Aspects of Thermo Scientific Nunc MicroWell Certification

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Key Words

Thermo Scientific™ Nunc™ MediSorp™ and MaxiSorp™, Thermo Scientific™ Nunc™ Immuno transparent plate, certification, quality test, ELISA, binding rabbit IgG, negative edge effect.

Goal

The goal of this application note is to describe the aspects of the Thermo Scientific Nunc certification using a rabbit IgG antibody in an ELISA assay and calculating the coefficient of variation (C.V.) in the assay. This to show that the adsorption is homogenous in the plates and fullfill the release criteria of a CV% below 5. Further to describe how a cold surrounding temperature could affect the results to generate the negative edge effect.

We certify the adsorption capability of Thermo Scientific Nunc MediSorp and MaxiSorp MicroWells F, U, C, and StarWell on the basis of the following test procedure (where the reagent volumes apply to F-wells, other well types using volumes proportionally adjusted to their respective contents).

1. Add to an 8 x 12 Nunc MicroWell matrix antibody coating mixture consisting of 10 µg rabbit IgG (Dako X 0903) and 65 ng rabbit IgG:HRP conjugate (Dako P 0128) per mL 0.05 M carbonate buffer, pH 9.6, 200 µL/well.

2. Seal with adhesive tape and incubate overnight in the dark at room temperature.

3. Wash 3 times (Nunc StarWell 5 times) with 0.15 M PBS, pH 7.2, containing an extra 0.2 M NaCl and 0.05% Triton X-100.

4. Add substrate solution consisting of 0.6 mg OPD·2HCl (Sigma P-1526) and 0.5 µL 30% H_2O_2 per ml 0.1 M phosphate-citrate buffer, pH 5.0, 200 µL/well.

5. Stop substrate reaction after 3.5-4 min. by adding 2N H_2SO_4, 150µL/well.

6. Read O.D. (Optical Density) at 490 nm against air in a MicroWell reader. U-wells are also read at 620 nm as a reference in order to eliminate any possible non-uniform extinction due to light refraction in the bottom curvatures. Consequently, dual wavelength reading is recommended in any assay using U-wells.

7. Calculate the C.V. (Coefficient of Variation) for the O.D. readings of the 8 x 12 MicroWell matrix according to the following formula:

$$C.V. = S \cdot \frac{100\%}{X} = \sqrt{\frac{\Sigma\,(X\text{-}X)^2}{N\text{-}1}} \cdot \frac{100\%}{X}$$

where: S = standard deviation of O.D. readings

X = mean of O.D. readings

X = individual O.D. readings

N = number of readings (wells)

This expression for S is equivalent to:

$$\sqrt{\frac{\Sigma\,(X^2) - \frac{1}{N}\,(\Sigma\,X)^2}{N\text{-}1}}$$

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The C.V. is a measure of the variation of the O.D. values between the wells in the matrix, and it is used as a measure of the adsorption uniformity between the wells. The smaller the C.V., the more uniform the adsorptive performance of the wells, all experimental uncertainties being equal.

According to the above procedure Nunc MediSorp and Nunc MaxiSorp certification guarantees a C.V. of less than 5% for an 8 x 12 well Nunc MicroWell matrix. However, as can be seen from Fig. 1, the 5% criterion does not necessarily exclude the possibility of some wells having large deviations from the mean O.D. For example, if just one well deviates, the deviation may reach almost 50% without invalidating the 5% C.V. requirement.

Of course, the fulfilment of the criteria not only assumes certain qualities of the wells per se, but also adequate technical skill, which involves a »human factor variable« to be considered in the matter of C.V. reproducibility.

However, at very low and very high O.D. values, inevitable experimental uncertainties may cause the C.V. to exceed 5%, as demonstrated by the results in (Fig. 2).

At low O.D. values minor interwell dissimilarities in substrate conversion, liquid volumes, measuring beam alignment, etc. will cause reading deviations which are large compared to the measured values thus resulting in a large C.V. Also at high O.D. values such dissimilarities will cause relatively large reading deviations due to the very steep course of the extinction curve at the upper extreme (Fig. 3).

Therefore, for the quality control, the O.D. mean is maintained between 1200 and 1300 mEU, which is in the middle of the constant C.V. range as it appears from Fig. 2. This may be adjusted by the substrate reaction time, which is generally in the area of 3.5-4 minutes using Dako standard conjugate preparations.

The influence of the substrate reaction time on C.V. has been checked, and the results are shown in Fig. 4. It is apparent that for reaction times longer than one minute, including the 3.5-4 minutes' range, the C.V. remains almost constant at a minimum of about 2%.

Part of the C.V. is due to photometric reading uncertainty, which means that the total C.V. includes the variation inherent in re-measuring one well 96 times.

The Nunc MicroWell reader here applied had 8 parallel detection channels, one for each well in a column. Assuming the same reading uncertainty on each channel, the C.V. due to this uncertainty alone has been determined at various O.D. values. It appears from the results shown in Fig. 2 that the elevated C.V. at extreme O.D. values is partially the result of enlarged reading uncertainties at the extremes. This is due to the limitations in photometric performance.
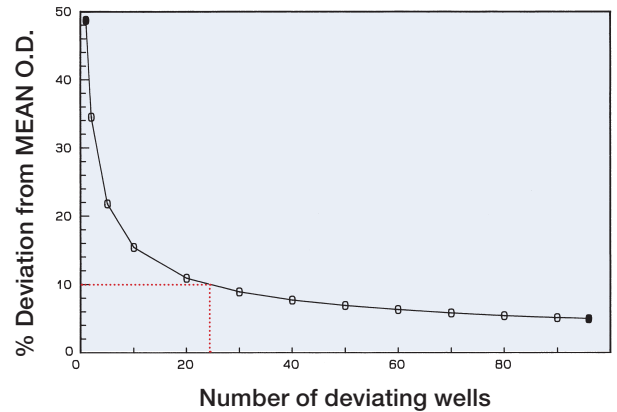

Fig. 1

Curve showing the number of wells, with a definite deviation from the mean O.D., required to produce a C.V. of 5% assuming that the remaining wells in the 8 x 12 matrix have zero-deviation. The dotted lines indicate 24 wells with 10% deviation as the upper limit for product acceptance. This curve was calculated from the C.V. formula given in the text.
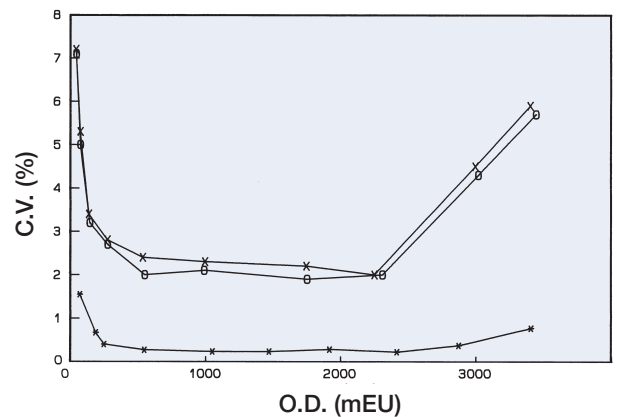

Fig. 2

C.V. of two 8 x 12 F-well matrices (X and O) at various O.D. values demonstrating C.V. elevations beyond the 5% acceptance level at the O.D. extremes. The curves were obtained by varying the amount of conjugate in the coating mixture while maintaining a constant substrate reaction time of 3.5 minutes. It should be noted that the same O.D./C.V. correlation was obtained by varying the substrate reaction time and keeping the amount of conjugate constant. This excludes the possibility of significant influence from conjugate dilution.

The bottom curve (*) represents the C.V. due to photometric reading uncertainty alone which is also elevated at the O.D. extremes thus contributing to the total C.V. elevations. This curve was produced by calculating the C.V. from 12 measurements of the same well. This was done at various O.D. values obtained through appropriate dilutions of converted substrate in an 8 x 12 F-well matrix, one column with each dilution. Each C.V. is the mean from 8 consecutive measurements in a column made by each detection channel in the Nunc MicroWell reader.
See text for further explanation.

A low O.D. value is measured by the ratio between two relatively large light intensities (from sample and blank), which implies a relatively large uncertainty. A high O.D. value is the result of very little transmitted light, which also implies enlarged uncertainty because the photometric sensitivity becomes inadequate.

Although not determined in this connection, possible calibration inaccuracies between the detection channels will also contribute to the total C.V.

In conclusion, for reproduction of the claimed qualities of Nunc MaxiSorp MicroWell products, one should observe the following precautions:

1. Use well-defined, standardised chemicals.

2. Keep the O.D. within the 1000-1500 mEU range.

3. Use sufficiently long substrate reaction times (but be sure that the substrate conversion has not levelled off).

4. Secure optimal photometer performance.

5. Use dual wavelength reading for U-wells.
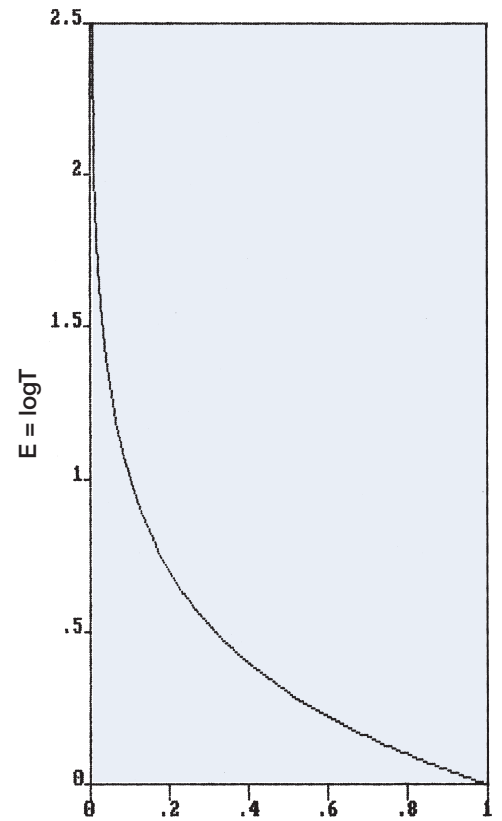
6. Use adequate technical skill.

Fig. 3

The relationship between photometric light transmission, T, expressed as the ratio between the light passing through the sample and through the blank, and extinction, E, according to the definition:

$E = -\log_{10} T$. One extinction unit, EU, is the extinction when $T = 0.1$, two EU the extinction when $T = 0.01$, etc. Optical density, O.D., is most conveniently displayed in EU (or mEU), because this figure is directly proportional to sample concentration according to the Lambert-Beer law. Note the very steep course of the curve when extinctions exceed 2 EU.

Fig. 4

C.V. of two 8 x 12 F-well matrices (X and O) for various substrate reaction times demonstrating that already beyond one minute, the C.V. has decreased to a minimum, leaving the routinely used 3.5-4 minutes well within the minimum range.

The curves were obtained by proportionally varying the amount of conjugate and substrate reaction time while maintaining the O.D. within the preferred 1200-1300 mEU range. The red curve is an average curve, adapted by eye.
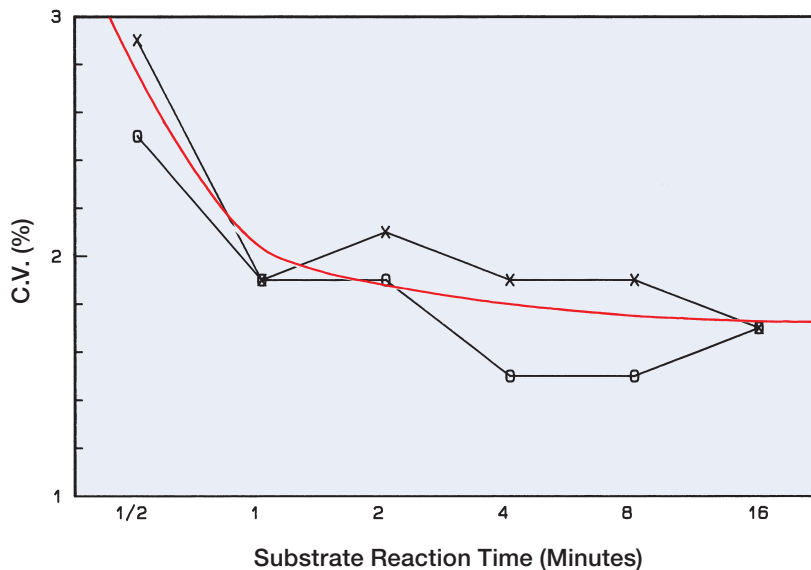
Negative Edge Effect in Thermo Scientific Nunc MicroWell ELISA

Here we will discuss negative edge effect, i.e. unexpectedly lower optical densities in peripheral wells than in central wells of a Thermo Scientific Nunc MicroWell matrix, due to temperature differences.

If, during some incubation in ELISA, one of the six conditions listed in Table 1 occurs, an edge effect of the corresponding sign (positive or negative) may be observed. It appears from Table 1 that the sign of the edge effect is dependent on the relative temperature of the surroundings, negative edge effect being the case when the surroundings are colder than the reactant liquids and/or the wells per se. An exception would be in "competitive" ELISA, where sample and labelled standard are incubated successively, starting with the sample. In this case relatively cold surroundings would give positive edge effect and vice versa.

Whereas positive edge effect easily occurs due to the short incubation time normally used in warm incubations (37°C), negative edge effect will be blurred due to the long incubation time normally used in cold incubations (4°C). Therefore, negative edge effect is a less common, or at least less pronounced, phenomenon than positive edge effect.

However, if negative edge effect is observed or suspected in an assay, one should always consider the causative conditions listed in Table 1.

If one of these conditions occurs during a brief substrate reaction, the negative edge effect may be expressed rather easily. Especially, this is likely to occur if the incubation prior to substrate reaction is a relatively warm one, which may establish the last case mentioned in Table 1.

To demonstrate the negative edge effect in this case, a Nunc Immuno Plate MaxiSorp F96 was incubated with 200 µL/well of IgG:peroxidase conjugate at 37°C for two hours (plate and conjugate were both preheated to 37°C), immediately followed by quick washings (within one minute) and H_2O_2/OPD substrate reaction for 3.5 minutes at room temperature. The plate showed negative edge effect, as illustrated in Fig. 5, compared with a plate which had been adjusted to room temperature before substrate reaction.

Obviously, to eliminate edge effects, not only the reactant liquid should be adjusted to the temperature intended for incubation, but also the wells per se.

Relative temperature of

Reactant Liquid	Wells	Surroundings (Incubator)	Sign of Edge Effect
Cold	Cold	Warm	+
Cold	Warm	Warm	+
Warm	Cold	Warm	+
Warm	Warm	Cold	-
Warm	Cold	Cold	-
Cold	Warm	Cold	-

Table 1

Dependence of edge effect sign on temperature conditions during (at the beginning of) incubation.
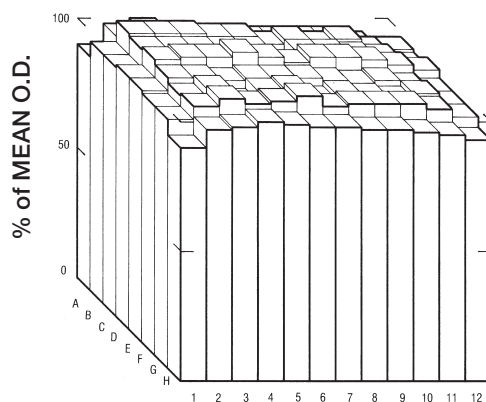

Fig. 5

Block diagram of the O.D. readings from H_2O_2/OPD substrate reactions in a Nunc MicroWell plate illustrating the edge effect after incubation with IgG:peroxidase conjugate at 37°C for two hours. Each column represents the O.D. reading of the respective well in percent of the plate mean value (1064 mEU). Note that the edge effect is most pronounced in the corner wells, A1 and H1 giving the minimum value = 90%, whereas the central well D5 gives the maximum value = 107%. See text for further explanation.

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