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Abstract

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Keywords

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

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A system consisting of a protein LG coated surface for the capture of mammalian antibodies (target), and an antigen fused to Tus and stoichiometrically linked to a DNA template *via* the Tus–Ter-lock sequence allowed the ultrasensitive detection of 5.5 attomol of target by real-time immunoPCR in complex media.

The early detection of life threatening diseases is essential for effective treatment and control of pandemics. Currently, the enzyme linked immunosorbent assay (ELISA) is still the gold standard for most immunodiagnostic methods.^{1,2} In immunoPCR (IPCR), the enzyme linked to the detection antibody (Ab) is replaced by a DNA template that can be amplified by PCR and visualised using an agarose gel.³ More recently, the use of quantitative real-time (rt) PCR has dramatically improved sensitivity and reproducibility, and so rt-IPCR has now been used to reliably detect a wide range of antigens such as tumour markers,^{4,5} viruses,⁶ prions⁷ and toxins.⁸ However, it has rarely been used to count antibody molecules in complex media.^{9,10} In a typical rt-IPCR setup for quantification of Abs (Fig. 1A), specific Abs bind to immobilised antigens and are detected by a secondary Ab–DNA conjugate.

The rt-IPCR method relies on the challenging synthesis of protein–DNA conjugates. Most commonly, a biotinylated DNA is linked to biotinylated Abs *via* a streptavidin bridge.^{6,11} While the biotin–streptavidin complex is strong ($K_d \approx 4 \times 10^{-14}$ M),¹² the resulting DNA–Ab conjugates are heterogeneous because streptavidin is tetrameric, affecting batch to batch reproducibility of the rt-IPCR. Moreover, this procedure requires multiple assembly and wash steps, and is expensive due to the high cost of reagents. DNA can also be covalently bound to Abs using bifunctional crosslinking agents or native chemical ligation.^{5,13} The latter covalently links the DNA site-specifically and with controlled stoichiometry, resulting in homogenous conjugates. However, all conjugation steps are complex, time-consuming and consequently preclude this technique from routine use in clinical settings.^{13,14} In addition, the non-specific binding of human anti-IgG has been shown to affect the sensitivity of IPCR dedicated to measure

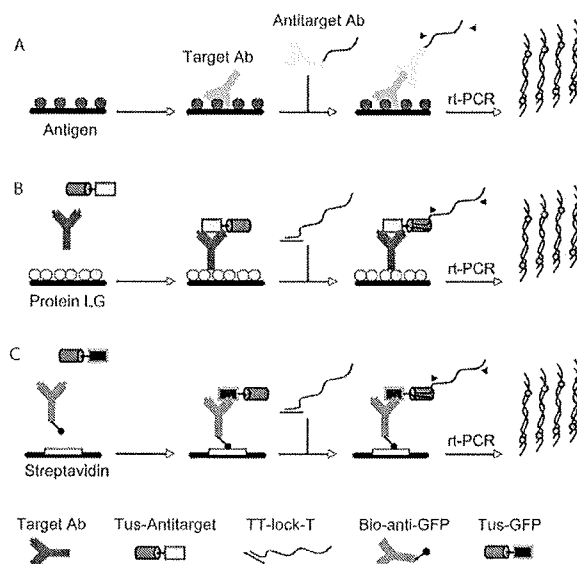


Fig. 1 A. Schematic representation of an rt-IPCR setup for the detection of a specific antibody (Ab).⁹ B. Schematic representation of the TT-lock based rt-IPCR setup for the detection of Abs. The target Ab contained in a sample is mixed with Tus–antitarget and the complex is captured by the protein LG coated surface. TT-lock extended DNA template (TT-lock–T) binds to the Tus domain and is further quantified by rt-PCR. C. TT-lock based rt-IPCR setup used for validation and for the competitive format. Biotinylated anti-GFP (Bio-anti-GFP) Abs are mixed with Tus–GFP and the complex is captured by the streptavidin-coated surface. TT-lock–T binds to the Tus domain and is further quantified by rt-PCR.

Abs, compromising the advantages gained by PCR amplification compared to ELISA.⁹ Consequently, a new stratagem is needed to develop a powerful diagnostic tool dedicated to the accurate quantification of Abs in a complex biological sample.

Tus is a monomeric replication terminator protein that binds tightly to short DNA sequences called *Ter*, present in the *E. coli* chromosome. The Tus–Ter-lock (TT-lock) sequence is a partially forked ds*Ter* DNA oligonucleotide that makes an extremely stable interaction with Tus.¹⁵ In recent work, Tus has been successfully used as a fusion tag to covalently link a modified TT-lock sequence to GFP.¹⁶ Here, we use the TT-lock to stoichiometrically and site-specifically link a DNA template with an antigen or epitope for the production of an antitarget–DNA conjugate to be used as a sensitive detection device dedicated to quantify specific Abs. In this new approach, protein LG coated wells are used to immobilise antibodies present in a sample since protein LG binds strongly

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to most mammalian Abs.^{17,18} The antitarget–DNA conjugate binds to a specific Ab and the DNA template is used to generate the rt-PCR signal (Fig. 1B).

As a proof of principle, we first used GFP as an antitarget protein to measure the concentration of biotinylated anti-GFP (Bio-anti-GFP) Abs immobilised on a streptavidin-coated support, using Tus–GFP¹⁶ as the detection device (Fig. 1C).

The binding of Tus–GFP, GFP and Tus to Bio-anti-GFP and the TT-lock sequence was first qualitatively assessed by SPR. As expected, Tus binds to the TT-lock, and GFP to Bio-anti-GFP Abs (see ESI,† Fig. S1A and B). Tus–GFP constructs (Nos 1259 and 1260) containing two different linker sequences were found by SPR to bind similarly to both the TT-lock and the anti-GFP surfaces, showing extraordinarily slow dissociation rate constants (Fig. S1C and D, ESI†). The size of the linker between Tus and GFP did not significantly affect the binding properties of Tus–GFP. Next, PCR amplification of the TT-lock extended DNA template (TT-lock–T) used for IPCR was optimised. The PCR standard curve of TT-lock–T displayed a correlation coefficient of 0.999, with a limit of quantification of 0.5 amol L⁻¹, over a 6-log dynamic range (Fig. 2A). The best rt-IPCR performance was obtained when Tus–GFP (No. 1259) at 0.4 nM was bound during 90 min to Bio-anti-GFP Abs at concentrations ranging from 33 fM to 0.3 nM in streptavidin-coated wells. After three initial wash steps, TT-lock–T at 50 pM was added for 30 min at RT. After five further wash steps, TT-lock–T was quantified

using rt-PCR (Fig. 2B). We were able to accurately quantify 111 fM of Bio-anti-GFP Abs. The detection limit was estimated to be 33 fM, corresponding to 1.6 amol of Bio-anti-GFP antibodies (~150 kDa) in 50 µL well volume. Similar results in terms of detection limit and reproducibility were obtained using the other Tus–GFP construct (No. 1260; see ESI,† Fig. S2).

At this stage, it is important to note that the PCR background signal represents the limit of detection of the TT-lock based rt-IPCR and is due to non-specific binding of the TT-lock–T DNA to the well of the plate. Initially, we tested the effect of constant concentrations of Tus–GFP with varying concentrations of TT-lock–T as well as varying concentrations of Tus–GFP with a constant concentration of TT-lock–T (data not shown). The main contributor to the rt-PCR background signal was found to be the concentration-dependent non-specific adsorption of the DNA to the well. The concentration of TT-lock–T (50 pM) that we used in our assays was found to be optimal; no improvement in signal to background ratio was obtained at lower concentrations.

We also tested the IPCR setup with an extended fully ds*TerB* DNA template (TerB–T; see ESI,† Fig. S3). Similar results were obtained as for TT-lock–T, since in low salt wash conditions (≤150 mM NaCl) neither ds*TerB* nor TT-lock dissociates during the time of the wash steps.¹⁵

Next, a competitive rt-IPCR was studied (Fig. 2C) to further validate the Tus–*Ter*-lock system as a basis for a specific and reliable immunoassay, and in addition, to quantify other GFP-fusion proteins. For this, we tested chloramphenicol acetyltransferase fused to GFP (CAT–GFP) and glycerol kinase fused to GFP (GK–GFP). Bio-anti-GFP antibodies (0.3 nM) and Tus–GFP (0.04 nM) were mixed in the presence of increasing concentrations (0.4–1000 nM) of CAT–GFP or GK–GFP in streptavidin-coated plates. As expected, CAT–GFP and GK–GFP competed with Tus–GFP binding sites, reducing the rt-PCR signal in a concentration-dependent manner (Fig. 2D). This further demonstrates the specificity and reliability of our rt-IPCR. The competitive assay is clearly limited in its application as a diagnostic tool as it cannot be used to measure low abundance proteins; nevertheless it could be a very practical research tool for the quantitative and specific detection of epitope-tagged fusion proteins originating from any cell line. It is important to note that *Staphylococcus aureus* sortase fused to GFP (SrtA–GFP) was also tested in the competitive rt-IPCR format but unfused SrtA was found to decrease the signal non-specifically at very high concentrations (Fig. S4, ESI†).

Next, we developed a high-density protein LG surface capable of capturing any mammalian antibody of interest. This enabled the Tus–*Ter*-lock IPCR assay to work in complex media, revealing the potential of this assay in diagnostics. The chimeric protein LG combines the light chain binding domains of protein L and an Fc-binding protein G domain.^{17,18} Purified LG was used to coat a 96 well plate at 4 °C overnight, after which all binding and wash steps were as with the streptavidin-coated plates (see ESI†). Using this LG-coated support, we could accurately quantify 333 fM of Bio-anti-GFP Abs in buffer (purified condition) or in cell culture medium supplemented with 10% foetal calf serum (Fig. 3A).

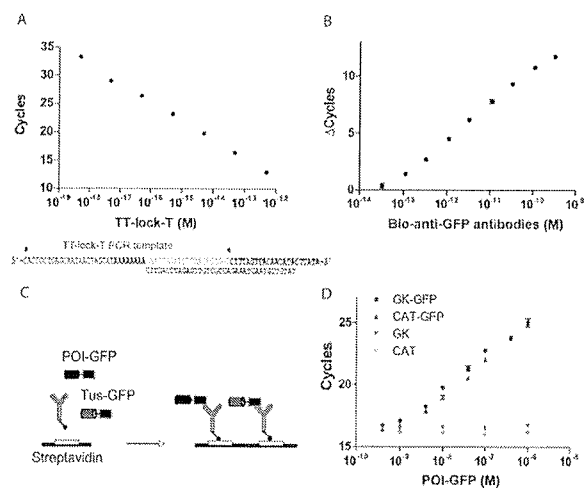


Fig. 2 Detection of Bio-anti-GFP antibodies with Tus–GFP. A. rt-IPCR of the TT-lock–T. Details of the TT-lock–T sequence and primers used for PCR amplification are shown (below) by arrowheads. B. Tus–GFP (0.4 nM) was treated with a range of concentrations of bio-anti-GFP (33 fM–0.33 nM) in streptavidin-coated wells. After the initial wash steps, the TT-lock–T (50 pM) was added to the well. After the final wash steps, rt-PCR was used to quantify the bound TT-lock–T. C. Competitive rt-IPCR format. TusGFP (0.04 nM) was treated with Bio-anti-GFP (0.33 nM) and competing POI–GFP (POI: protein of interest). After the initial wash steps, binding and detection of TT-lock–T was performed as usual. The POIs were CAT and GK. D. Concentration-dependent inhibition of rt-IPCR with increasing concentrations of competing POI–GFP. The control POIs, CAT and GK do not compete for Bio-anti-GFP sites. Duplicate measurements are shown.

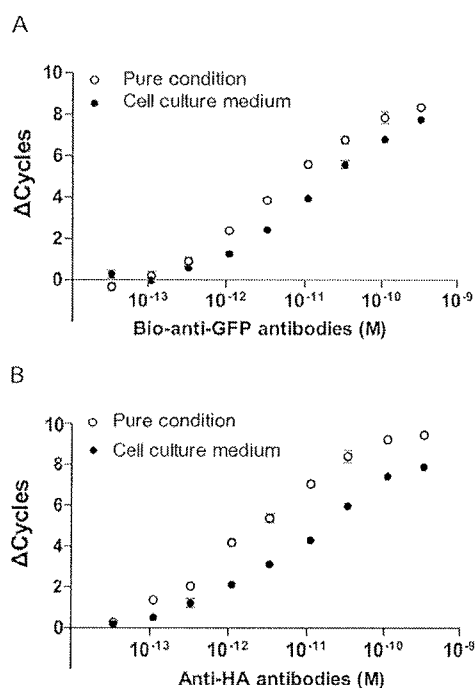


Fig. 3 Detection of Bio-anti-GFP and anti-HA antibodies using a protein LG coated surface in complex media. A. Tus-GFP (0.4 nM) was incubated with a range of concentrations of Bio-anti-GFP Abs (33 fM–0.3 nM) in LG-coated wells, either in blocking solution (pure condition) or in complete cell culture medium supplemented with 10% foetal calf serum. After washes, the TT-lock-T (50 pM) was added to the well. After further wash steps, rt-PCR was used to quantify the bound TT-lock-T. B. Tus-HA (0.4 nM) was incubated with a range of concentrations of anti-HA Abs (33 fM–0.3 nM) in LG-coated wells, either in blocking solution (pure condition) or in cell culture medium. All subsequent steps were as described for Tus-GFP. Each point is the average of triplicates.

The detection limit in both conditions was estimated to be 111 fM. A similar experiment was used also to measure anti-HA Abs, using Tus fused with the hemagglutinin A (HA) epitope (Tus-HA) as the detection device; 111 fM of anti-HA Abs (~150 kDa) could be accurately quantified, corresponding to 5.5 pg mL⁻¹, with a detection limit of 33 fM (Fig. 3B). In comparison,¹⁰ anti-E2 antibodies were previously detected by an ultrasensitive rt-PCR in cell culture medium at 120 pg mL⁻¹. This demonstrates the outstanding performance of our IPCR setup. It is interesting to note that when assays were carried out in one pot, the limit of detection increased by almost two orders of magnitude, which is still acceptable for many diagnostic applications with the advantage that assay time was nearly halved (data not shown).

The TT-lock-based rt-IPCR presented here has a wide linear dynamic range of four orders of magnitude. It does not require the use of streptavidin–biotin chemistry, which is known to result in non-specific binding. The technique requires reduced numbers of steps and incubation times. As a result, rt-IPCR data were obtained within 5 h, all steps included. In addition, the technique demonstrated a very high batch to batch

reproducibility as a direct result of the use of the monomeric TT-lock system for the production of homogeneous protein–DNA conjugates.

Although we have tested this new system with two specific Abs and demonstrated its robustness and reproducibility, there is still space for further developments. For instance, the antitarget–Tus reagent is currently expressed in *E. coli*, which does not support any posttranslational modifications. It is yet to be shown if Tus fusion proteins can be expressed in eukaryotic systems to produce posttranslationally modified antitarget fusion proteins, which would be very useful reagents. Additionally, although the TT-lock complex is extremely stable, it will still dissociate over time, limiting its use to monoplex rt-IPCR applications. We have previously shown that introduction of a 5-bromodeoxyuridine residue into the TT-lock enables simple production of irreversibly photocrosslinked Tus–DNA conjugates in high yields.¹⁶ This strategy could be used in our TT-lock based rt-IPCR to afford a route to multiplex rt-IPCR. Due to the simple methods for reagent preparation and its reproducibility, we expect TT-lock based rt-IPCR methods to become useful tools for a broad range of biomedical applications. We are currently developing other formats of this method for the detection of antigens using both monoplex and multiplex strategies.

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