APPLICATION NOTE

High throughput platforms to characterize a bispecific antibody subjected to force degradation.

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Introduction

Antibodies are one of the fastest growing classes of therapeutic drugs because of their high specificity and low toxicity. Recently, a new class of antibodies called bispecific antibodies (bsAbs) has emerged as one of the next generation antibody therapeutics. Unlike classical antibody formats in which the heavy and light chain are identical on both antigen binding sites, bsAbs are engineered to have two separate antigen binding sites. For example, some of these bispecific antibodies can bind different antigens expressed on two different cell types, bringing those cells together as a more effective treatment for complex diseases. There are also bispecific antibodies that can simultaneously bind and interfere with two different receptor-ligand interactions for a more effective blockage of proliferative or angiogenesis-associated pathways. As more bsAbs are produced for therapeutic applications, fast and accurate methods for evaluating and characterizing the stability of these antibody drugs are necessary during both discovery and development stages, as well as during quality analysis.

Forced degradation studies are an important part of therapeutic antibody research and development and are used throughout the product life cycle. They aid in understanding degradation pathways that may not be apparent during real time stability. They can be used for developing formulations and manufacturing methods enabling critical quality attributes (CQA). Even small amounts of degradation of a therapeutic antibody can affect product quality, safety and efficacy.[1]

Major degradation pathways seen for antibodies include aggregation, deamidation, oxidation, and fragmentation.[1,2] Thermal stress is one of the most common methods, during which the common temperatures used are 40 °C and 50 °C with 1-4 weeks of degradation time.[2]

Various methods to study the effects of thermal stress on therapeutic antibodies have been evaluated which include both analytical methods and functional assays. In this study, we evaluate the stability of a mouse bispecific antibody targeting mouse CD200 and mouse CD47 under thermal stress. Both CD47 and CD200 are overexpressed in many cancers to avoid immunosurveillance detection by binding receptors (SIRPα and CD200R, respectively) located on leukocytes.[3] The effect of thermal stress on the binding activity of this bispecific antibody was evaluated via a high throughput TR-FRET (time resolved fluorescence resonance energy transfer)-based HTRF® assay. Furthermore, an orthogonal technology was used downstream to assess fragmentation of the antibody after thermal stress. For this, the high throughput microfluidic LabChip® electrophoretic separation assay was run. LabChip assays have previously been used to assess monoclonal antibody degradation from thermal stress[4] as well as analysis of different bsAbs forms from cell culture supernatants.[5] Examples of places within the bispecific antibody discovery and development workflow where these types of assays may be used are shown in Figure 1. Here we report an orthogonal utilization of these two high throughput platforms as a workflow solution that enables a complete picture of bispecific antibody stability resulting in achieving this CQA as part of the successful development of a biologic prior to lot release.

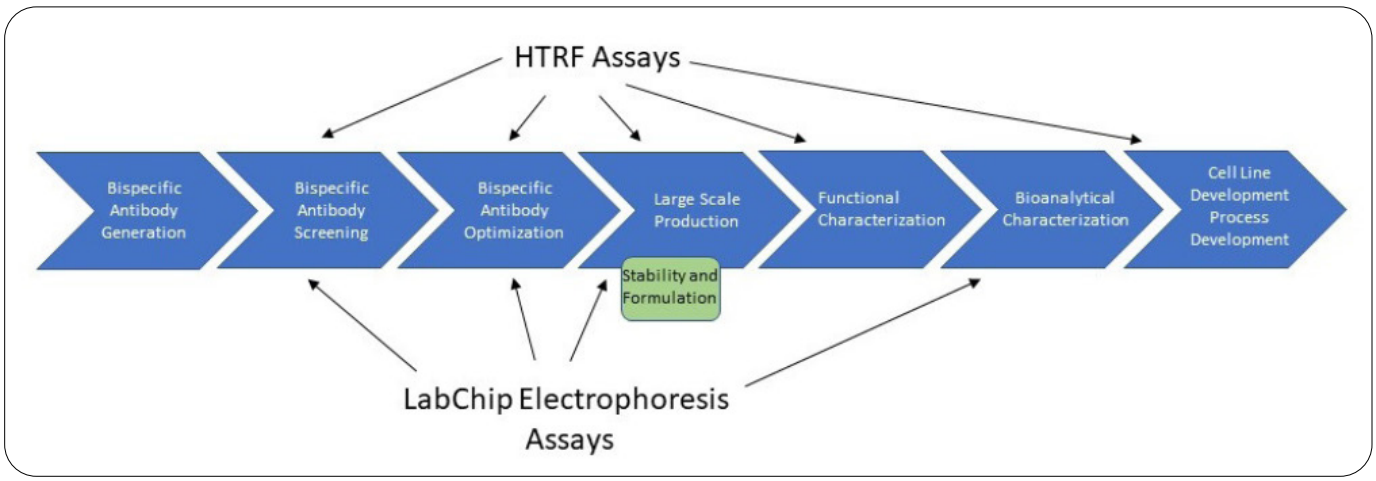


Figure 1: Example of Therapeutic Bispecific Antibody Discovery and Development Workflow.

Materials and methods

Antibodies and recombinant proteins

Bispecific antibody was purchased from Absolute Antibody. Anti-CD200/CD47 (cat# Ab00542-1.68) was used for the experiments performed in this study. Recombinant mouse His-CD200 (R&D Systems cat# 9867-CD-050), recombinant CD200-Fc (R&D Systems cat# 3355-CD-050), His-CD47 (ACRO Biosystems cat# CD7-M522b-100ug), and CD47-Fc (ACRO Biosystems cat# CD7-M5251-100ug) were used for experiments in this application notes.

Biotinylation of protein

CD200-Fc was biotinylated using the following protocol: 0.025 mg of the protein was incubated with a 2 mg/mL solution of ChromaLink™ biotin (SoluLINK cat# B1001-105) at a ratio of 10:1 biotin labeling molecule to protein for 2 hours at room temperature. The reaction was stopped by passing the reaction through a Zeba™ Spin Desalting Columns (Thermo Scientific™) 0.5 mL desalting column which had been buffer exchanged with PBS pH 7.4.

HTRF assay

HTRF assays are used to assess molecular interactions by coupling two biomolecules with either a donor (long-lived fluorescence) or an acceptor (short-lived fluorescence) fluorophore. When the biomolecules are in proximity, energy transfer between the two fluorescent dyes can be measured and is proportional to the number of biomolecules interacting. In this assay, the His-tagged mCD47 is captured by the anti-His Europium cryptate donor and the biotinylated mCD200 is captured by the Streptavidin d2 acceptor.

When it is present, the anti-mCD200/mCD47 bispecific antibody binds the mCD47 and mCD200, bringing the acceptor and donor dyes into proximity. Upon excitation at 320 nm of the donor, FRET occurs, resulting in light emission at 665 nm (see assay model in Figure 1).

All HTRF assays were performed using either Streptavidin-Eu cryptate (cat# 610SAKLA) donor, Streptavidin-d2 (cat# 610SADLF) acceptor, Mab Anti-6His-Eu Cryptate Gold (cat# 61HI2KLA) donor, or Mab Anti-6His-d2 (cat# 61HISDLF) acceptor in white ProxiPlate™-384 Plus microplates (Revvity) (see figure legends). All HTRF assays were run using PPI europium detection buffer (cat# 61DB9RDF) and were measured using a Revvity EnVision® 2105 Multimode Plate reader. The general protocol is shown in Figure 2.

The data were analyzed using GraphPad™ Prism version 7 (GraphPad™) software. Binding curves were generated using nonlinear regression (assuming there is one binding site and none of the signal comes from non-specific binding), using a 4-parameter logistic equation (sigmoidal dose-response curve with variable slope). Inhibition curves were generated using log (inhibitor) vs. response – Variable slope (four parameters). HTRF data is reported as the ratio of acceptor to donor signal = (665 nm/620 nm)*10,000.

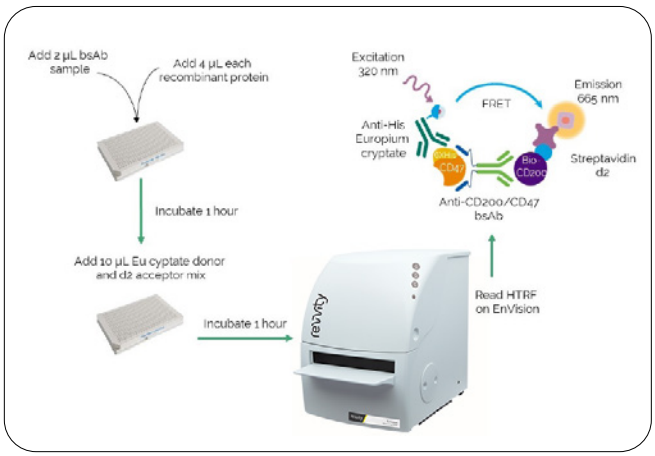


Figure 2: Protocol for bispecific antibody detection using HTRF assays.

LabChip assays

Fragment assessment experiments performed on a LabChip GXII Touch™ HT protein characterization system used microchip capillary electrophoresis-based separation. Molecular weight sizing, concentration analysis, and percent of total area for each species of the bsAb samples was evaluated using the ProteinEXact™ assay (cat # CLS150466) and HT LabChip (cat# CLS150337) which was developed for analyzing proteins ranging in size from 6.5 kDa to 250 kDa. All the reagents were part of the ProteinEXact reagent kit except DTT which was purchased from Sigma (cat #43815).

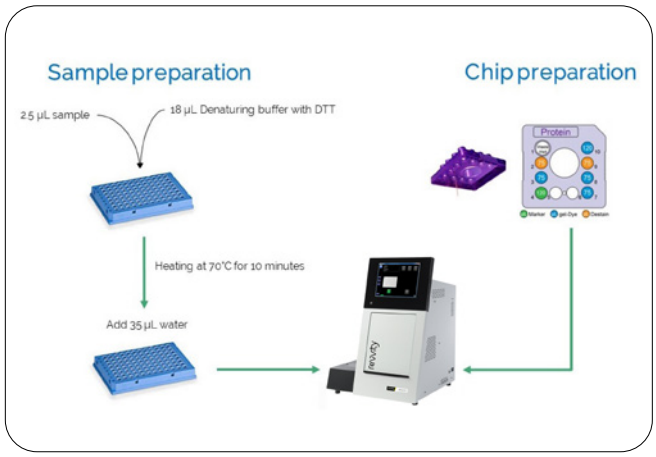


Figure 3: LabChip GXII Touch with ProteinEXact Workflow.

Samples were prepared following the protocol in ProteinEXact assay manual using HardShell PCR Plate-96 microplates (Revvity) (cat# 6008870) and run using default assay parameters on the LabChip GXII Touch instrument.

Binding assays and thermal stress evaluation

Development of binding assays using HTRF assay platform and subsequent evaluation post-thermal stress using HTRF assays as well as LabChip GXII Touch instrument as an orthogonal platform are discussed assay in the following section, results and discussion.

Results and discussion

1. Development of bispecific antibody binding assay with HTRF assay

In order to characterize the binding assay activity of the anti-mCD200/mCD47 bispecific antibody using a biochemical assay, it was important to first investigate good recombinant proteins with accessible tags for detection, determine the best binding buffer, and to confirm the binding of each individual arm of the bispecific antibody to show that each binding site is functional in the absence of the other protein. For the above bispecific antibody, the best two recombinant proteins were chosen based on experiments where we had confirmed that each recombinant protein bound in the absence of the other protein (data not shown).

The two recombinant proteins chosen for further characterization were a His-tagged mCD47 (His-mCD47) and a biotinylated mCD200 (bio-mCD200).

In order to determine the optimal donor-acceptor pair, two different donor-acceptor dye pairs were tested with four different concentrations of each protein. Bio-mCD200 recombinant protein was captured with either Streptavidin-Europium cryptate donor (SA-Eu) or Streptavidin-d2 (SA-d2) acceptor dye. The His-mCD47 was captured with either Mab Anti-6His-Eu Cryptate Gold donor (anti-His-Eu) or Mab Anti-6His-d2 acceptor (anti-His-d2) (Figure 4). A single concentration of 2 nM (final) for the bispecific antibody was chosen for this test based on preliminary experiments. Using the protocol from Figure 2, three different concentrations (5, 2, 1, or 0 nM) were tested for each protein and the signal to background was calculated using the signal of each concentration of the protein divided by the signal without the protein.

As shown in Figure 4B, the best donor-acceptor pair were the Mab Anti-6His-Eu Cryptate Gold donor and Streptavidin-d2 acceptor because it results in the greatest signal to background.

Once the donor-acceptor pair were chosen, the next step was to test a larger range of concentrations of the two proteins to find the optimal concentration for each protein. For this, eight protein concentrations were chosen and tested with 2 nM (final) of the bispecific antibody and tested using the assay schematic shown in Figure 2. Data from this experiment are shown as the calculated signal for each concentration and the calculated signal to background for selected data in Figure 4C and 4D, respectively. Concentrations above 15 nM for bio-mCD200 result in the signal plateauing and the signal for concentrations above 2.5 nM for His-mCD47 decrease in the assay. Therefore, from this experiment, we chose to move forward with 15 nM (final) of bio-mCD200 and 2.5 nM (final) of His-mCD47.

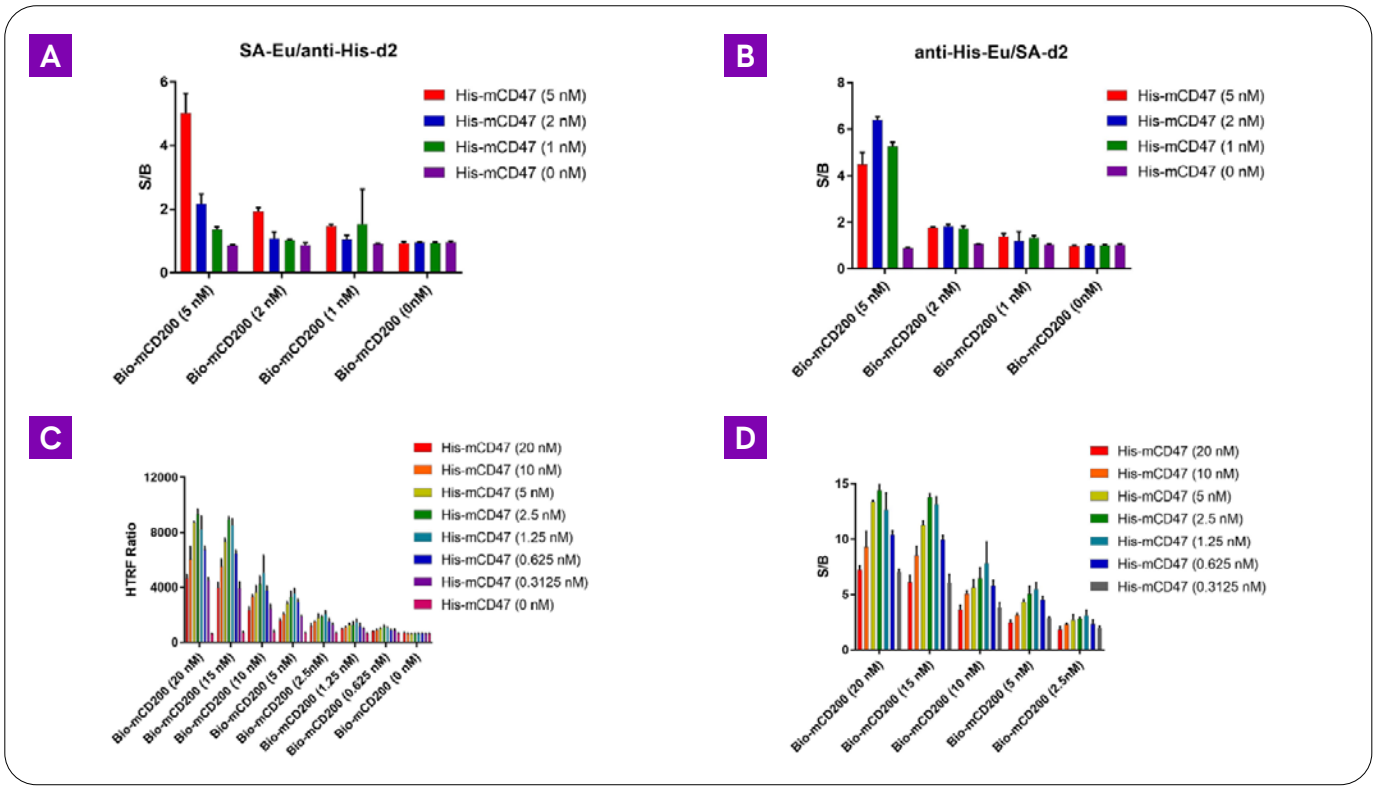


Figure 4: Optimizing reagents for bsAb HTRF assay. Experiment for finding best donor and acceptor dye pair to use with the bispecific antibody using SA-Eu/anti-His-d2 (A) or anti-His-Eu/SA-d2 (B) using a few different recombinant mouse bio-mCD200 and His-mCD47 protein concentrations. Larger cross-titration of recombinant proteins with raw data (C) or signal to background (D) shown. Assays performed in 384-well ProxiPlate-384 Plus, White 384-shallow well Microplate (Revvity) with each data point collected in triplicate.

Once suitable protein concentrations are determined, a full titration of the bispecific antibody can be performed. The concentrations of each recombinant protein are held constant and then a large range of concentrations of the bispecific antibody are tested. The EC_{50} and signal window should remain constant between batches of a bispecific antibody if both binding sites are functional and bin to the proteins similarly. Using the assay schematic shown in Figure 2 with 15 nM (final) Bio-mCD200 and 2.5 nM (final) His-mCD47, the bispecific antibody was titrated. As shown in Figure 5A, a titration of anti-mCD200/mCD47 bispecific antibody produces a dose dependent curve with an EC_{50} of 0.58 nM.

To show specificity of the signal and to show that each site binds similarly in the presence of the other protein, untagged proteins were used to compete for binding the bsAb. For this experiment, the EC_{50} of the bispecific antibody (0.6 nM final) was chosen for the assay. The protocol in Figure 2 was modified slightly: 2 µL of bsAb, 4 µL of a mixture of 15 nM (final) bio-mCD200 and 2.5 nM (final) His-mCD47 and 4 µL of a titration of either mCD200-Fc or mCD47-Fc were added to the plate and incubated for 1 hour prior to the addition of the donor-acceptor dye mixture. As shown in Figure 5B, there is a dose dependent decrease in signal for both untagged proteins. For mCD200, the IC_{50} is 1.5 nM and for mCD47, the IC_{50} is 2.6 nM. These data indicate that the signal is specific to the interaction of the bispecific antibody with both proteins.

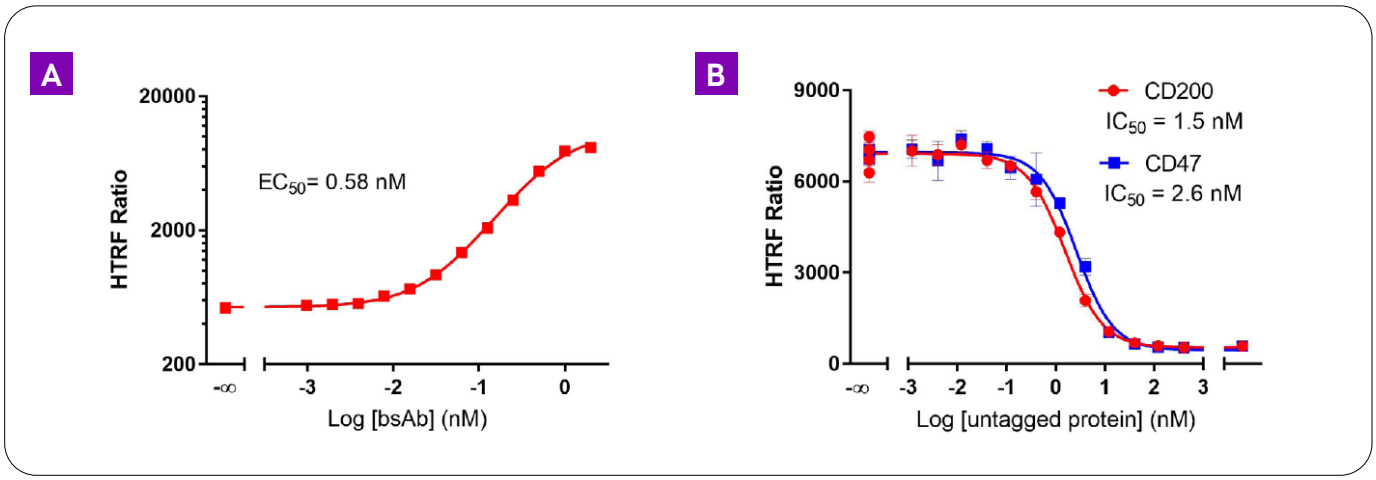


Figure 5: Bispecific antibody characterization assay. A) Titration of anti-mCD200/mCD47 bispecific antibody. B) Competition with untagged proteins for specificity testing. Assays performed with data points in triplicate in ProxiPlate-384 Plus, White 384-shallow well Microplate (Revvity).

2. Measurement of forced degradation of a bispecific antibody from thermal stress using high throughput platforms

High temperature can accelerate degradation pathways such as aggregation, fragmentation and deamidation. It is important to choose a temperature for forced degradation studies that is below the unfolding temperature. Above the unfolding temperature, the antibody will irreversibly denature, lose binding function, but may remain intact. To show this, an aliquot of the anti-mCD200/mCD47 bispecific antibody was stored at 65 °C and small samples were taken at 0, 15, 30, 45, and 60 minutes. Each time point was evaluated using the HTRF assay which reflects a significant loss of binding function over time (Figure 6A).

The reduction in binding activity seen in the HTRF assay could be explained by antibody degradation. Therefore, to determine if any antibody fragmentation had occurred during this time, the LabChip ProteinEXact assay was run under reducing conditions on the above time points. This assay separates the various species in the sample by size. Under reducing conditions, the heavy and light chains can be clearly resolved. The main antibody peaks (heavy and light chain) did not change during the 60 min time period at 65 °C. This suggested that at 65 °C, the bsAb is irreversibly denaturing or possibly aggregating resulting in a loss of binding function.

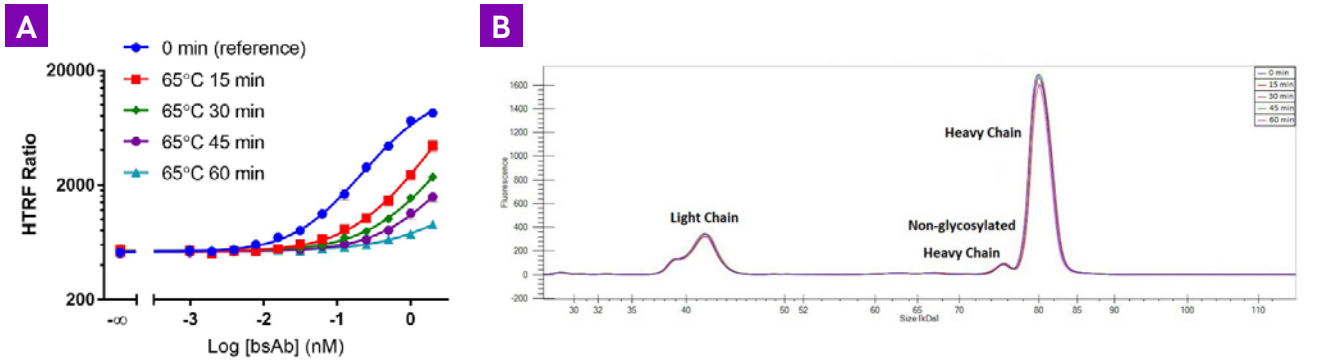
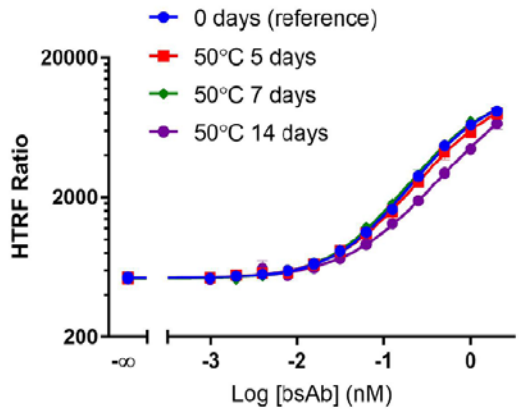


Figure 6: Monitoring of irreversible denaturation or aggregation of the bispecific antibody over time. A) HTRF bsAb binding assays on bsAb samples denatured by incubation at 65 °C for different timepoints up to 1 hour. Assay performed with data points in triplicate in 384-well ProxiPlate microplate. B) LabChip GXII profile of the stock concentration of bsAb (1 mg/mL) at each time point after incubation at 65 °C.

When assaying stability, it is important to look at a range of temperatures and timepoints to determine thermal stress conditions that give the most useful information. For this reason, we next chose to examine the effects of incubating the bsAb at 50 °C for a longer time period, which is a common temperature used for such assays.[2] The bsAb was incubated for days 5, 7, and 14 at 50 °C. Two concentrations (0.5 and 0.25 nM) which fall within the linear range of the assay were then chosen to determine a percent recovery. The signal for these two concentrations at days 5, 7, and 14 were interpolated from the binding curve generated from the control sample (day 0 reference). The concentration calculated was compared to the known input concentration to determine a percent recovery as tested by binding activity. As shown in Figure 7, there is no loss of binding activity at day 5 at 50 °C. However, at day 7 at the same temperature, there is a 12% loss of binding activity and a 40% loss of activity by day 14.



Average % recovery of activity	
5 days	104%
7 days	88%
14 days	60%

Figure 7: HTRF bsAb assay results after incubation at 50 °C at different timepoints up to two weeks. Assay performed with data points in triplicate in 384-well ProxiPlate. microplate.

Furthermore, to investigate whether any antibody fragmentation has occurred over the above time period, the LabChip ProteinEXact assay was run under reducing conditions (Figure 8A). This assay shows resolution of species into the light chain (LC), heavy chain (HC), non-glycosylated heavy chain (NGHC) and various degradation products. The profile shows small degradation products increasing over time. For example, a low molecular weight species (LMWS) migrating around 20 kDa, a medium molecular weight species (MMWS) seen migrating between the light and heavy chain, and two high molecular weight species (HMWS1 and HMWS2) which migrate slower than the heavy chain increase over time.

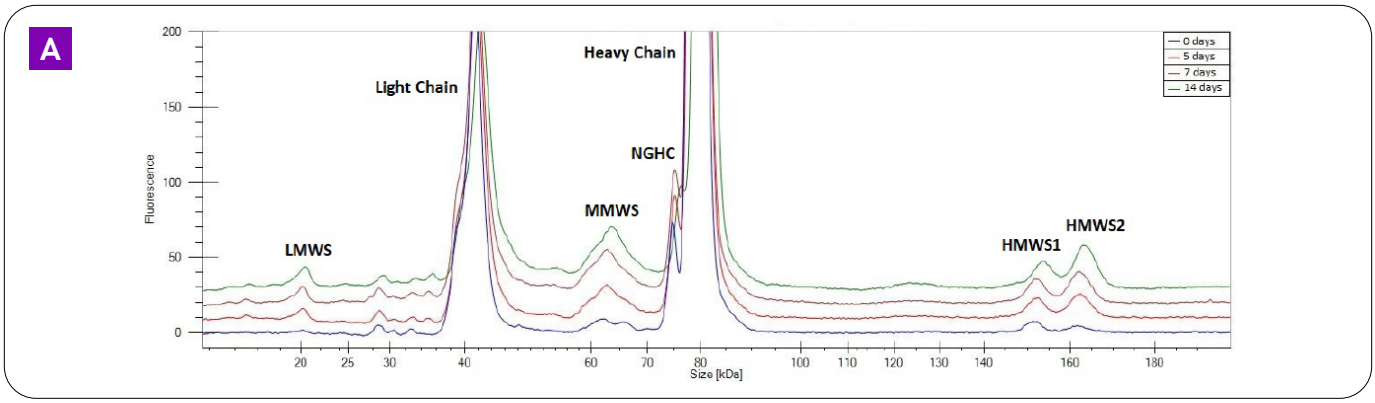
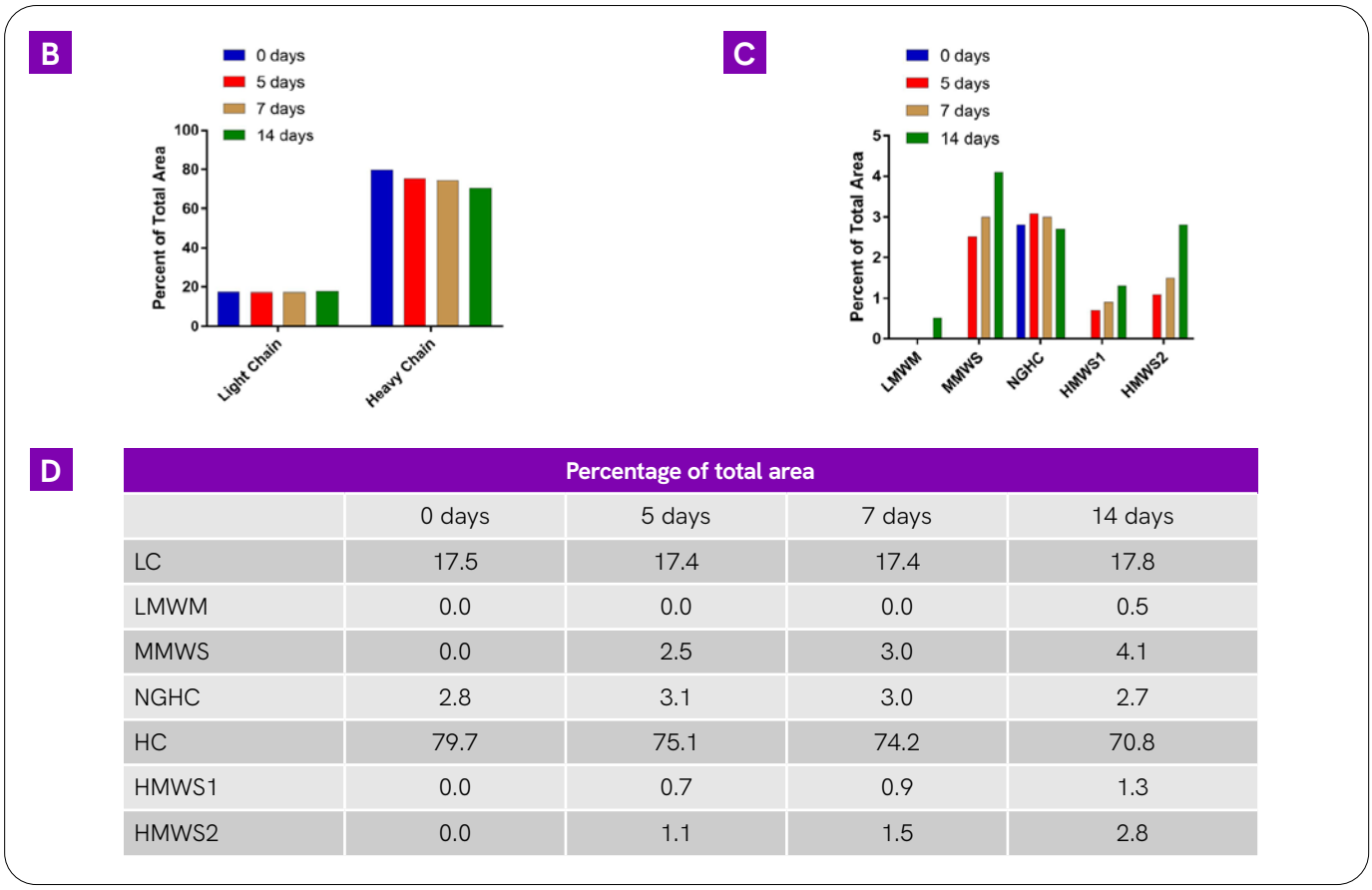


Figure 8: A) LabChip GXII profile of the stock concentration of bsAb at each time point after incubation at 50 °C.

The percentages of the area under each curve for each time point are shown (Figure 8B, C, and table). For day 0, the LMWS, MMWS, HMWS1, and HMWS2 had a low enough percentage of the total area that they were quantified as zero percent of total area. The percentage for the heavy chain peak decreases as the impurity peaks increase. However, the percentage of the non-glycosylated heavy chain (NGHC) and percentage of the light chain peak remains relatively constant. This suggests cleavage of the peptide chain of the heavy chain leads to the high, medium, and low molecular weight degradation products.



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Percentage of total area				
	0 days	5 days	7 days	14 days
LC	17.5	17.4	17.4	17.8
LMWM	0.0	0.0	0.0	0.5
MMWS	0.0	2.5	3.0	4.1
NGHC	2.8	3.1	3.0	2.7
HC	79.7	75.1	74.2	70.8
HMWS1	0.0	0.7	0.9	1.3
HMWS2	0.0	1.1	1.5	2.8

Figure 8: B) Percentage of total area for Light Chain (LC) and Heavy Chain (HC) peaks. C) Percentage of total area for other peaks. D) Table showing percentages of total area for each species.

Conclusions

The high throughput, HTRF and LabChip GXII Touch platforms, shown here provide complementary information on stability and insight into the degradation pathways of a bispecific antibody. Specifically, the biochemical HTRF binding assay is important for showing the function of the antibody, which could be used for assessing stability in different formulations.

The LabChip ProteinEXact assay, which leverages the microfluidics capillary electrophoresis technology, is highly sensitive and can separate small degradation products, hence can provide further insight into which part of the antibody is degrading (i.e., the light chain or the heavy chain). The two platforms used above constitute important tools for assessing bispecific antibody stability and variation when subjected to stress conditions. Therefore, the two orthogonal technologies can play a key role in a biologics workflow when determining the identity and stability of bispecific antibodies as one of the modalities in biotherapeutics.

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