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A Simple Method of Bead Counting for Next Generation DNA Sequencing

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Abstract.- Current methods of counting micron size beads (microshperes) for next generation DNA sequencing procedures either require use of expensive equipment and/or employ manual counting that may lead to variation of results among different users. This work presents the use of micro-capillary as a mean for efficiently counting beads used in different procedures where their number is important. The method employs taking the Known number of beads in micro-capillary through capillary action and packing them with the help of centrifugal force to make reference scale. Same procedure is used for unknown sample and compared to the reference scale. This counter provides bead count with comparable accuracy, ease, and is time and cost efficient. Moreover, the beads used for counting can be reused which is unlikely in other methods.

Keywords: Micro-capillary, bead counting, emulsion PCR, next generation DNA sequencing

INTRODUCTION

Current high throughput technologies of DNA sequencing and several other applications require small colonies of target DNA molecules evenly spaced on a solid surface. The most widely employed method to achieve this is to use micron size beads through emulsion PCR (emPCR) to coat them with multiple copies of DNA (Shendure and Ji, 2008). These micron size beads need to be counted at different steps in the process. The number of beads used for emPCR affects the amount, process and quality of sequencing data (Sandberg *et al*., 2009). There are different principles available for counting micron sized beads like Coulter principle, flow cytometery, spectrophotometery, image-based counting and hemocytometerey.

Coulter principle requires an expensive instrument with liquid handling, cleaning hardware and routine maintenance. During this process clogging is the major problem that requires de-clogging at different times. The aperture of the Coulter counter is extremely sensitive to invisible air bubbles, lint,debris and anything else that might clog it. Even bead suspensions made in pre-ultra-filtered buffer can clog the aperture. De-clogging is a procedure to remove debris from the aperture. (http://www.beckmancoulter.com/). Flow cytometery is an expensive procedure due to the cost of the instrument that requires a lot of maintenance. Flow cytometers are mainly used for cell counting, cell sorting, biomarker detection, diagnosis of health disorders and protein engineering research etc (Marti *et al*., 2001). Therefore, it is not effectual to purchase an expensive instrument for the sole purpose of bead counting. Spectrophotometric methods are used for small size beads (SOLiD sequencing beads ~1µm in size). These methods are not generally used for the beads larger than 1um (www.lifetechnologies.com/). The instrument may require from more (several hundred microliters using cuvettes) to less volume (a few microliters using NanoDrop). Spectrophotometery is the only method in bead counting that does not count beads directly rather it measures intensity of light after it passes through bead suspension. Therefore, spectrophotometeric measurements may have variable accuracy. Counting chambers (*e.g.*, Neubauer Chamber or Hemacytometers etc.) are specially designed microscopic slides to manually count the beads under microscope with complex calculations

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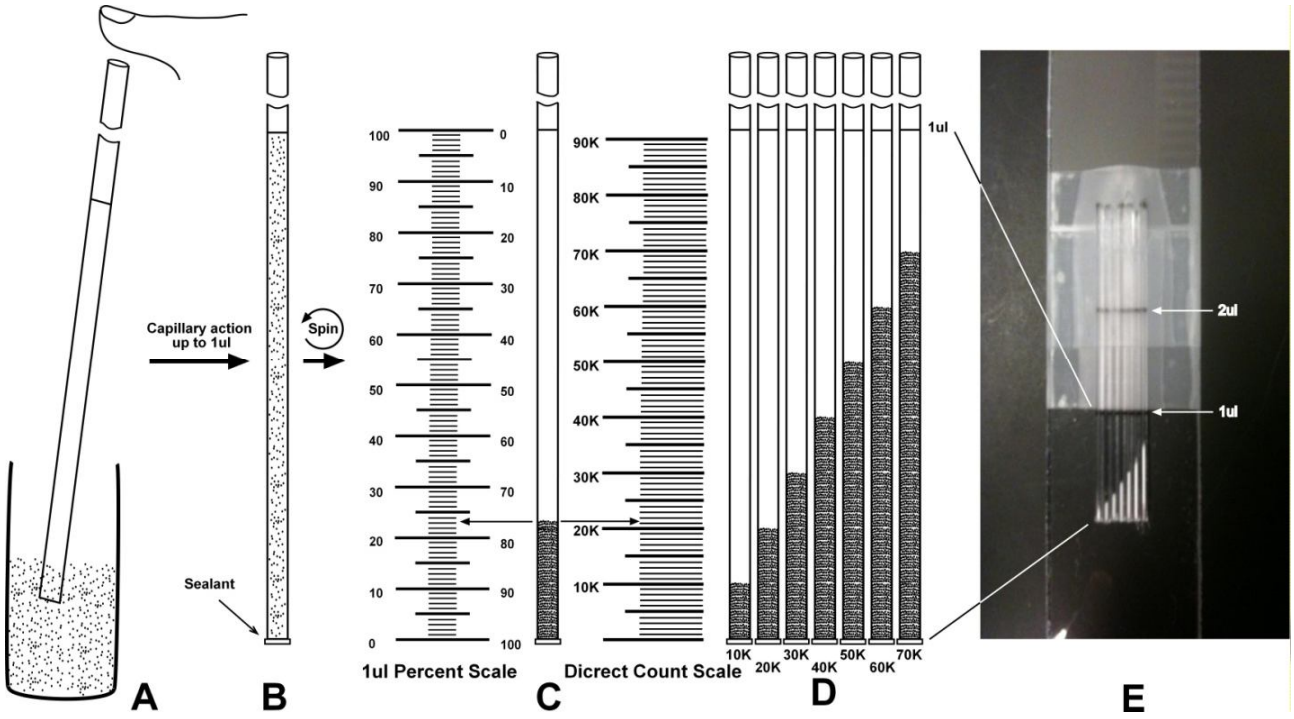

Fig. 1. Micro-capillary based bead counter and steps in making a reference scales (A-E) and counting the beads (A-C). (A) Aspiration of bead suspension through capillary action. (B) Sealing the bottom of micro-capillary. (C) Centrifugation to settle the beads down and reading from percentage scale or direct count scale deduced from (D) and (E). (D) Micro-capillaries with different bead concentrations after steps (A), (B) and (C) arranged from lower to higher concentration in order to make a reference scale. (E) Actual micro-capillaries arranged on transparent plastic slide from where formation of scales is explained in (C) in accordance with (D) and exemplary micro-capillary in (C) indicates 21,000 beads (see right arrow) being the 23% of 1µl volume (see left arrow) aspirated through capillary action.

(http://celeromics.com/en/resources/docs/Articles/Cell-counting-Neubauer-chamber.pdf). The major disadvantage of this method is to optimize suitable dilution of the beads. If the concentration of the beads is too high, then the cells will overlap on the counting chamber and are difficult to count. Therefore, suitable dilution of the bead suspension is required at this stage (Stone *et al*., 2009). Every dilution adds inaccuracy to the measurement. Manual counting under the microscope causes visual fatigue and may lead to variability among different users. Image-based counter uses bright-field microscope coupled with digital camera to capture images which are then analyzed with image analysis software. In this way, image-based counters provide automated way of bead counting. They save user from visual stress, however, it still needs sufficient dilution factor as well as costly instrument and especially designed counting chambers/slides that adds per count cost (Stone *et al*., 2009).

This communication presents a simple and efficient method for bead counting for next generation DNA sequencing as well as other applications. This method involves the uptake of bead suspension by capillary action into glass micro-capillary up to a certain mark, sealing its bottom, packing/centrifuging the beads and reading the number of beads from graduated marks on micro-capillary or a reference scale.

MATERIALS AND METHODS

A suitable glass micro-capillary with or without known internal diameter can be used for beads of known or unknown size. To prepare a reference scale, as an example, micro-capillaries of

No.	Method	Mean ± SD	CV, %
1	Micro-Capillary	1075000 ± 79057	7.35
2	Beckman Z2	1134800 ± 86053	7.58
3	BioRad TC-10	1050000 ± 50990	4.86

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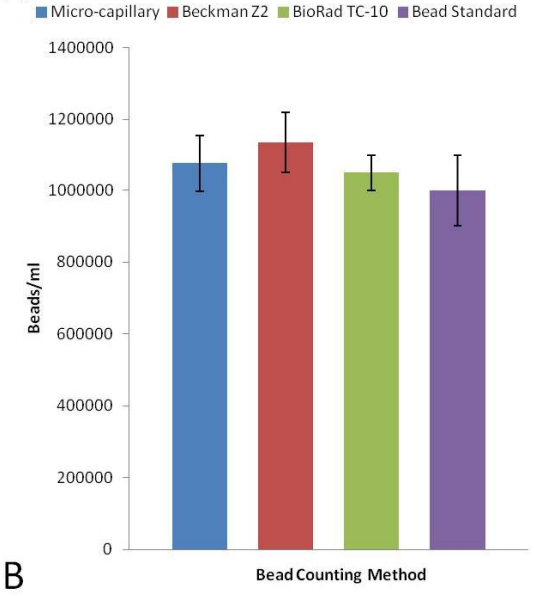

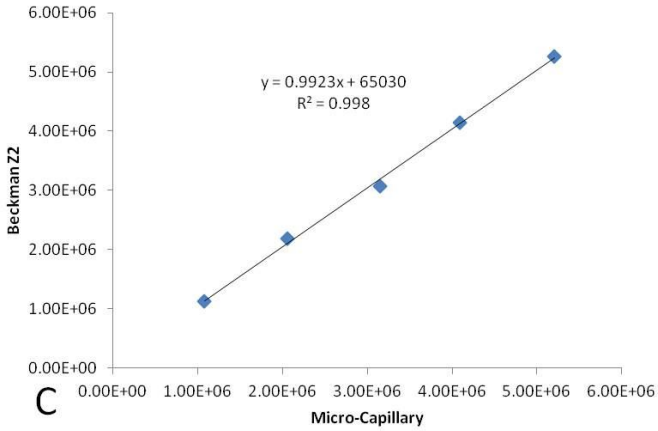

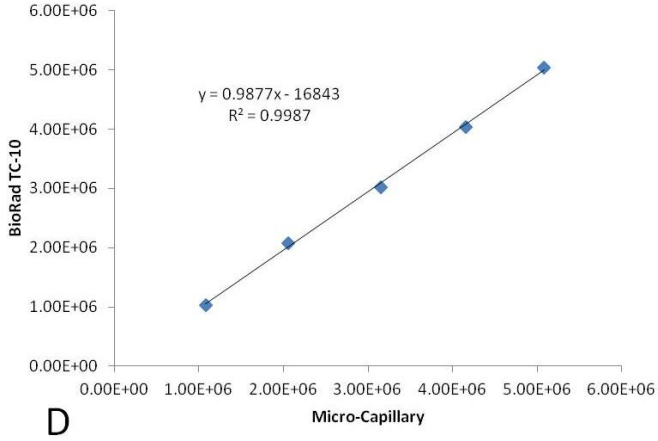

Fig. 2. An illustration of the relationship between bead counts performed with different methods. (A) Bead concentration statistics of same sample with five replicates obtained with different counting methods. (B) Accuracy and precision of the Micro-capillary counter compared to Beckman Z2 and BioRad TC-10 counting methods. A standard bead solution of 1.0 x 10^6 beads/ml was prepared from the known standard (2.4 x 10^6 beads/1170µl) as the starting material for counting measurements. Accuracy was evaluated by comparing the mean beads/ml measured by each method to the expected concentration of the standardized bead solution (last bar: 1.0 x 10^6 ± 10% beads/ml). Precision is indicated by the observed standard deviations as the error bars. (C) Correlation between samples with different bead concentrations measured using Micro-capillary and Beckman Z2 counters. (D) Correlation between samples with different bead concentrations measured using Micro-capillary and BioRad TC-10 counters.

known internal diameter of approximately 200µm (Sigma-Aldrich Corporation, St. Louis, MO, USA) and Sepharose DNA capture beads of approximately 20 µm diameter at the concentration of 2,400,000 beads per 1170 µl (Roche/454 Sequencing, Indianapolis, Indiana, USA) were used. Micro-capillary usually comes with graduation marks for every microlitre that assist the observation of capillary action up to a certain volume. The micro-vial containing Sepharose beads of known concentration, was shaken well to form a uniform suspension of beads. Different dilutions of the beads were made with Tris-EDTA buffer to the final concentrations of 10k (k = 1,000), 20K, 30k, 40k, 50k, 60k and 70k beads/µl (total volume of each dilution = 50µl).

First dilution of 10k beads/µl (total volume = 50µl) was mixed well on a vortex mixer to get

uniform distribution of bead suspension. One end of the capillary was dipped into the bead suspension near its surface. The suspension was allowed to rise into micro-capillary through capillary action up to the 1µl mark (Fig. 1A). Further rising of suspension into capillary was stopped by blocking the other end of the micro-capillary with the thumb or index finger. Micro-capillary was then sealed from the bottom with the help of very small circular piece of adhesive tape or sealing clay (Fig. 1B) and centrifuged for 3-5 seconds (Fig. 1C). The upper boundary of packed beads was taken as the measurement for 10k beads. Five of the total replicates were taken into measurement for each dilution. Average was taken for all five replicates for a single major scale reading. The same was done for all the rest of the dilutions (20k, 30k, 40k, 50k, 60k and 70k beads/µl). All these measurements will serve as the reference scale for later sample counts (Fig. 1D, E). Two scales were setup including direct bead count and percentage scale. Unknown sample of beads can now be performed as described above and compared to the reference scale (Fig. 1A-C). Count can be made on "direct count scale" as well as "percentage scale" which describes the ratio between the total volume and the packed beads volume (Fig. 1C).

RESULTS AND DISCUSSION

After preparation of reference scale, several counts were made to check the performance of the method. In order to observe the accuracy, the counts were also made with Coulter counter (Beckman Z2) as well as image analysis based counter (BioRad TC-10) with the same bead suspensions of unknown bead number used for micro-capillary bead counter. Accuracy and precision were found comparable between the Micro-capillary based counter and Coulter counter and BioRad TC-10 counter (Fig. 2). Emulsion PCR were performed using bead counts from Micro-capillary and results were comparable to the other method of counting (data not shown).

In comparison to other methods, no sophisticated instrument, liquid handling or maintenance is required and low cost of single count is an added advantage of micro-capillary based bead counting device. When bead sample is taken for measurement, the accuracy of bead counting depends on homogeneity of suspension. The higher bead concentration may hinder the proper capillary action at some point, so this should be performed right after thorough vortex mixing procedure. For simplicity, the procedure and results has been described with 1ul volume of test bead suspension aspirated into micro-capillary. However, it has been observed that aspirating more volume of test bead suspension in the micro-capillary (up to 2 or 3 µl) and calculating the concentration accordingly even lead to more accurate counts. Previously, CD4 cell counter for HIV/AIDS testing (from Zyomyx Inc., Freemont, CA) and Bullseye cell counting kit (from MidSci^{TM}) for bacterial cultures uses the similar approach.

The simplicity of the method allows the bead counter to be prepared in the lab in a short time and then can be efficiently used for unknown samples of the same beads. In case of precious bead sample or when the beads are already in fewer amounts, they can be retrieved after counting through micro-capillary. This retrieval seems unlikely in other methods including Coulter counter, image based counters, flow cytometry and counting chambers.

CONCLUSION

In conclusion, a micro-capillary based bead counting principles an affordable and robust method that is comparable with other expensive procedures mentioned above. The method is self explanatory and provides the user with efficient bead counting at very low cost. Furthermore, the beads can be reused after the count if required.

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Conflict of interest statement

N.A.

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