Samplix

Xdrop™ manual

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Chapter 1: Xdrop™ at a Glance

Targeted enrichment overview

Congratulations with your new Xdrop™ instrument, which we expect will facilitate groundbreaking research.

The Xdrop™ introduces a new approach for genomic analysis. Our innovative technology enables targeted enrichment of genomic regions in droplets. The Xdrop™ system offers sensitive and unbiased PCR-free sample enrichment and isothermal amplification prior to downstream analysis e.g. next generation sequencing.

Using the Xdrop™ instrument, cartridges, and reagents, DNA samples are partitioned into millions of picolitre size droplets. The workflow includes two steps:

1. Target enrichment in double emulsion droplets (Chapter 3, Droplet PCR – dPCR)
2. Isothermal amplification in single emulsion droplets (Chapter 5, Multiple Displacement Amplification in droplets – dMDA)

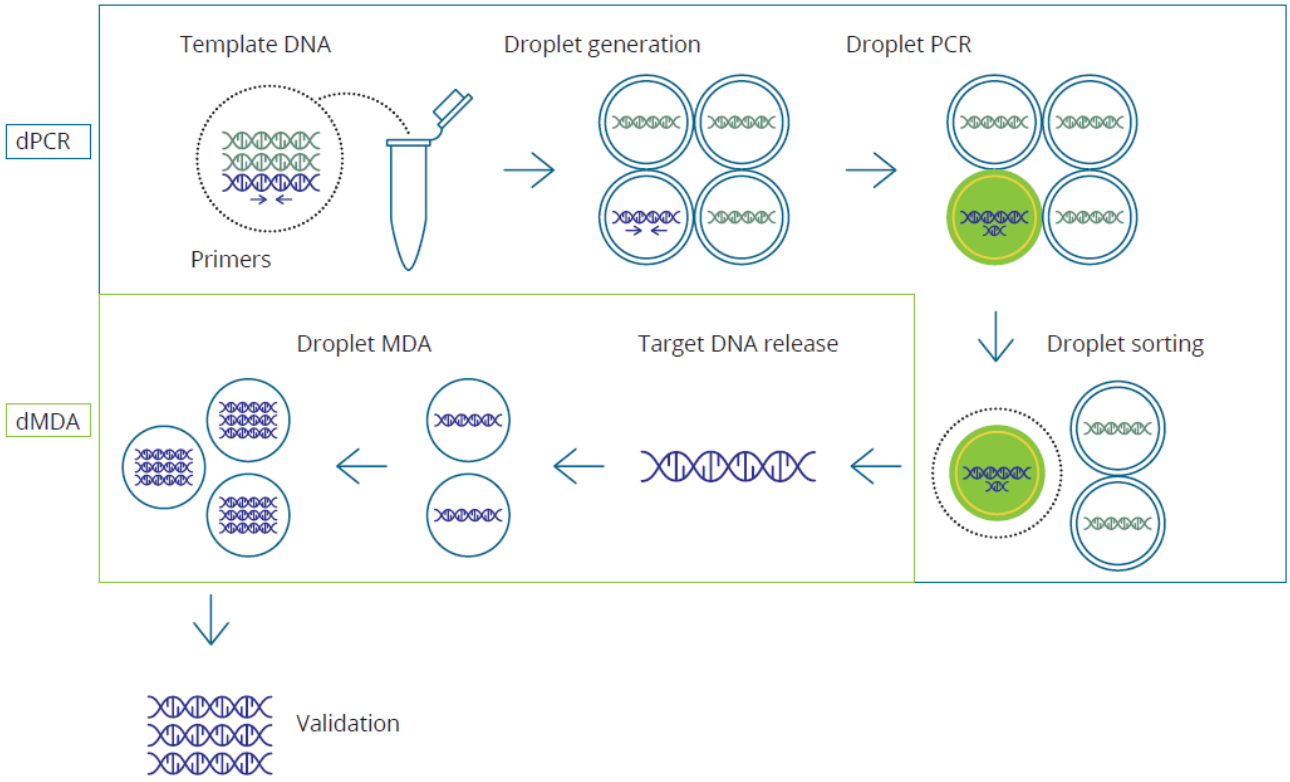


Fig. 1.1. Xdrop™ workflow including dPCR (droplet PCR) and dMDA (Multiple Displacement Amplification in droplets).

In the first step of enrichment, the sample is diluted and partitioned into millions of double emulsion droplets using the Xdrop™ instrument and the advanced microfluidics dPCR cartridge. These droplets are highly stable and are suitable for standard PCR cycling, flow cytometry analysis and sorting. Droplets containing the target DNA molecules are identified by a 120-160 bp targeted droplet PCR (dPCR) specific to a sequence (ID sequence) within or adjacent to the region of interest. Positive droplets are identified by their fluorescence and physically separated from negative droplets using a standard cell sorter. The result is an enrichment of long single molecules comprising kilobases of DNA information.

For downstream DNA amplification of the single molecules, Samplix has developed a proprietary technology. Each long fragment derived from the enrichment is partitioned into thousands of single emulsion droplets for high fidelity multiple displacement amplification in droplets (dMDA).
The Xdrop™ enrichment and amplification technology are compatible with downstream molecular biology techniques such as short- and long-read sequencing.

Workflow overview

Chapter 3		**Droplet PCR (dPCR)**
	~ 20 min	Prepare dPCR mix and cartridge set up
	~ 40 min	dPCR droplet generation in the Xdrop™ instrument
	~ 2 h	dPCR in thermal cycler
		Good pause point: store dPCR droplets after dPCR at 4ºC for up to 24 h
Chapter 4		**Flow cytometry**
	~ 15 min	Sample staining and setup
	~ 30 min/sample	Sorting (time depends on instrument)
		Proceed immediately to dMDA
Chapter 5		**Droplet Multiple Displacement Amplification (dMDA)**
	~ 10 min	dPCR droplets break
	~ 20 min	Prepare dMDA mix and cartridge set up
	~ 45 sec	dMDA droplet generation in the Xdrop™ instrument
	~ 16 h (overnight)	dMDA in thermal cycler
	~ 10 min	dMDA droplets break
		Verify enrichment and proceed to library preparation and sequencing

Xdrop™ instrument overview

The Xdrop™ droplet generator instrument is compatible with Samplix dPCR cartridges for production of double emulsion dPCR droplets and Samplix dMDA cartridges for the generation of single emulsion droplets for amplification of DNA. When using dMDA cartridges, always use the accompanying holder. The Xdrop™ droplet generator is used for generating both dPCR and dMDA droplets and is composed of the following parts (see figure below):

- A start button on the front of the instrument.
- Touch screen: provides the means to control the droplet generator with gloved or un-gloved hands.
- Status LED: purple when initializing and shutting down, green when in standby, blue when operating and yellow-green when opening and closing the drawer.
- Drawer: holds the dPCR or dMDA cartridge.
- USB port on the back of the instrument: connects to a USB flash drive for updating instrument firmware.
- Air vents on the back of the instrument: for ventilation.
- A hardware switch on the back of the instrument.

Specifications

Width: 25 cm / 9,8 inches
Height: 25 cm / 9,8 inches
Length: 48 cm / 18,9 inches
Weight 17 kg / 37,5 lbs.
Voltage requirements: 110 V-240 V (Line frequency; 50 - 60 Hz, Max current: 2,3 A)

Support

To find technical support, contact the support team at support@samplix.com

Warranty

The Xdrop™ instrument and associated accessories are covered by a standard Samplix ApS warranty. Contact your local Samplix ApS office for the details of the warranty.

Safety

We strongly recommend that you follow the safety specifications listed in this section and throughout this manual.

Xdrop™ is produced to comply with Safety Requirements for Electrical Equipment for Measurement, Control, and Laboratory Use (UL 61010-1) and complies with EU (CISPR 11, class A, group 1, 150 kHz – 30 MHz) EMC.

This equipment has also been tested and found to comply with the limits for a Class A digital device, pursuant to part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment. This equipment generates, uses, and can radiate radio frequency energy and, if not installed and used in accordance with the instruction manual, may cause harmful interference to radio communications. Operation of this equipment in a residential area is likely to cause harmful interference in which case the user will be required to correct the interference at his own expense.

FCC Caution: Any changes or modifications not expressly approved by the party responsible for compliance could void the user's authority to operate this equipment.

Environment and Power Requirements

Xdrop™ operating conditions are at temperatures 20-25°C, with a relative humidity (RH) 0-75%. Xdrop™ requires a stable power supply and can be powered using mains voltage of 100-240 VAC, 50-60 Hz. Main power fuses (F3A, 250 V, 5x20mm) are located behind a small black plastic cover between main switch and the power main plug at the back of the instrument. The Xdrop™ instrument must be installed on a flat surface where access to the main power outlet is not restricted. The instrument is not to be used against the manufacturers instructions. Failing to comply with the requirements can result in potential hazards to the instrument and the user.

Instrument safety warnings

The following warning labels refer directly to the safe use of the Xdrop™ instrument.

Icon	Meaning
	Warning about the risk of harm to body or equipment. Operating the Xdrop™ before reading this manual can constitute a personal injury hazard. Only qualified laboratory personnel should operate this instrument.
	Warning about the risk of harm to body or equipment from electrical shock. Do not attempt to repair or remove the outer case of this instrument, power supply, or other accessories. If you open these instruments, you put yourself at risk for electrical shock and void your warranty. All repairs must be done by an authorized repair service.
	Warning about the risk of harm to hands and fingers. Always keep hands and fingers away from the instrument when the drawer is in motion.

Intended use and intended users

The Xdrop™ instrument is intended for use by trained laboratory personnel in a clean laboratory environment for DNA sample preparation using droplet microfluidic technology.

Transportation and storage

Always transport the instrument in the original Samplix box. Before starting up the instrument, let it stay in room temperature for at least 2 hours.

Maintenance and cleaning

If the instrument is shipped back to Samplix for maintenance, please make sure that the outer surfaces are cleaned using a cloth and 70 % ethanol.

Xdrop™ Installation and Set-Up Quick Guide

1. Place the transport box on a flat surface.
2. Flip out the four lock twisters and turn them counterclockwise to unlock the lid of the transportation box.
3. Remove the lid to gain access to the instrument.
4. Slide a hand into the box on each side of the instrument and lift it out of the box.

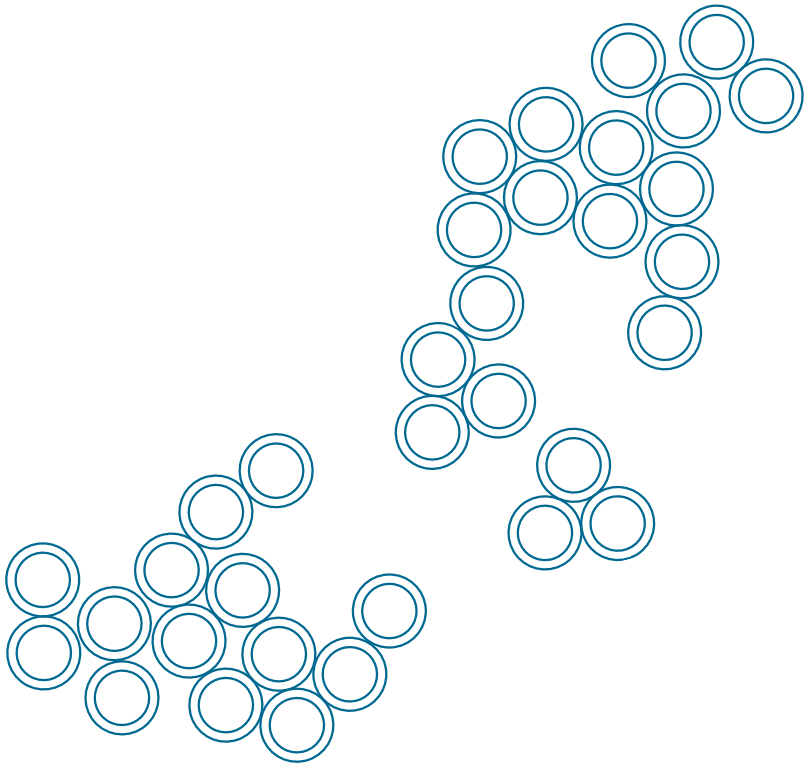
   Tip: If required, gently lift the back of the instrument 10-15 cm and place it against the foam/padding at the back. This should allow you to get a good grip placing both hands under the instrument.

5. Lift out the instrument and place it on a flat horizontal surface.
6. Leave the instrument unused for at least 2 hours.

   Note: Leaving the instrument at ambient temperatures allows the instrument to equilibrate and will reduce the risk of instrument failure.

7. Attach the power cord to the back of the instrument. Note: Use the included power cable only!
8. Plug the power cable into an appropriate power outlet.
9. Turn the main switch at the back of the instrument to the "|" position.
10. Press the Power button at the front to power up the instrument.

    Note: Power button needs to be fully pressed for the instrument to start up.



Required items for Xdrop™

dPCR

Name	Cat. No.			
Xdrop™ Instrument	IN00100			
dPCR cartridge	CA10100			
dPCR gasket	GA10100			
Storage film	FI00100			
dPCR kit	RE10100	Part 1 (store at -20°C) dPCR mix (2x) ● Droplet dye ●	Part 2 (store at -20°C) dPCR buffer (2x) ●	Part 3 (store at RT and 4ºC) dPCR oil ● (RT) Break Solution ● (RT) Break colour ● (4ºC)

dMDA

Name	Cat. No.		
Xdrop™ Instrument	IN00100		
dMDA cartridge	CA20100		
dMDA holder	HO10100		
dMDA gasket	GA20100		
Storage film	FI00100		
dMDA kit	RE20300	Part 1 (store at -20°C) dMDA mix (5x) ● dMDA enzyme ○	Part 2 (store at RT and 4ºC) dMDA oil ● (RT) Break Solution ● (RT) Break colour ● (4ºC)

Suggested Samplix items

Name	Cat. No.		
Cell sorter control kit	CO10100	Part 1 (store at -20°C) Droplet dye ● dPCR buffer (2x) ●	Part 2 (store at 4°C) Control droplets ○
Positive control primer kit	CO10200	Part 1 (store at -20°C) dPCR control primers ● Enrichment validation primers ● Positive control DNA ●	
Primer test PCR kit	RE10200	Part 1 (store at -20°C) dPCR mix (2X) ● qPCR dye (20x) ●	

Equipment and reagents for Xdrop™ enrichment and amplification supplied by the user

In addition to required and suggested Samplix products, the following items are required.

Equipment

Cell sorter instrument
Real-time PCR cycler
Thermal cycler with slow ramping function
Instrument for nucleic acid quantification e.g. Qubit™, Bioanalyzer™, TapeStation™, FEMTO Pulse™ or similar.
LAF (Laminar Air Flow) hood
Microcentrifuge
Vortex
Pipette set from P2 to P1000
Freezing blocks for both PCR tubes and microcentrifuge tubes

Consumables

Nuclease free PCR tubes and microcentrifuge tubes
Eppendorf DNA LoBind tubes 1,5 ml tubes (Catalog No. 0030108051)
Filtered pipette tips
Wide bore pipette tips for P200 pipette outer diameter of tip: 1 mm – 1,9 mm

Reagents

Nuclease-free water

Chapter 2: Droplet PCR Reaction Design for Target Selection

The Xdrop™ technology requires a simple assay design with the following components:

- A high molecular weight DNA sample (> 30 kb, depending on the assay) of high purity. Calculate the required amount of input DNA needed based on the desired enrichment and desired amount of output DNA using the online DNA input calculator at samplix.com
- One droplet PCR (dPCR) primer pair for enrichment. Please see the design guidelines below and use the online primer design tool at samplix.com
- One quantitative PCR (qPCR) QC primer pair for validation of Xdrop™ DNA enrichment. Please see the design guidelines below and use the online primer design tool at samplix.com

Target sequence

The Xdrop™ technology allows targeted enrichment by selection and amplification of a genomic region of interest without the need for long-range PCR. The target DNA of interest can contain repeat regions, GC-rich regions or other regions that are otherwise difficult to amplify. Specific primers amplifying a short fragment of 120-160 bp (ID sequence), within the target or in flanking regions, are used for capturing the kilobases-long region of interest. The Xdrop™ technology compartmentalizes the amplification reaction in small droplets and makes use of a highly processive DNA polymerase to enable amplification of almost all regions of the genome.

The length of the enriched target DNA will depend on the length of the input DNA. Consider using high molecular weight DNA as input with a DNA fragment size > 30 kb and of high purity. Calculate the optimal amount of input template DNA using the online sample input calculation tool at samplix.com

DNA sample preparation

When purifying the DNA sample, use a method that maintains the integrity and the purity of the DNA. Take all required measures to avoid contaminations. The Xdrop™ enrichment technology can be affected by contamination of the DNA sample by RNA, proteins, carbohydrates, salt and phenol among others. Purify the DNA to the same quality as required for long read sequencing.

Calculate the optimal amount of input template DNA using the online enrichment calculation tool at samplix.com

Primer design guidelines

The Xdrop™ enrichment technology relies on carefully designed and highly specific PCR primer pairs. Two sets of non-overlapping PCR primer pairs are required. The first one is called dPCR primer set and is responsible for creating a fluorescent signal used for target selection. The second set of PCR primers, called the qPCR QC primer set, 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 dPCR/qPCR primers are recommended.

Help for designing primers can be found in the online primer design tool at samplix.com

General guidelines for primer design

Apart from the dPCR primer pair, a qPCR QC primer pair is required for validation and calculation of the enrichment.

	dPCR primers	qPCR QC primers
Amplicon length	120-160 bp	80-120 bp
Melting temperature	~ 60°C	~ 60°C

Note: The qPCR QC primer pair must be different from the enrichment dPCR primer pair and placed within 2 kb distance from it. The qPCR QC and dPCR amplicons must not overlap.

The risk of false-negative enrichment prediction increases if the validation qPCR assay is placed further away from the dPCR assay.

The following guidelines apply to both dPCR and qPCR QC primer pairs:

- Avoid primer pairs with more than 2°C difference in melting temperature between 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 target and prior to generating droplets, both the dPCR enrichment primers and the qPCR QC primers must be optimized preferably by qPCR using your sample DNA and Samplix dPCR reagents ● and qPCR dye ●. For this assay, include a negative control with no template and at least three different concentrations of your template (or a biological replicate) in the range of the amount of input DNA suggested by the online sample calculation tool at samplix.com (see Fig. 2.1).

Use the Samplix primer test PCR kit (Cat. No. RE10200) that includes Samplix dPCR mix (2X) ● and Samplix qPCR dye ● to verify the primers and reaction efficiency.

The primer test reagents are optimized for the following reaction mix using Samplix Primer test PCR kit (Cat. No. RE10200):

dPCR reaction mix	1 reaction
H_2O (molecular grade)	7,2 µl
dPCR 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	2 min	
94°C	3 sec	40x
Annealing temp.	30 sec	
Data acquisition*	-	
Melting curve 70-90°C		1X

*Follow manufacturer's protocol for data acquisition program.

Note: The annealing temperature needs to be adjusted to the specific primer sets tested
Note: qPCR QC primer can be tested either in the Samplix dPCR reagents or in your preferred qPCR reagents

We recommend running a melting curve analysis with the template DNA, dPCR 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 supplied qPCR dye (20x) ● maximum values for excitation and emission are approximately 497 nm and 520 nm respectively (SYBR™ green settings). Make sure these values are selected in your qPCR instrument.

Calculate the PCR efficiency using the Ct values as input with the formula: (10^(-1/slope)-1) *100

Make sure that your designed primer pairs have an efficiency between 90-110 % and that the suggested DNA input amount yields a Ct value below 30.

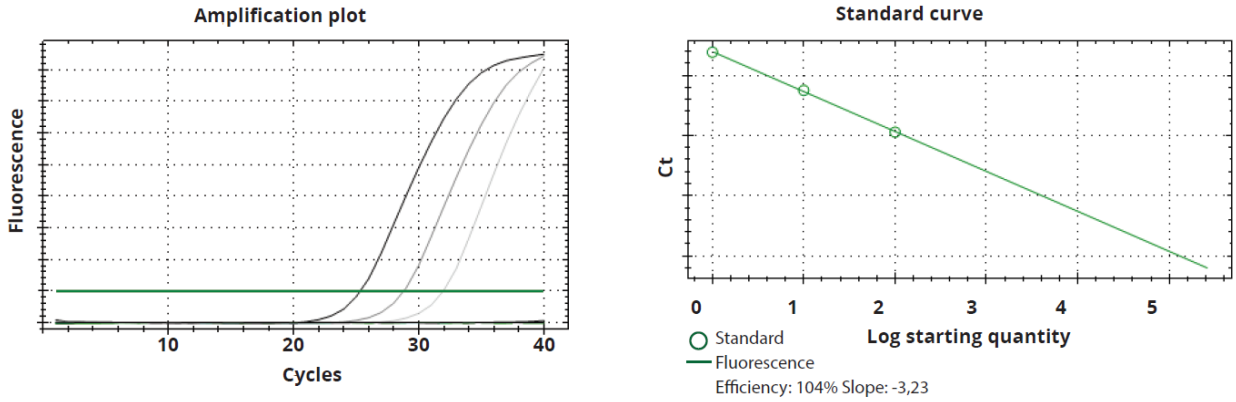


Fig. 2.1. Calculate PCR efficiency with at least three different concentrations of input DNA using the Samplix Primer test PCR kit (Cat. No. RE10200) and your designed primers. Left: Amplification plot of three DNA concentrations. Right: Standard curve and calculations of PCR efficiency.

Chapter 3: Droplet PCR (dPCR)

Preparations for dPCR

Use the Samplix Primer test PCR kit (Cat. No. RE10200) to determine the optimal primer concentration and annealing temperature for the dPCR reaction as described in the previous chapter (Chapter 2: Droplet PCR Reaction Design for Target Selection).

NOTE: Do not use any other reagents instead of Samplix dPCR kit (Cat. No. RE10100) for Xdrop™ dPCR droplet production as this may compromise droplet production, droplet stability, and downstream enrichment.

Samplix provides a Positive control kit (Cat. No. CO10200) that contains template DNA and two primer pairs; one for dPCR and one for validation qPCR after dMDA. See instructions on the next page.

Setup of dPCR reaction

1. Prepare the dPCR reaction mix following the table below.
2. Prepare mix for all reactions (40 µl reaction total per sample). Keep the reagents and the dPCR reaction mix on ice.
3. Prepare dilutions of the correct amount of template DNA. Calculate the optimal amount of input template DNA using the online sample calculation tool samplix.com
4. Add **2 µl** of the correct amount of template DNA to **38 µl** dPCR reaction mix to get 40 µl/reaction in total.
5. Allow the reaction mix to reach room temperature before loading on the dPCR cartridge followed by immediate insertion of the dPCR cartridge on the Xdrop™ instrument.

dPCR reaction mix	1 reaction
H_2O (molecular grade)	16,4 µl
dPCR mix (2x) ●	20 µl
dPCR Primer Forward 10 µM	0,8 µl
dPCR Primer Reverse 10 µM	0,8 µl
Total mix	**38 µl**

Template DNA	2 µl
Total volume	40 µl

Optional: Positive control dPCR reaction (Cat. No. CO10200)

Note that it is possible to include the Positive control kit (Cat. No. CO10200) provided by Samplix into the dPCR reaction. The kit includes both Positive control DNA ● and dPCR control primers ● (already mixed) with an annealing temperature of **60°C**. See table below.

dPCR reaction mix	1 reaction
H_2O (molecular grade)	16,4 µl
dPCR mix (2x) ●	20 µl
dPCR control primers ● mix	1,6 µl
Total mix	**38 µl**

Positive control DNA ●	2 µl
Total volume	40 µl

Validation primers ● (already mixed) are also provided within the kit to perform the enrichment validation (see Chapter 6).

Prepare dPCR cartridge

1. Dilute the 2x dPCR buffer ● to 1x with molecular grade water. Note: Dilute an entire vial of dPCR buffer. Precipitates potentially present in the 2x buffer will dissolve when diluted to 1x. Make sure to mix well by vortexing for 10 seconds or inverting the tube >20 times. Use leftover 1x dPCR buffer for sorting of droplets (chapter 4).
2. Unpack the cartridge from the plastic bag and place it on a clean flat surface in a LAF (Laminar Air Flow) hood or a similar clean, dust free environment. The layout of the cartridge is depicted in Fig. 3.1.
3. Handle the cartridge as follows:
    - Always use gloves when handling the cartridge.
    - Hold the cartridge by its sides.
    - Do not touch any of the input wells or droplet exit well.
    - Do not touch the microfluidic chip on the back of the cartridge.
    - Save the cartridge storage bag for later storage of the cartridge.

4. Be careful not to use the same lane more than once as this will disrupt droplet production. To avoid using the same lane more than once mark the storage plastic bag or the cartridge directly with a permanent marker once a lane has been used.
5. Load the cartridge with reagents in a LAF hood or a similar clean, dust free environment. Allow the reaction mix to reach room temperature before loading on the dPCR cartridge followed by immediate insertion on the Xdrop™ instrument.

Note: it is important to load the reagents on the cartridge in the order described on the next page.

Fig. 3.1. dPCR cartridge top view with wells (A-D) and lanes (1-8) marked.

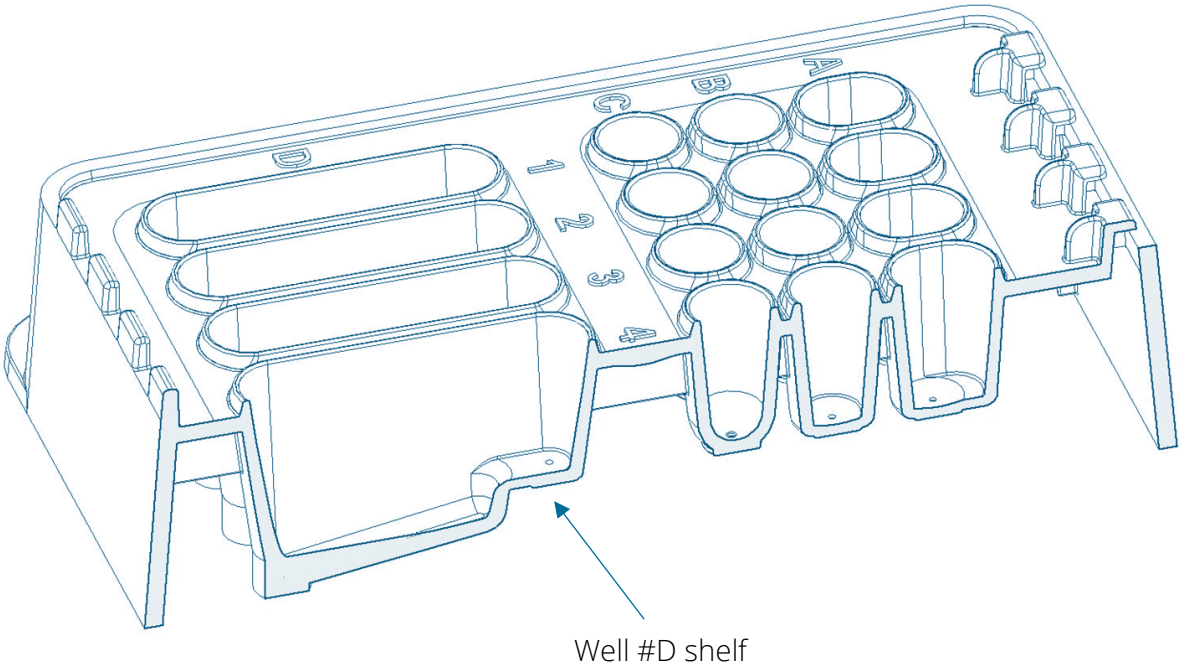


Fig. 3.2. Cross section of the dPCR cartridge. Notice the exact location of the shelf in well #D, where 40 µl of 1x dPCR buffer ● are added.

When loading the dPCR cartridge, avoid introducing air bubbles by pipetting on the side wall of the wells.

6. Load **300 µl** 1x dPCR buffer ● in the first well (**#A**). It is important to load the cartridge in the order described here and avoid air bubbles by pipetting carefully on the side of the well.
7. Load **40 µl** 1x dPCR buffer ● to the shelf of the collection well **#D** (Fig. 3.2).
8. Load **40 µl** dPCR reaction mix including primers and sample DNA into well **#C**.
9. Load **100 µl** dPCR oil ● into well **#B**.

Notice that the order of loading is:	**300 µl** 1x dPCR buffer ● in the well	**#A**
	40 µl 1x dPCR buffer ● to the shelf	**#D**
	40 µl 1x dPCR reaction mix in well	**#C**
	100 µl dPCR oil ● in well	**#B**

10. Add a white rubber gasket to the top of the cartridge. Orient the gasket to the cartridge using the angled corner. Attach to the pins first and then to the T-hooks (Fig. 3.3).

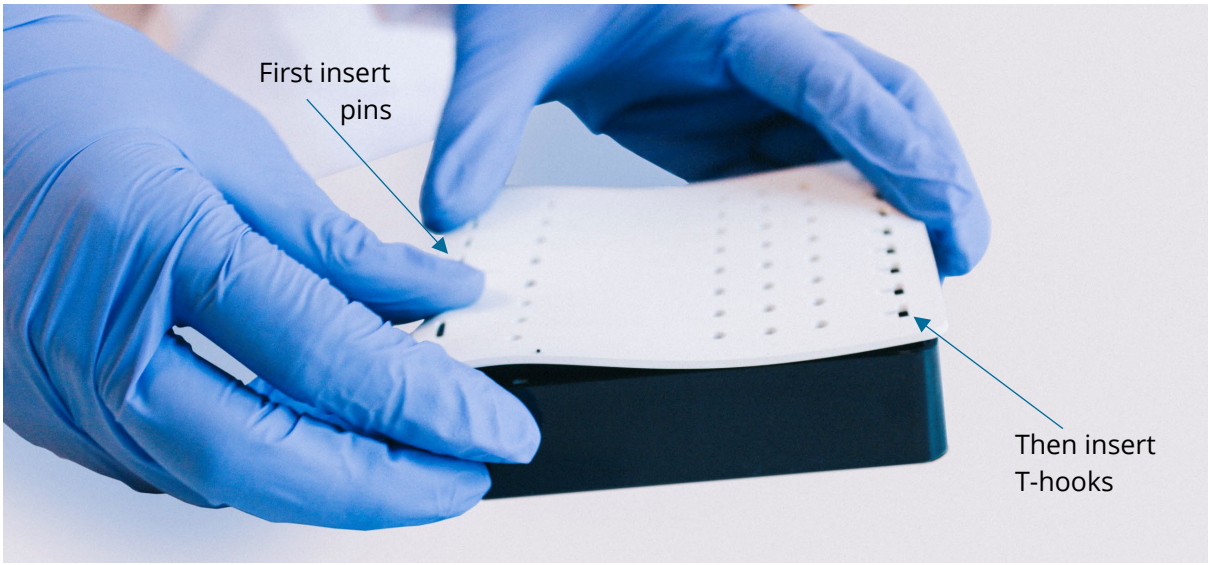


Fig. 3.3. Cover the cartridge with the white rubber gasket by orienting it correctly (angled corner on the gasket to angled corner on cartridge), then attach first the pins then pull gently to attach T-hooks.

Before turning on the instrument please make sure that the main power switch is in the "I" position. The main switch is located at the back of the instrument. Start the instrument by pushing the Start/Stop button at the front. The instrument will start initializing and will shift to the "Welcome screen".

11. Push "Open" on the instrument touch screen to eject the drawer (Fig. 3.4).

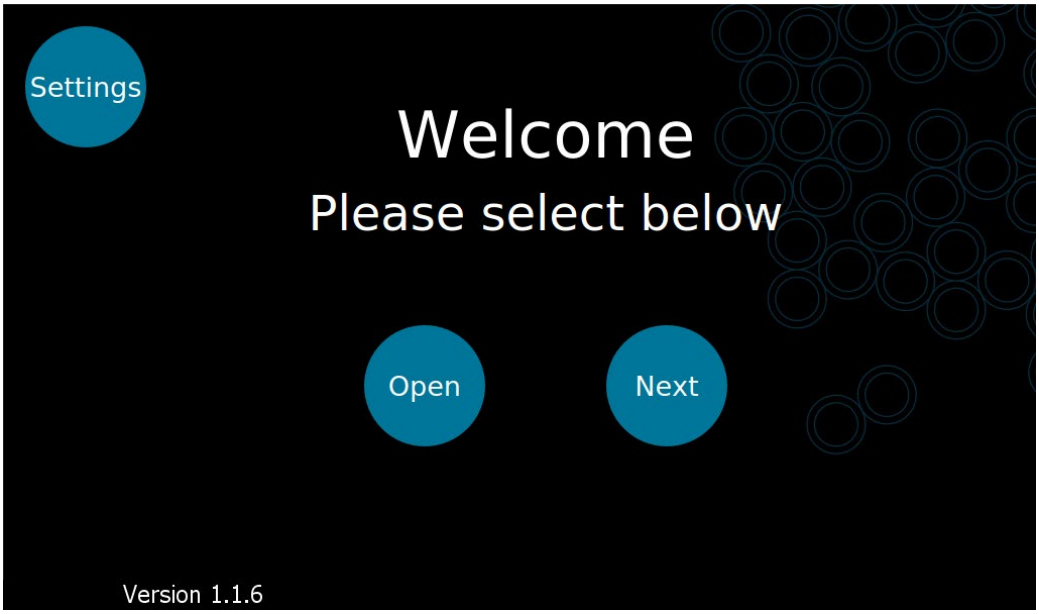


Fig. 3.4. The Xdrop™ instrument "Welcome" screen, press "open" to eject the drawer.

12. When the “Open” or “Next” button have been pressed, the screen shifts to “Please insert/remove cartridge” and “Close”. Make sure that the cartridge is correctly positioned into the drawer (Fig. 3.5) as it may otherwise cause damage to the instrument. Once the cartridge is correctly inserted, press “Close” to retract the drawer into the instrument.

Fig. 3.5. The Xdrop™ instrument with a correctly inserted dPCR cartridge. Be very careful to place the cartridge correctly as the instrument may otherwise damage the cartridge.

13. Once the drawer is fully closed, press “Next” on the touch screen.
14. The Xdrop™ instrument can operate with either dPCR or dMDA cartridges. Use the dPCR cartridge option by selecting “dPCR” on the touch screen (Fig. 3.6.).

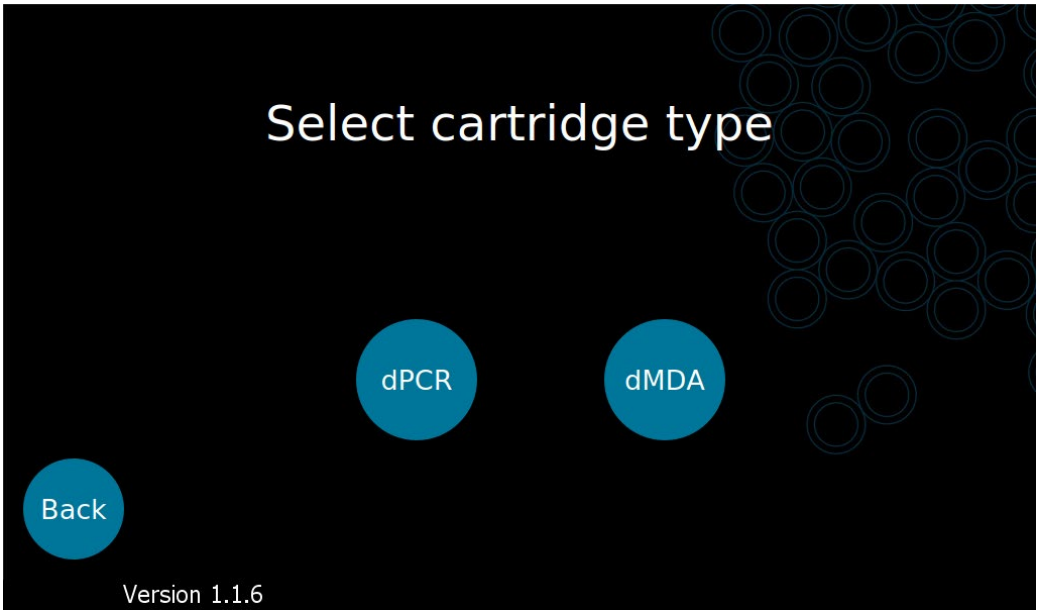


Fig. 3.6. The Xdrop™ instrument "Select cartridge type" screen, select dPCR cartridge.

15. The lanes to be processed are selected by pressing the corresponding numbers 1-8 on the screen. When selected, buttons turn green (green = selected and blue = not selected) (Fig. 3.7).

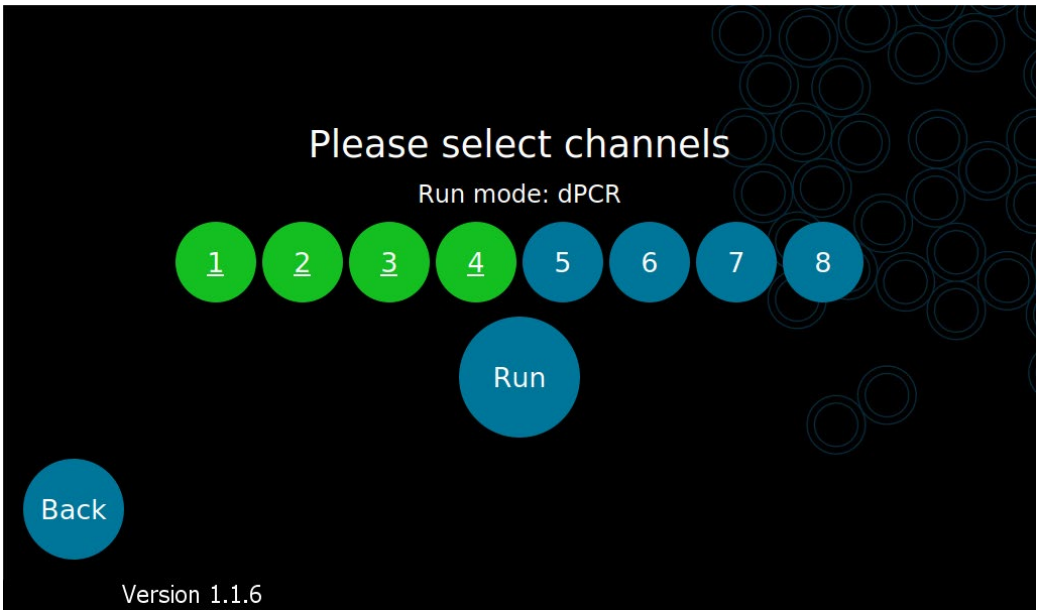


Fig. 3.7. Selecting the lanes to be used. Selected channels will be indicated by green buttons (here: 1 to 4). Blue buttons indicate channels not yet selected.

16. Press "Run".

The instrument will now build up pressure. This process can take up to 4 minutes.

Once the optimal pressures have been reached, the message “Making your droplets” and the remaining run time are displayed on the screen (Fig. 3.8). The Xdrop™ instrument will produce double emulsion dPCR droplets in approximately 40 minutes.

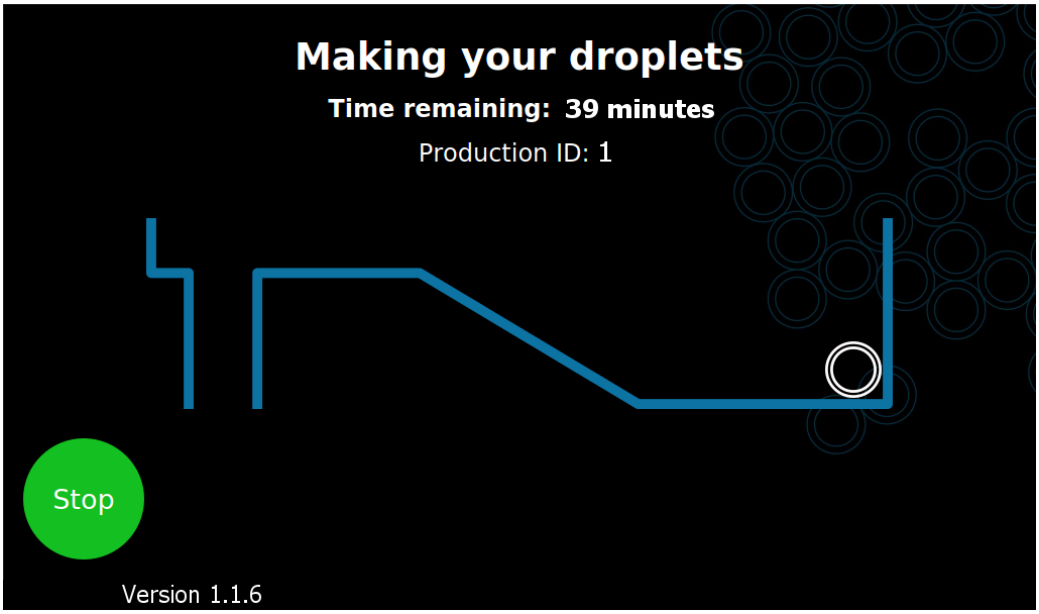


Fig. 3.8. Touch screen image while producing droplets.

17. When droplet production has been completed, the screen will change to “Your droplets are ready”.
18. Press “Open” to eject the cartridge.
19. Remove the cartridge from the instrument and place it in a LAF hood.
20. Press “Close” to make the instrument retract the drawer back into its closed position.
21. Press “Finish” to return to the Welcome screen.
22. Shut down the instrument after a completed droplet production to avoid damage to the instrument. If the instrument is not to be used for several hours, it should preferably be turned off. To turn the instrument off, push the ON/OFF button at the front to power off the instrument to initiate the automatic shutdown procedure.

Collect generated droplets

23. After droplet production, confirm that dPCR droplets have been produced. dPCR droplets will sink to the bottom of the collection well and form a white layer with a clear buffer phase on top.
24. Collect droplets from the collection well (#D) into a 0,5 ml or 1,5 ml tube. Use tips that minimize binding of droplets to the side of the tip.
25. 200 µl dPCR-buffer (surplus in the dPCR-buffer well #A) can be used to "wash" residual droplets from the shelf inside the collection well #D.

The droplets and buffer collected after droplet production should have a total volume of around 300-400 µl.

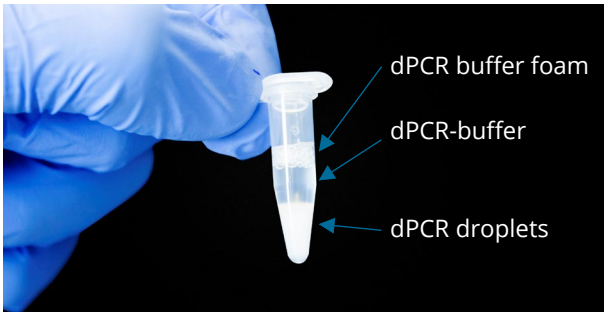


Fig. 3.9. Collection of dPCR droplets from the collection well (#D) into a 0,5 ml tube.

26. Mix gently and dispense the droplets from the 1,5 ml tube into four PCR-tube aliquots (of approximately 80-90 µl).

Note: dPCR droplets sediment rapidly during handling. To ensure equal distribution into the aliquots, be sure to mix gently by pipetting up and down between each pipetting step.

27. If any lanes are unused, the cartridge can be stored up to 1 week at room temperature. Discard the single-use gasket and remove excess liquid in wells #A and #B before storage of the cartridge.
28. Seal the cartridge with Storage film (Cat. No. FI00100) covering all wells and store protected from light and dust at room temperature in the provided bag.

Notice that the dPCR cartridge has a shelf-life of 1 week after the packaging has been opened, provided that this period does not exceed the expiry date indicated on the product.

Note: Each production lane and gasket are single-use and will not function properly if an attempt to re-use is carried out. Furthermore, attempting to reuse the gasket and cartridge lanes will increase the risk of cross-contamination of samples.

29. Place the PCR tubes in a thermal cycler and run the program described below. A block temperature ramp rate of 0,5°C/sec is recommended, with a total duration of the program of ~2 hours. This is to ensure slow temperature changes, allowing the reagents inside the droplets to reach the desired temperature.
30. Set the lid temperature to 105°C.

Temperature	Duration	Ramp rate	Cycles
30°C	5 sec	-	1x
94°C	2 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	∞	-	-

31. dPCR droplets can be stored after PCR at 4°C for up to 24 hours if proceeding with sorting and dMDA amplification. dPCR droplets are stable at 4°C up to 2 months, however, storage longer than 24 hours can compromise DNA quality and enrichment.


Good pause point: Store dPCR droplets after PCR at 4°C for up to 24 hours.

Chapter 4: Single DNA Molecule 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. 4.1).
- 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. 4.2) 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.

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 1ml 1x dPCR buffer to a flow cytometry tube (tubes depend on flow cytometer instrument).
    - Spin down Droplet dye at 5000 rpm ● 2 minutes.
    - Add 10 µl Droplet dye ● into the flow cytometry tube with 1ml 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 tube to transfer all droplets from the PCR tubes to the flow cytometry tube. Use tips that minimize binding of droplets to the side 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 tube containing the 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 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.

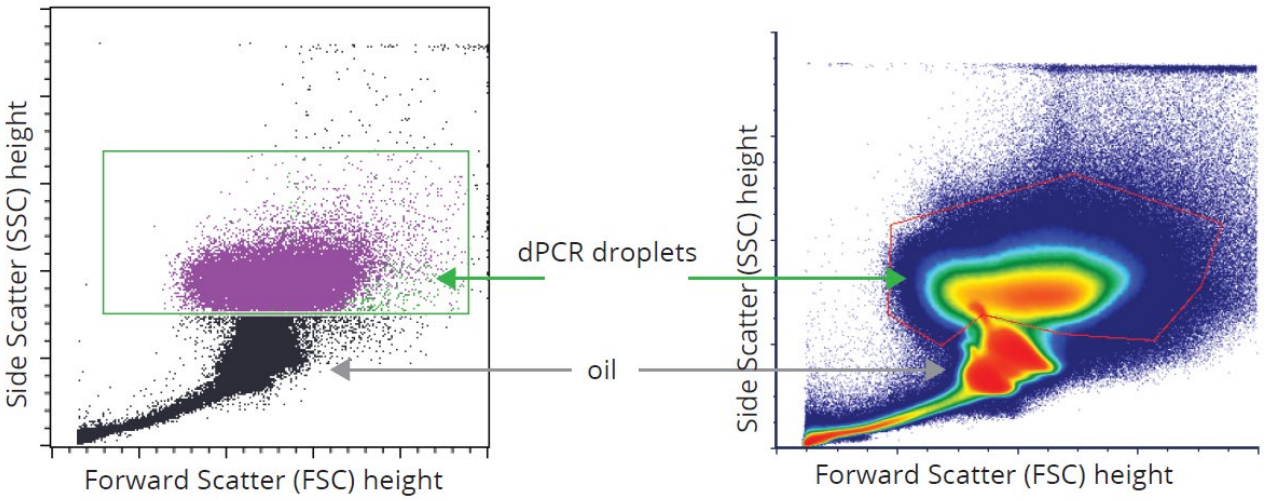


Fig. 4.1. Identify dPCR droplets on a plot showing forward scatter (FSC) versus side scatter (SSC) or back scatter (BSC). The "height" setting more clearly displays the population of correct dPCR droplets. The figure shows the same data on two different instruments. Left: Dot plot showing dPCR droplets 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).

8. 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 has a detectable fluorescent signal.

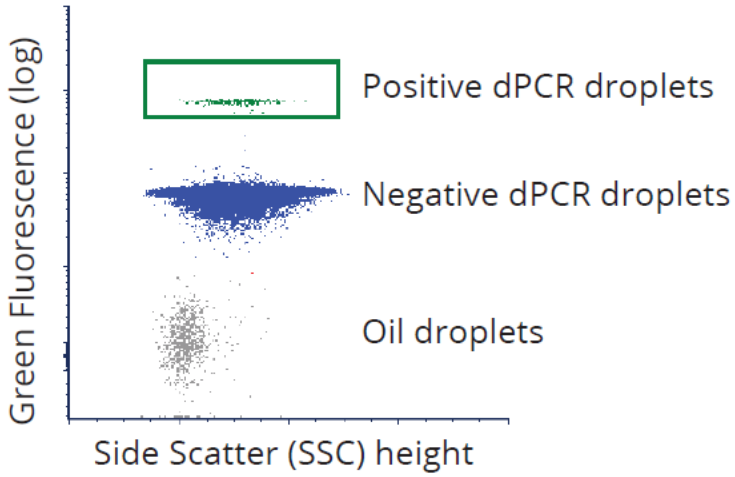


Fig. 4.2. Identify positive fluorescent population versus side scatter. The positive population is green, the negative population blue and the oil droplets grey in this figure. Set the software to show at least 100.000 events in the plot.

9. Set the gates as detailed above (Fig. 4.2), 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.
10. Start sorting the positive population into the collection tube containing 15 µl H_2O. Remember to acquire data during the sort for your records.
11. 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.

Optional: Set up flow cytometry with Cell sorter control kit (Cat. No. CO10100)

To easily set up flow cytometry of dPCR droplets, use the Samplix Cell sorter control kit (Cat. No. CO10100). This kit consists of ready-made dPCR droplets with a defined and large population of positive droplets. The kit allows you to establish the settings for dPCR droplet sorting.

1. 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.
2. Stain the Control droplets ○ with Droplet dye ● as follows:
    - Prepare flow cytometry buffer by adding 500 µl 1x dPCR-buffer to a flow cytometry tube (tubes depend on flow cytometer instrument).
    - Spin down the vial of Droplet dye ● at 5000 rpm for 2 minutes.
    - Add 5 µl Droplet dye ● into the flow cytometry tube with dPCR buffer. Mix gently to dissolve the dye in the dPCR-buffer.
    - Transfer 50 µl of Control droplets ○ to the prepared flow cytometry tube. Use tips that minimize binding of droplets to the side of the tip.
    - Leave mix of droplets and dPCR buffer at room temperature protected from light for 5 min to stain droplets.
3. Load the tube on a flow cytometer and start the analysis.
4. Identify the dPCR droplets as described above (see Fig. 4.1).
5. Identify positive and negative fluorescent droplets as described above (see Fig. 4.2). In the Cell sorter control droplets, you can expect the fraction of positive droplets to be about 10-15% of the total observed dPCR droplets.

Chapter 5: Multiple Displacement Amplification in droplets (dMDA)

Break sorted droplets (if applicable)

After sorting, keep the sorted dPCR droplets at 4°C and proceed immediately to dMDA amplification.
Do not store sorted dPCR droplets longer than 8 hours as this may lead to DNA degradation. If amplifying already purified DNA, continue directly to "Set up dMDA reaction", described on the next page.

After the enrichment step and sorting of selected DNA molecules from Xdrop™ dPCR droplets, release the DNA from the sorted dPCR droplets by breaking the droplets with Break solution ● and Break colour ● as described below.

1. Add 20 µl Break solution ● to each tube of sorted dPCR droplets.
2. Add 1 µl of Break colour ●. This will colour the water phase. If colouring is too weak, add 1 extra µl. Note: The water phase may be a colour ranging from yellow to purple as the Break colour functions as a pH indicator.
3. Flick tube gently, do not vortex.
4. Spin tube briefly (15-30 sec).
5. Remove the clear Break solution phase from the bottom of the tube and discard. Note: Be careful to remove all the Break solution as it may inhibit downstream enzymatic reactions.
6. Repeat steps 3-5 to remove all leftover Break solution.
7. Keep the coloured water phase, which will contain DNA from the positive dPCR droplets (Fig. 5.1).

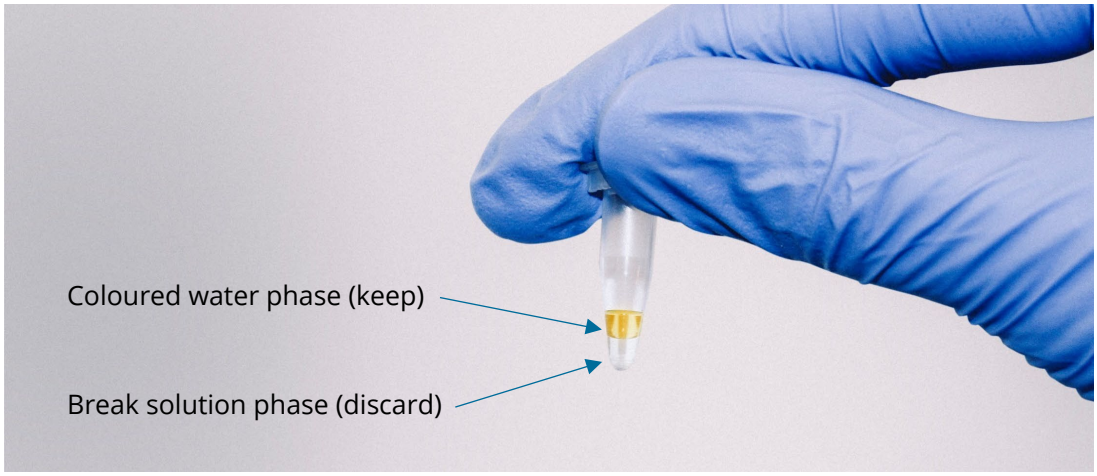


Fig. 5.1. Break sorted dPCR droplets with Break solution ● and Break colour ●. Discard the clear Break solution phase at the bottom of the tube. Keep the top coloured water phase, this phase will contain your DNA molecules.

Set up dMDA reaction

Note: Do not use any other reagents than Samplix dMDA kit (Cat. No. RE20300) for Xdrop™ dMDA 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 oil that should be kept at RT.

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 a LAF hood or similar clean, dust free environment.

1. Prepare the amplification mix (see table below). Mix gently, do not vortex.
2. Aliquot mix in cooling block. Important! Keep cold at 4°C until use.
3. Add 10 µl template (DNA from sorted dPCR droplets after break or genomic DNA 0,1 pg/µL).
4. We recommend including the following control reactions:
   - 10 µl molecular grade H_2O (negative control)
   - 10 µl sheath fluid from flow cytometer (contamination control)
   - 10 µl genomic DNA (0,1 pg/µL, positive control, provided by the user)
5. Mix gently and keep cold at 4°C until loading on the dMDA cartridge.

Note: When taking aliquots of your samples, always pipette from the center of the coloured phase of as an additional precaution to avoid carrying over remaining break solution.

Amplification mix	1X
H2O (molecular grade)	5 µl
dMDA mix (5x) ●	4 µl
dMDA enzyme O	1 µl
Total mix	**10 µl**
Template DNA solution	10 µl

Load the dMDA cartridge

The dMDA reaction takes place inside droplets formed in the dMDA cartridge inserted in the holder (Fig. 5.2). The cartridge must be sealed with a gasket on top during droplet production. Load samples in a clean LAF hood.

Note: The dMDA holder should be re-used for all the following runs.

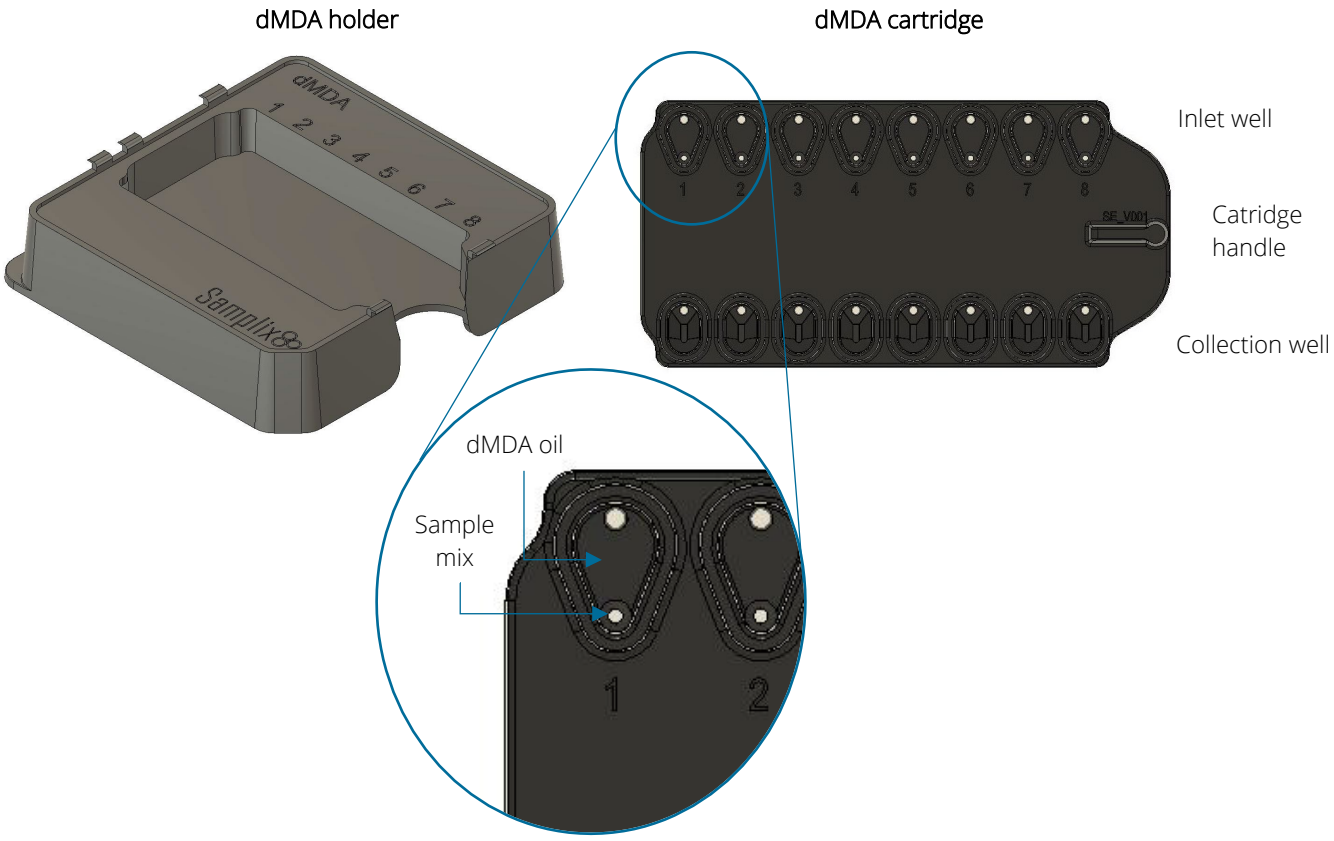


Fig. 5.2. Left: dMDA holder. Right: Top view of dMDA cartridge with inlet well on top and collection well below. Bottom: zoomed view of the holes of the inlet well.

Set up the cartridge:

1. Wearing gloves, unpack the dMDA holder and cartridge from their plastic bag.
2. Handle the cartridge as follows:

- Always wear gloves when handling the cartridge.
- Hold the cartridge by its sides or by the handle.
- Do not touch any of the inlet wells or droplet collection wells.

- Be cautious to avoid DNA contamination at all times.
- Store the cartridge (if partially used) covered by protective storage film in a clean, sealed plastic bag.

3. Be careful not to use the same sample lane more than once as this will disrupt droplet production and lead to contamination of your sample. In order to avoid using the same lane more than once, mark the storage plastic bag with a permanent marker once a lane has been used.

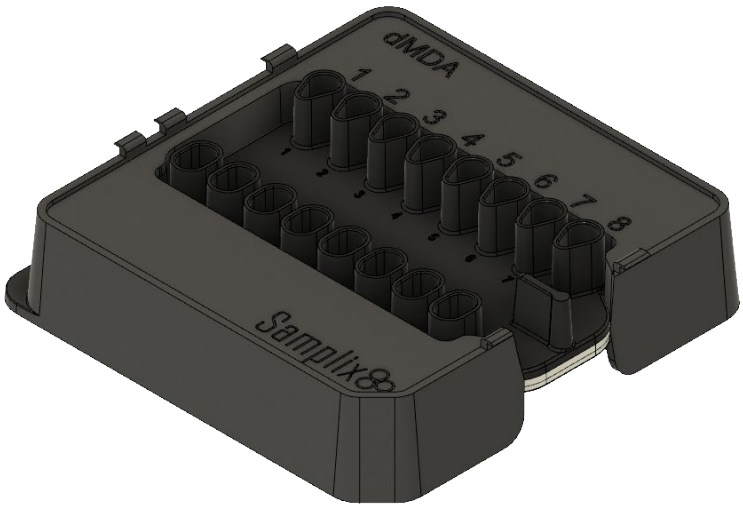


Fig. 5.3. The assembled dMDA cartridge with holder (dark grey) and dMDA cartridge (black) inserted.

Note: It is strictly mandatory that the sample is injected into the dMDA cartridge with wide bore pipette tips P200 with an outer diameter of 1 mm -1,9 mm.

4. Place the dMDA cartridge in the groove of the dMDA holder as shown in Fig 5.3. Inlet wells are placed closest to the numbers on the holder while the collection wells are placed closest to the side with the Samplix logo.
5. Place the wide bore pipette tip (with 20 µl sample mix) in the inlet hole at the bottom of the inlet well making a tight connection (Fig. 5.4 and Fig. 5.5).

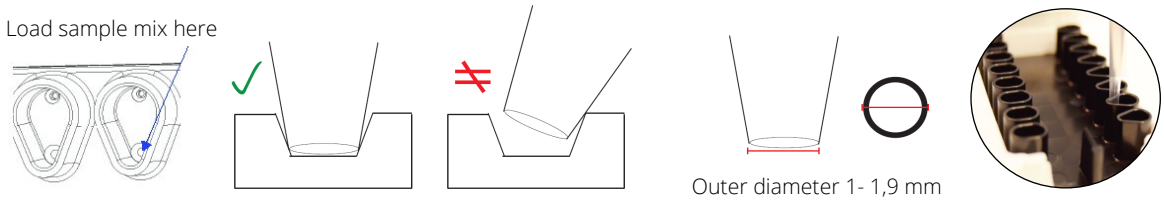


Fig. 5.4. Injection of the sample in the inlet well. From left to right: Cartridge inlet where to load the sample mix: the wide bore pipette tip should enter the hole in the pointy end of the tear shaped inlet well. Correct positioning of the wide bore pipette tip. Incorrect positioning of the wide bore pipette tip. Outer diameter of the wide bore pipette tip (1-1,9 mm). Picture of dMDA cartridge loading of the sample mix.

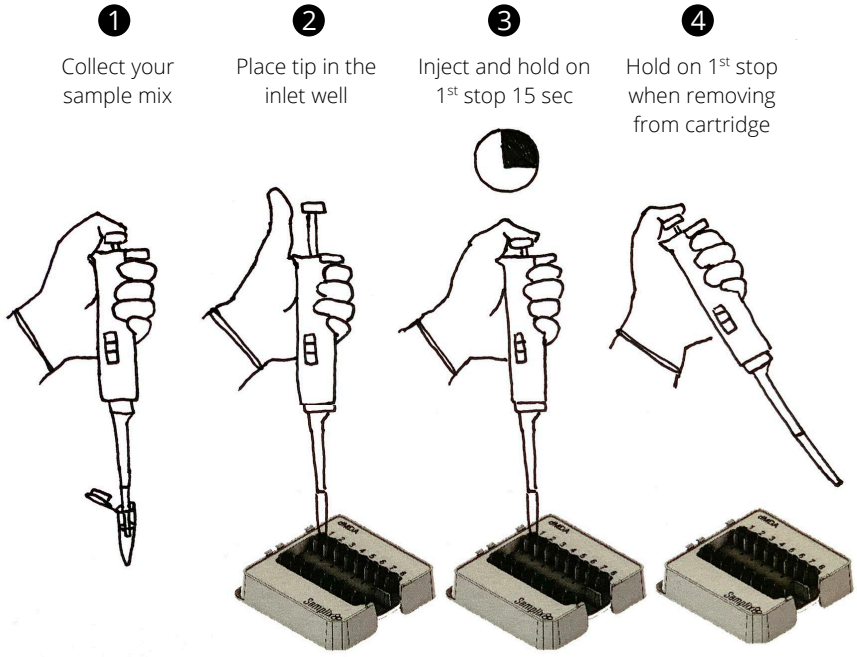


Fig. 5.5. Loading of the dMDA cartridge. 1) Collect your sample mix. 2) Place the wide bore pipette tip in the hole at the pointed end of the tear shaped inlet well. 3) Load the sample by slowly pressing the plunger to the first stop and keep it pressed for 15 seconds; this allows the mix to enter the microfluidic system. 4) Keep the plunger down at the first stop when removing the tip from the cartridge.

6. Slowly inject the sample and hold the pipette plunger in the first stop position for 15 seconds while still applying moderate pressure on the pipette tip to ensure a tight connection between the pipette and cartridge (see Fig. 5.4 and Fig. 5.5).
7. Remove the pipette while still holding the plunger button in the first position. The entire sample should have entered the channel in the chip, there should be no liquid visible in the well (see Fig. 5.5).
8. Repeat steps 5 to 7 for the next lane to use, if applicable.

9. Add 75 µl dMDA oil ● to the side of the inlet well allowing it to flow gently into the reservoir in the loaded lane(s). Do not inject the oil directly into the upper channel hole (see Fig. 5.2).
10. Place the gasket on top of the cartridge and fix using the pins and T-hooks (Fig. 5.6).

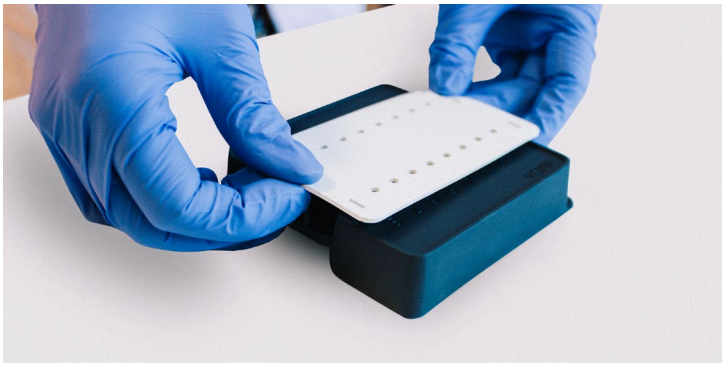


Fig. 5.6. Attach white rubber gasket to the dMDA cartridge. First insert pins and then T-hooks.

Generate dMDA droplets on the Xdrop™ instrument

Before powering up the instrument please make sure that the main power switch is in the "|" position. The main switch is located at the back of the instrument. Power up the instrument by pushing the Start/Stop button at the front. Instrument will start initializing and will shift to "Welcome screen".

1. Press "Open" on the Xdrop™ instrument touch screen to eject the drawer (Fig. 5.7).

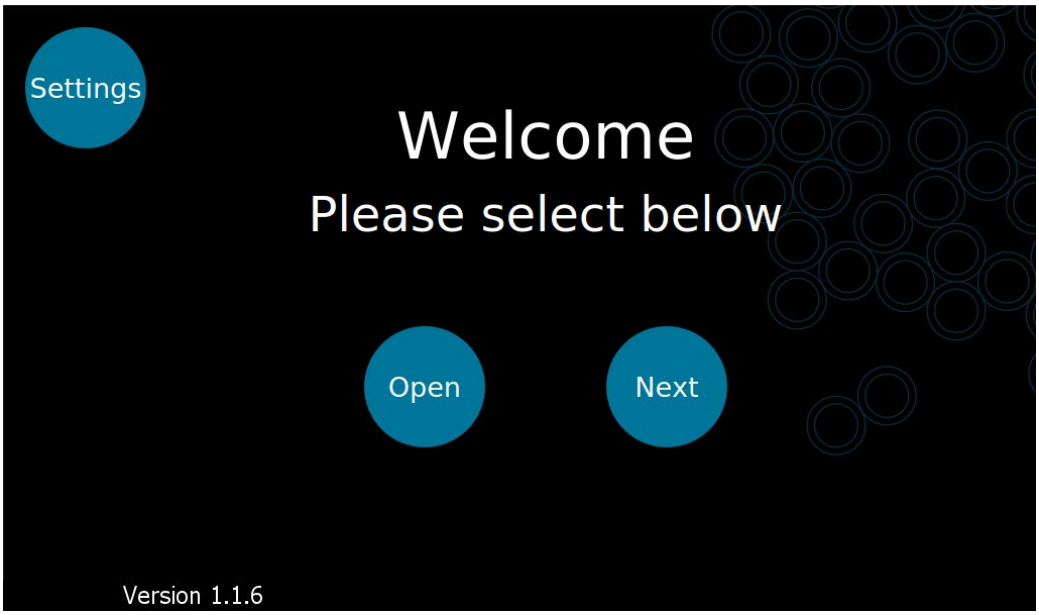


Fig. 5.7. The Xdrop™ instrument "welcome screen". Press open to eject the drawer.

2. When the “Open” or “Next” button has been pressed, the screen shifts to “Please insert/remove cartridge” and “Close”.

3. Place the loaded cartridge with the holder in the Xdrop™ instrument drawer. Make sure that the cartridge is correctly positioned into the drawer by aligning the rounded corner on the holder to the rounded corner on the instrument drawer (Fig. 5.8). Incorrect insertion of the cartridge may cause damage to the instrument. Once the cartridge is correctly inserted, press “close” to retract the drawer into the instrument.

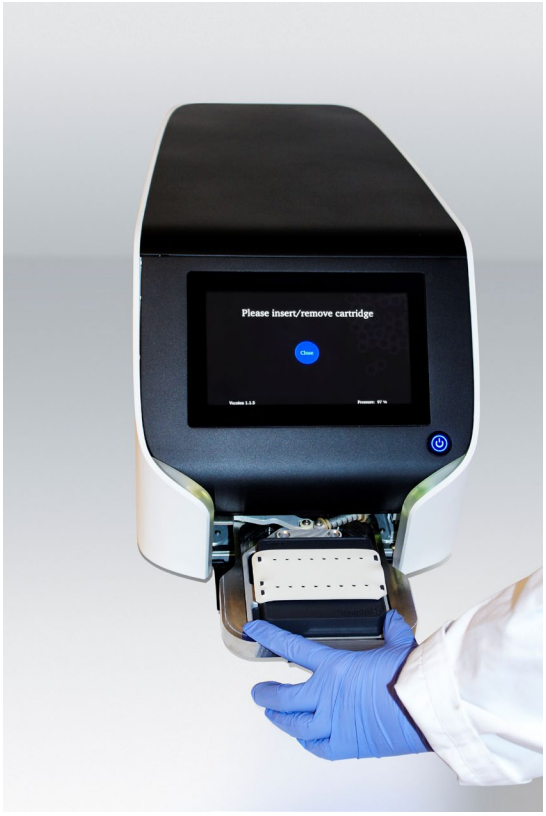


Fig. 5.8. Photo of Xdrop™ instrument with a correctly inserted dMDA cartridge.

4. Once the drawer is fully closed, press “next” on the touch screen.
5. The droplet generator instrument can operate with either dPCR or dMDA cartridges. Use the dMDA cartridge with the “dMDA” option on the touch screen (Fig. 5.9).

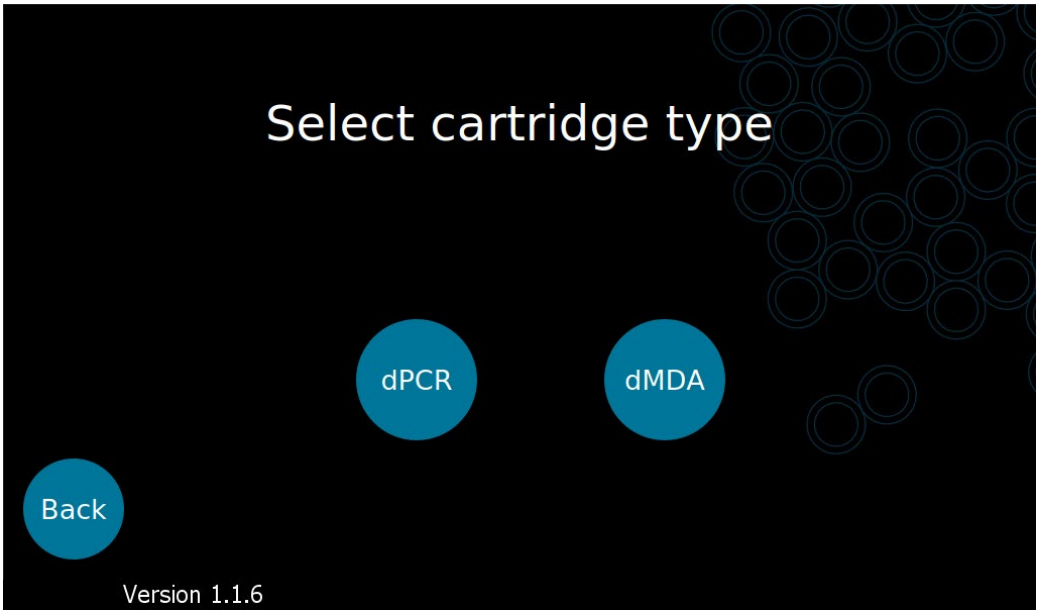


Fig. 5.9. The Xdrop™ instrument "Select cartridge type" screen. Select dMDA cartridge.

6. The channels to be processed are selected by pressing the corresponding numbers 1-8 on the screen. When selected, the button turns green (green = selected & blue = not selected) (Fig. 5.10). Deselect the channels not used.

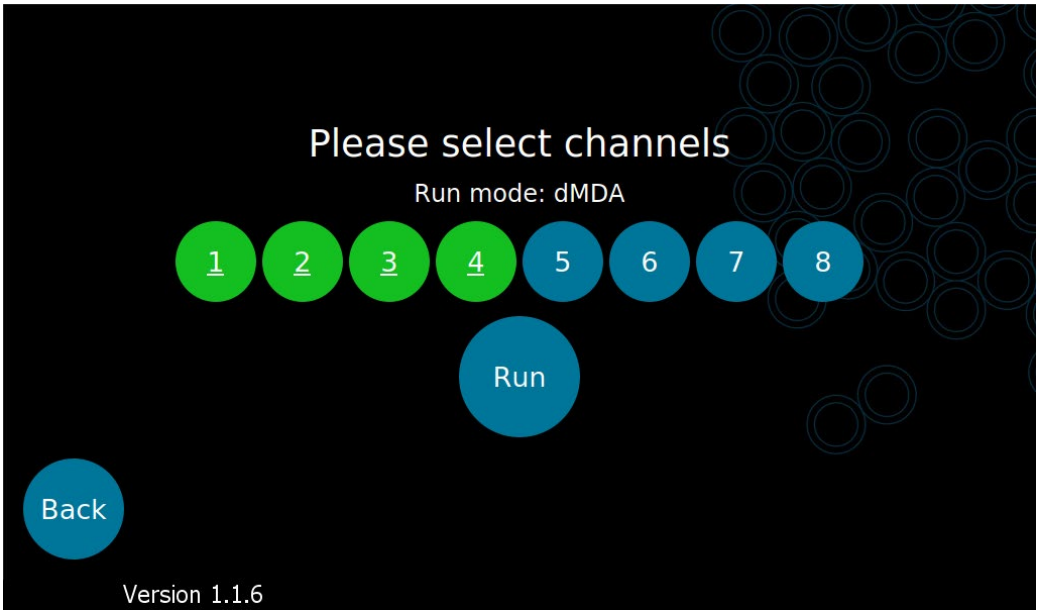


Fig. 5.10. Selecting the channels to be used. Selected channels will be indicated by green buttons (in this figure, channels 1 to 4). Blue buttons indicate channels not yet selected.

7. Press “run”.

The message “Making your droplets” and the remaining run time is displayed on the screen (Fig.5.11). The dMDA protocol will produce droplets in approximately 45 seconds.

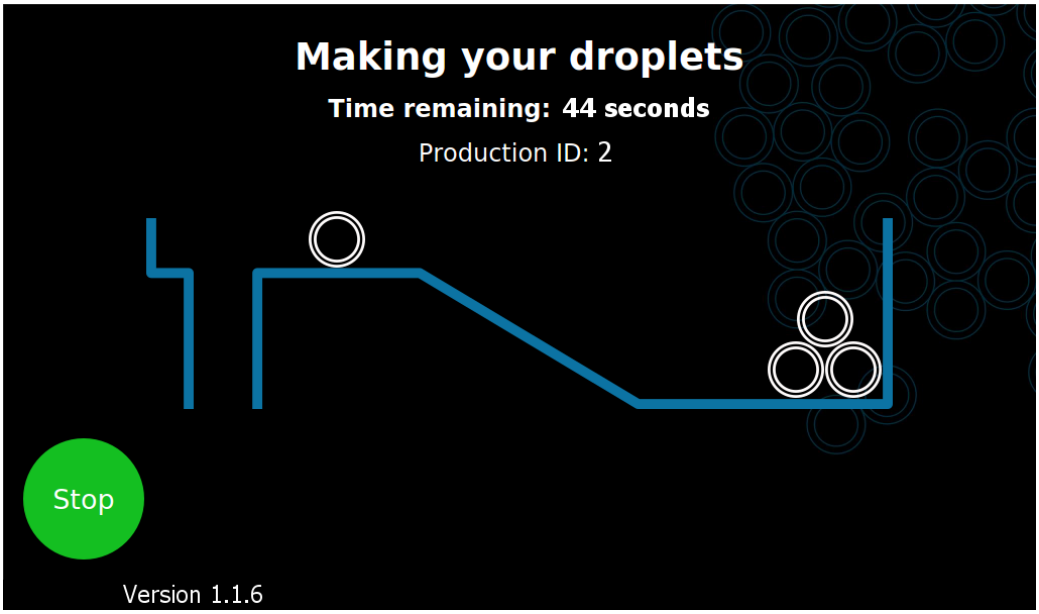


Fig. 5.11. Touch screen image while producing droplets.

8. Once droplet production has been completed, the screen will change to “Your droplets are ready”.
9. Press “Open” to eject the cartridge.
10. Remove the cartridge from the instrument and place it in a LAF hood.
11. Press “Close” to make the instrument retract the drawer back into its closed position.
12. Press “Finish” to return to the Welcome screen.
13. Shut down the instrument after a completed droplet production to avoid damage to the instrument. If the instrument is not to be used for several hours, it should preferably be turned off. To turn it off, push the ON/OFF button at the front to power off the instrument to initiate the automatic shutdown procedure.
14. Collect all the dMDA droplets from the collection well with a P200 pipette and transfer into a nuclease- and DNA-free PCR tube. Collect dMDA droplets by slowly pipetting from the sides towards the center/channel of the well. The total volume of dMDA droplets and oil in the collection well is 70-100 µl (Fig. 5.12).

Note: Each production lane and gasket are single-use and will not function properly if an attempt to re-use already processed lanes is carried out. Furthermore, attempting to reuse the cartridge and gasket will increase the risk of cross-contamination of samples.

15. Inspect the volume of collected droplets before removing the oil (step 16). You can expect approximately 2-3 mm layer of droplets on top of the oil phase.
16. Remove the excess dMDA oil from the bottom of the collection PCR tube. Only 1-2 mm of dMDA oil should be left in the bottom of the tube (see Fig. 5.12).

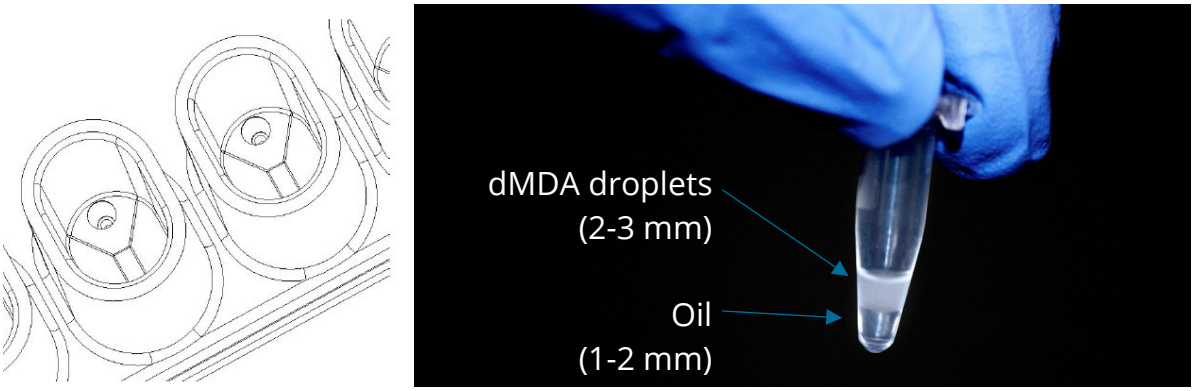


Fig. 5.12. Collection of dMDA droplets from the collection well. Left: Drawing of collection well. Notice that the sides are slightly slanting towards the inlet hole of the well. Collect the dMDA droplets by pipetting gently. Right: dMDA droplets in PCR tube after collection. dMDA droplets will form a white layer on the top with the excess oil at the bottom.

17. Incubate dMDA 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	∞

18. If some lanes are still unused when all samples have been run, place the Samplix Storage film (Cat. No. FI00100) over the cartridge while still in the holder (Fig. 5.13) and store the dMDA cartridge and holder in a Ziplock bag up to 1 week at room temperature. The Storage film should be placed so that all wells (used and unused) are sealed. Note that the dMDA cartridge has a shelf-life of 1 week after the packaging has been opened, provided that this period does not exceed the expiry date indicated on the product.

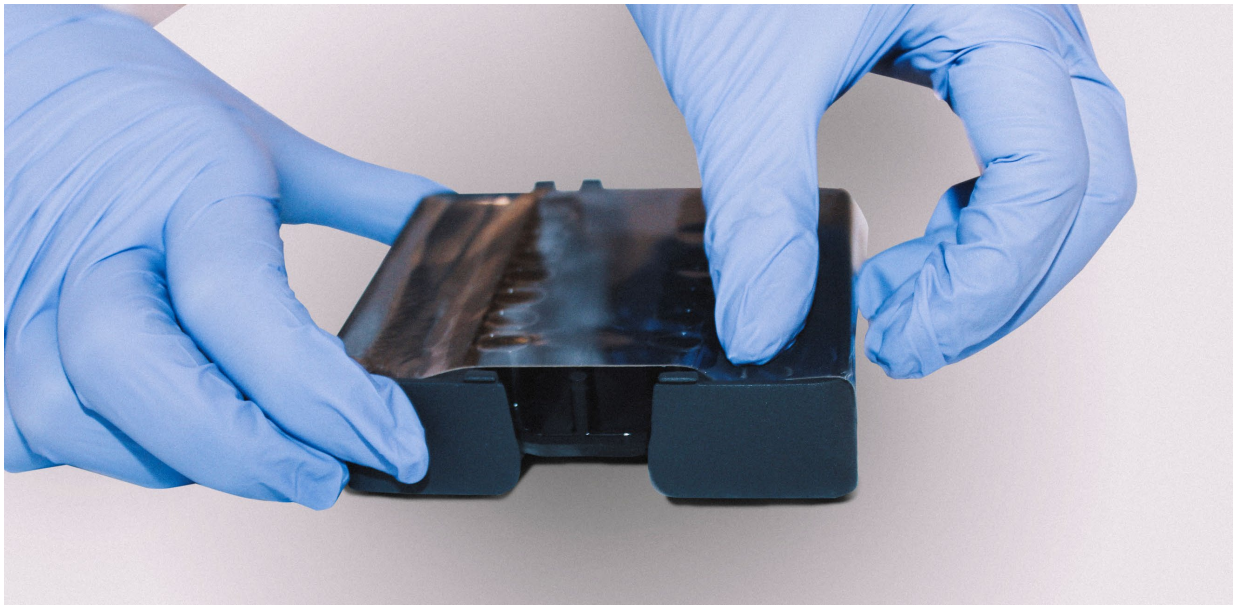


Fig. 5.13. Place a transparent protective film on the dMDA cartridge to seal the wells to avoid cross-contamination.

Chapter 6: Evaluation of Amplification and Target Enrichment

Quantify total DNA

After dMDA incubation, break the dMDA droplets with Break solution ● and Break colour ● (Fig. 6.1).

1. Add 20 µl Break solution ● to each tube.
2. Add 1 µl of Break colour ●. This will colour the water phase. If colouring is too weak, add 1 extra µl.
   Note: The water phase may be a colour ranging from yellow to purple as the Break colour is functioning as a pH indicator as well.
3. Flick tube gently, do not vortex.
4. Spin tube briefly (15-30 sec).
5. Remove the clear Break solution phase from the bottom of the tube and discard.
6. Repeat steps 3-5 to remove all leftover Break solution. It is important to remove all the Break solution as residual Break solution may inhibit downstream enzymatic reactions.
7. Keep the coloured water phase, which will contain the amplified DNA (Fig. 6.1).

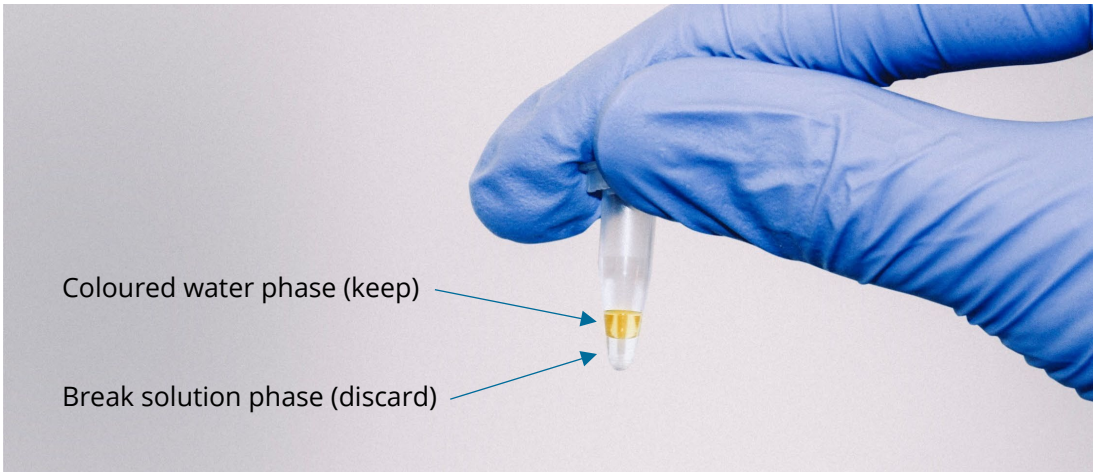


Fig. 6.1. Break post incubation dMDA droplets with Break solution ● and Break colour ●. Discard the clear break solution phase at the bottom of the tube. Keep the top coloured water phase, this phase will contain your DNA molecules.

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

Evaluate the enrichment of target DNA

After quantification of total DNA continue with measurement of target enrichment. An online tool for calculating the DNA enrichment based on qPCR is available at samplix.com

To determine fold enrichment of target DNA, perform a qPCR using the qPCR QC primers, not overlapping with the dPCR enrichment amplicon(s). See "Droplet PCR Reaction Design for Target Enrichment" (Chapter 2) or the online primer design tool at samplix.com. If a specific region of DNA is required, place the validation primer pair on the opposite side of this region but with maximum 5 kb distance from the dPCR primers. The enrichment measure is indicative of enrichment and might differ from the enrichment measured by sequencing.

To quantify the enrichment, perform a standard qPCR reaction using either the Samplix primer test PCR kit (Cat. No. RE10200) or your own preferred qPCR reagents (Fig. 6.2).

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

- dMDA amplified sorted positive population, 1:10 dilution (enriched sample, red curve in Fig. 6.2)
- dMDA H_2O, 1:10 dilution (negative control)
- Original sample input DNA in the same concentration as input in droplets. Run a standard curve of original sample to calculate PCR efficiency (grey curves in Fig. 6.2).

Suggested controls:

- dMDA sheath fluid from flow cytometer, 1:10 dilution (contamination control)
- dMDA 1 pg non-sorted input DNA, 1:10 dilution (positive control)
- H_2O (negative PCR control)

Note: When taking aliquots of your samples, always pipette from the center of the coloured phase as an additional precaution to avoid carrying over remaining break solution.

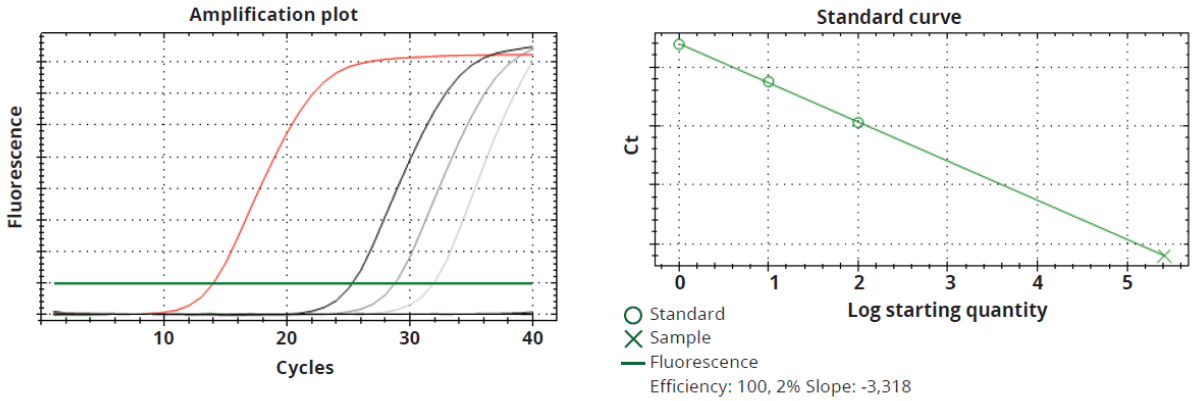


Fig. 6.2 Example of amplification and standard curves for qPCR QC validation of enrichment. Left: In the Amplification plot, the curve in red represents the dMDA amplified sorted positive population, while the grey shades represent 10x, 1x and 0,1x reference DNA (from darker to lighter). Right: Standard curve and calculations of PCR efficiency.

Calculate fold enrichment of target DNA

Use the "Enrichment calculator" in the "Tool" section under "Resources" on the Samplix homepage (Fig. 6.3) to calculate the fold enrichment of target DNA available at samplix.com

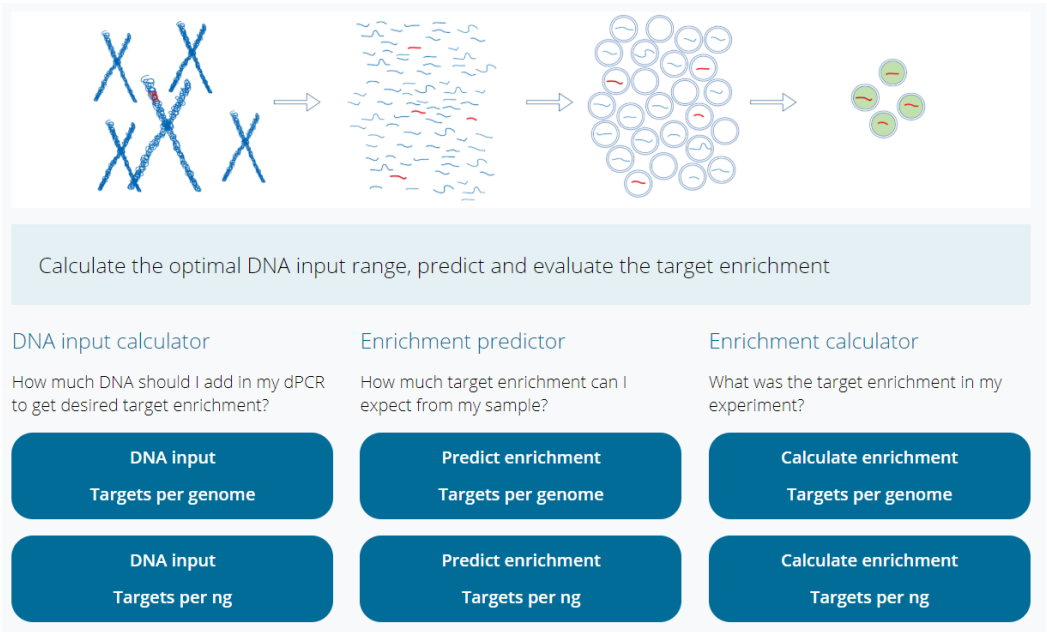


Fig. 6.3. Choose one of the two "Enrichment calculator" tools shown on the right. Enrichment can be calculated based on the number of targets per genome or the number of targets per nanogram of input DNA.

Example: Calculate Enrichment based on Targets per genome

If you select the tool "Calculate Enrichment based on Targets per genome", the following information is required:

- *Genome size*: Size of (host) genome in base pairs.
- *PCR efficiency*: Efficiency of PCR reaction in %. You can use the efficiency calculated when validating primers designed (if same qPCR reagents have been used). Calculate the PCR efficiency using the Ct values as input with the formula: (10^(-1/slope)-1) *100. If a % PCR efficiency has not been calculated, use 100% as input.
- *Targets per genome*: Copy number of the target in host genome disregarding off-targets.

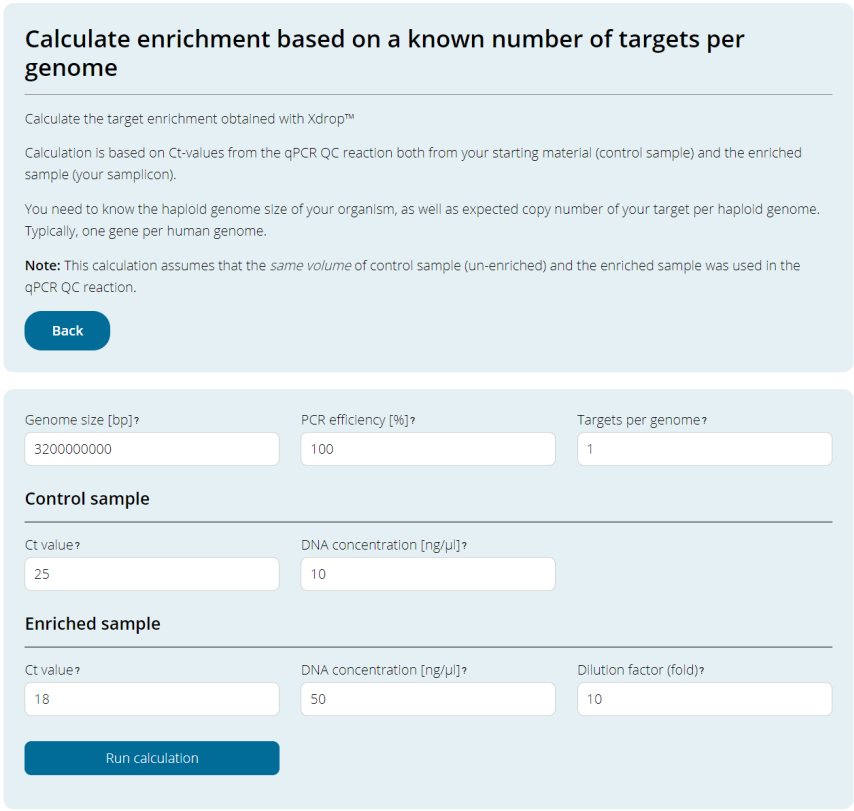


Fig. 6.4. Example of calculation using the online enrichment calculator tool. See explanation in the text.

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 dMDA reaction as input.
- *DNA concentration*: Concentration of DNA in the enriched sample (i.e. the concentration of DNA after the dMDA reaction) measured Qubit™, Bioanalyzer™, TapeStation™, FEMTO Pulse™ or similar.
- *Dilution factor*: Factor of dilution of dMDA reaction in qPCR (1:10 fold dilution recommended).

The calculator will provide you with the assessment of a successful enrichment of your DNA sample, as well as 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 technology.

Please contact us at support@samplix.com for library preparation and sequencing recommendations.