Samplix

DNA targeted enrichment with Xdrop® or Xdrop® Sort

Protocol

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Chapter 1: Introduction

DNA Targeted enrichment with Xdrop

The Xdrop targeted enrichment protocol enables high-resolution analysis of specific genomic regions by capturing and amplifying areas of interest without requiring long-range PCR. This process uses specific primers to amplify a short 120–160 bp detection sequence located within or adjacent to the target region. This short amplicon guides the capture of kilobase-scale regions of interest, including challenging features like repeat sequences, GC-rich areas, and other hard-to-amplify sections. The result is enriched, long DNA fragments—often exceeding 100 kb, depending on input sample—suitable for unbiased long-read or short-read sequencing (**Figure 1.1**).

Combined with sequencing technologies, the Xdrop or Xdrop Sort enrichment workflow supports a range of genomic investigations, such as:

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

Figure 1.1. Targeted enrichment with Xdrop or Xdrop Sort. The workflow enables the targeted enrichment of specific DNA sequences from complex samples, ensuring that only relevant regions are amplified for sequencing.

In this workflow, Xdrop or Xdrop Sort encapsulates individual DNA fragments, specific primers, and reagents into droplets, which are thermally cycled to amplify target-specific amplicons. When stained, these emit a detectable signal. Droplets containing the DNA sequence of interest are analyzed and sorted using Xdrop Sort or a flow cytometry-based cell sorter, with only droplets containing the DNA sequence of interest selected for further droplet amplification. After this final amplification, the enriched DNA is ready for long-read or short-read sequencing.

Materials

For DE20 droplet production and droplet PCR

Name	Item no.	
Xdrop instrument	IN00100	
or Xdrop Sort instrument	IN00200	
Xdrop DE20 Cartridge	CADE20A100	Shipped at room temp. Store at 4°C upon receipt
Xdrop DE Gasket	GADEA100	
Storage film	FI00100	
DE PCR Kit	RKDE20PCR100	
	Part 1 (Store at –20°C)	Part 2 (Store at RT)
	DE PCR mix (2x) ●	DE oil ●
	DE PCR buffer (2x) ●	
Cell sorter DE20 DNA sorting kit	RKCELLDNA100 (Store at –20°C)	
	DE PCR buffer (2x) ●	
	DE droplet dye ●	
Positive control DNA enrichment kit	RKPOSCO100 (Store at –20°C)	
	Positive control DNA ●	
	dPCR control primers ●	
	Enrichment validation primers ●	
Droplet break kit	RKBRESMVL100	
	Part 1 (Store at 4°C)	Part 2 (Store at RT)
	Droplet break color ●	Droplet break solution ●
Cell sorter control kit	RKCLLSRT100	
	Part 1 (Store at –20°C)	Part 2 (Store at 4°C)
	DE PCR buffer (2x) ●	Cell sorter control droplets ○
	DE droplet dye ●	

For SE85 droplet production for amplification of DNA

Name	Item no.	
Xdrop instrument	IN00100	
or Xdrop Sort instrument	IN00200	
Xdrop SE85 Cartridge	CASE85A100	
Xdrop SE85 Holder	HOSE85A100	
Xdrop SE Gasket	GASEA100	
Storage film	FI00100	
SE MDA Kit	RKSEMDA100	
	Part 1 (Store at –20°C)	Part 2 (Store at RT)
	SE MDA mix (5x) 🔴	SE oil 🟡
	SE MDA enzyme ○	
Droplet break kit	RKBRESMVL100	
	Part 1 (Store at 4°C)	Part 2 (Store at RT)
	Droplet break color 🟢	Droplet break solution 🟢

Assay design

This protocol provides the essential steps and guidelines for designing an effective targeted enrichment assay using Xdrop or Xdrop Sort.

Requirements for input DNA

For targeted enrichment, the input DNA must meet certain requirements:

Molecular Weight: DNA should have a molecular weight >30 kb to ensure it remains intact and suitable for amplification.

Purity: The DNA must be of high purity to prevent contamination with RNA, proteins, and other substances that could hinder amplification and enrich the region of interest.

Quantity: The required amount of input DNA can be calculated using the DNA calculator available at samplix.com/calculations.

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.

Key considerations:

Purification recommendations:

- Purify the DNA to meet the quality requirements for long-read sequencing.
- For input DNA >200 ng it is recommended to use a bead-based purification method. Perform the final elution from the beads at 55°C for 3 minutes to ensure that high molecular weight DNA is released.

Contamination:

- Take all necessary measures to prevent contamination. Enrichment can be affected by the following contaminants: RNA, proteins, carbohydrates, salts, and phenol, among other substances.

Primer design

Effective primer design is crucial to the success of the enrichment protocol. This workflow relies on designing two distinct sets of primer pairs (Figure 1.2):

Droplet PCR primers: A primer pair specific to the detection sequence, used to identify and enrich the region of interest during droplet PCR.

Validation qPCR primers: A separate primer pair targeting the validation sequence, is used to confirm the enrichment via quantitative PCR (qPCR).

Primer design guidelines

General:

- Avoid placing primers in low-complexity regions.
- Avoid primer pairs with unspecific target sequences.
- Avoid self-complementarity, stable secondary structures, or hairpins in primer sequence.

Amplicon Length:

- Droplet PCR primers: 120–160 bp
- Validation qPCR primers: 80–120 bp

Melting Temperature (Tm):

- Both droplet PCR and validation qPCR primers should have a Tm of ca. 60 °C for optimal performance.
- Ensure forward and reverse primers have a Tm difference ≤2°C.

Placement:

- Validation qPCR primers must be within 2 kb of droplet PCR primers, to avoid the likelihood of false-negative enrichment predictions.
- Ensure no overlap between droplet PCR and validation qPCR amplicons Figure 1.2).

Multiple primers for large ROIs:

- For ROIs >50 kb, multiple sets of droplet PCR and validation qPCR primers are recommended (Figure 1.2).

Additional help in designing primers can be found in the primer design tool at samplix.com/primer.

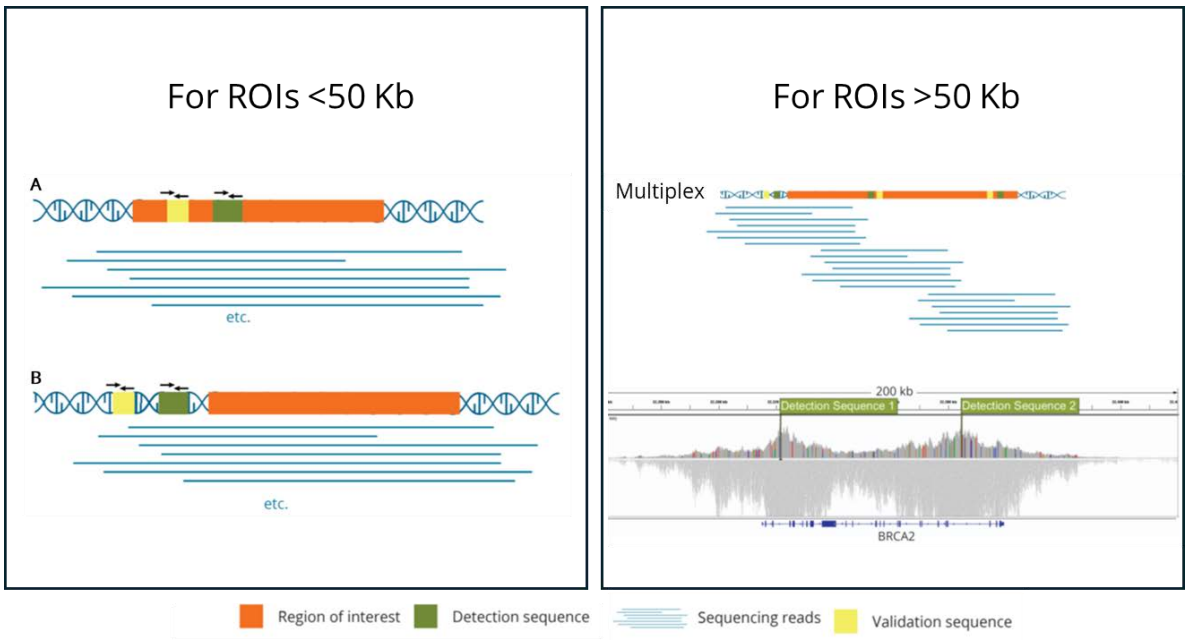


Figure 1.2. Targeted enrichment of a region of interest (ROI). Primer placement: a standard primer set should be designed for a short detection sequence (green) that is positioned either within (A) or flanking (B) the ROI, indicated in orange. Additionally, the validation sequence (yellow) should be designed in proximity to the detection sequence to enable assessment of enrichment at the end of the workflow. ROIs (>50 kb): For large regions, like the *BRC2* gene, a multiplex assay with a combination of two or more sets of droplet PCR and validation qPCR primers may be used. The validation sequence (yellow) should be designed near the corresponding detection sequence (green) to evaluate the enrichment at the end of the workflow.

Primer testing

For every new DNA target and before generating droplets for targeted enrichment, both the droplet PCR primer pair and the validation qPCR primer pair must be checked and optimized, via qPCR.

Key points for the assay:

- Include a negative control with no template
- Test at least three different concentrations of your template (or a corresponding biological replicate) (Figure 1.3, **A**)
- Use DNA input concentrations within the range suggested by the DNA input calculator at samplix.com/calculations.

Use your sample DNA or a corresponding biological replicate and DE PCR mix (2x) ● to verify the primers and reaction efficiency, following the steps below:

1. Prepare the PCR mix by combining the following 1X reaction:

   Keep cold at 4°C until use.

   *Use a DNA-binding dye such as SYBR®Green, EvaGreen® or similar.

PCR mix	1X
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
DNA binding dye (20x)*	1.0 µl
Total mix	**19 µl**
Template DNA	1.0 µl
Final volume	**20 µl**

2. Run the following qPCR program:

   *Adjust the annealing temperature to the Tm of the primer set being tested.

   **Follow the thermal cycler manufacturer's protocol for the data acquisition 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

Assay optimization and validation

1. Primer testing:
   - Test droplet PCR primers with the Samplix DE PCR mix ● as described above to ensure optimal performance.
   - The validation qPCR primers can be tested with either Samplix DE PCR mix ● or qPCR reagents from other suppliers.

2. Melting curve analysis:
   - Run 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.
   - Run a temperature gradient to optimize the annealing temperature.

3. Standard curve recommendation:

   For a single target in a genome the size of the human genome:
   - Suggested DNA amounts: 0.1, 1 and 10 ng; or 0.2, 2 and 20 ng.
   - Adjust the DNA amounts for smaller/larger genomes or different numbers of targets per genome.

4. PCR efficiency:
   - Calculate PCR efficiency using the Ct values with the formula:

$$Efficiency\ (\%) = \left(10^{\left(\frac{-1}{slope}\right)} - 1\right) \times 100$$

   PCR efficiency should fall between 80 - 120% with Ct value for the input DNA <30. (Figure 1.3).

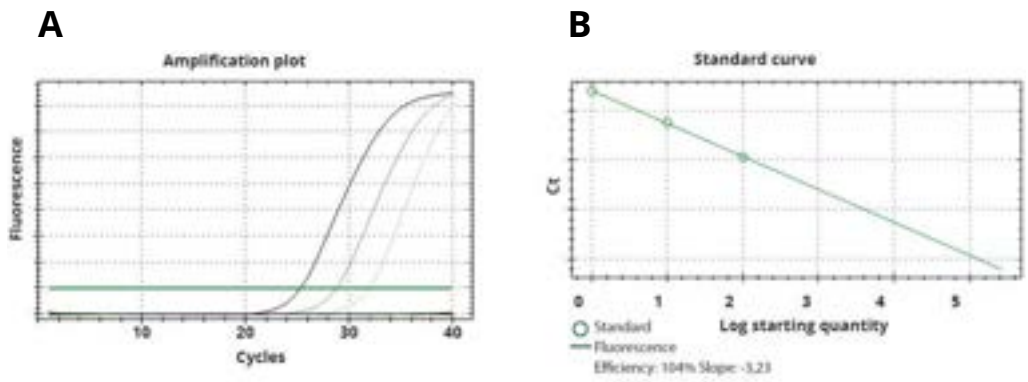


Figure 1.3. A: Amplification plots of three DNA concentrations. The middle curve is input DNA amount. B: Standard curve and calculations of PCR efficiency.

Chapter 2: DE20 Droplet PCR

After designing primer pairs as described in the Primer design section, proceed to encapsulate the PCR reagents and target DNA in double-emulsion droplets. For the detailed encapsulation protocol, refer to the Xdrop Manual or Xdrop Sort Manual.

Materials for DE20 droplet PCR

Name	Item no.	
Xdrop instrument or Xdrop Sort instrument	IN00100 IN00200	
Xdrop DE20 Cartridge	CADE20A100	Shipped at room temperature Store at 4°C upon receipt
Xdrop DE Gasket	GADEA100	
Storage film	FI00100	
DE PCR Kit	RKDE20PCR100 Part 1 (Store at –20°C) DE PCR mix (2x) ● DE PCR buffer (2x) ●	 Part 2 (Store at RT) DE oil ●
Droplet break kit	RKBRESMVL100 Part 1 (Store at 4°C) Droplet break color ●	 Part 2 (Store at RT) Droplet break solution ●

Additional materials

- Template DNA
- Droplet PCR forward and reverse primers at 10 µM
- Molecular grade water

Setting up droplet PCR

NOTE **Work in a dust-free environment, such as an LAF hood, to avoid contamination. Thaw all reagents at 4°C before use. This protocol is specific to Samplix's DE PCR kit.**

1. Prepare the droplet PCR mix as described in the table:

   Keep cold at 4°C until use.

Droplet PCR mix	1X
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	2.0 µl
Final volume	**40 µl**

2. Add **2 µl** of template DNA to the droplet PCR mix, making a final volume of **40 µl** per reaction. Calculate the optimal concentration template DNA using the online DNA input calculator.

3. Allow the mix to reach room temperature before loading on the Xdrop DE20 cartridge.

Positive control DNA enrichment kit

Suggested for validation, Samplix offers the Positive control DNA enrichment kit. This kit includes pre-mixed Positive control DNA ●, dPCR control primers ●, and Enrichment validation primers ● (annealing temperature: 60°C).

1. Prepare the Positive control droplet PCR mix (1X reaction):

   Keep cold at 4°C until use.

Positive control droplet PCR mix	1X
Water (molecular grade)	16.4 µl
DE PCR mix (2x) ●	20.0 µl
dPCR control primers ●	1.6 µl
Total mix	**38.0 µl**
Template (positive control DNA ●)	2.0 µl
Final volume	**40 µl**

2. Allow the mix to reach room temperature before loading on the Xdrop DE20 cartridge.

Xdrop DE20 cartridge preparation

NOTE **Allow cartridges to equilibrate to room temperature for at least 30 minutes before use.**

Produce double-emulsion droplets containing the complete droplet PCR mix as described in the Xdrop or Xdrop Sort manual.

Droplet recovery

1. Transfer the entire production volume from the cartridge collection well #D to a DNA low bind centrifuge tube. Double-emulsion droplets will be at the bottom of the well (Figure 2.1).
2. Rinse the shelf of the collection well #D of Xdrop DE cartridge with the remaining buffer from well #A. Transfer to the tube. The total volume in the collection well should be **300–400 µl**, variation may occur due to different buffers or media used.

Suggested: Verify droplet production using a microscope (see the Verifying droplet production section in the Xdrop or Xdrop Sort manual).

For PCR in DE20 droplets, aliquot sample into 200 µl PCR tubes. Ensure equal droplet distribution by gently shaking the tube between each **80 µl** aliquot.

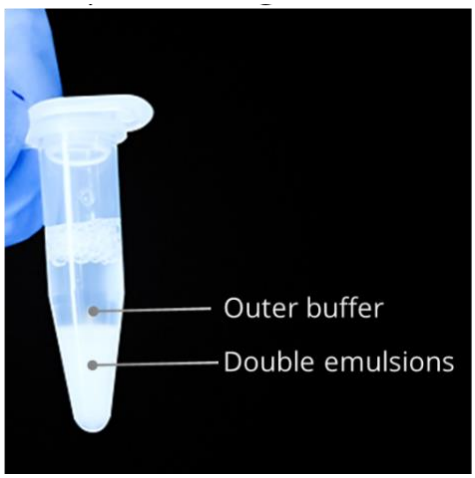


Figure 2.1. Droplet productions in Xdrop DE cartridges generate double-emulsion (DE) droplets, which have a higher density than the surrounding outer buffer, leading to sedimentation.

Droplet PCR

1. Place the PCR tubes in a thermal cycler and run the program described in the table:

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

2. Set the lid temperature to 105°C.

*For GC-rich sequences increase the initial denaturation step up to 5 min.

A block temperature ramp rate of 0.5°C/sec is recommended, with a total program duration of ≈2 hours to ensure slow temperature changes, allowing the reagents inside the droplets to reach the desired temperature.

3. Store at 4°C for up to 24 hours if further sorting and droplet MDA amplification is needed. Long-term storage may affect DNA quality and enrichment.

Chapter 3: Analyze and sort DE20 droplets

DE20 droplets can be sorted either on an Xdrop Sort instrument using the Xdrop DE20 Sort Cartridge or on a standard flow cytometry cell sorter. Droplet sorting allows the isolation of droplets containing the DNA of interest. In this process, droplets with the desired material are distinguished from negative ones based on the fluorescent signal from the detection sequence and the DE droplet dye ●.

Sorting on Xdrop Sort

Please refer to the Xdrop Sort manual for details on this workflow. It is important to include the steps described in the section "Staining DE20 droplets containing DNA." Note that flow cytometers are not required for this workflow. Once sorting is completed, proceed to break the droplets.

General recommendations for droplet flow cytometry

Below are instructions for analyzing and sorting DE20 droplets on flow cytometers or cell sorters.

Droplet density: Due to its high density, double-emulsion droplets will quickly sediment to the bottom of the sample tube. Adjust the sample probe to hover just above the bottom of the sample tube during analysis or sorting. Run with a high sample pressure to draw the droplets into the channels of the flow cytometer. It may take 5 to 10 minutes for the double-emulsion droplets to reach the point of interrogation and appear in the plot.

Droplet diameter: The outer diameter of DE20 droplets is 20 µm. Use a high sample pressure or fast flow rate to increase the width of the inner sample stream.

Assay chemistry: The oil shell of the double-emulsion droplets is transparent and will permit the detection of light from inside the droplet. Ensure that the assay chemistry within the droplets contains fluorophores compatible with the excitation-emission spectrum of your flow cytometer. The DE droplet dye ● emits green fluorescence upon binding to nucleic acids.

Threshold trigger channel: Use forward scatter (FSC) as the threshold channel to exclude unwanted events such as small particles and other contamination.

Doublet discrimination: Doublet discrimination can be performed but is not required as the double-emulsion droplets do not aggregate.

Sample reanalysis: As droplets may have broken or bleached during sorting, reanalysis of the sorted population is not recommended for assessing purity. A small volume of droplets can be sorted directly onto a microscope slide for microscopic inspection and purity assessment.

Flow cytometer instrument settings

Automatic bubble detection: Some instruments detect double-emulsion droplets as bubbles in the sample line, which automatically stops the flow cytometer. This can be prevented by disabling automatic bubble detection. If you disable bubble detection, be aware that the instrument will not automatically stop when the sample is empty, and you may risk air bubbles in the sample line.

Position of sample probe: Double-emulsion droplets will settle at the bottom of the sample tube. To ensure accurate analysis or sorting, the sample probe must be adjusted to submerge within the double-emulsion droplets, hovering just above the bottom of the tube (Figure 3.1). Some instruments allow user adjustments, while others require an engineer's assistance. *

Figure 3.1 Flow cytometer sample probe position.

Sort calibration: Adjustments to sort settings e.g. droplet delay or stream alignment may improve the sorting efficiency of double-emulsion droplets. *

Stream alignment: Ensure that the sorted double-emulsion droplets reach the collection tube. Access the sorting stream settings within the cytometer's software. In the sorting chamber, place a glass slide or another suitable target. Load a sample containing double-emulsion droplets and start analyzing. Identify the double-emulsion droplet population on a scatter plot as described below (see Figure 3.2). Sort a few hundred double-emulsion droplets onto the target slide to verify the stream settings.

Fine-tune the sorting streams (left, right, waste) to handle the double-emulsion droplets accurately.

Cleaning: Double-emulsion droplets do not easily break inside the instrument but always be diligent with cleaning. To remove suspected oil clutter, run a cleaning program with 70% ethanol. To remove DNA and cell debris, run a cleaning program with a 5-10% solution of sodium hypochlorite (bleach). Always wash with ddH_2O immediately after a bleach wash. Clean the sorting chamber and deflection plates regularly as recommended by the manufacturer.

* This adjustment may require assistance from the flow cytometer provider.

Staining DE20 droplets containing DNA

NOTE **Allow all reagents to equilibrate to room temperature for at least 30 minutes before use. Vortex and spin down reagents to ensure homogenization.**

Materials

Cell sorter DE20 DNA sorting kit	RKCELLDNA100 (Store at –20°C)
	DE PCR buffer (2x) ●
	DE droplet dye ●

After droplet PCR, stain droplets using the Cell sorter DE20 DNA sorting kit:

1. Dilute the DE PCR buffer (2x) ● to 1x in molecular-grade water and mix well. Add **1 ml** into a flow cytometry tube.
2. Centrifuge the DE droplet dye ● at 1700 g for 2 minutes and add **10 µl** DE droplet dye ● into the flow cytometry tube with 1x DE PCR buffer. Mix gently to dissolve the dye in the DE PCR buffer.
3. Remove the supernatant from the PCR tubes containing the DE droplets leaving the droplets untouched.
4. Transfer the droplets to the flow cytometry tube using **200 µl** of prepared buffer.
5. Incubate droplets for 5 minutes at room temperature, shielded from light, to stain the DNA inside the droplets.

DE20 droplet flow cytometry and sorting

Sample loading and analysis

1. Gently swirl or flick the flow cytometry tube containing the stained DE20 droplets and load to start analysis.
2. Set the flow cytometer to display at least 100,000 events on the live plot to ensure rare events are visible.
3. Set up an FSC-H vs. SSC-H plot and gate for DE20 droplets. These droplets will show higher side scatter compared to oil droplets (Figure 3.2 A).
4. To analyze or sort the droplets based on their fluorescent signal, plot the gate of the identified DE20 droplets in a green fluorescence vs. SSC plot (Figure 3.2 B).

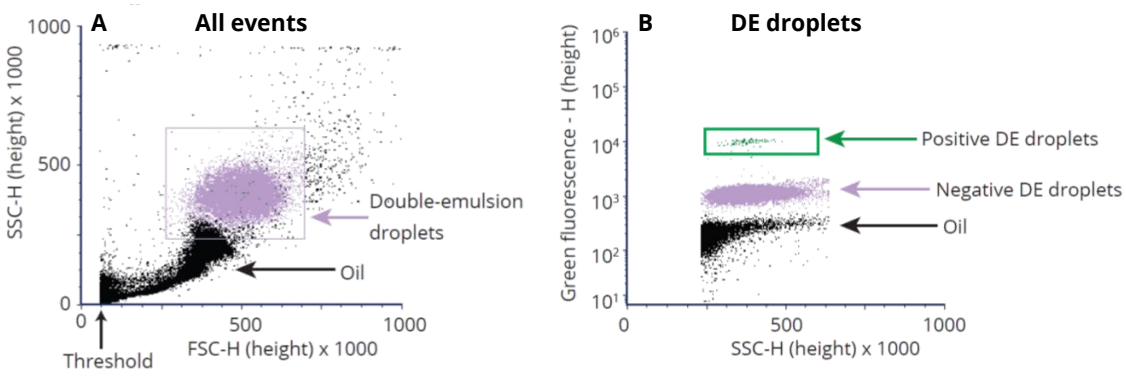


Figure 3.2. Identification of DE20 droplets. A: DE20 droplets are gated using a plot of FSC-H (Forward Scatter-Height) vs. SSC-H (Side Scatter-Height). The height parameter enhances the visualization of the DE20 droplet population. B: By plotting the DE droplets for fluorescence vs. SSC-H, positive (high fluorescence) and negative (low fluorescence) DE20 droplets can be distinguished. All DE20 droplets are slightly fluorescent after staining, including droplets not containing the target of interest ("Negative"), whereas contaminating pure oil droplets have very low green fluorescence. The plots were generated using a Sony® SH800S Cell Sorter. Plots may vary depending on the instrument, flow buffer, sample, or sample pressure.

NOTE **Plots may vary depending on the flow cytometer, the flow buffer, the encapsulated sample, and the diameter of the core stream as determined by the sample pressure.**

Sample sorting

1. Confirm the sort settings on the cell sorter. Ensure that the side stream is centered on the collection tube.
2. Add **15 µl** of molecular grade water into a 1.5 ml DNA LoBind collection tube and place the collection tube in the appropriate holder on the cell sorter instrument.
3. Set the sorting gate to select the desired population and begin sorting into the collection tube.
4. After sorting, store the droplets at 4°C. Do not store sorted DE20 droplets for more than a few hours to maintain DNA integrity. Prolonged storage may compromise the quality of the DNA.

Depending on the input, the DNA recovered from the sorted positive droplets is expected to be in the femtogram range. The expected number of positive droplets can be estimated using Samplix's online enrichment predictor.

Optional: Cell sorter control kit

Samplix offers the Cell Sorter Control Kit to help you establish the correct flow cytometry settings, ensuring that your system is properly calibrated. The kit contains pre-made DE20 droplets, which feature a large and well-defined population of positive droplets.

Materials

Cell sorter control kit	RKCLLSRT100	
	Part 1 (Store at –20°C)	Part 2 (Store at 4°C)
	DE PCR buffer (2x) ●	Cell sorter control
	DE droplet dye ●	droplets ○

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

1. Dilute the DE PCR buffer (2x) ● to one 1x in molecular-grade water and add **1 ml** into a flow cytometry tube.
2. Centrifuge the DE droplet dye ● at 1700 g for 2 minutes and add **10 µl** DE droplet dye ● into the flow cytometry tube with 1x DE PCR buffer. Mix gently to dissolve the dye in the DE PCR buffer.
3. Resuspend the Cell sorter control droplets ○ by vortexing gently. Transfer **50 µl** to the prepared flow cytometry tube.
4. Incubate droplets for 5 minutes at room temperature, shielded from light to stain the DNA inside the droplets.

5. Analyze the Cell sorter control droplets ○ by flow cytometry as described above.

NOTE **Approximately 10-20% of the total DE20 droplets should show positive fluorescence using the Cell sorter control kit.**

Breaking DE20 droplets

Release the DNA contents of DE20 droplets by using Droplet break solution ● and Droplet break color ●. The following procedures must be conducted in a controlled and clean laboratory environment, adhering to proper PPE guidelines.

Materials

Droplet break kit	RKBRESMVL100	
	Part 1 (Store at 4°C)	Part 2 (Store at RT)
	Droplet break color ●	Droplet break solution ●
For Xdrop Sort users only	Droplet sorting wash buffer ●	

NOTE **To use DE20 droplets sorted on Xdrop Sort for droplet-based MDA (dMDA), wash the droplets twice with 200 µl of fresh Droplet sorting wash buffer ● as described in the Xdrop Sort Manual.**

1. Vortex the Droplet break color ● tube upside down and spin the tube 15 to 30 seconds.
2. Add **20µl** Droplet break solution ●.
3. Add **1 µl** of Droplet break color ● to stain the aqueous phase (Figure 3.3). If the shade is too weak, adjust as needed.
4. Mix the contents of the tube by gently flicking the tube 10x.

Do not vortex.

5. Spin down the tube at 400g for 10 s.
6. Remove and discard the clear phase from the bottom of the tube.
7. Repeat steps 4–6 to remove any residual Droplet break solution ●.
8. Transfer the stained aqueous phase containing your sample to a DNA low bind centrifuge tube.

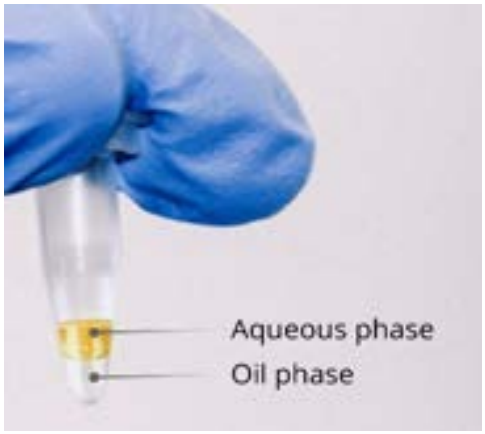


Figure 3.3 Break the droplets with Droplet break solution ● and Droplet break color ●. Keep the, colored aqueous phase.

Chapter 4: SE85 droplet MDA

Materials for SE85 droplet production and amplification of DNA

Name	Item no.	
Xdrop instrument	IN00100	
or Xdrop Sort instrument	IN00200	
Xdrop SE85 Cartridge	CASE85A100	
Xdrop SE85 Holder	HOSE85A100	
Xdrop SE Gasket	GASEA100	
Storage film	FI00100	
SE MDA Kit	RKSEMDA100	
	Part 1 (Store at –20°C)	Part 2 (Store at RT)
	SE MDA mix (5x) 🔴	SE oil 🟡
	SE MDA enzyme ○	
Droplet break kit	RKBRESMVL100	
	Part 1 (Store at 4°C)	Part 2 (Store at RT)
	Droplet break color 🟢	Droplet break solution 🟢

Setting up droplet MDA reaction

NOTE **Work in a dust-free environment, such as an LAF hood, to avoid contamination. Thaw all reagents at 4 °C before use. This protocol is for Samplix's SE MDA kit only!**

1. Prepare the Amplification mix:

   Keep cold at 4°C until use.

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

2. Add **10 µl** of DNA template (e.g., DNA from sorted DE droplets or controls).

3. **Recommended:** Include the following controls for reliable results:
   - **Negative control:** 10 µl molecular-grade water
   - **Contamination control:** 10 µl sheath fluid from the flow cytometer
   - **Positive control:** 10 µl of 0.1 pg/µl genomic DNA (dilute from 100 ng/µl in serial dilutions to reach 0.1 pg/µl)
4. Mix gently and keep the mix cold at 4°C until you're ready to load it on the Xdrop SE85 Cartridge.

Produce single-emulsion SE85 droplets containing amplification mix as described in the Xdrop or Xdrop Sort manual.

Droplet recovery

1. Transfer the entire production volume from the SE85 cartridge collection well to a nuclease- and DNA-free PCR tube. Single-emulsion droplets will be at the top of the collection well (Figure 4.1).
2. Wash the collection well of the cartridge with the oil that sediments to the bottom of the tube after harvest.
3. The total volume in the collection well, should be **70 – 100 µl**, variation may occur due to different buffers or media used.
4. Carefully remove all but 1–2 mm of oil from the bottom of the collection tube, see Figure 4.1.

Figure 4.1. Droplet productions in Xdrop SE cartridges produce single-emulsion (SE) droplets with lower density than the oil they are dispersed in, resulting in suspension.

Droplet MDA

1. Place the PCR tube with droplets in a thermal cycler.
2. Set the lid temperature to 75°C to prevent overheating.
3. Incubate the droplets in a thermal cycler at 30°C for 16 hours followed by 10 minutes at 65°C.

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

Breaking SE85 droplets

Release the DNA contents of the SE85 droplets by using Droplet break solution ● and Droplet break color ●. The following procedures must be conducted in a controlled and clean laboratory environment, adhering to proper PPE guidelines.

NOTE **Allow all reagents to equilibrate to room temperature for at least 30 min before use. Vortex and spin down reagents to ensure homogenization.**

1. Vortex the Droplet break color ● tube upside down and spin the tube 15 to 30 seconds
2. Add **20µl** Droplet break solution ●.
3. Add **1 µl** of Droplet break color ● to stain the aqueous phase (Figure 4.2). If the shade is too weak, adjust as needed.
4. Mix the contents of the tube by gently flicking the tube 10x.

Do not vortex.

Figure 4.2. Break the droplets with Droplet break solution ● and Droplet break color ●. Keep the, colored aqueous phase.

5. Spin down the tube at 400g for 10 s.
6. Remove and discard the clear phase from the bottom of the tube.
7. Repeat steps 4–6 to remove any residual Droplet break solution ●.
8. Transfer the stained aqueous phase containing your sample to a DNA low-bind centrifuge tube.

NOTE **The color of the Droplet break color ● may range from yellow to purple it is pH dependent**.

NOTE **When taking aliquots of your sample DNA after breaking, always pipette from the center of the colored phase to avoid any carryover of the Droplet break solution.**

Quantification and further amplification

Quantify the total DNA using a sensitive method such as a fluorometer or electrophoresis-based system. If possible, assess the size of sorted and amplified DNA fragments.

Reamplification: If the DNA yield is insufficient for downstream sequencing, reamplify the DNA:

- Prepare a 10 µl amplification mix with enzyme as described above and add it to the recovered DNA.
- Set the thermal cycler's lid temperature to 75°C for the MDA reamplification.
- Incubate at 30°C for 2 hours, followed by 10 minutes at 65°C to inactivate the droplet MDA enzyme.

This short reamplification step should increase the yield by 3- to 5-fold without increasing DNA chimeras or reducing fragment length.

Chapter 5: Evaluating target DNA enrichment

Once the concentration of total DNA is determined, an assessment of the levels of enrichment of your target region can be made. Use quantitative PCR (qPCR) and Samplix' online calculator at samplix.com/calculations to estimate the enrichment.

Estimating target DNA enrichment

To estimate the fold enrichment of your target DNA, perform a qPCR using the qPCR validation primers. Please refer to the section and the Samplix Primer Design Tool for guidance on designing these primers. Ensure the qPCR Assay design primer pair is placed within 5 kb of the detection sequence defined by the droplet PCR primers.

NOTE **PCR-based enrichment estimates are indicative and may vary from sequencing-based measurements of the entire enriched region.**

Setting up a standard qPCR for enrichment estimation

Prepare a qPCR using the following DNA templates, the numbers refer to the schematic workflow in Figure 5.1:

- **(1) Enriched sample:** 1:10 dilution of droplet MDA amplified enriched DNA.
- **(2) Non-Template Control (NTC):** 1:10 dilution, negative control.
- **(3) Original input DNA:** Input DNA at the same concentration as in the original input in the experiment.

Additional Controls:

- **(4) Sheath fluid:** 1:10 dilution of flow cytometer sheath fluid, contamination control.
- **(5) Non-enriched amplified DNA:** 1 pg of droplet MDA from non-enriched DNA at a 1:10 dilution, positive control.
- **(6) Molecular grade water:** Negative PCR control.

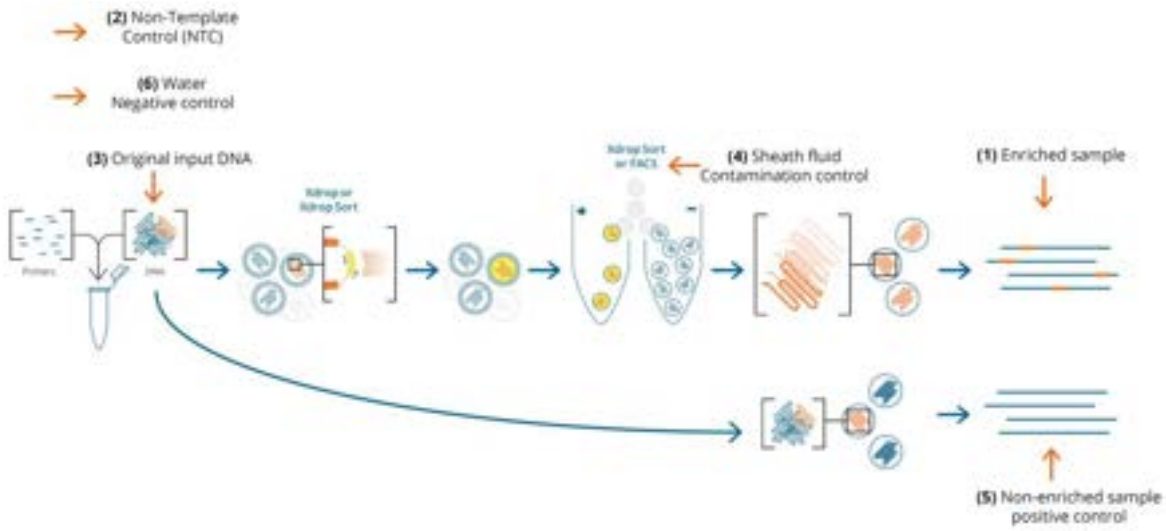


Figure 5.1. Xdrop DNA enrichment workflow. Orange arrows indicate where DNA should be sampled for the qPCR to estimate the fold enrichment.

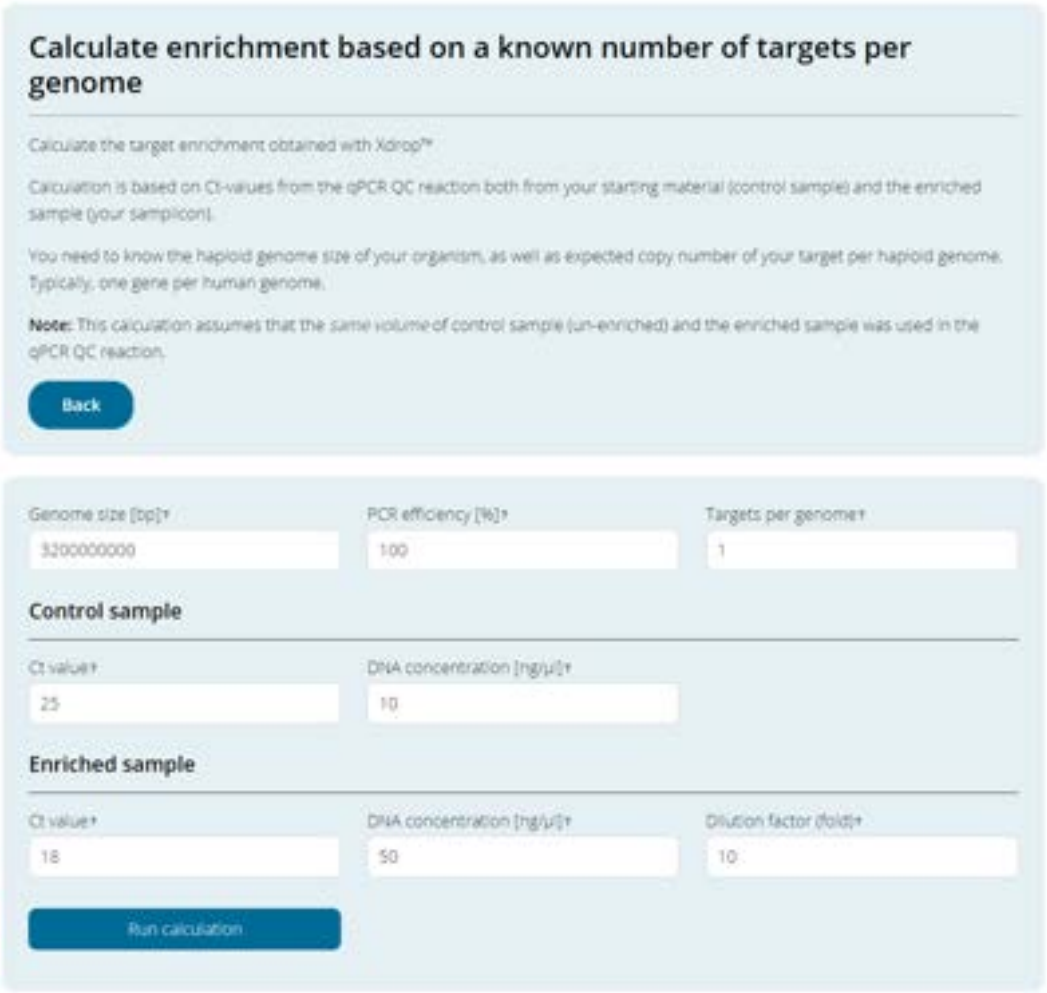
Calculate fold enrichment of target DNA

Use the Samplix "Enrichment calculator" tool at samplix.com/calculations to determine the fold enrichment. This tool offers two methods:

1. **Targets per genome:** Estimates enrichment based on the number of targets per genome. See example below.
2. **Targets per ng of DNA:** Calculates enrichment per nanogram of input DNA.

Calculation of enrichment based on the number of targets per genome

Select "Calculate enrichment targets per genome". The following information is required to give you an enrichment estimate:



Genome size: Enter the size of the host genome in base pairs.

PCR efficiency: Enter the PCR efficiency of the qPCR validation primer set as estimated in the initial testing.

Targets per genome: Specify the target copy number in the host genome, ignoring off-targets.

Control Sample (Reference DNA):

- Ct Value: From qPCR of 1x reference DNA (original input).
- DNA Concentration: Concentration of the reference DNA in the non-enriched sample.

Enriched Sample:

- Ct Value: From qPCR of the droplet MDA reaction product.
- DNA Concentration: DNA concentration in the enriched sample (estimated by a fluorometer or electrophoresis-based system).
- Dilution Factor: Factor of dilution of droplet MDA reaction in qPCR (1:10 fold dilution recommended).

The calculator will estimate the target DNA fold enrichment, with sequencing suggested for values above 100-fold enrichment.

DNA library preparation

After verifying enrichment, prepare DNA libraries for your chosen sequencing platform. Xdrop-enriched DNA is compatible with both long-read and short-read sequencing technologies. Remember, qPCR-based enrichment estimates are indicative and may differ from those obtained through sequencing.

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