# The Avidin-Nucleic Acids Nano Assemblies (ANANAS), as powerful molecular amplifiers in *in vitro* diagnostics

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## Background and Scope of the Work

The **A**vidin-**N**ucleic **A**cids **N**ano-**AS**semblies (ANANAS) are a novel kind of poly-avidin nanoparticles obtained upon a nature driven self-assembly process, whose preparation has been optimised in recent years to yield a novel tool with multiple applications in biomedicine [1, 2].
Avidin is a tetrameric protein that is capable of binding with high affinity ($K_d$~$10^{-15}$M) four biotin molecules. This property represents the basis for its exploitation as a molecular tool in many biotechnological applications among which immunodiagnostics and drug delivery [3]. However, classic avidin-biotin technology potentials are limited by the maximum number (4) of ligands that can be brought together by the individual avidin unit.
The ANANAS particles are nanoparticles (about 100 nm in diameter) potentially capable of overcoming such limits since they have a "core" composed of several avidin units and, consequently, they are characterized by higher and precisely defined biotin loading capacity (**Figure 1**).

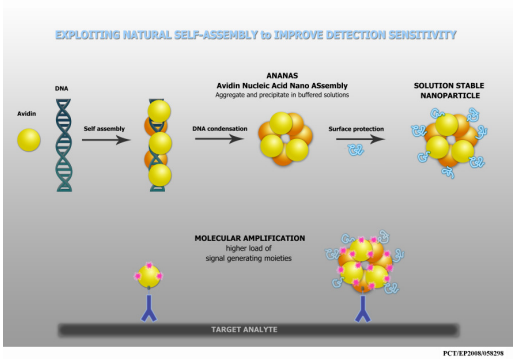


***Figure 1.*** *The ANANAS concept.*

In this work we evaluated the performance of the ANANAS particles in avidin-based *in vitro* diagnostics configurations, namely ELISA and Blot systems. The ANANAS-based analytical sensitivity was compared to that of benchmark reagents currently in the market in three analytical set-ups.

## Methods

Goat-IgGs, rabbit-anti-Goat IgGs were purchased from KPL. Horseradish Peroxidase (HRP), Avidin-HRP conjugate and TMB were from Sigma Aldrich. ANANAS particles containing about 300 avidin units/particle were prepared and provided by ANANAS Nanotech [1, 2]. Detection antibodies and horseradish peroxidase (HRP) were biotinylated according to standard protocols. Anti-mouse IgG ABC Vectatstain detection reagents were purchased from Vector Labs (USA); Anti-bovine IgG1 monoclonal antibody (MAb) and BHV1-antigen coated microwell plates were kindly provided by IZSLER (Brescia, Italy); IBR Positive and negative cow milk samples were provided by IZV (Legnaro, Padova, Italy).

*Performance on a generic ELISA platform*

96 well polystyrene plates (Nunc Maxisorp) were conditioned with rabbit anti-goat IgG. After washing, serial (1/4) dilutions of biotinylated goat IgGs (from 55 to 0.023 pg/well) were incubated for 1 h. After well washing, detection was performed using commercial avidin-HRP conjugate or the ANANAS particles followed by biotin-HRP. After the final wash, detection was achieved using TMB as the HRP substrate and reading the plate at 450 nm after 2-20 minutes incubation.

*Performance on a blot platform*

Serial amounts of biotinylated goat IgG were spotted on a PVDF membrane. After quenching, detection was achieved with the Vectastain-ABC system or ANANAS particles + biotin-HRP, followed by colour development with the diaminobenzidine (DAB) substrate.

*Detection of IBR positive cows from milk samples*

BHV1 antigen was trapped onto Nunc Maxisorp plates by a virus specific MAb coated to the solid phase. Positive cow milk samples were serially diluted into negative milk and incubated for 1h at 37°C. After washing, an anti-bovine IgG1 MAb, HRP conjugated (IZSLER) or biotinylated and, followed by ANANAS and biotin-HRP, were delivered; colour development was finally achieved with TMB.

## Results

**Figure 2** shows the results of an ELISA experiment in which detection of a biotinylated antibody was achieved with either a commercial avidin-HRP conjugate or the ANANAS integrated system. The latter gives rise to a positive response (characterized by signal/standard deviation > 2) at all points tested. This corresponds to a detection limit of less than 0.023 pg/well. In the same analytical set-up, the positive signal onset for the commercial competitor avidin-HRP was at 6.17 pg/well. The ANANAS enhanced sensitivity is due to both lower

noise and higher signal intensities. The enhancement factor in this analytical set-up (calculated from the ratio of the two onset values) was of about 240 fold.

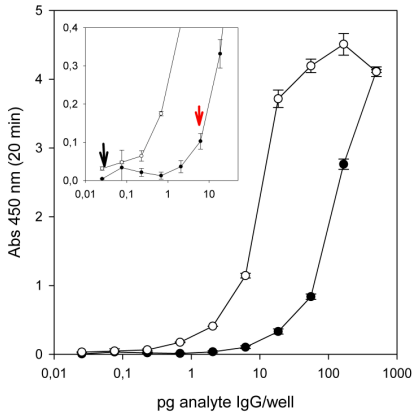


***Figure 2** ANANAS (o) vs commercial avidin-HRP (●) in a model ELISA assay. Arrows indicate the positive signal onsets (signal/stdv >2).*

**Figure 3** shows the analytical performance of the ANANAS system in a blot assay where the analyte was mouse IgG from serum, which was diluted serially into PBS/BSA. In this case, the ANANAS performance was compared to that of the commercial competitor Vectastain ABC from Vector Labs, which relies on a patented avidin-based amplifying technology. The results of this assay can be used to predict the efficacy of detection in Western or Southern blots.

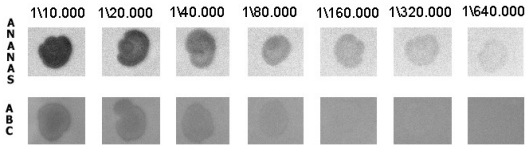


***Figure 3**. Detection of mouse IgGs from serum in a dot blot experiment using the ANANAS system and commercial enhanced Vectatstain ABC system. Spots (1µl) were made with mouse serum serially diluted from 1:10000 to 1:640000 in PBS/BSA.*

As in ELISA, the ANANAS system shows significantly higher sensitivity than the commercial competitor, due to both reduced noise and higher signal. In this experimental set-up the ABC system positive onset was at mouse serum dilution of 1/80.000, which corresponds to about 125 pg of IgG/spot, whereas positive signal with the ANANAS system was observed also at the higher dilution tested (1/640000), which corresponds to about 16 pg of IgG . Therefore, the enhancement factor is > 8 fold. This result is of particular interest, since the ABC system itself already relies on a signal amplifying technology.

**Figure 4** shows some preliminary data obtained in a real analytical context, namely in the detection of anti-BHV1 (Bovine herpesvirus type 1) IgGs in cow milk. The presence of these antibodies in milk is due to either infection or immunization with BHV1**.** Known positive milk samples were diluted in negative milk to simulate a real stable situation, in which bulk milk is obtained upon pooling together the product of several cows. In this situation the presence of an infected animal (and thus the early diagnosis of an infected stable) may be masked by diluting seropositive milk with that of healthy animals.

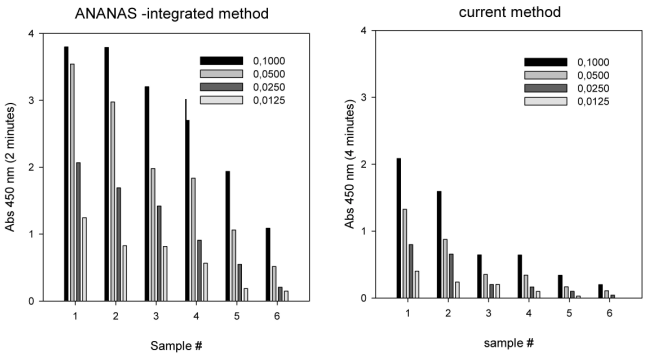


***Figure 4.** Detection of anti-BHV1 IgGs in six positive cow milk samples, each diluted with negative milk. The dilutions tested are 1 to 10; 1 to 20, 1 to 50 and 1 to 125*

The results show that the ANANAS integrated detection system yields to significantly higher reading values than the competitor system, despite the shorter development time used. In all milk tested, a clear positive response was observed in all of the dilutions investigated (up to 1 to 125). Experiments with a larger pool of samples, aimed at estimating the actual sensitivity and specificity of the integrated system are currently underway.

## Conclusions

The novel Nanoassembled ANANAS system improves the performance of present *in vitro* diagnostics technologies within classic assay platforms. The system can be easily integrated in the majority of the immunodiagnostics set-ups, where it allows improving the sensitivity without the need for the user to invest in novel instrumentation. The sensitivity reached with colour-based developments is on the same scale as the one obtained with ECL integrated with classic avidin-based or HRP-antibody conjugates. The ANANAS technology then represents an easy and low cost alternative to improve research and diagnostic laboratories sensitivity.

## References

[1] M. Pignatto, N. Realdon, M. Morpurgo, Bioconjug Chem, *In press* (2010).
[2] M. Morpurgo, M. Pignatto, D. Teoli, patent # PD2007A000223, PCT WO 2009/00395 A1, 2007.
[3] J. Abelson, M. Simon, W. M., E. Bayer, eds. Methods in Enzymology. Vol. 184. 1990.

## Biosketch

*Margherita Morpurgo* is assistant professor at the School of Pharmacy at the University of Padova. Her research interests focus on the development of organic and inorganic assemblies for drug delivery and diagnostic use. Since 2006 she is the scientific leader and co-founder of ANANAS Nanotech, a University Spin-off dedicated at developing avidin-based nanoparticles for *in vitro* diagnostics and drug delivery application.