

# HDX-MS characterisation of biotinylated immunoassay conjugates

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## Introduction

Immunoassays are a key bioanalytical technique for the quantification of analytes for *in vitro* diagnostics (IVD) and pre-clinical pharmaceutical applications. Immunoassay performance is dependent on the quality of labelled antibody (Ab) conjugate used. Biotin is routinely used as an antibody-conjugate, due to its small size and high affinity towards avidin/streptavidin, however, little is known on the effects of this type of conjugation on antibody higher order structure (HOS) and ultimately the antibody-antigen interaction itself. Better understanding of changes of HOS as a result of conjugation and how this relates to immunoassay activity is a key step towards better design and optimisation of immunoassay conjugates.

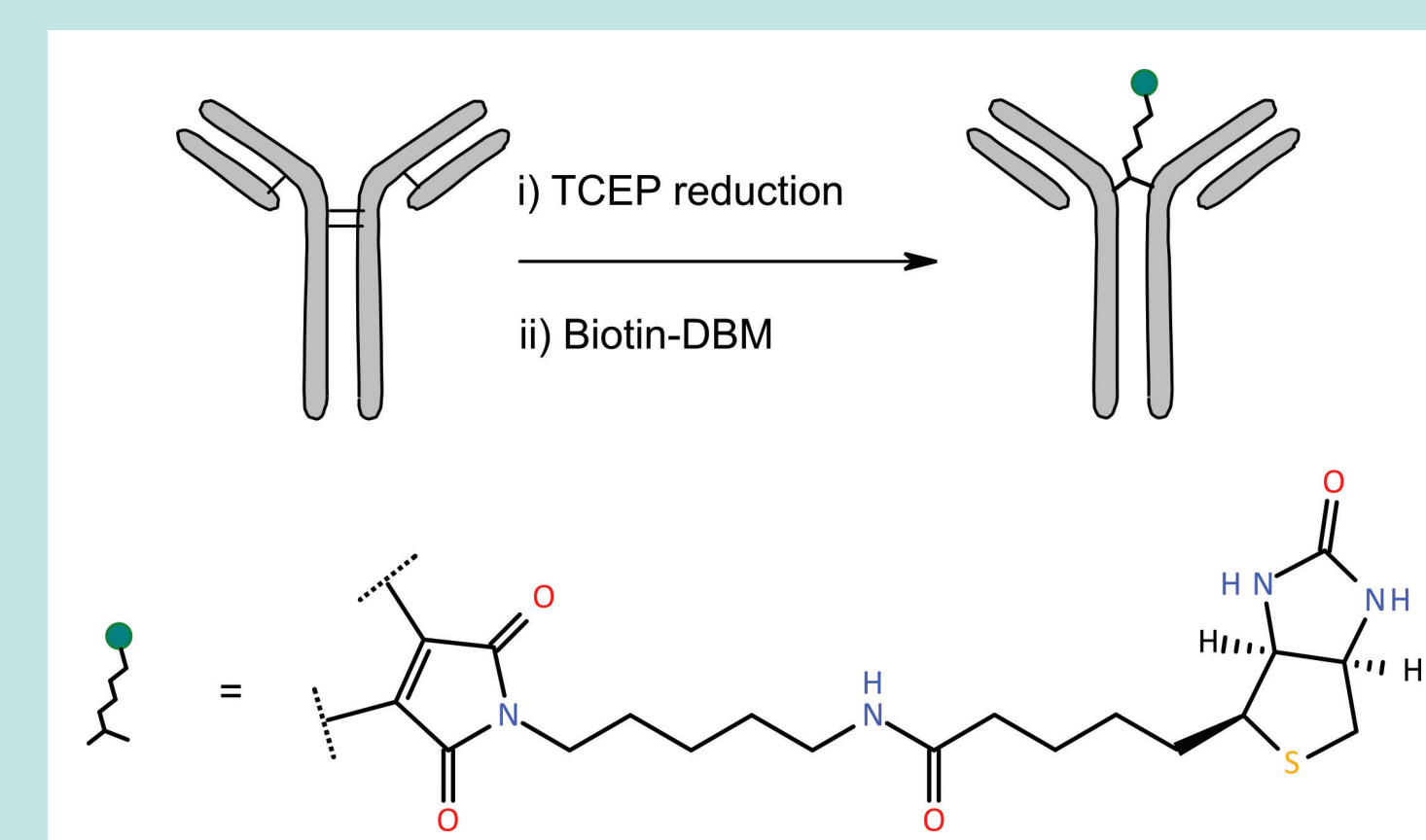


Figure 1. Site-directed antibody-biotin conjugation using Biotin-DBM

Using *site-directed biotin-dibromomaleimide (Biotin-DBM) conjugation* of cysteine residues (Figure 1), a series of biotin conjugates of the therapeutic monoclonal antibody *Herceptin*<sup>™</sup> was prepared under a range of conjugation conditions. The performance of the conjugates was assessed using two complementary immunoassays. A conjugate showing some impairment of assay performance was selected, and Hydrogen deuterium exchange mass spectrometry (HDX-MS) analysis applied to compare HOS structures with unconjugated *Herceptin*.

## Immunoassay

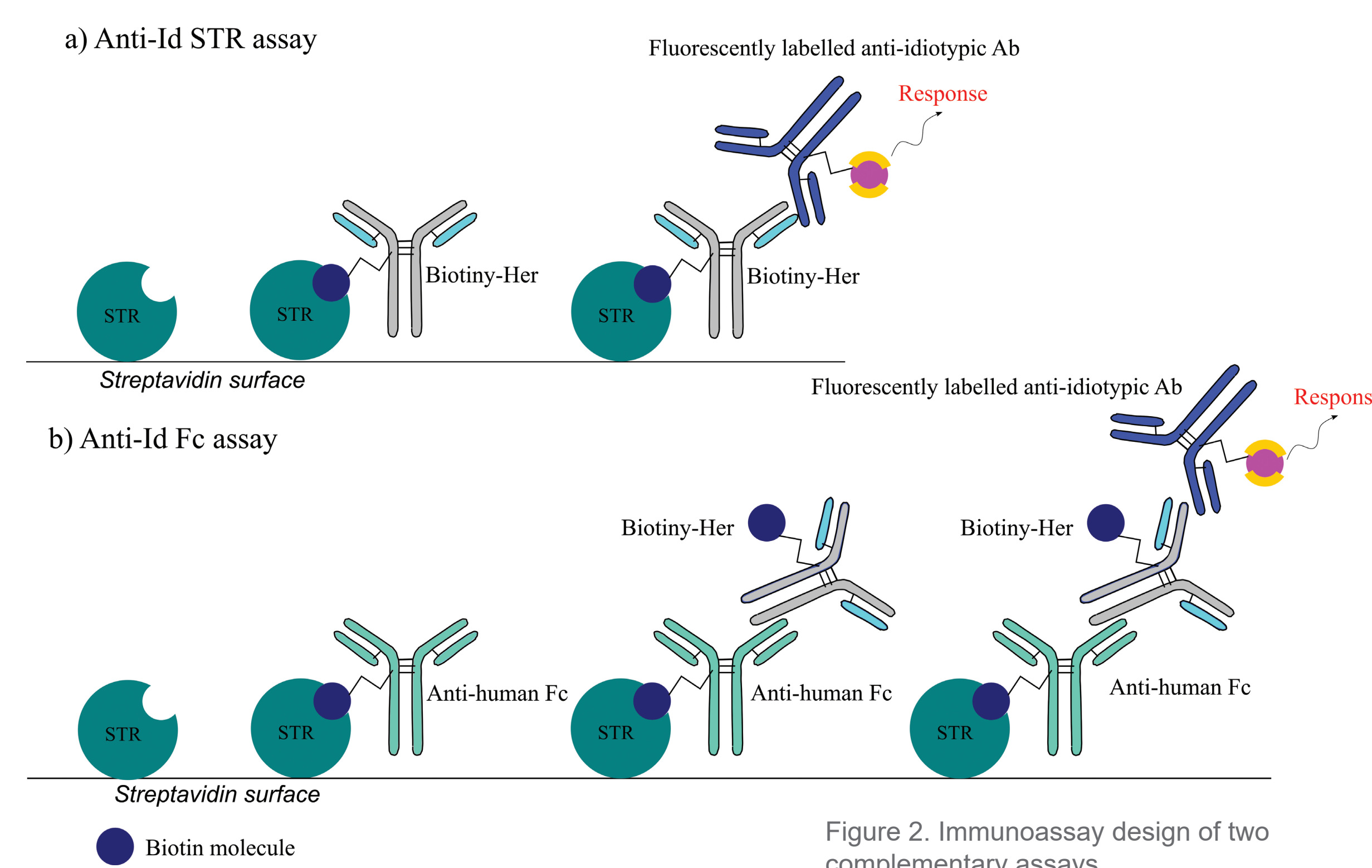


Figure 2. Immunoassay design of two complementary assays

The *Herceptin*-conjugates were employed in two different immunoassays formats using the Gyrolab<sup>™</sup> platform (Figure 2).

- 1. Biotin Capture (Anti-id STR):** conjugate is captured onto the surface by biotinylation. Detection occurs via a fluorescently labelled anti-idiotypic antibody targeting the Fab region of the conjugated antibody. **Modification of Fab region of the conjugate reduces binding of anti-idiotypic and hence reduces signal.**
- 2. Fc capture (Anti-id Fc):** conjugate is captured onto the surface via a biotinylated Anti-human Fc intermediate then detected with the fluorescently labelled anti-idiotypic antibody. **Modification of Fab region of the conjugate will reduce signal as will Fc modification due to reduced binding to surface.**

Candidate DV147/205-1 exhibited reduced signal in both immunoassays (Figure 3), indicating potential HOS damage to both the Fc and Fab regions of *Herceptin* structure. This candidate was therefore selected for further HDX-MS characterisation.

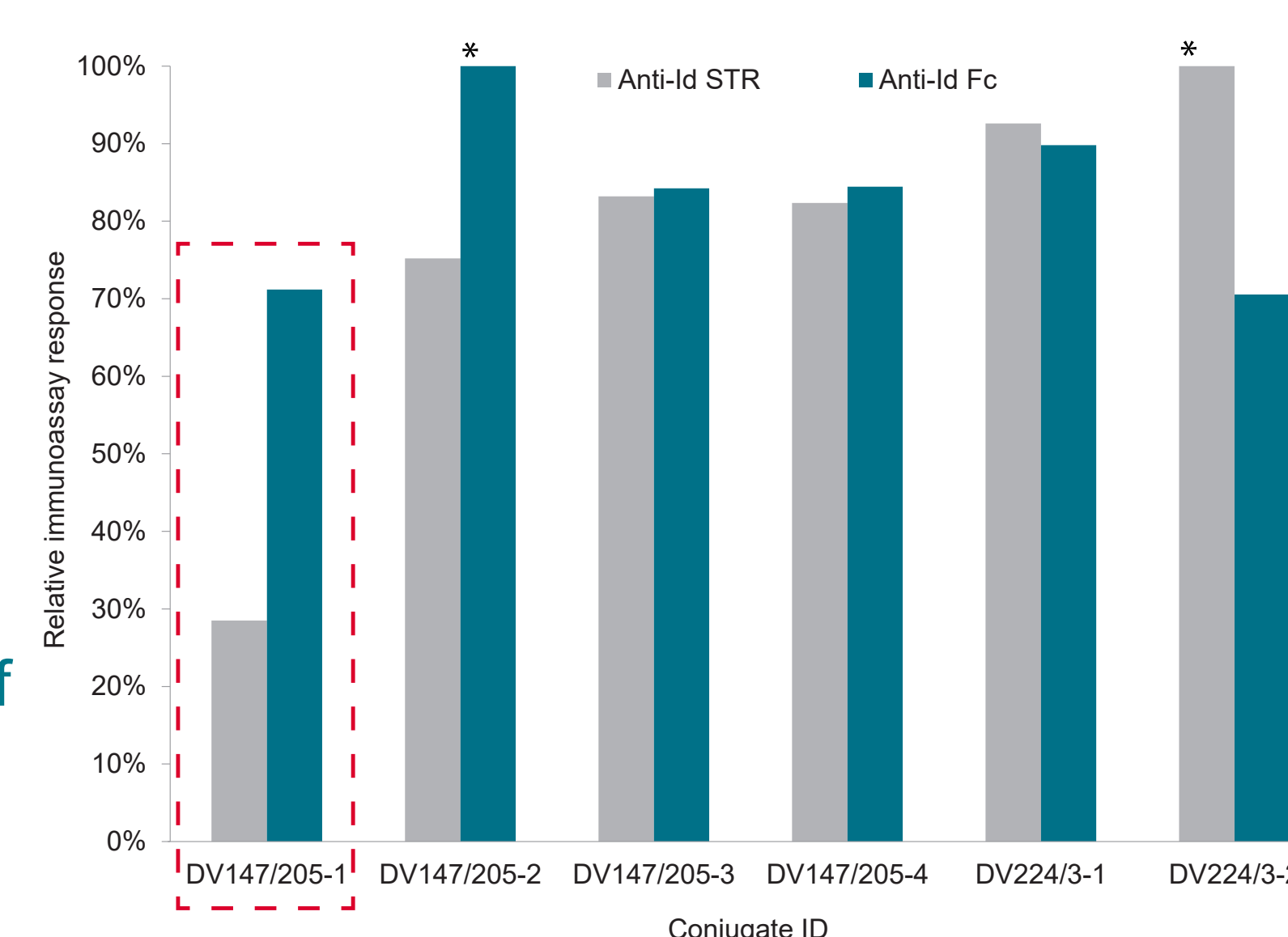


Figure 3. Relative immunoassay responses for biotin-DBM conjugates, assay responses are displayed relative to the highest responding sample indicated by \*.

## HDX-MS and conjugation characterisation

*Herceptin* and the DV147/205-1 conjugate were deglycosylated with PNGase F then subjected to mild reducing conditions (600  $\mu$ M TCEP, 2 hours, 37°C) before analysis by intact LC-HRMS.

**LC:** Thermo Scientific<sup>™</sup> Vanquish UHPLC, Thermo Scientific<sup>™</sup> MAbPac<sup>™</sup> RP, 0.5% formic, 65°C

**MS:** Thermo Scientific<sup>™</sup> Orbitrap Q Exactive<sup>™</sup> Plus in HMR mode

For DV147/205-1, biotin-DBM conjugation was observed on both the heavy and light chain plus on a series of reduced intermediates indicating heterogeneous conjugation. Higher ratios of conjugate were observed for HH containing fragments suggesting the presence of inter-chain conjugation in the hinge region. The presence of unconjugated *Herceptin*<sup>™</sup> observed in DV147/205-1 will contribute to the low relative response in Anti-STR immunoassay as only biotinylated Ab's will contribute a response in the assay.

*Herceptin* control and the conjugate DV147/205-1 were analysed by differential HDX-MS.

**Instrumentation:** Waters nanoACQUITY UPLC with HDX-MS linked to Synapt G2Si

**Quench:** 8M Urea, 1M TCEP, pH 2.5, 10 min quench hold

**Digestion temp:** 15°C Incubation points: T = 0, 5, 30, 60, 4hrs

Multiple regions of increased uptake (highlighted in red Figure 5), both in the Fc and Fab regions, were identified in the DV147/205-1 conjugate relative to the *Herceptin* control. No regions of reduced uptake were observed suggesting overall a decrease in HOS as a result of conjugation.

In particular, accelerated HDX was observed for the sequence 244-254 located in the C<sub>H2</sub> domain, which has previously been identified as linked to destabilisation of other IgG1 based therapeutics as a result of oxidation<sup>3</sup> and deglycosylation<sup>4</sup>.

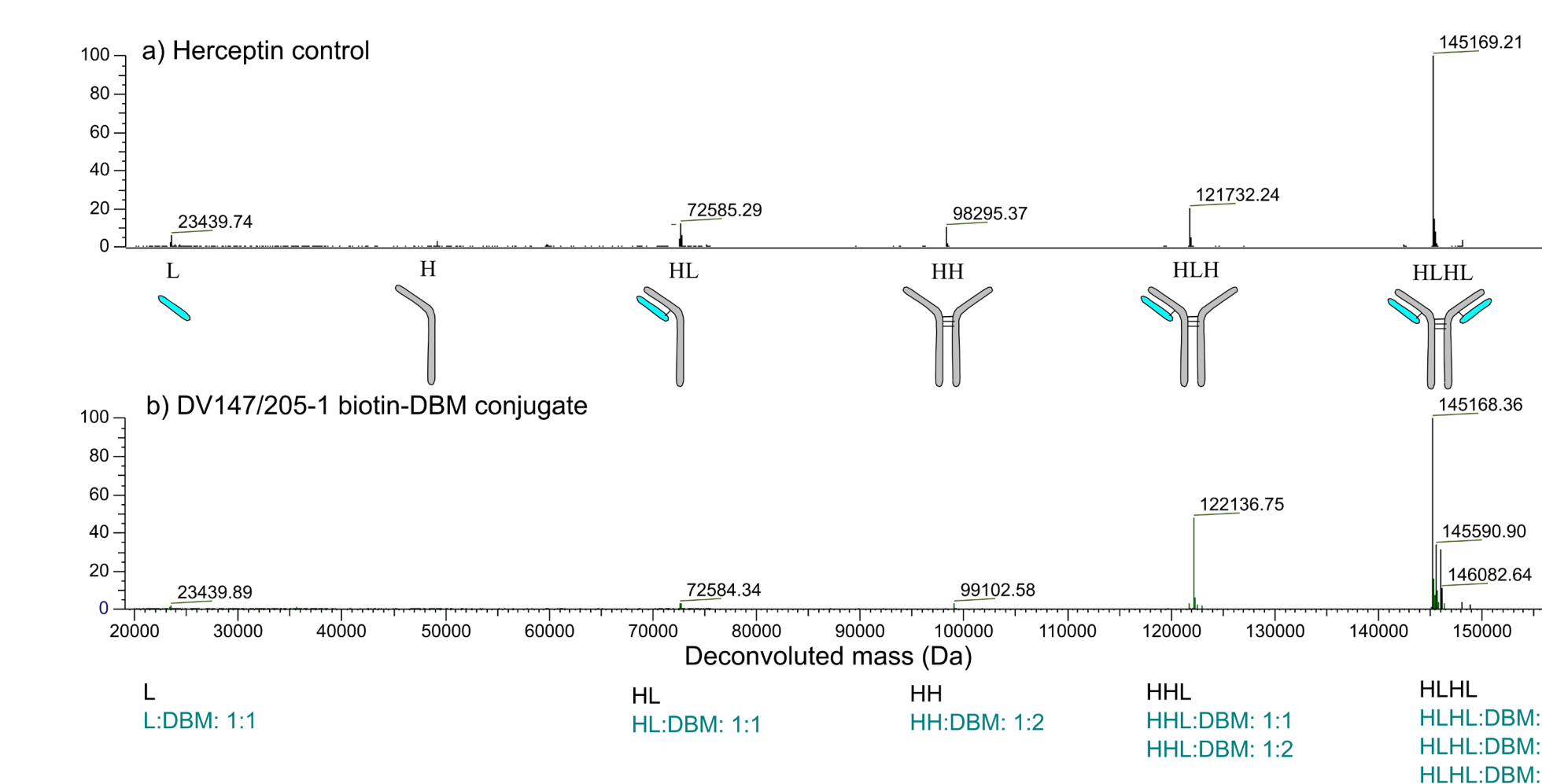


Figure 4. Deconvoluted spectra of partially reduced samples. H and L denote heavy and light chain structures respectively, the presence of biotin-DBM conjugates is indicated where present.

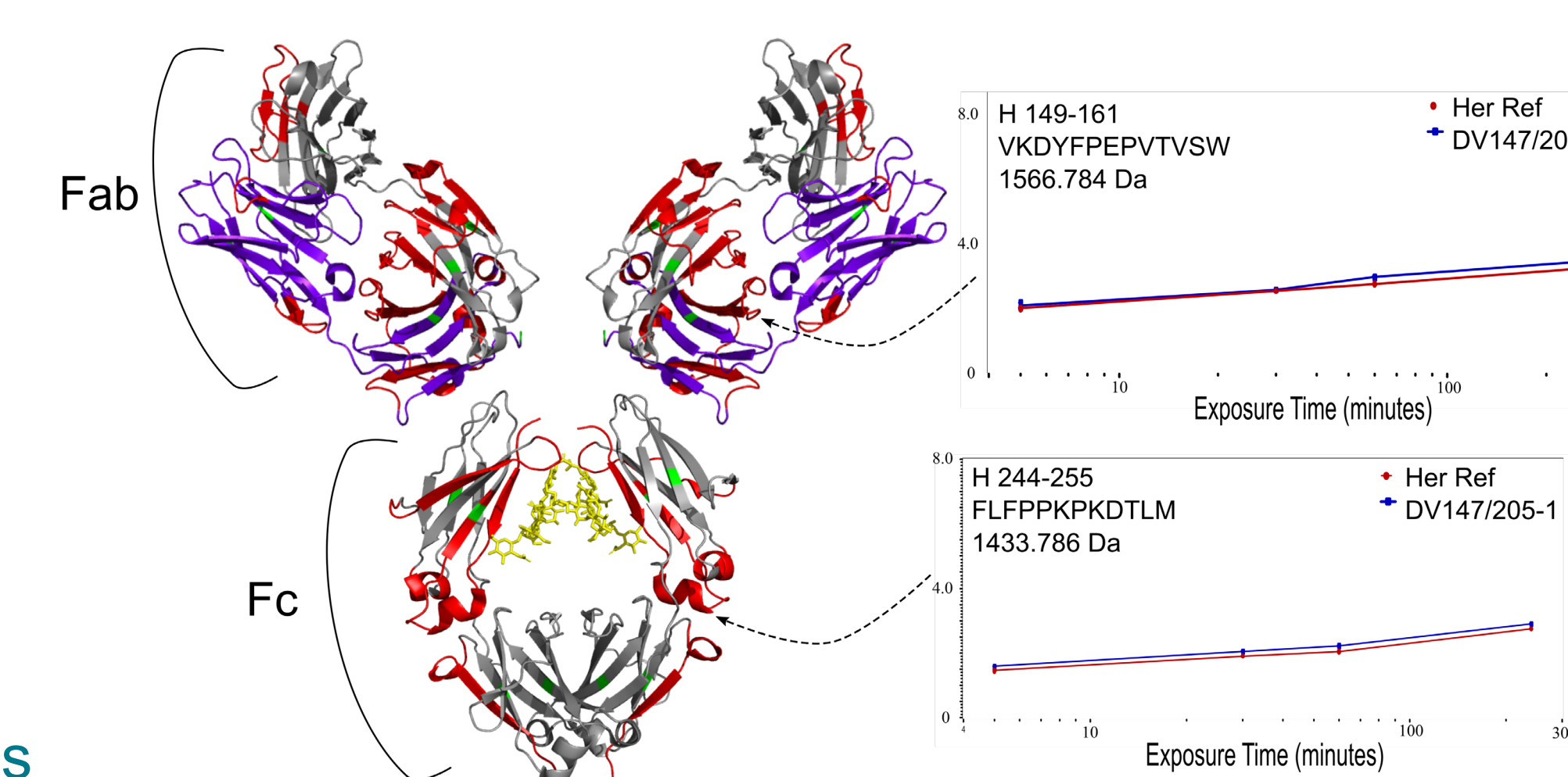


Figure 5. Regions of increased HDX-MS uptake (highlighted in red) identified as a result of conjugation of *Herceptin*, as mapped on to the crystal structures (PDB: Fab 1n8z<sup>1</sup>, and Fc 3D6G<sup>2</sup>). Unchanged heavy and light structure is indicated in grey and purple respectively. Cysteine sites are highlighted in green.

## Conclusions/future work

Reduced immunoassay performances were observed for the DV147/205-1 conjugate indicating structural change in both Fc and Fab regions of *Herceptin* structure. HDX-MS analysis corroborated this, indicating changes in both these regions.

HDX-MS identified multiple regions of loss of HOS for the DV147/205-1 conjugate. This reflects the heterogeneous nature of conjugation as identified by HRMS analysis of Ab fragments.

HDX-MS epitope mapping experiments of the HerAb-HER2 complex, using the *Herceptin* control and DV147/205-1 conjugate will be performed to understand how conjugation has altered binding to the drug target HER2 and relate HOS changes to antibody-antigen interactions.

## References

- Cho, H. S, *et al*, 2003, *Nature* 421, 756-760 | 2. Moiani, D. *et al*, To be published | 3. Zhang, A, *et al*, 2014, *Anal. Chem.* 86, 3468-3475 | 4. Pan, J. *et al*, 2016, *Chem Sci.* 7, 1480-1486