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# Design and optimization of a turn-on fluorescence assay for the identification of improved ADC linkers

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# Design and Optimization of a Turn-on Fluorescence Assay for the Identification of Improved ADC Linkers

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## Abstract

Antibody-drug conjugates (ADCs) are a class of drugs used for targeted delivery in the treatment of cancer. The prototypical linker used for such ADCs the lysosomally cleaved Val-Cit-PABC linker. This system emerged as a result of its rapid cleavage rate by the lysosomal enzyme cathepsin B as well as its stability in human plasma. However, recent studies have shown that this system is frequently unstable in the presence of various enzymes including neutrophil elastases and carboxylesterases. To mitigate this issue, we have designed a peptide library that can be readily screened in order to identify sequences with improved properties. In short, the library was designed to utilize a turn-on fluorescence assay: a simple assay made possible by a fluorophore, AMC (7-Amino-4-methylcoumarin), known to be non-fluorescent when the 7-amino group is bound as an amide but is highly fluorescent upon cleavage of the amide bond. Therefore, AMC can be employed as a fluorescent probe for rapid determination of amide bond cleavage – specifically that of ADC linkers. Lysosomal ADC processing relies on cleavage of the amide bond between the linker and the payload, and therefore the turn-on fluorescence assay provides a simple method for determining whether peptide linkers are susceptible to such cleavage.


## Turn-on Fluorescence with 7-Amino-4-methylcoumarin (AMC)

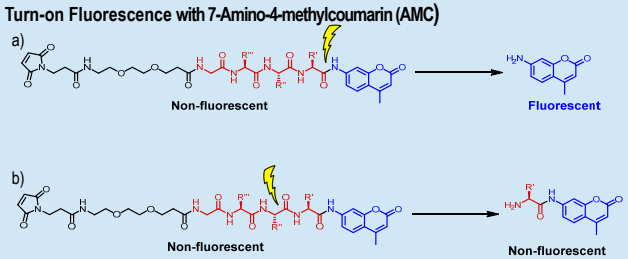



Figure 1. Turn-on fluorescence with AMC (blue) and tetrapeptide linker (red). AMC known to be non-fluorescent when the 7-amino group is bound as an amide. a) Upon cleavage of the P1-P1' amide bond, fluorescence is observed. b) Cleavage of any other peptide bond would not result in fluorescence.

## Synthesis of Turn-on Fluorescence Probe

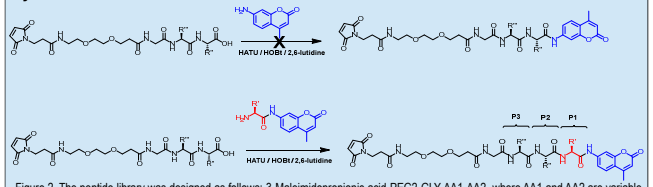
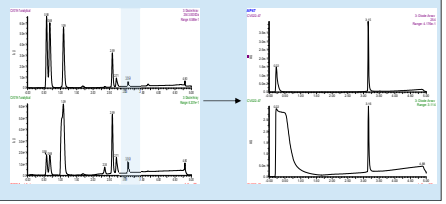



Figure 2. The peptide library was designed as follows: 3-Maleimidopropionic acid-PEG2-GLY-AA1-AA2, where AA1 and AA2 are variable amino acids representing a mixture of polar and nonpolar amino acids of various sizes. Due to the poor nucleophilicity of the AMC, it cannot be directly coupled to the tripeptide. Instead, the AMC is attached to a single amino acid (AA3) and subsequently coupled to the tripeptide linker. Eight amino acid-AMC molecules were each coupled to roughly twenty tripeptide linkers, resulting in a library of ~160 peptides. The preparation of the library required the optimization of various coupling reactions. All individual compounds were purified by preparative high-performance liquid chromatography (HPLC) and were characterized by liquid chromatography-mass spectroscopy. (LC-MS).

## Purification through Preparative HPLC-MS

Figure 3. All tetrapeptide-AMC linkers were analyzed for product and purified through preparative HPLC-MS. After purification, 500 µM stock solutions of each product in DMA were created. The purity of these stock solutions was determined through analytical LC-MS. Tetrapeptide-AMC linkers with a purity less than 70% were deemed not usable for future assays.

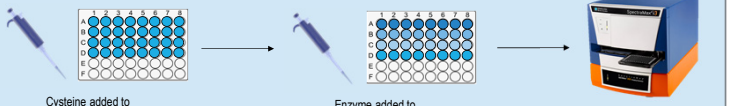


## Peptide-AMC Linker Library

| AMC Peptide | Chemistry | Purity (%) | AMC Peptide | Chemistry | Purity (%) | AMC Peptide | Chemistry | Purity (%) | AMC Peptide | Chemistry | Purity (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AP1 | Gly-Ala-Ala-Ala-AMC | 93 | AP35 | Gly-Ser-Cit-Gly-AMC | 70 | AP69 | Gly-Gly-Inal-Pro-AMC | 98 | AP104 | Gly-Ser-Ala-Asn-AMC | 97% |
| AP2 | Gly-Val-Cit-Ala-AMC | 99 | AP36 | Gly-Ser-Trp-Gly-AMC | 98 | AP70 | Gly-Gly-2nal-Pro-AMC | >99 | AP105 | Gly-Ser-Phe-Asn-AMC | 77% |
| AP3 | Gly-D-Val-D-Cit-Ala-AMC | 98 | AP37 | Gly-Pro-Asn-Gly-AMC | >99 | AP71 | Gly-Gly-Cit-Pro-AMC | 98 | AP106 | Gly-Ser-Cit-Asn-AMC | 100% |
| AP4 | Gly-Gly-Val-Ala-AMC | >99 | AP38 | Gly-Pro-Alb-Gly-AMC | >99 | AP72 | Gly-Gly-Trp-Pro-AMC | Imp. | AP107 | Gly-Ser-Trp-Asn-AMC | > 95% |
| AP5 | Gly-Gly-Alb-Ala-AMC | 74 | AP39 | Gly-Pro-Phe-Gly-AMC | 98 | AP73 | Gly-Ser-Asn-Pro-AMC | Imp. | AP108 | Gly-Pro-Asn-Asn-AMC | 85% |
| AP6 | Gly-Gly-1nal-Ala-AMC | 98 | AP40 | Gly-Pro-Cit-Gly-AMC | N/A | AP74 | Gly-Ser-Phe-Pro-AMC | >99 | AP109 | Gly-Pro-Phe-Asn-AMC | > 95% |
| AP7 | Gly-Gly-2nal-Ala-AMC | 98 | AP41 | Gly-Pro-Trp-Gly-AMC | 98 | AP75 | Gly-Ser-Cit-Pro-AMC | 98 | AP110 | Gly-Pro-Cit-Asn-AMC | 20% |
| AP8 | Gly-Gly-Cit-Ala-AMC | 82 | AP42 | Gly-Ala-Ala-Leu-AMC | 92 | AP76 | Gly-Ser-Trp-Pro-AMC | 98 | AP111 | Gly-Pro-Trp-Asn-AMC | 68% |
| AP9 | Gly-Gly-Trp-Ala-AMC | 98 | AP43 | Gly-Val-Cit-Leu-AMC | 98 | AP77 | Gly-Pro-Asn-Pro-AMC | 98 | AP112 | Gly-Gln-Asn-Asn-AMC | 93% |
| AP10 | Gly-Ser-Asn-Ala-AMC | 99 | AP44 | Gly-D-Val-D-Cit-Leu-AMC | 20 | AP78 | Gly-Val-Cit-Ser-AMC | < 70% | AP113 | Gly-Gln-Phe-Asn-AMC | > 95% |
| AP11 | Gly-Ser-Alb-Ala-AMC | 77 | AP45 | Gly-Gly-Val-Leu-AMC | 10 | AP79 | Gly-D-Val-D-Cit-Ser-AMC | < 70% | AP114 | Gly-Gln-Cit-Asn-AMC | > 95% |
| AP12 | Gly-Ser-Phe-Ala-AMC | 98 | AP46 | Gly-Gly-Alb-Leu-AMC | 81 | AP80 | Gly-Ala-Ala-Ser-AMC | < 70% | AP115 | Gly-Gln-Trp-Asn-AMC | > 95% |
| AP13 | Gly-Ser-Cit-Ala-AMC | 99 | AP47 | Gly-Gly-1nal-Leu-AMC | >99 | AP81 | Gly-Gly-Alb-Ser-AMC | < 70% | AP116 | Gly-Val-Cit-Phe-AMC | 13% |
| AP14 | Gly-Ser-Trp-Ala-AMC | >99 | AP48 | Gly-Gly-2nal-Leu-AMC | 98 | AP82 | Gly-Gly-1nal-Ser-AMC | 66% | AP117 | Gly-D-Val-D-Cit-Phe-AMC | > 95% |
| AP15 | Gly-Pro-Asn-Ala-AMC | >99 | AP49 | Gly-Gly-Cit-Leu-AMC | 93 | AP83 | Gly-Gly-Val-Ser-AMC | < 70% | AP118 | Gly-Ala-Ala-Phe-AMC | 95% |
| AP16 | Gly-Pro-Alb-Ala-AMC | 99 | AP50 | Gly-Gly-Trp-Leu-AMC | >99 | AP84 | Gly-Gly-Cit-SerAMC | < 70% | AP119 | Gly-Gly-Alb-Phe-AMC | 100% |
| AP17 | Gly-Pro-Phe-Ala-AMC | 97 | AP51 | Gly-Ser-Asn-Leu-AMC | 97 | AP85 | Gly-Gly-Trp-Ser-AMC | 70% | AP120 | Gly-Gly-1nal-Phe-AMC | 87% |
| AP18 | Gly-Pro-Cit-Ala-AMC | 99 | AP52 | Gly-Ser-Alb-Leu-AMC | 90 | AP86 | Gly-Ser-Asn-Ser-AMC | 85% | AP121 | Gly-Gly-2nal-Phe-AMC | > 95% |
| AP19 | Gly-Pro-Trp-Ala-AMC | 98 | AP53 | Gly-Ser-Phe-Leu-AMC | 99 | AP88 | Gly-Ser-Alb-Ser-AMC | 96% | AP122 | Gly-Gly-Val-Phe-AMC | > 95% |
| AP20 | Gly-Gln-Asn-Ala-AMC | 99 | AP54 | Gly-Ser-Cit-Leu-AMC | 98 | AP89 | Gly-Ser-Phe-Ser-AMC | 96% | AP123 | Gly-Gly-Cit-Phe-AMC | > 95% |
| AP21 | Gly-Gln-Alb-Ala-AMC | 92 | AP55 | Gly-Ser-Trp-Leu-AMC | 98 | AP90 | Gly-Ser-Cit-Ser-AMC | 100% | AP124 | Gly-Gly-Trp-Phe-AMC | > 95% |
| AP22 | Gly-Gln-Phe-Ala-AMC | >99 | AP56 | Gly-Pro-Asn-Leu-AMC | 98 | AP91 | Gly-Ser-Trp-Ser-AMC | 100% | AP125 | Gly-Ser-Asn-Phe-AMC | 82% |
| AP23 | Gly-Gln-Cit-Ala-AMC | 98 | AP57 | Gly-Pro-Alb-Leu-AMC | 99 | AP92 | Gly-Pro-Asn-Ser-AMC | 74% | AP126 | Gly-Ser-Alb-Phe-AMC | > 95% |
| AP24 | Gly-Gln-Trp-Ala-AMC | 99 | AP58 | Gly-Pro-Phe-Leu-AMC | 99 | AP93 | Gly-Pro-Phe-Ser-AMC | 100% | AP127 | Gly-Ser-Phe-Phe-AMC | 73% |
| AP25 | Gly-D-Val-D-Cit-Gly-AMC | 99 | AP59 | Gly-Pro-Cit-Leu-AMC | 98 | AP94 | Gly-Pro-Cit-Ser-AMC | < 70% | AP128 | Gly-Ser-Cit-Phe-AMC | > 95% |
| AP26 | Gly-Ala-Ala-Gly-AMC | 98 | AP60 | Gly-Pro-Trp-Leu-AMC | 98 | AP95 | Gly-Val-Cit-Asn-AMC | <75% | AP129 | Gly-Ser-Trp-Phe-AMC | > 95% |
| AP27 | Gly-Gly-Alb-Gly-AMC | Imp. | AP61 | Gly-Gln-Asn-Leu-AMC | 98 | AP96 | Gly-D-Val-D-Cit-Asn-AMC | <75% | AP130 | Gly-Pro-Asn-Phe-AMC | 68% |
| AP28 | Gly-Gly-2nal-Gly-AMC | 98 | AP62 | Gly-Gln-Phe-Leu-AMC | 98 | AP97 | Gly-Ala-Ala-Asn-AMC | <75% | AP131 | Gly-Pro-Alb-Phe-AMC | > 95% |
| AP29 | Gly-Gly-Cit-Gly-AMC | 99 | AP63 | Gly-Gln-Cit-Leu-AMC | 99 | AP98 | Gly-Gly-Val-Asn-AMC | 74% | AP132 | Gly-Pro-Phe-Phe-AMC | > 95% |
| AP30 | Gly-Gly-Val-Gly-AMC | 95 | AP64 | Gly-Gln-Trp-Leu-AMC | >99 | AP99 | Gly-Gly-2nal-Pro-AMC | 100% | AP133 | Gly-Pro-Cit-Phe-AMC | 80% |
| AP31 | Gly-Gly-Cit-Gly-AMC | 98 | AP65 | Gly-Ser-Alb-Leu-AMC | 98 | AP100 | Gly-Gly-Val-Asn-AMC | 74% | AP134 | Gly-Pro-Trp-Phe-AMC | 92% |
| AP32 | Gly-Gly-Trp-Gly-AMC | >99 | AP66 | Gly-Val-Cit-Pro-AMC | Imp. | AP101 | Gly-Gly-Cit-Asn-AMC | 80% | AP135 | Gly-Gln-Asn-Phe-AMC | > 95% |
| AP33 | Gly-Ser-Asn-Gly-AMC | 70% | AP67 | Gly-D-Val-D-Cit-Pro-AMC | 70% | AP102 | Gly-Gly-Trp-Asn-AMC | 74% | AP136 | Gly-Gln-Phe-Phe-AMC | > 95% |
| AP34 | Gly-Ser-Phe-Gly-AMC | 98 | AP68 | Gly-Ala-Ala-Pro-AMC | 98 | AP103 | Gly-Ser-Asn-Asn-AMC | 85% | AP138 | Gly-Gln-Trp-Phe-AMC | > 95% |

Figure 4. Current Peptide-AMC Linker Library. 77 AMC-peptide linkers were synthesized and characterized by purity using LC//MS. Purities were characterized as follows: green indicates high purity, yellow/orange indicates medium purity, red indicates low purity, Imp. indicates that an impurity elutes at the same time as the product, and N/A indicates that there was not enough material to check its purity.

## Turn-on Fluorescence Assay Set-Up



Cysteine added to peptide-AMC linker to quench maleimide.

Enzyme added to peptide-AMC linker

Fluorescence analyzed at excitation 360 nm and emission 460 nm.

Figure 5. Set-Up of Turn-on Fluorescence Assay. This is a high throughput assay performed in a 96-well plate. Cysteine is added to the peptide-AMC linker to quench the reactivity of the maleimide. Then the enzyme is added to the peptide-AMC linker and their fluorescence is measured over time in the plate reader at an excitation of 360 nm and an emission of 460 nm.

## Kinetic Study with Cathepsin B

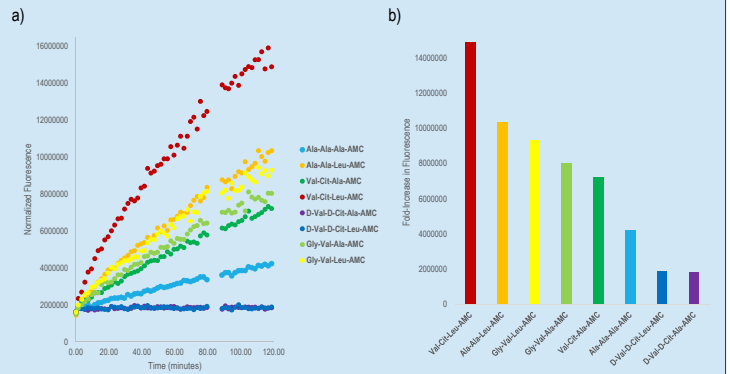



Figure 6. Kinetic Study with 8 Peptide-AMC linkers and Cathepsin B. a) Fluorescence of 8 peptide-AMC linkers (125 nM) was measured at excitation 360 nm and emission 460 nm over time in the presence of cathepsin B. This data translates to cleavage of these linkers over time. A positive control, Val-Cit-Leu-AMC, and a negative control, D-Val-D-Cit-Ala-AMC were identified. The positive control exhibited the most cleavage by cathepsin B while the negative control exhibited hardly any cleavage at all. b) Fluorescence of 8 peptide-AMC linkers after being in the presence of cathepsin B for two hours.

## Potential Applications

The turn-on fluorescence assay provides a simple method for screening linker stability in various enzymes. So far, around 150 peptide-AMC probes have been synthesized and characterized by their purity. The screening conditions for cathepsin B cleavage have been optimized, and a positive control (Val-Cit-Leu-AMC) and negative control (D-Val-D-Cit-Leu-AMC) have been identified from the initial peptide-AMCs tested. This assay can be used to analyze internalization and processing of an ADC. ADCs will be prepared with AMC attached then cells will be treated with these ADCs. The plate reader will be used to measure the fluorescence of these cells over time. In theory, the fluorescence should be proportional to the internalization and processing of the ADC. The application is outlined below.

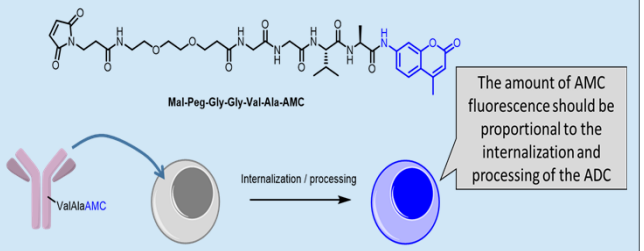
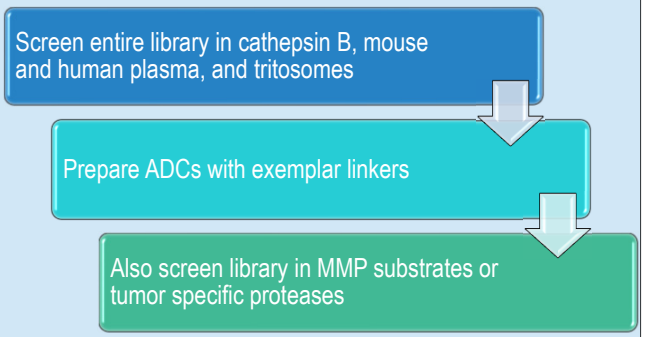



Figure 7. Future potential applications of the turn-on fluorescence assay include measuring ADC internalization and processing. ADCs with AMC mimicking the payload will be prepared. Cells will be treated with these AMC-ADCs and, using the plate reader, the internalization and processing of the ADC will be measured over time. The amount of AMC fluorescence should be proportional to the internalization and processing of the ADC.

## Further Study of Linker Stability in Different Enzymes





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## References


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