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A Simple Method for *in situ* Quantification of Cells on Carriers

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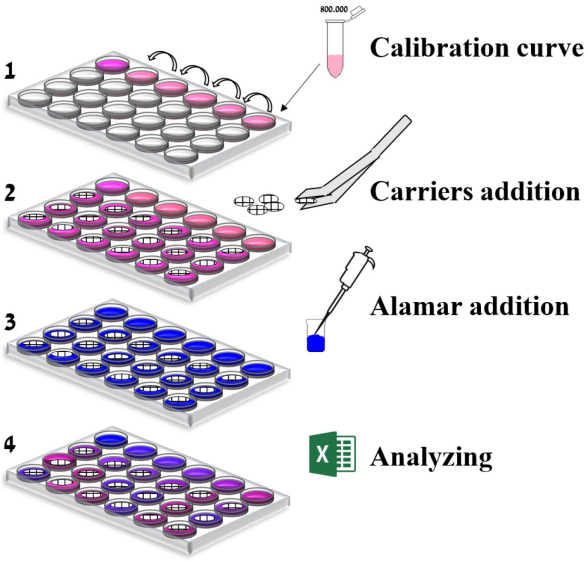
[Abstract] The technology of cell carriers was developed as a response to the need for high cell density to enable higher production levels in cell-based production processes. To follow the production process, quantifying the number of cells on these carriers is required, as well as tracking their viability and proliferation. However, owing to various carriers' unique structures, tracking the cells is challenging using current traditional assays that were originally developed for monolayers of adherent cells. The current "gold standard" method is counting cell nuclei, which is tedious and counts both live and dead cells. A few other techniques have been developed, but they are all specific to a carrier type and involve specialized equipment. Here, we describe a broad ranging method for counting cells on carriers. The method is based on the Alamar blue dye, a well-known, common marker for cell activity. No separation of the cells from the carriers is needed, nor is any specialized equipment. The method is simple and rapid, and provides comprehensive details necessary for control of production processes in cells. This method can be easily implemented in any cell-based process and other unique platforms for measuring growth of cells.

Graphic abstract:



Schematic of the *in situ* quantification method.

Keywords: Alamar blue, Cells, Carriers, Quantification, Fluorescence, High-throughput quantification, Plate reader

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[Background] Cell culture-based production is gaining increasing attention owing to the need for new manufacturing strategies. The advantages of cell-based production processes include the use of defined and serum-free cell cultures that allow greater consistency. Moreover, cell-based processes can be adapted to manufacturing processes involving bioreactors that are scalable and need less space (Aubrit *et al.*, 2015). The mounting use of cell culture-based vaccine production, together with a quest for a more efficient and scalable process, have stimulated the development and implementation of carrier technology. Micro and macrocarriers are small compact surface support matrices for growing adherent cells. Those carriers (from the micrometer to the millimeter size range for micro and macrocarriers, respectively) and their density allows their maintenance in suspension with gentle stirring (Huang *et al.*, 2020).

To better follow and understand a production process, it is necessary to quantify the number of cells on these carriers, as well as to track their viability and proliferation. The gold standard method for quantification of cells on micro and macrocarriers is nuclear staining (Sanford *et al.*, 1951). In this method, cells are separated from the carriers, stained with crystal violet, and counted visually under a microscope. The method is tedious (Berry *et al.*, 1996) and counts both live and dead cells, making it unsuitable for tracking cell condition. As a result, a method that can better quantify cells on micro and macrocarriers is desirable.

Counting cells on carriers poses a challenge to traditional assays due to the carriers' shape. The main feature of the widely used methods to measure cell quantity (Riss *et al*., 2013) is dyeing the cells with a fluorescent or colorimetric dye and quantifying the results based on fluorescence or absorbance. Examples of such dyes are glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1), and resazurin (Alamar blue). These assays are relatively inexpensive and have a homogeneous format, but their use is limited to single layer cultures (Mosmann, 1983; Berridge and Tan, 1993; Marshall *et al.*, 1995). A limited number of assays for cell quantification on carriers have been developed, but they all require specialized equipment that is not commercially available, and are "matched" with specific micro or macrocarriers (Farrell *et al.*, 2016). Thus, to date, no assays for quantification of cells on carriers that are simple, rapid, reliable, and low-priced are commercially available.

Here, a method for quantification of cells on carriers is described. The assay utilizes Alamar blue, a well-known and common marker for cell activity (Rampersad, 2012), and includes a calibration curve with known cell numbers. When added to cell cultures, the oxidized form of Alamar blue enters the cytosol and is converted to the reduced form by mitochondrial enzyme activity. This redox reaction is accompanied by a shift in the color of the culture medium from indigo blue to fluorescent pink, which can be easily measured by fluorometry (Al-Nasiry *et al.*, 2007). The method can quantify cells *in situ* on the carriers without requiring separation of cells from the carriers. The method is simple, rapid, elegant, does not require specialized reagents or equipment, and results are strongly correlated with the gold

standard assay. The method can be easily adapted for any cell-based process or other unique platforms for cell growth, including additional carriers and other scaffolds for culturing cells such as hydrogels.

Materials and Reagents

1. 1.5 ml Eppendorf tubes (Sarstedt, catalog number: 72.692.005)
2. Pipette tips (Filtered) (Thermo, catalog numbers: TF112-1000-Q [1,000 µl], T104RS-Q [10 µl], TF140-200-Q [200 µl])
3. 24-well clear plates (Corning, catalog number: 3524-ND)
4. 75 cm^2 T-flasks (Grenier, catalog number: 658-975)
5. 500 ml vacuum filter bottle system, Sterile (Corning, catalog number: 431097)
6. Counting vessels (Invitrogen, catalog number: C10283)
7. Aluminum foil (Nidix)
8. Vero cells (WHO, catalog number: RCB 10-87)
   Note: Cells used for the calibration curve must be the same as cells on carriers.
9. L-alanine-L-glutamine (Biological Industries, catalog number: 03-022-1B)
10. NutriVero FLEX-20 (Biological Industries, catalog number: 05-069-1A)
11. Pen-strep antibiotics (Biological Industries, catalog number: 03-031-1C)
12. Phosphate buffered saline (PBS) (Biological Industries, catalog number: 02-020-1A)
13. Recombinant trypsin-EDTA solution (Biological Industries, catalog number: 03-079-1A)
14. Trypan blue solution (Biological Industries, catalog number: 03-102-1B)
15. Alamar blue (Promega, catalog number: G808)
16. Cell medium (see Recipes)

Equipment

1. Sterile tweezers (United Scientific Supplies, catalog number: 20A00R146)
2. Sterile scalpel (Asp medical, catalog number: LDT455)
3. Pipettors 200 and 1,000 µl (ThermoFisher Scientific, catalog numbers: 4652140, 4652080)
4. Incubator at 37°C with 5% CO_2 (ThermoFisher Scientific, model: Heracell 150i)
5. Laminar flow hood (ThermoFisher Scientific, model: Msc-advantage)
6. Refrigerator 4°C (Mondial Elite, model: BEV PV 40 MED)
7. Centrifuge (Eppendorf, model: Minispin plus spin)
8. SPARK plate reader (NEOTEC BIO, TECAN)
9. Cytometer cell countess II (Invitrogen, model: AMQAX1000)
10. Heater (Talboys, microplate heater)

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Software

1. Microsoft Excel 2010

Procedure

A. Preparing the cells

1. Maintain the cells in cell medium (see Recipes) in 75 cm^2 T-flasks at 37°C under 5% CO_2.
2. Harvest the cells by washing the flask one time at RT with 5 ml of PBS at room temperature (RT), followed by incubation at 37°C with 2 ml of trypsin-EDTA for 5 min.
3. Transfer the cells from the flask to a 1.5 ml Eppendorf tube.
4. Centrifuge the cells at RT for 5 min at 1,200 × *g*.
5. Remove the supernatant with a 1 ml pipette.
6. Resuspend the pellet in 1 ml of cell medium at 37°C, by pipetting the solution up and down five times.
7. Take a sample of 50 µl from the cell solution, and mix 1:1 with trypan blue in a 1.5 ml Eppendorf tube.
8. Count the cells with a cytometer cell countess.
9. Dilute as necessary by adding cell medium to reach a concentration of 800,000 cells/ml (stock for calibration curve). A total of 2 ml is necessary.
10. If the cells to be quantified were cultured and grew in different media than the one described here, the exact same conditions should apply to cells for the calibration curve.

B. Preparing the calibration curve

1. Add 1 ml of fresh medium to wells 1A, 2A, 3A, 4A, and 5A of a 24-well plate.
2. Place 2 ml of 800,000 cells/ml (stock for calibration curve, see A9) in well 6A.
3. Take 1 ml from well 6A, and transfer it to the adjacent well (5A).
4. Mix by gently pipetting five times with a 1 ml pipette.
5. Take 1 ml from well 5A, and transfer it to the adjacent well (4A).
6. Mix by gently pipetting five times with a 1 ml pipette.
7. Take 1 ml from well 4A, and transfer it to the adjacent well (3A).
8. Mix by gently pipetting five times with a 1 ml pipette.
9. Take 1 ml from well 3A, and transfer it to the adjacent well (2A).
10. Mix by gently pipetting five times with a 1 ml pipette.
11. Take 1 ml from well 2A and dispose of it.
12. Well 1A contains only 1 ml of fresh medium for negative control.
13. Wells 2A-6A contain 50,000, 100,000, 200,000, 400,000, and 800,000 cells, respectively (Figure 1).
14. Use another 24-well plate if there are more than 18 samples. In that case, prepare another

calibration curve in the new plate as described above.

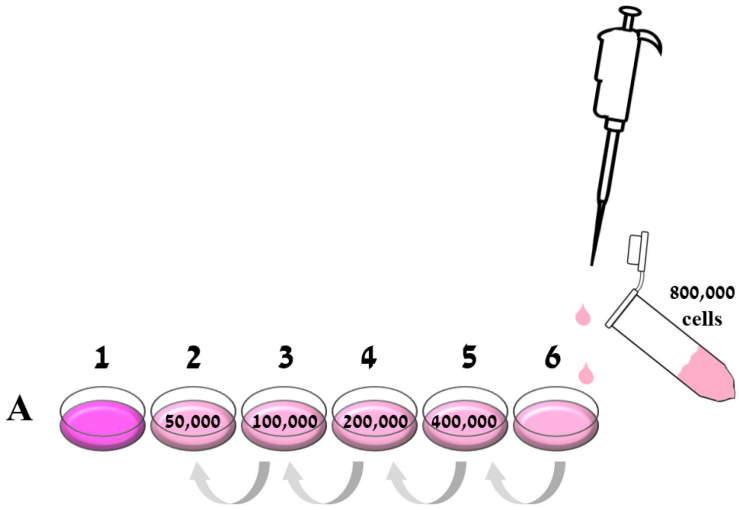

Figure 1. Preparation of the calibration curve.

The cell stock was brought to 800,000 cells/ml, and 2 ml of it were added to well 6A in a 24 well plate. In the same row, 1 ml of culture medium was placed in the other five wells (1A-5A). A serial dilution was performed by taking 1 ml of 800,000 cell/ml from well A6 to the adjacent well (5A), and serially across the entire row so that wells contained 800,000, 400,000, 200,000, 100,000, and 50,000 cells. The last well in the row (1A) was left with 1 ml fresh medium as a blank control.

C. Determining the number of cells on carrier

1. Add 1 ml of cell medium to all empty wells in rows B, C, and D in the plate.
2. Place one carrier in a well containing 1ml of fresh medium.
3. Add 100 µl of Alamar blue to all wells in the plate.
4. Cover the plate with aluminum foil (protect from light).
5. Incubate the plate for 1 h at 37°C.
6. Read the plate in the SPARK plate reader, with Ex 550 nm, Em 580 nm filters.

Data analysis

1. Transfer the data to an Excel file.
2. In the Excel file, write the number of cells that were used in the calibration curve (50,000-800,000) on column A, and include the blank (no cells, from well A1).
3. Insert the corresponding fluorescence intensity values in the adjacent column (B).
4. Subtract the value of the blank from all fluorescence values, and write the results in the adjacent column (C). see example below (Figure 2).

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	A	B	C
1	**Amount of cells**	**Fluorescence Intensity**	**Blank reduction**
2	blank (no cells)	14919	0
3	50000	16947	2028
4	100000	18342	3423
5	200000	21387	6468
6	400000	27562	12643
7	800000	41433	26514

Figure 2. Example of data layout for analysis.

The number of cells that were used for the calibration curve and their fluorescence intensity values are in columns A and B in an excel file. The fluorescence of the blank is subtracted from all values and written in column C.

5. Write the fluorescence results from all other wells (containing cell-carriers). If several carriers were taken from the same vessel/reactor, write all results in the same column.
6. Average the results from step 5 (if more than one carrier from the same experiment) and subtract the value of the blank.
7. Calculate the number of cells on each carrier using the excel FORECAST formula based on the calibration curve (Figure 3).

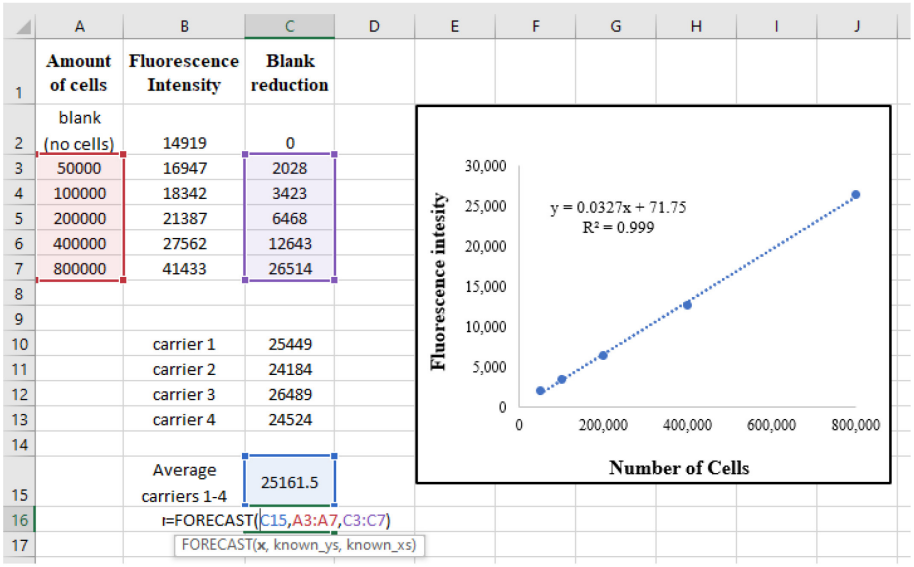

Figure 3. Example of data analysis.

The fluorescence results from all wells (containing carriers) are written and their average is calculated. The amount of cells on the carriers is calculated by the FORECAST formula based on the calibration curve.

Notes

1. Every type of cells and carriers may be used.
2. Every validated 24-well plate may be used.
3. Every type of plate reader with suitable filters may be used.
4. For each 24-well plate, use its own calibration curve.
5. Cells for the calibration curve must be fresh and may be counted in every validated cytometer.
6. The method can quantify cells in different media, but the calibration curve must be performed under the same conditions.

Recipes

1. Cell medium
   Take 500 ml of FLEX-20 medium
   Add 5 ml of 200 mM L-alanine L-glutamine
   Add 0.5 ml of pen-strep antibiotics
   Filter the solution with a filter bottle

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The protocol described here has been derived from the work of Rosen *et al.* (2021).

Competing interests

The authors declare not to have any competing interests

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