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A universal immuno-PCR platform for comparative and ultrasensitive quantification of dual affinity-tagged proteins in complex matrices.

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Electronic Supplementary Information

Materials and Methods

Cloning, expression and protein purification.

The Tus-HA, Tus-GFP, Tus and GFP-HA were prepared as previously described.[1-4] The vector harboring the coding sequence for SrtA-GFP-HA was prepared by digesting the vector pIM018 (SrtA-GFP)[4] with NdeI to obtain a DNA fragment containing the sequences coding for SrtA and a portion of GFP. The DNA fragment was then ligated into the recipient vector pIM057 (GFP-HA),[3] which had been digested with NdeI and dephosphorylated to remove the same portion of GFP coding sequence. Insertion of the SrtA coding sequence was confirmed by PCR and plasmid sequencing.

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SrtA-GFP-HA was expressed and purified using a routine procedure previously described.[1] The protein was further purified by gel filtration chromatography using a Superdex 200 10/300 GL column (GE Lifesciences) and Biologic Duoflow Chromatography System (Biorad) in buffer A (45 mM Na_2HPO_4, 5 mM NaH_2PO_4, 10% v/v glycerol, 2 mM β-mercaptoethanol, 300 mM NaCl). Concentration of SrtA-GFP-HA was determined by fluorescence quantification of the fusion protein against a GFP-HA standard using a fluorescence plate reader (Victor3V Wallac 1420, Perkin–Elmer) set to 460 nm excitation/535 nm emission.

Determination of protein and nucleic acid content

Total protein content of the samples was determined by SDS-PAGE (1 µl of each sample). The gel was then stained with Coomassie Blue. Total nucleic acids content of the samples was determined by agarose gel electrophoresis (1 µl of each sample).

Washing of microplate wells

Wells were washed with BW (20 mM Tris-HCl (pH 8), 150 mM NaCl and 0.005% Tween-20) in an Inteliwasher 3D-IW8 Microplate Washer (Biosan). The well washing parameters were set as follows: aspiration first; 02 level aspiration rate; 1 s aspiration time; 2 s final aspiration time; 200 µl dispensed wash buffer; 01 level dispense rate; no soaking of wells; no shaking of wells.

Affinity profiling of Tus variants with matching antibodies by Tus-Ter-lock qIPCR

Polyclonal goat anti-GFP IgG (Abcam, ab6673, 1 mg/ml), monoclonal mouse anti-His_6 (Abcam, 18184, 1 mg/ml) or monoclonal rat anti-HA IgG (Roche, 11867423001, 0.1 mg/ml) were diluted to 1 µg/ml in 50 mM phosphate buffer (pH 7.8) and adsorbed to microplate wells (50 µl/well; Nunc, Maxisorp) overnight at 4 ºC. Wells were then washed once with the

microplate washer as described below. All wells were blocked with 50 µl block buffer for 1 h at RT and washed once more with the microplate washer. After washing, 50 µl of the tagged-Tus variants (Tus, Tus-GFP or Tus-HA) described above and ranging in concentration from 10 fM-100 nM were added for 10 min at RT. Reactions were performed at least in triplicate. All wells were then washed three times with a microplate washer. TT-lock-T (1 µM stock) was diluted in block buffer to 0.5 nM and 50 µl were added to each well for 10 min at RT, after which the wells were washed five times with the plate washer. The PCR primers (0.5 µM each in water) were then added to the wells (50 µl/well) and left for 1 h at RT to allow dissociation as previously described.[4]

For the qPCR, 10 µl aliquots were taken from each well after the dissociation step and combined with 10 µl of 2X SensiMix SYBR and Fluorescein real-time PCR mix (Bioline). Real-time qPCR was performed using a Bio-Rad IQ5 Thermocycler. The positive control consisted of 10 µl real-time PCR mix, 9 µl primer mix and 1 µl of the diluted TT-lock-T, and the no-template control contained only 10 µl real-time PCR mix and 10 µl of the primer mix to detect contaminants. qPCR was performed according to the following parameters: initial denaturation at 95 ºC for 10 min, denaturation at 95 ºC for 10 s, annealing at 55 ºC for 10 s, and extension at 60 ºC for 10 s (40 cycles).

ΔCt values were the absolute values obtained by subtracting background Ct values (no analyte) from reaction Ct values (with analyte).

Preparation of TTA

Tus-HA (50 µM) in buffer B (buffer A without NaCl) was freshly diluted to 500 nM in block buffer (BW supplemented with 1% w/v BSA). Two volumes of Tus-HA were bound with one volume of TT-lock-T (1 µM stock) for 10 min at RT to produce the TT-lock-T:Tus-HA complex. Two volumes of monoclonal rat anti-HA IgG (660 nM, Roche, 11867423001) were

added to the complex for 5 min at RT to form the TTA. The fully assembled TTA was then diluted in block buffer to a final concentration of 0.5 nM.

Effect of incubation time on TTA qIPCR signal to noise ratio

GFP-HA (1 nM) in 50 mM phosphate buffer (pH 7.8) was adsorbed to microplate wells (50 μl/well) overnight at 4 ºC. Wells were then washed once with the microplate washer as described below. All wells were blocked with 50 μl block buffer for 1 h at RT and washed once more with the microplate washer. Following blocking, 50 μl of the pre-assembled TTA described above were added for 2-30 min at RT, and wells were washed five times with the plate washer. The addition of primers and the qPCR were performed as described above.

Preparation of CLS

E. coli BL21*(DE3)*RIPL cells were inoculated into 20 ml Overnight Express Instant TB Medium (Novagen) and incubated at RT with shaking at 100 rpm for two days. The cultures were centrifuged at 3,148 *g* for 10 min, the supernatant discarded and cell pellets resuspended in buffer A containing 10 mM imidazole, at a ratio of 10 ml/g of wet cell pellet. The resuspended cells were lysed by passing twice through a French pressure cell at 11,000 psi. Lysates were then centrifuged at 40,000 *g* and 4 ºC for 1 h with the addition of 0.05% Tween-20, and finally the CLS was frozen with liquid nitrogen and stored at -80 ºC prior to use.

GFP-HA and SrtA-GFP-HA quantification in various media by TTA qIPCR

Polyclonal goat anti-GFP capture IgG was diluted to 10 μg/ml in 50 mM phosphate buffer (pH 7.8)and adsorbed to microplate wells (50 μl/well) overnight at 4 ºC. Wells were then washed once with the microplate washer as described below. All wells were blocked with 50 μl block buffer for 1 h at RT and washed once more with the microplate washer. Following blocking, GFP-HA (100 μM in buffer B) was diluted to concentrations ranging from 0.1 pM-

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100 nM in either block buffer, neat human male AB serum (Sigma), CCM (Cell Culture Medium, Invitrogen, 12491-023), CLS, or CCS. The CCS was obtained from A549 human lung epithelial cells grown for 48 h in RPMI 1640 media supplemented with 10% FBS (a kind gift from Dr Andreas Lopata). 50 μl of each analyte were added to the wells and incubated for 2 h at RT. Reactions were performed in triplicate. After incubation, wells were washed three times with the microplate washer and 50 μl of the pre-assembled TTA (~ 0.5 nM) were added for 5 min at RT. All wells were then washed five times with the microplate washer. The addition of primers, qPCR and normalization were performed as described above. The LOD for each assay was determined as the ΔCt value occuring at three times the standard deviation of the mean negative control value. For quantification of SrtA-GFP-HA, all steps were performed as described above for GFP-HA in undiluted serum.

References

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