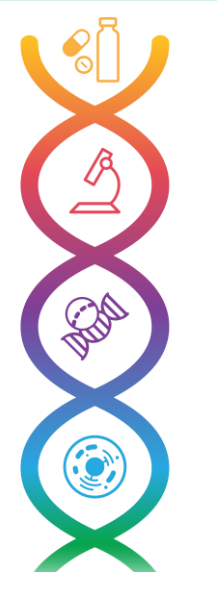# Quantifying fetal DNA in maternal blood plasma by ddPCR using DNA methylation

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Abstract

The proportion of cell-free DNA (cfDNA) circulating in maternal blood originating from the fetus, the fetal fraction, is an important quality control metric for tests on fetal-derived cfDNA. Epigenetic differences produce dissimilar DNA methylation patterns, and high-contrast regions are leveraged via methylation-sensitive digestion to quantify fetal and maternal DNA via droplet digital PCR (ddPCR). This advancement positions ddPCR as a faster and less expensive alternative to NGS for fetal fraction estimation.

## Droplet digital PCR can distinguish fetal & maternal methylation

- Differences in fetal and maternal DNA methylation are visualized in ddPCR using methylation-sensitive restriction enzymes (MSREs).

- MSRE digestion is performed in droplets without disruption to ddPCR workflow.
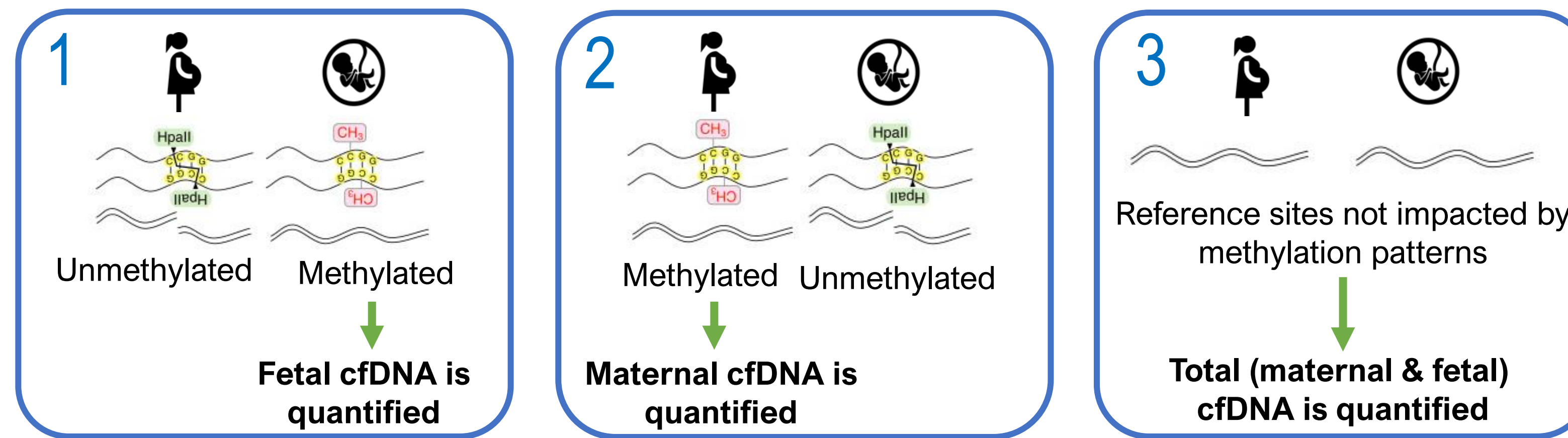

These steps happen simultaneously in the same ddPCR reaction: 1) Hypermethylated fetal DNA is quantified after digestion of hypomethylated maternal DNA. 2) Maternal DNA is similarly quantified. 3) Total DNA is quantified by targeting non-digested regions, allowing for calculation of the maternal blood's fetal fraction.

- A TaqMan assay targeting 3 hypermethylated fetal and 2 hypermethylated maternal sites produced strong correlation between ddPCR and NGS NIPT on clinical samples.

$$\% \, Fetal \, DNA = \frac{Fetal_{Norm}}{Fetal_{Norm} + Maternal_{Norm}}$$
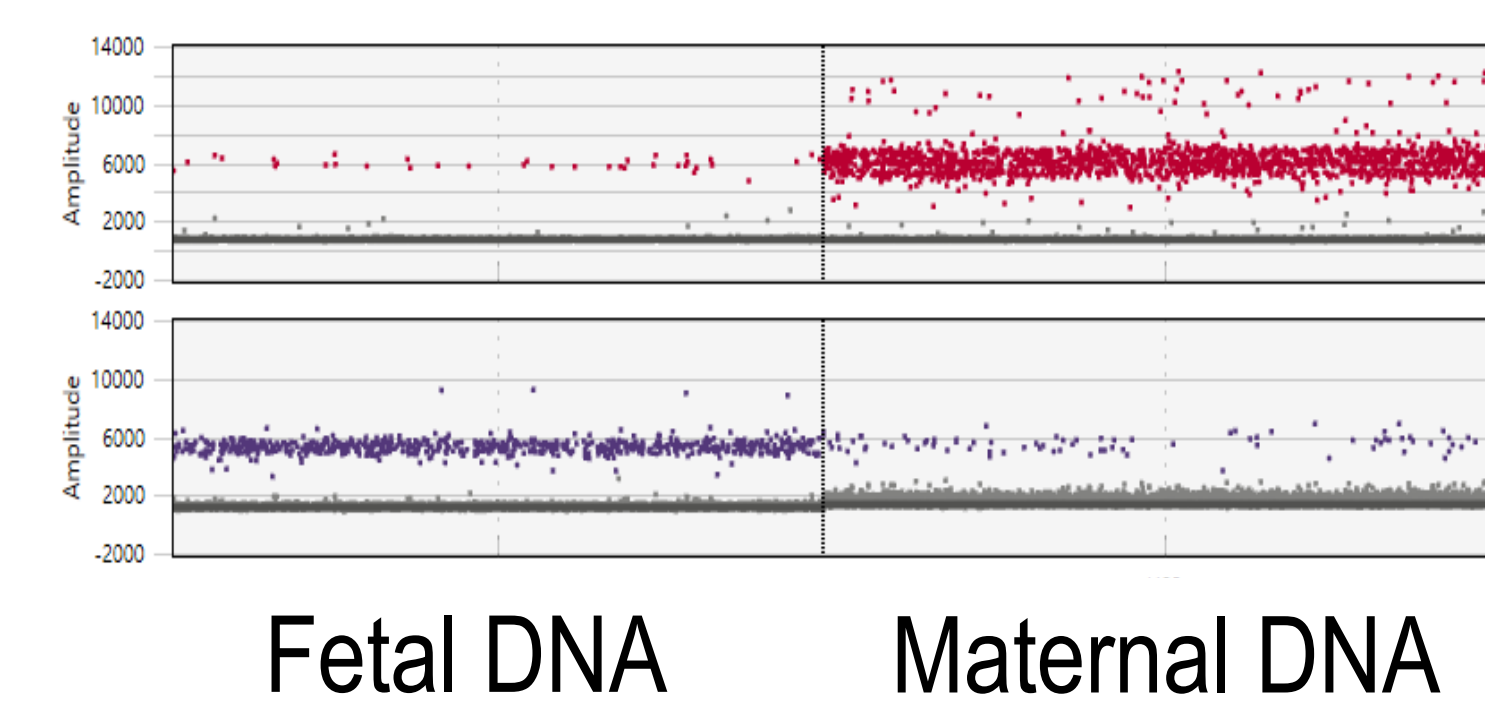
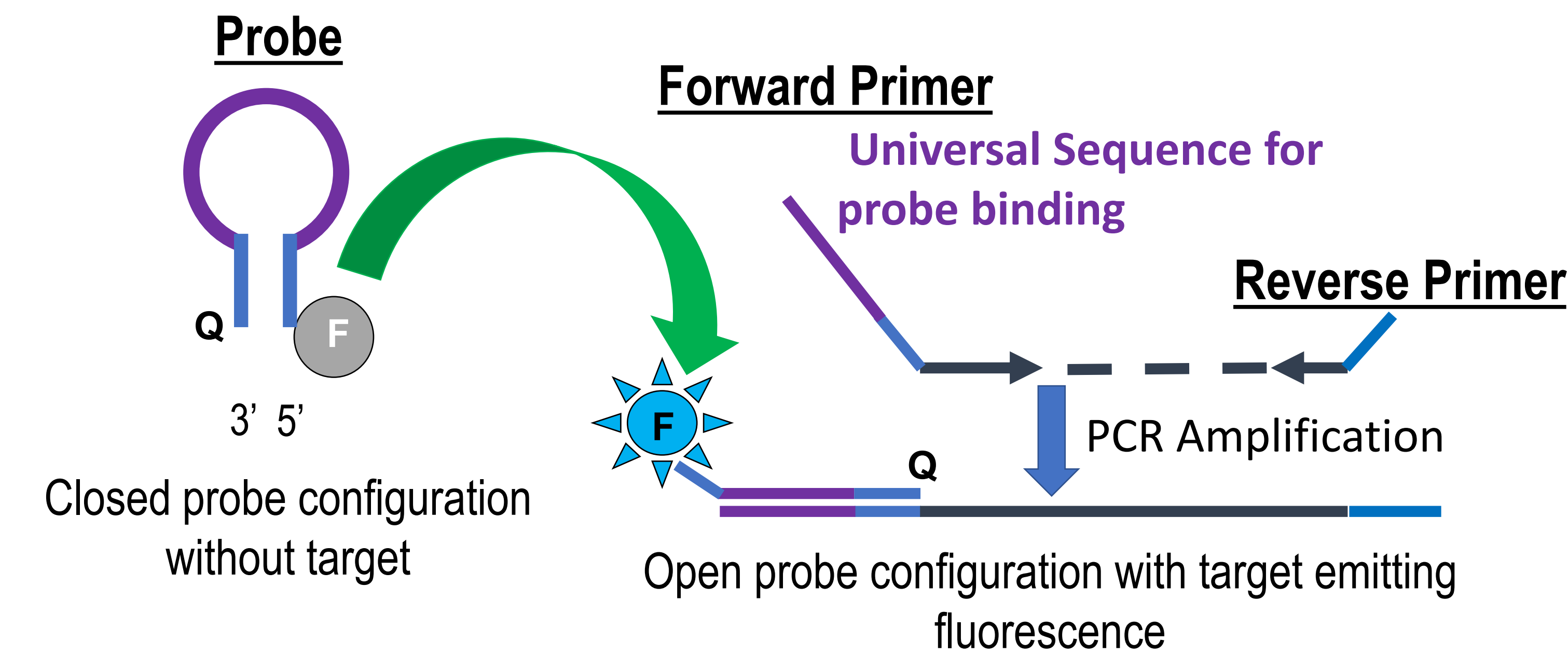
Following MSRE digestion, ddPCR signal is retained for maternal assays (top) for Maternal DNA and fetal assays (bottom) for Fetal DNA

Pregnant Clinical Samples

y = 1.3015x + 0.0592
R² = 0.9466
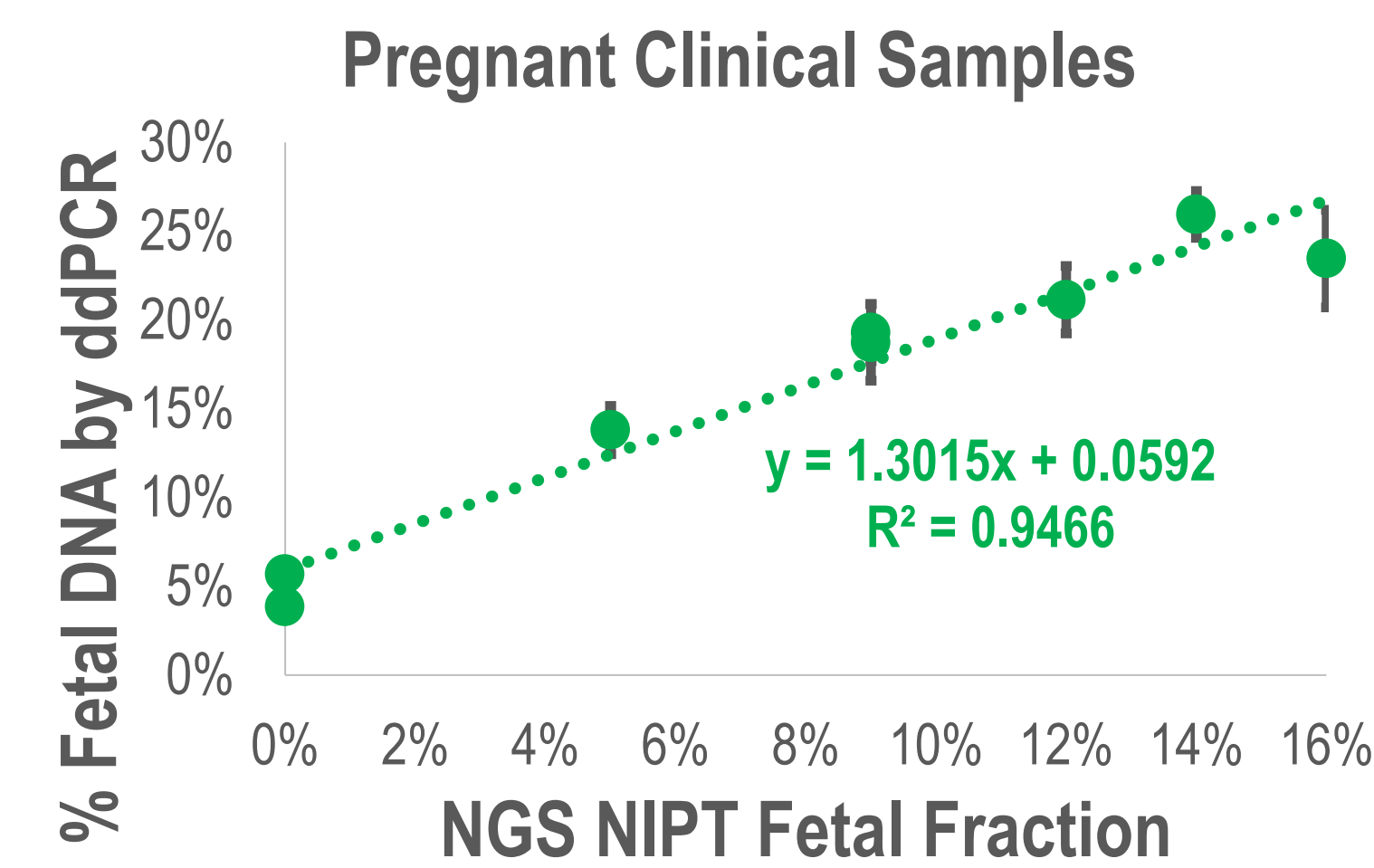
ddPCR % fetal DNA for a set of 2 non-pregnant and 6 pregnant clinical samples (n = 3 replicates).

## Universal probes allow for high-level multiplexing on QX600

Probe

Forward Primer
Universal Sequence for probe binding

Reverse Primer

3' 5'

Closed probe configuration without target

Open probe configuration with target emitting fluorescence

PCR Amplification

All forward primers within a target group have the same universal sequence and are assigned to the same universal probe.

Channel	Probe Target Group	Assays per Group	Probes per Group
FAM	Hypomethylated Reference	5	1
HEX	Hypermethylated Maternal DNA	5	1
Cy5	Hypermethylated Reference	5	1
Cy5.5	Hypermethylated Reference	5	1
ROX	Y Chromosome	5	1
ATTO	Hypermethylated Fetal DNA	5	1

## Improved assay selection with an automated design engine

- An internally developed assay design engine performs ultra-fast generation of primer pairs for high level multiplexing, minimizing off-target and heterodimer events.

- Integration of NGS methylation data allows the engine to quickly design large multiplexes of compatible assays targeting regions showing high fetal/maternal methylation contrast.
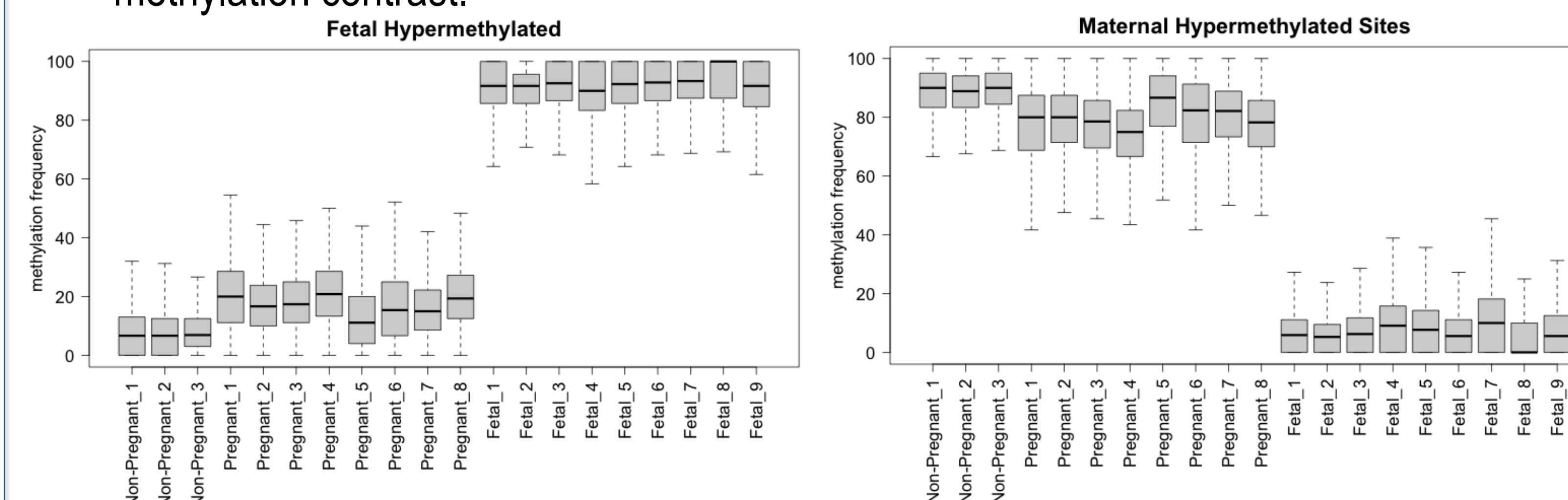
Methylation frequency of sites with high methylation contrast between fetal and maternal DNA. Non-pregnant and pregnant DNA were isolated from human plasma while fetal DNA was extracted from placental tissue.

A ddPCR 30-plex for estimating fetal DNA in maternal blood plasma

- A 30-plex (5 assays / target group) utilizing **one universal probe per channel** was tested on a set of 30 clinical samples on a Bio-Rad QX600 ddPCR system.

- Positive droplet clusters were identified using an automated thresholding algorithm, after dynamic color calibration for each fluorescence channel.

- Correlation (r^2 =0.87) was observed between ddPCR fetal fraction and attached NGS NIPT values, following assay normalization and correction. Note: NGS NIPT FF values were obtained at earlier gestational ages than the sample collected for ddPCR.

Pregnant Clinical Samples

y = 0.8557x
R² = 0.8774
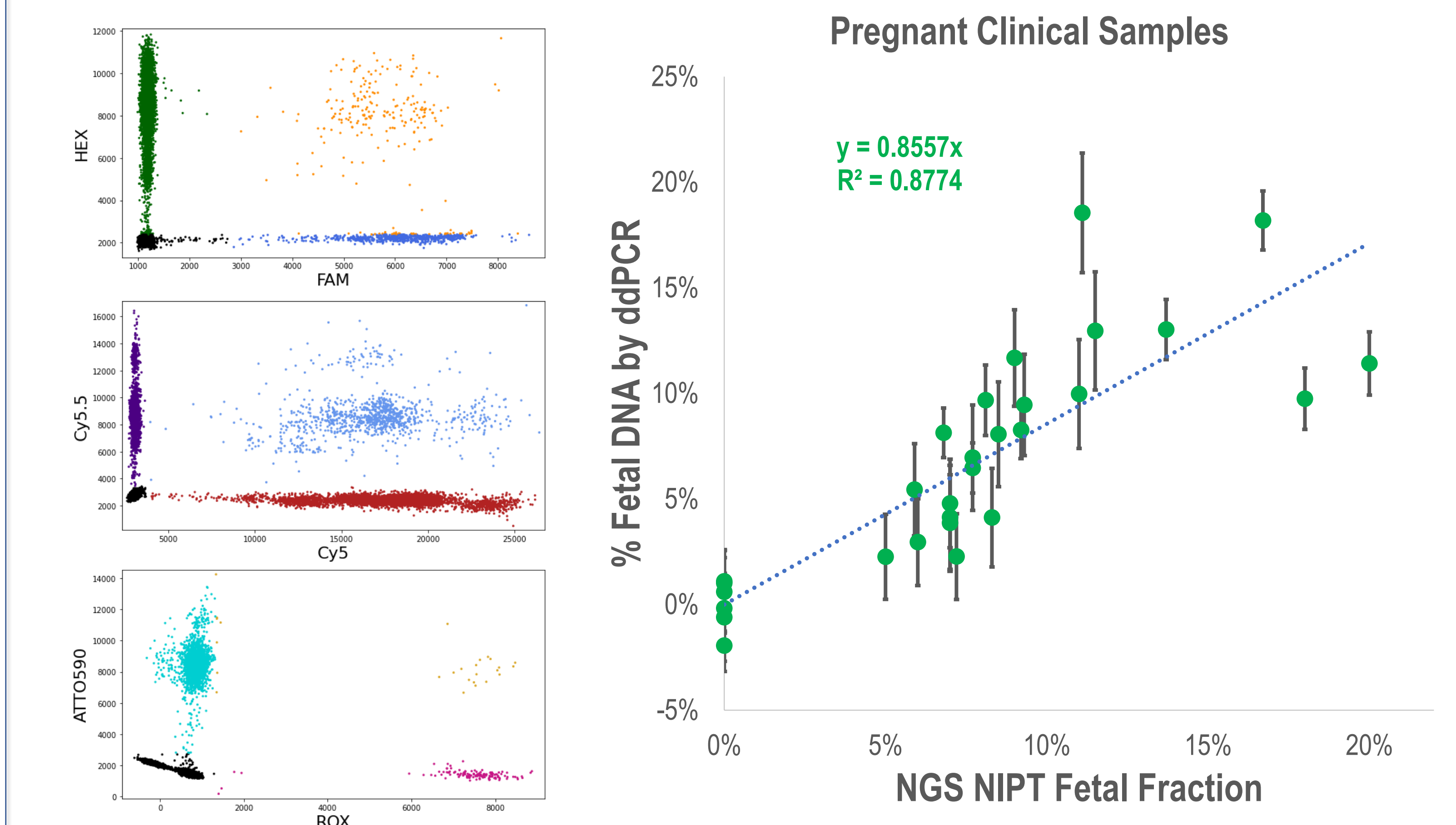
A 6-channel ddPCR multiplex with universal probes was tested on 30 clinical samples (6 non-pregnant, 24 pregnant, n=1 replicate). 2D ddPCR plots are shown for one sample well with a 7% FF male pregnancy (left)). Samples showed good correlation with attached NGS NIPT results when corrected by non-pregnant samples.

Methylation-based analyses can be performed in ddPCR

- **Detection of methylation differences across otherwise similar DNA can be done with no impact to the ddPCR workflow.**

- **The use of universal probes allows for higher levels of multiplexing with only one probe used across several assays per fluorescence channel.**

- **Integration of NGS methylation data with an automated high-plex design engine resulted in faster assay design targeting regions of high methylation contrast.**

- **Efficient design of multiplexes targeting differentially methylated sites also has utility in other methylation-based biomarker analyses, including some cancers.**

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