Supporting information

Sensitive label-free thermal stability assay for protein denaturation and protein-ligand interaction studies

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1. Experimental section

1.1 Materials and instrumentation

Target proteins; IgG\textsubscript{1} antibody (anti-h TSH 5409 SPTNE-1), streptavidin (SA), carbonic anhydrase (CA), and malate dehydrogenase (MDH) were purchased from Medix Biochemica (Espoo, Finland), BioSpa (Milan, Italy), Merck (Darmstadt, Germany), and Roche (Basel, Switzerland), respectively. Triethylammonium acetate (TEAAc) was from PanReac Applichem (Darmstadt, Germany). Acetazolamide (AZA) was from Alfa Aesar, Thermo Fisher Scientific (Haverhill, MA, United States). The nonadentate Eu\textsuperscript{3+}-chelate, \(\{2,2',2'',2'''-(4'-4''-isothiocyanatophenyl)-2,2',6',2''-terpyridine-6,6''-diyl][bis(methylene-nitrilo)]tetrakis(acetate)\)europium(III) was from QRET Technologies (Turku, Finland) and conjugated according to manufacturer's instruction to the probe peptide. Probe peptide (NH\textsubscript{2}-EYEEEEEVEEEVEEE) was purchased from Pepmic Co., Ltd (Suzhou, China). Black Framestar 96-well microtiter plates, used in all assays, were from 4titude (Surrey, U.K.). EuCl\textsubscript{3} standard, and DELFIA enhancement solution (DES) were purchased from PerkinElmer Life and Analytical Sciences, Wallac (Turku, Finland). All other reagents, including analytical-grade solvents, buffer components, SYPRO Orange (5000x), 1,1,3,3,3',3'-hexamethylindodicarbocyanine iodide, and biotin were from Sigma-Aldrich (St. Louis, MO).

Eu-probe purification was carried out using reversed-phase adsorption chromatography, Dionex ultimate 3000 LC system from Thermo Fischer Scientific, Dionex, and Ascentis RP-amide C18 column from Sigma-Aldrich, Supelco Analytical. Time-resolved luminescence (TRL) emission signals were measured at 615 nm, using 340 nm excitation wavelength, and 400 µs delay and decay times. SYPRO Orange luminescence was measured using 485 nm excitation and 590 nm emission wavelengths. All measurements were performed using Victor 1420 multilabel counter from PerkinElmer Life and Analytical Sciences, Wallac or Spark 20M from Tecan Life Sciences (Männedorf, Switzerland). Temperature cycling was performed with a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA).

1.2 Eu-probe preparation

Isothiocyanate activated Eu\textsuperscript{3+}-chelate was conjugated to the N-terminus of the peptide sequence to prepare the Eu-probe. Eu\textsuperscript{3+}-chelate conjugations was performed in labeling buffer containing pyridine/H\textsubscript{2}O/triethylamine in 9:1.5:0.1 ratio. Eu\textsuperscript{3+}-chelate (1 mg) was dissolved in 100 µL of water and mixed with the probe peptide (0.5 mg) in 100 µL of the labeling buffer. Reaction solution was incubated at room temperature (RT) for 18 h. Eu-probe was purified under the following
conditions: eluent system containing 50 mM TEAAc pH 7.0:ACN 100%, linear gradient (1 ml/min from 10:90 to 50:50 in 17 min). After purification, Eu-probe concentration was determined based on the Eu$^{3+}$-ion concentration by comparing observed TRL-signal to a commercial Eu$^{3+}$-standard (DELFIA), assuming that the probe peptide and Eu$^{3+}$-chelate are in 1:1 ratio.

1.3 Eu-probe functionality and stability

Eu-probe functionality was first studied in terms of pH and signal stability. In all assays, the modulation solution was prepared from disodium hydrogen phosphate 200 mM and citric acid 100 mM buffer (citrate-phosphate buffer) using varying ratios of the two assay components to adjust the pH. Thereafter the buffer was diluted 10-fold for the assay. Phosphate-citrate buffer with pH 10 was further adjusted with NaOH. All assays were performed as triplicates using two-step protocol. Eu-probe (1 nM) pH stability (pH 2-10) was first studied with and without IgG$_1$. To understand the TRL-signal stability at varying pH, we monitored the stability of the Eu-probe without IgG$_1$ over 130 min time period (Figure S1). Sample buffer (0.1x PBS pH 7.5, 0.001% Triton X-100) was added in 8 µL to the 96-well plate and thereafter 65 µL of the modulation solution in citrate-phosphate buffer (pH 2-10) supplemented with 0.01% Triton X-100. Reaction with IgG$_1$ (80 nM) was studied using the same pH range (Figure 2A). IgG$_1$ was kept at room temperature (RT, 25 °C) or heated at 80 °C for 3 min to denature the protein before addition of the modulation solution, citrate-phosphate buffer (pH 2-10), 0.01% Triton X-100, 3.5 µM 1,1,3,3,3′,3′-hexamethylindodicarbocyanine iodide,$^{1,2}$ and 1 nM Eu-probe, in 65 µL volume. TRL-signal stability was monitored during the 60 min time period. The TRL-signal stability was further studied after selection of the pH 4-modulation solution. IgG$_1$ (0-1670 nM) was denatured for 3 min at 80 °C and triplicate reactions were monitored multiple times during 60 min incubation at RT (Figure 2B). After selection of the final modulation solution (disodium hydrogen phosphate 7.7 mM citric acid 6.1 mM (pH 4), 0.01% Triton X-100, 3.5 µM 1,1,3,3,3′,3′-hexamethylindodicarbocyanine iodide, and 1 nM Eu-probe), protein denaturation was studied in the following sample buffers: HEPES (10 or 100 mM), Tris (10 or 100 mM) or PBS (0.1x or 1x) all with and without 0.001% Triton X-100 (Figure S2). IgG$_1$ thermal melting at 80 nM concentration was measured after ramping the temperature in intervals of 5 °C. The modulation solution was added after 3 min incubation at each temperature and TRL-signals were monitored after 5 min.

Final sample buffer used in all following assays was 0.1x PBS (pH 7.5) supplemented with 0.001% Triton X-100. Under the selected condition, thermal denaturation was studied with four model proteins, MDH (100 nM), CA (200 nM), IgG$_1$ (50 nM), and SA (400 nM) (Figure S3). Denaturation at
8 µL was performed using temperature step interval of 2-5 °C from 25 to 90 °C. TRL-signals were monitored 5 min after modulation solution (65 µL) addition.

1.4 Protein-Probe and SYPRO Orange assay comparison

Next, the developed Protein-Probe assay was performed side-by-side with the SYPRO Orange control method. With both methods, the IgG₁ was added in 8 µL to the 96-plate and heating was performed using 5 °C interval and 3 min denaturation time. IgG₁ concentrations used with the Protein-Probe and SYPRO Orange methods were 80 nM and 2 µM, respectively (Figure 2C). In the Protein-Probe assay, the modulation solution was added in 65 µL and TRL-signals were monitored after 5 min. SYPRO Orange was added in 2 µL in 0.1x PBS (pH 7.5) supplemented with 0.001% Triton X-100. In the final volume, SYPRO Orange was in 1x concentration and luminescence signals were monitored after 5 min.³⁴

In the sensitivity study of the Protein-Probe and SYPRO Orange assays, IgG₁ was titrated from 0 to 5 µM, and denatured using a single temperature (80 °C, 3 min) selected based on the previous assays (Figure 2D). After thermal denaturation, the modulation solution or SYPRO Orange was added as previously and the luminescence signals were monitored after 5 min.

1.5 Protein-Probe in protein-ligand interaction studies

Protein-ligand interaction was studied using two models, CA with AZA and SA with biotin. Assays were performed using the same buffer as in the protein thermal denaturation studies. Functional concentrations for AZA and biotin were first determined (Figure 3A). CA (200 nM) and SA (400 nM) were added in 4 µL volume to the 96-plate and mixed with equal volume of the relevant ligand, AZA (0-90 µM) or biotin (0-20 µM). Both reactions were heated at protein-specific single temperature, 70 °C and 90 °C for CA and SA reactions, respectively. TRL-signals were measured 5 min after addition of 65 µL of the modulation solution. Thereafter, thermal shift assays for CA and SA were performed with and without saturating concentration of the corresponding ligand, 5 µM AZA and 10 µM biotin, using temperature step interval of 2-5 °C from 55 to 90 °C. TRL-signals were monitored after 5 min as previously (Figure 3B).

1.6 Protein-Probe method robustness

The robustness of the Protein-Probe methods was tested by running several individual temperature curves for IgG₁ using two thermal cycler devices. Five separate dilutions of IgG₁ (80 nM) were prepared for heated with each instrument in 96-well plate using three replicates. 8 µL of IgG₁ was
heated using 2.5 °C intervals for 3 minutes before the addition of the modulation solution individually prepared for each 5 dilution set of IgG, and added in 65 µL volume. The TRL-signals were measured after 5 min.

1.7 Data analysis

The S/B ratio was calculated as $\mu_{\text{max}}/\mu_{\text{min}}$ and coefficient variation (CV%) as $(\sigma/\mu) \times 100$. In these formulas, $\mu$ is the mean value and $\sigma$ is the standard deviation (SD). The data was analyzed using Origin 8 (OriginLab, Northampton, MA). The denaturation temperatures and half-maximal effectivity concentration ($\text{EC}_{50}$) were obtained using standard sigmoidal fitting functions.
2. Results

![Figure S1](image)

**Figure S1.** Eu-probe pH stability. The stability of the Eu-probe (1 nM) was tested in pH controlled citrate-phosphate buffer at various pH ranging from 2 to 10. TRL-signals were monitored at various time points during the 130 min incubation period. In all assays, the protein sample buffer was 0.1x PBS (pH 7.5), 0.001% Triton X-100. At pH 2 (black) and in some extent at pH 3 (red), TRL-signal of the Eu-probe was reduced over time, indicating instability of the Eu$^{3+}$-chelate at low pH. At pH 4 (blue), pH 5 (magenta), pH 6 (green), pH 7 (dark blue), and pH 8 (violet), the monitored TRL-signal was stable. At pH 10 (purple), the overall TRL-signal was lowered compared to the pH giving the highest signal, although the TRL-signal stability was well retained. These results indicate no pH related effects on Eu-probe signal in detection buffer above pH 4. All signals were normalized to maximal TRL-signal at pH 7, and the data represents mean ± SD (n=3).
Figure S2. Functionality of the Protein-Probe method in various protein sample buffers. Buffer composition is known to affect protein denaturation, and this was briefly studied in terms of main buffer components in two concentrations at pH 7.5. As a model system, 80 nM of IgG, melting curves were measured at multiple temperatures from 25 to 80 °C. Denaturation was performed in 1x PBS (black), 0.1x PBS (red), 100 mM Tris (blue), 10 mM Tris (magenta), 100 mM HEPES (green) or 10 mM HEPES (dark blue) with (A) and without (B) 0.001% Triton X-100. No major change in $T_m$ were monitored either with ($T_m$ 70-73 °C) and without Triton X-100 ($T_m$ 70-73 °C). Triton X-100 had no effect on $T_m$, but it improved S/B ratios and standard deviations (SD) compared to sample buffer without Triton X-100. The highest S/B ratios were monitored with Tris-buffers and HEPES gave the lowest S/B ratios. In all experiments, the S/B ratio was over 19 reaching at the maximum of 50 with Tris-buffer. These rather small changes were considered insignificant. There were more differences between sample buffer SD values than S/B ratios. The 0.1x PBS with 0.001% Triton X-100 was chosen due to its low SD values compared to other sample buffers. Deeper study on buffers was not performed, as buffer composition is often case specific. The data represents mean ± SD (n=3).
Figure S3. Protein melting curves for reproducibility analysis of the Protein-Probe method. Temperature melting curves of the 10 individual IgG₁ (80 nM) dilutions (each dilution in different color) were monitored using 2.5 °C intervals from 25 to 90 °C. All denaturation cycles were performed in 8 µL volume using two separate temperature-cycling instruments, 5 dilutions per instrument. Eu-probe (1 nM) was added in 65 µL as a part of modulation solution (pH 4), which was prepared separately for the two dilution sets heated with different instruments. TRL-signals were monitored 5 min after the Eu-probe addition. The Tₘ values obtained of these melting curves were on average of 70.3 ± 0.2 °C and 71.5 ± 0.1 °C depending on the instrument used, which clearly divided curves in two populations. The average CV% of the individual IgG₁ dilutions was 8%, and the average CV% of the three replicates within each individual measurement point was 4%. Data represents mean ± SD (n=3).
Figure S4. Protein melting curves for model proteins. Temperature melting curves for the selected model proteins, malate dehydrogenase (MDH, blue), carbonic anhydrase (CA, black), IgG₁ (red), and streptavidin (SA, magenta), were monitored using temperature step interval of 2-5 °C from 25 to 90 °C. All denaturation cycles were performed in 8 µL volume using 0.1x PBS supplemented with 0.001% Triton X-100. MDH, CA, IgG₁, and SA were measured at concentrations of 100, 200, 50, and 400 nM, respectively. Eu-probe (1 nM) was added in 65 µL as a part of modulation solution (pH 4) and TRL-signals were monitored 5 min after the Eu-probe addition. The observed Tᵣᵣ for MDH, CA, IgG₁, and SA were 44.6, 67.9, 70.7, and 75.7 °C, respectively. Data represents mean ± SD (n=3).
3. References

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