Supplementary Information

Ultrasensitive antibody detection by agglutination-PCR (ADAP)

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Antibody	Sequence	Diluent	Detection limit	
			ng/ml	*attomoles*
Anti-biotin	4	buffer	0.89 ±0.39	12 ±5
Anti-biotin	4	serum	3.7 ±2	49 ±27
Anti-biotin	1	buffer	0.072 ±0.008	0.96 ±0.11
Anti-Mouse IgG	4	buffer	1.9 ±0.6	25 ±8
Anti-Mouse IgG	4	serum	2.3 ±0.3	31 ±4
Anti-Mouse IgG	2	buffer	0.096 ±0.023	1.3 ±0.3
Anti-Mouse IgG	2	serum	0.060 ±0.008	0.8 ±0.1
Anti-GFP pAb	4	buffer	2.0 ±1.1	27 ±15
Anti-GFP pAb	4	Serum	1.7 ±0.6	23 ±8
Anti-GFP pAb	4	Human plasma	3.6±0.5	48±7
Anti-GFP mAb	4	Buffer	0.16 ±0.05	2.1 ±0.7
Anti-GFP mAb	4	cell culture media	0.12 ±0.05	1.6 ±0.7
Anti-Insulin	2	buffer	0.39 ±0.48	4.3 ±5.3
Anti-Insulin	ELISA	buffer	6.7 ±1.9	3722 ±1056
Anti-Insulin	2	serum	0.015 ±0.004	0.17 ±0.04
DNP	4	antisera	370 ±83	*N/A*

Table S1. *Summary of limits of detection for various antibodies in different diluents and with different DNA sequences using ADAP*. Antibodies were resuspended in the indicated diluent and then incubated with 2 µL of 0.5-1 nM solution of the appropriate antigen-DNA conjugate. The limit of detection for the ADAP is defined as the average ΔC_t value of the buffer C only blank plus 3 standard deviations of the blank[41]. The limit of detection is calculated relative to the blank.

Name	5' mod	Sequence	3' mod	Notes
Set 1A	thiol	CAGGTAGTAGTACGTCTGTTTC ACGATGAGACTGGATGAA	none	
Set 1B	phosphate	TCACGGTAGCATAAGGTGCAAG ATAATACTCTCGCAGCAC	thiol	
Set 1 bridge	none	CUACCGUGAUUCAUCCAG	none	Same as Set 2, U = deoxyribouracil
Set 1 F	none	GGCCTCCTCCAATTAAAGAA	none	Same as Set 2
Set 1 R	none	GTGAACCGTTATTTGGGTAC	none	Same as Set 2
Set 2A	thiol	GGCCTCCTCCAATTAAAGAATC ACGATGAGACTGGATGAA	none	
Set 2B	phosphate	TCACGGTAGCATAAGGTGCAGT ACCCAAATAACGGTTCAC	thiol	
Set 2 bridge	none	CUACCGUGAUUCAUCCAG	none	Same as Set 4, U = deoxyribouracil
Set 2 F	none	GGCCTCCTCCAATTAAAGAA	none	Same as Set 4
Set 2 R	none	GTGAACCGTTATTTGGGTAC	none	Same as Set 4
Set 4A	thiol	TCGTGGAACTATCTAGCGGTGT ACGTGAGTGGGCATGTAGCAAG AGG	none	
Set 4B	phosphate	GTCATCATTCGAATCGTACTGC AATCGGGTATTAGGCTAGTGAC TACTGGTT	thiol	
Set 4 bridge	none	GAAUGAUGACCCUCUUGCUA	none	U = deoxyribouracil
Set 4 F	none	CGTGGAACTATCTAGCGGTGTA	none	
Set 4 R	none	ACCCGATTGCAGTACGATTC	none	

Table S2. *Oligonucleotide sequences*[19-22] *used in ADAP*. Set 1A, 1B, 2A, 2B, 4A and 4B represent the sequences for oligonucleotides on the antigen-DNA conjugates. Set 1F, 1R, 2F, 2R, 4F and 4R represent the primer sequences for qPCR quantification of the full length amplicons. Set 1 bridge, Set 2 bridge and Set 4 bridge represent the sequences for the bridge oligonucleotides as shown in **Figure 1a**.

Technology	ADAP	ELISA	RIA
Detection limit (attomole)	4.3	3722	32*
Sample volume	2 µl	100 µl	25 µl
# of assays 100 µg antigen	1.7 million	10-50	N/A
Assay time	< 3hr	< 4hr	3-24 hr
Multiplexability	2-50	<9	N
Solid support	N	Y	N
Washing/centrifugation	N	Y	Y
Radioisotope	N	N	Y
Detection device	PCR thermocycler	Plate reader	Gamma counter

Table S3. *ADAP enjoys many advantages over common antibody detection methods*. ADAP uses very low quantities of sample with ultralow reagent consumption in a solution-phase,

wash-free, radioisotope-free assay. ADAP and ELISA values were calculated from in-house experiments, while the value for RIA was determined from literature [Falorni, A.; Ortqvist, E.; Persson, B.; Lernmark, A. Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using 35S or 3H recombinant human ligands. *J. Immunol. Meth.* **1995**, *186*, 89-99.].

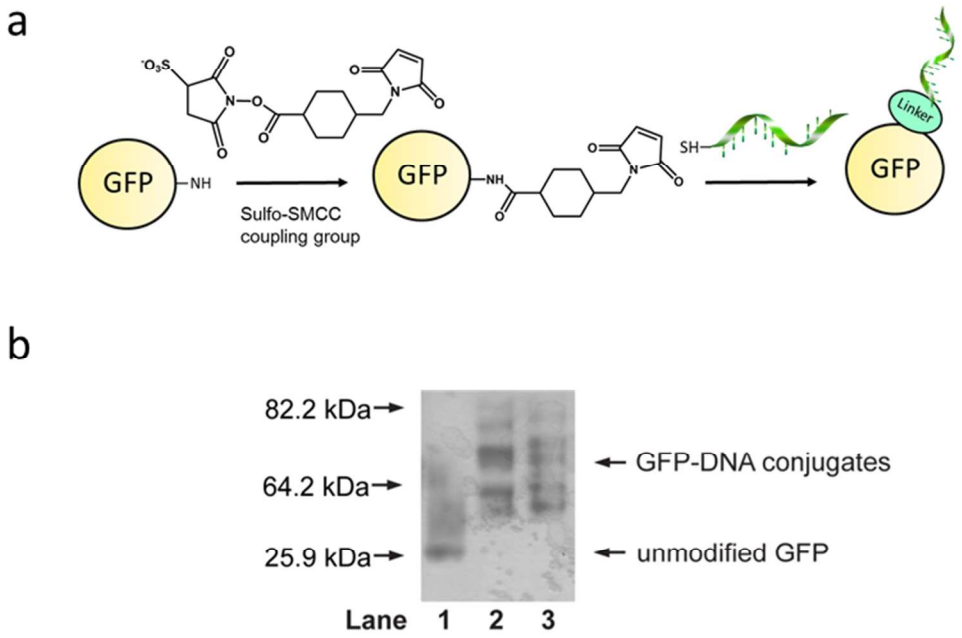

Figure S1. *Representative silver stain of an antigen-DNA conjugate*. (**a**) Reaction schemes for GFP-DNA conjugation by sulfo-SMCC. (**b**) Samples were resolved by SDS-PAGE and then total protein/ DNA was visualized by silver staining. Lane 1 is unmodified GFP, lane 2 is GFP-2A conjugate and lane 3 is GFP-2B conjugate. A significant mass shift was observed in lanes 2 and 3 due to the addition a 14 kD oligonucleotide to the protein. Laddering of lanes 2 and 3 is a result of the addition of multiple oligonucleotides to a single protein.

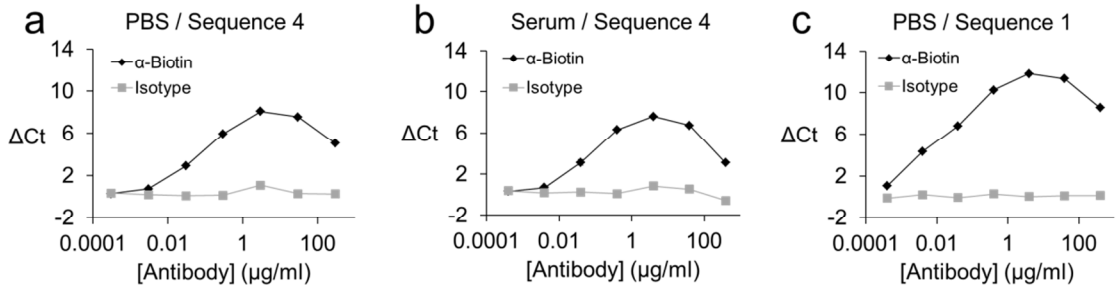

Figure S2. *Representative ADAP curves from anti-biotin detection experiment*. (a) Anti-biotin antibodies were incubated with biotin-DNA conjugates and analyzed by ADAP. An isotype antibody was also tested as a negative control. (b) Antibodies were then spiked into fetal bovine serum and analyzed. (c) Finally, biotin-DNA conjugates bearing alternate sequences were used to show replicability. Error bars represent the standard deviation from triplicate but are too small to be visualized for many data points.

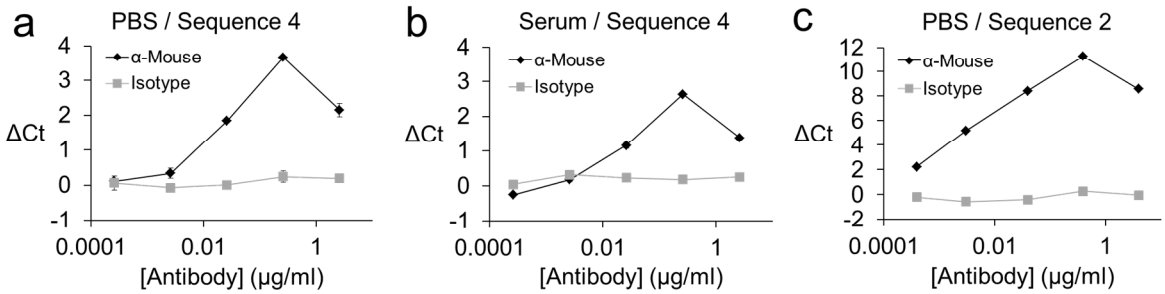

Figure S3. *Representative ADAP curves from anti-mouse detection experiment*. (a) Anti-mouse antibodies were incubated with mouse IgG-DNA conjugates and analyzed by ADAP. An isotype antibody was also tested as a negative control. (b) Antibodies were then diluted into fetal bovine serum and analyzed. (c) Finally, mouse IgG-DNA conjugates bearing alternate sequences were used to show replicability. Error bars represent the standard deviation from triplicate but are too small to be visualized for many data points.

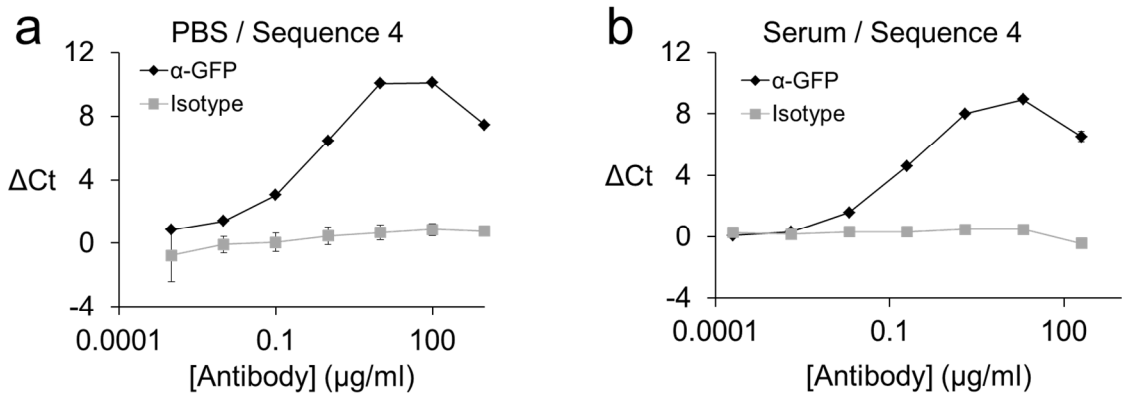

Figure S4. *Representative ADAP curves from anti-GFP detection experiment*. (a) Anti-GFP antibodies were incubated with GFP-DNA conjugates and analyzed by ADAP. An isotype antibody was also tested as a negative control. (b) Antibodies were then diluted into fetal bovine serum and analyzed. All experiments were performed in triplicate. Error bars represent the standard deviation from triplicate but are too small to be visualized for many data points.

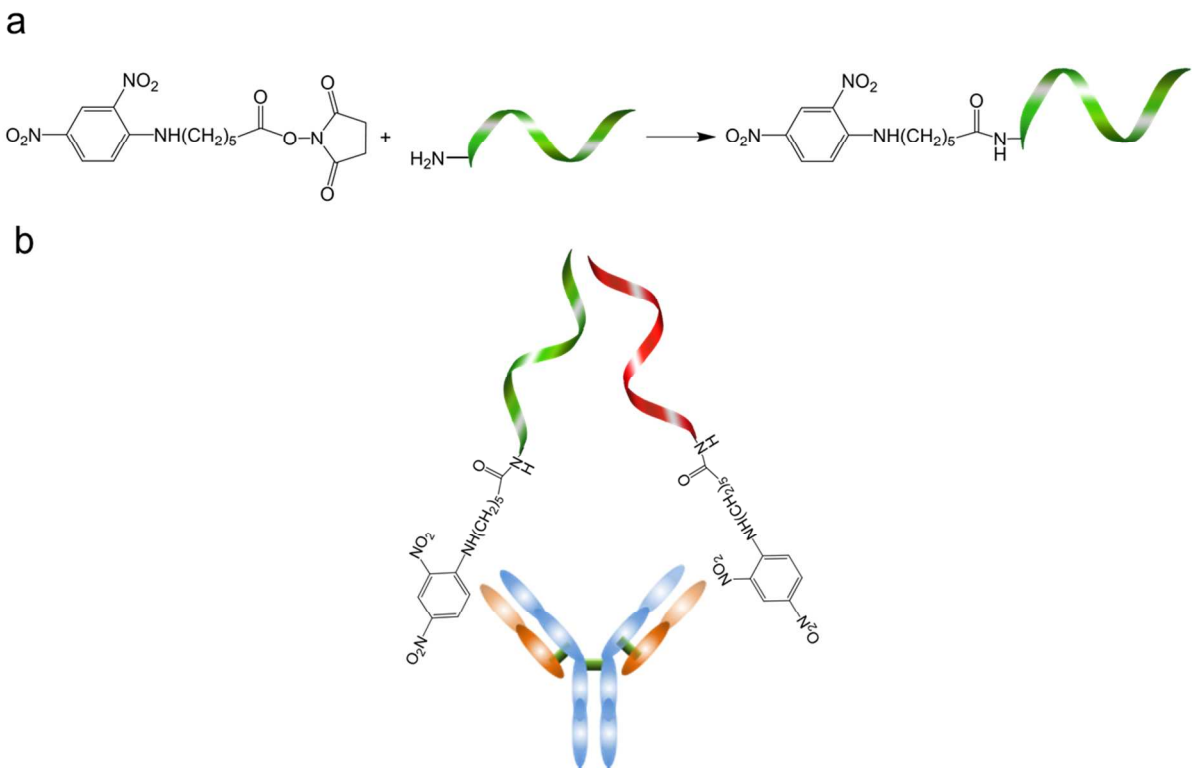

Figure S5. *Schematic diagram for small molecule-DNA conjugate ADAP*. (a) Reaction scheme for small molecule-DNA conjugate synthesis. DNP-DNA conjugates are synthesized by reacting succinimidyl ester-activated DNP with amine modified DNA in one step. The conjugation product is then characterized by mass spectrometry (b) Small molecule-DNA conjugate pairs are brought in close proximity by binding to the complementarity determining region on the antibodies. A short bridge oligonucleotide bind onto the complementary sequences, and a DNA ligase ligates the two-halves of DNA into a full length amplicon, which can be further amplified and quantified by qPCR.

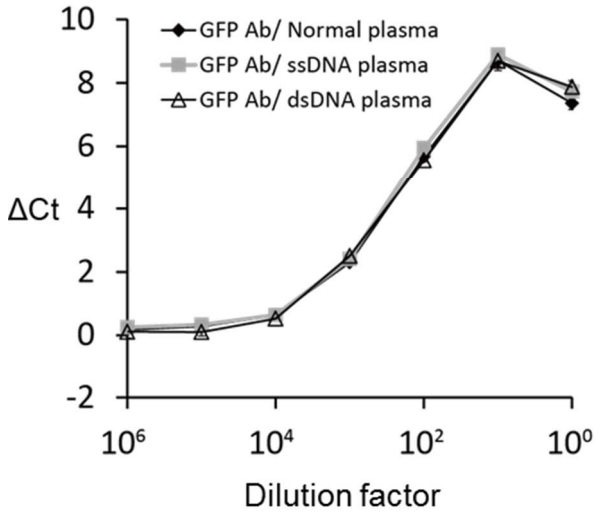

Figure S6. *Detection of anti-GFP antibodies in anti-DNA and normal plasma without competition DNA*. Anti-GFP antibodies were diluted into anti-DNA plasma or normal plasma and then analyzed by ADAP with GFP-DNA conjugates as the probes. Though interference is observed for anti-DNA and normal plasma at 1:1 and 1:10 dilutions as shown in **Figure 5a**, no interference is observed in the presence of anti-GFP antibodies. This might be attributed to high affinity anti-GFP antibodies dominate the agglutination of GFP-DNA conjugates, which thus masks the interference from anti-DNA antibodies.

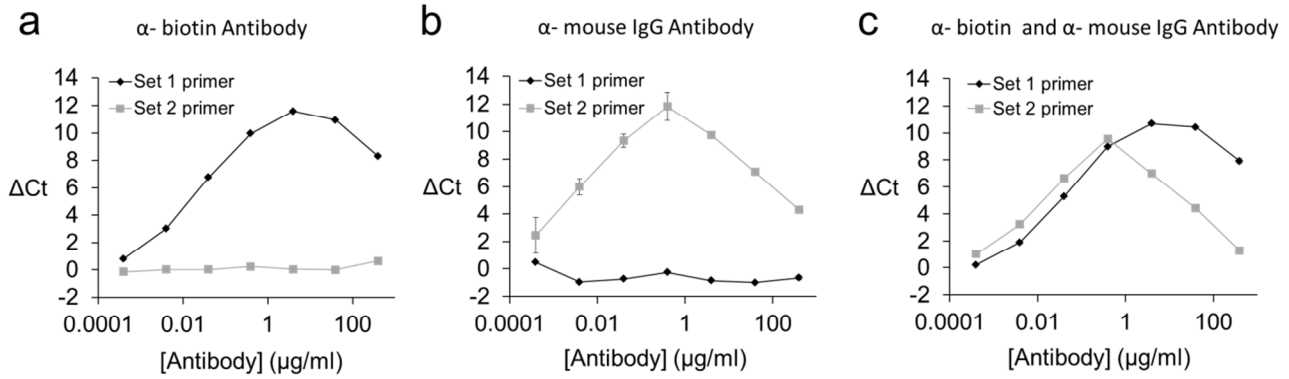

Figure S7. *Representative multiplex ADAP curves for multiple antibodies*. (a) Both biotin-DNA (Sequence Set 1) conjugates and mouse-IgG-DNA (Sequence Set 2) conjugates are incubated with anti-biotin antibody and analyzed by ADAP using both primer sets. Only sequence 1 shows detectable signal. (b) Both conjugates are incubated with anti-mouse-IgG antibody. Only sequence 2 shows detectable signal. (c) Both conjugates are incubated with both antibodies. Both sequence set 1 and set 2 show signal. These results demonstrate the orthogonality of multiplexed antibody detection using ADAP.

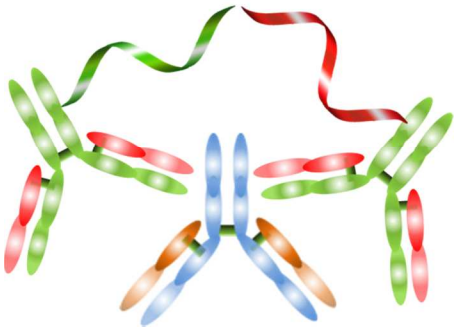

Figure S8. *Schematic diagram for proximity ligation assay*. In proximity ligation assay, a single batch of polyclonal antibodies is split into two pools. Each antibody pool is conjugated to unique DNA oligonucleotides (green and red). In the presence of the target molecule (in this case the blue and orange immunoglobulin molecule), the two antibody-DNA conjugates bind to different epitopes on the target molecules and are brought into close proximity. Upon the addition of a bridge oligonucleotide and DNA ligase, the two halves of DNA on the conjugate are linked together and regenerate the full length amplicon, which can be further quantified by qPCR.

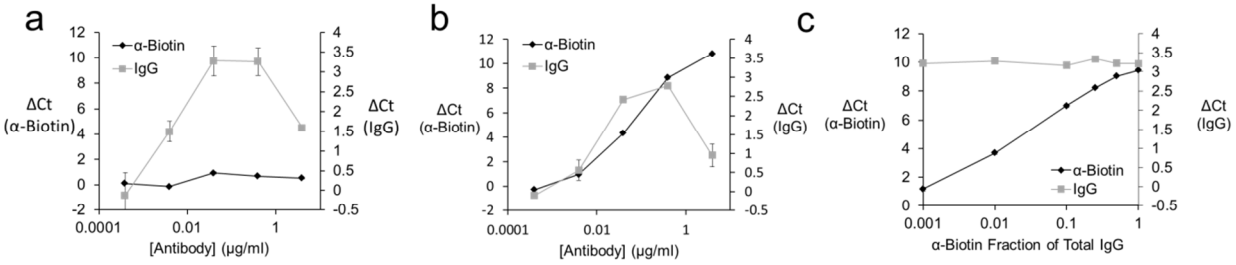

Figure S9. *Representative multiplex ADAP and PLA curves for antigen-specific antibodies and total IgG*. (a) Both biotin-DNA conjugates (ADAP probes) and anti-goat-IgG-DNA conjugates (PLA probes) are incubated with goat IgG. Only the PLA probe shows detectable signal. (b) Both biotin-DNA and anti-goat-IgG-DNA conjugates are incubated with goat anti-biotin antibody. Both ADAP and PLA probes show detectable signal. (c) Both biotin-DNA and anti-goat-IgG-DNA conjugates are incubated with varied fractions of anti-biotin antibodies under fixed total IgG condition. ADAP probes show concentration dependent signal while the signal derived from the PLA probes shows high signal that does not vary with the concentration.

These results demonstrate the orthogonality of multiplex antibody and total antibody detection using ADAP and PLA.

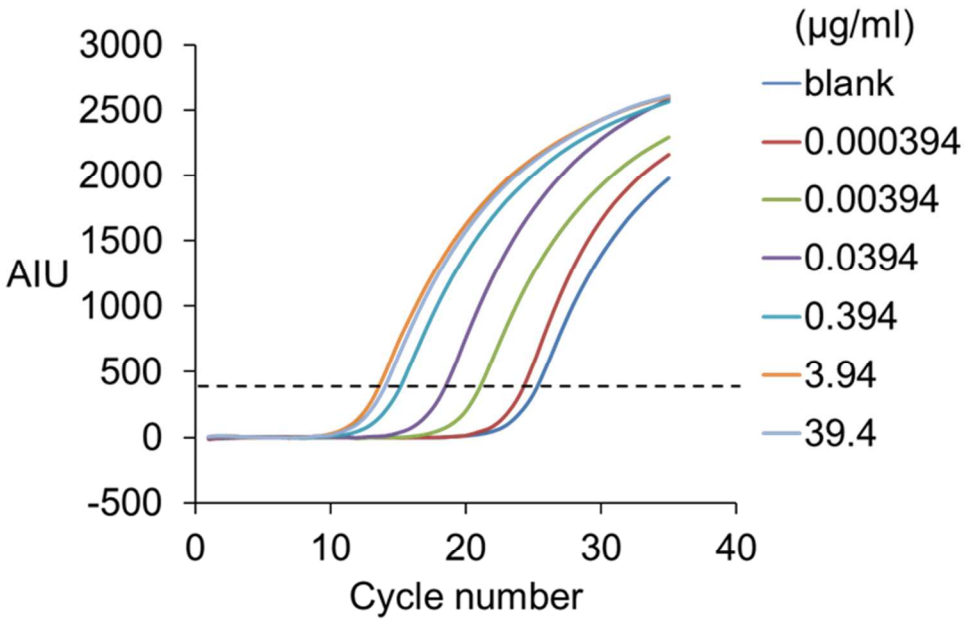

Figure S10. *Raw fluorescence qPCR data from a representative ADAP experiment*. Serially diluted antibodies are analyzed by ADAP. The threshold fluorescence value is indicated by the dashed line. The C_t value is defined as the cycle number where the fluorescence value corresponds to the threshold value. Amplification curves with higher antibody concentrations reach exponential amplification earlier and thus have smaller C_t values. ΔC_t is defined as the C_t value of the blank minus C_t value of the sample. The higher the antibody concentration, the larger the ΔC_t value.

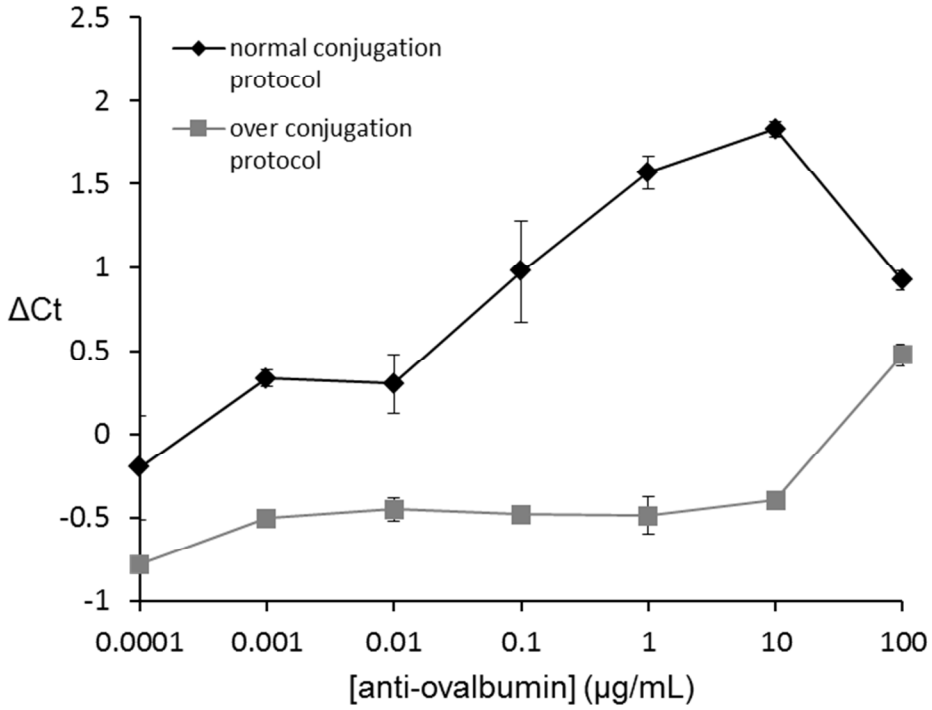

Figure S11. *Effect of antigen:DNA conjugation ratio on ADAP performance*. Ovalbumin (OVA)-antigens DNA conjugates were synthesized with either low or high antigen:DNA ratios (1:2 or 1:6 respectively). The ovalbumin conjugates are incubated with a dilution series of monoclonal anti-OVA antibodies in buffer and subjected to ADAP analysis. The overconjugated ovalbumin (grey squares) show significantly reduced assay performance while the normally conjugated ovalbumin (black diamonds) shows concentration dependent signal as expected. We suspect that this is the result of epitope masking when antigens are overconjugated with oligonucleotides.

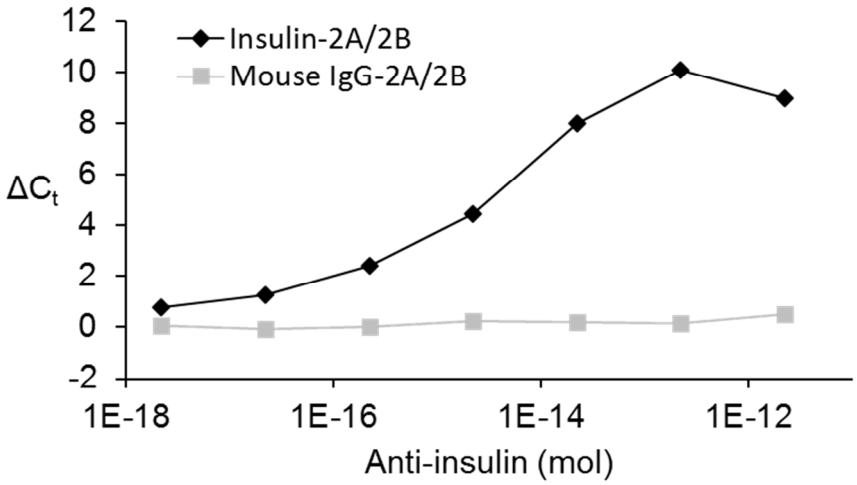

Figure S12. *ADAP detection of antibodies is specific to the cognate antigen*. Anti-insulin antibodies were incubated with insulin- or mouse IgG- DNA conjugates in bovine serum and then analyzed by ADAP. As expected, the anti-insulin antibodies agglutinate the insulin-DNA conjugates and generate signal (black diamonds). They do not agglutinate the mouse IgG-DNA conjugates, as they have no affinity for this unrelated protein, and therefore generate no signal (gray squares). This result demonstrates that ADAP is specific for the cognate antigen-antibody pair.