# Detection and Sorting of droplets

Double emulsion droplets generated in the Xdrop instrument with the dPCR cartridge can be sorted and collected in a standard cell sorter, thereby 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 Detection Sequence (see Chapter 3 in the Xdrop™ manual).

## Requirements for flow cytometry 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 just above and near the bottom of the sample tube.

## Notes to operator

- dPCR droplets are stable and relatively heavy. Start with a high sample pressure to get the dPCR droplets into the channels. Be aware that it can take up to 5-10 min before the dPCR droplets reach the point of interrogation and appear on the plot.
- Due to the density of the dPCR droplets, they will quickly sediment at the bottom of the sample tube and will not remain in suspension during sorting. Make sure that the sample probe is positioned near the bottom of the sample tube.
- Use either SSC or FSC as threshold channel to exclude contaminating events (e.g. particles, pure oil droplets) (see Fig, 4.1).
- dPCR droplets have a diameter about 20 µm, therefore the correct events are high in Forward Scatter (FSC) and Side Scatter (SSC) (see Fig. 4.1, left). **Note:** The "height" setting more clearly displays the population of correct dPCR droplets.
- After staining with Droplet dye ● all dPCR droplets are fluorescent even droplets not containing the target ("negative" droplets) (see Fig. 4.1, right and Fig. 4.2).
- Doublet discrimination can be performed but is not required. Doublet discrimination will slightly decrease the number of dPCR droplets analyzed while not contributing greatly to sorting quality.
- 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. 4.2) A histogram plot is not recommended.
- A negative control sample is not required since an internal negative control will be present in all samples (see Fig. 4.2).

## Preparation of droplets for flow cytometry

1. Remove tubes with dPCR droplets from the PCR machine.
2. Make sure the 2x dPCR buffer ● is diluted with molecular grade water to 1x. Mix well by vortexing for 10 seconds or inverting the tube at least 20 times.
3. Stain droplets with Droplet dye ● as follows:

   - Prepare flow cytometry buffer by adding **1 ml** 1x dPCR buffer to a flow cytometry sample tube or 1,5 ml tube (tubes depend on flow cytometer instrument).
   - Spin down Droplet dye ● at 1,7g (5000 rpm with a minifuge) for 2 minutes.
   - Add **10 µl** Droplet dye ● into the flow cytometry tube with **1 ml** dPCR buffer. Mix gently to dissolve the dye in the dPCR-buffer.
   - Remove the supernatant from the PCR tubes containing the dPCR droplets leaving the droplets undisturbed at the bottom.
   - Use **200 µl** buffer from flow cytometry sample tube to transfer all droplets from the PCR tubes to the flow cytometry sample tube. Use tips that minimize binding of droplets to the sides of the tip.
   - Leave at room temperature protected from light for 5 min to stain droplets.

4. Add **15 µl** of molecular grade $H_2O$ into the bottom of a 1,5 ml DNA LoBind collection tube and place the collection tube in the appropriate holder in the cell sorter instrument.
5. Confirm that the sort settings for your cell sorter are correct. Check that the side stream is centered on the collection tube.
6. Load sample tube containing stained dPCR droplets in buffer on the flow cytometer and start analysing.

## Flow cytometry analysis and sorting of dPCR droplets

7. Identify the dPCR droplet population on a plot of FSC (height) versus SSC (height) (Fig. 4.1). dPCR droplets have higher side scatter (SSC) and fluorescence than oil droplets after staining. Note that it could take a few minutes before the heavy dPCR droplets are analyzed and visible in the plot.
8. Use side scatter (SSC) or forward scatter (FSC) as threshold channel and exclude events that are not dPCR droplets (Fig. 4.1).

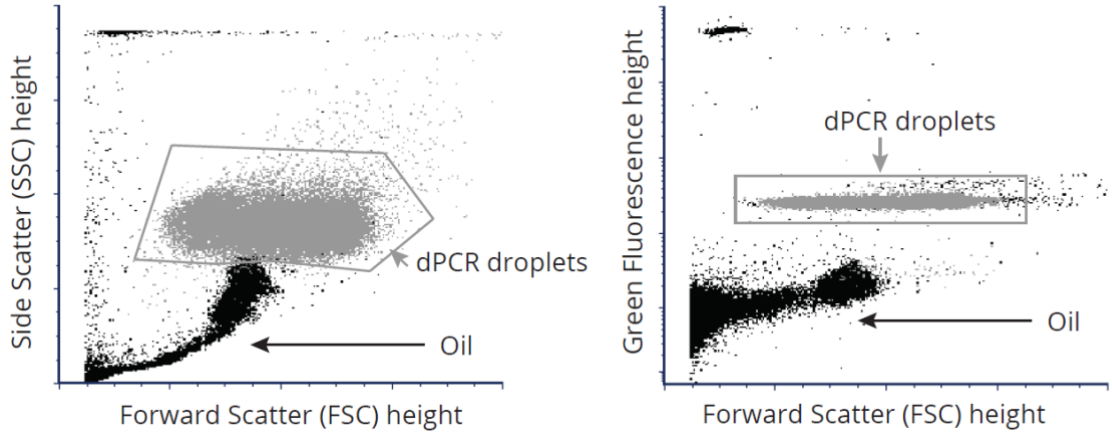



**Fig. 4.1. Identification of dPCR droplets.** Identify dPCR droplets. Left: on a plot showing forward scatter (FSC) versus side scatter (SSC) or back scatter (BSC). Right: on a plot showing green fluorescence as a function of FSC. Note that all dPCR droplets are slightly fluorescent after staining, including droplets not containing the

target of interest, whereas contaminating pure oil droplets have very low green fluorescence. The "height" setting more clearly displays the population of correct dPCR droplets.

9. 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. 4.2). Plot fluorescence versus side scatter (SSC). Please notice that the "negative" population of dPCR droplets has a detectable fluorescent signal.

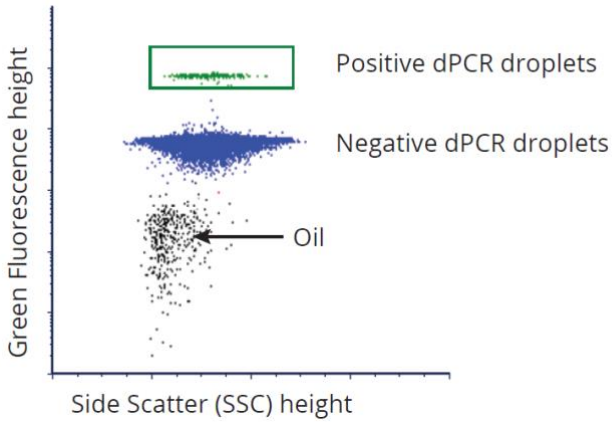



**Fig. 4.2.** Identify positive fluorescent population versus side scatter. In this figure, the positive population is shown in green, the negative population in blue and the oil droplets in grey. Set the software to display at least 100.000 events in the plot.

10. Set the gates as detailed above (Fig. 4.2), taking care to draw a strict gate around the population to be sorted. **Note:** be aware of potential drift in fluorescence over time and be prepared to move the sorting gate during the sorting if required.
11. Start sorting the positive population into the collection tube containing 15 µl molecular grade $H_2O$. Remember to acquire data during the sort for your records.
12. After sorting keep the tube with sorted droplets at 4°C.

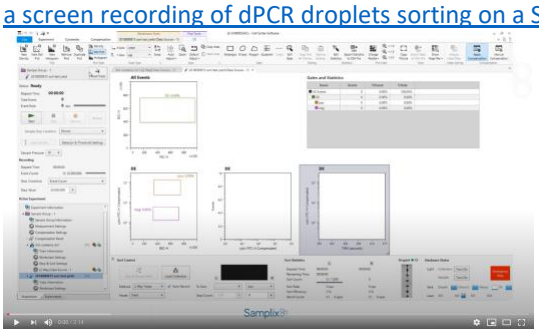
**Note:** Do not store sorted dPCR droplets longer than a few hours at 4°C as this will lead to loss of material and DNA integrity. Continue to Multiple Displacement Amplification in droplets (dMDA) as soon as possible.

Depending on the input, the amount of 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 multiple displacement amplification dMDA kit developed for the Xdrop™ technology. Please refer to the protocol as described in Chapter 5 in the Xdrop™ manual.

## Additional Information

You can watch here:

- a screen recording of dPCR droplets sorting on a SONY SH800 FACS



- a screen recording of dPCR droplets sorting on a BD Melody

## Frequently asked questions

1. **How long time to sort and wash 8 samples and 4 samples**?
   In a SONY SH800 sorter a standard run takes around 30 min to an hour 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 backflush in between the samples provides acceptable results. The washing time is approximately 15-20 min. in a SONY sorter. If there are concerns about oil deposits in the sample line a wash step with detergent can be performed however this is not required between runs.

4. **How should sorter temperature be set?**
   Cell sorters should run at room temperature, both sample and collection tube, 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 not be able to handle 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 will sediment fast (< 1 min), therefore positioning of the probe at the bottom of the tube is recommended for higher yield droplet sorting.

7. **Does dPCR samples lead to oil clogs/oil contamination of the lines?** We have never observed clogs or oil deposit in the sample. The dPCR droplets do not seem to break inside the instrument and we have not observed any problems with blocked nozzles on either a Sony SH800 sorter, BioRad S3e sorter or on BD sorters (Aria series and FACSMelody).