with the same amount of each reagent.

8. Using a pipet tip, add a small amount of resin (~100 beads) to the test tube. No resin is added to the control tube.
9. Heat the mixtures at 100 °C for 3 min.
10. Examine the color of each mixture. A yellow to brown color indicates a negative test (no free $-NH_2$ is present, coupling is complete). The color of sample tube should be very similar, if not identical, to the color of the control tube. (If the coupling is incomplete, *see* **Note 2**.)
11. Remove the N-terminal Fmoc group by treating the resin with 20 % piperidine in DMF for 20 min.
12. Wash the resin with DMF (five times), DCM (four times).
13. Perform a Kaiser test to ensure the 20 % piperidine in DMF solution is functioning properly. A dark purple solution after heating should be observed (presence of a free $-NH_2$ group).
14. Repeat **steps 5–12** for the rest of the linker positions.

3.1.2 Synthesis of Random Positions: X_5ZX_5-Linker by Split-and-Pool Synthesis

1. After the removal of the N-terminal Fmoc group of the linker sequence, the resin is now ready to be split (*see* **Note 3**).
2. Suspend the resin in 20 mL of 1:1 DCM/DMF mixture in a reaction vessel. The resin density should be uniform throughout the solvent. The reaction vessel can be inverted regularly to ensure uniformity of resin density.
3. Split the resin into 19 (for Tyr library) or 20 (for Ser/Thr library) 2 mL reaction vessels using a 1000 μL pipet. The volume of each split should be around 800 μL.
4. Add 1:1 DCM/DMF mixture to the remaining resin and repeat the splitting until no resin is left in the 30 mL reaction vessel (*see* **Note 4**).
5. Each 2 mL reaction vessel should be clearly labeled with the name of an amino acid.
6. Treat the resin with 4 eq. of Fmoc-AA-OH, HBTU, HOBt and 8 eq. of NMM for 90 min, where AA is the appropriate amino acid (*see* **Note 5**).
7. Wash the resin with DMF (3 times).
8. Treat the resin with 4 eq. of Fmoc-AA-OH, HBTU, and HOBt and 8 eq. of NMM for 60 min, where AA is the appropriate amino acid.
9. Wash the resin with DMF (three times), and DCM (three times).
10. Perform Kaiser test on three representative reactions. Ile is usually chosen due to the low coupling efficiency. The other two are randomly chosen. A control test (no resin) should also be performed. Proceed if all three Kaiser tests are negative.

11. Suspend the resin in 1:1 DCM/DMF mixture.
12. Transfer all the resin as slurry into the 30 mL reaction vessel using a 1000 μL pipet. Repeat the transfer until no resin is left in each 2 mL vessel.
13. Treat the resin with 20 % piperidine in DMF for 20 min.
14. Wash the resin with DMF (5 times) and DCM (4 times).
15. Repeat **steps 2–14** for four positions.
16. For coupling of fixed amino acid, resin was treated with 4 eq. of HBTU, and HOBt; 4 eq. of appropriate Fmoc-protected amino acid; and 8 eq. of NMM in DMF for 90 min. For Tyr library, 2 g of resin was treated with 4 eq. of Fmoc-Tyr-OH. For Ser/Thr library, resin was split into two equal portions; each was coupled with 4 eq. of Fmoc-Ser-OH or Fmoc-Thr-OH.
17. Wash the resin with DMF (three times) and DCM (three times).
18. Perform Kaiser test to ensure that the coupling is complete.
19. Perform split and pool synthesis (**steps 2–14**) for the 5N-terminal random positions.
20. After library synthesis and removal of N-terminal Fmoc group, the library is treated with 15 eq. of Alloc-OSu and 5 eq. of NMM in DMF for 30 min.
21. Wash the library with DMF (five times) and DCM (ten times).
22. Side-chain deprotection was carried out by treating the resin with 20 mL of modified reagent K for 2 h.
23. Wash the resin with TFA (three times), DCM (ten times), and DMF (ten times).
24. Suspend the resin in DMF and store at –20 °C. The library is now ready for screening.

3.2 Screening the Library Against Kinase

3.2.1 Synthesis of Fluorescence Label

1. Dissolve TMR-NHS in DMF at 40 mM concentration (10.5 mg in 0.5 mL).
2. Add 1.1 eq. of N-Boc-ethylenediamine (3.3 μL).
3. Stir the reaction overnight in the dark.
4. Remove DMF by SpeedVac concentrator.
5. Add 1 mL of toluene and mix well.
6. Remove the solvent by SpeedVac concentrator.
7. Dissolve the crude product (TMR-NH_2-Boc) in 1 mL of TFA.
8. Stir the reaction for 2 h in the dark.
9. Remove TFA by SpeedVac.
10. Add 1 mL of toluene and remove the solvent by SpeedVac.
11. Dissolve the crude product (TMR-NH_2) in 400 μL of anhydrous DMSO (50 mM concentration).

12. Weigh out 1.2 mg of SPDP into a 1.5 mL microcentrifuge tube.
13. Add 78.5 μL of TMR-NH_2 solution.
14. Add 0.55 μL of triethylamine.
15. Incubate for 2 h in the dark.
16. Store the crude product (TMR-S-S-Py) in DMSO at –20 °C.

3.2.2 Library Screening

1. Transfer 100 mg of resin (as slurry in DMF) into a 2 mL polypropylene spin column.
2. Wash with DMF (three times), ddH_2O (five times), and kinase buffer (five times).
3. Transfer the resin into a 1.5 mL microcentrifuge tube as slurry in kinase buffer.
4. The resin should settle at the bottom of the tube, leaving a clear supernatant. Carefully pipet and discard the supernatant, leaving the resin in minimal (~290 μL) amount of kinase buffer (*see* **Note 6**).
5. Prepare 100 mM solution of [γ-S]ATP in 50 mM HEPES (pH 7.4). Dissolve 2 mg of [γ-S]ATP in 36 μL of 50 mM HEPES. Mix well with a pipette.
6. Add 6 μL of 100 mM [γ-S]ATP solution to the resin.
7. Add the kinase to the reaction mixture. The amount of kinase (and incubation time) depends on the enzymatic activity. Usually a final concentration of 5 μM of kinase is recommended for initial screening.
8. Invert the microcentrifuge tube and lightly tap the side, so the whole reaction mixture settles in the cap and top of the tube.
9. Wrap the cap of the tube with parafilm.
10. Fix the microcentrifuge tube (in the inverted position) onto an orbital shaker using masking tape.
11. Mix the reaction at 200 rpm, 30 °C for 3–40 h, depending on the enzymatic activity of the kinase.
12. Invert the tube back to its upright position and lightly tap the side so the reaction mixture settles at the bottom.
13. Transfer the reaction mixture to a 2 mL spin column.
14. Wash the resin with kinase buffer (five times), ddH_2O (five times) and 4 M guanidine-HCl, pH 7 (three times).
15. Incubate the resin in 4 M guanidine-HCl three times, 15 min each time on a rotary shaker.
16. Wash the resin with water (ten times) and labeling solution (five times).
17. Prepare 100 μM solution of TMR-NH_2 in DMSO.

18. Add 2 μL of TMR-NH_2 solution to 1.6 mL of labeling solution (0.125 μM) and mix well.
19. Add this mixture to the resin and incubate for 2 h in the dark.
20. Wash the resin with labeling solution (five times), DMF (five times), and selection solution (five times).
21. Transfer the resin to a petri dish using selection solution.
22. Visualize the beads under a fluorescence microscope, isolate, and discard all fluorescent beads using a micropipette (*see* **Note 7**).
23. Transfer all the non-fluorescent beads into a 2 mL spin column.
24. Prepare 100 μM solution of TMR-S-S-Py in DMSO.
25. Add 2 μL of TMR-S-S-Py solution to 1.6 mL of labeling solution (0.125 μM) and mix well.
26. Add this mixture to the resin and incubate for 2 h in the dark.
27. Wash the resin with labeling solution (five times), DMF (five times), and selection solution (five times).
28. Transfer the resin to a petri dish using selection solution.
29. Visualize the beads under a fluorescence microscope and isolated the fluorescent beads.
30. Prepare 50 mM TCEP solution. Transfer TCEP solution to a petri dish.
31. Transfer fluorescent beads to the petri dish containing TCEP solution and visualize the disappearance of fluorescence signal. Positive beads should lose their fluorescence upon TCEP treatment.
32. Transfer the positive beads (now non-fluorescent) to a microcentrifuge tube and store at 4 °C.

3.3 Sequencing of Hit Peptides by PED-MS

3.3.1 Removal of N-Terminal Alloc Group

1. Add 500 μL of DMF to the microcentrifuge tube containing hits from the screening.
2. Transfer the mixture to a PED vessel.
3. Add 500 μL of DCM to the microcentrifuge tube.
4. Pour the mixture into the PED vessel. All beads should now be in the PED vessel.
5. Wash the beads with DMF (three times) and THF (three times).
6. Treat the beads with 5 mg of $Pd(PPh_3)_4$, 50 mg of PPh_3, and 40 μL of N-methylaniline in 1 mL of THF for 2 h.
7. Wash the beads with THF (five times) and DMF (five times).
8. Wash the beads with 1 mL of 1 % (w/v) SDDC in DMF.
9. Incubate the beads with 1 mL of 1 % SDDC in DMF for 15 min.
10. Wash the beads with DMF (five times) and pyridine (five times). The peptides are now ready for sequencing by PED/MS.

3.3.2 PED

1. Prepare 2:1 (v/v) pyridine/water mixture containing 0.1 % triethylamine (mix 8 mL of pyridine, 4 mL of water and 12 μL of triethylamine).
2. Prepare 7.8 mM solution of Fmoc-OSu in pyridine.
3. Mix 160 μL of Fmoc-OSu solution with 148 μL of 2:1 pyridine/water mixture.
4. Add 12 μL of PITC and mix briefly (10 s).
5. Add this mixture (320 μL) to the PED vessel.
6. Allow the reaction to proceed for 6 min and then wash with pyridine (two times) and DCM (three times).
7. Wash the beads with TFA (one time) and incubate in 1 mL of TFA for 6 min.
8. Drain the solvent, add 1 mL of TFA, and incubate for 6 min.
9. Drain the solvent and wash the beads with DCM (three times) and pyridine (two times).
10. Repeat **steps 3–9** for the desired number of cycles.
11. After the second TFA treatment of the last PED cycle, suspend the beads in 500 μL of TFA.
12. Add 30 μL of dimethyl sulfide to the reaction.
13. Add 500 μL of TFA and 30 mg of ammonium iodide and let the reaction proceed for 20 min.
14. Wash the beads with TFA (two times), water (ten times), DMF (three times), and 20 % piperidine in DMF (one time).
15. Treat the beads with 1 mL of 20 % piperidine in DMF for 20 min.
16. Wash the beads with DMF (three times) and water (five times).
17. Suspend the beads in 1 mL of water and transfer them to a petri dish using a pipette. Repeat this transfer until all beads are present in the petri dish (*see* **Note 8**).
18. Transfer the beads into individual 1.5 mL microcentrifuge tubes (one bead/tube).
19. Add 20 μL of 40 mg/mL CNBr in 70 % TFA in water to each tube and allow the reaction to proceed in the dark for 12–14 h.
20. Dry the samples in SpeedVac and store at 4 °C.

3.3.3 MALDI-TOF MS Analysis

1. Add 5 μL of 50:50:0.1 (v/v) acetonitrile/water/TFA to each sample tube.
2. Vortex the tubes for 10 s and centrifuge at 870 × *g* for 10 s.
3. Repeat **step 2** twice.
4. Mix 1 μL of this solution with 2 μL of α-CCA solution.
5. Spot 1 μL of the mixture onto a MALDI sample plate.

6. Perform mass spectrometric analysis on a Bruker Microflex MALDI-TOF instrument (or any other MALDI-TOF model).
7. Mass spectra can be analyzed with Moverz or FlexAnalysis software.

4 Notes

1. The choice of PEGA or other resins that are permeable to relatively large proteins is crucial for successful on-bead enzymatic reactions. Popular resins such as TentaGel are not permeable to macromolecules and should be avoided.
2. Repeat any incomplete coupling reaction with 4 eq. Fmoc-amino acid, 4 eq. of HATU, and 8 eq. of NMM.
3. It is not recommended to dry PEGA resin and therefore resin splitting is best carried out by pipetting the resin slurry suspended in solvents.
4. Despite uniformity during the splitting, the first few vessels usually get slightly more resin than the subsequent ones. The second round of splitting should be done in reverse order (transfer resin to last vessel first) to achieve an equal amount of resin in each vessel.
5. 3.5 % (mol/mol) of CD_3CO_2D is added to the coupling solution of Fmoc-Leu-OH and Fmoc-Lys-OH, while 3.5 % (mol/mol) of $CH_3CD_2CO_2D$ is added to the solution of Fmoc-Nle-OH. This allows for the differentiation of isobaric amino acids during MS analysis [9].
6. The resin itself has substantial volume. It is important that the total reaction volume be large enough to ensure proper mixing of the reaction contents and yet minimal in order to conserve enzyme and reagents. A final volume of 300 μL works well for 100 mg of resin.
7. The number of false-positive beads, caused by binding of the fluorescent dye directly to the peptides, was typically 10–30 for 100 mg of resin.
8. Alternatively, hold the PED vessel upside down above a petri dish and spray it with a water bottle to wash the beads out of the vessel and into the petri dish.

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