Novel method for quantifying cells on carriers and its demonstration during SARS-2 vaccine development

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Abstract

The most effective way to prevent and control infectious disease outbreak is through vaccines. The increasing use of vaccines has elevated the need to establish new manufacturing strategies. One of the major approaches is cell-based production, which creates a need for high cell density to enable higher cell production levels. This has led to development of the technology of cell carriers, including micro and macro cell carriers. To follow the production process, quantifying the number of cells on these carriers is required, as well as the tracking of their viability and proliferation. However, owing to various carriers' unique structures, tracking the cell's is challenging using current traditional assays that were originally developed for monolayers of adherent cells. The current "gold standard" method is counting cell nuclei, separating cells from the carrier, staining with crystal violet and visually counting under a microscope. This method is tedious and counts both live and dead cells. A few other techniques were developed but were specific to the carrier type and involved specialized equipment. In this study, we describe a broadly ranging method for counting cells on carriers that was developed and employed as part of the production of a vaccine for use in the SARS-CoV-2 pandemic. The method is based on the Alamar blue dye, a well-known, common marker for cell activity, and was found to be successful in tracking cell adsorption, cell growth and viability on carriers. 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 process control of viral vaccine production in cells. This method can be easily implemented in any of a number of cell-based processes and other unique platforms for measuring growth of encapsulated cells.

Keywords: Alamar blue, cells, macro-carriers, quantification, Fibra-Cel, vaccine

1. Introduction

Pandemic diseases, such as SARS-CoV-2, can sweep through human populations and potentially cause hundreds of millions of deaths. To date, vaccination is the most effective way to prevent and control infectious disease outbreaks. Research to discover new vaccines, or to develop and improve existing vaccines against viral diseases, is a worldwide high priority (Kiesslich & Kamen, 2020). Cell culture-based production of vaccines is gaining increasing attention owing to the need for new manufacturing strategies. The advantages of cell-based vaccines production include the independence from supply of, for example, chicken eggs along with minimization of cross contamination and allergenic components. In addition, use of defined and serum-free cell cultures allow greater consistency. Moreover, cell-based processes can be adapted to manufacturing process involving bioreactors that are scalable and need less space. Taken together, such advantages allow rapid and accurate manufacturing, especially during times of urgent need for vaccines (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 macro carries are small compact surface support matrices for growing adherent cells. Those carriers (from the μm to mm size range for micro and macro carriers, respectively) and their density allows their maintenance in suspension with gentle stirring. They can be made of any of a variety of materials, such as DEAE-dextran, glass, polyesters, PET, polypropylene, polystyrene plastic, acrylamide, collagen and others. The carriers are ideally used for high density cell culture in batch or perfusion modes which minimize time and resources. Fibra-Cel® is one example of a macro carrier used in vaccine production. It enables sustained periods of high-density cell growth in perfusion, has high surface-to-volume ratios, which greatly increases the yield of cellular products. The entrapped cells are shielded from turbulence and are less susceptible to shear forces from impeller blades and sparger gas bubbles. Fibra-Cels have been found suitable for many anchorage-dependent cell types, including Vero cells (Cino et al., 2011).

The Vero cell line was established from kidney cells of an African green monkey in 1962 (Y Yasumura, 1963). This cell line can be passaged indefinitely, allowing extensive cell characterization and creation of large cell banks. The cells are grown while adhering to surfaces, can be adapted to grow in serum-free conditions (Merten et al., 1996), and are

widely used in many research areas including virology, bacteriology and toxicology (Ammerman et al., 2008). The cells lost their ability to express interferon, *i.e.* the cells do not secret the signal peptide interferon so that, upon infection with viruses, this anti-viral defense mechanism is impaired (Emeny & Morgan, 1979). This feature makes them susceptible to many viruses. Vero was the first continuous cell line to be approved by the WHO for viral vaccine manufacturing for human use under regulatory guidelines. It is considered non-tumorigenic below a certain passage number and safe to use as a substrate for vaccines (Biologicals & Organization, 1987; Unit & WHO Expert Committee on Biological Standardization (1985 : Geneva, 1985). While other cell lines are frequently used for production of viral vaccines, such as HEK293, PER C6 and EB66, the long-standing experience with the Vero line together with its approval by regulatory authorities, continues to make it the first choice for many vaccine manufacturers (Barrett et al., 2009; Genzel, 2015). Several candidate vaccines were product in Vero cell; SARS (Liu et al., 2018), influenza (Merten et al., 1996) and polio virus (Paillet et al., 2009). Moreover, when compared with other cell lines, Vero has shown superior virus productivity for a range of viruses including Dengue virus, Enterovirus type 71, Japanese encephalitis virus and Measles virus (Grein et al., 2017; Lu et al., 2008; Silva et al., 2008).

Establishment of a viral vaccine platform using Vero cells is based on the recombinant strains of vesicular stomatitis virus (rVSV), where the native surface glycoprotein (VSV-G) has been replaced by the surface glycoprotein of another virus. For example, the approved Ebola virus vaccine is based on an rVSV which expresses the Zaire Ebola virus glycoprotein and is produced in Vero cells (Monath et al., 2019). With respect to the ongoing COVID-19 pandemic, some inactivated SARS-CoV-2 based vaccine candidates are produced in Vero cells and are currently in clinical trials (WHO, 2021). Recently, our institute has generated an efficient and cost-effective vaccine for SARS-CoV-2 that is based on rVSV, in which its native surface glycoprotein was replaced with the surface glycoprotein of SARS-CoV-2. Vaccination of hamsters with this rVSV resulted in rapid and potent induction of neutralizing antibodies against SARS-CoV-2. Single dose vaccination was able to protect hamsters against SARS-CoV-2 challenge, and immunized hamsters' lungs showed only minor pathology and no viral load (Yahalom-Ronen et al., 2020). Based on these promising results, human clinical trials now in progress.

The vaccine generated by our institute is produced in Vero cells grown on Fibra-Cel. To better follow and understand the production process, it is necessary to quantify the number of cells on these carriers, as well as to track their viability and proliferation. Furthermore, since the multiplicity of infection (MOI) is the ratio of rVSV to Vero cells, it is essential to know the number of cells at the time of infection. The gold standard method for quantification of cells on micro and macro carriers is nuclear staining. In this method, cells are separated from the carriers, stained with crystal violet and counted visually under a microscope. The method is tedious and counts both live and dead cells, making it unsuitable for tracking the cells' condition nor for MOI calculations. Furthermore, the accuracy of the nuclear staining method is questionable (Berry et al., 1996). As a result, a method that can better quantify cells on micro and macro carriers is desirable.

Counting cells on carriers poses a challenge to traditional assays, due to the carriers' dimensional shape. Widely used methods to measure cell quantity are known (Riss et al., 2013). The main feature of these assays is dyeing the cells with a fluorescent or colorimetric dye and quantifying the results based on fluorescence or absorbance. Examples of such dyes are GF-AFC, MTT, MTS, XTT, WST-1, resazurin (Alamar blue). These assays are relatively inexpensive, have a homogeneous format, but were all limited to single layer cultures (Berridge & Tan, 1993; Marshall et al., 1995; Mosmann, 1983). 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 macro carriers (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 describe and exemplified using Fibra-Cel® macro carriers. The assay utilizes Alamar blue, a well-known and common marker for cell activity, and includes calibration curve with known cells numbers. The method can quantify cells in situ on the carriers without requiring separation of cells from the carriers. This method was first optimized and then tested with a variety of cell growth processes, during our SARS-2 vaccine development. It was shown to successfully track cell adsorption, cell growth and viability. 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 platform for cell growth, such as hydrogels.

2. Material and methods

2.1 Materials

Vero cells were purchase from ATCC (ATCC©, CLL-81). NutriVero FLEX-20, L-alanine-L-glutamine, Pen-strep antibiotics, recombinant trypsin-EDTA solution, Trypan blue solution, Phosphate buffered saline (PBS), Crystal violet 0.1% were purchase from Biological Industries (05-069-1A, 03-022-1B, 03-031-1C, 03-079-1A, 03-102-1B, 02-020-1A, 01-986-1A respectively). Alamar blue was purchased from Promega (Cell titer blue, G808). Calcein AM was purchase from Sigma (C1359). Fibra-Cel disks were purchased from Eppendorf (New Brunswick Scientific, M1292-9984).

2.2 Cells

Vero cells were maintained at 37°C under 5% CO_2 in T-flasks (COSTAR) in FLEX 20 medium supplemented with 2 mM L-alanine-L-glutamine and 0.1% pen-strep antibiotics. Cells were harvested by washing the flask with PBS and incubating with trypsin-EDTA. The cells were centrifuged for 5 minutes at 1200 RPM. The supernatant was removed, and the pellet resuspended in fresh medium. A sample was taken, mixed 1:1 with trypan blue, and counted with cell countss^{tm} (Invitrogen).

2.3 Absorption of cells to Fibra-Cels in shake flasks

Fibra-Cels were weighed and 1.25 gr (~270 disks) were added to a flask containing 30 ml PBS and autoclaved in 121°C for 1 hour. After sterilization, PBS was replaced with growth medium (FLEX 20, 2mM L-alanine-L-glutamine, 0.1% Pen Strep) and incubated overnight in shaking incubator (37°C, 5% CO_2, 70% Humidity, 45 RPM). In the next day, $13.5x10^6$, $27x10^6$ and $54x10^6$ Vero cells (equivalent to 50,000, 100,000 and 200,000 cells/Fibra-Cel) were added to different flasks and allowed to adsorb.

To follow cells adsorption, samples of Fibra-Cels were taken at 30, 60, 90 and 180 min and quantified. At each time point, the percentage of adsorption was calculated by dividing the number of cells that quantified on the Fibra-Cel by the theoretical maximum absorption

values (50,000, 100,000 and 200,000 cells/Fibra-Cel). In parallel, at the same time points, Fibra-Cels were also quantified by nuclear staining (described below).

In addition to direct quantification of cells on Fibra-Cels by Alamar and nuclear staining, the following indirect quantification was also performed. At time zero, all cells were found in the medium. As time progressed, cells were absorbed to Fibra-Cels and disappeared from the medium. Therefore, at each time point, samples of medium were taken and the cells remaining in the medium was quantified by regular cell counting (described in 2.2, above). The percentage of absorption was calculated by subtracting the number of cells remaining in the medium from the number that were seeded ($13.5x10^6$, $27x10^6$ and $54x10^6$ cells) and dividing the results by the seeding amount.

2.4 Growing cells on Fibra-Cels in shake flasks

Fibra-Cels were treated as described in Sec. 2.3. Over the following days, $6.75x10^6$, $13.5x10^6$, $27x10^6$ and $54x10^6$ Vero cells (equivalent to 25,000, 50,000, 100,000 and 200,000 cells/Fibra-Cel) were added to different 125 ml flasks and allowed to adsorb. After adsorption, medium with Fibra-Cels were transferred to 500 ml shake flasks and medium was added to total of 300 ml. The flasks were returned to the incubator, and cells were grown for 6 days. Replacement of 70% medium with fresh medium was done once daily. The number of cells on Fibra-Cels were quantified after 1, 2, 3, 4, 5 and 6 days. In addition, cells on Fibra-Cels were stained with Calcein AM. 4 μM of Calcein was added to each well containing Fibra-Cel in a 24 well plate and incubated at 37°C for 10 minutes followed by fluorescence microscopic analysis.

2.5 Quantification of cells on Fibra-Cel

For calibration curves, cells were brought to 800,000 cells/ml and 2 ml were added to one well of a 24 well plate (COSTAR). 1 ml of fresh medium was placed in the other 5 wells in the same row. Then, 1 ml of 800,000 cell/ml was taken to the adjacent well, and serially diluted across the entire row so that wells contained 800,000, 400,000, 200,000, 100,000, and 50,000 cells/ml. The last well in the row was left with 1 ml fresh medium as a negative control. To assess the number of cells grown on Fibra-Cel, each Fibra-Cel was added, separately, to a well containing 1 ml media. All wells were supplemented with 100 μl Alamar blue. The plate was covered by aluminum foil to protect it from light and incubated for 1 hour

at 37°C without CO_2. After incubation, the plate was read in SPARK plate reader, TECAN, NEOTEC BIO, with Ex 550 nm, Em 580 nm filters. The number of cells grown on Fibra-Cel was calculated based on FORCAST of the calibration curve. All data were analyzed by Excel and Prism.

2.6 Nuclear staining

Five Fibra-Cels were taken from shake flasks using sterile tweezers and placed in a 50 ml sterile test tube (Greiner). One ml Acetic Acid 100% was added and the tube was vortexed at maximum speed for 1 min (to separate cells from the carriers). Then, 1 ml of 0.1% crystal violet was added (for a total volume 2 ml) and vortexed for 1 min at maximum speed followed by incubation at 37°C, 5% CO_2, 70% humidity for 1 hour (for staining the nuclei). After incubation, a short rapid vortex at maximum speed was performed and a 50 µl sample was taken from the test tube and diluted 1:4 with PBS.

Fifty µl of crystal violet diluted sample was inserted into a Hemocytometer. Stained nuclei were counted under a light microscope (Nikon). The surface of the hemocytometer is divided to 9 square fields, each divided to 4X4 squares. The number of nuclei in 4 fields was count, and the total number of cells in one Fibra-Cel was calculated using this equation: (Number of nuclei in 4 fields x 10^4 x 2)/5 (2 is the volume of the original test tube, 5 is the Number of Fibra-Cels).

3. Results and discussion

The basic concept underlying the new method is described in Fig. 1. In the upper row of a 24 well plate, 1 ml medium or 1 ml of 50,000-800,000 cells/well are seeded. These cells constitute a calibration curve. All other rows contained 1 ml of medium, and each well contained 1 carrier with an unknown number of cells. One hundred µl of Alamar was added to each well, the plate was covered by aluminum foil to protect from light and incubated for 1 hour at 37ºC. After incubation, the plate was read with Ex 550 nm, Em 580 nm. Quantification of each carrier was done individually based on the calibration curve, using the FORCAST Excel function.

3.1 Method optimization

3.1.1 Incubation time and calibration curve range

Various numbers of cells are tested and quantified. To allow quantification of as many quantities as possible, the dynamic range of the method should be tested. The calibration curve defines the limit of this range and, therefore different numbers of cells were initially tested. One ml of 25,000, 50,000, 100,000, 200,000, 400,000, 600,000, 800,000, and 1,000,000 cells/ml were seeded in triplicates in three plates, and 100 µl of Alamar blue was added. In addition, the incubation time of Alamar blue with the cells determines the appropriate time for this method, which ideally is as short as possible, but still able to determine of a wide range of cell numbers. Therefore, to determine the optimal incubation time, the incubation was allowed to continue for 90 minutes, and the fluorescence results were read every 30 min.

As shown in Fig. 2, seeding of 25,000 cells resulted in a very low signal, even with longer incubation times. In contrast, the signal from 1,000,000 cells seemed to fall outside the linear range. For those reasons, the calibration curve was set to be from 50,000 to 800,000 cells. In our vaccine development processes, at least 100,000 cells are seeded on each Fibra-Cel, and even if only 50% of the cells are adsorbed, the lower limit of the assay will suffice to quantify the number of cells. Cell growth on Fibra-Cel usually reaches up to 10^6 cells/Fibra-Cel at the end of the growth process. Hence, the upper limit of the method will be capable of covering and quantifying the entire process. For rare cases, when cell growth reaches >800,000

cells/Fibra-Cel, two slightly different versions of the method can be used (described in Sec. 3.2.1 and 3.2.2). As for duration, incubation for 60 min was found to be the optimal. A slight difference was found between 60 and 90 minutes, while 30 min, showed reduced signal. The duration of the method (less than 1.5 hours including cell seeding, incubation and fluorescence reading) is comparable to, or shorter than, other quantification assays.

3.1.2 Pre-incubation time of the calibration curve cells

The cells that provide the calibration curve are raised in a flask, and just before performing the method, they trypsinized, counted, and plated as described above. To ensure that no pre-incubation of the cells with the plate is needed, two different calibration curves were prepared. In the first, cells were seeded, in duplicate, in the upper rows of a 24 well plate and incubated for an hour at 37ºC before addition of Alamar. In the second, cells were seeded, in duplicate, in a different 24 well plate and Alamar was added immediately. As shown in Fig. 3. The two curves are within one SD of the other, and are thus considered statistically identical. There is no major difference between direct and prior incubation. Due to the desirability of a rapid method, from this point forward, no prior incubation was done.

3.2 Expanding the dynamic range of the method

3.2.1 Alamar blue "dosage"

To allow quantification of higher numbers of cells, the effect of double "dose" of Alamar (200 µl) was tested. Cells were trypsinized, counted and seeded with 100,000 to 1,600,000 cells and 200 µl of Alamar was added to each well. In parallel, the "standard" calibration curve with 50,000-800,000 cells and 100 µl Alamar was done. As can be seen in Fig. 4, slopes of both calibration curves were linear in the chosen cell number range. In addition, the slope for 200 µl Alamar was 0.0253 ± 0.0005 - twice the slope for 100 µl Alamar which was 0.0132 ± 0.0006. These results indicate that for higher cell numbers, a higher dose of Alamar can be used, and demonstrate that the dynamic range of the method can be expanded simply by adding more reagent.

3.2.2 Is the whole equal to the sum of its parts?

In this newly developed method, each Fibra-Cel is quantified individually, based on change in fluorescence, which occurs inside cells. The amount of cells on Fibra-Cel can be very high

(up to a few millions), if the cells are overly crowded, Alamar may not react with all the cells. To test this possibility, the following experiment was conducted: Cells were seeded, absorbed and grown for 5 days on Fibra-Cels in a shake flask. Several Fibra-Cels were taken, some were inserted individually into a well with 1 ml media as described above. Some were sliced to uneven pieces (2-3) and each piece was inserted into a well with 1 ml media. A calibration curve was done as above. Alamar blue was added to all wells, the plate was incubated for one hour and fluorescence was read. Representative results are shown in Fig. 5. As can clearly be seen, the sum of the Fibra-Cel pieces adds up to the whole. These results were very encouraging as they demonstrated an altered version of the method that would allow quantification of high cell numbers. It also shows that the method is robust and that Alamar can react with large numbers of cells, even if a Fibra-Cel is very crowded. As shown in Sec. 3.2.1, adding more Alamar can also be used for high cell numbers, as can shorter incubation times (data not shown). Together, these experiments demonstrate that scope of this method can be expanded and tailored for its specific purpose.

3.3 Application of the new method

3.3.1 Quantification of cells on Fibra-Cel with the presence of additives

To test whether the method is influenced by additives to the medium, we tested different concentrations of trypsin diluted in medium. The presence of trypsin was tested because it is used normally to harvest adhesive cells from flasks. First, a calibration curve of 50,000 to 800,000 cells diluted in either fresh medium (0% trypsin) or different concentrations of trypsin (40, 100%) was prepared. All other steps were conducted as above. The results show an influence of trypsin addition on fluorescence signals. Medium with 40% trypsin has a slightly different calibration curve, whereas 100% trypsin resulted in a totally different curve. The substantial influence of trypsin on the method, dictate the use of same medium and additives for calibration curve as for the sample tested. To demonstrate the ability to quantify cells on Fibra-Cel using different media and additives, cells were grown on Fibra-Cels as described, and several Fibra-Cels were taken for quantification. Each set of Fibra-Cel were placed into wells inside 3 different 24 well plates. In each plate, cells used for calibration were diluted in 0/40/100% trypsin and 1 ml of this 0/40/100% trypsin in medium was added to the wells containing Fibra-Cel. Although different fluorescence signals were measured in each plate, the relative florescence was the same, and quantification resulted in the same

numbers (data not shown). Hence, the method can quantify cells in different media, but calibration curves must be performed under the same conditions.

3.3.2 Quantification of cells on Fibra-Cels in the presence of 70% alcohol

Our vaccine development process required that the number of cells found on Fibra-Cels be quantified after production of the VSV-based vaccine. However, because live viruses should not be inserted into the fluorescence device, in which the fluorescence signal is read, a 70% alcohol can be used to inactivate the virus. However, 70% alcohol can influence cells' metabolic parameters, and therefore should not be used until after the Alamar reaction is performed. Hence, 70% alcohol can be added only after incubation with Alamar. Two 24 well plates were used. In both, a regular 50,000-800,000 cells calibration curve was seeded and Alamar was added. After completion of incubation, one plate was read as usual. 300µl of the well contents of the second plate were transferred to an equivalent plate (fluorescence was read) and 700µl of 100% alcohol was added (resulting in 1ml of 70% alcohol). The plate was mixed and fluorescence was read. As can be expected, the fluorescence of the wells containing 300µl decreased (to ~20% of the source fluorescence). Surprisingly, addition of 700µl alcohol restored the fluorescence to values ~90% of the source. The slope of the regular calibration curve was comparable to the slope of the curve with 70% alcohol (Fig. 7). Fibra-Cels with the same number of cells were quantified; some were put in 1 ml media and were quantified with the “standard” method based on the regular curve. Others were put in 70% alcohol in medium and were quantified based on the 70% alcohol calibration curve. Quantification was the same (data not shown). These results enable expanded use of this method to quantify cells on Fibra-Cel from many sources.

3.3.3 Observation of cell adsorption to Fibra-Cels in shake flasks

In an attempt to challenge the newly developed method in another way, adsorption of cells to Fibra-Cel was examined. Increasing amounts of cells, $13.5x10^6$, $27x10^6$ and $54x10^6$, were added to different shake flasks and were allowed to adsorb to the Fibra-Cels for 180 min. To follow adsorption, a sample of ten Fibra-Cels was taken at times 30, 60, 90 and 180 min. For each time point, the number of cells on the carrier was determined by the method and the percentage of adsorption over fluorescence was read time was calculated (Fig. 8). Adsorption

percentage was similar irrespective of the initial number of cells. All reached ~60% adsorption after 60 min. and an average of ~80% after 3 hours.

Cells adsorption was calculated in parallel by two other methods: 1. Nuclear staining and 2. Cells counts in the medium. For each of these methods, adsorption percentages were calculated and compared to those obtained using our new method. Comparable results were obtained by all methods, indicating the broad applicability of the new method. Moreover, as opposed to other methods, the new method quantifies the number of cells in each Fibra-Cel and allows exploration of cells's scattering on different Fibra-Cels within shake flasks.

The method was further tested in numerous cell adsorption experiments and the results were repeatable and comparable to other methods. The pattern of absorption was similar with around 60% absorption after one hour.

3.3.4 Observation of cell growth on Fibra-Cels in shake flasks

Cell growth on Fibra-Cels was also studied with the present method. $6.75x10^6$, $13.5x10^6$, $27x10^6$ and $54x10^6$ cells (equivalent to 25,000, 50,000, 100,000 and 200,000 cells/Fibra-Cel) were added to shake flasks and allowed to adsorb. After adsorption of cells to Fibra-Cels the flasks were incubated in 37ºC and 5% CO_2 for several days to allow cell growth. A sample of ten Fibra-Cels was taken every 24 hours and examined with the method and in parallel with nuclear staining (5 Fibra-Cels in each method). As can be seen in Fig. 9a, after a lag time of ~24 hours, all cells grew and duplicated themselves in 24 hours. These results were compared to results obtained by nuclear staining. In addition, these results correlate with the cells' known growth rate. Cells on Fibra-Cels were also characterized by Calcein staining (Fig. 9b). The number of cells on each Fibra-Cel grew over time. Altogether, the result demonstrates the applicability of the method as a way to follow cell growth.

3.4 Universality of the method

The newly developed method is rapid and easy to perform, the Alamar is available commercially, and its use was demonstrated in several applications. To enable universal applicability, the method was tested in a different lab in our institute. All reagents (cells, Alamar, media, flasks, plates) and equipment (fluorescence reader) were different and in this other lab. Two groups of ten Fibra-Cels with known numbers of cells ($8.5x10^5$ and $1.1x10^6$

cells/Fibra-Cel) were taken and divided equally into two. Quantification of each group was performed simultaneously in the two labs. The results obtained by our lab were $8.1(\pm0.8)x10^5$ and $1.2(\pm0.1)x10^6$ cells/Fibra-Cel while the results obtained by the other lab were $8.9(\pm0.9)x10^5$ and $1.1(\pm0.1)x10^6$. These results demonstrate the applicability of this newly developed method by any appropriately outfitted lab.

4. Conclusion

In this study, we described an innovative, simple and rapid method to quantify the number of cells on carriers. The method was developed in response to a challenge faced during development of our VSV-based vaccine for use in the SARS-CoV-2 pandemic. The vaccine is produced in Vero cells grown on Fibra-Cel, one of the common macro-carries used in the vaccine industry, in order to increase their biomass (One carrier can accommodate one million cells and even more). However, traditional cell quantification assays are either tedious or are unsuitable for quantifying cells on carriers. The method based on the use of Alamar, which is a non-toxic dye that does not damage cell attachment to carriers, is readily available and whose use is uncomplicated, is thus superior to traditional assays. The dimensional shape of the carriers, which poses a challenge to traditional assays, has no impact in this new method. The method was capable of tracking cell adsorption and growth. Besides the ease of performance, the method is rapid, does not require specialized reagents or equipment, and yields results which correlate well with the “gold standard” assay. Additionally, the method can be easily adapted for any cell-based process or other unique platform for cell growth, such as hydrogels (Cohen et al., 2018).

Acknowledgements

We would like to thank Dr. Sandy Livnat for editorial assistance.

Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Fig. 1. Basic concept of the new method for quantification of cells on carriers. 1 ml with different numbers of cells were seeded in the upper row of a 24 well plate. All other wells contained 1 ml media with 1 Fibra-Cel with an unknown number of cells. After 1 hour of incubation with Alamar, fluorescence was read. The number of cells was quantified based on the calibration curve.

Fig. 2. Duration of incubation and dynamic range of the method. A 24 well plate was seeded in triplicate with 25,000, 50,000, 100,000, 200,000, 400,000, 600,000, 800,000 and 1,000,000 cells per well. The reaction of cells with Alamar blue was allowed to continue for 30, 60 or 90 minutes. Analysis of 3 plates with those triplicates is shown.

Fig. 3. Direct vs. prior incubation of cells for calibration curves. Cells were trypsinized, counted, and seeded, in duplicate, on the upper rows of a two 24 well plates. The first plate was incubated for 1hour at 37ºC before addition of Alamar. Alamar was added immediately to the second plate. After 1 hour of incubation with Alamar, the plates were read and calibration curves were plotted.

Fig. 4. Comparison between calibration curves with 100 µl vs. 200 µl Alamar. Different numbers of cells were added with either 100 µl or 200 µl Alamar and incubated for 1 hour at 37ºC. Fluorescence was read and calibration curves were plotted.

Fig. 5. Whole vs. sliced Fibra-Cel quantification. Several Fibra-Cel, originating from the same experiment, were placed in a 24 well plate either as a whole Fibra-Cel or as pieces of a sliced Fibra-Cel. Each option was quantified by Alamar as compared to a calibration curve.

Fig. 6. Calibration curves in different trypsin concentrations. 50,000-800,000 Cells were diluted in media that contain either 0, 40 or 100% trypsin. Alamar was added and fluorescence was read after 1-hour incubation. Different slopes are seen for the diverse trypsin concentrations.

Fig. 7. Regular calibration curve vs. a calibration curve with 70% alcohol. Two plates with 50,000-800,000 cells per well were diluted in media, Alamar was added followed by 1-hour incubation. The fluorescence of one curve was read. 300µl of the other plate was transfer to yet a different plate to which wells 700µl of pure alcohol was added, and the plate mixed and read.

Fig. 8. Adsorption of cells to Fibra-Cels over time. Several shake flasks with Fibra-Cels were seeded with different numbers of cells and adsorption on Fibra-Cels was monitored for 180 min. The percentages of cells adsorbed of the cells seeded was calculated to each time point. Mean and SDs are shown.

Fig. 9. Cells growth on Fibra-Cels over time. Several shake flasks with Fibra-Cels were seeded with different numbers of cells which were allowed to grow for 6 days. At each time point (a) the amount of cells on Fibra-Cel was quantify and (b) cells on Fibra-Cel were stained with Calcein.