Samplix

Targeted enrichment of DNA with Xdrop® or Xdrop® Sort

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Xdrop and related Samplix technologies are for research use only, not for use in diagnostic procedures.

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Required Samplix items for the Xdrop or Xdrop Sort targeted enrichment workflow

For 20 µm DE20 droplet production

Name	Item no.		
Xdrop instrument or Xdrop Sort instrument	IN00100 IN00200		
Xdrop DE20 Cartridge	CADE20A100		Shipped at room temperature (RT) Store at 4°C upon receipt
Xdrop DE Gasket	GADEA100		
Storage film	FI00100		
DE PCR Kit	REFKITDE100		
	Part 1 (Store at –20°C) DE PCR mix (2x) ● DE droplet dye ●	Part 2 (Store at –20°C) DE PCR buffer (2x) ●	Part 3 (Store at RT and 4°C) Droplet oil (DE) ● (RT) Droplet break solution ● (RT) Droplet break color ● (4°C)
Primer test PCR kit	REFKITPRMPCR100 (Store at –20° C) DE PCR mix (2x) ● qPCR dye ●		
Positive control DNA enrichment kit	REFKITPOSCO100 (Store at –20°C) Positive control DNA ● dPCR control primers ● Enrichment validation primers ●		
Cell sorter control kit	REFKITCLLSRT100		
	Part 1 (Store at –20°C) DE PCR buffer (2x) ● DE droplet dye ●		Part 2 (Store at room temperature) Cell sorter control droplets ○

For single-emulsion droplet production for amplification of DNA

Name	Item no.	
Xdrop instrument	IN00100	
or Xdrop Sort instrument	IN00200	
Xdrop instrument	IN00100	
Xdrop SE85 Cartridge	CASE85A100	
Xdrop SE85 Holder	HOSE85A100	
Xdrop SE Gasket	GASEA100	
Storage film	FI00100	
SE MDA Kit	REFKITSEMDA100	
	Part 1	Part 2
	(Store at –20°C)	(Store at RT and 4°C)
	SE MDA mix (5x) 🔴	Droplet oil (SE) 🟡 (RT)
	SE MDA enzyme ○	Droplet break solution 🟢 (RT)
		Droplet break color 🟢 (4°C)

Targeted enrichment of DNA with Xdrop or Xdrop Sort

This workflow enables high-resolution exploration of any genomic region or gene cluster. It captures and amplifies a region of interest without the need for long-range PCR. Specific primers amplify a short fragment of 120–160 bp (the detection sequence) within the target or in flanking regions. This short amplicon is used to capture the kilobases-long region of interest, which can contain repeat regions, GC-rich regions, or regions that are otherwise difficult to amplify. The yield is enriched long DNA fragments (over 100 kb, depending on the size of the starting material) from small amounts of sample input, suitable for unbiased long-read or short-read sequencing (Fig. 1).

Combined with the appropriate sequencing technology, the targeted enrichment workflow with Xdrop or Xdrop Sort supports a range of genomic investigations, including:

- Characterization of edits in engineered genes
- Identification of insertions, gene duplications, and conserved regions
- Enrichment and amplification of poorly characterized regions
- Discrimination of genes and pseudogenes
- Resolution of tandem repeats
- Closure of gaps in gene sequences

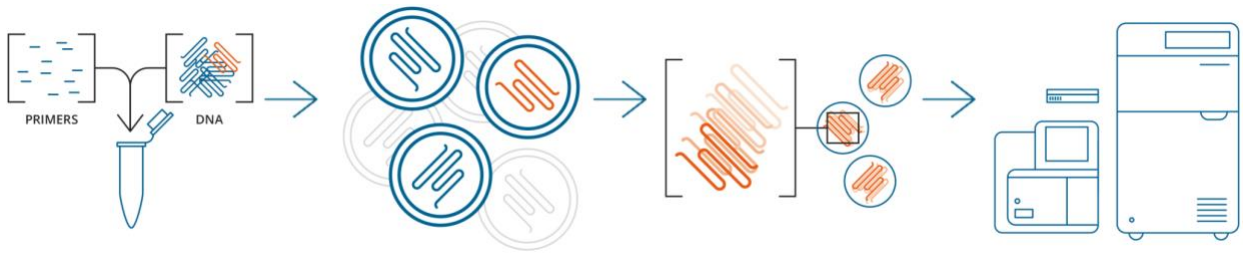

Fig. 1. The Xdrop targeted enrichment workflow. Start with high molecular weight DNA. The yield is enriched long DNA fragments suitable for long- or short-read sequencing.

Assay design and DNA sample preparation

The length of the final enriched target DNA depends on the length of the input DNA fragments. When extracting the DNA sample, use a method that maintains the integrity and purity of the DNA.

Note: If more than 200 ng of input DNA is available, we recommend a bead-based purification before starting with the targeted enrichment workflow. Any bead-based purification kit is suitable. Make sure to follow the manufacturer's recommendations. We recommend performing the final elution at 55°C for 3 minutes to ensure that high molecular weight DNA is released from the beads.

Take all required measures to avoid contamination. Enrichment can be affected by contamination of the DNA sample with RNA, proteins, carbohydrates, salts, and phenol, among other substances. Purify the DNA to the same quality as required for long-read sequencing.

Targeted enrichment with Xdrop or Xdrop Sort has a simple assay design with the following components:

- High molecular weight DNA samples (>30 kb, depending on the assay) of high purity. Calculate the required amount of input DNA using the DNA input calculator at samplix.com/calculations.
- One primer pair for the detection sequence, used for identifying and enriching the region of interest during droplet PCR (Fig. 2). Please see the design guidelines below and use the primer design tool at samplix.com/primer.
- One primer pair for the validation sequence, used to validate the enrichment via quantitative PCR (qPCR; Fig. 2). Please see the design guidelines below and use the primer design tool at samplix.com/primer.

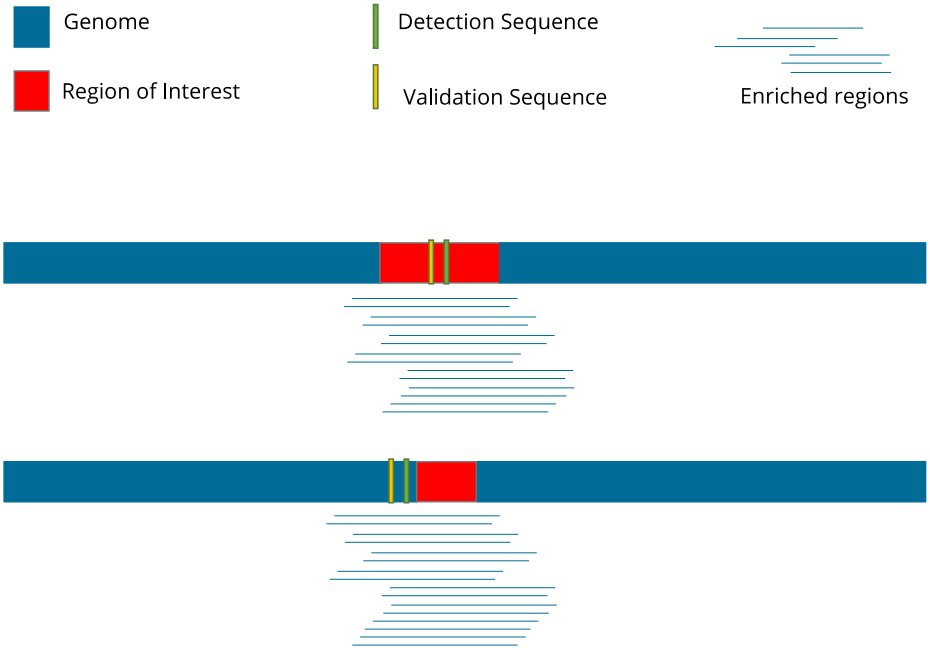

Fig. 2. Targeted enrichment of a region of interest. Design a standard primer set for the short detection sequence within (top) or flanking (bottom) your region of interest. A primer set for a validation sequence is designed to evaluate the enrichment at the end of the workflow.

Primer design guidelines

Targeted enrichment with Xdrop or Xdrop Sort relies on two sets of carefully designed, highly specific and non-overlapping PCR primer pairs. The first one, called the droplet PCR primer set, amplifies the detection sequence. After staining, this amplicon generates a fluorescent signal that is used for selection of the target. The second set of PCR primers, called the validation qPCR primer set, amplifies the validation sequence, which is used to validate the assay and quantify the number of target fragments in the pool of enriched fragments.

If the target spans over a region of 50 kb or longer, a combination of two or more sets of droplet PCR and validation qPCR primers are recommended. Additional help to design primers can be found in the primer design tool at samplix.com/primer.

	Droplet PCR primers (Detection sequence)	Validation qPCR primers (Validation sequence)
Amplicon length	120–160 bp	80–120 bp
Melting temperature	~60°C	~60°C

Note: The validation qPCR primer pair must be different from the detection droplet PCR primer pair and placed within 2 kb of it. The amplicons of the droplet PCR and the validation qPCR must not overlap.

The risk of false-negative enrichment prediction increases if the validation sequence is placed more than 2 kb away from the detection sequence.

The following general guidelines apply to both droplet PCR and validation qPCR primer pairs:

- Avoid primer pairs with more than 2°C difference in melting temperature between the forward and reverse primer.
- Avoid placing primers in low complexity regions.
- Primers need to be specific. Avoid primer pairs that amplify sequences not related to the target sequence.
- Follow the general recommendations for PCR primer design: avoid self-complementarity, stable secondary structures, hairpins, etc.

Primer testing

For every new DNA target and prior to generating droplets for targeted enrichment, both the droplet PCR primer pair and the validation qPCR primer pair must be optimized and checked, preferably via qPCR, using your sample DNA and Samplix droplet PCR reagents: DE PCR mix (2x) ● and qPCR dye ●. For this assay, include a negative control with no DNA template and at least three different concentrations of your DNA template (or a biological replicate of this) in the range of the amount of input DNA suggested by the DNA input calculator at samplix.com/calculations.

Use the Samplix Primer test PCR kit (Item no. REFKITPRMPCR100), which includes DE PCR mix (2x) ● and qPCR dye ●, to verify the primers and reaction efficiency.

Droplet PCR mix	**1 reaction**
Water (molecular grade)	7.2 µl
DE PCR mix (2x) ●	10 µl
Primer forward 10 µM	0.4 µl
Primer reverse 10 µM	0.4 µl
qPCR dye (20x) ●	1 µl
Total mix	**19 µl**
Template DNA	1 µl
Total volume	20 µl

And the following qPCR program:

Temperature	**Duration**	**Cycles**
30°C	5 sec	1x
94°C	3 min	
94°C	3 sec	40x
Annealing temp.	30 sec	
Data acquisition*	-	
Melting curve 70–90°C		1X

*Follow the manufacturer's protocol for the data acquisition program. The maximum excitation and emission values for the supplied qPCR dye (20x) ● are respectively approximately 497 nm and 520 nm (SYBR™ green settings).

Note: The annealing temperature needs to be adjusted to the specific primer sets tested.

Note: The validation qPCR primers can be tested with either Samplix DE PCR mix as above or qPCR reagents from other suppliers.

We recommend running a melting curve analysis with the template DNA, droplet PCR primers, and Samplix reagents to check for the presence of alternative amplicons and primer–dimers. Consider running a temperature gradient to determine the optimal annealing temperature.

The maximum excitation and emission values for the supplied qPCR dye (20x) ● are found at approximately 497 nm and 520 nm respectively (SYBR™ green settings). Make sure these values are selected on your qPCR instrument.

Calculate the PCR efficiency by plotting the logarithm of the starting quantity of DNA on the X axis and the corresponding Ct values on the Y axis as shown the right panel of Figure 3. Calculate the slope of the trend line for this plot and use this as input for the formula: Efficiency = (10^(-1/slope)-1) *100%

Make sure that your designed primer pairs have an efficiency between 80 and 120%, and that the suggested DNA input amount yields a Ct value below 30 (Fig. 3).

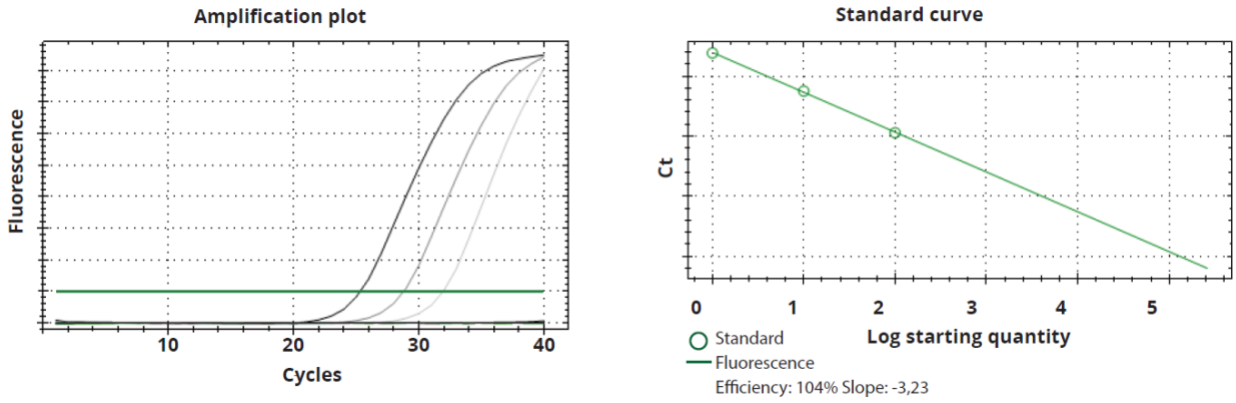

Fig. 3. Calculate PCR efficiency with at least three different concentrations of input DNA using the Samplix Primer test PCR kit (Item no. REFKITPRMPCR100) and your designed primers. Left: Amplification plot of three DNA concentrations. Right: Standard curve and calculations of PCR efficiency.

For a single target in a genome that is the size of the human genome, we recommend the following DNA amounts for the standard curve: 0.1, 1 and 10 ng; or 0.2, 2 and 20 ng. For smaller or larger genomes or a different number of targets per genome, these amounts need to be adjusted.

Samplix provides a Positive control DNA enrichment kit (Item no. REFKITPOSCO100) that contains template DNA and two primer pairs: one for droplet PCR and one for validation qPCR after droplet MDA. See instructions on how to use the Positive control DNA enrichment kit on the next pages.

Droplet PCR

Use the Samplix Primer test PCR kit (Item no. REFKITPRMPCR100) to determine the optimal primer concentration and annealing temperature for droplet PCR, as described in the previous chapter.

Note: For this workflow, do not use reagents other than the Samplix DE PCR Kit (Item no. REFKITDE100) for Xdrop or Xdrop Sort DE droplet production as this may compromise droplet production, droplet stability, and downstream enrichment.

Setup of droplet PCR

1. Prepare the droplet PCR mix following the table below.

2. Prepare one single master mix for all reactions (38 µl mix per sample). Keep the reagents and the master mix on ice (except the Droplet oil (DE) ●, which should be kept at room temperature).

3. Prepare dilutions of the correct amount of template DNA. Calculate the optimal amount of input template DNA using the online DNA input calculator.

4. Add **2 µl** of the correct amount of template DNA to **38 µl** droplet PCR mix to get 40 µl/reaction in total.

5. Allow the reaction mix to reach room temperature before loading it onto the Xdrop DE20 Cartridge as described in Xdrop Manual v. 6.1 or later (for Xdrop users) or in Xdrop Sort Manual v. 4.0 or later (for Xdrop Sort users). Immediately insert the loaded cartridge into the instrument.

Droplet PCR mix	**1 reaction**
Water (molecular grade)	16.4 µl
DE PCR mix (2x) ●	20 µl
Droplet PCR primer forward 10 µM	0.8 µl
Droplet PCR primer reverse 10 µM	0.8 µl
Total mix	38 µl

Template DNA	2 µl
Total volume	40 µl

Optional: Positive control DNA enrichment droplet PCR (Item no. REFKITPOSCO100)

Samplix provides a Positive control DNA enrichment kit (Item no. REFKITPOSCO100) that can be used as a control reaction along with your samples and primers. The kit includes both positive control DNA ● and pre-mixed dPCR control primers ● with an annealing temperature of **60°C**.

Droplet PCR mix	1 reaction
Water (molecular grade)	16.4 µl
DE PCR mix (2x) ●	20 µl
dPCR control primers ●	1.6 µl
Total mix	**38 µl**

Positive control DNA ●	2 µl
Total volume	40 µl

The Enrichment validation primers ● are also provided with the Positive control DNA enrichment kit to validate enrichment. Like the dPCR control primers, forward and reverse primers are already mixed.

DE droplet production

Produce DE20 droplets containing your droplet PCR mix as described in Xdrop Manual v. 6.1 or later (for Xdrop users) or in Xdrop Sort Manual v. 4.0 or later (for Xdrop Sort users).

After production, as noted in the manuals, collect the droplets into a 0.5 ml or 1.5 ml tube, then mix gently and dispense into four PCR-tube aliquots (of approximately **80–90 µl** each).

DE20 droplets sediment rapidly during handling. To ensure equal distribution into the aliquots, mix gently by pipetting up and down between each pipetting step.

Droplet PCR reaction

1. Place the tubes in a thermal cycler and run the program shown in the table. While we recommend a block temperature ramp rate of 0.5°C/sec, with some cyclers, ramp rates of up to 1.5°C/sec may be necessary. This depends on the ramp rate for the cycler. The total program duration should be 2 hours. This slow ramping ensures the reagents inside the DE20 droplets reach the desired temperature.
2. Set the lid temperature to 105°C.

Temperature	Duration	Ramp rate	Cycles
30°C	5 sec	-	1x
94°C	3 min*	Block ramp 0.5°C/sec	
94°C	3 sec	Block ramp 0.5°C/sec	40x
Annealing temp.	30 sec	Block ramp 0.5°C/sec	
4°C	∞	-	-

*Increase initial denaturation step run at 94°C up to 5 min for GC-rich detection sequences.

3. Samplix DE20 droplets can be stored after PCR at 4°C for up to 24 hours if proceeding with sorting and droplet MDA amplification. DE droplets are stable at 4°C for up to 2 months, but storage longer than 24 hours can compromise DNA quality and enrichment.

Detection and sorting of DE20 droplets

DE20 droplets generated using Xdrop or Xdrop Sort with the Xdrop DE20 Cartridge can be sorted and collected on Xdrop Sort using the Xdrop DE20 Sort Cartridge or on a standard cell sorter. This sorting ensures that you capture the DNA of interest. In this step, the positive DE20 droplets containing the material of interest are identified and separated from the negative droplets using the fluorescent signal created by the detection sequence and the DE droplet dye 🟠.

Workflow for Xdrop Sort

Please refer to chapter 3 of the Xdrop Sort Manual v. 4.0 or later for the details of this workflow. It is important to include the steps described in the section "Staining of DNA inside DE20 droplets" in that chapter. No expertise with flow cytometry is needed for the Xdrop Sort workflow. The sorting of DE20 droplets containing DNA with a detection sequence follows the procedure described in the manual. After sorting, proceed to breaking the droplets.

Workflow for flow cytometry sorting

Requirements from a flow cytometry cell sorter

- A 488 nm (blue) laser
- Optical configuration (i.e., laser, filters, mirrors, detectors) set to detect fluorophores excited at 488 nm, such as FITC or GFP (or PE)
- 100 µm nozzle tip or sorting chip
- A sample probe that can be positioned just above the bottom of the sample tube

Notes to operator

- DE20 droplets are stable and relatively heavy. Start with a high sample pressure to draw the droplets into the channels of the flow cytometer. It can take up to 5–10 min before the DE20 droplets reach the point of interrogation and appear on the plot.

- Due to the density of the DE20 droplets, they will quickly sediment at the bottom of the sample tube and will not remain in suspension during sorting. This is usually not a problem. Make sure that the sample probe is positioned just above the bottom of the sample tube.

- Use forward scatter (FSC) as threshold channel to exclude contaminating events (e.g., particles or pure oil droplets; see Fig. 4, left).

- DE20 droplets have a diameter of about 20 µm, the correct events are high in forward FSC and side scatter SSC (Fig. 4, left). **Note:** The "height" setting more clearly displays the population of DE20 droplets.

- After staining with DE droplet dye ●, all DE20 droplets are fluorescent, even droplets not containing the target ("negative" droplets). However, "positive" droplets are 10- to 30-fold brighter (Fig. 4, right).

- Doublet discrimination can be performed but is not required. DE20 droplets do not stick together.

- Positive fluorescent droplets are likely to be very rare (possibly less than 0.02% of the total number of DE20 droplets, depending on the assay). Therefore, a positive population can be difficult to identify. Make sure the live plot of fluorescence shows at least 100,000 events (Fig. 4, right). A histogram plot is not recommended as the positive fluorescent droplets will be difficult to identify.

- A negative control sample is not required since an internal negative control is present in all samples in the form of droplets that do not contain the target region (Fig. 4, right).

Preparation of droplets for flow cytometry

1. Remove the tubes with the DE20 droplets from the PCR cycler.

2. Make sure the 2x DE PCR 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 DE droplet dye ● as follows:

    - Prepare flow cytometry buffer by adding **1 ml** 1x DE PCR buffer to a flow cytometry sample tube or 1.5 ml tube (depending on the flow cytometer instrument).

- Spin down DE droplet dye ● at 5,000 rpm (minifuge, or 1,700 g) for 2 minutes.
- Add **10 µl** DE droplet dye ● into the flow cytometry tube with **1 ml** 1x DE PCR buffer. Mix gently to dissolve the dye in the DE PCR buffer.
- Remove the supernatant from the PCR tubes containing the DE droplets leaving the droplets undisturbed at the bottom.
- Use **200 µl** buffer from the flow cytometry sample tube to transfer all droplets from the PCR tubes to the flow cytometry sample tube. Use tips that minimize the binding of droplets to the sides of the tip.
- Leave the mix of DE20 droplets and DE PCR buffer + DE droplet Dye at room temperature and protected from light for 5 minutes to stain droplets.

4. Add **15 µl** molecular-grade water 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 the sample tube containing the stained DE20 droplets in buffer onto the flow cytometer and start analyzing.

Flow cytometry analysis and sorting of DE20 droplets

1. Identify the DE20 droplet population on a plot of FSC (height) versus SSC (height) (Fig. 4, left). DE20 droplets have higher side scatter (SSC) and fluorescence than oil droplets. Note that it could take a few minutes before the heavy DE20 droplets are analyzed and visible in the plot.

2. Use side scatter (SSC) or forward scatter (FSC) as threshold channel and exclude events that are not DE20 droplets (Fig. 4, left).

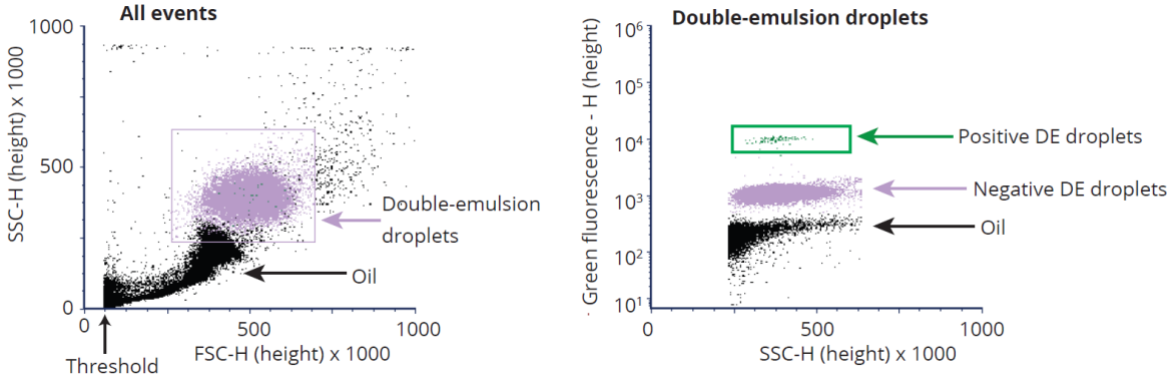

Fig. 4. Identification of DE20 droplets. Left: Gate DE20 droplets on a plot showing forward scatter (FSC) versus side scatter (SSC) or back scatter (BSC). Right: Identify positive fluorescent DE20 droplets on a plot showing green fluorescence versus SSC. Note that all DE20 droplets are slightly fluorescent after staining, including droplets not containing the target of interest ("negative"), whereas contaminating pure oil droplets have low green fluorescence. The "height" setting more clearly displays the population of DE20 droplets.

3. Gating the identified DE20 droplets in a new plot, identify a positive and a negative green-fluorescent population of droplets. The positively fluorescent population can be excited with a 488 nm laser and detected in the green channel (Fig. 4, right). Plot fluorescence versus side scatter (SSC). Please notice that the "negative" population of DE20 droplets has a detectable fluorescent signal.

4. Set the gates as detailed above (Fig. 4, right), 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.

5. Start sorting the positive population into the collection tube containing 15 µl molecular-grade water. Remember to acquire data during the sort for your records.

6. After sorting, keep the tube with sorted droplets at 4°C.

Note: Do not store sorted DE20 droplets longer than a few hours at 4°C as this will lead to a loss of material and decreased DNA integrity. Continue with multiple displacement amplification in droplets (droplet MDA) 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. To amplify the sorted DNA, continue with the multiple displacement amplification droplet MDA kit developed for Xdrop. Please refer to the protocol as described in Xdrop Manual v. 6.1 or later (for Xdrop users) or in Xdrop Sort Manual v. 4.0 or later (for Xdrop Sort users) for producing singe-emulsion droplets and see below for setting up droplet MDA reaction.

Optional: Set up flow cytometry with Cell sorter control kit (item no. REFKITCLLSRT100)

To easily set up flow cytometry of DE20 droplets, use the Samplix Cell sorter control kit (Item no. REFKITCLLSRT100). This kit consists of ready-made DE20 droplets with a defined and large population of positive droplets. The kit should assist you to establish the flow cytometry settings for DE20 droplet sorting.

1. Make sure the 2x DE PCR buffer ● is diluted with molecular-grade water to 1x. Mix well by vortexing for 10 seconds or inverting the tube at least 20 times.

2. Stain the Cell sorter control droplets ○ with DE droplet dye ● as follows:

   - Prepare flow cytometry buffer by adding **500 µl** 1x DE PCR buffer to a flow cytometry tube (tubes depend on flow cytometer instrument).

   - Spin down the vial of DE droplet dye ● at 5,000 rpm (minifuge, or 1,700 g) for 2 minutes.

   - Add **5 µl** DE droplet dye ● into the flow cytometry tube with 1x DE PCR buffer. Mix gently to dissolve the dye in the droplet PCR buffer.

   - Resuspend the Cell sorter control droplets ○ by vortexing gently and transfer **50 µl** to the prepared flow cytometry tube. Use tips that minimize the binding of droplets to the side of the tip.

   - Leave the mix of DE20 droplets and DE PCR buffer + DE droplet dye at room temperature and protected from light for 5 minutes to stain droplets.

3. Load the tube onto a flow cytometry cell sorter and start the analysis.

4. Identify the DE20 droplets as described above (see Fig. 5, left).

5. Identify positive and negative fluorescent droplets as described above (see Fig. 5, right). With the Cell sorter control droplets, you can expect the fraction of positive droplets to be about 10–30% of the total observed DE20 droplets.

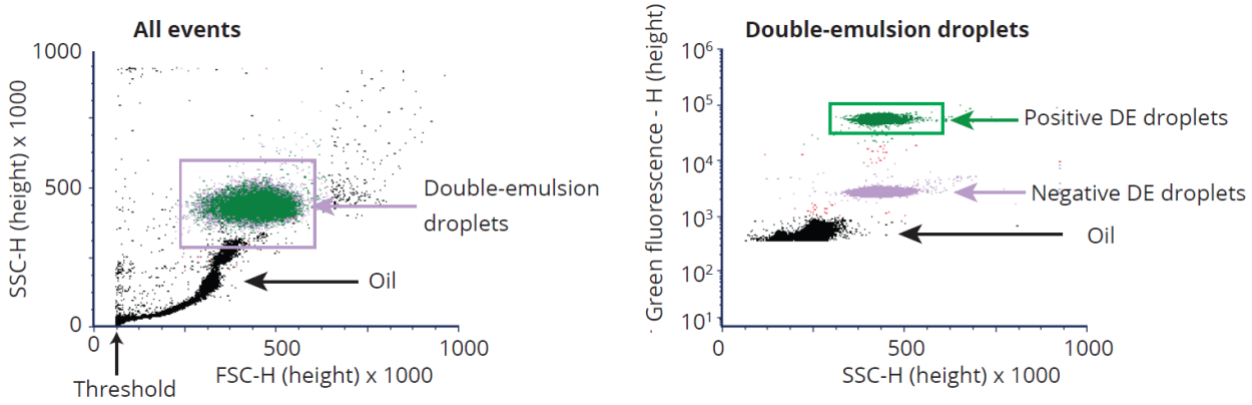

Fig. 5. Set up droplet sorting with Cell sorter control kit. Left: Identify DE20 droplets. Gate DE20 droplets on a plot showing forward scatter (FSC) versus side scatter (SSC) or back scatter (BSC). Right: Identify positive and negative DE20 droplets on a plot showing green fluorescence versus SSC. Note that all DE20 droplets are slightly fluorescent after staining, including droplets not containing the target of interest ("Negative"), whereas contaminating pure oil droplets have low green fluorescence. The "height" setting more clearly displays the population of DE20 droplets.

Break the sorted DE20 droplets

For targeted enrichment of DNA, the contents of sorted DE20 droplets must be released by breaking the droplets. This is done with Droplet break solution ● and Droplet break color ● as described here.

Note: Before starting, vortex the Droplet break color ● tube upside down and then spin the tube. This is required to ensure that the reagent is fully homogenized.

1. Add **20 µl** Droplet break solution ● to each tube of sorted DE20 droplets.

2. Add **1 µl** of Droplet break color ●. This will color the water phase. If coloring is too weak, add 1 µl extra of Droplet break color. **Note:** The water phase may be a color ranging from yellow to purple as the Droplet break color is functioning as a pH indicator as well.

3. Flick tube gently, **do not vortex**.

4. Spin tube briefly (15–30 seconds).

5. Remove the clear Droplet break solution phase from the bottom of the tube and discard.

6. Repeat steps 3–5 to remove all leftover Droplet break solution. It is important to remove all the Droplet break solution as residual Droplet break solution may inhibit downstream enzymatic reactions.

7. Keep the colored water phase, which will contain the amplified DNA (Fig. 6).

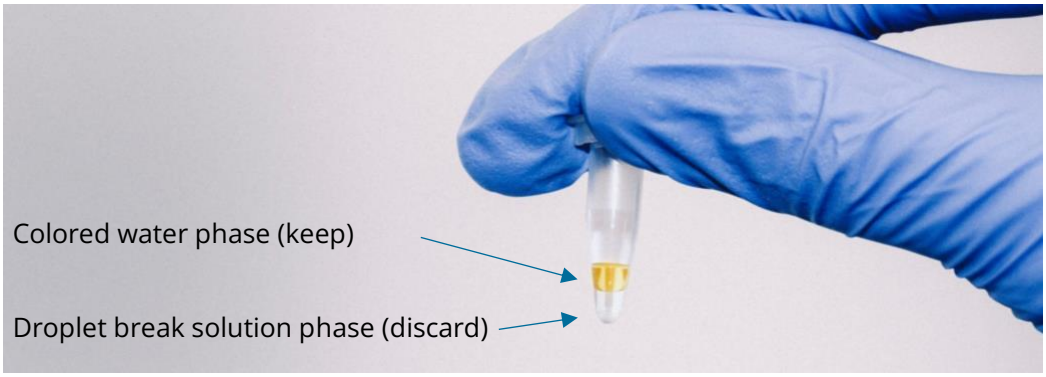

Fig. 6. Break the sorted DE20 droplets with Droplet break solution ● and Droplet break color ●. Discard the clear Droplet break solution phase at the bottom of the tube. Keep the top, colored water phase that will contain your DNA.

Droplet MDA

Produce single-emulsion droplets

Produce single-emulsion droplets as described in the Xdrop Manual version 6.1 or later (for Xdrop users) or in the Xdrop Sort Manual version 4.0 later (for Xdrop Sort users).

Set up droplet MDA reaction

Note: Do not use any other reagents than Samplix SE MDA Kit (Item no. REFKITSEMDA100) for Xdrop SE droplet production as this may compromise droplet production, droplet stability, and downstream enrichment. Thaw and keep all reagents at 4°C or in a cooling block while setting up the reactions, except the Droplet oil (SE) 🟡, which that should be kept at room temperature.

Note: The MDA reaction is very susceptible to contamination. Make sure to avoid DNA contaminations of any kind.

Prepare the amplification mix following the table below in an LAF hood or similar clean, dust-free environment.

1. Prepare the amplification mix (see table below). Mix gently, do not vortex.

2. Aliquot the mix in a cooling block. **Important!** Keep cold at 4°C until needed.

3. Add **10 µl** template, which can be either the DNA from sorted DE droplets after breaking or 1 pg total genomic DNA.

4. The breaking of droplets described in the previous chapter will result in the volume of the DNA phase being larger than 10 µl. Dilute the template with molecular-grade water to a total volume of **20 µl** to include an extra MDA reaction for each sample. This will increase the DNA yield from the droplet MDA.

5. We recommend including the following control reactions in addition:

   10 µl of molecular-grade water (negative control)

   10 µl of sheath fluid from cell sorter (contamination control), if a cell sorter was used to sort the DE20 droplets

   10 µl of 0.1 pg/µl genomic DNA (positive control) *

   *Ensure the positive control is prepared from a commercial DNA sample of known concentration and is diluted using molecular-grade water to 0.1 pg/µl through serial dilutions, in at least two steps. If you start from a 100 ng/µl stock, dilute 1:1000 to 0.1 ng/µl followed by another 1:1000 dilution to obtain 0.1 pg/µl. Note that you can also use the DNA from the Positive Control Kit (REFKITPOSCO100): Make a dilution 1:50 from the 5 ng/µl stock solution to 0.1 ng/µl followed by a 1:1000 dilution to obtain 0.1 pg/µl.

6. Mix gently and keep cold at 4°C until you're ready to load it on the Xdrop SE85 Cartridge as described in the Xdrop Manual v. 6.1 or later (for Xdrop users) or in the Xdrop Xdrop Sort Manual v. 4.0 or later (for Xdrop Sort users).

Note: When pipetting from your DNA sample after droplet break, always pipette from the center of the colored phase as an additional precaution to avoid carrying over remaining Droplet break solution.

Amplification mix	**1X**
Water (molecular grade)	5 µl
SE MDA mix (5x) ●	4 µl
SE MDA enzyme ○	1 µl
Total mix	**10 µl**
Template DNA solution	10 µl

Produce single-emulsion droplets as described in the Xdrop Manual v. 6.1 or later (for Xdrop users) or in the Xdrop Sort Manual v. 4.0 or later (for Xdrop Sort users).

When droplets are produced, collect them in a PCR tube as described in the Xdrop Manual v. 6.1 or later (for Xdrop users) or in the Xdrop Sort Manual v. 4.0 or later (for Xdrop Sort users).

Incubate the droplets in a thermal cycler at 30°C for 16 hours followed by 10 minutes at 65°C. Run the following program on a thermal cycler:

Temperature	Duration
30°C	16 hours
65°C	10 min
4°C	∞

Note: Set the lid temperature of the thermal cycler to 75°C to avoid over-heating of the sample.

Break the SE droplets and quantify total DNA

After SE droplet incubation, release DNA from the SE droplets with Droplet break solution ● and Droplet break color ● as described below (Fig. 7).

Note: Before starting, vortex the Droplet break color ● tube upside down and then spin the tube. This is required to ensure that the reagent is fully homogenized.

1. Add **20 µl** Droplet break solution ● to each tube.
2. Add **1 µl** of Droplet break color ●. This will color the water phase. If coloring is too weak, add 1 µl extra of Droplet break color. **Note:** The water phase may be a color ranging from yellow to purple as the Droplet break color is functioning as a pH indicator as well.
3. Flick tube gently, **do not vortex**.
4. Spin tube briefly (15–30 seconds).
5. Remove the clear Droplet break solution phase from the bottom of the tube and discard.
6. Repeat steps 3–5 to remove all leftover Droplet break solution. It is important to remove all the Droplet break solution as residual Droplet break solution may inhibit downstream enzymatic reactions.
7. Keep the colored water phase, which will contain the amplified DNA (Fig. 7).

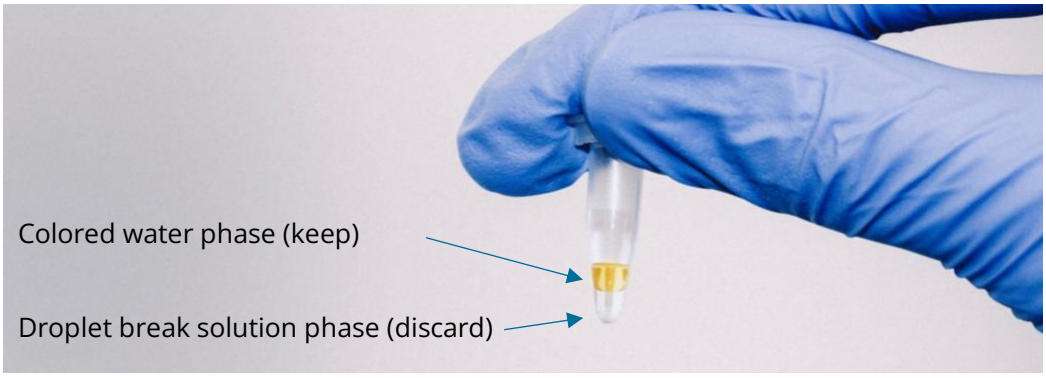

Fig. 7. Break the sorted DE20 droplets with Droplet break solution ● and Droplet break color ●. Discard the clear Droplet break solution phase at the bottom of the tube. Keep the top, colored water phase that will contain your DNA.

Measure the total amount of enriched and amplified DNA using a sensitive method such as with Qubit™, Bioanalyzer™, TapeStation™, FEMTO Pulse™, or similar. If possible, evaluate the size of sorted and amplified DNA fragments.

Note: If the total yield of DNA recovered after amplification is too low for your subsequent sequencing requirements, you can perform a reamplification. Prepare a 10 µl amplification mix with enzyme as described above and add it to the broken single-emulsion droplets. Incubate for 2 hours at 30°C, then for 10 min 65°C to inactivate the droplet MDA enzyme. Set the lid temperature of the thermal cycler for the MDA reamplification to 75°C. This should increase the yield by 3- to 5-fold. This short reamplification of the DNA outside droplets for 2 hours should not increase the number of DNA chimeras or decrease the DNA fragment length.

Evaluate the enrichment of your target DNA

After quantification of total DNA, continue with measurement of targeted enrichment. Calculate the DNA enrichment based on qPCR using the online tool at samplix.com/calculations as described below.

To estimate the fold enrichment of target DNA, perform a qPCR using the validation qPCR primers, not overlapping with the droplet PCR enrichment amplicon(s). See the section Assay design and DNA sample preparation in the first chapter of this guide and the online primer design tool at samplix.com/primer. If you require a specific region of DNA, place the validation qPCR primer pair on the opposite side of this region but within a maximum 5 kb distance from the detection sequence defined by the droplet PCR primers. The enrichment measured by qPCR is only indicative and might differ from the enrichment measured by sequencing of the entire enriched region.

To quantify the enrichment, perform a standard qPCR using the Samplix primer test PCR kit (Item no. REFKITPRMPCR100) or your own preferred qPCR reagents.

Set up a qPCR using the following DNA as a template:

- Droplet MDA amplified DNA, 1:10 dilution (enriched sample)
- Non-template control (NTC), 1:10 dilution (negative control)
- Original sample input DNA in the same concentration as input in droplets

Suggested controls:

- Sheath fluid from cell sorter, 1:10 dilution (contamination control) if droplets are sorted by a cell sorter
- Droplet MDA of 1 pg non-sorted input DNA, 1:10 dilution (positive control)
- Water (negative PCR control)

Note: When taking aliquots of your sample DNA from SE droplets after breaking, always pipette from the center of the colored phase as an additional precaution to avoid carrying over remaining droplet break solution.

Calculate fold enrichment of target DNA

Use the “Enrichment calculator” at samplix.com/calculations (Fig. 8) to calculate the fold enrichment of target DNA.

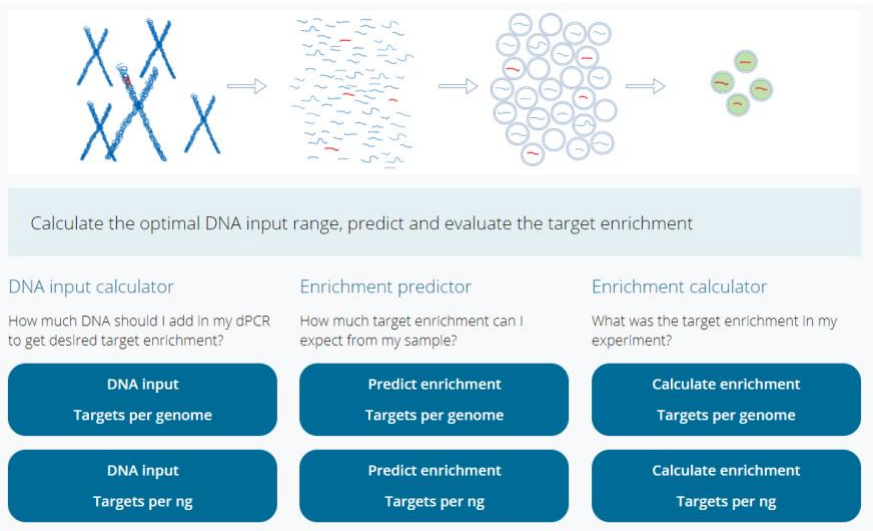

Fig. 8. At samplix.com/calculations , choose one of the two “Enrichment calculator” tools shown on the right. Enrichment can be calculated based on the number of targets per genome or the number of targets per nanogram of input DNA.

Example: Calculate enrichment based on targets per genome

If you select the tool “Calculate enrichment based on targets per genome” at samplix.com/calculations (Fig. 8), the following information is required (Fig. 9):

- *Genome size*: Size of (host) genome in base pairs.
- *PCR efficiency*: Efficiency of PCR in %. You can use 100% efficiency if the primers used initially provided an efficiency of 80–120% during the initial testing.
- *Targets per genome*: Copy number of the target in host genome disregarding off-targets.

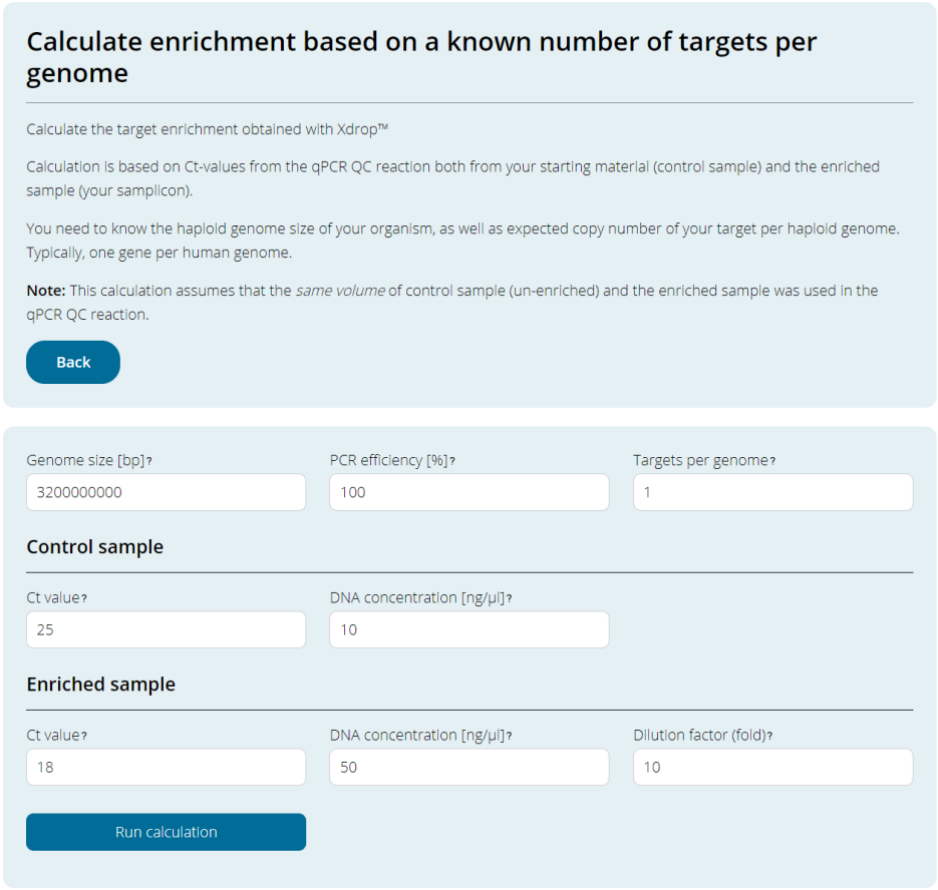

Fig. 9. Example of a calculation using the online enrichment calculator tool at samplix.com/calculations. See explanation in the text.

Control sample

- *Ct value*: Ct value from qPCR of 1x reference DNA (original sample input DNA).
- *DNA concentration*: Concentration of reference DNA in control/un-enriched stock solution.

Enriched sample

- *Ct value*: Ct value from qPCR using the product from droplet MDA reaction as input.
- *DNA concentration*: Concentration of DNA in the enriched sample after amplification (i.e., concentration of DNA after the droplet MDA reaction) measured using Qubit™, Bioanalyzer™, TapeStation™, FEMTO Pulse™ or similar.

- *Dilution factor*: Factor of dilution of droplet MDA reaction in qPCR (1:10 fold dilution recommended).

The calculator will provide you with the assessment of a successful enrichment of your DNA sample and the estimated concentration of target after enrichment.

After enrichment verification, you can proceed to prepare your DNA libraries for the desired sequencing platform. Xdrop enriched DNA is compatible with both long- and short-read sequencing technologies. Note that the enrichment measured by qPCR is only indicative and might differ from the enrichment measured by sequencing of the entire enriched region.