Application Note

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High throughput generation of bacterial isolate libraries using direct liquid cultivation with cellenONE

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Introduction

The proficiency of the cellenONE® single cell isolation instrument for high-throughput generation of isolate libraries was demonstrated by isolating single cells of *Escherichia coli* directly in liquid broth. The cellenONE system relies on the combination of cell detection by microscopy imaging and cutting-edge image analysis, and cell dispensing by high precision microdroplet generation. Using cellenONE and its bacteria-dedicated software microLIFE, monoclonality can be checked on recorded images and reaches 91% and 100% in bright-field and fluorescence isolation mode, respectively, which has no equivalent on the market. We demonstrate here an outstanding post-cellenONE cultivability rate, with up to 85% of single bacteria engendering cultures.

Now that microbiologists (almost) sequenced the world, there is a need for mechanistic insights into interactions between microbiota and their environment, that only culture-based approaches

can bring, hence the need to increase throughput in generation of pure microbial isolates (1). Furthermore, there is growing interest and activity worldwide in the establishment of biofoundries, core facilities dedicated to the development and production of bioengineered products and (micro)organisms (2). Advances in synthetic biology research and applications in such core facilities similarly require increased throughput for the generation of clone libraries. The cellenONE has the capability of generating 1000 monoclonal and growing isolates in less than two hours, including instrument and sample preparation time. It also skips the colony formation step on solid medium needed by classical Petri dish-based methods, which are tedious and time-consuming (Fig. 1). It therefore greatly accelerates microbial cultivation workflows and opens unprecedented scaling-up possibilities for high-throughput cultivation of microbes, from clone library construction in bioengineering platforms to culturomics applications.

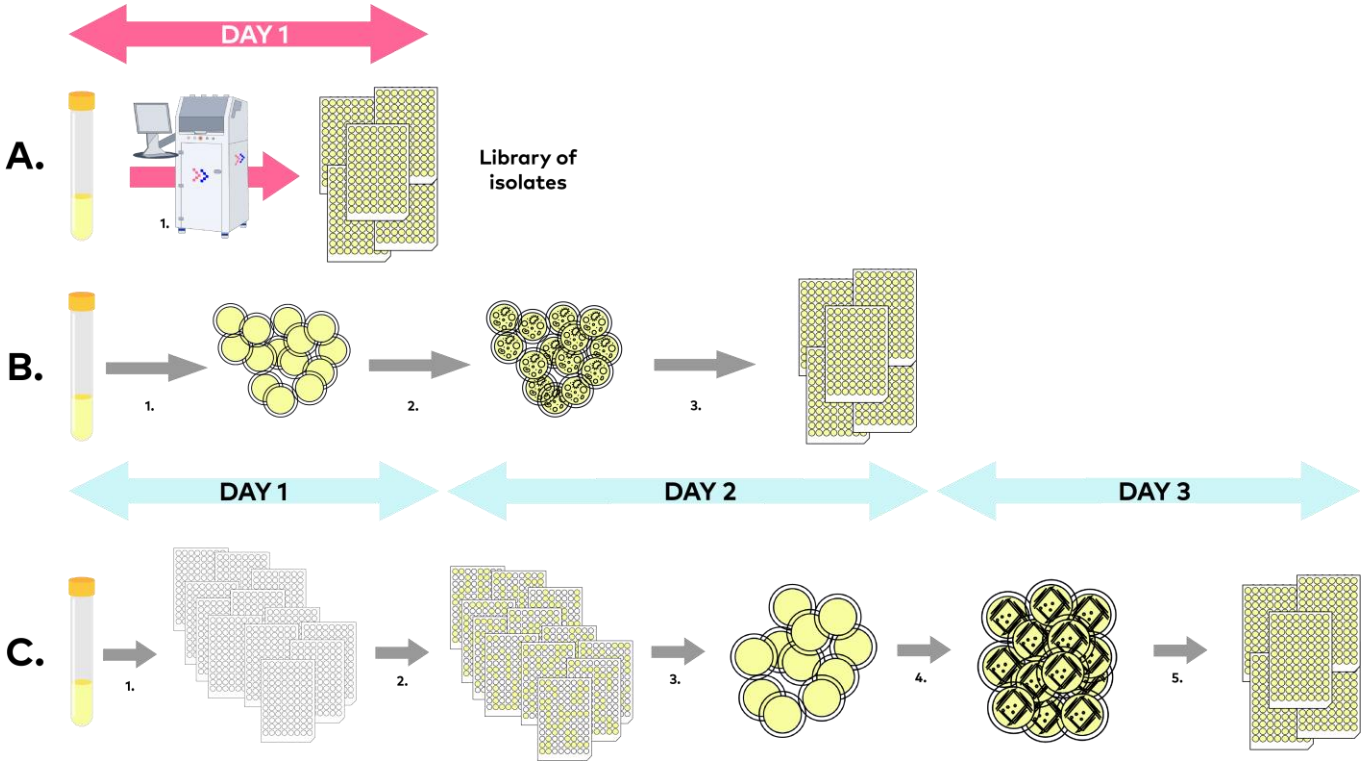

Figure 1. Workflows for the generation of libraries of isolates or 100's clones from a sample, and their duration*. A. cellenONE: 1. Sample preparation and cellenONE isolation. B. Direct plating: 1. Sample dilution and plating, 2. Plate incubation, 3. Colony picking. C. Dilution to extinction: 1. Sample preparation and multi-dilution inoculation of liquid medium, 2. Culture incubation, 3. Purification by plate streaking, 4. Plate incubation, 5. Colony picking. Note that duration increases greatly with throughput and with incubation time for Petri dish-based methods (B and C) (see Fig. 2).*

Materials and methods

Bacterial culture and sample preparation

An *E. coli* strain derived from *E. coli* DH5α (Thermo Fisher), a versatile strain commonly used in biotechnology processes for cloning and subcloning, was used as model. On the day before the experiments, low-temp freezer glycerol stocks were used to inoculate LB medium (Miller formulation, Dutscher) precultures, which were grown overnight. Fresh subcultures were inoculated on the day of the experiment and incubated for 2 hours to reach exponential growth. A volume equivalent to 10^7 cells was pelleted by centrifugation (9000 rcf, 3 min) and bacterial cell pellets were washed with sterile saline buffer (PBS, Gibco), then resuspended and serial diluted to 10^5 cells/mL PBS.

cellenONE isolation

All bacterial cloning experiments were performed in bright-field isolation mode in a cellenONE® X1 BSC (Cellenion, France), enabling work in sterile conditions, running microLIFE software (Cellenion, France), which implements a detection algorithm dedicated to microbial cells. A diluted cell suspension was aspirated, and cellenONE runs were automated so that each well of 96-well microtiter plates, containing 20 or 60 µL of liquid medium, was inoculated with a single bacterium. Several studies showed that using conditioned medium, i.e., medium in which cells were previously grown, had a beneficial effect on cell clonal outgrowth after isolation (3, 4). In line, we assessed the hypothesis that metabolites and signal molecules excreted by bacteria during full exponential phase would function as growth factors and accelerate single bacterium outgrowth. We evaluated clonal outgrowth in LB supplemented or not with 30% of conditioned LB medium, obtained by filtrating exponential phase culture. The experiment was repeated 6 times, on 6 different days. After cell isolation experiments, all cell images taken during cellenONE runs were analyzed using microLIFE Viewer.

Clonal outgrowth monitoring

To be able to detect bacterial growth in small volume cultures from its most early stages, 0.5 µL culture from each well of the cellenONE-inoculated microplates were deposited on LB agar medium in LPS tray plates (Dutscher) at regular time intervals. Agar plates were then incubated until colony formation to evaluate cultivability in the liquid cultures. This read-out method requires at least one colony forming unit in 0.5 µL in the liquid cultures, i.e., 30 cells per 60-µL well, to detect growth on agar plates. The detection threshold of this method was much lower than optical density-based methods (5) as only 5 replication cycles were required to measure clonal outgrowth.

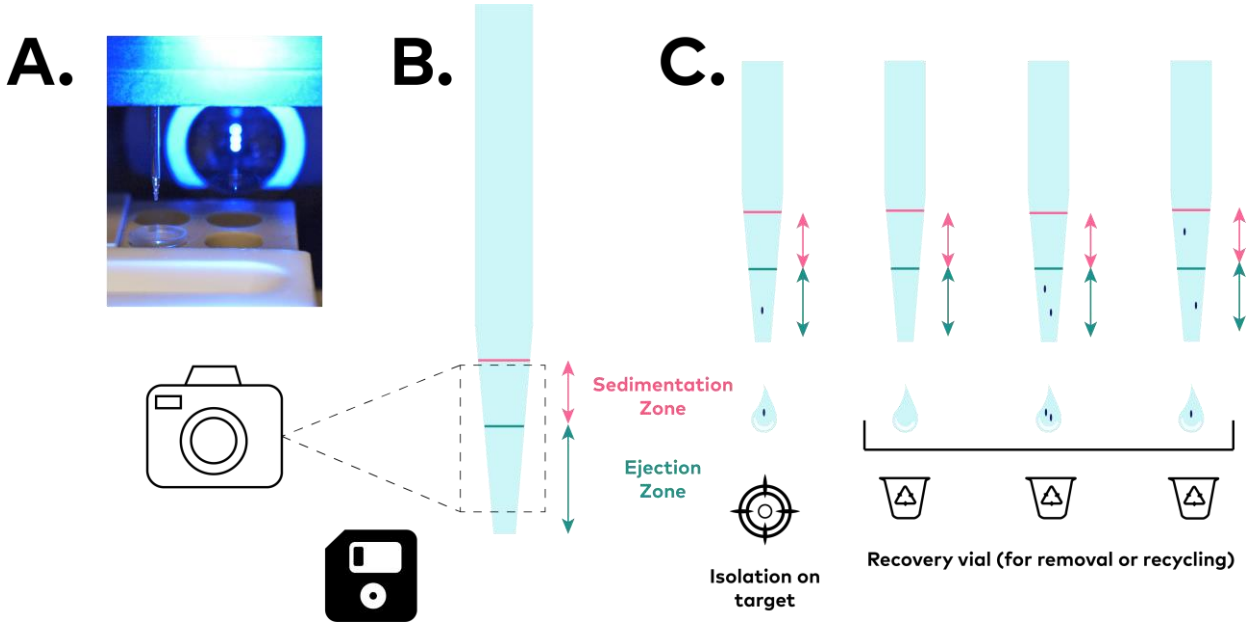

Figure 2. *Principle of cellenONE technology. A. cellenONE piezo dispense capillary (PDC) in front of microscopy camera. B. cellenONE Mapping. C. cellenONE decision scheme in cell isolation mode.*

Results and discussion

cellenONE isolation accuracy

For single cell isolation, cellenONE first aspirates the bacterial cell suspension through a glass capillary (piezo dispense capillary, or PDC, Fig. 2A). The PDC generates 200-800 pL droplets (with less than 0.2% CV) thanks to gentle piezo-acoustic waves. Image analysis of the tip of the PDC allows the system to automate a Mapping (Fig. 2B), delimiting the ejection zone, corresponding to the volume of liquid that is expulsed in each droplet, as well as a safety zone above (sedimentation zone). While generating droplets, the system evaluates the presence of zero, one or more cell(s) in both zones, and triggers the ejection of the droplet in any user-defined target if, and only if, it detects one cell in the ejection zone and no cell in the sedimentation zone (Fig. 2C). The software records images of isolated cells for post-processing image analysis. In the present experiment, monoclonality rates (i.e., the proportion of wells which received one and only one cell), were 88% on average, representing 1,522 wells out of 1,728. Thanks to image analysis, we could remove from further analysis the wells where no cell or more than one cell were dispensed. Clone or isolate libraries are typically obtained using either direct plating (Fig. 1B) or dilution-to-extinction based methods (Fig. 1C). When generating cultures from natural environments where microbes grow naturally in mixed communities, cultures obtained by either of these methods may contain several distinct populations (coculture of different strains, species, genera, or even higher phylogenetic levels). Streaking steps or multiple plating and colony picking cycles are typically required to guarantee the monoclonality of isolates (6, 7, 8). The isolation accuracy level of cellenONE and microLIFE software, combined with the opportunity to check monoclonality of each well directly on images, has no equivalent on the market for bacteria.

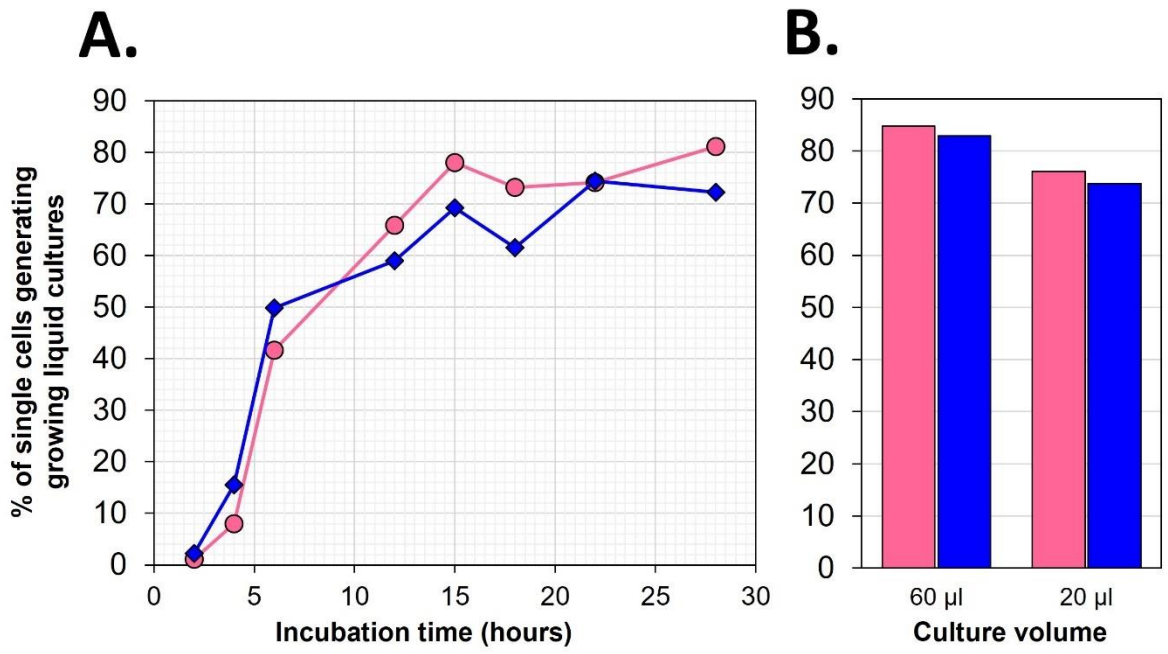

***Figure 3. Outgrowth of single cell-inoculated liquid cultures**. Typical clonal outgrowth dynamics (A) and maximum cultivability rates (B) following inoculation with single cells in LB supplemented (blue) or not (pink) with 30% conditioned medium.*

Cultivability rates

Cultivability rates, i.e., the proportion of wells in which growth was detected, were calculated on wells where monoclonality was qualified by cellenONE run images, i.e., which were inoculated by one, and only one, cell. Along liquid culture incubation, cultivability rate overall followed typical microbial growth dynamics (Fig. 3A). At early stages of incubation, the addition of conditioned medium had a positive effect on cultivability: growth was detectable in more wells after 4 h with 30% conditioned medium (15%) than without (9%). This suggests an induction of *E. coli* cell replication after isolation, and a reduction of the lag phase, by the addition of conditioned medium. Between 6 and 15 hours after inoculation, growth became progressively detectable in more and more wells, in all treatments. This is the reflection of the intra-populational lack of synchronicity of cellular states in cultures (9): when placed in fresh medium, the duration of lag phase is not equal for all single cells. Cultivability rate in 60 µL cultures reached 85% in non-supplemented LB and, 83% in LB supplemented with 30% conditioned medium (Fig. 3B). The observed cultivability following single bacteria isolation was outstanding compared with other single cell isolation techniques (11, 12, 13).

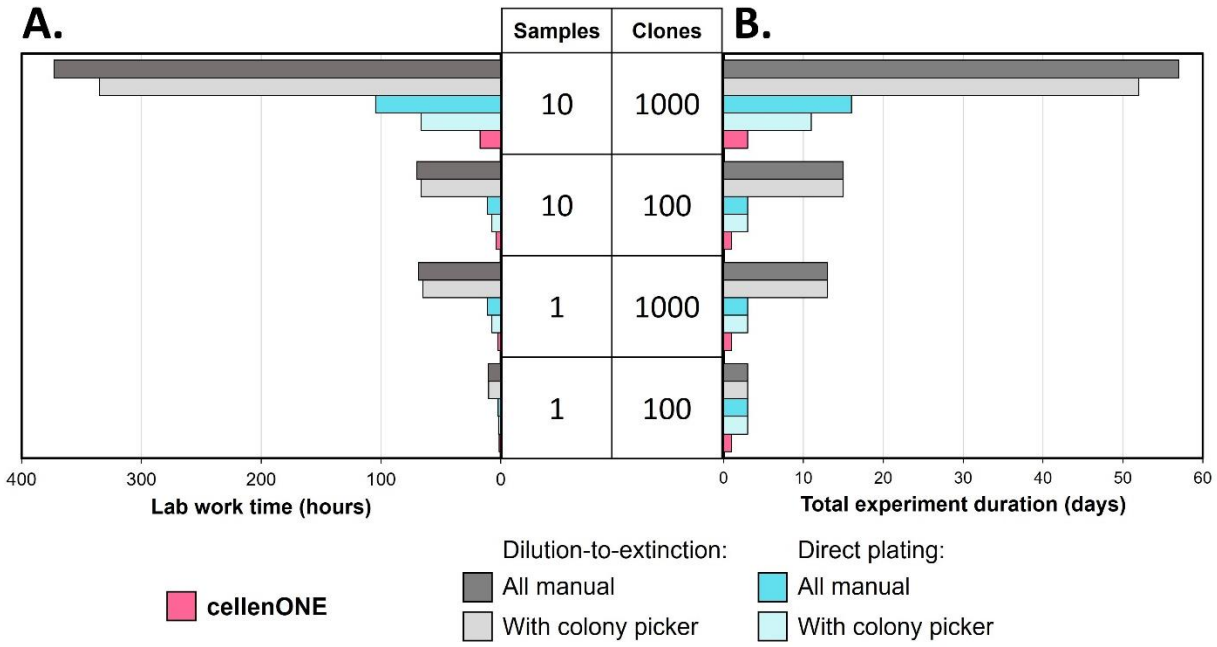

Figure 4. Working time and duration for the isolation of 100 or 1,000 pure liquid cultures from 1 or each of 10 samples using cellenONE (pink), direct plating (purple) or dilution-to-extinction (grey) methods. *A. Lab work time, accounting for cellenONE: single-cell isolation accuracy, cultivability rate, cellenONE preparation and run setting time, cell washing, and isolation run duration, vs. for Petri dish-based methods: Petri dish or microplate preparation, plating or streaking and colony picking time. B. Total duration, accounting in addition for liquid dilutions or agar plate incubation time for Petri-dish based methods.*

Hands-on time and total duration of workflows for generation of isolate or clone libraries

We generated axenic liquid cultures without intermediate incubation steps, on agar plates like in direct plating-based methods (Fig. 1B) or in liquid dilutions like in dilution-to-extinction methods (Fig. 1C), saving tedious and long work. With cellenONE, generating 1000 clones in liquid cultures from one sample represents a hands-on time of approximately 2.5 hours, while dilution-to-extinction methods would require 17 hours (Fig. 4A). Using direct plating-based method, typically used for mutant library construction, for isolating 100 clones from each of 10 samples represents a lab work time of 11 hours vs. 3.5 hours using cellenONE and, importantly, a total duration of 3 days instead of less than 1 day for cellenONE (Fig. 4B). One should note that the time gained using cellenONE over classical Petri dish methods increases exponentially with an increase in number of samples and/or clones processed per experiment (Fig. 4).

Conclusions and future directions

We demonstrated here that cellenONE is an optimal platform for the generation of bacterial isolates, thanks to a combination of outstanding single-cell isolation accuracy, exceptionally good clonal outgrowth, and its capacity of dispensing single cells directly into liquid cultures. Additionally, it was demonstrated that using direct liquid cultures over traditional petri dish methods represents a significant gain in hands-on time. On one hand, cellenONE opens scaling-up opportunities for culturomics approaches on natural samples, allowing rapid screening of multiple media and much bigger isolate library construction. On the other hand, integration of cellenONE in clone generation workflows significantly increases throughput, for more efficient and cost-effective biofoundries, especially using the HT version of the cellenONE for fully autonomous processing of multiple samples and target plates.

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