# iFlow™ Droplet Generation Application (v1.5)

## Introduction

Droplet generation is the production of micron/sub-micron sized droplets with highly reproducible capacity. Usually, the system requires two or more immiscible liquid phases: the dispersed phase, sometimes called the droplet phase, and the continuous phase. The size of the generated droplets is mainly controlled by the channels' geometry, the flow rate ratio of two phases, interfacial tension, etc.

PreciGenome Microfluidic Droplet Generation System PG-DG with droplet generator chips can be used for preparation of water-in-oil or oil-in-water droplets with high uniformity at thousands of droplets per second. Compared to conventional methods, it can generate droplets with much higher robustness, precision, and repeatability in a quick and cost-effective way. The droplets are highly uniform in diameter (CV 0.1-5%), and both size and production rate can be fine tuned by adjusting the reaction environment. Different packages are available for both small and large scale production while retaining a compact and user-friendly platform based on the iFlow pressure controllers.

**Figure 1.** Droplet Generation Package with High Speed Imaging System and iFlow Touch Controller

## System Specs

- Pressure sensor accuracy of ±0.25 %FSS BFSL (Full-Scale Span Best Fit Straight Line), sensor resolution: 0.0061 %, pressure stability: 0.05 %
- Integrated UI with 10” touch screen
- Droplet generation with pressure or vacuum in one channel
- Air leakage detection with integrated airflow rate monitoring, range of 200sccm
- Liquid flow rate monitoring and control with external flow rate sensor (optional)
- Flow rate repeatability: <1% of the measured value
- Highest sensitivity: <1µl/min

## System Benefits

- Tunable and controllable droplet size
- High droplet uniformity
- Wide range of production rates/volumes
- OEM service and module integration available

## Applications

- Cell encapsulation
- Single cell analysis
- Hydrogel, microparticles and polymer synthesis
- Foams
- Drug delivery
- Cell culture
- Digital PCR, DNA/RNA sequencing

## Microfluidic Chips

PreciGenome offers a variety of microfluidic chips in different materials to meet most of our customers' application requirements for droplet generation. Two types of materials, plastic and glass, are commonly used to fabricate microfluidic chips. Chip material selection depends on the application requirements, including chip design, types of reagent used for experiments, needs of applications, budget, fabrication time, etc.

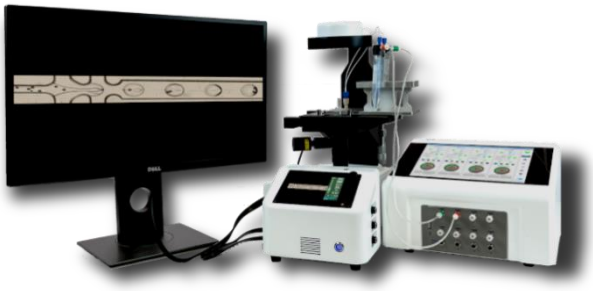
| Catalog # | Material | Inlet # | Droplet Size | Applications |
|---|---|---|---|---|
| CHP-DG-SEMUL-4-COC | TOPAS | 2 | 30-100 µm | Single emulsion. |
| CHP-DG-SEMUL-8-M1 | TOPAS | 2 | 80-100 µm | Single emulsion |
| CHP-DG_SEMUL-8-M2 | TOPAS | 2 | 65-145 µm | Single emulsion |

| | | | | |
|---|---|---|---|---|
| CHP-DG-DEMUL | TOPAS | 3 | 30-100 µm | Single/double emulsion |
| CHP-DG-DEMUL-3-COC | TOPAS | 3 | 100-200 µm | Single/double emulsion |
| CHP-DG-DEMUL1-1-COC | TOPAS | 4 | 70-210 µm | Single/double emulsion |
| CHP-DG-DEMUL2-1-COC | TOPAS | 4 | 140-420 µm | Single/double emulsion |
| CHP-DG-SEMUL-8-PG | TOPAS | 2 | 70-110 µm | Single emulsion |
| Glass chip | Glass | 2 & 3 | 20-100 µm | Single/double emulsion |

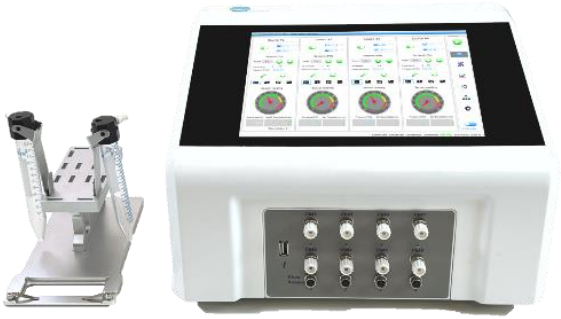
**Table 1.** Droplet Generation Chip information

## System Components

- iFlow Controller, high speed imaging system, mount



- Setup with large volume reservoir kits



- Setup with small volume chip tanks

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- High speed imaging system (optional)

**Chips and Accessories**

- Microfluidic chips
- Reagent tanks: 500 µl and 1 ml
- Reservoir kit: 1.5ml, 15ml, 50ml, and 100+ ml
- Flow rate sensor (optional): 7 µl/min, 80 µl/min, 1ml/min, 5ml/min (extend to 10ml/min)
- Tubing, Connectors, etc.

## Working Mechanism

The following section will describe results of experiments done in-house by PreciGenome to show the performance of our product.

**Working mechanism for large reservoirs**:

Aqueous and oil solutions are loaded in sample reservoirs (1.5ml, 15ml, 50ml, or 100+ ml). Pressure is directly applied to the reservoir kits where solutions are pushed into the PTFE tubing connected to the microfluidic chip. Pressure is tuned to achieve desired droplet size and production rate and droplets are collected from the product tank.

**Working mechanism for tubing free reagent tanks**:

Reagent tanks are plugged directly to chips. Aqueous and oil solutions are loaded in reagent tanks. The chip mount is sealed and pressure is directly applied to the reservoir tanks and tuned to achieve desired droplet size and production rate. The solutions are pushed into channels in the microfluidic chip and droplets are collected from the product tank.

## Materials and Experimental Procedure

### Materials

Reagent list included polystyrene microspheres (10 and 20 µm diameter, Bangs Labs), Triton X-100 (Sigma), TWEEN-20 (Sigma), BSA (≥ 98%, Sigma), (Krytox 157-FSH (DuPont), EvaGreen oil (Bio-Rad), Novec 7500 oil (3M), PBS (1x, Cytiva), and deionized water (MiliQ).

Instrument list included iFlow Touch pressure controller (PG-MFC, PreciGenome), high speed imaging system (PG-HSV-M, PreciGenome), plasma treater (BD-20, ETP) and ZOE fluorescent cell imager (Bio-Rad).

### Surfactant solution preparation

Surfactant of choice was mixed and dissolved to desired concentration in corresponding phase. Triton X-100 and TWEEN-20 (Sigma) were prepared separately in DI water, while Krytox (DuPont) was prepared in Novec 7500 oil (3M). Bovine serum albumin (BSA, ≥ 98%, Sigma) in phosphate buffer saline (PBS, 1x, Cytiva) may be substituted for surfactant solution if encapsulating live cells. A 0.22 µm PES filter was used to remove dust or large particles from the resulting solution before use.

### Bead solution preparation

A 0.22 µm PES filter was used to remove dust or large particles from DI water before addition of other reagents. Polystyrene/divinylbenzene microspheres (10 and 20 µm diameter, Bangs Labs) were then dissolved to desired concentrations in DI water, with different size microspheres prepared separately. For double emulsion application, TWEEN-20 was dissolved to 0.5% w/v at same time as beads. In both single and double emulsion, bead solution was vortexed for 30 s immediately before use.

### Plasma treatment of microfluidic chips

For double emulsion applications, Scotch tape was used to cover DG-DEMUL chip inlets for the inner water and oil phases, as well as all inlets for channels not immediately used. The chip was then placed directly under an oxygen plasma treater (BD-20, ETP) fixed in place with clamps. Plasma treatment was conducted for 5 minutes followed by channel usage within 1 minute.

### Large volume production using reservoir kits (>0.5 ml)

Mount the reservoir kits (1.5ml, 15ml, 50ml, or 100+ ml) and connect the mount to the iFlow Touch pressure controller. Load a droplet generation chip on the chip holder. Load DI water to the aqueous reservoirs and oil solutions to the oil reservoirs. Enable one pressure controller channel in use at a time and run for several seconds to prime the reservoir tubing. When done for all reservoirs, connect the reservoir tubing directly to the chip. Run the pressure controller to start producing droplets.

### Small volume production using chip tanks (<0.5 ml)

Mount the reagent tanks and connect the mount to the iFlow Touch pressure controller. Load a droplet generation chip on the chip holder. Load DI water to the aqueous reservoirs and oil solutions to the oil reservoirs. Run the pressure controller to start producing droplets.

### Case Study 1 – Single bead encapsulation

For single emulsion, follow procedure for large volume production with DG-DEMUL chip, loading DI water in aqueous reservoirs and oil in oil reservoir. After priming reservoir tubing and producing stable droplets, stop the pressure controller. Replace DI water with 10 µm bead solution in the aqueous reservoir connected to the central water inlet. Run without changing tubing setup. When beads begin to flow into the chip junction, discard currently produced droplets without stopping the controller. When ready, stop and collect newly produced droplets.

For double emulsion, load surfactant mixes in appropriate reservoirs instead of DI water and oil: 0.5% TWEEN-20 or 2% BSA should go to the inner aqueous reservoir, 2.2% Krytox should go to the oil reservoir, and 1% Triton X-100 should go to the outer aqueous reservoir. After priming reservoir tubing and producing stable droplets, stop the pressure controller. Replace inner water phase reservoir with 10 µm bead solution. Run without changing tubing setup. When beads begin to flow into the chip junction, discard currently produced droplets without stopping the controller. When ready, stop and collect newly produced droplets.

**Case Study 2 – Drop-Seq application**

Follow procedure for large volume production with DG-DEMUL-3-COC chip, loading DI water in aqueous reservoirs and oil in oil reservoir. After priming reservoir tubing and producing stable droplets, stop pressure controller and replace DI water with bead solutions. The central water inlet received the larger beads (20 µm) to mimic barcoding beads, and the peripheral water inlet received the smaller beads (10 µm) to mimic cells. Run without changing tubing setup. When beads begin to flow into the chip junction, discard currently produced droplets without stopping the controller. When ready, stop and collect newly produced droplets.

## Results and Discussion

**Droplet Generation with Reservoir Kits (>1 ml):**

Adjusting the pressure ratio is the simplest method for adjusting droplet size and production rate in a certain range). In general, a lower W:O (**W**ater to **O**il) ratio leads to smaller droplets, and higher total pressure or larger W:O ratio leads to greater production rate. An overview of droplet size and production rate is provided in **Tables 2 and 3**. For the DG-DEMUL chip, the pressure of both water phases was kept the same, and the chip was configured for single emulsion applications. Backflow occurs as the W:O ratio approaches 1:2 for the DG-SEMUL chip, and 1:3 or 3:1 for the DG-DEMUL chip. Droplets as small as the nozzle size (30 µm) can be attained at a pressure setting of 2.5 psi for water and 5.7 psi for oil for the DG-SEMUL chip and 4 psi for water and 11.9 psi for oil for the DG-DEMUL chip Production rates at these pressure settings were less than 100 droplets per second. Values may change based on liquid tubing length and inner diameter (ID). For the DG-SEMUL chip, all liquid tubing had an ID of 1/32”; for the DG-DEMUL chip, inlet tubing had an ID of 300 µm and outlet tubing 1/32”. For both chips, tubing length was 36 cm for water, 38 cm for oil, and 28 cm for collection; both water phases had the same length in the DG-DEMUL experiment.

| W (psi) / O (psi) | 1 | 2 | 3 | 4 | 6 |
|---|---|---|---|---|---|
| 1 | Inert | Chaotic | | | Backflow (W-O) |
| 2 | | 52 µm 950 dps | 60 µm 1047 dps | 72 µm 1116 dps | Chaotic |
| 3 | | 47 µm 350 dps | 50 µm 1346 dps | 55 µm 1674 dps | |
| 4 | | Backflow (O-W) | 45 µm 1488 dps | 47 µm 2046 dps | 56 µm 2976 dps |
| 6 | | | | 40 µm 2046 dps | 41 µm 5022 dps |

**Table 2.** Droplet generation with reservoir kits by adjusting pressure with iFlow Touch controller (CHP-DG-SEMUL chip)

| W (psi) / O (psi) | 1 | 2 | 3 | 4 | 6 |
|---|---|---|---|---|---|
| 1 | Inert | Jetting | Backflow (W-O) | | |
| 2 | | 86 µm 279 dps | Chaotic | Jetting | |
| 3 | | 59 µm 465 dps | 86 µm 558 dps | Chaotic | Jetting |
| 4 | | 52 µm 37.2 dps | 73 µm 930 dps | 86 µm 1302 dps | |
| 6 | | Backflow (O-W) | 51 µm 1488 dps | 57 µm 2976 dps | Chaotic |
| 9 | | | | 44 µm 2976 dps | |

**Table 3.** Droplet generation with reservoir kits by adjusting pressure with iFlow Touch controller (CHP-DG-DEMUL chip)

| W (psi) / O (psi) | 1 | 2 | 3 | 4 | 6 |
|---|---|---|---|---|---|
| 1 | | Chaotic | | | Backflow (W-O) |
| 2 | | 53 µm<br>858 dps | 60 µm<br>1002 dps | 98 µm<br>400 dps | Chaotic |
| 3 | | 45 µm<br>353 dps | 50 µm<br>1410 dps | 53 µm<br>2000 dps | |
| 4 | | Backflow (O-W) | 44 µm<br>1169 dps | 46 µm<br>2200 dps | 54 µm<br>3445 dps |
| 6 | | | | 38 µm<br>2200 dps | 40 µm<br>5830 dps |

**Table 4.** Droplet Generation with reagent tanks by adjusting pressure with iFlow Touch controller (CHP-DG-SEMUL chip)

| W (psi) / O (psi) | 1 | 2 | 3 | 4 | 6 |
|---|---|---|---|---|---|
| 1 | | | Backflow (W-O) | | |
| 2 | | Chaotic | | | Backflow (W-O) |
| 3 | Backflow (O-W) | 60 µm<br>651 dps | 81 µm<br>933 dps | | Jetting |
| 4 | | 53 µm<br>1302 dps | 62 µm<br>1679 dps | | |
| 6 | | | 51 µm<br>2425 dps | | 98 µm<br>1436 dps |
| 9 | | | 39 µm<br>1119 dps | 35 µm<br>9954 dps | 84 µm<br>2154 dps |

**Table 5.** Droplet Generation with reagent tanks by adjusting pressure with iFlow Touch controller (CHP-DG-DEMUL chip)

| | | | |
|---|---|---|---|
| **Baseline**<br>**(3.2 psi W1, 2.4 psi O, 6.2 psi W2)** | Inner: 32 µm<br>Outer: 50 µm | | |
| **Decrease W1**<br>**(-1.0 psi)** | Inner: 25 µm<br>Outer: 55 µm | **Increase W1**<br>**(+0.2 psi)** | Inner: 33 µm<br>Outer: 49 µm |
| **Decrease O1**<br>**(-0.4 psi)** | Inner: 38 µm<br>Outer: 47 µm | **Increase O2**<br>**(+0.4 psi)** | Inner: 30 µm<br>Outer: 51 µm |
| **Decrease W2**<br>**(-0.4 psi)** | Inner: 35 µm<br>Outer: 55 µm | **Increase W2**<br>**(+1.4 psi)** | Inner: 30 µm<br>Outer: 42 µm |

**Table 6.** Droplet Generation with reservoir kits in double emulsion configuration (CHP-DG-DEMUL chip)

**Droplet Generation with Reservoir Tanks (<1 ml ):**

Pressure ratio has a similar effect for the reagent tanks compared to the reservoir kits, though the DG-DEMUL chip is more prone to chaotic flow behavior at certain pressure ratios. An overview of droplet size and production rate is provided in **Tables 4 and 5**. For the DG-DEMUL chip, the pressure of both water phases was kept the same, and the chip was configured for single emulsion applications. Backflow occurs as the W:O ratio approaches 1:2 for the DG-SEMUL chip, while no major trend is present for the DG-DEMUL chip.

**Double Emulsion Droplet Generation:**

Double emulsion tests were conducted with the same reservoir kit setup as previously described. Pressure balancing is often required before droplet production is stable, as this ensures all three phases are distributed uniformly and can produce a stable water-in-oil-in-water emulsion. Final stable pressures tend towards a 1.3:1.0:2.5 ratio (inner water:oil:outer water). An overview of droplet shell tuning is provided in **Table 6.** Further increases to oil pressure or decreases to outer water pressure create unstable second emulsions (not shown.)

**Case Study 1 – Single Bead Encapsulation:**

10 µm polystyrene beads have a similar density and size to cells, and so their behavior in encapsulation experiments can be a useful model to the droplet generation system's viability for single cell studies. Cell encapsulation follows the Poisson distribution:

$$Pr(x = k) = \frac{\lambda^k e^{-\lambda}}{k!}$$

where $Pr(x=k)$ is the probability of encapsulation events, k is the number of occurrences (or cell number per droplet), λ is the expected value of x (or average cell number per droplet)

$Pr(x)$ has its maximum value at $k = \lambda$. So the maximum value of $Pr(x=1)$ is approximately 0.368 (or 36.8%). For single emulsion droplets of 60 µm diameter, PreciGenome's droplet generation system with the DG-DEMUL chip was found to approach this value at approximately 21 million beads/ml. The theoretical Poisson distribution values matched with bead concentrations approximately one-third of the starting value, which can be explained by the reagent flow behavior in the DG-DEMUL chip **(Table 7)**. Two aqueous phases meet at the junction where they are encapsulated by the oil phase, but only one aqueous phase contained beads; therefore, the bead concentration during and after droplet generation would be lower than that in the initial solution.

This also applied to a lesser extent with double emulsion droplets. For those with an inner shell of 30 µm diameter, a starting concentration of approximately 18 million beads/ml resulted in 14.8% single encapsulations, which is close to a bead concentration one-half the starting value. The lower overall encapsulation rate is due primarily to the smaller volume of the inner shell, which is the only one that could encapsulate beads in the aqueous phase.

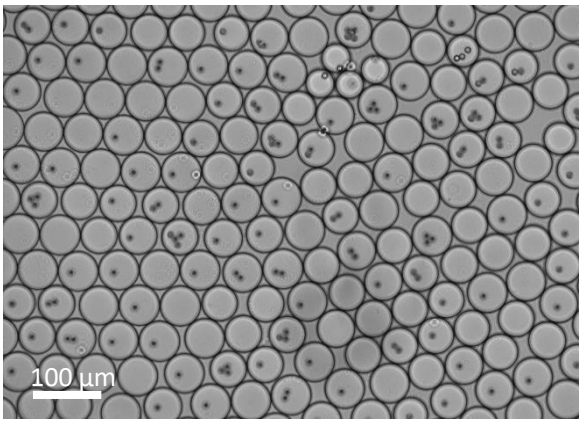


**Figure 2.** Droplets encapsulating 10 µm beads (single emulsion)

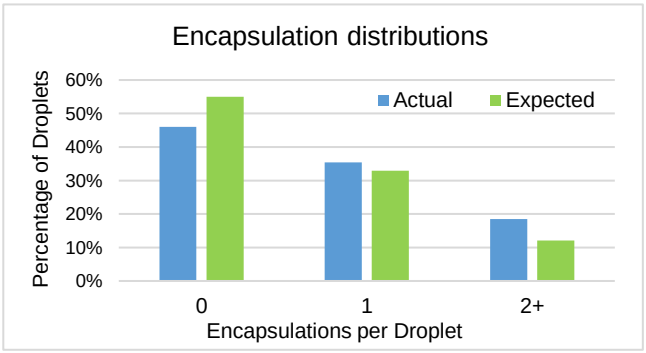


**Figure 3.** Encapsulation distribution for 10 µm beads (single emulsion)

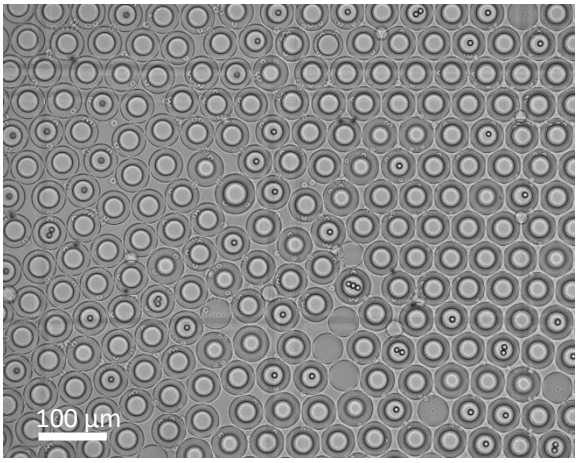


**Figure 4.** Droplets encapsulating 10 µm beads (double emulsion)

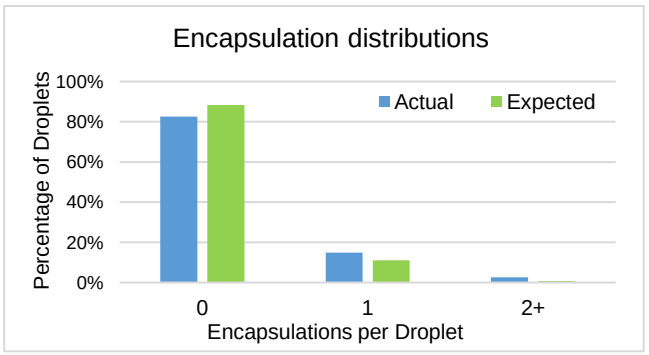


**Figure 5.** Encapsulation distribution for 10 µm beads (double emulsion)

| Initial Bead Concentration | 0 bead Encapsulations | | 1 bead Encapsulations | | 2+ beads Encapsulations | | All encapsulations | |
|---|---|---|---|---|---|---|---|---|
| (beads/ml) | Actual (%) | Expected (%) | Actual (%) | Expected (%) | Actual (%) | Expected (%) | Actual (%) | Expected (%) |
| 7,470,000 | 80.3 | 75.5 | 18.1 | 21.2 | 1.6 | 3.3 | 100.0 | 100.0 |
| 12,900,000 | 69.0 | 69.0 | 24.0 | 25.7 | 7.0 | 5.3 | 100.0 | 100.0 |
| 20,580,000 | 46.1 | 55.0 | 35.4 | 32.9 | 17.5 | 12.1 | 100.0 | 100.0 |

**Table 7.** Single emulsion bead encapsulation data with reservoir kits (CHP-DG-DEMUL chip).

**Case Study 2 – Drop-Seq Application:**

Drop-Seq is a common application of single cell studies. Cells may be isolated in droplets alongside barcoded beads to form miniature reaction chambers. 20 µm polystyrene beads may be used to mimic these barcoded beads due to their similar size and density, and their behavior can be predicted with the Poisson distribution as described in the previous section. With two types of beads being encapsulated, the maximum probability of two simultaneous single-bead encapsulation events is the square of the probability for one such event, approximately 13.5%.

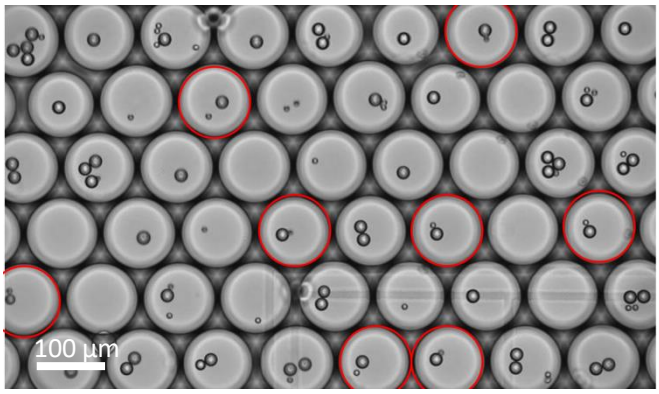


**Figure 4.** Drop-Seq model with 10 and 20 µm beads.

For droplets of 100 µm diameter, PreciGenome's droplet generation system with the DG-DEMUL-3-COC chip was found to approach this value at approximately 4 million beads/ml for 20 µm beads and 2.4 million beads/ml for 10 µm beads. The theoretical Poisson distribution values matched with bead concentrations approximately one-half the starting value for 20 µm beads and two-thirds for 10 µm beads. With both bead solutions at 4 million beads/ml, a higher frequency of encapsulation events is observed for 10 µm beads. Given their smaller size, the 10 µm beads may have aggregated more readily, allowing more to be encapsulated than if each phase contributed half the total volume.

## Conclusions

The iFlow platform is a robust and convenient tool for droplet generation. By tuning the pressure during operation, users can achieve excellent control over droplet size and production rate. From here, encapsulation can reach the upper limits of the Poisson distribution with the appropriate concentration of particles and careful preparation of the microfluidic setup.

PreciGenome offers two different microfluidic mounts for droplet generation, which together provide a wide throughput range between 0.2–1000 mL or more. With the same microfluidic chip, both scales may achieve similar droplet size and production rates with appropriate pressure tuning. Combined with our high speed imaging system for easy visualization, PreciGenome thus provides an economical solution from early discovery to pre-clinical studies and scaled up production.

More information can be found on our website: Droplet Generation w. Microfluidic Generator Chips | PreciGenome, US

## References

Pan, X., et. al., CRISPR-Cas9 Extracellular Vesicles for Treating Hearing Loss. Sept 2023, DOI: 10.1101/2023.09.14.557853