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Thermo Scientific Nunc Immuno Stick Methods

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The use of precipitating enzyme substrates implies the opportunity of running ELISA with positive and negative controls at the same time on one immunostick.

This opportunity adds to the desired simplicity of the possible use of sticks in doctor's office and OTC tests.

This article describes how immunosticks can be used for assaying one or two analytes with controls at the same time.

Assaying one analyte

Rabbit IgG was used as analyte in a double antibody sandwich assay using swine anti-rabbit antibodies (SaR) and SaR conjugated with alkaline phosphatase (AP).

Thermo Scientific Nunc MaxiSorp sticks were coated with 1 mL saturating SaR (e.g. 5 µg/mL corresponding to approx. 1 µg per cm^2 of the 5.2 cm^2 total stick surface), washed in running tap water, and blocked with 1 mL 0.5% BSA. After another washing and drying of the sticks, their tips were incubated with 0.25 mL saturating analyte, and the sticks were washed again.

The sticks thus prepared were incubated half the way up with 0.5 mL analyte dilutions from a 1:2 dilution series, washed, incubated all the way up with 1 mL AP conjugated SaR, washed again and immersed in BCIP precipitating enzyme substrate (see below) for color development.

The result was a series of 3-banded sticks presenting the positive control in the tip band, the analyte test in the middle band, and the negative control in the upper band, as shown in Fig. 1.

Assaying two analytes

1. **Using AP conjugate with BCIP substrate**
   Rabbit anti-fibronection (RaFBN) and rabbit anti-a-foetoprotein (RaAFP) were used as analytes in a 3-layer assay.
   Nunc™ MaxiSorp™ sticks were coated on the tips with 0.25 mL 1:1 mixture of saturating fibronectin (FBN) and a-foetoprotein (AFP), half the way up with 0.5 mL saturating FBN, ¾ way up with 0.75 mL saturating AFP, and blocked all the way up with 1 mL 0.5% BSA, with washing between each step. After another washing and drying of the sticks, their tips were incubated with 0.25 mL 1:1 mixture of saturating analytes, and the sticks were washed again.
   The sticks so prepared were incubated ¾ way up with 0.75 mL of either separate or 1:1 mixed analytes in final concentrations of 25 ng/mL. After washing, the sticks were incubated all the way up with 1 mL AP conjugated SaR, washed again and immersed in BCIP substrate.
   This resulted in 4-banded sticks presenting the positive control(s) in the tip (first) band, the RaFBN test in the second band, the RaAFP in the third band, and the "negative control(s)" (see discussion) in the upper band as shown in Fig. 2(A).
2. **Using horse radish peroxidase (HRP) conjugates with H_2O_2 / ODN precipitating substrate (see below)**
   FBN and AFP were used as analytes in a double antibody sandwich assay.
   The set-up principle was the same as above, but using saturating RaFBN and/or RaAFP in the coating layer, 25 ng/mL FBN and/or AFP in the second layer, and a 1:1 mixture of HRP conjugated RaFBN and RaAFP in the third layer.

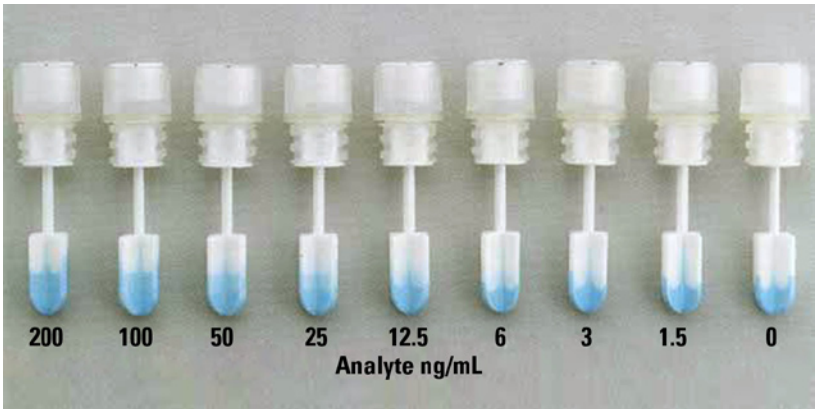

Fig.1
Immunostick ELISA of analyte in a 1:2 dilution series ranging from 200 to 1.5 ng/mL signalled by AP-conjugate and precipitating BCIP enzyme substrate in the middle bands of the sticks with adjacent positive and negative controls in the tip and upper bands, respectively. See text for further explanation.

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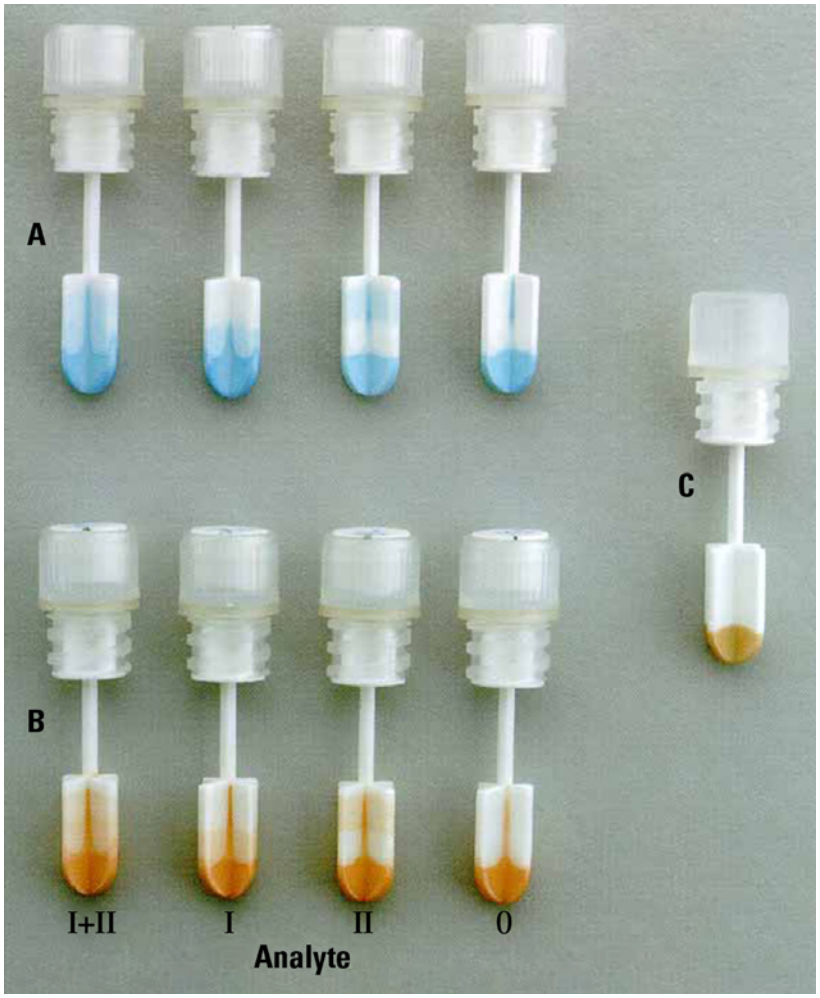

Fig. 2
Immunostick ELISA of 2 analytes (I an II) either mixed 1:1 or separate in final concentrations of 25 ng/mL using AP/BCIP (A). HRP/ODN (B), or a combinatin of both detection systems (C). The analyte signals are presented in the two middle bands flanked with positive and negative controls in the tip and upper bands, respectively. See text for further explanation.

This again resulted in 4-banded sticks, but this time positive signals were represented by red bands instead of blue as shown in Fig. 2(B).

Discussion

It would be more elegant to assay two analytes by use of different enzyme/substrate systems in order to get different colors for different analytes.

Despite the fact that the HRP/ODN and AP/BCIP systems run at pH 7 and 10, respectively, we have proved that the two systems can be used in the same assay if the substrate reactions are done successively. If the ODN substrate reaction is done before the BCIP reaction, a color between red ODN and blue BCIP arises in the tip band where both analytes are present (positive controls) as shown in Fig. 2(C), instead of the ambiguous unmixed color when only one system is used.

The "negative controls" in the 2-analyte assays should have missed only the 2nd layer, instead of missing both the 1st and 2nd layer, to be true negative controls. This could have been achieved if the top band of the sticks had also been coated with a mixture of 1st layer reagents.

However, this would have implied the risk that an analyte had also combined with a 1st layer reagent in the top band thus spoiling the negative controls. To avoid such undesired invasion by reagents from below, it is necessary that the sticks are dried before incubation with sample analytes - as it is before incubation with saturating analytes for positive controls as mentioned above.

The given assay designs were determined by the selection of easily available immuno reagents (Dako, Denmark) and were merely intended as inspiring examples of more sophisticated uses of the Thermo Scientific Nunc Immuno Stick.

Precipitating substrates

A list of precipitating enzyme substrate chromogens is given below with the combined risk claims from Aldrich and from Sigma (1989):

For peroxidase

1. 3,3-Dimethoxybenzidine, o-dianisidine (ODN)
   Turns reddish
   IRRITANT; POSSIBLY CARCINOGENIC
2. 3,3-Diaminobenzidine
   Turns reddish
   POSSIBLY CARCINOGENIC
3. 3-Amino-9-ethyl carbazole
   Turns reddish
   TOXIC; POSSIBLY CARCINOGENIC
4. 4-Chloro-1-naphtol
   Turns bluish
   IRRITANT

For alkaline phosphatase

5. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP)
   Turns bluish
   NO RISK CLAIMS
6. Nitro blue tetrazolium (NBT)
   Turns bluish
   NO RISK CLAIMS

We have so far successfully experienced Nos. 1 and 5, respectively, as well as Nos. 5 and 6 in combination, which gives additional color intensity,
- according to the following recipes:

ODN

30 mg o-dianisidine dihydrochloride (Sigma D-3252) in 5 mL DMSO per 45 mL H_2O + 50 mL PBS, added with 40 µL 30% H_2O_2 just before use. Turns red within 30 minutes.

BCIP (Sedgwick & Holt, 1983)

100 mg 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma B-8503) per 100 mL AMP-buffer (95.8 mL 2-amino-2-methyl-1-

propanol (Merck 801465) + 150 mg $MgCl_2 \cdot 6H_2O$ + 0.1 mL Triton X-100 in 800 mL H_2O, pH adjustment to 10.25 with conc. HCl, overnight standing, pH readjustment to 10.25 and H_2O addition to 1000 mL). Turns blue within 30 minutes.

BCIP + NBT (Kimball & al., 1988)
0.4 mm 5-bromo-4-chloro-3-indolyl phosphate (Sigma B-8503) and 0.046 mm nitro blue tetrazolium (Sigma N-6639) in 100 mm Tris-HCI buffer, pH 8.8, with 100 mm NaCl and 5 mm $MgCl_2$. Turns violet within 30 minutes.

References

Sedgwick J.D. & Holt P.G. (1983).
A solid phase immunoenzymatic technique for enumeration of specific antibody secreting cells.
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Kimball S.R. & al (1988).
Quantitation of proteins by dot-immuno-binding assay.
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