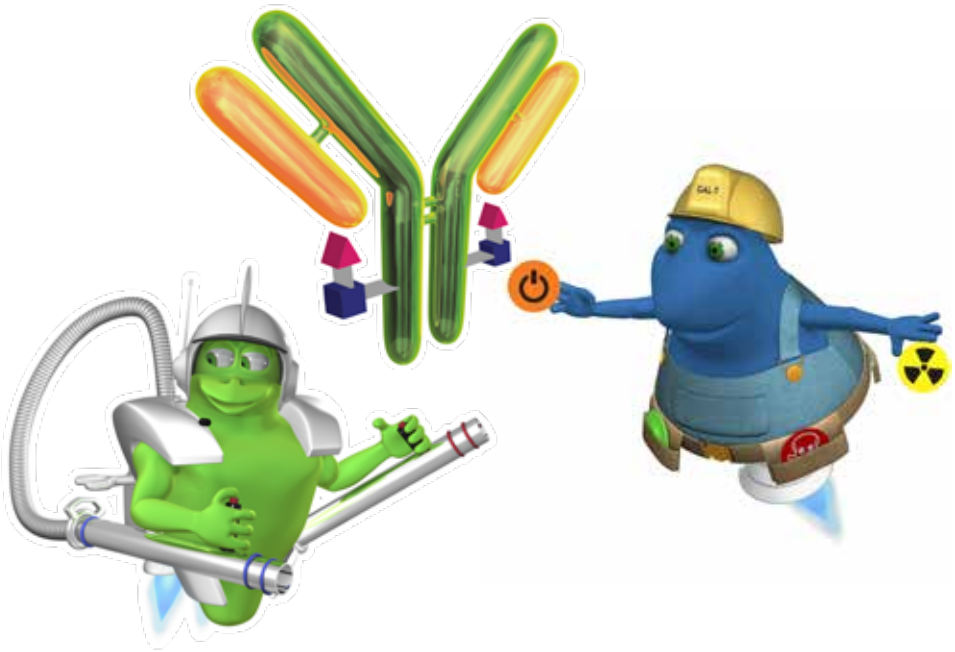
GlyCLICK®



Site-specific Antibody Conjugation Technology

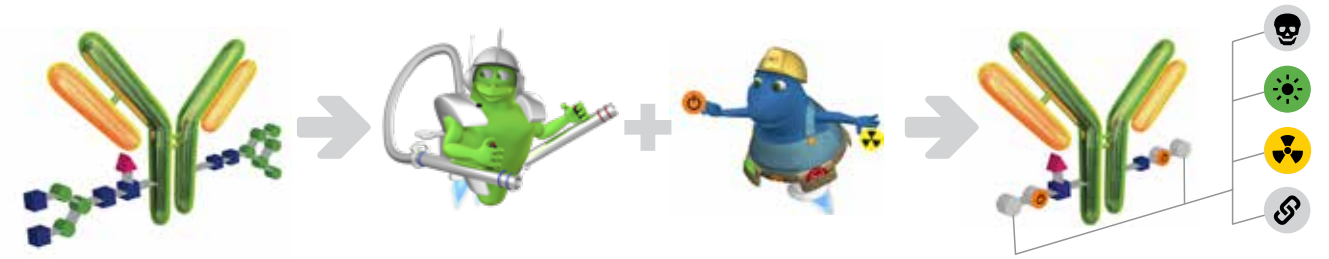
SmartEnzymes™

GENOVIS

SmartEnzymes™

GENOVIS

GlyCLICK®



GlyCLICK® is a site-specific conjugation technology for IgG using enzymatic remodeling and click-chemistry.

GlyCLICK is a simple three-step technology that enables quantitative incorporation (conjugation) of functional groups at the Fc-glycan sites of IgG from several species and subclasses (*Fig. 1*). The technology utilizes the Fc-specific endoglycosidase GlycINATOR® for Fc-glycan remodeling. This is combined with biocompatible click-chemistry for complete and robust labeling of the target antibody. GlyCLICK is highly specific and incorporates two labels per IgG, a degree of labeling (DOL) of 2. This results in stable and homogenous antibody conjugates suitable for sensitive applications.
The GlyCLICK technology is available in various kit formats to facilitate tailored and site-specific labeling of antibodies for a diverse range of applications. Available formats are the Azide activation kit for conjugation using custom cyclooctyne-functionalized labels, and kit formats with sDIBO-labels: AlexaFluor®488, Biotin or Deferoxamine (DFO).

Human IgG1-4, IgG from mouse, rabbit, rat, monkey, sheep, goat, cow and horse

Available conjugates: Alexa Fluor®488, biotin and DFO. Azide activation kits are available for custom conjugation

Available GlyCLICK formats and sDIBO labels

Kit format	Kit size	Name	Examples of Applications
Fluorophore	250 µg	AlexaFluor® 488	*In vitro* imaging, IHC, FCM
Affinity	250 µg	Biotin	Immuno assays, ELISA, western blot
Chelator	250 µg	Deferoxamine	*In vivo* immuno imaging
Azide activation	250 µg 2 or 10 mg	No label	Custom conjugation

GlyCLICK conjugation overview

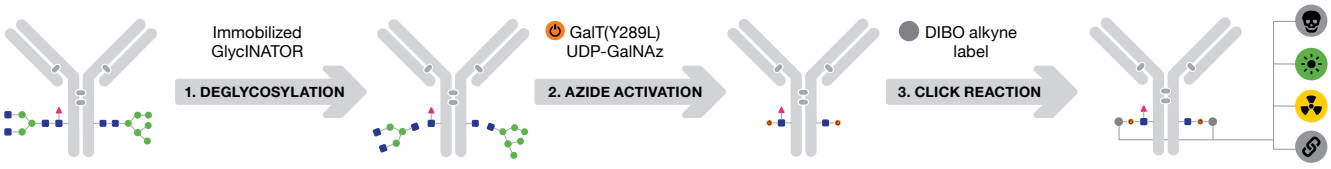


Figure 1. Schematic presentation of the GlyCLICK conjugation process.

1. **Deglycosylation**
   The IgG-specific endoglycosidase GlycINATOR (EndoS2) digests Fc-glycans, exposing the core GlcNAc. The enzyme removes all glycoforms, including high-mannose, hybrid, complex, and bisecting type glycans.

2. **Azide activation**
   Azide-containing UPD-GalNAz is enzymatically attached to the exposed GlcNAc at the Fc-glycan sites by the enzyme β-1,4-Galactosyltransferase, GalT(Y289L), making the antibody azide-activated for conjugation.

3. **CLICK reaction**
   Azide-activated antibodies are conjugated at the Fc-glycan sites using a DBCO or sDIBO-label through Strain-Promoted Copper-free Click-Chemistry (SPAAC), resulting in a stable attachment of one label on each Fc/2.

GlyCLICK characteristics

Site-specific GlyCLICK conjugation

GlyCLICK allows quantitative and complete labeling with a constant degree of label (DOL=2) for reproducible results. Trastuzumab was labeled with AlexaFluor®488 using the GlyCLICK kit (T-GlyCLICK-AlexaFluor®488) and digested with FabRICATOR® to evaluate the specificity of the conjugation process. The resulting F(ab')2 and Fc/2 fragments were separated using RP-HPLC with the fluorescent signal detected for the Fc domain only (*Fig. 2*), indicating site-specific conjugation.

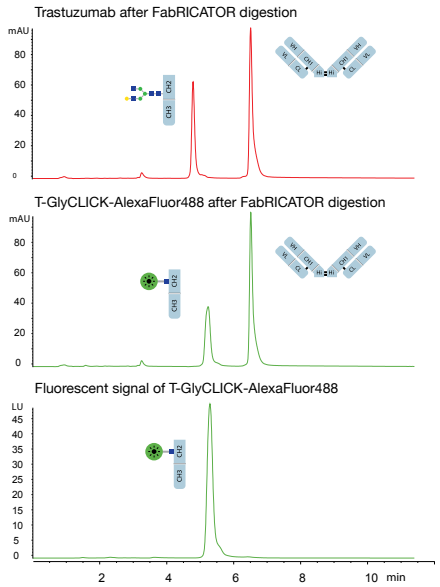


Figure 2. RP-HPLC analysis of trastuzumab performed on an Agilent 1290 using Waters Acquity UPLC® BEH C4, 1.7 µm, 2.1x100 mm column in an acetonitrile/isopropanol gradient at 65°C.

Modular technology

GlyCLICK is a versatile and scalable tool for the conjugation of IgG with a selection of labels and functional groups such as dyes, affinity tags and chelators. For example, stable conjugation of chelating agents and toxins is possible (*Fig. 3*). In addition, any custom label or payload with suitable click-chemistry can be conjugated using the Azide activation kit. All conjugates will have the same reproducible mode of incorporation, independent on the label or payload.

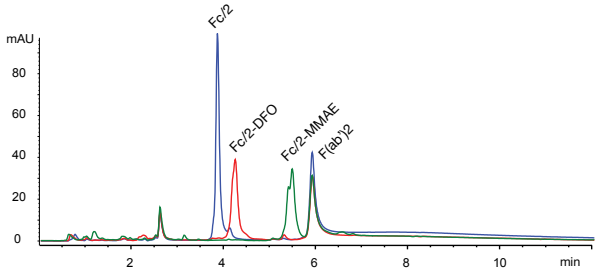


Figure 3. RP-HPLC separation of panitumumab (Vectibix®) unmodified and conjugated with a DFO chelator or MMAE toxin (Monomethyl aurostatine E) after digestion with FabRICATOR to F(ab')2 and Fc/2 fragments. The analysis was performed on an Agilent 1290 using Waters Acquity UPLC® BEH C4, 1.7 µm, 2.1x100 mm column in an acetonitrile/isopropanol gradient at 65°C.

Preserved affinity

Site-specific conjugation at the Fc-glycan sites ensures intact immuno-reactivity by preserving the antigen-binding capability of the antibody. Surface Plasmon Resonance (SPR) was performed on trastuzumab with various GlyCLICK conjugates as compared to random conjugates carrying the DyLight™488 label. The results show overlapping curves between the GlyCLICK conjugates and trastuzumab (*Fig. 4*). Although the affinity is unchanged for randomly conjugated material, the level of binding is severely impared.

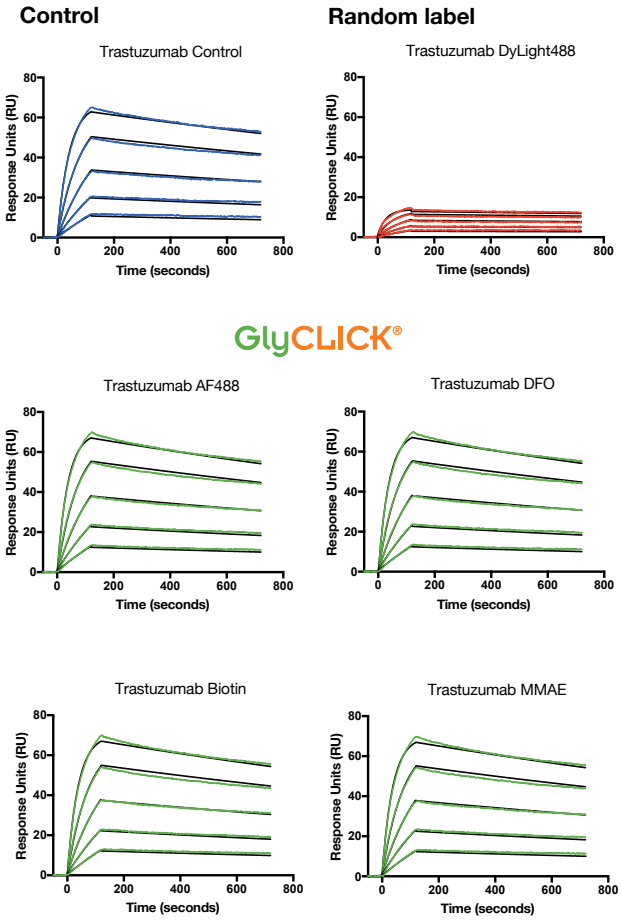


Figure 4. Affinity analysis of native and conjugated trastuzumab. Anti-human IgG (Fc) was used as the capturing molecule for trastuzumab, random DyLight™488 (DAR=10) conjugates and GlyCLICK conjugates: AlexaFluor®488, DFO, Biotin and MMAE (DAR=2). HER2 was injected at a range ensuring sufficient curvature. All data was fitted against a 1:1 mathematical model.

Fluorescent imaging applications

The modular and site-specific technology in GlyCLICK allows the generation of tailored conjugates suitable for fluorescent imaging applications such as *in vitro* cell imaging, immunohistochemistry (IHC) and flow cytometry (FCM).

Fluorophore
In vitro cell imaging, IHC, FCM

Azide activation
Custom conjugation

High-affinity binding for flow cytometry applications

Fluorescent antibodies are valuable tools in qualitative and quantitative analysis using Flow Cytometry (FCM). Indirect detection methods require several incubation steps and secondary antibodies are a potential source of unsolicited background staining due to unspecific binding or cross- reactivity. The performance of directly labeled antibodies conjugated using GlyCLICK was evaluated by FCM (*Fig. 5*). Trastuzumab was labeled with AlexaFluor®647 (T-GlyCLICK- AlexaFluor®647) or Cy5 (T-GlyCLICK-Cy5) using the GlyCLICK technology for direct detection of HER2(+) cells and analyzed using FCM. An indirect detection method was also evaluated using a commercially available labeling method optimized for FCM analysis. The results show that the GlyCLICK conjugates provide a 10-fold better separation index (SI) due to the lower background signal when compared to the indirect detection method.

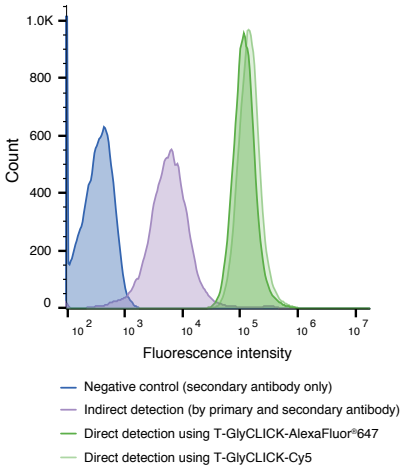


Figure 5. Flow Cytometry (FCM) of HER2(+) cells (MDA-MB-435) treated with human Fc blocking mix (Miltenyi Biotech) and incubated with GlyCLICK conjugates or mouse primary antibody (3.7 ug/ml) for 30 minutes. Cells incubated with mouse primary antibody were incubated with secondary donkey anti-mouse antibody solution (1:200) for 30 minutes. Resuspended (PBS+ 1% BSA) cells were then analyzed using a CytoFlex flow cytometer.

Site-specific fluorescense labeling using GlyCLICK

The GlyCLICK technology generates site-specific quantitative conjugation (*Fig. 6*) that enables direct detection of cells with a single incubation step and increased flexibility in multiplexing experiments.

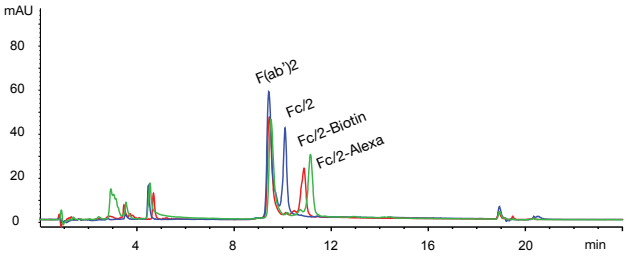


Figure 6. RP-HPLC separation of Mouse IgG2a unmodified and conjugated with Biotin or AlexaFluor®488 followed by digestion with FabRICATOR Z into F(ab')2 and Fc/2 fragments. The analysis was performed on an Agilent 1290 using Waters Acquity UPLC® BEH C4, 1.7 µm, 2.1x100 mm column in an acetonitrile/isopropanol gradient at 65°C.

High-quality *in vitro* cell imaging using GlyCLICK

The site-specific GlyCLICK technology enables quantitative conjugation for the detection of cells and increased flexibility for multi-staining analyses in fluorescent imaging. Confocal microscopy of cells with HER2 high (+) and low (-) expression was performed using a direct detection method with GlyCLICK-conjugated trastuzumab-AlexaFluor®647 and indirect detection using secondary labeled antibodies optimized for imaging applications (*Fig. 7*). The site-specific GlyCLICK conjugates provided high-quality images with distinct cell membrane labeling. Direct detection using GlyCLICK conjugates also showed a high signal-to-noise ratio superior to that of indirect labeling.

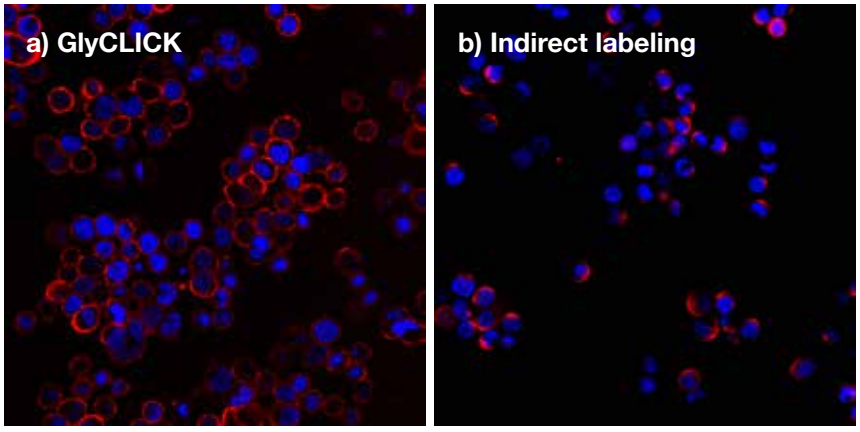


Figure 7. Cells fixated with PFA (2%) were incubated with GlyCLICK conjugated antibodies (1 µg/ml) for 1 hour **(a)**, or mouse primary antibody (0.2 µg/ml) for 1 hour and donkey anti-mouse secondary antibody (1:200) for 30 minutes **(b)**. Cells were stained with DAPI (0.2 µM) for 2 hours. Confocal microscopy was performed using the detection threshold obtained from a control sample.

Quantitative conjugation for histological imaging

The quality and affinity of the conjugated antibody is crucial in IHC methods in order to obtain reliable and reproducible results. Since GlyCLICK generates conjugates with a degree of label that equals two and preserves full antigen binding affinity, reliable and quantitative results in sensitive imaging applications can be achieved. GlyCLICK was used for site-specific conjugation of trastuzumab-AlexaFluor®647. Data was compared to a standard commercial kit for random labeling using LC-MS (*Fig. 8*). GlyCLICK conjugates displayed a constant DOL of 2 compared to the heterogenous pool of conjugates generated by the random labeling approach.

The labeled antibodies (*Fig. 8*) were used for imaging with confocal and wide-field epi-fluorescense microscopy in HER2(+) human breast tissue to assess the performance of the different conjugates (*Fig. 9*). The GlyCLICK site-specific labeled antibody allowed for detection with confocal and wide-field imaging of highly expressed antigens with good visualization of distinctly labeled membranes in the tissue sections.

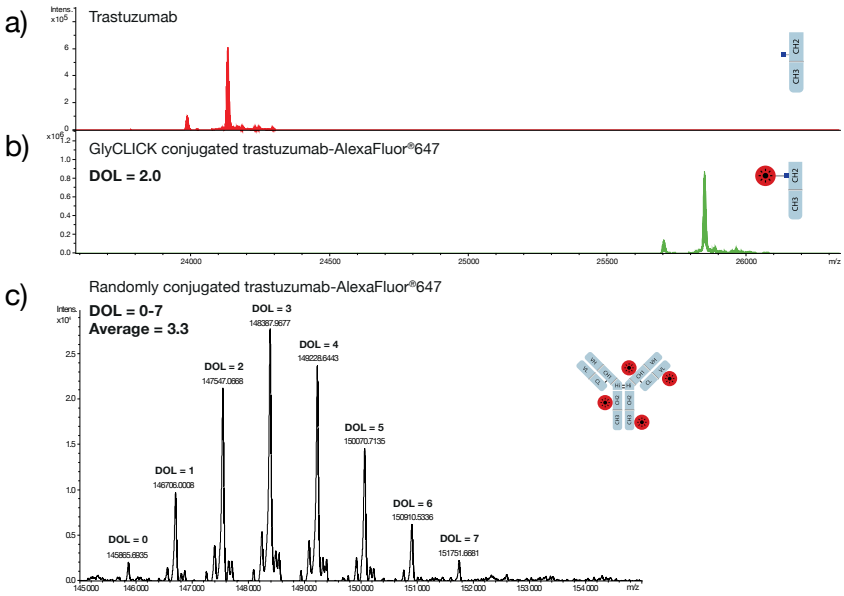


Figure 8. LC-MS analysis of GlyCLICK and random antibody conjugates. **a)** Fc subunit analysis by LC-MS after digestion with FabRICATOR of trastuzumab, deglycosylated to the inner GlcNAc using GlycINATOR, **b)** Fc subunit analysis by LC-MS after digestion with FabRICATOR of T-GlyCLICK-AlexaFluor®647, **c)** Intact mass analysis of randomly conjugated trastuzumab-AlexaFluor®647. All samples were separated by PR-LC on Protein BEH C4 column (Waters) and analyzed on a Bruker Impact II ESI Q-TOF. Mass spectra were deconvoluted using the MaxEnt algorithm.

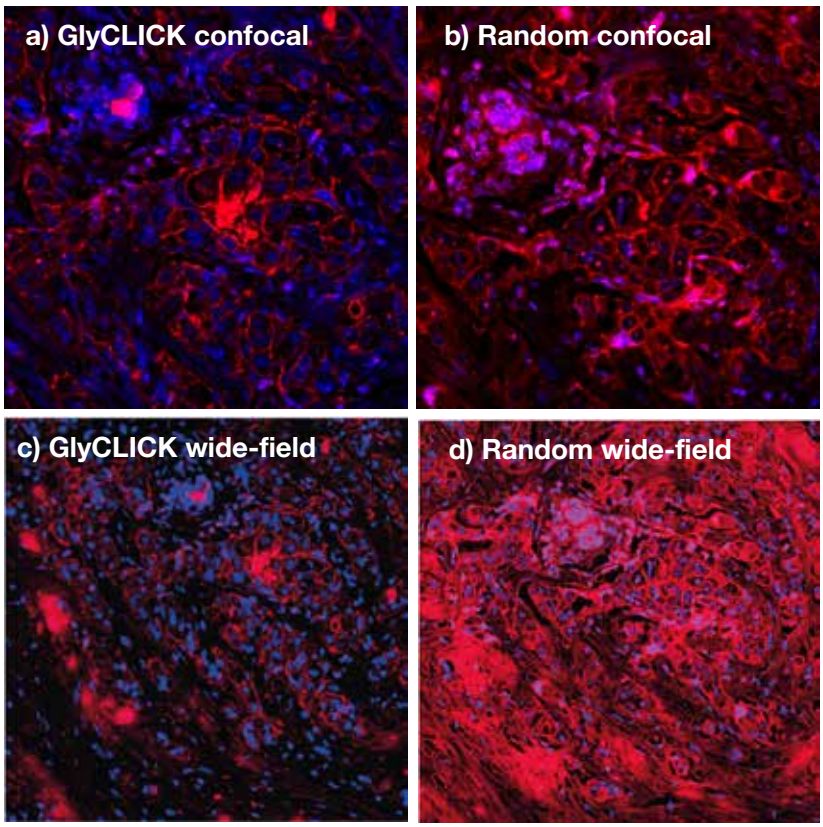


Figure 9. Confocal **(a, b)** and wide-field epi-fluorescense microscopy **(c, d)** of paraffin embedded HER2(+) human breast tissue with trastuzumab-AlexaFluor®647 conjugates using GlyCLICK and random labeling (10 ug/ml). The HIER processed tissue was blocked (BSA) and incubated with GlyCLICK and random conjugated antibodies (10 µg/ml) for 90 minutes prior to DAPI incubation (0.2 µM) and mounting in antifade solution.

In vivo immuno imaging

Since GlyCLICK is site-specific and gives quantitative labeling at the Fc-glycan sites, conjugates give reproducible results in imaging applications. They retain full affinity and display increased tumor uptake *in vivo*, with low batch-to-batch variations.

Chelator (DFO)
In vivo immuno imaging

Azide activation
Custom conjugation

Site-specific and homogenous conjugates

MS analysis was performed on trastuzumab conjugated with sDIBO-DFO using GlyCLICK (T-GlyCLICK-DFO) or random conjugates labeled at lysine residues using p-SCN-Bn-DFO (T-DFO) (*Fig. 10*). Results show a distinct mass shift for the intact antibody which verifies site-specific labeling by GlyCLICK with DOL of 2.0. Random labeling provided heterogenous conjugates with a varied DOL of 0-6. The conjugates were analyzed to estimate the difference in hydrophobicity. GlyCLICK conjugates eluted as a single peak with only a minor shift in hydrophobicity compared to unlabeled and randomly labeled trastuzumab which displayed a broad peak with more hydrophobic and heterogenous material (*Fig. 11*).

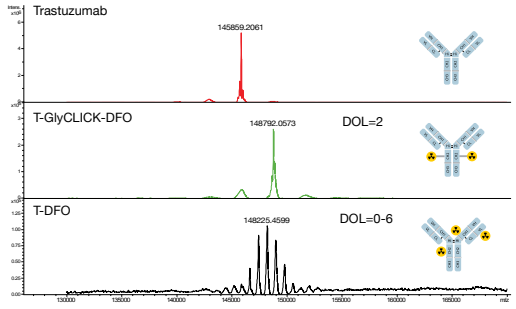


Figure 10. Intact mass analysis of trastuzumab, GlyCLICK and random DFO-conjugated trastuzumab. Trastuzumab and randomly conjugated material was deglycosylated with GlyCINATOR. All samples were separated by RP-LC on BEH C4 column (Waters) and analyzed on Bruker Impact II ESI-Q-TOF. Mass spectra were deconvoluted using the MaxEnt algorithm.

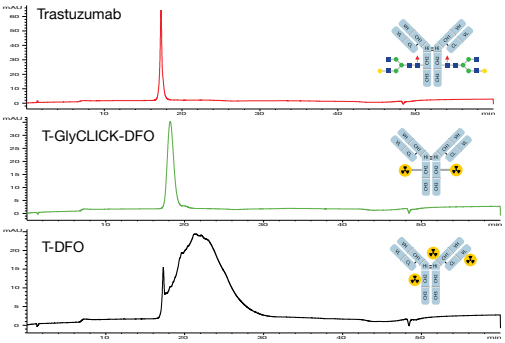


Figure 11. Hydrophobic interaction analysis of trastuzumab, GlyCLICK and random DFO conjugates injected on TSKgel® Butyl-NPR column (Tosoh Bioscience) in 25 mM NaP pH 7, 1.5 M ammonium sulfate and eluted with a salt gradient with 20% isopropanol. The random lysine DFO-conjugates were more hydrophobic and not resolved in the analysis due to heterogeneity.

GlyCLICK enhances tumor uptake *in vivo*

PET imaging was used to evaluate the effect of GlyCLICK conjugates *in vivo* by analyzing ^{89}Zr-DFO-trastuzumab labeled using GlyCLICK conjugation (DAR=2.0) or random labeling at lysines (DAR=0-6, average 3). Tumor bearing mice were injected with ^{89}Zr-DFO-trastuzumab and PET/CT images were obtained (*Fig. 12*). A fivefold increase in tumor uptake and a significantly longer circulation time was observed in mice injected with GlyCLICK conjugates (*Fig. 13*).

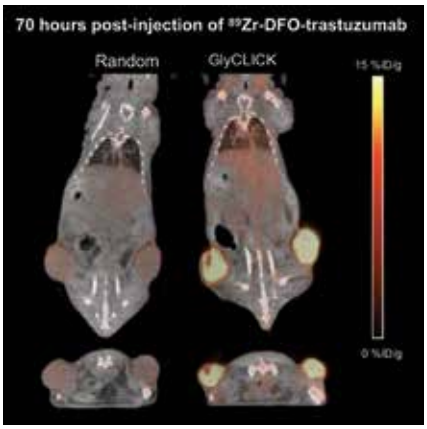


Figure 12. Longitudinal PET/CT imaging in coronal (top) and axial (bottom) images of SK-OV-3 tumor bearing mice (N=4/tracer).

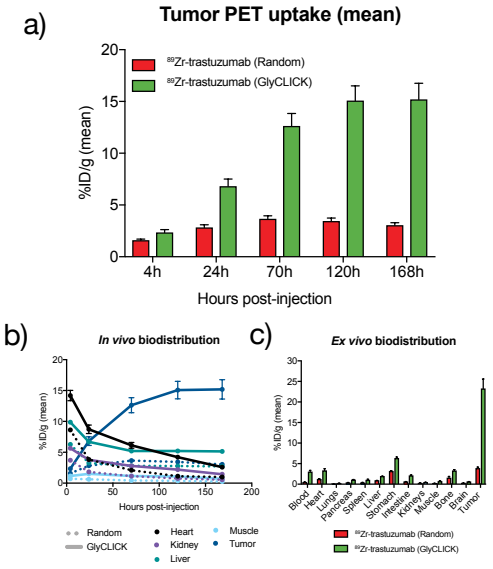


Figure 13. PET/CT imaging analysis of SK-OV-3 tumor bearing mice showing **a)** mean tumor uptake, **b)** image-derived biodistribution of ^{89}Zr-DFO-trastuzumab in major organs and **c)** biodistribution *ex vivo*.

ADC development

Traditional conjugation strategies for cytotoxic drug development include lysine conjugation or reduced cysteine chemistry. These approaches result in heterogeneous conjugation and may impact the *in vivo* performance. GlyCLICK however, preserves the affinity and increases tumor uptake *in vivo* compared to the randomly conjugated antibodies.

Azide activation
Custom conjugation

Reliable payload quantitation using GlyCLICK

The quality and efficacy of an antibody conjugation method is a crucial aspect of ADC development. To evaluate the antibody-drug-ratio (DAR) of conjugates produced with GlyCLICK, trastuzumab was site-specifically conjugated with DM1, DBCO-PEG4-Ahx-DM1 (T-GlyCLICK-DM1), and compared to randomly conjugated trastuzumab-DM1 (T-DM1). Trastuzumab and the conjugates were analyzed by HIC separation using brentuximab vedotin as a control (*Fig. 14*). Homogenous conjugation with DAR=2.0 was observed for T-GlyCLICK-DM1 compared to the heterogenous T-DM1 and brentuximab-vedotin.

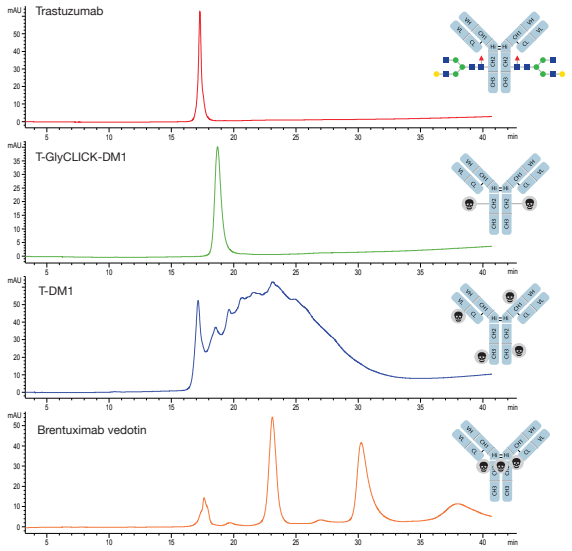


Figure 14. Characterization of T-GlyCLICK-DM1 and T-DM1. HIC samples were analyzed on a TSKgel®Butyl-NPR column (Tosoh Bioscinece) in 25 mM NaP, 1.5 M ammonium sulfate and eluted with decreasing salt gradient with 20 % isopropanol. The DAR variants of T-DM1 were not resolved due to the heterogeneity in hydrophobicity of the material.

Preserved affinity with minor drug loss in serum

T-GlyCLICK-DM1 was tested for cytotoxicity using SK-BR-3 cells which yielded a dose-response curve (*Fig. 15*). The curvature is similar to that of T-DM1, however, as 2.0 instead of 3.53 drugs are conjugated using the GlyCLICK kit, these conjugates were less cytotoxic.
To evaluate the *in vivo* characteristics, serum stability was evaluated for affinity to HER2 using ELISA and loss of drug using HIC analysis. The antigen binding assay showed similar relative binding after 168 h incubation (Fig. 16a). The HIC profile indicates that GlyCLICK is less hydrophobic compared to T-DM1 and retains binding to its antigen in serum (Fig. 16b).

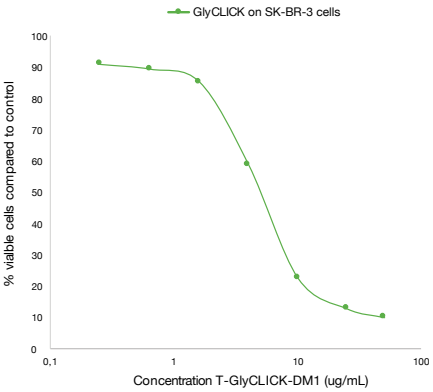
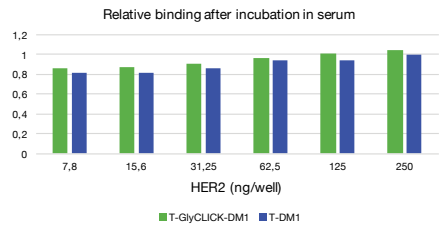


Figure 15. Cytotoxicity analysis of T-GlyCLICK-DM1. Cell viability was measured using CellTiter-Glo® Luminescent assay (Promega). HER2(+) cells (SK-BR-3), 5000 cells/well were incubated with 0-10 ug/ml T-GlyCLICK-DM1 in medium at 37°C, 5% CO2 for 72 hours prior to addition of reagent.



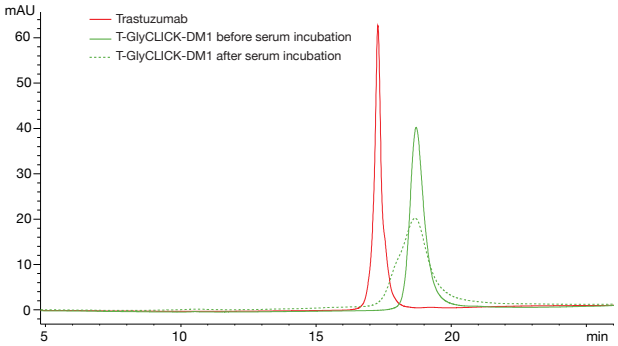


Figure 16. Serum stability analysis. **a)** Antigen binding after serum incubation. T-GlyCLICK-DM1 and T-DM1 (0.2 mg/ml) were incubated in rat serum, 85% in TBS for 168 h at 37°C. Diluted samples (1:2000) were analyzed against HER2(+) using ELISA with binding visualized using a secondary antibody of goat anti-human IgG HRP using ABTS as substrate. **b)** Qualitative HIC analysis after serum incubation. T-GlyCLICK-DM1 was affinity purified by CaptureSelect™ IgG CH1 (ThermoFisher) after rat serum incubation for 144 hours. Samples were analyzed as described in *Fig. 14*.

GlyCLICK®

GlyCLICK contains all reagents and materials needed to azide activate or label the IgG.

	Product ID	Description	Size	EUR	USD
	L1-F01-025	GlyCLICK Alexa Fluor® 488	Conjugates 250 µg IgG	885	940
	L1-C01-025	GlyCLICK DFO	Conjugates 250 µg IgG	885	940
	L1-A01-025	GlyCLICK Biotin	Conjugates 250 µg IgG	885	940
	L1-AZ1-025	GlyCLICK Azide Activation	Activates 250 µg IgG	790	835
	L1-AZ1-020	GlyCLICK Azide Activation	Activates 1 x 2 mg IgG	1,195	1,295
	L1-AZ1-100	GlyCLICK Azide Activation	Activates 1 x 10 mg IgG	4,900	5,900

Place an Order

Genovis accepts orders from all over the world. Orders from US and Canada are handled by Genovis Inc, and orders from EMEA and Asia are handled by Genovis AB.
You can place your order via email, fax or telephone, or you can order directly online using a credit card.

US and Canada

Send your purchase order to:
orders.us@genovis.com

Order phone (toll free):
1-855-782-0084
Order fax:
1-858-524-3006

Place your order online:
www.genovis.com/store

EMEA and Asia

Send your purchase order to:
order@genovis.com

Order phone:
0046 (0)46 10 12 30
Order fax:
0046 (0)46 12 80 20

Place your order online:
www.genovis.com/store

Partnering

For partnering opportunities related to the GlyCLICK technology please contact:
Jonathan Sjögren, PhD
VP Sales & Business Development
Jonathan.sjogren@genovis.com

Legal and Disclaimers

Genovis products are intended for research use only. They are not intended to be used for therapeutic or diagnostic purposes in humans or animals.

FabRICATOR®
This product is provided under an exclusive world-wide intellectual property license from Hansa Medical AB derived from international publication WO03051914, including granted US Patent No US 7,666,582 and granted European Patent No 1458861. The license encompasses IdeS from Streptococcus pyogenes for biotechnical industrial applications which are neither therapeutic nor diagnostic, other than the following exception which is included within the license: digesting IgG in vitro in clinical samples for diagnostic purposes.

SiteClick™ included in GlyCLICK®
This product is provided under an intellectual property license from Life Technologies Corporation. The transfer of this product is conditioned on the buyer using the purchased product solely in research conducted by the buyer, excluding contract research or any fee for service research, and the buyer must not (1) use this product or its components for (a) diagnostic, therapeutic or prophylactic purposes; (b) testing, analysis or screening services, or information in return for compensation on a per-test basis; or (c) manufacturing or quality assurance or quality control, and/or (2) sell or transfer this product or its components for resale, whether or not resold for use in research. For information on purchasing a license to this product for purposes other than as described above, contact Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, CA 92008 USA or outlicensing@lifetech.com.

GB1910-1

US & Canada

Genovis Inc.
245 First Street, Suite 1800
Cambridge, MA 02142
USA

Customer service: 1-617-444-8421
Order phone (toll free): 1-855-782-0084
Order fax: 1-858-524-3006
Email: orders.us@genovis.com

EMEA & Asia

Genovis AB
Box 790
SE-220 07 Lund
Sweden

Customer service: 0046 (0)46 10 12 30
Order phone: 0046 (0)46 10 12 30
Order fax: 0046 (0)46 12 80 20
Email: order@genovis.com

GENOVIS

info@genovis.com | www.genovis.com