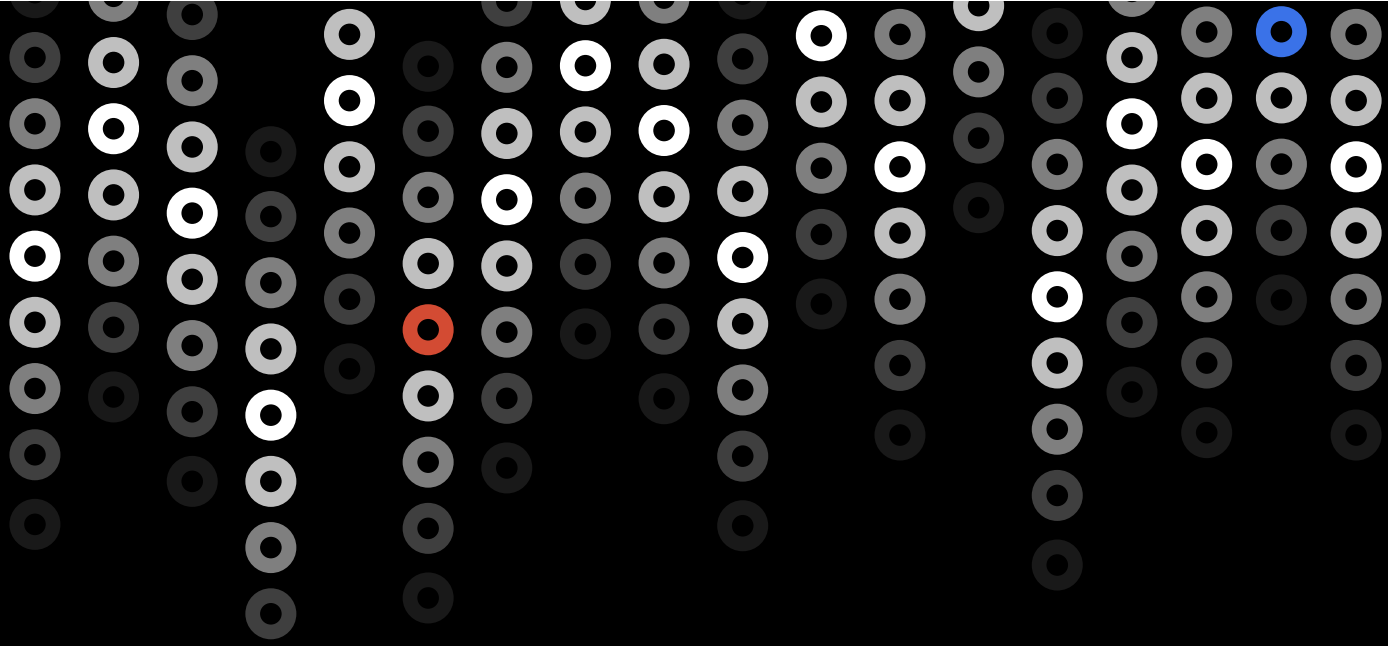

YOUR GROUND CONTROL IN HIGH-THROUGHPUT BIOLOGY

APPLICATION NOTE

DG-AN0001 - Rev A - Application Note

DROPLET-BASED HIGH-THROUGHPUT SCREENING SYSTEMS FACILITATE *IN VITRO* EVOLUTION OF ENZYMES

Droplet Genomics

DROPLETGENOMICS.COM

Droplet-based high-throughput screening systems facilitate in vitro evolution of enzymes

"(...) From so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved." (Charles Darwin, "On the Origin of Species")

SUMMARY:

- *In vitro* evolution mimics the process of natural evolution in a laboratory setting, and is useful for the engineering of proteins to improve their properties or analyze the structure-function relationships.
- Droplet-based microfluidics streamlines the *in vitro* selection process by providing controlled compartmentalization and droplet manipulation capabilities that substantially reduce reagent demand and increase the speed of high-throughput screening experiments as compared to the conventional approach.
- Droplet Genomics Onyx and Styx systems support the microfluidics-assisted *in vitro* evolution workflow.

INTRODUCTION

In 2018 the Nobel Prize in Chemistry was awarded for efforts in the development of directed evolution of enzymes and phage display of peptides and antibodies. Artificial molecular evolution that utilizes principles similar to natural evolution has emerged as a powerful tool for protein engineering and fundamental studies of protein structure-function relationships. Engineering of enzymes aiming to improve their properties or discover new-to-nature reaction pathways is important not only for scientific advancement but also for industrial processes and environmental applications[1,2].

An ideal *in vitro* selection system would enable the identification of enzyme variants exhibiting desired properties from a large library of mutants based on a precise assay readout. Conventional screening assays based on the use of multiwell plates would require processing 652 plates in 1536-well format for every million variants in each screening round[3]. This approach is impractical even with sophisticated robotic automation as screens would take weeks and would consume tens of liters of reagents. Droplet-based microfluidics enabling controlled generation and manipulation of picoliter droplets has offered a new paradigm for high-throughput screening by providing substantially increased throughput, reduced reagent demand, and scalability. It was demonstrated that microfluidic systems allow screening ~10^8 individual enzymatic reactions in a matter of hours using <150 µL of reagents meaning a 1000-fold increase in speed and a 10^6-fold reduction in cost as compared to the conventional approach[4].

An essential requirement for a successful directed evolution study is linking the desired activity of enzyme (=the phenotype) to its genetic information (=the genotype). Compartmentalization of mutant variants within water-in-oil emulsion had previously restricted *in vitro* evolution to the engineering of polymerases or DNA modifying enzymes utilizing methods such as compartmentalized self-replication[5] or compartmentalized self-tagging[6]. The readout of such techniques consists of amplified DNA encoding improved variants which are gradually enriched over the background in each subsequent

round of selection. With the development of on-chip droplet sorting devices[7], this constraint was relieved as efficient separation of positive droplets based on the presence of fluorescence signal retained the phenotype-genotype linkage throughout the selection procedure and enabled the use of a broader range of reporter systems.

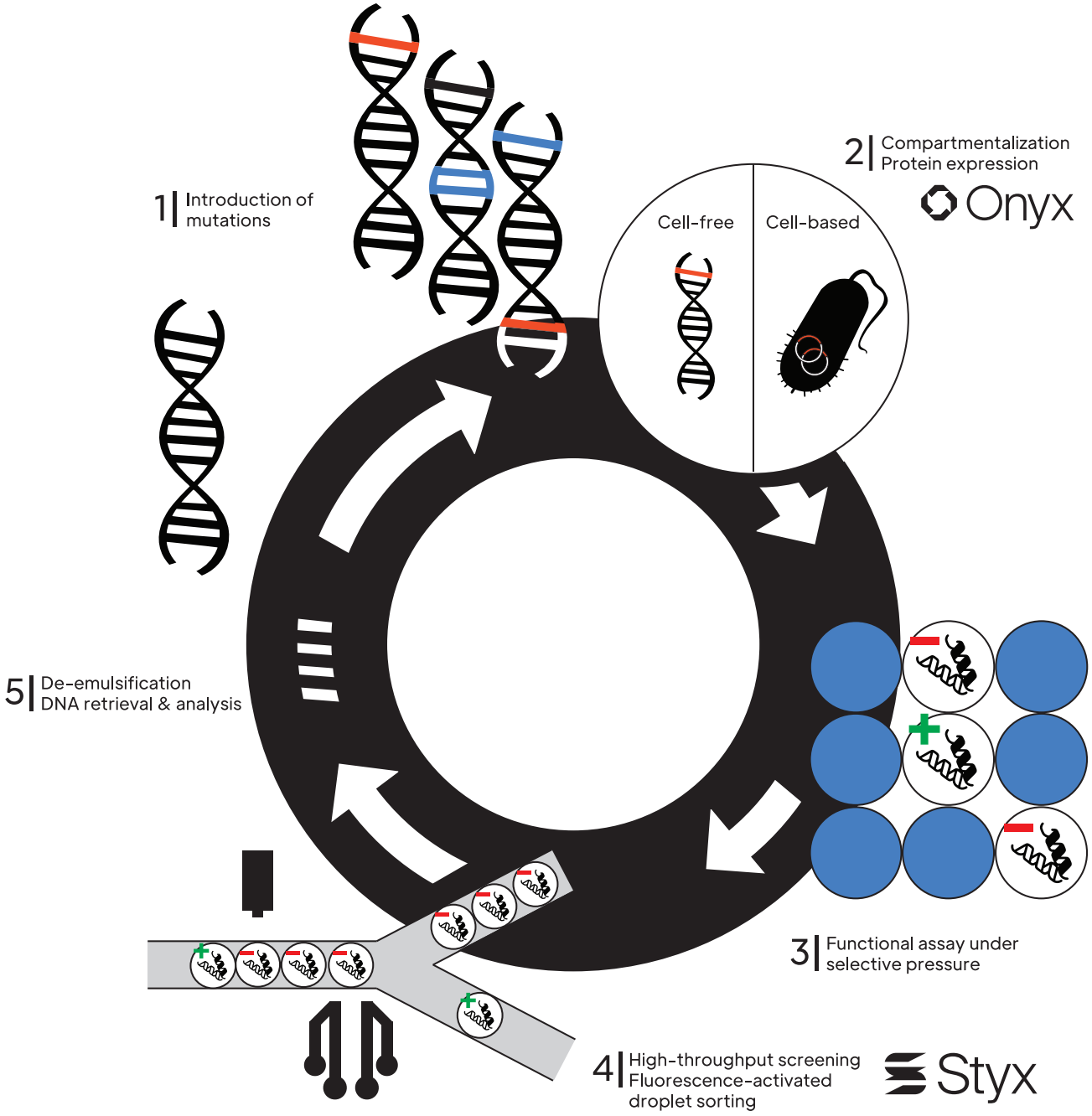

Figure 1. The overview of microfluidics-assisted *in vitro* compartmentalization and fluorescence-activated droplet sorting for enzyme engineering.

MICROFLUIDICS-ASSISTED *IN VITRO* EVOLUTION WORKFLOW

1. Introduction of mutations

The choice of mutagenesis strategy is critical for the success of an *in vitro* evolution study. If the enzyme of interest is well characterized, structural or biochemical information can be leveraged to focus mutagenesis on functionally relevant sites. This strategy generates smaller libraries and allows for a lower throughput screening. Alternatively, if the structure-function relationships of the parent enzyme are poorly understood, more mutants should be screened to discover a desired phenotype. This is usually achieved via more thorough random mutagenesis that results in an exponential growth in the number of unique variants to be screened. Researchers have developed many methods to perform both forms of gene diversification, and the most successful strategies often integrate both random and focused approaches[8].

2. Compartmentalization & protein expression

Linking the genotype to phenotype is typically achieved by compartmentalization of a single mutant gene variant per compartment. Partitioning is a stochastic process that obeys Poisson distribution where the probability of having a compartment with *k* genes follows the equation

$P(k,\lambda) = \frac{e^{-\lambda}}{k!}\lambda^k$, with λ being the average number of genes per compartment. For example, at λ = 0.2, 82% of compartments will be empty, 16% will have one gene and only 2% will have two genes. Mutant genes can be compartmentalized as DNA templates or as single cells transformed with a gene library. Once inside the compartment, *in vitro* transcription-translation system or cells will drive the protein expression.

Microfluidics-assisted compartmentalization into monodisperse water-in-oil microdroplets offers the precise retention of phenotype-genotype linkage, high throughput, and compatibility with subsequent protein expression and functional assays. Droplet Genomics Onyx system provides a user-friendly and flexible solution for the generation of monodisperse emulsions. Moreover, integrated droplet manipulation capabilities are useful for assays involving reagent addition steps.

3. Functional assay under selective pressure

The assay aiming to identify improved variants depends on the nature of a particular enzyme and engineering goal. Droplets serve as isolated reaction vessels in which discrete assays can take place.

Most enzymes are not associated with directly observable phenotypes and require a fluorescent, luminescent, colorimetric, or another detectable reporter. To generate an observable signal that correlates with the desired enzymatic activity, appropriately labeled or surrogate substrates should be used.

4. High-throughput screening & fluorescence-activated droplet sorting

High-throughput screening and sorting aim to recover “positive” droplets and discard the background. Fluorescence-activated droplet sorting (FADS) allows sorting water-in-oil droplets by dielectrophoresis. Droplets are introduced into a microfluidic sorting device and exposed to a laser. If fluorescence is detected, a pulse high voltage alternating current is applied across the electrodes adjacent to the sorting junction. This leads to the deflection of a polarized fluorescent droplet towards the designated arm of a sorting junction. Droplet Genomics has developed the Styx system to make high-throughput screening and droplet sorting accessible to anyone interested in adopting advanced droplet microfluidics workflows.

5. De-emulsification, DNA retrieval & analysis

"Positive" droplets that contain enzyme variants exhibiting the desired properties as well as genes encoding these enzymes are broken to purify DNA which afterward is sequenced to identify the beneficial mutations. These improved variants may be further diversified and subjected to subsequent rounds of screening.

HIGH-THROUGHPUT SCREENING AND DROPLET SORTING WITH THE STYX SYSTEM

Droplet Genomics Styx system is a platform for droplet analysis and sorting that can accommodate a broad range of experimental designs.

After the generation of monodisperse emulsion with the Onyx instrument and functional assay in the presence of a fluorescent reporter, the emulsion is analyzed with the Styx system using an appropriate laser at a typical measurement throughput of >1000 droplets per second. During droplet analysis, Styx visualizes the fluorescence data either as scatter plots or histograms. Target droplet population can be manually selected to define sorting parameters. The progress of droplet sorting can then be monitored with the help of two cameras placed at the sorting junction and outlet channels of the microfluidic chip (Figure 2).

Microscopy analysis of sorted emulsion might be employed to confirm the enrichment of fluorescent droplets (Figure 3). As droplet sorting parameters can be as stringent as needed, efficient removal

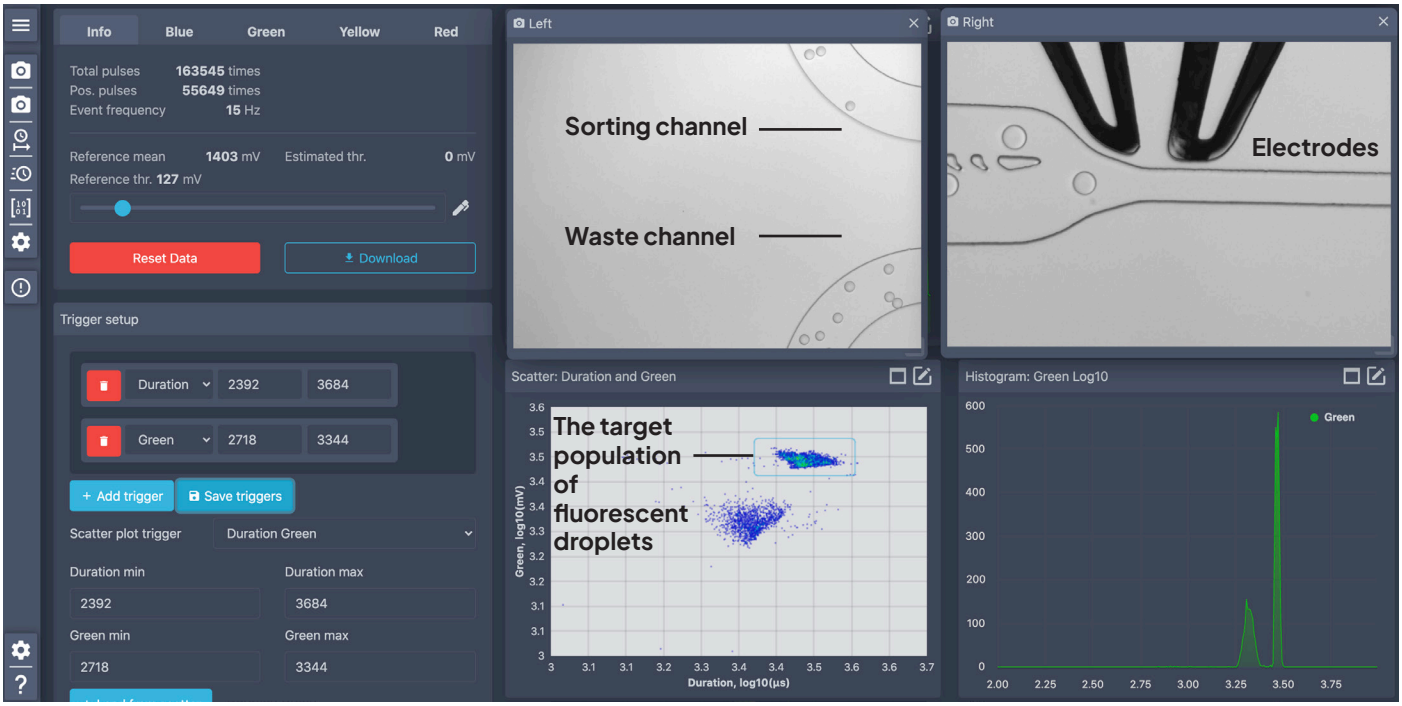

Figure 2. An overview of fluorescence data provided by the Styx system.

of background in principle allows selecting best performers in a single round. In practice, some background might sneak into sorted populations due to the presence of multiplets – droplets with two or more co-encapsulated variants.

Is it possible to enrich extremely rare variants (<<1% of the initial population)? While this is technically achievable, the duration of the sorting run that would result in a reasonable amount of sorted emulsion would be impractical. This issue can be overcome by performing a round of enrichment based on the basic activity (e.g., select enzyme variants that still possess the wild type activity despite the introduced mutations), and a second iteration would then be functional screening under the selective pressure.

FADS has already been successfully applied for the engineering of various enzymes, for example, horseradish peroxidase with improved catalytic rates[4], esterase with improved enantioselectivity[9], T7 RNA polymerase able to recognize synthetic promoters[10], and many more.

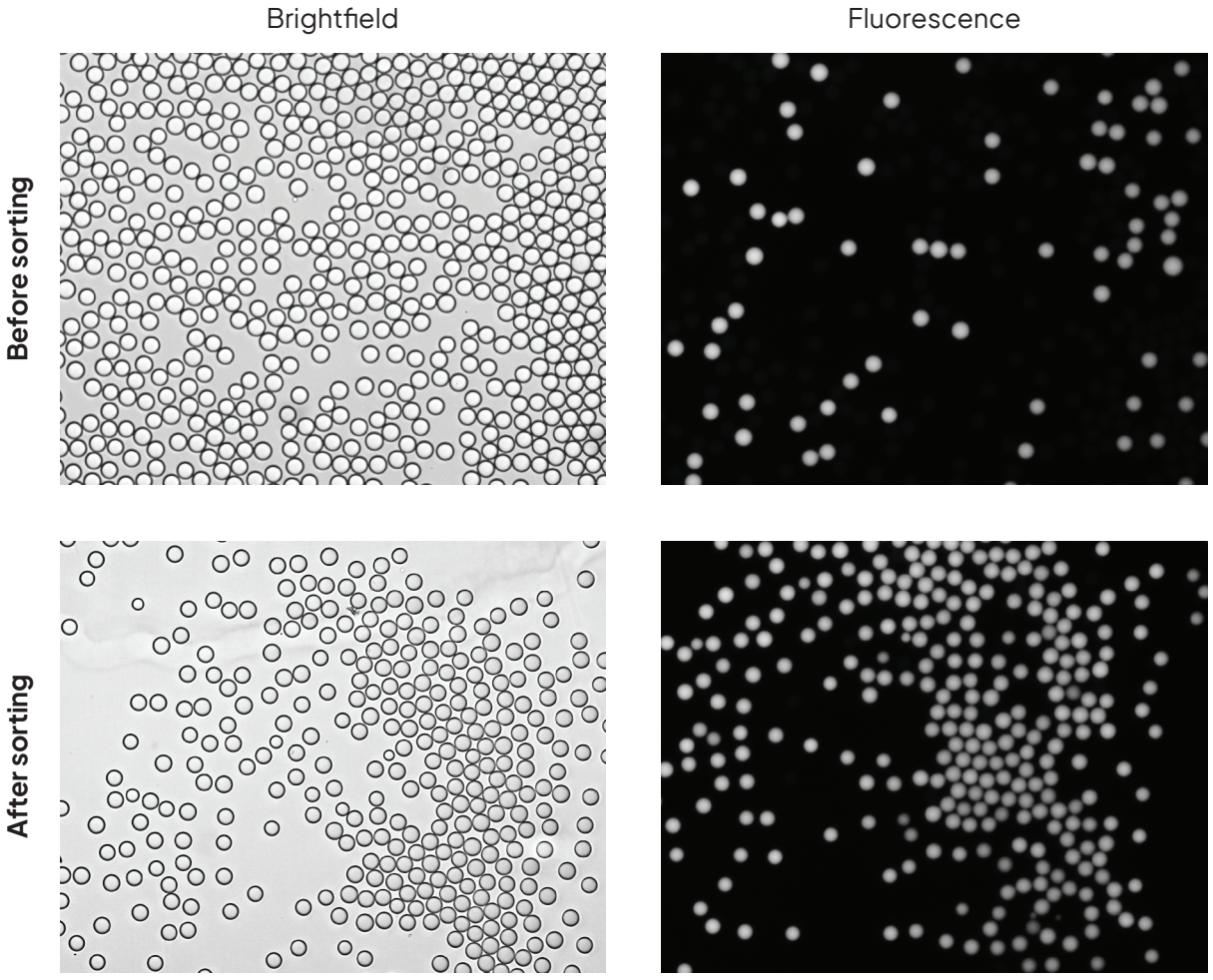

Figure 3. Microscopy analysis of emulsion before and after the sorting of fluorescent droplets.

CONCLUSIONS

- A typical *in vitro* evolution campaign consists of the following iterative steps: introduction of mutations, compartmentalization and protein expression, functional assay under selective pressure, high-throughput screening and droplet sorting, de-emulsification and DNA retrieval. Enzyme variants exhibiting the desired properties should enrich over the background.
- Microfluidic technologies facilitate *in vitro* evolution experiments by providing controllable encapsulation and droplet manipulation functionalities.
- Droplet Genomics Onyx and Styx systems are robust and flexible tools for any *in vitro* evolution experimental design.

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ORDERING INFORMATION

Product	Quantity	Cat. No.
Instruments		
Onyx	1 instrument	DG-ONYX
Stand-Alone Microfluidic Pump	1 instrument	DG-PMP
Styx	1 instrument	DG-STYX
Modules for Styx System		
405 nm Laser	1 unit	DG-STX-405
561 nm Laser	1 unit	DG-STX-561
633 nm Laser	1 unit	DG-STX-633
Kits		
Droplet Generation Kit	5 runs	DG-KIT-DM
Co-Flow Droplet Generation Kit	5 runs	DG-KIT-CF
Droplet Selection Kit	5 runs	DG-KIT-SLC
Styx Microfluidic Tubing Kit	5 runs	DG-KIT-STX-MT
Reagents & Consumables		
Droplet Stabilization Oil	15 mL	DG-DSO-15
Spacing Oil	15 mL	DG-SPO-15
Sample Loading Oil	15 mL	DG-SLO-15
Emulsion Breaker	1 mL	DG-EB-1
Microfluidic Tubing	30 m (OD 0.76 mm)	DG-MT0.3-30
	30 m (OD 1.07 mm)	DG-MT0.56-30
Droplet Selection Device	1 chip	DG-STX-SRT
Custom Microfluidic Chips	1 chip	Please inquire

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APPLICATION NOTE

DG-AN0001 – Rev A – Application Note

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