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The importance of biotin presentation in avidin-based assays

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Introduction

Biotin-labeled peptides have many important applications in immunology and histochemistry, such as affinity purification [1], FRET-based flow cytometry [2], solid-phase immunoassays [3], and receptor localization [4].

The biotin label is most frequently located directly on the N-terminal group of the peptide, often without any regard to how this may affect peptide-target interactions, biotin-avidin binding, and the solubility properties of the resultant peptide. In many instances the products are poorly soluble, and have little biological activity and poor affinity for avidin. Problems can also arise during the synthesis of such N-terminally biotinylated peptides due to the poor solubility and reactivity of many of the reagents used for biotin introduction.

To overcome these limitations, we have designed a range of tools (Biotin NovaTag™ resins), which provide a simple and elegant solution to these problems [5, 6]. Using these resins, biotinylated peptides are obtained directly following TFA cleavage, without the need for any additional biotinylation steps (Figure 1). Resins incorporate either an ethylenediamine or a 15 atom PEG spacer between the peptide and biotin to reduce steric hindrance.

The use of Biotin-PEG NovaTag™ resin is particularly advantageous because not only does the hydrophilic PEG chain confer better solubility to the peptide biotin conjugate, but its extended conformation leads to better avidin binding which can dramatically improve assay sensitivity. Furthermore as the biotin is an integral part of the linker, its presence in every peptide chain is assured from the outset.

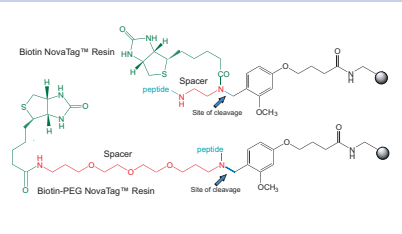

Fig. 1: Loaded Biotin NovaTag™ resins, showing point of attachment of peptide and site of cleavage.

Results and discussion

Biotinylated-peptide design

When designing biotinylated peptides for use in assays, two of the most important considerations are the position of the biotin moiety and the nature of the spacer group between the peptide and biotin. This is because these can profoundly effect the strength of peptide-protein and biotin-avidin interactions and consequently the sensitivity of the assay. To exemplify the utility of our new resins in ligand optimization, we prepared C-terminally and conventional N-terminally biotinylated peptides and evaluated their efficacy in protein-binding and kinase assays.

Peptide synthesis

The peptide sequences shown in Table 1 were all prepared on a Protein Technologies' Symphony peptide synthesizer using HCTU/NMM activation. N-terminal biotinylation of peptides was effected using Biotin-ONp (01-63-0116) or N-Biotinyl-NH-$(PEG)_2$-COOH (01-63-0133). C-terminally biontinylated peptides were prepared directly on the appropriate Biotin NovaTag™ resin. In the case of Biotin-PEG NovaTag™, the first residue was coupled using HATU, since this reaction involves acylation of a less reactive secondary amine. The syntheses of peptides **5** & **7** are described in detail.

Table 1: Biotnylated peptides.

Peptide	Sequence
1	N-Biotin-RXXXXF-NH_2
2	H-RXXXXF-$NHCH_2CH_2$NH-biotin
3	H-RXXXXF-NH-PEG-NH-biotin
4	Biotin-KKKKXXXXXXXXXXXXXXXXKDEE-NH_2
5	H-KKKKXXXXXXXXXXXXXXXXKDEE-NHCH$_2$CH$_2$NH-biotin
6	Biotin-NH-PEG-KKKKXXXXXXXXXXXXXXXXKDEE-NH_2
7	H-KKKKXXXXXXXXXXXXXXXXKDEE-NH-PEG-NH-biotin

Peptide 5

	Conditions
Resin	Biotin NovaTag™ resin (0.15 mmol, 0.41 mmol/g)
Instrument	Protein Technologies, Inc. Symphony
Coupling	Fmoc-Aaa-OH: HCTU:DIPEA (3:3:6), 30 min
Deblock	20% Piperidine in DMF
Cleavage	TFA/thioanisole/EDT/phenol/water (82.5:5:2.5:5:5) for 2.5 h

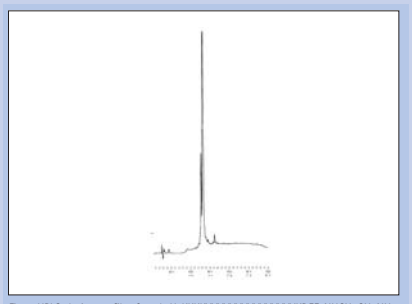

Fig. 2: HPLC elution profile of crude H-KKKKXXXXXXXXXXXXXXXXKDEE-NHCH$_2$CH$_2$NH-biotin prepared with Biotin NovaTag™ resin. Minor polar by-product is methionine sulfoxide peptide.

Peptide 7

	Conditions
Resin	Biotin PEG NovaTag™ resin (0.15 mmol, 0.42 mmol/g)
Instrument	Protein Technologies, Inc. Symphony
Coupling	1st Residue: Fmoc-Glu(OtBu)-OH: HATU:DIPEA (3:3:5), 30 min Fmoc-Aaa-OH: HCTU:NMM (3:3:5), 30 min
Deblock	20% Piperidine in DMF
Cleavage	TFA/thioanisole/EDT/phenol/water (82.5:5:2.5:5:5) for 2.5 h

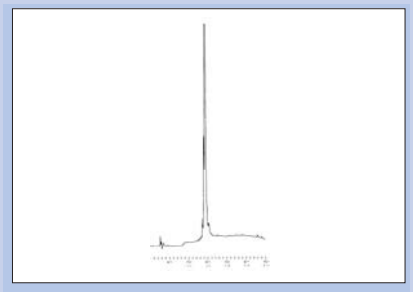

Fig. 3: HPLC elution profile of crude H-KKKKXXXXXXXXXXXXXXXXKDEE-NH-PEG-NH-biotin prepared with Biotin-PEG NovaTag™ resin. Minor polar by-product is methionine sulfoxide peptide.

AlphaScreen™ protein-binding assay

The peptide-protein binding assay was conducted using the AlphaScreen™ technology as shown in Figure 4. N- and C-terminally biotin-labeled versions of the native peptide ligand immobilized on streptavidin-coated donor beads were screened against acceptor beads loaded with target protein. Only the peptide which was C-terminally labeled with PEG-biotin had acceptable solubility in the test buffer and showed significant levels of protein binding (Figure 5).

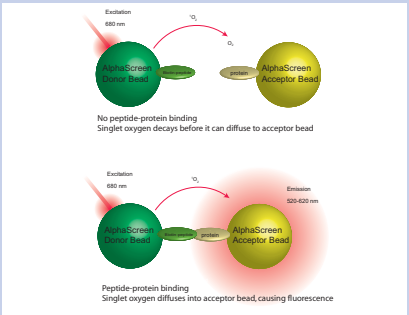

Fig. 4: Principles of the Alpha Screen assay.

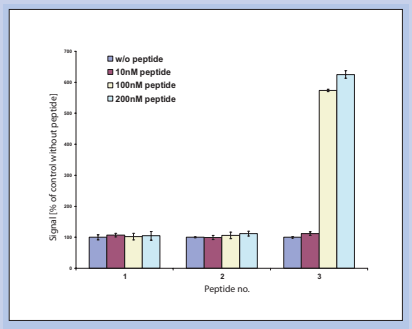

*Fig. 5: AlphaScreen protein-binding assay. Peptide **1**: N-biotin-RXXXXF-NH_2; peptide **2**: H-RXXXXF-NHCH$_2$CH$_2$NH-biotin; peptide **3**: H-RXXXXF-NH-PEG-NH-biotin.*

Kinase assay

N- and C-terminally biotin-labeled versions of a kinase substrate were evaluated in the assay shown in Figure 6. Peptides that were C-terminally labeled with biotin were found to give better responses than those labeled on the N-terminus, whilst inclusion of a PEG spacer between the peptide and biotin appeared to have little effect (Figure 7).

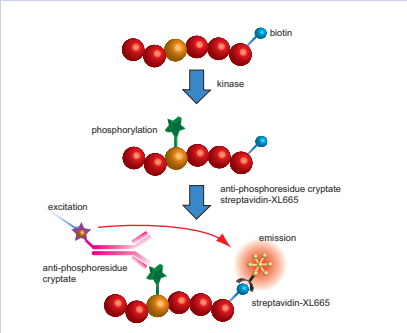

Fig. 6: Principle of the kinase assay.

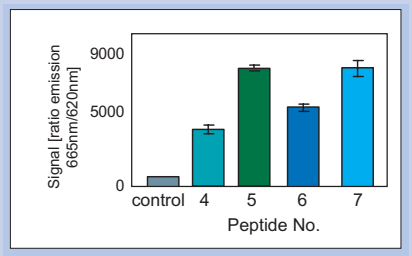

*Fig. 7: Kinase assay. Peptide **4**: biotin-KKKKXXXXXXXXXXXXXXXXKDEE-NH_2; peptide **5**: H-KKKKEXXXXXXXXXXXXXXXXKDEENHCH$_2$CH$_2$NH-biotin; peptide **6**: biotin-NH-PEG-KKKKXXXXXXXXXXXXXXXXKDEE-NH_2 ; peptide **7**: H-KKKKXXXXXXXXXXXXXXXXKDEE-NH-PEG-NH-biotin.*

Conclusions

- The positioning of the biotin label within a peptide sequence can profoundly influence the sensitivity of an assay.
- Ideally, both N-terminal and C-terminal biotinylated peptides, incorporating spacers of different lengths and types, should be screened to determine the optimal combination for a given assay.

References

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