

Quantifying small numbers of antibodies with a 'near-universal' protein-DNA chimera

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We present general means to greatly increase the sensitivity of antibody-based assays. Augmentation relies on a 'tadpole' protein-DNA chimera whose protein moiety binds most classes of mammalian antibodies but not avian immunoglobulin Y (IgY). We used this tadpole in affinity capture assays followed by real-time PCR to quantify numerous molecules, including prostate-specific antigen (PSA) in human serum, with great sensitivity and accuracy.

Antibodies conjugated to isotopes¹, enzymes² and dyes³ are widely used to detect antigens and amplify measurement signals. Although assays based on antibody-DNA fusions^{4–6} are more sensitive than the state-of-the-art enzyme-linked immunosorbent assays (ELISAs)², the performance of all these methods is limited by the quality of the antibody conjugates. Unfortunately, the chemical means used to make such conjugates often diminish antigen-binding activity and yield product mixtures with a range of stoichiometries, typically 3–6 reporter molecules per antibody⁷. Using such heterogeneous conjugates requires calibration of each preparation. Moreover, the range of stoichiometries increases signal noise, which reduces assay precision, and ultimately reduces the sensitivity and dynamic range of antigen measurement, especially near the limit of detection. We previously addressed these issues by site-specifically conjugating DNA to recombinant affinity proteins yielding homogeneous reagents with 1:1 stoichiometries (known as 'tadpoles') for measuring small numbers of individual molecules⁸.

Here we describe the construction a tadpole that can be used to quantify mammalian antibodies and opens the possibility

of enhancing the sensitivity of existing immunological assays with the amplification power of PCR. We expressed an intein fusion (Fig. 1a) of a recently described polypeptide⁹ assembled from domains of *Streptococcus* sp. protein G, which binds mammalian immunoglobulin γ (IgG) Fc chains¹⁰, and *Peptostreptococcus* sp. protein L, which binds mammalian immunoglobulin κ and λ light chains¹¹, in *Escherichia coli* (Rosetta-gami B; Novagen) and joined it to a cysteine-containing oligo via intein-mediated ligation in solution (Supplementary Fig. 1a online). The accumulation of the protein-DNA fusion reached a maximum in 4 h at 37 °C compared with the 16 to 20 h we had previously observed for solid-phase synthesis at 22 °C (ref. 8). We purified the resulting LG tadpole by serial anion exchange and hydrophobic-interaction chromatography.

We verified that the LG tadpole (Fig. 1b) consisted of protein covalently linked to dsDNA and that it bound mammalian immunoglobulins. On native polyacrylamide gels, the electrophoretic mobility of SYBR gold-stained tadpole was reduced compared to that of the DNA alone (Supplementary Fig. 1a). Notably, this slower-migrating species shifted back to the faster mobility observed for the DNA tail after proteinase K treatment (Supplementary Fig. 1b). We observed that the LG tadpole bound mouse and rabbit IgG-coated beads, but not ethanolamine-coated beads (Supplementary Fig. 1c) with a measured dissociation constant of 3.4 nM for mouse immunoglobulin (Supplementary Fig. 2 online). This dissociation constant is close to the range of the reported affinities of unconjugated LG for human IgG (5.9 nM), human IgG Fc fragments (2.2 nM) and human immunoglobulin κ light chains (2.0 nM)⁹, and is consistent with our observation that the dissociation constant of a single-chain antibody domain for

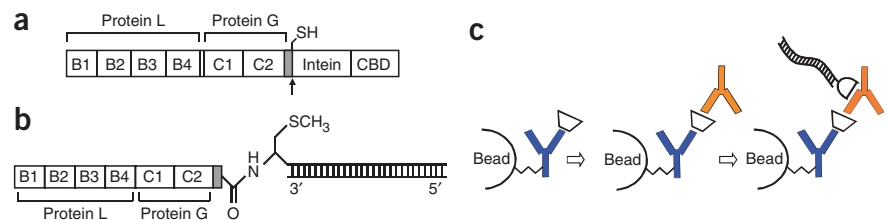


Figure 1 | LG-intein fusion protein, LG tadpole and measurement assay. **(a)** Structure of the protein LG intein fusion protein with the protein L (B1–4) and protein G (C1–2) domains, the polyhistidine domain (gray box), the *Methanobacterium thermoautotrophicum* rir1 intein domain, and the chitin-binding domain (CBD). The arrow indicates the position of the intein active-site cysteine (sulfhydryl is labeled) and the polypeptide hydrolysis site. **(b)** LG antibody-binding polypeptide is covalently attached via amide linkage to a single 5' terminus on the dsDNA. Gray box represents the 6 histidine residues proximal to the DNA conjugation site. **(c)** Scheme of antigen capture using IgY-coupled (blue) magnetic beads: bound antigen is detected with a second mammalian antibody (gold), and quantified by the amount of bound detection antibody in the antigen sandwich using the LG tadpole and real-time PCR.

anthrax protective antigen was not substantially altered by DNA attachment⁸. Neither the oligonucleotide tail alone, nor the tadpole pretreated with proteinase K, heat or 0.1 M DTT bound mouse IgG-coated beads, indicating that tertiary protein structure was necessary for immunoglobulin binding (data not shown).

The LG tadpole binds mouse monoclonal antibodies typically used as antigen capture reagents in conventional sandwich immunoassays¹². However, we discovered this tadpole does not bind chicken¹³ IgY-coated beads (**Supplementary Fig. 3** online). We used this fact to test improvements on detection limit and linear range for assays (**Fig. 1c**) measuring an *E. coli* transcriptional initiation factor 1 fusion protein (INFA-TAP)¹⁴ and human interleukin 6 (IL6). For these measurements, and throughout this work, we used IgY as a capture antibody. We controlled for cross-reactive binding of the detection IgG and nonspecific binding of the tadpole by subtracting the value of the signal of replicates containing no detection IgG and no antigen, and confirmed equivalent low levels of nonspecific signal even in the presence of a nonspecific detection antibody. First, we coated M280 tosyl beads (Dyna) with affinity-purified chicken polyclonal anti-protein A IgY (Immunology Consultants Laboratory), captured INFA-TAP from 50- μ l serial dilutions, detected bound antigen with polyclonal rabbit anti-TAP IgG (Open Biosystems), and quantified the bound antibodies with the LG tadpole and real-time PCR (**Supplementary Fig. 4** online). We detected as few as 21,559 INFA-TAP molecules (36 zeptomoles) in 50- μ l samples (equivalent to a 716 attomolar detection limit). Next we measured IL6 from serial dilutions in mouse serum using affinity-purified chicken anti-IL6 IgY (Genway), rabbit polyclonal anti-IL6 IgG (Pierce) and LG tadpole as above (**Supplementary Fig. 5** online). After correcting for nonspecific signal, as above, we detected as few as 1,016 human IL6 molecules (1.7 zeptomoles) in 50 μ l (equivalent to a 34 attomolar detection limit). The tadpole-enhanced INFA-TAP and IL6 assays exhibited wider linear range and were considerably more sensitive than the 2.6 nanomolar (**Supplementary Fig. 6** online) and 18 picomolar (**Supplementary Fig. 7** online) detection limits obtained by ELISA using the same antibodies, respectively.

To extend this technique to quantifying proteins in clinical samples, we first created a calibration for measuring human PSA diluted into calf serum. We verified that calf serum had undetectable levels of PSA and contributed little to the nonspecific signal beyond that generated by beads alone (data not shown). Here we used affinity-purified chicken anti-PSA IgY (Lee Biosolutions), polyclonal rabbit anti-PSA IgG (Abcam) and the LG tadpole to measure PSA over five orders of magnitude to a 1.6 picomolar detection limit with a precision (defined as the percentage coefficient of variation) of 4.8% in the middle of the linear range of measurement (**Fig. 2a**). We also used the same antibodies to create a matched ELISA that measured PSA in these dilutions over three orders of magnitude to a nanomolar detection limit with a precision of 9.8% (**Fig. 2a**).

In parallel experiments, we calibrated this method using human serum. We first verified the absence of substances that interfere with PSA quantification by spiking pure PSA into human serum and recovering 102–112% of the spiked PSA from these samples, compared with that observed from a buffer control (**Supplementary Table 1** online). These measurements indicated that human serum did not have a negative impact on the limit of detection greater than did calf serum. These measurements exhibited a 4.1 pM

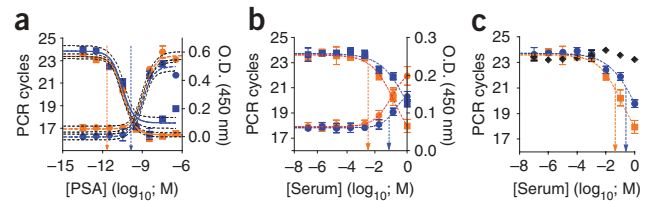


Figure 2 | Quantification of PSA in human serum. **(a)** Calibration measurement of purified PSA added to human (blue) or calf (gold) serum using either tadpoles (squares) or ELISA (circles). Gold and blue dashed vertical arrows indicate limits of detection for the tadpole assays and ELISAs, respectively. Solid and dashed curves indicate means and 95% confidence belt, respectively, as in reference 8. **(b)** PSA concentration measured in serum from an individual with prostate cancer (gold) and serum from a patient with congestive heart failure (blue) using either tadpoles (squares) or ELISA (circles). Gold and blue dashed vertical arrows indicate limits of detection for the cancer serum measurements using tadpole assays and ELISA, respectively. **(c)** Tadpole quantification of PSA in serum of a healthy individual (black diamonds), an individual with prostate cancer (gold squares) and an individual with congestive heart failure (blue circles). Dashed curves and arrows indicate mean values and the middle of the assay range, respectively. Error bars, 2 s.d. ($n = 3$).

limit of PSA detection and five orders of magnitude dynamic range with a measurement precision of 6.6% (**Fig. 2a**). Both the ELISA and tadpole assays exhibited a slight so-called ‘hook-effect’¹⁵ at higher PSA concentrations (that is, the assays detected fewer antigen-bound antibodies at high PSA concentrations), and this effect was more pronounced in human serum, which necessitated greater dilution of the sample to accurately quantify PSA.

We then measured PSA in human sera from three adult males: one healthy, one with congestive heart failure and one with prostate cancer. We obtained sera from a clinical laboratory (Asterand, Inc.). This laboratory had determined PSA concentrations in the prostate cancer serum to be 10.1 ng/ml using the Elecsys PSA assay (Roche Diagnostics) operating within US Food and Drug Administration (FDA)-approved specifications. To confirm that these sera lacked substances that interfered with PSA quantification, we spiked known quantities of PSA into these samples and recovered 80–120% of the spiked PSA with the tadpole-augmented immunological assay (**Supplementary Table 1**).

We serially diluted each serum sample identically to the calibration standards (above) and measured PSA in these dilution series using tadpoles and a matched ELISA. We determined a limiting dilution of 1:500 and 1:30 for the tadpole and ELISA measurements, respectively, corresponding to detection limits of 220 attomoles (4.4 picomolar or 131 pg/ml) and 4.8 femtomoles (95.5 picomolar or 2 ng/ml), respectively (**Fig. 2b**). To determine absolute PSA concentration in these sera, we prepared 1:10 serum dilutions and quantified PSA in three replicates by comparing responses to the human serum calibration control. We measured 0.09, 4.2 and 10.8 ng/ml PSA in the ‘healthy’, ‘heart failure’ and ‘prostate cancer’ sera, respectively (**Fig. 2c**). These measurements were reproducible: within each dilution series, the coefficient of variation was 3.8%, and among separate assays performed on three successive days, it was 6.1%. These results show that, when performed on human serum, assays using the LG tadpole were about 100 times more sensitive than the matched ELISA.

We described a ‘near-universal’ mammalian antibody counting tadpole, comprised of a protein head, LG, covalently linked to a

PCR-amplifiable DNA tail in a 1:1 molar ratio. Our solution-phase conjugation method is superior to our previously reported 'on-column' method⁸ (and conventional chemical techniques) because the mild reaction conditions preserve binding activity and are amendable to large-scale synthesis. Given the number of mammalian antibodies now in use and the increasing availability of IgYs, we believe this tadpole holds substantial near-term promise for enhancing the sensitivity, precision and dynamic range of existing assays. In the long term, we suspect that this tadpole will be an interim technical development, which will eventually be superseded by methods based on non-antibody affinity molecules. In the meantime, we hope this LG tadpole can become a useful standard secondary reagent for immunological assays and increase their sensitivity and precision.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

I.B. synthesized and purified LG tadpoles, conjugated antibodies to magnetic beads, performed binding assays and quantified data. K.Y. made the expression constructs, performed polyacrylamide gel analysis, control ELISAs and PCR measurements. R.Y. suggested the use of the LG protein and had input into project design. O.R. and R.B. had input into project design and interpretation. I.B. and R.B. wrote the manuscript and guarantee its integrity.

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