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Perfect order plating: principle and applications

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The raw quality of data is one of the first and most desirable requirements of Good Laboratory Practice (GLP) and Good Statistics Practice (GSP). In quantitative microbiology, this implies that cell and colony counts should follow the 'ideal' Poisson distribution (Ref. 1). Since the classical works by Student (Ref. 2) and Fisher *et al.* (Ref. 3), it has been known that under 'ideal conditions', microbial cell counts taken using a haemocytometer or colony counts on parallel plates will vary in accordance with Poisson distribution. When these conditions are fulfilled, the mean count is a direct measure of the density of the bacterial population, and such an estimate is the most accurate. 'Any significant departure from the theoretical [Poisson] distribution is a sign that the mean may be wholly unreliable' (Ref. 3).

Modern studies show that the plate counts from, for example, the Ames *Salmonella* assay (Ref. 4–6) or the microbiological quality control of food and drug products (Ref. 7) too often (~50% of experimental data sets) exhibit significant extra-Poisson variability, also called 'over-dispersion'. This phenomenon remains a serious problem in quantitative microbiology. Biometricians try to solve it by inventing sophisticated statistical models and corresponding tests for this phenomenon. However, introducing additional parameters into such models complicates the analysis and reduces its efficacy and reliability (Ref. 5–8 and references therein). The problem is so serious that, in a recent publication (Ref. 7), German microbiologists and biometricians suggested the introduction of non-Poisson statistical models into national GLP standards for microbiological (epidemiological) control of the safety and quality of foods and drugs.

One of the main reasons for all this might be the low precision of traditional plating techniques. Traditionally, two plating procedures are the most common.

1. Small volumes (drops) of microbial cell suspension are pipetted onto the medium surface in Petri dishes and spreading with a bent glass rod (spatula) as uniformly as possible.
2. Melted top agar with embedded microbial cells is poured over the base agar layer (Ref. 9).

To provide 'ideal' conditions to achieve uniform colony size or the Poisson distribution for plate counts, a technique called 'perfect order plating' (POP) and a special device called the 'poissoner' were proposed in 1973 (Ref. 10,11). Since that time, they have been used in several laboratories in Russia, the USA, the UK (Ref. 10–21; N.N. Khromov-Borisov, PhD thesis, Leningrad State University, USSR, 1976) and, now, Brazil. They are most popular among molecular geneticists, who need precise estimates of mutation, recombination, transformation, survival rates and so on.

To date, however, only brief descriptions of the method and the device have existed in English, in the review on measuring the spontaneous mutation rate in yeast, published in 1978 (Ref. 13, 14). All other descriptions are in Russian – three abstracts (Ref. 10–12), a PhD thesis (N.N. Khromov-Borisov, PhD thesis, Leningrad State University, USSR, 1976) and a textbook on genetics (Ref. 15). The applications described previously were mainly concerned with the use of concentrated cell suspensions and the registration of rare events (with frequencies up to 10^{-6}), such as secondary revertant/recombinant colonies on special selective media. Here, we report revised protocols, the optimized construction of a poissoner and some new applications and illustrations of the method.

1. Perfect order principle

The fundamental concept is to have a perfectly ordered, equidistant arrangement of small drops of microbial cell suspension on the surface of solidified (agar) medium in

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Fig. 1. Perfect order principle: the application of POP to colony growth studies. Very small drops (<1 μl in volume and no more than 1 mm in diameter) of concentrated yeast cell suspension ($\sim 10^6$–10^8 cells ml^{-1}) were placed onto the agar surface with a point pen following a perfect trigonal dot pattern (drawn on the diagram laid under the plate). The colonies are uniform in size and shape after growing under standard incubation conditions. The variability of the colony diameter on a dish is extremely low, with the relative standard error of the measurements being ~1%.

Petri dishes. This provides uniform conditions for the cell growth and, as a result, uniform colony size or the ideal Poisson distribution for their counts.

2. Protocols

2.1. Generating uniform colony size using the POP principle

Very small drops (<1 μl in volume and no more than 1 mm in diameter) of concentrated cell suspension ($\sim 10^6$–10^8 cells ml^{-1}) are placed onto the agar surface following a perfect trigonal dot pattern (like a honeycomb with an additional dot at the centre of each cell). This might be performed either by hand or with a special inoculator that resembles the poissoner except that the pins have pointed tips. In the first case, any common device such as a micropipette or a point pen can be used, and the drops are placed onto the medium following a diagram laid under the plate. During the incubation, the small spots containing thousands of cells grow into colonies that are uniform in size and shape (Fig. 1). Damage to the agar surface should be avoided, because this will disturb the colony shape.

2.2. Poissoner

The poissoner is a microbiological inoculator designed to provide a Poisson distribution for colony counts on the Petri dishes. It is made of stainless metal and looks like a circular brush-like stamp with cylindrical pins (prongs). Pins

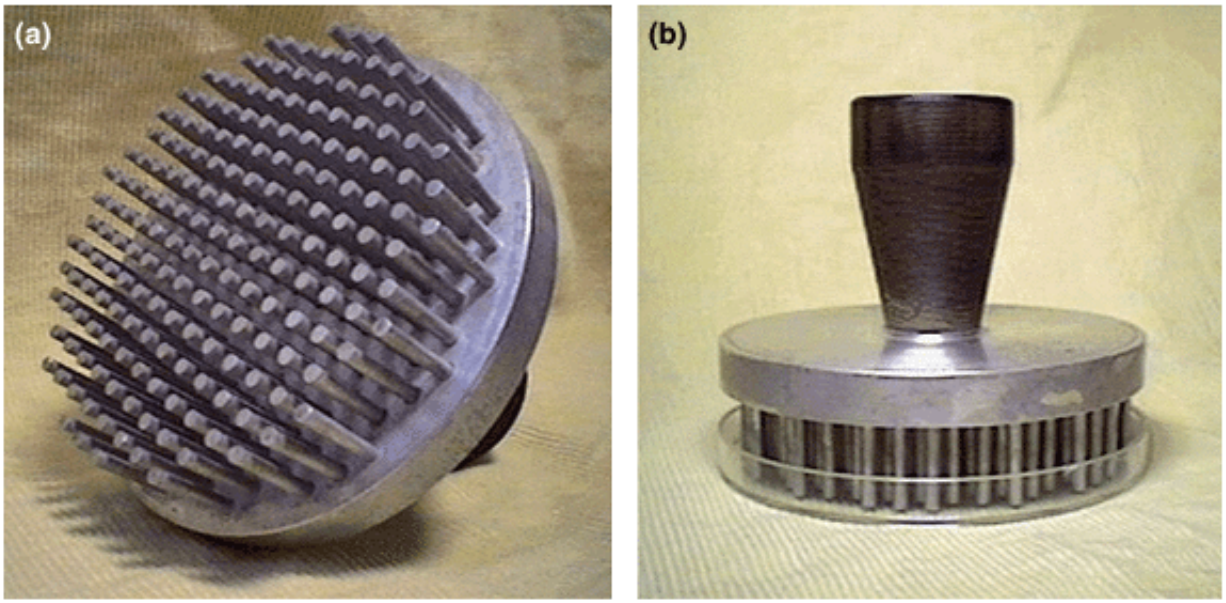

Fig. 2. General view of the 'poissoner' (a) and one of its working positions: face down (b).

can be made of rivets (~2.0 mm in diameter and 1.5–2.0 cm long) with carefully levelled open ends. They are fixed equidistantly and concentrically on the metallic base to fit the inside of a standard Petri dish as completely as possible. A total of 163 or 187 pins, with 3.0 mm intervals between them, can be recommended as optimal parameters (Fig. 2).

Poissoner can be flame sterilized after dipping its working part (pins) in alcohol. Overheating of its metallic parts should be avoided. For the sake of safety, its handle is made of incombustible material with a low heat conductivity (usually plastic).

There are two working positions for the poissoner: face down (Fig. 2b) and face up (inverted), like in replica plating (not shown). The first is used for fast work when many plates must be seeded without high precision. The inverted position is preferable for higher precision. Correspondingly, the end of the handle is flattened so that the inverted poissoner can stay stably on the desk.

2.3. *Generating Poisson-distributed colony counts using poissoner*

Put 20–25 ml of the microbial cell suspension in a sterile Petri dish and dip the working part (pins) of the sterile poissoner in it. Carefully stir the suspension by rotating the poissoner and then transfer it onto a Petri dish with solidified agar medium. Before plating, check whether each pin carries a hemispherical drop of suspension on the end. If any pins do not carry a drop, clean the poissoner from the hydrophobic contamination with detergent and/or alcohol. The preferred working positions are face up for the poissoner and face down for the dish. Put the dish on the working part of the inverted poissoner and gently press to imprint visually distinct, small, circular dints (traces, spots) on the agar surface, but avoid damaging the agar. Check through the transparent dish bottom whether each pin leaves a drop and a dint. Carefully remove the dish to prevent the merging of the drops. After incubation, it will be possible to calculate the sample distribution of resulting colonies and to test the goodness-of-fit with the expected Poisson distribution on each dish. To do this, count the numbers of dints (spots) without any colony, then with one colony, with two, etc., and present the data as in Table 1. Repeat the procedure with other dishes.

3. Advantages of the POP technique

When using the POP technique for measuring colony growth (according to the above protocol), the variability of the colony diameter on parallel dishes is extremely low, with the relative standard error of the measurements being ~1% (Fig. 1). Such precision is comparable with that of a good experiment in physics or engineering.

The POP technique permits low variability and therefore high precision in the colony counts. When using the poissoner, the colonies are much better separated (non-overlapping) and their arrangement is obviously more

Table 1. Distributions of the yeast colonies on ten Petri dishes generated with a poissoner and comparison of corresponding plate counts with those obtained using the traditional plating procedure

I	Number of dints with *i* colonies in dish number										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**Total**
0	21	23	19	27	25	18	24	23	26	25	231
1	48	48	57	51	48	43	41	54	45	43	478
2	56	54	54	49	59	49	63	54	47	48	533
3	29	29	35	37	32	38	32	34	36	38	340
4	18	17	13	14	11	23	14	13	20	20	163
5	13	15	5	8	8	7	11	7	11	11	96
6	2	1	2	1	2	8	0	1	2	1	20
7	0	0	0	0	0	1	2	1	0	1	5
8	0	0	0	0	2	0	0	0	0	0	2
9	0	0	1	0	0	0	0	0	0	0	1
10	0	0	1	0	0	0	0	0	0	0	1
Total	187	187	187	187	187	187	187	187	187	187	1870
Poisson fit[a]	0.77	0.98	0.39	0.61	0.47	0.89	0.79	0.51	0.95	1.00	0.97
	Total number of colonies per dish obtained with poissoner[b]										
P_P=0.66	396	392	378	362	374	437	388	364	394	401	3886
	Total number of colonies per overdispersed dish obtained with the traditional plating procedure[c]										
$P_T \approx 10^{-32}$	308	443	391	372	341	320	435	381	328	315	3634

[a]*P* values for the exact dispersion test of conformance to a Poisson distribution (Ref. 27). For all ten dishes (as well as for the total colony counts, *P*=0.97), departure from the Poisson distribution is negligible. Poisson distributions generated with POP on all ten dishes are homogeneous and/or reproducible: *P*=0.689±0.002, obtained as the Monte Carlo estimation for the Fisher (probability) exact test (5×10^6 iterations) using a 10×10 contingency table and StatXact-4.

[b]Total plate counts generated with POP are also homogeneous (Poisson distributed): P_P=0.66 (Bol'shev-Volodin test, Z_P=0.44).

[c]These values were obtained with the same cell suspension using the traditional plating procedure. Their heterogeneity is extremely significant owing to extra-Poisson variability: $P_T = 8.3 \times 10^{-33}$ (Bol'shev-Volodin test, Z_T = 11.93) (Ref. 4–8).

uniform than with the traditional plating method using a pipette and a spatula (Figs. 3 and 4). The procedure allows several hundred (500 and even more) non-overlapping, Poisson-distributed colonies to be obtained on a single dish. As a result, two to three dishes seem to be sufficient to estimate the mean counts with a relative error of ~5%.

POP generates an 'ideal' Poisson distribution with high reproducibility (homogeneity of counts on parallel plates) (Table 1, Figs. 4 and 5) compared with heterogeneous (over-dispersed) plate counts obtained with the same cell suspension using traditional plating technique (Table 1) and with the data published in the literature (Fig. 5) (Ref. 4–8).

Using the poissoner makes it possible to test statistically how well the observed colony count distributions fit the Poisson distribution, because each of 163 (or 187) spots (dints) imprinted by the pins can be considered as a small dish (Table 1). Several hundred Petri dishes have been plated in our laboratories using this technique and no statistically significant departure from the Poisson distribution was ever observed. Moreover, when the number of colonies per dish is too large, so that some colonies are merged and undistinguishable, a fast estimation of the mean value can be used (Ref. 14,17).

POP is much faster, simpler and cheaper than traditional procedures. It can be a good alternative to the common plating techniques. By using poissoner, one can treat one dish per second. Traditional spreading technique requires several dozens of seconds (and even 1–2 min) per dish. Additional economy is achieved because sterilization of the poissoner is very cheap compared with the use of disposable as well as re-sterilized plates, pipettes, spreaders, tips and so on.

Thus, POP can be used in any microbiological laboratory. It will be of particular importance in those fields of experimental and applied microbiology in which GLP and GSP are required, such as the genetics and breeding of microorganisms, environmental, epidemiological, clinical, pharmaceu-

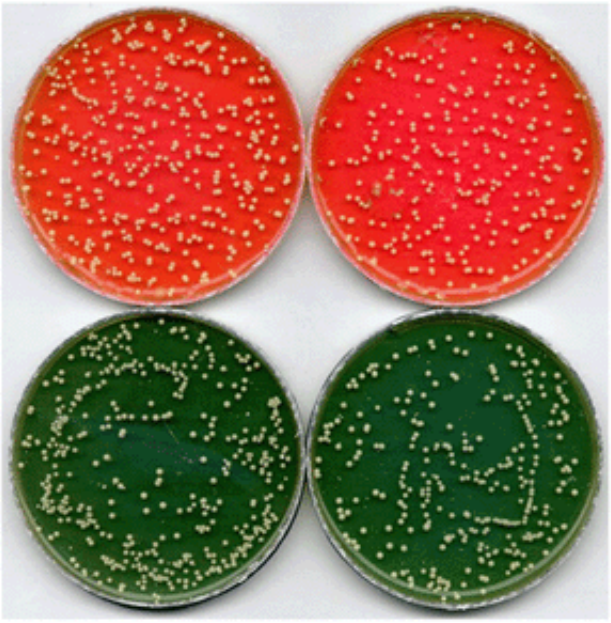

Fig. 3. Colony arrangements generated using a poissoner (red) and traditional plating with pipette and spatula (green). Two pairs of plates with nearly equal numbers of colonies are presented, ~440 per dish in the first column and ~300 in the second.

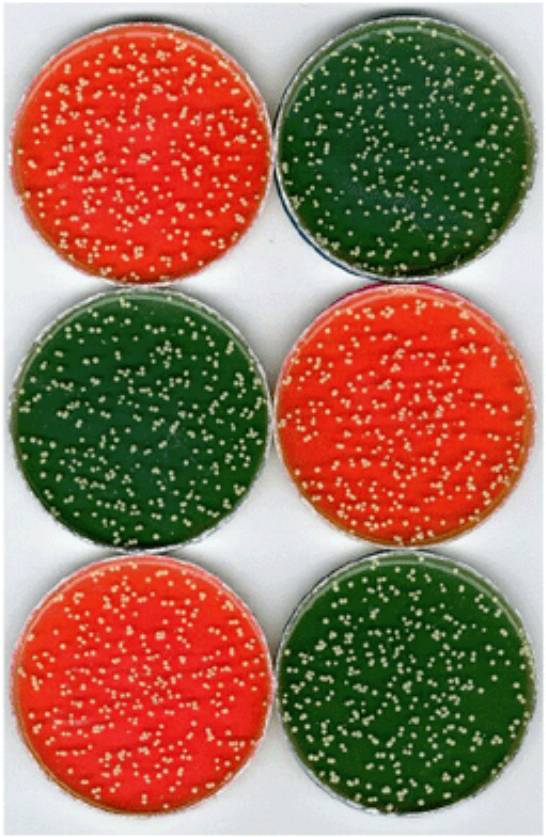

Fig. 4. Reproducibility of the results generated by a poissoner. Using the same poissoner, cell suspension and medium batch, two people independently plated three dishes each (green and red). The variability of plate counts is very small: 360–390 colonies per dish.

tical and industrial microbiology, biotechnology, genetic toxicology, many kinds of microbiological toxicity assay, and microbiological safety and quality control. It can provide more safety when working with pathogenic microbes.

4. Disadvantages of the POP technique

Like any other laboratory technique, POP requires from the experimenter some kind of definite skill and training. Some psychological obstacles can also become apparent. For instance, to an experimenter who is accustomed to the traditional techniques, the need to work with relatively large volumes of the cell suspension (20–25 ml) placed in a Petri dish (not in a tube) might become uncomfortable owing to its rarity. Nonetheless, almost all miniaturized microbiological procedures can be easily modified to be adapted for use with the poissoner.

5. Products and equipment used

5.1. Species, strains, reagents and media

The yeast used was *Saccharomyces cerevisiae*, haploid strain N123 (used as a wild type in this laboratory), genotype *MATa his1*. This was grown in the standard complete YEPD medium for yeast (Ref. 22).

6. Data analysis

To test the goodness-of-fit of the observed variation in colony counts on each Petri dish with the expected Poisson distribution, several relevant statistical procedures can be used (Ref. 23–26). We used the exact and Monte Carlo versions of the Fisher's variance (dispersion index) test, calculated using the programs created by Papworth (Ref. 27) and available from the author.

For graphical visualization of the goodness-of-fit, C.A.MAN software was used (Ref. 26; http://www.medizin.fu-berlin.de/sozmed/caman.html). For higher-quality presentation, all graphs were re-drawn with Prizm-3 software (http://www.graphpad.com/prism/Prism.htm). To test the homogeneity within a given data set, any relevant procedure for the analysis of contingency tables can be used. We used the exact and Monte Carlo tests in the StatXact-4 software (http://www.cytel.com/new.pages/SX.2.html). To fix

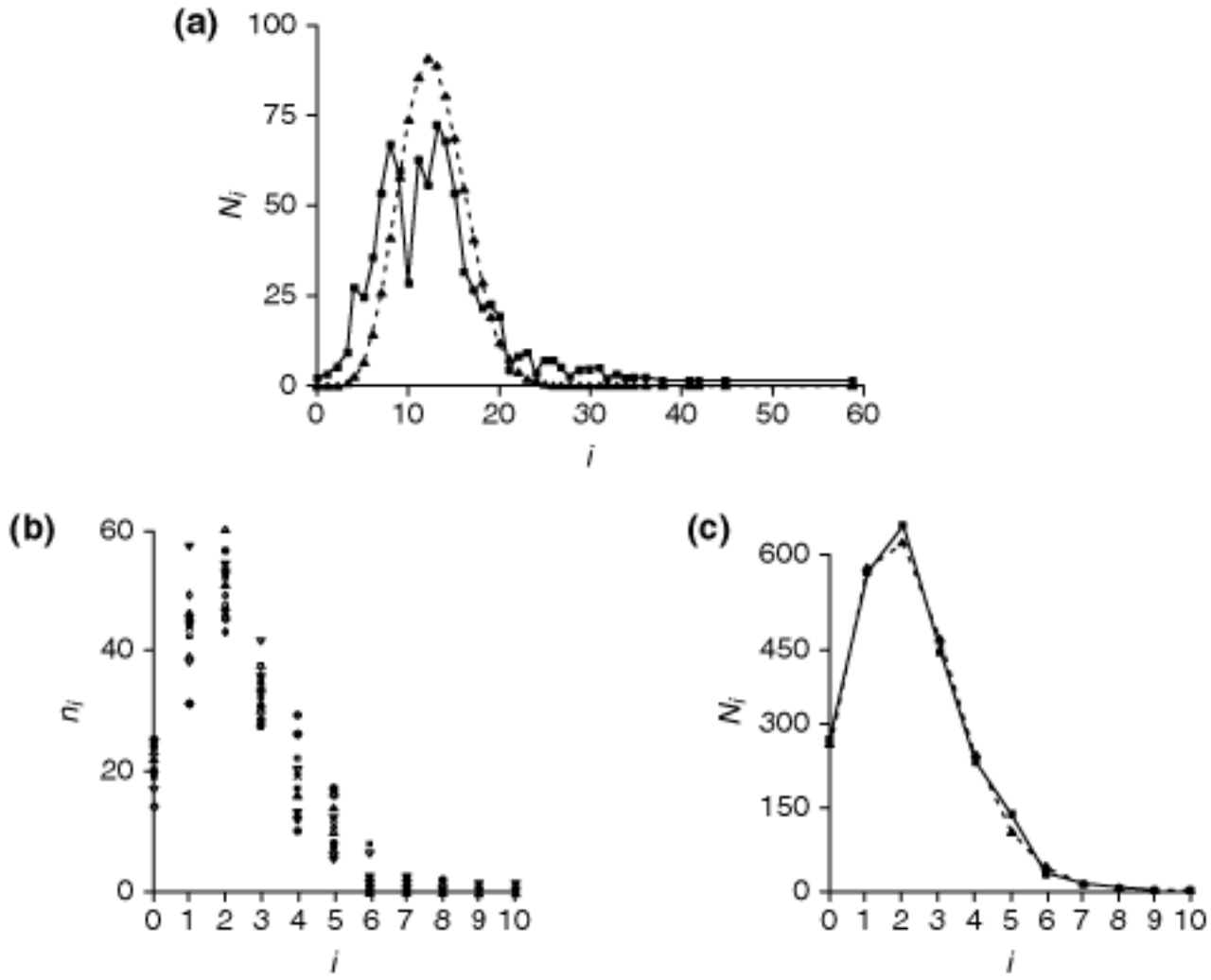

Fig. 5. Visualized goodness-of-fit to Poisson distribution for the published data (Ref. 4) (a) and the data generated with a poissoner (c) and their reproducibility (b). Squares show the observed distribution and triangles the expected theoretical distribution under the Poisson assumption.

the images of the dishes the Hewlett-Packard ScanJet4 scanner and corresponding software were used.

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