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Supplementary materials

Plasmonic ELISA for the detection of gp120 at ultralow concentrations with the naked eye

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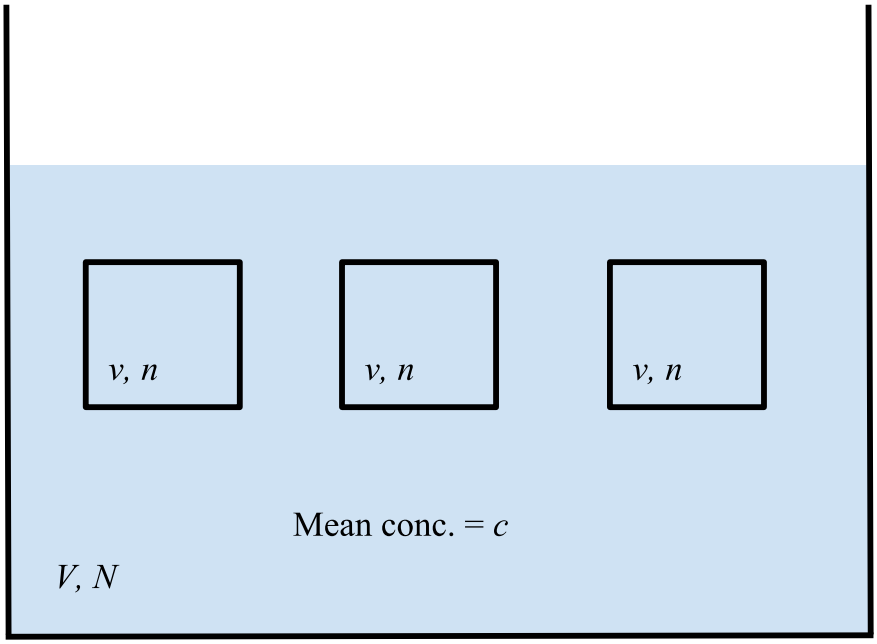
Contents

1. Notation

v	Volume of a small sample
V	Volume of a large reservoir
n	Number of molecules in small sample
N	Number of molecules in large reservoir
λ	Mean concentration in molecules per unit volume
$\bar{n}$	Mean number of molecules in volume v
p_0	Probability of obtaining no molecules in a single well
$p(X = r\|R)$	Probability of r out of R wells containing at least one molecule
k	Dilution factor
σ	Standard deviation
c_d	Concentration in dilution d
S	Nominal volume of the sample
B	Nominal volume of the blank
Δs_r	Random error in the sample volume
Δb_r	Systematic error in the sample volume
d	Number of dilutions
Δk_r	Fractional dilution error resulting from random errors
Δk_s	Fractional dilution error resulting from systematic errors

2. Poisson-Binomial model



Figures S1: Schematic of sampling of very dilute solutions

Given three samples of very dilute solution in volumes v = 100µl, containing on average c molecules of solute per unit volume (Figure S1), what is the probability of there being no molecules in one, two or all three of the samples? We use 'molecules' as shorthand for 'molecules of solute', since the solvent molecules play no part in the analysis. The samples were prepared by a series of dilutions from a more concentrated solution, but this process is irrelevant if we know the mean number of molecules that occupy a final volume v.

First let us ask how many molecules you expect to find in one specific region of volume v within a big reservoir of volume $V >> v$ containing a total of N molecules randomly distributed throughout the volume. The mean concentration in any sample taken from the reservoir is then related to N and V by $c = N/V$.

The mean number of molecules in a volume v is given by

$$\bar{n} = vc$$

Imagine dropping the molecules into the big reservoir one at a time. The probability that the first molecule ends up in v is v/V, and therefore the probability that it doesn't end up in v is

$$\left(1 - \frac{v}{V}\right)$$

Since the dropping of molecules is independent, the probability p_0 that *none* of them ends up in the volume v is

$$p_0 = \left(1 - \frac{v}{V}\right)^N = \left(1 - \frac{\bar{n}}{N}\right)^N$$

At this point we can use the fact that N is very large compared to $\bar{n}$, and a well-known mathematical formula tells us that:

$$\lim_{n\to\infty} \left(1 + \frac{\bar{n}}{N}\right)^N = e^{-\bar{n}}$$

from which we deduce that, for practical purposes

$$p_0 = e^{-\bar{n}} = e^{-cv}$$

We are interested in four outcomes of the three final wells, namely: the probability of none, one, two or three wells containing no solute molecules. However, more generally there may be up to R wells at a given dilution and we wish to know the probability that r of these contain at least one molecules of solute. If they are uncorrelated, the Poisson distribution applies to each of the R independent (Binomial) trials and we have:

$$p(X = r|R) = \binom{R}{r}(p_0)^{R-r}(1 - p_0)^r$$

This Poisson-Binomial model is widely used in the interpretation of serial dilution experiments[1]. Poisson models have also been applied in the context of single molecule detection[2].

3. Application to plasmonic ELISA

For a nominal concentration of 10^{-n} g/mL, the expected number of molecules per well can be determined using the molecular weight of the compound. In the case of gp120, the molecular weight is approximately 120 kDa. In 100 µL, we therefore have on average 5 molecules per well at 10^{-17} mg/l and 0.5 molecules per well at 10^{-18} g/mL. The probability of a given number of wells containing at least one molecule can then be derived from the Poisson-Binomial distribution. For example, given a mean value of 0.5 molecules per well (10^{-18} g/mL) the chance of obtaining one well with at least one molecule of solute is given by:

$$P(X = 1|3) = \binom{3}{1}(e^{-0.5})^2(1 - e^{-0.5})^1 = 0.434$$

The corresponding number of molecules for p24 (24 kDa) as used by de la Rica and Stevens[3] is five times that of gp120. As a consequence, single molecules are more likely to be obtained in lower concentrations for p24 than gp120.

In Table 1 (reproduced below for convenience), the probability of *r* wells containing at least one biomarker at the nominal concentration is reported for concentrations in the range 10^{-16} g/mL to 10^{-19} g/mL. Outside this dilution range the probability of obtaining either no wells with molecules ($<10^{-19}$ g/mL) or all wells containing molecules ($>10^{-16}$ g/mL) is the most likely outcome. The results from de la Rica and Stevens[3] using p24 are included for comparative purposes. In both cases we find that the most likely outcome (highlighted in bold) has been observed. These calculations suggest that the results observed in the two experiments are consistent. For three replicates, the relationship between concentration and probability of *r* of the final pipettes containing at least one molecule is shown in Figure S2.

Table 1: Probability distribution of wells with at least one biomarker.
Probabilities calculated using the Poisson-Binomial model and based on nominal concentrations at the 10^{-18} g·mL^{-1} dilution of 2.5 per well for p24 and 0.5 per well for gp120. Probabilities calculated using the Poisson-Binomial model and based on nominal concentrations at the 10^{-18} g·mL^{-1} dilution of 2.5 per well for p24 (as in de la Rica and Stevens[3]) and 0.5 per well for gp120. Probabilities of the most likely outcome corresponding to results observed in here for gp120 and in de la Rica and Stevens[3] for p24 are highlighted in bold, and in both cases this is precisely the outcome observed.

Biomarker	Wells containing biomarker	Dilution (g/ml)		
		10^{-17}	10^{-18}	10^{-19}
gp120	0	0.000	0.223	-
	1	0.000	**0.434**	-
	2	0.020	0.282	-
	3	**0.980**	0.061	-
p24[1]	0	0.000	0.001	**0.472**
	1	0.000	0.019	0.402
	2	0.000	0.207	0.114
	3	**1.000**	**0.773**	0.011

[1]As reported in de la Rica and Stevens[3] and included for comparative purposes.

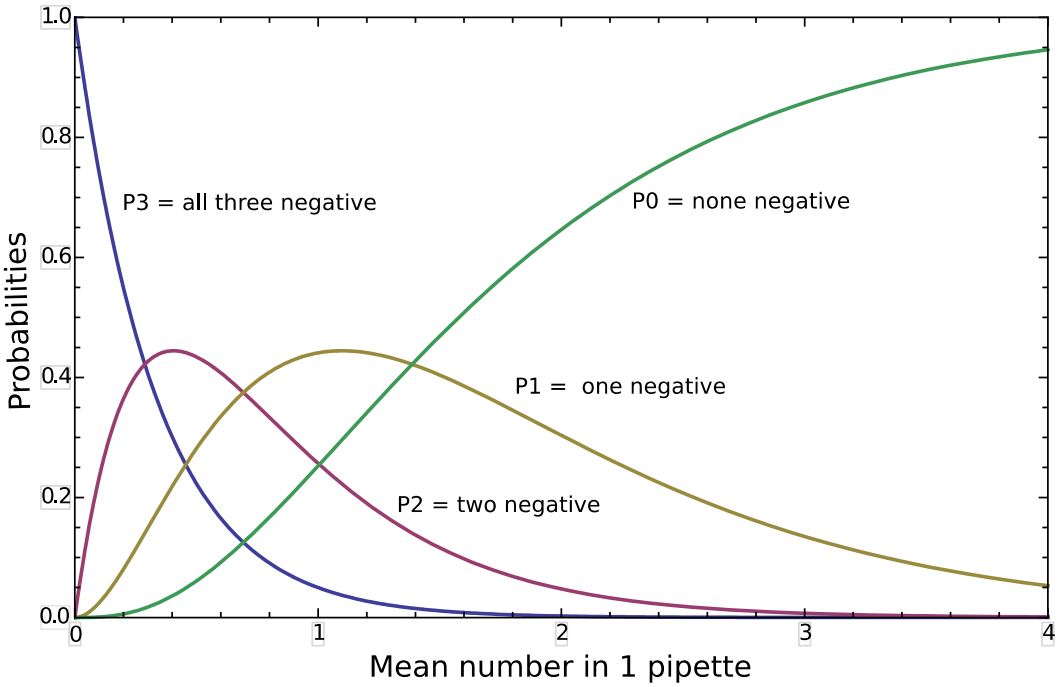


Figure S2: Probability of *r* pipettes out of 3 containing no molecules, for r = 0, 1, 2, and 3

4. Dilution error

At each stage of the dilution, a volume of sample *S* is added to a volume of blank *B* such that the concentration per unit volume at the d^{th} dilution, c_d is related to the concentration at the previous stage as follows

$$c_d = \frac{c_{d-1}S}{S+B} = kc_{d-1}$$

where *k* is the dilution factor, nominally 0.1 in the case of a ten-fold dilution.

Dilution error arises due to a combination of *random* ("imprecision") and *systematic* ("inaccuracy") errors in pipetting for both the sample (100 µL) and blanks (900 µL). We define these errors as *s* and *b* with subscripts to denote whether the errors are random *(e.g.* Δs_r) or systematic (e.g. Δs_s).

The pipettes used in these experiments (ThermoScientific Finnpipette™) have according to the manufacturer an inaccuracy (tolerance limit) of 1% for the 100µl and 0.6% for the 1000µl pipettes. The corresponding random errors, reported as coefficients of variation (CoV), are respectively 0.4% and 0.2%. In calibration experiments (n=20) using both pipettes, we found our errors to be somewhat lower with the exception of the inaccuracy of blanks (7.4 µL v*ersus* 6 µL). We use the manufacturer values for our reported error bars and therefore have in µL:

$\Delta s_r = 0.4$
$\Delta b_r = 2$
$\Delta s_s = 1$
$\Delta b_s = 6$

In this experiment, solutions for calibrating the system were obtained by serially diluting a stock solution containing gp120 with the concentration of 10^{-4} g/mL (10^{-5} g per 100 µL). A total of 14 ten-fold dilutions were required to obtain the final dilution of 10^{-18} g/mL.

4.1. Random dilution error

The random errors can be modeled as independent Gaussian distributions, whose combined variance is summed using standard error propagation theory

$$\sigma_{dilution,\ random}{}^2 = d\Delta k_r{}^2$$

where f_r is the fractional dilution error factor resulting from random errors in the sample and blank and *d* is the number of dilutions.

As above, the dilution factor *k* is related to the sample and blank volumes

$$k = \frac{S}{S+B}$$

For small errors in S and B of Δs_r and ΔB the standard formula for deriving errors uses partial differentiation and combination of errors by quadrature

$$\Delta k_r{}^2 = \left(\frac{\partial k}{\partial S}\Delta s_r\right)^2 + \left(\frac{\partial k}{\partial S}\Delta b_r\right)^2$$

By partial differentiation of *k* and quadrature:

$$\Delta k_r{}^2 = + \left(\frac{-B}{(S+B)^2}\right)^2 \Delta s_r{}^2 + \left(\frac{S}{(S+B)^2}\right)^2 \Delta b_r{}^2$$

$$\frac{\Delta k_r{}^2}{k^2} = \left(\frac{S+B}{S}\right)^2 \frac{S^2\Delta b_r{}^2 + B^2\Delta s_r{}^2}{(S+B)^4}$$

Which simplifies to:

$$\frac{\Delta k_r{}^2}{k^2} = \frac{S^2\Delta b_r{}^2 + B^2\Delta s_r{}^2}{S^2(S+B)^2}$$

And is equivalent to[4]:

$$\frac{\Delta k_r{}^2}{k^2} = k^2(1-k)^2\left(\frac{\Delta b_r{}^2}{B^2} + \frac{+\Delta s_r{}^2}{S^2}\right)$$

Using the manufacturers values for random error, we obtain a value of 0.4411% for Δk_r. The random error therefore ranges from 0.9% after 5 dilutions (10^{-8} g/mL) to 1.6% after 15 successive dilutions (10^{-18} g/mL).

4.2. Systematic dilution error

The influence of the systematic errors can be investigated by examining the influence of the manufacturers' reported tolerance limits and their influence on the value of the dilution factor *k,* which for a ten-fold dilution is nominally 0.1.

For the upper limit we have:

$$k(1+\Delta k_{s,\,upper}) = \frac{S+\Delta s_s}{S+\Delta s_s+B-\Delta b_s} = 0.101446$$

therefore the systematic error is:

$$\left(\frac{\Delta k_{s,\,upper}}{k}\right)^d = 1.01446^d$$

Correspondingly for the lower limit we have:

$$\left(\frac{\Delta k_{s,\,lower}}{k}\right)^d = 1.01443^d$$

As this is a geometric series, the error diverges in percentage terms with the upper limits exceeding those of the lower limits. At the 10^{-18} g/mL dilution the systematic error in the upward direction reaches 25.1% and is substantially greater than the random error. If we instead use the experimental value of the inaccuracy of the blank (-7.4 μL) and sample (+0.4 μL), the error is within these bounds.

4.3. Combined dilution error

In order to combine dilution errors we add the predicted confidence intervals (95%) for the random error based on a Gaussian distribution to the systematic error limits (Table S2). Equivalent calculations were performed for Bovine Serum Albumin (BSA).

Table S2: Error analysis for dilution series used in plasmonic ELISA. Based on manufacturer's reported imprecision and inaccuracy. Systematic errors are propagated and combined using quadrature. Systematic error is combined with 95% CI for random errors based on the Gaussian distribution.

Dilution (g/mL)	**Dilution** (mol. per well)	**Random dilution error** (CoV, %)	**Systematic dilution error[1]** (±%)	**Combined dilution error[1]** (±%)	**Combined dilution error** (± mol.)
10^{-14}	5000	1.37	17.9	20.8	1040
10^{-15}	500	1.43	19.7	22.7	114
10^{-16}	50	1.48	21.5	24.7	12.4
10^{-17}	5	1.54	23.3	26.6	1.33
10^{-18}	0.5	1.59	25.2	28.6	0.14

[1]We report the systematic error for the upper limits as these are larger than for the lower limits due to their geometric nature. For example in the 10^{-18} mg/L dilution the lower limit is 20.1%, compared with 25.2% for the upper limit

5. Sampling variability

In addition to the aforementioned errors, uncertainty in the number of molecules per well also arises due to pure sampling. Since sampling follows the Poisson distribution, the standard deviation is equal to the square root of the expected number of molecules. Consequently the coefficient of variation is small at high concentrations, but can be very large for dilute solutions.

In Table S2, we provide 95% confidence interval for the number of molecules per well at the nominal concentration according to the Poisson distribution. Contrasting Tables S2 and S3, it can be seen that the sampling variability is the dominant form of uncertainty for the number of molecules per well at the lowest dilutions.

Table S3: Sampling variability and total uncertainty. Sampling variability based on Poisson sampling**.** Total uncertainty derived by convoluting Gaussian dilution errors with Poisson sampling distribution.

Dilution	**Dilution**	**Sampling variability**	**Sampling variability**
(g/mL)	(mol. per well)	(CoV,%)	(mol. per well; 95% CI)

			Lower	Upper
10^{-14}	5000	1.4	4900	5200
10^{-15}	500	4.5	460	550
10^{-16}	50	14	36	64
10^{-17}	5	45	1	9
10^{-18}	0.5	140	0	2

6. Supplementary references

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4. W. Hyk and Z. Stojek, *Analytical Chemistry*, 2013, 85, 5933-5939.