

# Application Note

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## Use of UltraAmp™ Reagents in ELISAs

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## Introduction

The Enzyme-linked immunosorbent assay (ELISA) is a simple and inexpensive method used to determine the presence of biomarkers for the diagnosis of multiple diseases and drug development. High sensitivity is crucial to a better understanding of cellular mechanisms and to decrease the amount of sample required per assay. In non-radioactive ELISAs the output signal depends on the number of enzyme molecules or fluorophores that can be indirectly attached per analyte molecule. To increase the assay's sensitivity some researchers have tried signal amplification by doing successive incubations of enzyme labeled avidin and biotinylated anti-avidin antibodies<sup>1</sup>, while others have improved efficiency of the antigen extraction from cells<sup>2</sup>. The main drawbacks of the former approach are an increase in assay time and decrease in reproducibility due to the multiple incubations and washes. The latter approach is just an improvement on the sample preparation methods to recover more target (equivalent to target amplification), and is not improving the sensitivity of the ELISA. We developed an indirect and sandwich ELISA using DNA dendrimer<sup>3</sup> conjugates that deliver a higher number of labels per target molecule. We have prepared DNA dendrimers conjugated to anti-biotin antibodies containing up to 200-300 molecules of horseradish peroxidase (UltraAmp Anti-biotin HRP), and compared these reagents to streptavidin-HRP (~2HRP/streptavidin). The assay format consisted of a standard microtiterplate ELISA in which the UltraAmp conjugate is a "drop-in" reagent replacing the streptavidin, so that no extra steps are involved. Interleukin 1 alpha (IL-1a) was selected as the model analyte. Upon optimization we were able to detect less than 2 pg/mL of IL-1a, corresponding to greater than 20-fold signal amplification respect to streptavidin-HRP (SA-HRP). The UltraAmp conjugate was also used to detect IL-6, TNF alpha, antibodies against beta 2 glycoprotein I (b2 GPI) and gastric parietal cell antibody (GPA). For these markers, signal amplifications ranged from 3 to >200 fold compared to streptavidin-HRP detection.

## Materials and Methods

### Plate Coating and Blocking:

Coating antibody was diluted in the Coating Buffer (10mM PBS) to the concentration recommended by the manufacturer. Otherwise, the optimal antibody concentration was determined by running a titration series of the coating antibody. Each well was incubated with 100µL of diluted antibody overnight at room temperature (20-25°C). The next day wells were emptied and blotted dry on paper towels. Unbound sites in the wells were blocked with 200-300µL of Blocking Buffer (1-4%BSA, 10mM PBS) per well for 1-2 hours at room temperature. The wells were washed three times with ELISA Wash Buffer 1 (50mM Tris-HCl pH 7.4, 0.2% Tween 20) and blotted dried on paper towels. For commercially coated plates, from Pierce (Endogen Kit), BioSource, and/or INOVA kits only the ELISA Method section was followed.

### Sandwich ELISA Method:

All reagents were brought to room temperature (20-25°C) prior to use. Standards and samples were prepared in Assay Buffer (1%BSA, 10 mM PBS). Immediately after the coating and blocking procedure, 50µl of standards/samples were added per well. The plate was covered and incubated at room temperature for 1 hour. With the standards/samples still in the wells, 50µl of diluted biotinylated detecting antibody were added to each well, and the plate was covered and incubated at room temperature for an additional hour. The wells were emptied, washed 3 times with ELISA Wash Buffer I (50mM Tris-HCl pH 7.4, 0.2% Tween 20), and blot dried.

### UltraAmp reagent hybridization:

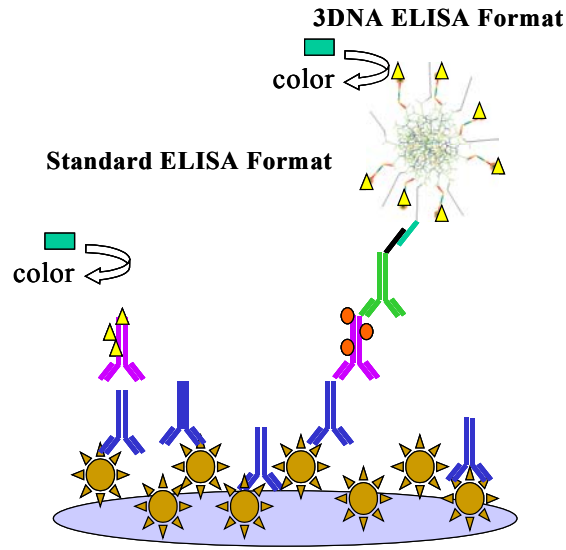
The UltraAmp Anti-biotin HRP conjugate was diluted in Binding Buffer (48% SA-HRP buffer (Pierce), 48% Wash Buffer and 2% dextran sulfate) to a final dendrimer concentration of 0.6 ng/µL and stored at room temperature until use. Each well was incubated with 30-50 µL of diluted UltraAmp Anti-biotin HRP Conjugate, covered and incubated for 1-1.5 hours on a rotary shaker at approximately 100 rpms at room temperature. The wells were emptied, washed 4-5 times with Wash Buffer II (50mM Tris-HCl pH 7.4, 0.2% Tween 20, 50mM NaCl), and blot dried on paper towels. The wells were then incubated with 100µL of substrate (TMB HRP Solution, Pierce, Cat # N301) at room temperature in the dark. The reaction was stopped after 20 minutes and the absorbance read at 450 nm.

### Indirect ELISA for pre-coated plates:

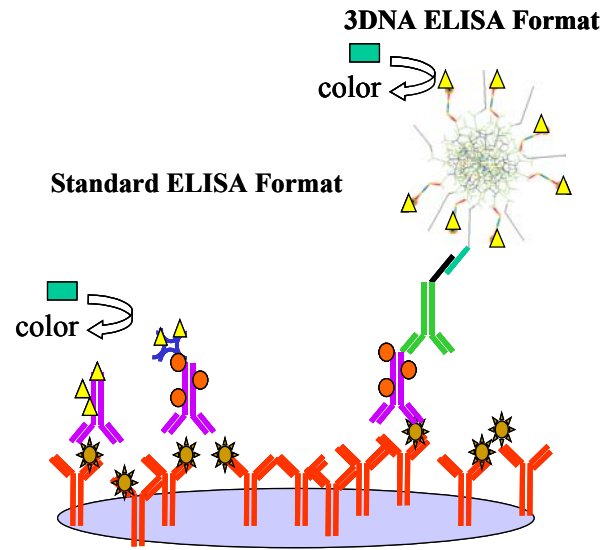
Antigen-coated plates from INOVA were used to test indirect ELISAs. All assays were run according to the manufacturer. For the UltraAmp approach, the incubation with the HRP labeled anti-human antibody was substituted by biotinylated anti-human antibody. Unbound antibodies were washed off and samples were incubated with the UltraAmp Anti-biotin HRP conjugate (30ng as UltraAmp reagent) for 1 hour as described above. The wells were washed 3-5 times with Wash Buffer II and incubated with HRP substrate. The reaction was stopped after 20 minutes and the absorbance read at 450 nm.

### Sandwich ELISA for pre-coated plates:

Pre-coated plates from Pierce were used to test the sandwich ELISA approach. All assays were run according to the manufacturer. For UltraAmp detection, streptavidin-HRP was substituted by incubation with UltraAmp Anti-biotin HRP (18 ng as UltraAmp reagent per well) for 1 hour at room temperature. The wells were washed with Wash Buffer II and incubated with substrate. The reaction was stopped after 20 minutes and the absorbance read at 450 nm.



Indirect ELISA



Sandwich ELISA

## Results and Discussion

In order to determine the optimal ELISA method for using the UltraAmp reagents, we studied a variety of assay conditions, different size UltraAmp reagents, and a variety of assays formats. All tests were run in replicate and UltraAmp reagents were compared to the standard detection method (usually SA-HRP or HRP directly attached to an antibody) to assess performance of these reagents.

### Incubation/Assay Conditions:

Dextran sulfate has been known to improve the DNA-probe hybridization to target by increasing the relative concentration of the probe in the aqueous solution. With this in mind the effect of dextran sulfate on dendrimer conjugate hybridization to target was evaluated. Figure 1 shows the results of a dextran sulfate titration curve, where 5% was found to be the optimal concentration in the binding buffer used for a UltraAmp Anti-biotin HRP (30) (2-layer dendrimer) reagent incubation. For the UltraAmp Anti-biotin HRP (200) (4-layer dendrimer) 2-2.5% is the optimal concentration (data not shown). The difference in the optimal dextran sulfate concentrations for the two UltraAmp reagents could be attributed to their size or mass of DNA. A 4-layer dendrimer has a molecular weight about 9 times greater than the 2-layer, so is more susceptible to precipitate/aggregate at higher dextran sulfate concentrations.

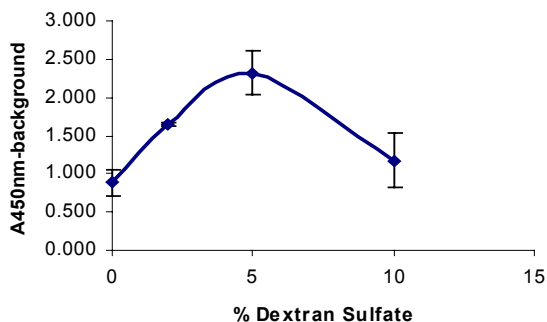


Figure 1. **Dextran sulfate titration:** Effect on signal response from UltraAmp Anti-biotin HRP (30). 20 ng per well of UltraAmp reagent were used for the detection of 64 pg/mL of IL-1 alpha.

To determine the optimal UltraAmp conjugate concentration, titration experiments for UltraAmp Anti-biotin HRP 30 and 200 were done. The results for the Anti-Biotin HRP 30 mass titration (see Figure 2) show that after 20ng per well the signal starts to plateau.

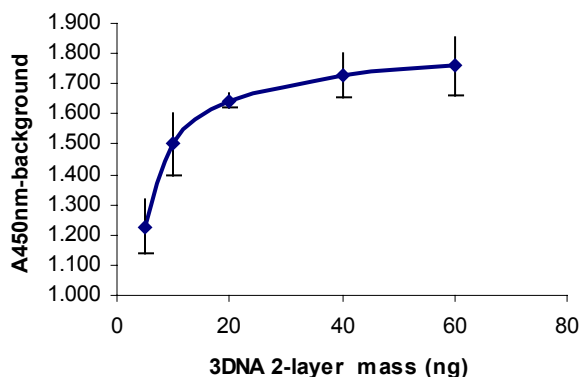


Figure 2. **Dendrimer mass titration:** Effect on signal response from Anti-biotin HRP (30) conjugate. The UltraAmp conjugate was used for the detection of 64 pg/mL of IL-1 alpha.

The salt concentration plays an important role in the hybridization of the UltraAmp conjugate because it stabilizes the DNA bridge between the dendrimer and the anti-biotin antibody conjugate as well as hybridized the HRP tagged oligonucleotides. Some commercial buffers might already have the optimal salt concentration, therefore it is important to do a salt titration as part of the assay optimization. Figure 3 shows 200mM as the optimal NaCl concentration when 95% Guardian Peroxidase (Pierce, cat # 37548) with 2% dextran sulfate was used as the binding buffer.

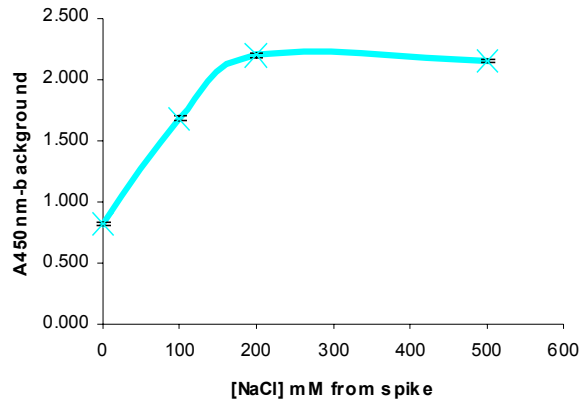


Figure 3. **NaCl titration in Guardian Peroxidase** as the UltraAmp binding buffer. UltraAmp reagents were used at 0.6 ng/mL in binding buffer. UltraAmp conjugates were tested for detection of 64 pg/mL of IL-1 alpha.

In addition to the salt concentration, we also tested other binding buffer formulations. Figure 4 shows the signal response from a Anti-biotin HRP (200) UltraAmp conjugate in different types of buffers. Binding Buffer I (reference buffer) containing SA-HRP dilution buffer, a proprietary buffer from Pierce (cat# 1857451), gave the best signal response from the UltraAmp conjugate. The same formulation with R&D systems buffer (R&D) and the Guardian Perodixase buffer (BB2), when spiked with 200 mM NaCl, demonstrated similar results to our reference SA-HRP (BB1) buffer.

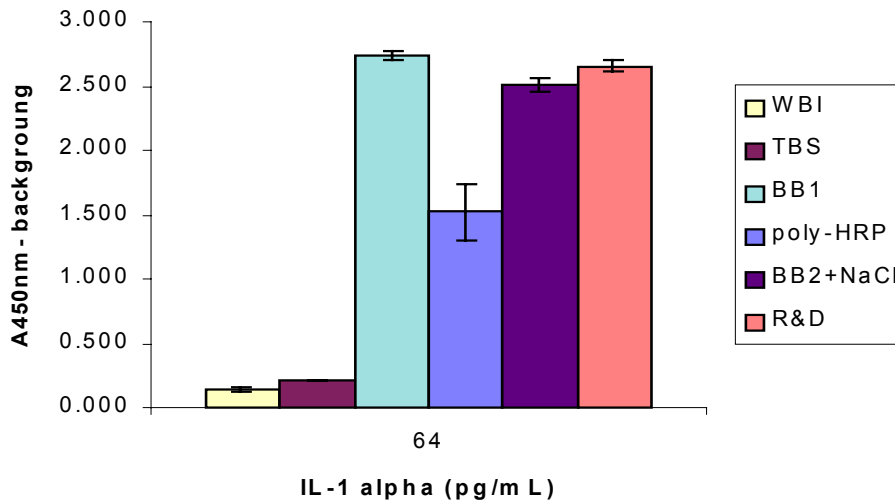


Figure 4. **Evaluation of hybridization buffer:** Effect on signal response from 4-layer anti-biotin dendrimer conjugates in the detection of IL-1 alpha with 18 ng per well of conjugate as dendrimer. WBI: wash buffer I (50mM Tris-HCl pH 7.4, 0.2% Tween 20, no NaCl), BB1: binding buffer I (48% SA-HRP dilution buffer from Pierce cat# 1857451, 48% wash buffer I, and 2% dextran sulfate), poly-HRP: Pierce buffer cat #N500, BB2+NaCl: 2% dextran sulfate and 200 mM NaCl in Guardian Peroxidase (Pierce, cat# 37548), R&D: same formulation as BB1 but use R&D systems SA-HRP dilution buffer instead of that from Pierce.

### SA-HRP comparison to UltraAmp Anti-biotin HRP 30 and 200:

We also studied the relative amplification of UltraAmp reagents compared to Streptavidin-HRP. As expected the more HRP labels per detection molecule, the greater the signal response. Figure 5 compares the signal from both types of UltraAmp conjugates in the detection of IL-1 alpha to that produced by SA-HRP. At 10.24 pg/mL of IL-1 alpha, the signal from UltraAmp Anti-biotin HRP 30 was about 9 times greater than that from SA-HRP, while the signal from UltraAmp Anti-biotin HRP 200 was about 26 times greater. Based on the estimated number of HRP molecules per UltraAmp reagent, we expected about 50-100 fold improvement over SA-HRP for the Anti-biotin HRP (200) reagent, but only observed 26 fold. It is likely that the size of the UltraAmp reagent impact the binding constant ( $K_d$ ) of the antibody pairs (other supportive data not shown).

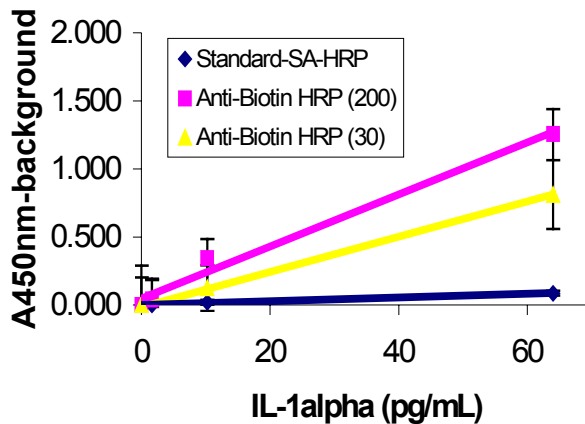


Figure 5. **Comparison of signal response from anti-biotin HRP (30) and (200) UltraAmp reagents:** Detection of IL-1 alpha with 18 ng per well of UltraAmp conjugate.

### Detection scheme design:

Two different approaches were evaluated for the detection of human gastric parietal cell ( $H^+/K^+$ ) ATPase antibodies. In the first, the human antibodies were detected with a biotinylated anti-human IgG, which was then detected with an anti-biotin HRP UltraAmp reagent. The second approach consisted of detection of the human antibodies with an anti-human IgG HRP UltraAmp reagent. As shown in Figure 6, the anti-biotin UltraAmp reagent detecting the biotinylated Anti-human IgG antibody performed more robustly than the direct detection method. Two possible explanations for the difference in output signal between the two detection schemes are: (1) difference in avidity of the antibody pair, or (2) the proximity of the UltraAmp to the target could block other binding sites.

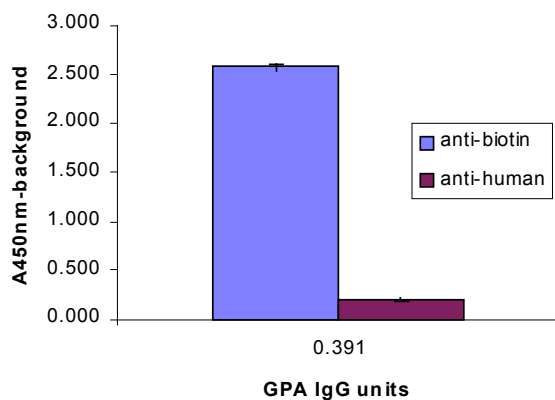


Figure 6. **Detection of gastric parietal cell (H<sup>+</sup>/K<sup>+</sup>) ATPase antibodies by UltraAmp reagents.** Comparison of the response from an anti-biotin UltraAmp reagent to that of an anti-human IgG UltraAmp reagent.

We have also studied the potential for amplification of a variety of ELSIA type assays (listed in Table 1) and have observed a range of fold amplification depending on the assay. Amplification folds ranged from 3 fold for a standard sandwich ELISA detecting TNF to >200 fold for Gastric Parietal antibody (GPA) without observing any loss of assay linearity in the UltraAmp detection range and with little increase in background signal. Backgrounds typically increased by no more than 20-50% , for example Abs 450 0.065 for standard SA-HRP detection to 0.090 for the corresponding UltraAmp reagents.

Table 1. Summary of signal amplification obtained with UltraAmp reagent detection relative to the standard method.

ELISA	Kit/Ab pair	Highest amplification	Correlation (R <sup>2</sup> )
Indirect	H pylori	10	0.999
Indirect	Histone	7	0.995
Indirect	GPA	218	0.985
Indirect	ACA	17	0.999
Indirect	B2GPI	30	0.996
Sandwich	IL-1a	80	0.997
Sandwich	IL-6	15	0.996
Sandwich	TNF	3	0.999

## Conclusions

- UltraAmp reagents are compatible with a variety of commercial assay (binding) buffers.
- Salt concentration, dextran sulfate percentage, and overall conjugate concentration in the binding buffer were determined to be important parameters in the optimization of UltraAmp reagent binding to target.

- As would be expected, the UltraAmp Anti-biotin HRP 200 gave greater signal amplification than UltraAmp Anti-biotin HRP 30 reagents. However, this may not always be the case for all assays (data not shown).
- Signal amplification may vary among assay types and antibody pairs.

## References

- (1) O'Connor, E., Roberts, E. M., and Davies, J. D. Amplification of cytokine-specific ELISAs increases the sensitivity of detection to 5-20 picograms per milliliter. *J. Immunol. Methods* (1999) 229:155-160.
- (2) Jones, J. B., Somodi, G. C., and Scott, J. W. Increased ELISA sensitivity using a modified extraction buffer for detection of *Xanthomonas campestris* pv. *Vesicatoria* in leaf tissue. *J. Appl Microbiol.* (1997) 83:397-401.
- (3) Nilsen, T.W., Grayzel, J., and Prensky, W. Dendritic Nucleic Acid Structures. *J. Theor. Biol.* (1997) 187: 273-284.
- (4) *Immunoassay: A Practical Guide*, Chan and Perlstein, Eds., 1987, Academic Press: New York.