Supporting Information

Nanomolar protein-protein interaction monitoring with label-free Protein-Probe technique

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1. Experimental section

1.1 Materials and instrumentation

EZ-Link™ NHS-PEG4-Biotin and EDTA-free Pierce Protease Inhibitor were purchased from Thermo Fisher Scientific (Waltham, MA, USA). NAP-5 columns were acquired from GE Healthcare (Chicago, IL, USA). Streptavidin (SA) was purchased from Biospa (Milan, Italy). Biotin, Adenosine triphosphate (ATP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), bovine serum albumin (BSA), Cy3-RNA (GAGAGAAGGAAG), RNA-BHQ2 (CUUCCUUCUC), and DNA (CTTCTCCTTCTC) were acquired from Sigma Aldrich (St. Louis, MO, USA). C-reactive protein (CRP) was from Merck (Kenilworth, NJ, USA). Antibodies (mAbs; anti-h CRP, anti-h Hemoglobin, and anti-h Lp-PLA2) were a kind gift from Oy Medix Biochemica Ab (Espoo, Finland). SYPRO Orange was purchased from Invitrogen (Carlsbad, CA, USA). Eu3+-GTP and Eu3+-probe labeling was performed using the 9d chelate, and Eu3+-biotin was labeled with the 7d chelate. Thermal ramping plates, black Framestar 96, were from 4titude, Surrey, UK. Other assay plates were black OptiPlate 384-well microtiter plates from PerkinElmer (Groningen, Netherlands) and low volume black or white 384-well plates from Corning (Corning, NY, USA). All the other reagents were from Sigma Aldrich. Eu3+-GTP and Eu3+-biotin were prepared and characterized as described before. The Eu3+-probe (NH2-EYEEEEEVEEEVEEVEE) was prepared and purified as described earlier. The Protein-Probe was prepared by mixing Eu3+-probe and 1,1,3,3,3',3'-hexamethyldiindocarbocyanine iodide (HIDC) in citrate-phosphate buffer (7.7 mM Na2HPO4, 6.1 mM citric acid, pH 4) supplemented with 0.01% Triton X-100. Fresh Protein-Probe was prepared for all individual assays. Protein purification was performed using an ÄKTA system (GE Healthcare) and HisTrap, MBPTrap, or Superdex 75 (GE Healthcare) columns. Additionally, Dionex ultimate 3000 LC system (Dionex, Sunnyvale, CA, USA) and Ascentis RP-amide C18 column (Sigma-Aldrich, Supelco Analytical) was used for reverse phase chromatography in all Eu3+-labelings. Thermal ramping was performed using PTC-200 Peltier Thermal Cycler (MJ Research, St. Bruno, Canada). Time-resolved luminescence (TRL) signals were measured with either Victor 1420 multilabel counter (PerkinElmer Life and Analytical Sciences, Wallac, Turku, Finland; excitation 340 nm, emission 615 nm) or Tecan Spark M20 (Tecan, Männedorf, Switzerland; excitation 340 nm, emission 620 nm), using delay times of 400-800 µs and integration time of 400 µs. SYPRO Orange luminescence was measured using Tecan Spark M20 (485 nm excitation and 590 nm
emission). Excitation and emission spectra were also measured using Tecan Spark M20. Luminescence lifetime measurements were performed using Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). FRET (Förster resonance energy transfer)-measurements were performed using Tecan Infinite200Pro with 530 nm excitation and 590 nm emission wavelengths. Qubit Fluorometer (Invitrogen, excitation 485 nm, emission 590 nm) was used in protein concentration determination. All biochemical assays were performed in a 10 µL final volume using triplicate reactions and three individual experiments unless otherwise indicated.

1.2 Eu³⁺-probe and Protein-Probe spectral characterization and lifetime measurements

The excitation and emission spectra of 1 nM Eu³⁺-probe and 5 µM HIDC were measured on OptiPlate 384-well plate in 65 µl. The excitation scans for the Eu³⁺-probe, Eu³⁺-probe with mAb/CRP sample, Protein-Probe solution (1 nM Eu³⁺-probe, 5 µM HIDC), and Protein-Probe solution with mAb/CRP sample were performed at 250-500 nm, with 620 nm emission. The emission spectra from 550 to 800 nm were measured using excitation at 340 nm. The samples were prepared by combining 30 nM mAb with 50 nM CRP at RT, followed by 3 min incubation at 85 °C, using 8 µl sample volume. These measurements were performed using 400 µs delay and integration times. The excitation scan for 5 µM HIDC (at 450-680 nm) was performed using emission at 700 nm, and the emission spectra (at 630-850 nm) was measured using excitation at 618 nm. All spectra were measured using 1 nm step and 5 nm bandwidth.

The lifetimes of 50 nM Eu³⁺-probe, the Protein-Probe solution (50 nM Eu³⁺-probe, 3.5 µM HIDC), and the Protein-Probe solution with sample containing 1000 nM anti-CRP mAb and 200 nM CRP were measured in a quartz cuvette in 40 µl. The sample was prepared by incubating the mAb and CRP first at RT, then 3 min at 85 °C before addition to the Protein-Probe solution. Measurements were performed using 340 nm excitation and 615 nm emission, delay and gate times were 0.1 ms, and the total measurement time was 3 ms.

1.3 Protein production and purification

Eukaryotic initiation factor 4A (eIF4A), eukaryotic translation initiation factor (eIF4H), and programmed cell death protein 4 (PDCD4) were produced and purified at the Medical Research Council (MRC) Toxicology Unit, using their constructs and protocols with minor changes. Briefly, His-eIF4A, maltose-binding protein (MBP)-eIF4H, and MBP-PDCD4 were expressed using E. coli BL21 (DE3), and the biomass was produced applying standard protocols for IPTG-induction. Cells were harvested, resuspended, and lysed in a lysis buffer containing 20 mM sodium phosphate (pH 7.4) 100 mM NaCl, 10% glycerol, 1 mM DTT supplemented with EDTA-free Pierce Protease Inhibitor. After debris removal by centrifugation, the filtered (0.45 µm) supernatants were transferred in buffer A (eIF4A) containing 20 mM sodium phosphate (pH 7.4), 500 mM NaCl, 20 mM imidazole, 1 mM DTT or buffer B (eIF4H and PDCD4) 20 mM Tris (pH 7.5),
200 mM NaCl, 1 mM EDTA, 1 mM DTT. Proteins were collected using HisTrap or MBPTrap affinity chromatography. Bound proteins were eluted with a linear imidazole gradient (HisTrap) or using maltose (MBPTrap). Affinity tag was cleaved from the pooled fractions by TEV-protease overnight at +4 °C (1:200 TEV-protease:target protein ratio). Affinity chromatography was repeated and the flow through was collected and pooled. Further purification by size exclusion chromatography was performed, and collected, pooled, concentrated, and liquid nitrogen snap-frozen fractions were stored at -80 °C in storage buffer containing 20 mM Tris (pH 7.5), 100 mM KCl, 2 mM DTT, 0.1 mM EDTA, 10% glycerol. Protein concentrations were determined using Qubit Protein Assay Kit. Protein purity was estimated further by monitoring A280/A260 ratio and by running SDS-PAGE. A280/A260 ratio of ≥ 1.9 indicate negligible amounts of contaminations by nucleic acids and nucleotides, and purity over 90% for eIF4A (46 kDa), eIF4H (29 kDa), and PDCD4 (52 kDa) was estimated based on SDS-PAGE.

KRAS proteins, designed ankyrin repeat protein (DARPin) K27, and SOS catalytic domain (SOS\textsuperscript{cat}) were produced, purified, and analyzed as described before, and were a kind gift from Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research 6.

BSA was biotinylated with 2x molar excess of EZ-Link NHS-PEG4-Biotin in 100 µL of 50 mM phosphate buffer, pH 8.5, for 1 h. The biotinylated BSA (bio-BSA) was purified using a NAP-5 column, according to the manufacturer’s instructions. The protein concentration and labeling degree were determined using absorbance at 280 nm, and by comparing the bio-BSA to free biotin. The biotin concentration was determined in 20 mM HEPES (pH 7.5), 10 mM NaCl, 0.001 % Triton-X 100, and 0.005 % γ-globulins, utilizing QRET technique. Biotin standard (0-1 µM) was assayed in the presence of SA (2 nM), Eu\textsuperscript{3+}-biotin (5 nM) and MT2 (2.2 µM) in 10 µL\textsuperscript{3}. Concentration of an unknown bio-BSA was determined similarly and compared to the biotin standard. The TRL-signals were monitored after 5 min incubation at RT.

1.4 Proof-of-concept streptavidin/bio-BSA assay

To observe SA binding to bio-BSA, assay using 20 nM BSA or bio-BSA was performed with 0-600 nM SA in Assay Buffer (10 mM HEPES, pH 7.5, 0.001 % (v/v) Triton X-100). After 5 min incubation, 65 µL of the Protein-Probe (7.7 mM Na\textsubscript{2}HPO\textsubscript{4}, 6.1 mM citric acid, pH 4, 0.01% Triton X-100, supplemented with 6 µM HIDC, and 4.5 nM Eu\textsuperscript{3+}-probe), was added on top of 8 µL of protein-protein interaction (PPI) reaction. TRL-signals were monitored after 5 min incubation at room temperature (RT). The SA/bio-BSA interaction was confirmed in biotin titration, using 20 nM BSA or bio-BSA in the presence of 200 nM SA and 0-10 µM biotin. PPI was monitored using the Protein-Probe and TRL-signals were monitored as above.

1.5 Antibody-antigen interaction at RT and elevated temperatures
Anti-CRP and non-specific mAb assays were performed in Assay Buffer. For detection, the Protein-Probe contained 3.5 µM HIDC and 1 nM Eu³⁺-probe. To monitor CRP interaction with mAb, 0-100 nM CRP was combined with 0-500 nM anti-CRP mAb or two non-specific mAbs (hemoglobin or Lp-PLA2 mAb). After 10 min incubation at RT, the Protein-Probe was added in 65 µL on top of the 8 µL PPI reaction, and TRL-signals were monitored after 5 min.

For measurements in elevated temperatures, the thermal stability of the anti-CRP mAb (0.5-120 nM) was monitored using thermal ramping from 50 to 95 °C and 5 °C increments. All samples were prepared at RT and thereafter incubated for 3 min at each temperature before the Protein-Probe was added. TRL-signals were monitored after 5 min of incubation at RT. The CRP/mAb interaction was monitored using the same assay protocol as with the mAb alone, by combining 0-50 nM CRP with 2-30 nM anti-CRP mAb. TRL-signals were monitored as above. The same protocol was also used to monitor interaction with non-specific anti-hemoglobin mAb (10 nM) with 5 or 10 nM CRP.

1.6 eIF4A interaction control, thermal stability, and interactions measured at elevated temperatures

Assay conditions. All biochemical assays were performed in a 10 µL final volume using triplicate reactions and three individual experiments unless otherwise indicated. All the thermal ramping assays were performed with a sample volume of 8 µL, and the Protein-Probe was added in a volume of 65 µL or SYPRO Orange in a volume of 2 µL in the used reference assays. All reactions were mixed at RT before incubation in heating. All eIF4A assays were performed in 20 mM HEPES, pH 7.5, 100 mM KCl, 1 mM MgCl₂ with or without 0.001 % Triton X-100 supplement. The Protein-Probe contained 3.5 µM HIDC and 1 nM Eu³⁺-probe, and the SYPRO Orange final concentration in well was 5x.

eIF4A interaction control and helicase activity. eIF4A (1.5 µM) functionality was first assayed in the presence or absence of eIF4H (1.5 µM) in a helicase assay. Both eIF4A and eIF4H were added in 3 µL volume and mixed together with 2 µL of preannealed Cy3-/BHQ2-RNA (50 nM) complex and Cy3-RNA-complementary DNA (1 µM). The preannealing was performed by heating Cy3-/BHQ2-RNA (1 µM) up to 85 °C for 2 min and slowly cooling down to RT. The preannealed complex was stored at -20 °C. The helicase reaction was initiated by adding 2 µL of Mg²⁺/ATP complex (2/5 mM), and FRET-signals were monitored every 5 min for 45 min. Using the same concentrations, PDCD4 function as an eIF4A inhibitor was assayed. eIF4A, eIF4H, and Cy3-/BHQ2-RNA/DNA complex were all added in 2 µL volume. PDCD4 (0-10 µM) was also added in 2 µL and components were incubated for 5 min before reactions were initiated with 2 µL of Mg²⁺/ATP complex. FRET-signals were monitored as above.

eIF4A thermal stability and interactions. The thermal stability of eIF4A was monitored using a protein concentration of 75 or 150 nM with the Protein-Probe and using a protein concentration of 2 µM with SYPRO Orange. Additionally, the thermal stability of the interacting partners was studied using both
methods. In Protein-Probe assays, PDCD4 was monitored using a protein concentration of 150 and 300 nM and eIF4H at 500 and 1000 nM concentrations. In SYPRO Orange assays the concentrations were 2 µM for PDCD4 and 2 or 6 µM for eIF4H. Samples were incubated with heating for 3 min (25-70 °C using 5 °C increments) before Protein-Probe addition, followed by TRL-signal monitoring after 5 min. SYPRO Orange assays were performed in a single step using 5x SYPRO Orange directly in heating. For further assays with the Protein-Probe, we selected PPI buffer without Triton X-100 supplement. Interaction of eIF4A (75 nM) with PDCD4 (0-300 nM) or eIF4H (0.5 or 1 µM), and interaction of 0.5 µM eIF4H with 150 nM PDCD4 were observed. The samples were incubated for 3 min at 45-75 °C using 5 °C increments, after which the Protein-Probe was added. TRL-signals were monitored after 5 min as described previously.

1.7 KRAS interaction control and measurement of thermal stabilization

**Assay conditions.** The DARPin K27 QRET assay was performed in 20 mM HEPES (pH 7.5) 1 mM MgCl$_2$, 10 mM NaCl, 0.01 % Triton-X 100, and 0.005 % γ-globulins in a final volume of 15 µL. All the thermal ramping assays were performed with a sample volume of 8 µL, and the Protein-Probe was added in a volume of 65 µL. In the reference assays, 1x or 5x SYPRO Orange was used in a final volume of 10 µL. The reactions were prepared at RT before the heating. All KRAS Protein-Probe and SYPRO Orange assays were performed in 10 mM HEPES, pH 7.5, 0.001 % (v/v) Triton X-100, 1 mM MgCl$_2$, and 20 mM NaCl. The Protein-Probe contained 4 µM HIDC and 1 nM Eu$^{3+}$-probe.

**KRAS interaction control.** DARPin K27 binding and ability to block KRAS nucleotide exchange was determined using a quenching resonance energy transfer (QRET) assay and Eu$^{3+}$-GTP, as described earlier$^{2,6}$ Briefly, GDP- or GMPPNP (5'-Guanylyl imidodiphosphate)-loaded KRAS (50 nM) was incubated with equal volume of GDP or K27 (2-2000 nM). After 5 min, detection complex (10 nM Eu$^{3+}$-GTP and 2.5 µM MT2) was added and the 5 min incubation was repeated. Eu$^{3+}$-GTP association was initiated by SOS$^{cat}$ (10 nM) addition, and the increased TRL-signal was monitored multiple times during 60 min incubation at RT.

**KRAS concentration testing.** To determine the appropriate KRAS concentration for the following assays, a GDP-loaded KRAS dilution series was prepared (0-500 nM for the Protein-Probe, 0-15 µM for SYPRO Orange) and the samples were incubated at 70 °C for 3 min. The Protein-Probe or SYPRO Orange were added, and luminescence or TRL-signals were monitored after 5 min. Additionally, the Protein-Probe was used to perform a similar titration for DARPin K27 (0-500 nM), using the same incubation time and temperature as with GDP-KRAS.

**KRAS thermal stability and interaction.** The thermal stability of GMPPNP-KRAS and GDP-KRAS was monitored using 50 nM KRAS with the Protein-Probe or 3 µM KRAS for SYPRO Orange. The thermal stability of 100 nM K27 was measured with the Protein-Probe. The samples were incubated for 3 min at
25-95 °C and TRL-signals were monitored using 5 °C increments. SYPRO Orange assay was monitored at the same temperatures but using single-step protocol. The interaction between 50 nM GMPPNP-KRAS or GDP-KRAS and 100 nM K27 was monitored in thermal ramping using the Protein-Probe. Samples were incubated and monitored as above from 25 to 95 °C.

### 1.8 Data analysis

The S/B ratio was calculated as \( \mu_{\text{max}} / \mu_{\text{min}} \), specific signal 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 were analyzed using Origin 2016 (OriginLab, Northampton, MA). The denaturation temperatures \( (T_m) \), half-maximal inhibitory concentration \( (IC_{50}) \), and half-maximal effectivity concentration \( (EC_{50}) \) were obtained using standard sigmoidal fitting functions. \( \Delta T \) was obtained from the difference between the calculated protein \( T_m \) values. Detection limits were defined as 3 x SD of the blanks (buffer).
2. Supporting results

Figure S1. Eu\textsuperscript{3+}-probe and HIDC spectra and luminescence lifetimes for the Protein-Probe method. (A) Excitation and emission spectra of the Eu\textsuperscript{3+}-probe (1 nM) and HIDC (5 µM). The Eu\textsuperscript{3+}-probe excitation scan was performed at 250-500 nm, with emission wavelength of 620 nm, and the emission scan was performed at 550-800 nm, using excitation at 340 nm. The HIDC excitation spectra was scanned at 450-680 nm, using emission wavelength of 700 nm, and the emission spectra was scanned at 630-850 nm, with excitation at 618 nm. The Eu\textsuperscript{3+}-probe excitation and emission maxima were 341 and 616 nm, respectively. The corresponding maxima for HIDC were 637 and 657 nm, respectively. The HIDC excitation spectrum overlaps with the Eu\textsuperscript{3+}-probe emission spectrum for efficient Eu\textsuperscript{3+}-probe quenching. (B) The luminescence lifetimes were measured for the Eu\textsuperscript{3+}-probe (50 nM) and the Protein-Probe (containing 50 nM Eu\textsuperscript{3+}-probe and 3.5 µM HIDC) without or with the denatured mAb sample (1.2 µM). In the presence of HIDC (red), significantly reduced lifetime is detected compared to Eu\textsuperscript{3+}-probe alone (black). Sample addition produced intermediate life (blue), indicating Eu\textsuperscript{3+}-probe interaction with the sample. Measured lifetimes are context depend and only directive as high Eu\textsuperscript{3+}-probe concentration was used.
Figure S2. Detection of anti-CRP mAb interaction with CRP. 100 nM of anti-CRP mAb and two non-specific mAbs (marked together) were assayed with or without CRP (20 nM). All individual components produced similar TRL-signal, but high TRL-signal was observed only from the anti-CRP mAb in complex with CRP. At these concentrations, reflecting 5:1 mAb binding to CRP, the S/B ratio was 4.3 and 13, when the complex signal was compared to mAb and CRP signals alone, respectively. Data from a single representative assay showing individual reactions (mean ± SD, n=3).
Figure S3. Anti-CRP mAb concentration has an impact on the TRL-signal level but not on the melting point. (A) Anti-CRP mAb (0.5-120 nM) was monitored in thermal ramping using the Protein-Probe technique. Thermal denaturation of the mAb led to increased TRL-signal at elevated temperatures for all concentrations except 0.5 nM, which could not be detected. The maximum S/B ratio of 21, calculated from the highest and the lowest mAb signal (measured at 90 and 55 °C, respectively), was monitored with 120 nM mAb. (B) The mAb concentration did not affect the observed T_m. The average T_m using 2-120 nM mAb was 77.1 ± 1.2 °C. The highest calculated T_m was obtained with 5 nM mAb (76.8 ± 0.1 °C) and the lowest using 120 nM mAb (74.8 ± 0.2 °C). Protein concentration increase has only minor T_m-decreasing effect. The data are individually normalized to the maximum signal within each mAb concentration, showing non-optimal behavior at the lowest (2 nM) mAb concentration. Data from a single representative assay showing individual reactions (mean ± SD, n=3).
Figure S4. Interaction between CRP and anti-CRP mAb can be detected in thermal ramping. (A) Thermal ramping was performed using 30 nM mAb with or without 5, 50 or 150 nM CRP. With 50 nM CRP and mAb, a two-phase curve is observed, whereas 5 nM CRP followed the mAb curve and 150 nM CRP the CRP alone curve, respectively. (B) Using 10 nM anti-CRP mAb (mAb1, black and red) or non-specific mAb (mAb2, blue and magenta) with 5 or 10 nM CRP in thermal ramping, clear interaction-specific TRL-signal increase was detected. When the anti-CRP mAb was combined with 5 or 10 nM CRP, the signal increase at 90 °C was 7.4-fold or 4.0-fold, respectively, compared to the increase observed with the non-specific anti-hemoglobin mAb. The results are presented as specific signal, where the TRL-signal of the mAb is subtracted from the TRL-signal of the combination samples, producing the CRP-specific increase. Data from a single representative assay showing individual reactions (mean ± SD, n=3).
Figure S5. eIF4A is an activity helicase whose function can be induced with eIF4H and blocked by PDCD4. (A) eIF4A (1.5 µM) was assayed without or with an equal concentration of eIF4H in a kinetic assay using preannealed blunt end RNAs (12 bp) labeled with either Cy3 or BHQ2 (50 nM). The assay was performed in the presence of DNA (1 µM) complementary to Cy3-RNA, and the assay was initiated with ATP (2 mM) added in complex with MgCl$_2$ (5 mM). Helicase activity was monitored every 5 min from the increasing FRET signal. (B) To monitor PDCD4 inhibitory activity, its concentration was titrated (0-10 µM) in a similar reaction as the helicase activity by using 1:1 complex of eIF4A/eIF4H (1.5 µM). The reaction was initiated by ATP/Mg$^{2+}$ addition and the complex was incubated for 30 min, before the FRET-signal was monitored. IC$_{50}$ value of 330 ± 8 nM was monitored for PDCD4. Data from a single representative assay showing individual reactions (mean ± SD, n=3).
Figure S6. Thermal stability of eIF4A was measured using the Protein-Probe technique and SYPRO Orange. eIF4A thermal stability was monitored in thermal ramping using 2 µM eIF4A with SYPRO Orange, and 75 or 150 nM with the Protein-Probe assay. The Protein-Probe assay was performed in two buffers, with or without 0.001% Triton X-100. The Protein-Probe technique showed increased sensitivity compared to SYPRO Orange, and further increase in eIF4A detectability could be obtained using Triton X-100. Maximum S/B ratio monitored from 75 nM eIF4A at 60 °C increased from 23 to 2.3 when reaction with or without Triton X-100 was compared. Triton X-100 had no major effect on eIF4A stability, giving T_m values of 54.5 ± 0.1, 53.6 ± 0.3, and 54.3 ± 0.5 °C with eIF4A (75 or 150 nM) in the presence or absence of Triton X-100, respectively. With SYPRO Orange, the calculated T_m for 2 µM eIF4A was 50.3 ± 0.1 °C, which is slightly higher than reported previously. Data from a single representative assay showing individual reactions (mean ± SD, n=3).
Figure S7. No increase in TRL-signal is detected from non-interacting proteins in heating. eIF4H (500 nM) and PDCD4 (150 nM), which do not interact, were observed in thermal ramping using the Protein-Probe. Combining the proteins did not lead to an increase in the S/B compared to the S/B of the individual components, unlike when eIF4A is measured with either of these proteins. Thus, the increase in the signal can be attributed to specific PPI and is not the result of non-specific interactions of partially unfolded proteins or caused by increased total protein concentration. Data from a single representative assay showing individual reactions (mean ± SD, n=3).
Figure S8. K27 can bind and efficiently block the GDP-KRAS nucleotide exchange. K27 and GDP were titrated (0-6000 nM) in a quenching resonance energy transfer (QRET) based nucleotide exchange assay using GDP- or GMPPNP-loaded KRAS (50 nM). With GDP-KRAS, both K27 and GDP blocked the Eu$^{3+}$-GTP association in the presence of 10 nM SOS$^{cat}$ and 2.5 µM MT2, used as a quencher. The monitored IC$_{50}$ values for K27 and GDP were 26.4 ± 0.7 and 20.5 ± 1.4 nM, respectively. On the other hand, no clear nucleotide exchange blocking with even the highest K27 concentration (6000 nM) was monitored with GMPPNP-loaded KRAS, as with the GDP control the monitored IC$_{50}$ value was 28.5 ± 4.2 nM. These low IC$_{50}$ values reflect the used KRAS concentration and not the true IC$_{50}$ value, which is thus lower than 50 nM, but cannot be determined exactly. However, the data demonstrate the high affinity interaction between K27 and GDP-loaded KRAS and specificity over GMPPNP-KRAS. Data from a single representative assay showing individual reactions (mean ± SD, n=3).
Figure S9. The Protein-Probe technique showed improved detectability for KRAS compared to SYPRO Orange. To determine the appropriate KRAS concentration for the PPI assays, GDP-KRAS was titrated (0-500 nM) using the Protein-Probe or up to 15,000 nM using SYPRO Orange. Data were monitored at a single point after 3 min incubation at 70 °C. The lower limit of detection, calculated as 3 x SD of the blanks (buffer), were 4.2 nM and 117 nM for Protein-Probe and SYPRO Orange, respectively. Based on the data, we selected 50 and 3000 nM KRAS for further assays with Protein-Probe and SYPRO Orange, respectively. Data from a single representative assay showing individual reactions (mean ± SD, n=3).
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