Method development of UltraAmp™ reagents as signal amplification molecules in ELISAs

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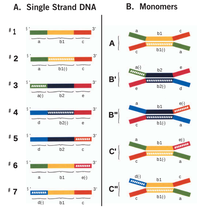
Abstract

Diagnosis of diseases such as Hepatitis, acquired immunodeficiency syndrome (AIDS), and Lyme disease, and detection of biomarkers such as interleukines are commonly done by an enzyme-linked immunosorbent assay (ELISA). ELISAs are relatively cheap, high throughput assays, but are often limited by sensitivity. In order to improve the limits of detection of a variety of ELISAs, we have developed a method to implement the use of horseradish peroxidase (HRP) multi-labeled UltraAmp™ reagents. UltraAmp™ reagents are DNA dendrimers conjugated with a variety of enzymes, haptens and antibodies. The labels and targeting moieties are conjugated to oligonucleotides that are complementary to the outer layers of the dendrimer, so the DNA dendrimers are functionalized by hybridization with the modified oligonucleotides. This creates a multi-labeled molecule ideal for signal amplification. We selected IL-6 and IL-1 alpha as models for the development of the assay and observed variable signal amplification (up to 200-fold) among different HRP labeled UltraAmp™ reagents (anti-biotin, anti-IL-6, anti-IL-1a) and among antibody pairs compared to streptavidin-HRP detection. We found that binding of the UltraAmp™ reagent to targets stacked farther away from the plate resulted in greater signal amplification. Furthermore, UltraAmp™ reagents with greater number of HRP molecules attached gave greater signal with a low increase in background, but the amplification was not proportional to the theoretical number of HRP molecules attached. UltraAmp™ reagents can provide greater signal to noise ratios than those obtained with streptavidin-HRP detection.

Keywords: DNA dendrimers, ELISA, cytokines, HIV antibodies, signal amplification

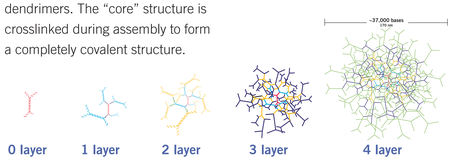
Dendrimer Components

Dendrimers are made from 7 single stranded DNAs. The strands are hybridized pair-wise to produced building block units called monomers.



Dendrimer Technology

Monomers are hybridized together to assemble various size DNA dendrimers. The "core" structure is crosslinked during assembly to form a completely covalent structure.

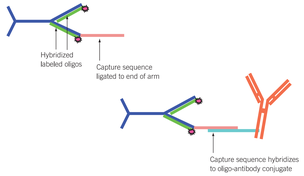


Methods

Preparation of UltraAmp™ reagents

Enzyme and Antibody labeling

The dendrimers and HRP and anti-biotin antibodies labeled oligonucleotides complementary to the outer core of the dendrimer are incubated at room temperature for 30 minutes in a buffer that would allow hybridization. The end product is a dendrimer conjugated to the desired antibody and labeled with HRP enzymes



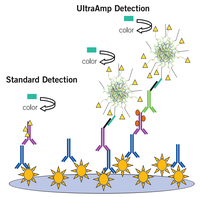
ELISA Assays

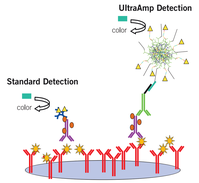
Indirect ELISA

- Antigen-coated plates from various manufacturers were used to test indirect ELISAs.
- For UltraAmp detection, incubation with the HRP labeled anti-human antibody was substituted by anti-human UltraAmp HRP or biotinylated anti-human antibody.
- For detection with biotinylated anti-human antibodies, unbound antibodies were washed off and detected with anti-biotin UltraAmp HRP

Sandwich ELISA

- Antibody pre-coated plates were used to test the sandwich approach.
- For UltraAmp detection, streptavidin-HRP was substituted by incubation with anti-biotin UltraAmp HRP

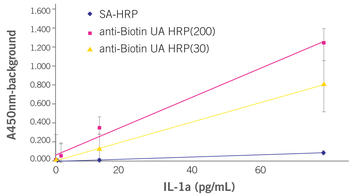




Results

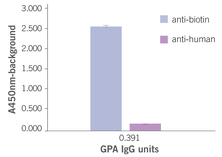
Sandwich ELISA for detection of IL-1a

- Comparison of signal response from anti-biotin UltraAmp HRP (30) and (200) compared to signal from streptavidin HRP. 18 ng of UltraAmp reagent were used for the detection in a 50 µL volume.



Indirect ELISA for detection of GPA antibodies

- Comparison of signal response between anti-human UltraAmp (direct) vs anti-biotin UltraAmp (indirect) – HRP detection
- Signal enhancement is significantly better when human antibodies are detected first with biotinylated anti-human antibody followed by anti-biotin HRP labeled UltraAmp



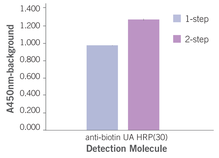
Amplification in HIV test

HIV positive controls and samples were incubated in HIV antigen coated plates, followed by detection with anti-human HRP labeled UltraAmp reagents

Sample	Detection	A @ 450 nm	Result
control 1	Manufacturer's	1.383	+
control 2	Manufacturer's	1.627	+
negative	Manufacturer's	0.037	-
negative	Manufacturer's	0.035	-
negative	Manufacturer's	0.039	-
blank	Manufacturer's	0.039	-
S/4	Manufacturer's	0.161	+
S/8	Manufacturer's	0.104	-
S/16	Manufacturer's	0.072	-
negative	anti-Human HRP(30)	0.039	-
blank	anti-Human HRP(30)	0.039	-
S/8	anti-Human HRP(30)	0.339	+
S/16	anti-Human HRP(30)	0.256	+
S/32	anti-Human HRP(30)	0.218	+
S/64	anti-Human HRP(30)	0.222	+

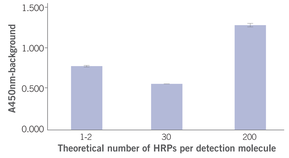
One-step vs two-step UltraAmp Detection

In this detection approach, anti-biotin antibodies are first bound to target, then chased with HRP labeled dendrimer. This results in increased signal compared to UltraAmp reagents that have the anti-biotin antibodies pre-bound to the dendrimer



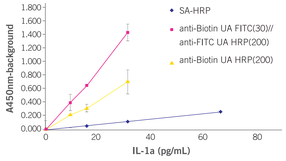
Amplification vs Theoretical HRP number

Detection of 37.5 pg/mL of IL-6 in a sandwich ELISA: Comparison of three different detection molecules according to their HRP theoretical number

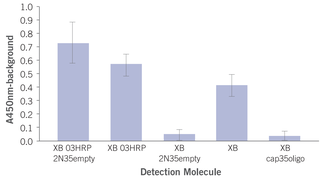


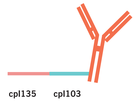
UltraAmp Detection of UltraAmp Reagents (Sequential Hybridization)

Biotinylated anti- IL-1a was detected with FITC labeled anti-biotin UltraAmp (30). FITC labeled anti-biotin UltraAmp was then detected by HRP labeled anti-FITC UltraAmp.



Is the UltraAmp complex more prone to shear force (stripping)?





Nomenclature: XB 03 HRP 2N35 empty: anti-biotin HRP oligo in cpl03 position, naked 2-layer dendrimer in cpl35 position. XB 03 HRP: anti-biotin HRP oligo in clp03 position. XB 2N35 empty: anti-biotin antibody with empty 2-layer dendrimer in cpl35 position. XB: anti-biotin antibody with no labels. XB cap35oligo: anti-biotin antibody with cpl35 position blocked.

Anti-biotin antibody with empty 2-layer dendrimer in cpl35 position, anti-biotin antibody with no labels, and anti-biotin antibody with the cpl35 position blocked were all chased with HRP labeled cap35 oligo to determine if the dendrimer was stripping or dissociating from position cpl35. The lack of signal for anti-biotin antibody with empty 2-layer dendrimer in cpl35 position indicates that the complex is not stripping from the surface. The equivalent signal of anti-biotin HRP oligo in cpl03 position with naked 2-layer dendrimer in cpl35 position, and anti-biotin HRP oligo in clp03 position further supports this observation.

Conclusions

- **Anti-biotin HRP UltraAmp reagents provided up to 200-fold signal amplification in IL-1a and IL-6 sandwich ELISAs, as well as GPA and HIV indirect ELISAs**
- **Unbound-excess antibody competes with dendrimer conjugates for biotin binding.**
- **Antibody stacking results in greater signal amplification.**
  - **There is a great improvement in signal when human antibodies are detected first with anti-human biotinylated followed by anti-biotin HRP labeled UltraAmp**
  - **Detection of biotinylated antibody with an anti-biotin FITC labeled UltraAmp reagent followed by detection with an anti-FITC HRP labeled UltraAmp reagent gives greater signal than detection with anti-biotin HRP labeled UltraAmp reagent alone**
- **An UltraAmp reagent labeled with 200 HRPs yields greater signal than one labeled with 30 HRPs, but amplification is not proportional to the increase in the number of HRPs**
- **Dendrimer binding to the detection antibody conjugate does not cause it to dissociate ("strip") from its target**