Detection and Sorting of droplets

Double emulsion droplets generated with the dPCR cartridge can be sorted and collected in a standard cell sorter, capturing the DNA of interest. In this step, the positive dPCR droplets containing the region of interest are identified and separated from the negative droplets using the fluorescent signal provided by the dPCR amplification of the ID sequence (see Chapter 3).

Requirements for cell sorter

- A 488 nm (blue) laser.
- Optical configuration detecting fluorophores excited at 488 nm such as FITC, GFP, and PE.
- 100 µm nozzle tip/sorting chip minimum.
- Sample probe should be positioned at the bottom of the sample tube.

Notes to operator

- dPCR droplets are large, therefore the correct events are high in Forward Scatter (FSC) and Side Scatter (SSC) (see Fig. 1).
- Smaller events and events with low SSC represent pure oil droplets and should be gated out.
- dPCR droplets are stable and relatively heavy. It can take up to 5-10 min depending on the sample pressure before the dPCR droplets reach the point of interrogation and appear on the plot.
- Positive fluorescent droplets are likely to be very rare (possibly less than 0,02% of total dPCR droplets). Therefore, a positive population can be difficult to identify. Make sure the live plot of fluorescence shows at least 100.000 events (see Fig. 2) A histogram plot is not recommended.
- A threshold setting on FCS of about 5% or similar should be set to avoid disturbance by small particles.
- dPCR droplets are relatively robust, set sample pressure to aim for a rate equal to 5000 events/sec.

Flow cytometry analysis and sorting of dPCR droplets

1. Identify the dPCR droplet population on a plot of FSC (height) versus SSC (height) (Fig. 4.1). dPCR droplets are higher in side scatter (SSC) than oil droplets. Note that it could take a few minutes before the heavy dPCR droplets are analyzed and visible in the plot.

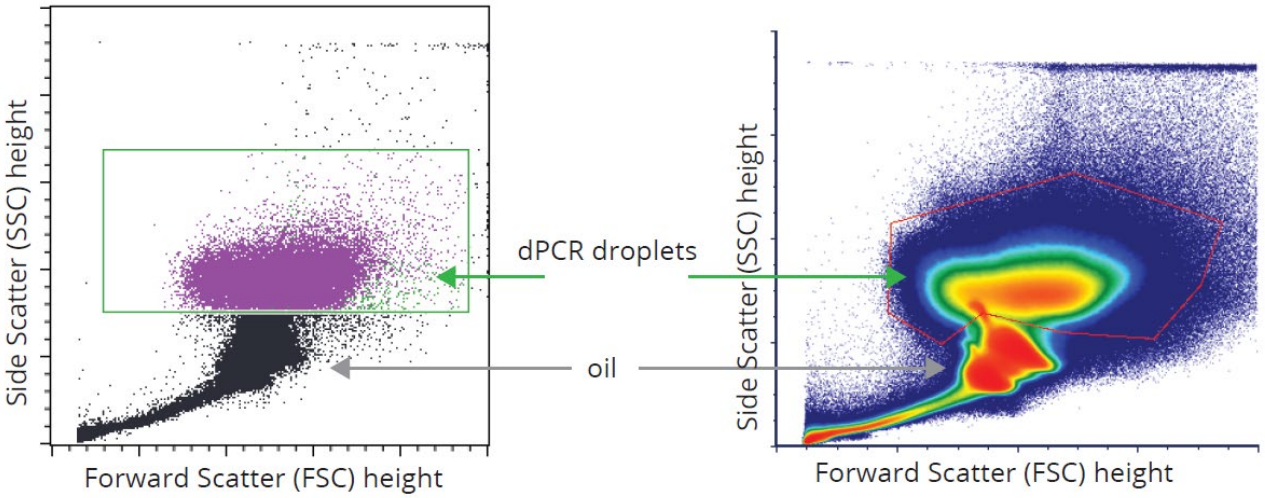

Fig.1. Identify dPCR droplets on a plot showing forward scatter (FSC) versus side scatter (SSC) or back scatter (BSC). The "height" setting more clearly displays the population of correct dPCR droplets. The figure shows the same data on two different instruments. Left: Dot plot showing dPCR droplets inside the green gate (SONY SH800 Cell sorter, software SONY). Right: Density view of all events from a flow cytometry analysis software (FCS Express 6).

2. Gating the identified dPCR droplets in a new plot, identify a positive and a negative green fluorescent population of droplets. Using a green filter, the positive fluorescent population can be excited with a 488 nm laser and detected in the green channel (Fig.2). Plot fluorescence versus side scatter (SSC). Please notice that the "negative" population has a detectable fluorescent signal.

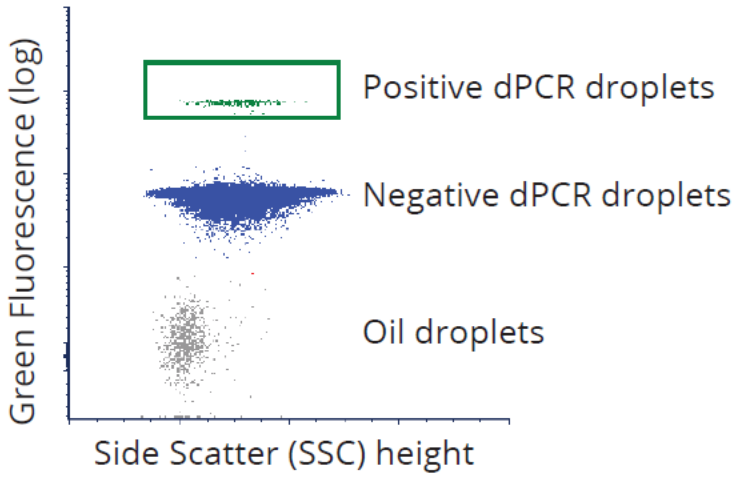

Fig. 2. Identification of positive fluorescent population versus side scatter. The positive population is green, the negative population blue and the oil droplets grey in this figure. Set the software to show at least 100.000 events in the plot.

3. Set the gates as detailed above (Fig.2), taking care to draw a strict gate around the population of interest. Note: be aware of potential drift in fluorescence over time and be prepared to move the sorting gate during the sorting if required.
4. Start sorting the positive population into the collection tube containing 15 µl H_2O. Remember to acquire data during the sort for your records.
5. After sorting keep the tube with sorted droplets at 4°C.

Note: Continue immediately to Multiple Displacement Amplification in droplets (dMDA)!

Note: Do not store sorted dPCR droplets longer than a few hours at 4°C as this will lead to loss of material and DNA integrity!

Depending on the input, the DNA recovered from the sorted positive droplets will be in the range of femtograms. The expected number of positive droplets can be calculated using Samplix online Enrichment Predictor at samplix.com. To amplify the sorted DNA, please continue with the isothermal amplification dMDA kit developed for the Xdrop™ technology. Please refer to the protocol as described in Chapter 5.

Frequently asked questions

1. How long time to sort and wash 8 samples and 4 samples?
   In a SONY sorter a standard run takes around 30 min to sort each sample. Washes in between samples take 15-20 min. This means that 8 samples can be processed in 6 hours and 40 min, 4 samples in 3 hours and 20 min. This timeframe is indicative for DB cell sorters and varies depending on cell sorter.

2. Can Samplix provide an example of FCS file in advance?
   Yes, an FCS file from a run with Samplix cell sorter control kit can be provided. Contact us at support@samplix.com.

3. Can Samplix provide specific guidance regarding wash settings?
   Washing with water and bleach in between samples provides the optimal cleaning, but line back-flush in between the samples provides acceptable results. The washing time is approximately 15-20 min. in a SONY sorter.

4. How should sorter temperature be set?
   Cell sorters should run at room temperature, please turn the temperature setting off.

5. Does the full run need to be recorded? Too many events might make the software crash.
   The instrument software (e.g. DB) might crash during a long recording of cell sorting. You can record at the beginning of the sorting, a couple of times in between the sorting and at the end. This is enough for troubleshooting.

6. Is sample agitation during sorting required? If available, turn the agitation on. Given the weight of the droplets, they can deposit on the tube, therefore the position of the probe at bottom of the tube is recommended.