ELECTRONIC SUPPLEMENTARY INFORMATION

Ultrasensitive detection of antibodies using a new Tus-*Ter*-lock immunoPCR system

Isabelle Morin, Nicholas E. Dixon and Patrick M. Schaeffer

Materials and methods

Cloning, expression and purification

The LG coding sequence was amplified from the plasmid pHD389,[1] a kind gift of Prof. Lars Björck, using the following primers psJCU47: 5'-AAAAAAAGATATCGGATCCGGCGG-TCATGGCACCAAAAAAACTGCTATCGCTATCGCTG-3' and psJCU148 5'-AAAAAA-AGTCGACGCCGCCTGTTTCCGGCAGAGCGGCCGCCATTTCAGTTACCGTAAAGG-TCTTAGTCG-3'. The PCR fragment was digested with EcoRV and SalI and ligated into pET-20b(+) (Novagen) to yield pET-LG. The LG coding sequence was checked by DNA sequencing.

Cloning of Tus-HA was carried out as follows. The plasmid pPMS1259[2] coding for Tus-GFP was digested with BamHI and EcoRI to discard the GFP coding region. Oligonucleotides, psJCU 150 5'-GATCCGGACCTCAAGGGTTGGGTACCTACCCATAC-GATGTTCCAGATTACGCTTAAG-3' and psJCU151 5'-AATTCTTAAGCGTAATCTGG-AACATCGTATGGGTAGGTACCCAACCCTTGAGGTCCG-3' coding for a linker sequence (SGPQGLGT) and the C-terminal HA epitope (YPYDVPDYA) were mixed to obtain a final concentration of 1 μM, denatured for 2 min at 90°C and slowly cooled down for optimal annealing. This double-stranded DNA linker presenting the BamHI and EcoRI overhangs was then ligated into the vector to yield p-Tus-HA.

The cloning of coding sequences for SrtA-GFP and SrtA was performed as follows. Primers psJCU94 5'-AAAAAAGATATCCAAGCTAAACCTCAAATTCCGAAAGAT-AAATCG-3' and anti-sense psJCU95 5'-AAAAAAGCTAGCTTTGACTTCTGTAGCT-

ACAAAGATTTTACG-3' were used to amplify the *srtA* gene from *Staphylococcus aureus* genomic DNA, kind gift of Dr Natkunam Ketheesan. The PCR product was then digested by EcoRV and NheI and ligated into pET-GFP[3] to yield pET-SrtAGFP. Sequencing of pET-SrtAGFP indicated a deletion of the G in the NdeI site resulting in a frameshift in the reading frame. To restore the correct reading frame, pET-SrtAGFP was digested with SpeI and protruding ends were endfilled and religated to yield the correct pETc-SrtAGFP. The plasmid pETc-SrtAGFP was used to express SrtA-GFP. pETc-SrtAGFP was then simply digested by NheI, endfilled and religated to introduce a stop codon at the end of SrtA open reading frame. The product plasmid pET-SrtA was used to express SrtA. The vectors containing the coding sequences for CAT-GFP, GK-GFP, CAT and GK were cloned using the same strategy as for SrtA-GFP and SrtA, and are described elsewhere.[3]

The cloning of pPMS1260 for the production of Tus-GFP(1260) containing a poly-seryl-alanyl linker was performed as follows. Plasmid pND1100 (NED, unpublished), was constructed by a series of gene and linker insertions and is a derivative of the vector pETMCSIII[4]. It contains a modified tus gene under control of the phage T7 promoter. The modified gene encodes, in-frame and in order: an N-terminal Met-(His)6-Ser tag, followed by wild-type Tus from Met1 to Ala308, followed by a 37-residue linker with sequence Asn-Leu-Val-Asn-(Ser-Ala)15-Met-His-Met. The final His-Met codons (CATATG) comprise a unique NdeI site at which a gene of interest can be fused in-frame, and this is followed ~0.5 kb downstream by a unique EcoRI site. The complete e-gfp gene used for the construction of pPMS1259[2] (coding for Tus-GFP(1259)) was inserted between these two restriction sites to yield pPMS1260.

All proteins were expressed in *E. coli* BL21(DE3)RIPL and purified with Ni-charged resin Profinity IMAC (Bio-Rad) using a previously described strategy.[2]

Bio-TT-lock, TT-lock-T, TerB-T and primer mixes

Bio-TT-lock was assembled as follows, oligonucleotides psJCU30 5’-BiotinAAAAAAAAA-AGGGGATATGTTGTAACTAAAG-3’ and psJCU12 5’-CTTTAGTTACAACATACTTAT-3’ were diluted in dilution buffer (20 mM Tris pH 8, 150 mM NaCl) to reach a final concentration of 50 μM of annealed DNA. The mixture was denatured for 2 min at 80°C, slowly cooled to obtain optimal annealing and stored at –20°C.

TT-lock-T was assembled as follows, oligonucleotides psJCU45 5’-CACCGCTGAG-CAATAACTAGCATAAAAAAAAGAACTGGATCTCAACAGCGGTCTTTAGTTACAA-CATACTTATA-3’ and psJCU46 5’-TATGTTGTAACTAAAGACCGCTGTTGAGATC-CAGTTC-3’ were diluted in dilution buffer to concentrations of 1 and 5 μM, respectively. The mixture was denatured for 2 min at 80°C, slowly cooled to obtain optimal annealing and stored at –20°C.

TerB-T was assembled as described above with the oligonucleotides psJCU57 5’-TACGATCAATAACGAGTCGCCACAAAAAAAATGGCGACAACTCTAGGTCAAGCT-TTAGTTACAACATACTTATA-3’ and psJCU58 5’-ATAAGTATGTTGTAACTAAAGC-TTGACCTAGAGTTGTCGCCA-3’. The amplification primer mix used for TT-lock-T was obtained by diluting primers psJCU39 5’-CACCGCTGAGCAATAACTAGCAT-3’ and psJCU40 5’-ACCGCTGTTGAGATCCAGTTC-3’ to 0.5 μM in water. The amplification primer mix used for TerB-T was obtained by diluting the primers psJCU60 5’-TACGATC-AATAACGAGTCGCCAC-3’ and psJCU 61 5’-CTTGACCTAGAGTTGTCGCCA-3’ to 0.5 μM in water.

Surface plasmon resonance (SPR)

Surface plasmon resonance experiments used a ProteON XPR36 protein arrayer (Bio-Rad), operating at 22˚C to qualitatively evaluate the binding of the Tus-GFP fusion proteins to Bio-anti-GFP antibodies and to the TT-lock (Fig. S1). The first set of experiments qualitatively evaluated the dissociation rate of the Bio-anti-GFP antibodies with Tus-GFP (Nos 1259 and

1260) and parts thereof in rt-IPCR buffer conditions. Here, Bio-anti-GFP antibodies were diluted in PBS buffer pH 7.4 supplemented with 0.005% of Tween 20 (PBST from Bio-Rad Cat176-2720) at a concentration of 1 µg/mL and immobilised onto the surface of a NLC ProteOn Sensor chip (Bio-Rad; contact time of 600 s; flow rate of 25 µL/min). The various analyte proteins diluted in PBST buffer at a concentration of 60 nM were then made to flow over the surface for 300 s with a flow rate of 25 µL/min. The second set of experiments qualitatively evaluated the dissociation kinetics of Bio-TT-lock with the same analyte proteins. Here, Bio-TT-lock was diluted in PBST buffer at a concentration of 20 nM and immobilised onto the surface of the same chip on a different flow cell (contact time of 600 s with a flow rate of 25 µL/min). The various analyte proteins diluted in PBST buffer to 60 nM were then made to flow over the surface for 300 s at a flow rate of 25 µL/min.

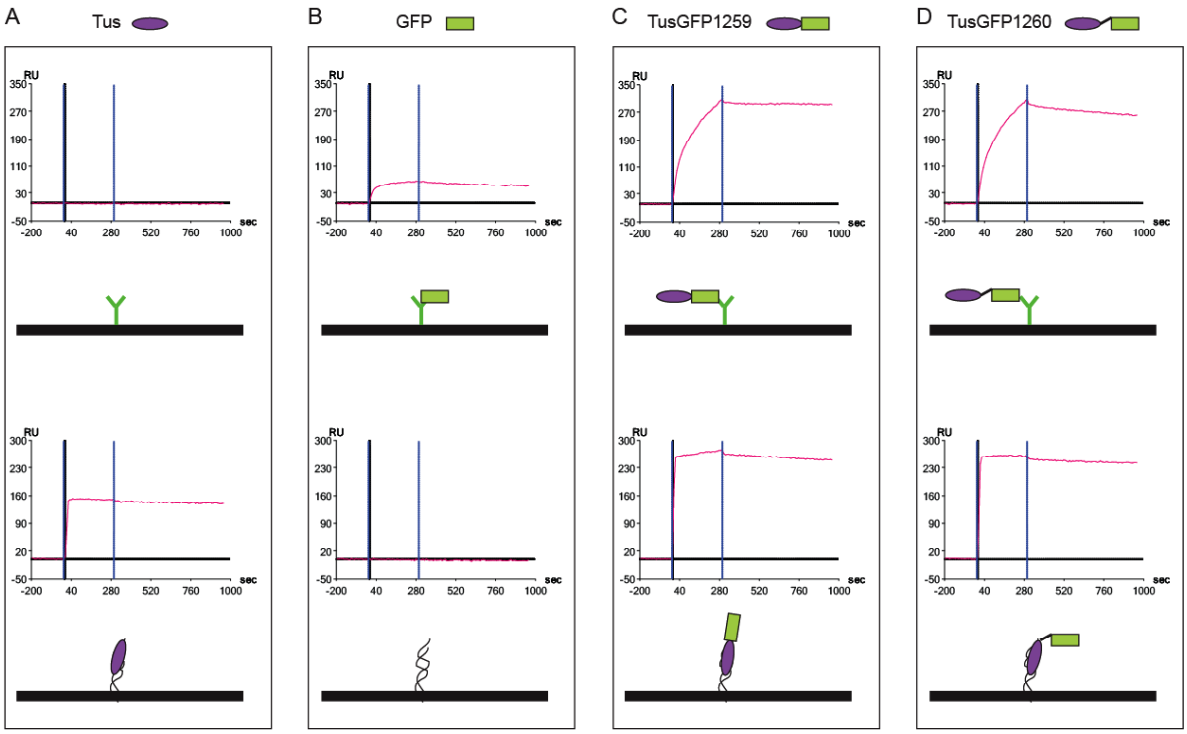

Figure S1: Binding of Tus (panel A), GFP (panel B), Tus-GFP/1259) (panel C) and Tus-GFP/1260 (panel D) to immobilized Bio-anti-GFP (top row) and Bio-TT-lock (bottom row).

Real-time Immuno-PCR with streptavidin-coated plates (direct format)

Wells of Reacti-Bind streptavidin coated HBC black 96-well plates with SuperBlock blocking buffer (Thermo Scientific) were initially washed once with 200 μL of BW buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 0.005% Tween-20). Bio-anti-GFP antibodies (Ab 6658, Abcam) were serially diluted in BW buffer (0.5 μg/mL to 50 pg/mL). Tus-GFP was diluted to 0.4 nM in BW supplemented with 1% BSA (block solution). In each well, 45 μL of Tus-GFP (0.4 nM) were incubated with 5 μL of each of the antibody dilutions during 90 min at room temperature (RT). Wells were washed 3 times with 200 μL of BW. Then, 50 μL of TT-lock-T or TerB-T at 50 pM were added to each well and incubated 30 min. Wells were washed 5 times with 200 μL of BW buffer. Finally, 50 μL of amplification primer mix were added to each well to dissociate the DNA template during 60 min at RT. The rt-PCR used the Corbett Rotor Gene RG6000 thermocycler with 10 μL of well content and 10 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The no template control (NTC) was performed as before without the antibody and measures the background signal (direct and indirect non-specific binding of DNA to the well surface). Negative controls used 10 μL of amplification primer mix to check for template contaminations. The PCR was set up as follows: a first cycle at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s and 60°C for 10 s. For data analysis, the fluorescence threshold was manually adjusted. The points at which the fluorescence crosses the threshold (Ct) were extracted for all experiments. The normalisation of the Ct values was achieved as follows. The mean of NTC values was established for each set of experiments and was subtracted from the Ct values, generating ΔCycles values. The ΔCycles values increase with increasing amounts of TT-Lock-T as a result of increasing concentrations of antibodies (Figs S2, S3).

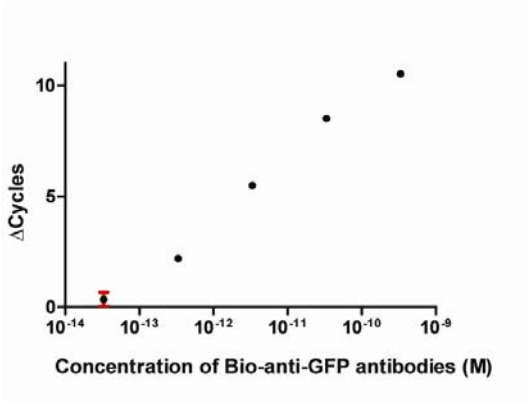

Figure S2: Detection of Bio-anti-GFP antibodies with Tus-GFP(1260)/TT-lock-T conjugates (averages of duplicate measurements).

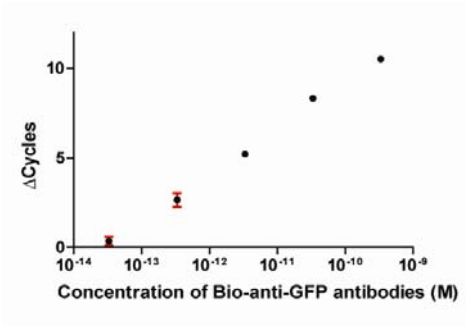

Figure S3: Detection of Bio-anti-GFP antibodies with Tus-GFP(1259)/TerB-T conjugates (averages of duplicate measurements).

Real-time Immuno-PCR with streptavidin-coated plates (competitive format)

A protocol similar to that described above was applied for the competitive assay, with the following modifications: Tus-GFP was diluted to a final concentration of 0.04 nM in the presence of Bio-anti-GFP antibodies at 333 pM and varying concentrations of a POI-GFP (CAT-GFP, GK-GFP, or SrtA-GFP in concentrations ranging from 0.4 nM to 1 μM) and incubated in streptavidin-coated wells for 90 min. The remaining steps where then performed as described above. For the control experiments, CAT, GK and SrtA were used in place of CAT-GFP, GK-GFP, and SrtA-GFP (SrtA data shown in Fig. S4).

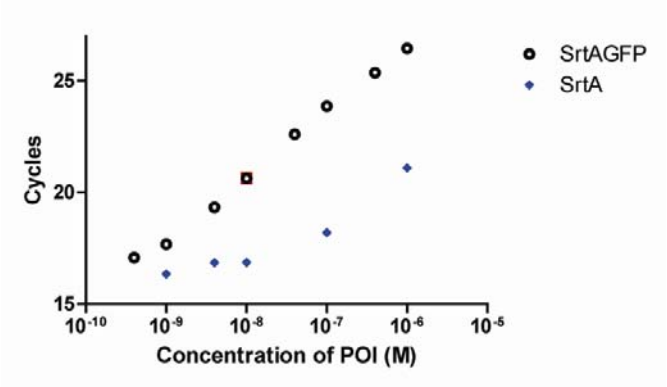

Figure S4: Concentration dependent inhibition of rt-IPCR with increasing concentrations of competing SrtA-GFP. The control SrtA proteins do compete for Bio-anti-GFP sites at higher concentrations.

Real-time Immuno-PCR with LG-coated plates

Each well of Maxisorp U96 plate (Nunc) was incubated overnight at 4°C with 2 μg of LG protein, diluted in 50 μL of phosphate buffer (45 mM Na_2HPO_4, 5 mM NaH_2PO_4, 10% v/v glycerol). Wells were washed once with 200 μL of BW followed by 60 min incubation at RT with 200 μL of block solution. Wells were then washed three times with 200 μL of BW to obtain LG-coated plates. Bio-anti-GFP or anti-HA antibodies (Roche, rat monoclonal antibody 11867423001) were serially diluted in BW buffer (0.5 μg/mL to 50 pg/mL). For the experiments in pure conditions, Tus-GFP or Tus-HA was diluted to 0.4 nM in block solution. For the experiment in cell culture medium, Tus-GFP or Tus-HA were diluted to 0.4 nM in Dulbecco's modified Eagle Medium DMEM High Glucose (GIBCO) supplemented with 10% foetal calf serum (PAA Laboratories, Morningside, Australia), L-glutamine (2 mM), penicillin (10 U/mL) and streptomycin sulphate (100 μg/mL), a kind gift of Nicole Gerlach. In each well, 45 μL of Tus-GFP or Tus-HA at 0.4 nM were incubated with 5 μL of each of the antibody dilutions for 90 min. All remaining steps were done as for the streptavidin-coated plates. Controls, real-time PCR and data analysis were as described above.

References :

1. B. M. Kihlberg, U. Sjobring, W. Kastern and L. Bjorck, *J Biol Chem*, 1992, **267**, 25583-25588.
2. D. B. Dahdah, I. Morin, M. J. Moreau, N. E. Dixon and P. M. Schaeffer, *Chem. Commun.*, 2009, 3050-3052.
3. M. J. J. Moreau, I. Morin and P.M. Schaeffer, Submitted.
4. C. Neylon, S. E. Brown, A. V. Kralicek, C. S. Miles, C. A. Love and N. E. Dixon, *Biochemistry*, 2000, **39**, 11989-11999.