

# AN AUTOMATED METHODOLOGY FOR ASSESSMENT OF PROTEIN STABILITY USING NANOGRAMS OF PROTEIN

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## Introduction

We demonstrate how the Fida Instrument can be utilized for probing conformational stability of therapeutic proteins using as little as 40 nano grams of protein per measurement<sup>1</sup>.

**40<sub>ng</sub>** of protein per measurement<sup>1</sup>

We report the free energy of unfolding ( $\Delta G^\circ$  (H<sub>2</sub>O)) and denaturation midpoint values ( $C_m$ ) for adalimumab, human serum albumin (HSA), and rituximab at different pH-values. The method measures the change in hydrodynamic radius and intrinsic fluorescence of a protein using Taylor dispersion analysis (TDA) during in-line denaturation with guanidinium chloride (GuHCl).

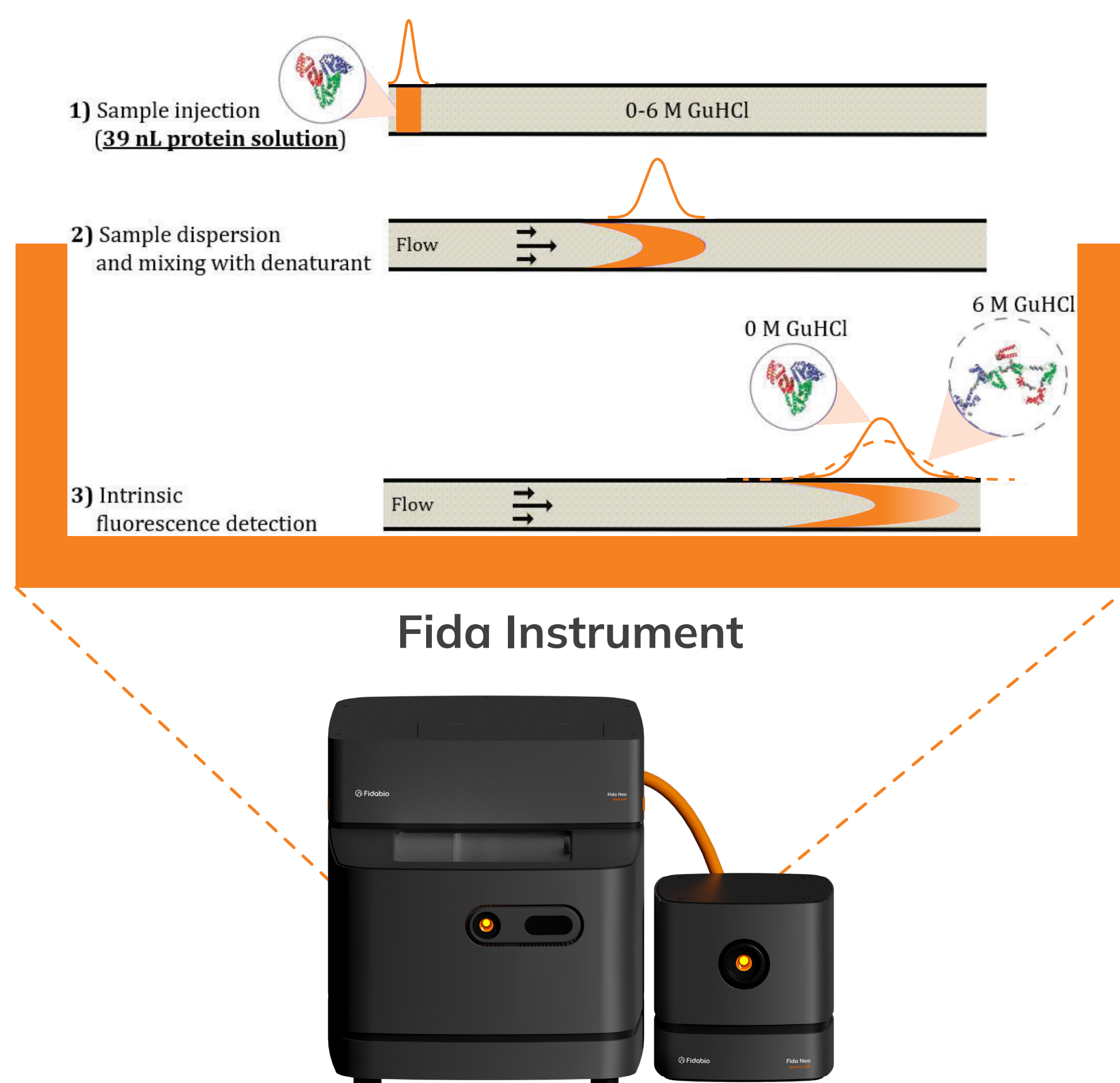
Conformational protein stability is typically probed by observing relative changes in optical properties such as fluorescence and/or scattering upon thermal or chemical perturbations. The Fida Instrument relies on absolute measurements of hydrodynamic radius ( $R_h$ ) and intrinsic fluorescence utilizing TDA and UVfluorescence detection. TDA is a first principles methodology thus the assay readout ( $R_h$ ) can be accurately predicted from solved or simulated protein structures such as PDB files or AlphaFold, respectively. The simple workflow and low sample consumption (40 nL) of the Fida Instrument make it ideal for assessing protein stability in early drug development or when having scarce amounts of sample.

## Methods

Experiments were performed on a Fida Instrument employing 275 nm LED-UV detection using a high-sensitivity coated capillary (Fida Biosystems).

The hydrodynamic radii and intrinsic fluorescence of adalimumab, HSA, and rituximab were measured as a function of denaturant concentration (GuHCl, 0-6 M) at pH 4.0, 7.0, and 10.0. Each data point consumed only 40 nL of protein sample (~1 mg/mL) with an analysis time of 6 min. The assay buffer was 20 mM HEPES, 20 mM histidine-HCl, and 20 mM sodium succinate following pH-adjustment with NaOH or HCl. Data analysis was conducted using Fida software (V 2.4).

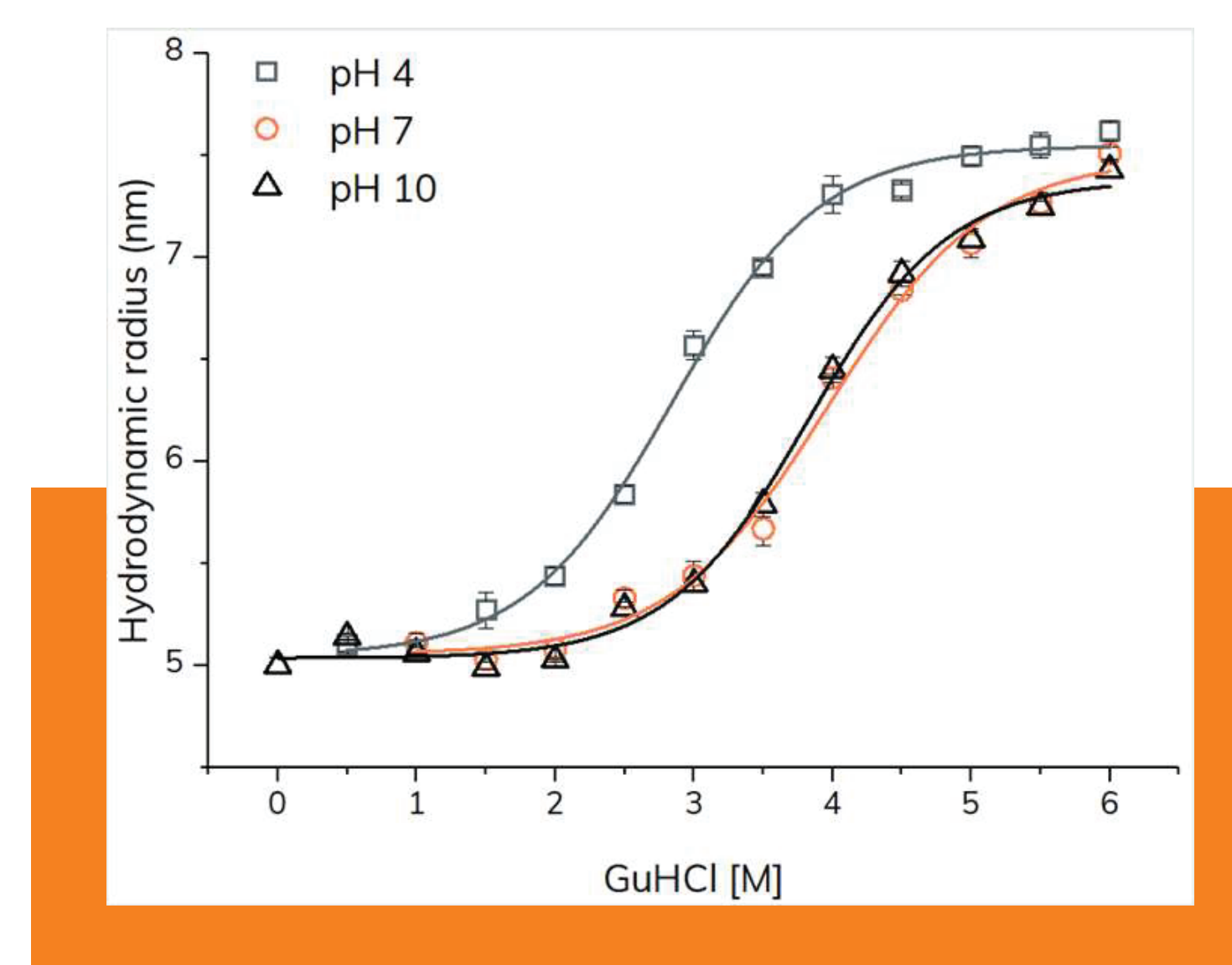
### AUTOMATED WORKFLOW



## Results

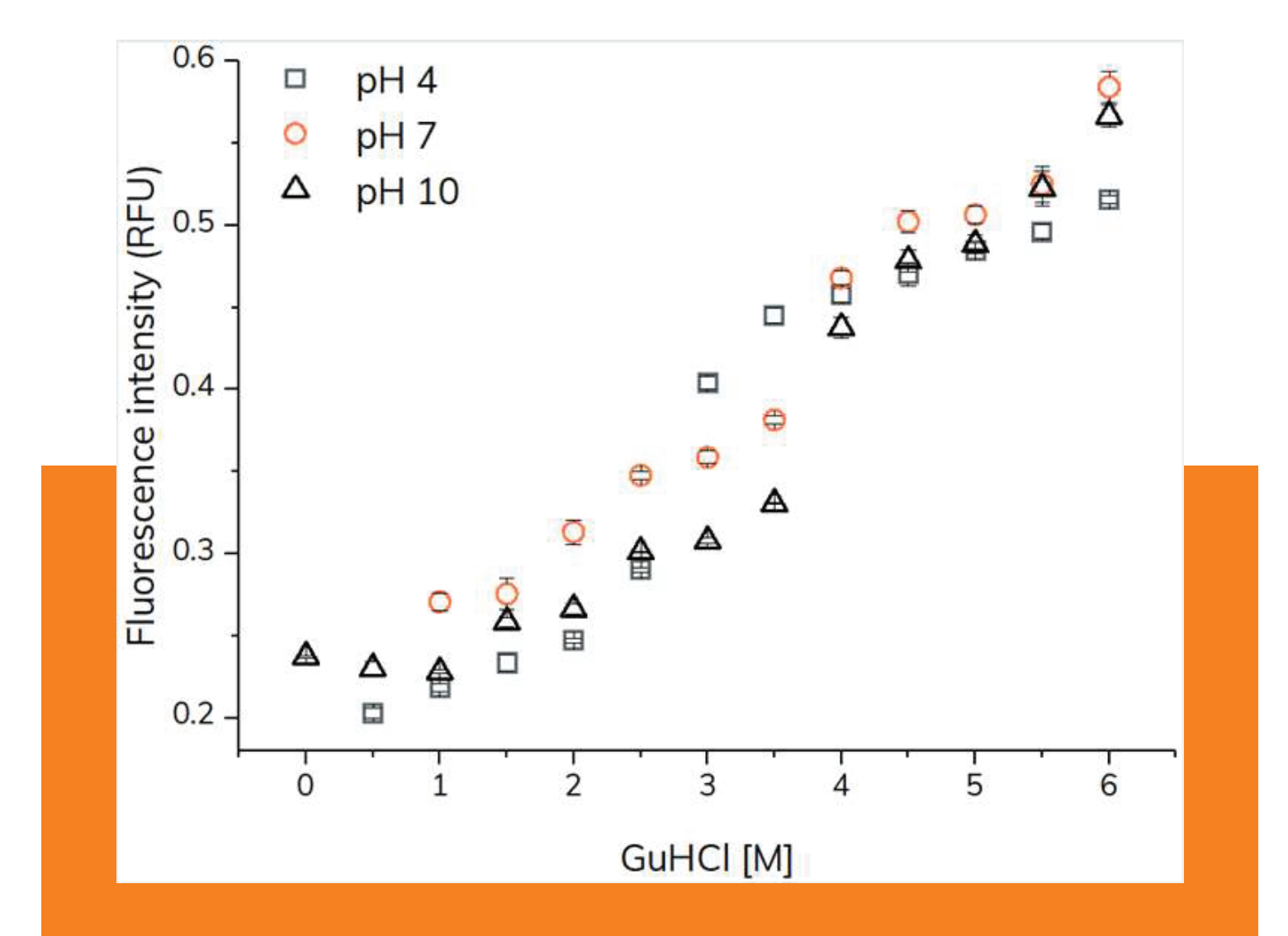
### CONFORMATIONAL STABILITY

#### 1 HYDRODYNAMIC RADIUS ( $R_h$ )



The size (hydrodynamic radius) of adalimumab (1 mg/mL) as a function of GuHCl concentration (0-6 M) at pH 4, 7, and 10. Measured using the Fida 1 consuming only 40 ng of protein per data point. Solid lines represent fitting to the Fidabio protein unfolding model for obtaining stability parameters (see table below).

#### 2 INTRINSIC FLUORESCENCE



Intrinsic fluorescence intensity of adalimumab (1 mg/mL) as a function of GuHCl concentration (0-6 M) at pH 4, 7, and 10, obtained simultaneously with the  $R_h$  measurements above. Thereby providing an additional measure of conformational stability as well as structural changes around the tryptophan and tyrosine residues.

#### 3 STABILITY PARAMETERS $\Delta G^\circ$ (H<sub>2</sub>O) & $C_m$

pH	Adalimumab	HSA	Rituximab
	$C_m - M$ [GuHCl]		
4.0	2.6	3.5	2.4
7.0	3.7	2.2	3.2
10.0	3.6	2.6	3.2
pH	$\Delta G^\circ$ (H <sub>2</sub> O) (kcal/mol)		
	Adalimumab	HSA	Rituximab
4.0	2.9	3.6	2.8
7.0	3.9	2.0	3.4
10.0	4.3	3.0	3.0

Stability assessment of adalimumab, HSA, and rituximab at pH 4, 7 and 10 based on the denaturation midpoint ( $C_m$ ) and standard free energy change in absence of denaturant ( $\Delta G^\circ$ (H<sub>2</sub>O)). Data obtained from the Fidabio protein unfolding model.

## Conclusions

In this work, we report a generic Fida Instrument method for assessing the conformational stability of proteins utilizing two concurrent readouts, protein size ( $R_h$ ) and intrinsic fluorescence intensity. The method was developed using the Fida Instrument which provides straightforward assay development, walkway automation, absolute measurements ( $R_h$ ) as well as low sample consumption (nL). Furthermore, a Fidabio unfolding model was derived for convenient determination of  $C_m$  and  $\Delta G^\circ$  from the resulting unfolding curves.

### REFERENCES

[1] Morten E. Pedersen, Jesper Østergaard, and Henrik Jensen; Rapid Assessment of Protein Stability Utilizing the Fida 1. In-preparation.