# Early target and functional affinity insights guide candidate selection during lead optimization

## Accelerated Trastuzumab clone selection by early all-in-one insights into yield, Fab & functional Fc affinity, confirmed by structural data

White paper 1 | Version 1 | Hannah Dewerchin[1], Thomas Martens[2], Sarah Parmentier[1], Filip Delport[1], Koen Sandra[2]

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## Abstract

Monoclonal antibody (mAb) therapeutics, such as Herceptin (commercial name Trastuzumab), remain powerful tools in various cancer therapies, especially in HER2 positive breast cancer. Alternative clones to Trastuzumab are still in development in the continued rapidly growing market of biosimilars, as economically it allows for fast-tracked validation and NDA submission. However, lead optimization of the clone candidates still involves iterative and costly processes for design, production, purification and characterization. With a combined study from Vixen Bio and RIC Biologics on Trastuzumab clones, we show an all-in-one data-rich approach to reduce optimization rounds by early pivotal insights into antibody yield, target binding efficacy, functional potency, stability and structural integrity. This allows us to select only the highest performing candidates for in-depth analysis and avoid late-stage failures.

## Introduction

Trastuzumab is the originator monoclonal antibody therapeutic targeting Human Epidermal Growth Factor Receptor 2, otherwise known as "HER2". HER2 is a cell transmembrane receptor that is expressed on many different tissue cells. Its main function is initiating signaling pathways within the cell that stimulate cell proliferation. On specific tissue cells it is overexpressed and serves therefore as a powerful predictive marker and therapeutic target for immunotherapy in breast, ovarian, and gastric cancers. Trastuzumab specifically binds to the extracellular region (domain IV) of the HER2 receptor and interferes with the signaling pathways, such as MAPK, that drives cell proliferation and survival, therefore interfering with the oncogenic functions and even provoking immune clearance responses against overexpressed HER2 cells [1,2]. For biosimilar candidate development it's vital that the same biological functions hold through the lead optimization phases. Comparability to the originator is even mandatory in the regulatory filing and can impact the clinical trial design.

Where the optimisation decisions are usually tackled during separate phases for all characteristics – Fab target, Fc-receptor binding potency, and structural analysis a risk lies in missing good candidates or ones that showcase lower performance in later development.

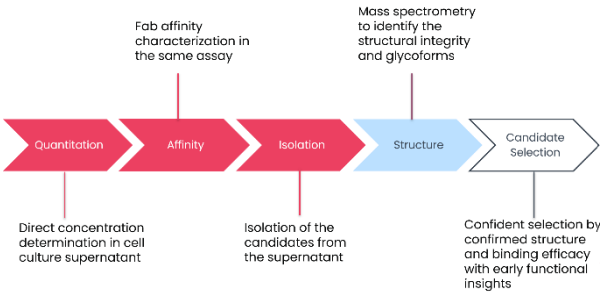


*Figure 1: Workflow and benefits of the Lead Optimisation assay*

In this combined study on Herceptin variants, RIC and Vixen tested 4 different clones against the originator and a degraded version in an alternative data rich method that allows **to assess yield while confirming Fc receptor and Fab target binding affinity**. The build-in isolation enables an integrated process for **direct total structural MS** analysis on the lead antibodies. These early insights into biological activity showed that hidden Post Translational Modifications (PTM's) impacting functional potency can be identified early on instead of in later phases only. (Figure 1)

## Methodology

In an integrated workflow, protein G sensor probes are applied to quantify 4 Herceptin clones and degraded Herceptin versus the originator in the supernatant of CHO medium. This step also provides early insights into Fc-functionality changes by the real time monitored Fc capture method. This is followed by a HER2 protein binding step to assess the Fab region affinity and to rank the candidates in target binding performance. In a last step the sensor probes isolated through multiple cycles of capture and release the produced antibodies for total structure analysis by mass spectrometry (MS) by RIC. (Figure 2)

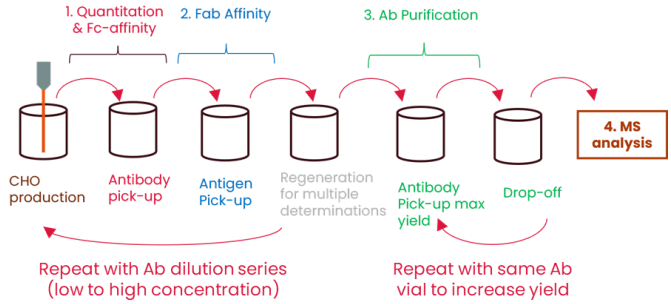


*Figure 2: Assay methodology for quantification, affinity and isolation by Vixen Bio and MS analysis by RIC.*

1. Antibody quantification and Fc affinity

A calibration curve was constructed by capturing purified Herceptin on protein G sensors in a 6-point serial dilution series spiked into blank CHO supernatant (0.19 to 6 µg/ml) in duplo. The calibration curve was obtained by linear regression of the label-free association endpoints (Figure 3). The mAb clones of interest were determined directly from the CHO supernatant by a blind dilution series. The obtained concentrations were compared to a UV280 measurement (data not shown). Both Herceptin and clone 8 were also quantified with an anti-human IgG sensor (anti-hIgG).

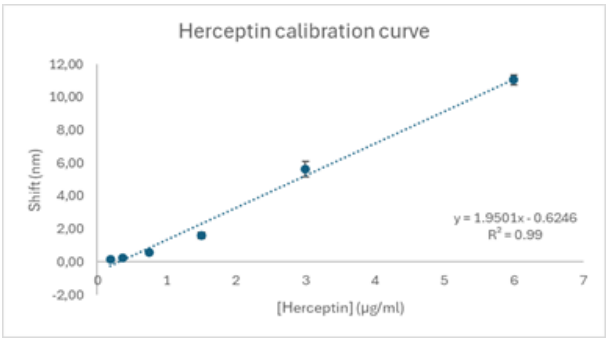


*Figure 3: Calibration curve by linear regression of purified Herceptin spiked in blank medium*

2. Antibody affinity

The antibody captured on the sensors were dipped in a solution containing a fixed concentration of HER2 to measure association and dissociation rates. The kinetic profiles were fitted with a bivalent model using the measured concentrations resulting in $k_{on}$, $k_{off}$ and $K_D$ values.

3. Antibody purification

Purification of antibodies was performed via multiple drop-off cycles from crude medium. Regeneration demonstrated high repeatability (20 cycles) and successful recovery of antibody for downstream MS.

4. MS analysis:

Isolated antibodies were desalted via Reverse Phase Liquid Chromatography (RPLC) and analysed on a Q Exactive Plus Orbitrap MS system (Size exclusion chromatography -MS) for total structure analysis. Degraded Herceptin was further studied on mutations by liquid chromatography – mass spectrometry (LC-MS) based peptide mapping.

## Results

**Concentration analysis & Fc affinity**

The measured Herceptin concentration was identical to the expected value. The Herceptin clones showed higher concentrations when protein G was used but not when anti-human IgG probes were used. The specific region of binding by our biosensor revealed that the clones have distinct differences in affinity towards the protein G

sensor compared to an anti-hIgG capture of Herceptin. Protein G binds specifically on the CH2-CH3 hinge region on the Fc heavy chain of antibodies [3-6]. This is the same region that the e.g. FcRn-receptor on lymphocytes, FcγRIIa on myeloid cells, FcγRIIIa on natural killer cells, or the complement pathway will recognize that binds to the Fc-region of the therapeutic antibody. Affinity differences in this region caused by post translational modifications (PTM's) can thus highly impact the functional effect and half-life of the antibodies.

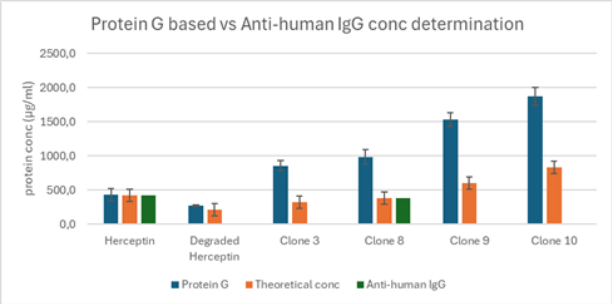


*Figure 4: Concentration determination of samples, obtained by protein G compared to anti-hIgG capture for Herceptin and clone 8.*

**Kinetic Fab affinity analysis**

The following kinetic profiling of mAb's Fab binding to HER2 (Figure 5) showed that all clones have a comparable $k_{on}$, $k_{off}$ and $K_D$ values compared to Herceptin, as seen in the On-/Off-rate map (Figure ). Clone 9 shows less affinity maturation compared to the other clones. The degraded version exhibited reduced affinity caused by a significant higher $k_{off}$, indicating that affinity can be applied as measure for stability validation.

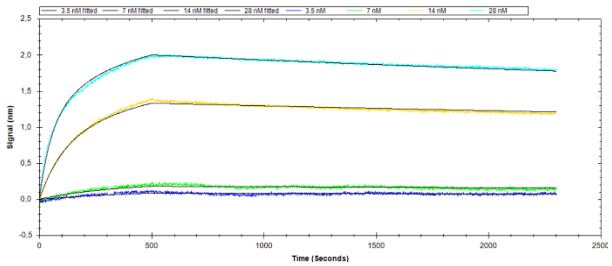


*Figure 5: Bivalent fit of Herceptin real-time binding curves, resulting in $k_{on}$, $k_{off}$ and $K_D$ analysis.*

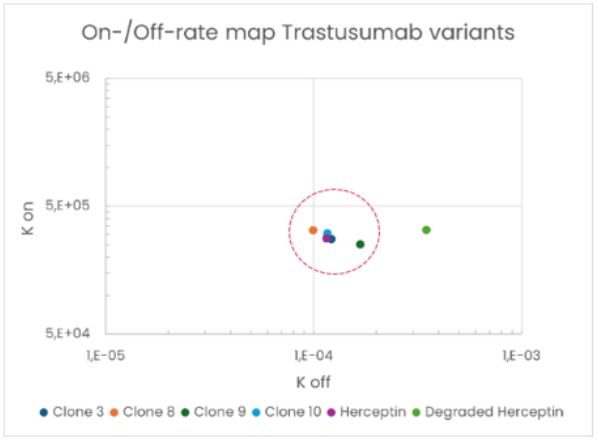


*Figure 6: On/Off rate map showing the target binding affinity of Herceptin, the clones (circle) and degraded Herceptin (light green).*

**Built-in Isolation for downstream analysis**

The 20 pick-up cycles at the end of the assay showed a high repeatable maximum endpoint shift for all clones. An average accumulative yield was achieved of 350 ng per clone, sufficient for MS. Additional pick-up cycles can be included to increase the yield if required by the downstream analysis method. (Figure 7)

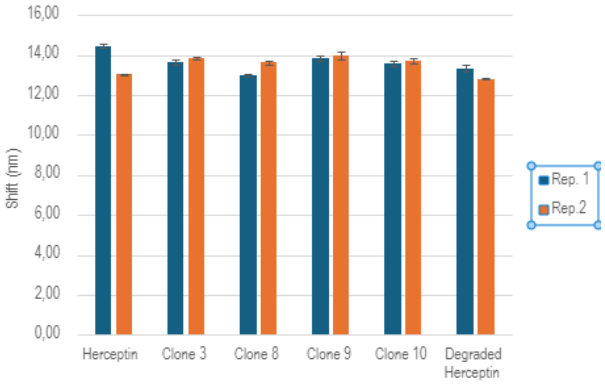


*Figure 7: Average endpoint shifts of the candidates during each cycle of isolation on White Fox. Repeats are separate sample preparations and probes.*

**Mass Spectrometry Analysis**

The isolated mAb's were successfully analysed by MS thereby confirming molecular weight, glycosylation pattern and structural integrity.

Differences in Fc glycosylation were revealed between Herceptin and the study samples: a shift from G1F/G0F to G0F/G0F forms indicate less galactose glycosylation. This can explain the change in affinity towards protein G and can result in changes of Fc tail functionality, like FcRIIa, FcRIIIa, FcRn affinity and complement activation which will determine effector cell activation, function and antibody half-life in the patient. Lower affinities inadvertently increase the need for higher dosing and therefore increase safety risks.

Degraded Herceptin could not be differentiated from intact Herceptin by MS at intact mAb level. Peptide mapping on isolated mAb revealed increased isoaspartate levels in the Complementary Determining region 3 (CDR3). This modification impacts HER2 binding, resulting in higher $k_{off}$ as observed by Vixen Bio. Having affinity data available before running MS analysis will ensure all the proper testing can be done or that the right candidates can be excluded from following optimisation rounds. (**Error! Reference source not found.**)

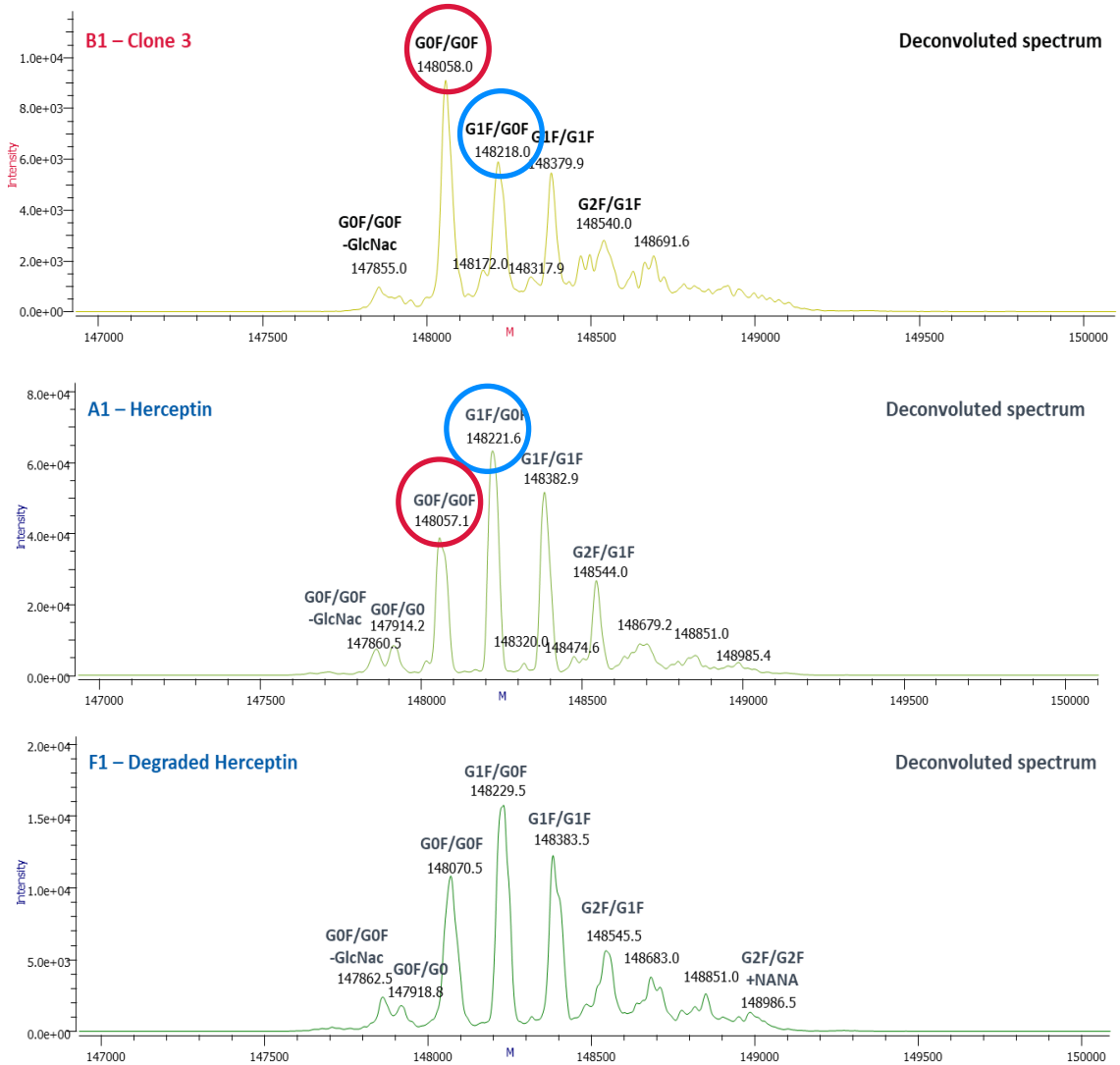


*Figure 8: Deconvoluted spectra by SEC-MS on Clone 3, Herceptin and degraded Herceptin, where clone 3 shows a shift from G1F/G0F towards G0F/G0F indicative for less galactose glycosylation. Degraded Herceptin cannot be distinguished from Herceptin in total MS and required additional peptide mapping.*

## Conclusion

This methodology enables a streamlined mAb development workflow from purification to comprehensive early-stage characterization across all functional domains. This integrated approach provides robust, data-driven insights into antibody performance. By delivering complete functional profile insights at an early stage, it significantly increases the understanding of your designs and processes and thus reduces the number of candidates advancing and allows for less and smarter optimization rounds, effectively de-risking the lead optimization process. As a result, only the highest-performing candidates are selected for further in-depth physicochemical analysis, leading to substantial cost savings. At the same time, early detection of hidden functional PTMs minimizes late-stage surprises and enables more targeted optimisation, ultimately maximizing return on R&D investment.

## References


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## Acknowledgements


The authors are grateful to RIC Biologics for collaborating in this study and providing the samples and mass spectrometry data.