1   **A single droplet digital PCR for *ESR1* activating mutations detection in plasma**

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16   **Running title:** Detection of circulating *ESR1* mutations by ddPCR

17   **Keywords:** *ESR1* mutation, ddPCR, circulating tumor DNA, breast cancer, monitoring

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1   **Disclosure:** C.P., E.J., M.H.S., and F.C.B. have ongoing patent applications relating to

2   circulating tumor DNA analysis.

1 **Statement of translational relevance:**

2 Exons 5 and 8 mutations in *ESR1* are recurrent mechanisms of resistance to aromatase

3 inhibitors (AI) in estrogen receptor (ER)-positive metastatic breast cancer and may be

4 targeted by selective ER down-regulators. We implemented a novel droplet digital PCR,

5 which allows for the detection of the most frequent *ESR1* mutations in circulating cell-free

6 DNA. In prospectively collected plasma samples, *ESR1* mutations were found in 29% of AI-

7 resistant patients, with excellent concordance and higher sensitivity to next generation

8 sequencing. Moreover, circulating *ESR1* mutations appear to be reliable markers for ctDNA

9 monitoring in order to predict treatment response. Ultimately, the short turnaround time, high

10 sensitivity and limited cost of the *ESR1*-ddPCR are compatible with repeated samplings to

11 detect the onset of resistance to AI before the radiological progression. This opens a window

12 of opportunity to develop new clinical strategies for breast cancer hormone therapy, as tested

13 in an ongoing phase 3 trial.

List of abbreviations:

Aromatase Inhibitor (AI)

Cell-free DNA (cfDNA)

Circulating tumor DNA (ctDNA)

Droplet digital PCR (ddPCR)

ER+ HER2-negative Metastatic Breast Cancer (ER+ HER2- MBC)

Estrogen Receptor (ER)

Estrogen Receptor positive (*ER*+)

Limit of blank (LOB)

Limit of detection (LOD)

Mutant Allele Frequency (MAF)

Peripheral blood mononuclear cells (PBMC)

Progressive disease (PD)

Standard deviation (SD)

Time of progression (ToP)

Wild type (WT)

Human genes:

ESR1: Estrogen Receptor 1

HER2: Human Epidermal Growth Factor Receptor 2

EGFR: Epithelial Growth Factor Receptor

KRAS: KRAS proto-oncogene, GTPase

BRAF: B-Raf Proto-Oncogene, Serine/Threonine kinase

1 **Abstract:**

2 Background: Activating mutations in the estrogen receptor 1 (*ESR1*) gene are recurrent

3 mechanisms of acquired resistance to aromatase inhibitors (AI), and may be the target of other

4 selective estrogen receptor down-regulators. To assess the clinical utility of monitoring *ESR1*-

5 resistant mutations, a droplet digital PCR (ddPCR)-based assay compatible with body fluids is

6 ideal due to its cost-effectiveness and quick turnaround.

7 Methods: We designed a multiplex ddPCR, which combines a drop-off assay, targeting the

8 clustered hotspot mutations found in exon 8, with another pair of probes interrogating the

9 E380Q mutation in exon 5. We assessed its sensitivity *in vitro* using synthetic oligonucleotides,

10 harboring E380Q, L536R, Y537C, Y537N, Y537S or D538G mutations. Validation of the assay

11 was performed on plasma samples from a prospective study and compared to next generation

12 sequencing (NGS) data.

13 Results: The multiplex *ESR1*-ddPCR showed a high sensitivity with a limit of detection ranging

14 from 0.07 to 0.19% in mutant allele frequency depending on the mutation tested. The screening

15 of plasma samples from patients with AI-resistant metastatic breast cancer identified *ESR1*

16 mutations in 29% of them with perfect concordance (and higher sensitivity) to NGS data

17 obtained in parallel. Additionally, this test identifies patients harboring polyclonal alterations.

18 Furthermore, the monitoring of ctDNA using this technique during treatment follow-up predicts

19 the radiological response to palbociclib-fulvestrant.

20 Conclusion: The multiplex *ESR1*-ddPCR detects, in a single reaction, the most frequent *ESR1*

21 activating mutations and is compatible with plasma samples. This method is thus suitable for

22 real-time *ESR1* mutation monitoring in large cohorts of patients.

1 INTRODUCTION

2   In most breast cancer cases, the estrogen receptor (ER) exerts a key survival signaling, and

3   ER-positive (ER+) immunostaining is a well-characterized predictive biomarker of hormone

4   therapy efficiency at both early and advanced stages. In 2013, several studies reported

5   activating point mutations in the estrogen receptor gene, *ESR1* (1-4). These oncogenic

6   mutations activate the ER in a ligand-independent manner and were proposed to be a potential

7   mechanism of resistance to hormone depletion, by aromatase inhibitors (AI) for example. In

8   ER+ HER2-negative metastatic breast cancer (ER+ HER2- MBC) patients, *ESR1* mutations

9   are rarely detected in primary tumors or at first metastatic relapse (5,6). However, their overall

10   incidence increases alongside the exposure to AI (3-5,7), suggesting that *ESR1* mutations are

11   a positively selected resistance mechanism to this treatment. It has been reported that 30 to

12   40% of ER+ HER2- MBC patients who are resistant to AI carry *ESR1* mutations, some even

13   harboring polyclonal *ESR1* alterations (8,9). These mutations occur in the ligand-binding

14   domain, typically in two hotspot locations: codon 380 (exon 5) and codons 536, 537 and 538

15   (exon 8) (10-12). Importantly, other hormone therapy agents, such as selective ER down-

16   regulators (e.g., fulvestrant, elacestrant…), retain a significant activity on mutated ER

17   (Supplementary Table 1) (12). This indicates that mutations in *ESR1* could be a predictive

18   biomarker in the management of ER+ MBC (11). Accurate and sensitive detection tools are

19   therefore needed to initiate clinical trials that investigate the clinical utility of *ESR1* mutation

20   monitoring. In that context, a liquid biopsy approach based on circulating tumor DNA (ctDNA)

21   detection is a most promising tool to detect the onset of resistance acquisition.

22   Our group, and others, have shown that drop-off droplet digital PCR (ddPCR) is an efficient

23   tool to screen for clustered mutations that can be covered by a single TaqMan probe, unlike

24   conventional ddPCR, which requires a specific probe for each targeted mutation. The drop-off

25   assay relies on the concept that a single mismatch is sufficient to destabilize the complex

26   'probe / target' sequence. Hence, any mutation in the region covered by the 'drop-off' probe

27   leads to a loss of signal when compared to wild-type (WT) samples. All previous studies

1 reported high sensitivity and reproducibility for hotspot mutations in the *EGFR, KRAS,* and

2 *BRAF* genes (13-15).

3 Here, we present the validation of a novel ddPCR assay, which combines a drop-off ddPCR,

4 targeting the clustered hotspot mutations found in *ESR1* exon 8, with another pair of probes

5 interrogating specifically the E380Q mutation, located in exon 5. This multiplex test detects, in

6 a single reaction, up to 95% of the described activating *ESR1* mutations, based on previous

7 studies (3,4,10,12,16), and is compatible with body fluid samples.

8

9 **MATERIALS AND METHODS**

10 **Droplet digital PCR assay**

11 *TaqMan® probes and primer design*

12 For the E380Q mutation in exon 5, the assay used a probe specifically targeting the E380Q

13 mutation (E380Q probe, FAM-labeled) and a reference probe (REFex5 probe, VIC-labeled)

14 annealing to an adjacent invariant region (Figure 1A). For the clustered hotspot mutations in

15 exon 8, a drop-off assay was designed using a probe targeting the WT sequence of codons

16 536, 537 and 538 where mutations are likely to be found (Hotspot probe, VIC-labeled) and a

17 reference probe (REFex8 probe, FAM-labeled) annealing to an adjacent invariant region

18 (Figure 1B). For compatibility with ctDNA detection, primers were designed to generate

19 amplicons under 150 bp (101 bp and 125 bp for exons 5 and 8, respectively). The following

20 primers were used to amplify (*a*) exon 5: Fwd primer: 5'-TTGCTTGTTTTCAGGCTTTGTGGA-

21 3'; Rev primer 5'-AGCGCCAGACGAGACCAATCAT-3'; and (*b*) exon 8: Fwd primer: 5'-

22 ACAGCATGAAGTGCAAGAACGT-3'; Rev primer 5'-TGGCTTTGGTCCGTCTCCTC-3'. The

23 following TaqMan probes (Life Technologies) with a 5' fluorophore and a 3' non-fluorescent

24 quencher (NFQ) were designed as follows: REFex5 5'-(VIC)-TGACCCTCCATGATC-(MGB

25 NFQ)-3'; E380Q 5'-(6-FAM)-ACCTTCTACAATGTGCCTG-(MGB NFQ)-3'; REFex8 5'-(6-

26 FAM)-CTAGCCGTGGAGGGGC-(MGB NFQ)-3'; Hotspot 5'-(VIC)-CCTCTATGACCTGCTGC-

27 (MGB NFQ)-3'.

1   *ddPCR mix preparation*

2   ddPCR reactions were performed in 20 µL according to the manufacturer's protocol. Briefly,

3   20 µL mastermix solution containing ddPCR™ Supermix for probes without dUTP (Bio-Rad

4   Laboratories) at a final concentration of 1x, relevant primers at 450 nM each and relevant

5   TaqMan® probes (E380Q + REFex5 or Hotspot + REFex8 in simplex conditions or both assays

6   combined in multiplex conditions) at 250 nM each (Applied Biosystems), DNA template (up to

7   8 µL) and nuclease-free water were loaded into a disposable droplet generator cartridge (Bio-

8   Rad). After adding 70 µL of droplet generation oil (Bio-Rad), the cartridge was loaded into a

9   QX100 Droplet Generator (Bio-Rad). Generated droplets were transferred to a 96-well PCR

10 plate and PCR reactions were run on a C1000 thermal cycler (Bio-Rad) under the following

11 program: 95°C 10 min, 40 cycles of (94°C 30 sec, 58°C 60 sec), 98°C 10 min. For optimization

12 experiments, we used 20 ng (6,060 genome equivalent) of DNA per reaction. For the mutation

13 screening in patient samples, we used 8 µL of cell-free DNA (cfDNA). Negative controls with

14 no DNA and positive controls (E380Q and D538G mutations) with 6,060 WT genome-

15 equivalent from peripheral blood mononucleated cells (PBMC) were included at each run.

16 Reactions were analyzed on the Bio-Rad QX100 droplet reader.

17 *ddPCR data analysis*

18 The concentration of mutant DNA copies was estimated using the dedicated workflow

19 available in the QuantaSoft v1.7.4 software. For the E380Q mutation, the number of mutant

20 copies per droplet is equivalent to the number of VIC$^+$/FAM$^+$ droplets in simplex conditions

21 and to clouds #4 and #6 in multiplex conditions (Figure 1). The mutant allele frequency

22 (MAF) was calculated as follows: (VIC$^+$/FAM$^+$ droplets / (VIC$^+$/FAM$^+$ droplets + VIC$^+$/FAM$^-$

23 droplets)) for simplex conditions and (clouds #4 + #6/(clouds #4 + #6 + #2 + #5)) for multiplex

24 conditions. For mutations in exon 8, the number of mutant copies per droplet is equivalent to

25 the number of VIC^{low}/FAM$^+$ droplets in simplex conditions and to cloud #4 in multiplex

26 conditions. The MAF was calculated as follows: (VIC^{low}/FAM$^+$ droplets / (VIC^{low}/FAM$^+$ droplets

27 + VIC$^+$/FAM$^+$ droplets)) for simplex conditions and (cloud #4 /(clouds #4 + #3 + #5)) for

28 multiplex conditions. Samples were run in triplicates and were considered to be positive if the

8

1   merged replicates presented a minimum of 3 mutant droplets for E380Q or 8 mutant droplets

2   for exon 8 and if the average MAF was higher than the defined LOD of the multiplex assay.

3   *In vitro* **performance**

4   The limit of blank (LOB) was determined as previously reported (13,15,17,18). Briefly, we

5   defined the false-positive mean and associated standard deviation (SD) of the E380Q and

6   Drop-off Ex8 assays, in simplex or multiplex conditions by analyzing 48 replicates of WT

7   genomic DNA extracted from PBMC. Then, the calculated 95% confidence interval was used

8   to define the LOB. To assess the limit of detection (LOD) of the E380Q and Drop-off Ex8

9   assays, we used synthetic oligonucleotides harboring E380Q, or the most frequent mutations

10   in exon 8 (L536R, Y537C, Y537N, Y537S or D538G). Serial dilutions in 10 ng (3,030 genome

11   equivalent) of WT DNA from PBMC, reproducing MAFs from 0.8% to 0.04%, were analyzed in

12   8 replicates for each mutated oligonucleotide.

13   **Validation in clinical samples**

14   All plasma samples analyzed for *ESR1* mutation status were collected from patients with AI-

15   resistant ER+ HER2- MBC. Patients were prospectively enrolled at the Institut Curie (Paris,

16   France) in the ethically-approved ALCINA study (NCT02866149, cohort 6) after signed

17   informed consent and that were included prior to the initiation of a new line of therapy with

18   palbociclib and fulvestrant. Progression-free survival (PFS), defined as the time from inclusion

19   in the study to progression disease (PD) or death from any cause, was collected prospectively.

20   Survival analysis was performed using Kaplan–Meier plots with significance tested using the

21   log-rank test. Plasma was isolated from fresh blood collected in EDTA blood collection tubes

22   (BD Vacutainer®) within 3 hours as previously performed in the laboratory (19,20) and stored

23   at -80°C until needed. For *ESR1* screening, 2 mL of plasma were thawed and cfDNA extracted

24   using the QIAamp® Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's

25   protocol and stored at -20°C until use. In parallel to ddPCR experiments, targeted-NGS on a

26   panel of 39 cancer-related genes was performed in a blind fashion to allow head-to-head

27   comparison with the *ESR1*-ddPCR assay. Following library preparation, samples were

1 subjected to ultra-deep sequencing on Illumina HiSeq2500 using a 2 x 100 bp paired-end

2 configuration. Read depth obtained for the two *ESR1* hotspots regions was higher than 5,800X.

3 Paired-end read alignments were performed on GRCh37 (hg19) human reference with

4 Bowtie2 (v2.1.0). Once aligned, paired-end reads that map to multiple locations or with poor

5 mapping quality (score <6) were removed. Pileup files were generated using samtools (v.1.1)

6 and variant calling was performed using Varscan2 (v2.3.6). A minimum base quality of 15 was

7 required to count for a read at a position, and only variants supported by a minimum of 5

8 mutated reads at a position with a minimum read depth of 12 were selected. Additionally, the

9 nucleotide composition for each position of the regions containing the two *ESR1* hotspots was

10 extracted from the BAM alignment files using GATK (v.3.5).

11

12 **RESULTS**

13 **Screening of multiple *ESR1* hotspot mutations by ddPCR**

14 Based on the evidence that more than 80% of the activating *ESR1* mutations found in ER+

15 HER2- MBC patients resistant to AI alters codon 380 in exon 5 and the codons 536, 537 and

16 538 in exon 8 (19% and 64% respectively, see Supplementary Table 1), we developed a

17 ddPCR assay targeting these two regions. This assay is composed of only two pairs of TaqMan

18 probes, which can be combined in a single ddPCR reaction. The first pair targets specifically

19 the E380Q mutation in exon 5, using a probe complementary to the E380Q mutant allele

20 (E380Q probe, FAM-labeled) and a reference probe spanning an adjacent invariable region

21 (REFex5 probe, VIC-labeled) (Figure 1A, left panel). In this unconventional E380Q assay,

22 droplets containing WT alleles are VIC-positive only, because the E380Q probe cannot

23 hybridize onto WT sequences, whereas droplets containing E380Q mutant alleles are double

24 positive (VIC$^+$/FAM$^+$) (Figure 1A, right panel). The second pair of probes, constituting the Drop-

25 off Ex8 assay, targets the clustered mutations in exon 8 using a probe complementary to the

26 WT sequence of the altered region (Hotspot probe, VIC-labeled), which detects all the

27 mutations occurring at codons 536, 537 and 538, together with a reference probe spanning an

1   invariable region within the same amplicon (REFex8 probe, FAM-labeled) (Figure 1B, left

2   panel). Therefore, droplets containing WT alleles are double positive (FAM$^+$/VIC$^+$) whereas the

3   mismatch induced by the mutations leads to a VIC signal decrease (FAM$^+$/VIC^{low}), resulting in

4   a shift of the droplet cloud (MUT Ex8) toward a single FAM-positive population (Figure 1B,

5   right panel).

6   The multiplexed assay, combining primers and probes from the E380Q and the Drop-off Ex8

7   assays, allows to screen for mutations in exons 5 and 8 in a single reaction. In a test using

8   synthetic E380Q or D538G oligonucleotides, we could distinguish each WT and mutant clouds

9   for exons 5 and 8 in multiplex conditions (Figure 1C). We observed an additional cloud of

10   droplets containing both WT Ex5 and WT Ex8 amplicons (Figure 1C, Cloud #5), which we also

11   find in WT samples (data not shown), as well as a cloud containing E380Q and WT copies

12   when testing for the E380Q mutation (Figure 1C, left panel, Cloud #6), and not observed when

13   testing for the D538G mutation.

14   **Detection of polyclonal alterations**

15   In addition to the D538G mutation, we tested four other synthetic oligonucleotides harboring

16   the Y537C, Y537N, Y537C or L536R mutations found in exon 8. We observed that the position

17   of the MUT Ex8 cloud was dependent on the mutation tested (Figure 2A). This suggests that

18   each nucleotide change does not equally destabilize the complex 'probe / target sequence'.

19   The largest shifts were observed for the mutations D538G and Y537C. Both clouds of droplets

20   containing these mutant alleles were localized in the same area and were therefore

21   indistinguishable from each other; whereas the clouds for Y537N, Y537S and L536R displayed

22   smaller shifts and were distinct from the D538G/Y537C cloud. We further tested combinations

23   of two mutant oligonucleotides in multiplex conditions confirming that multiple mutations in

24   exon 8 (e.g., D538G + Y537N, D538G + Y537S, D538G + L536R or Y357N + L536R) display

25   a specific pattern (Figure 2B). We clearly detected the combination of one mutation in exon 8

26   (e.g., D538G) with E380Q (Figure 2B). The multiplex *ESR1*-ddPCR can thus identify patients

27   carrying polyclonal alterations, as previously reported (8, 9).

1   *In vitro* **performances**

2   We further estimated the specificity and sensitivity of the *ESR1*-ddPCR by analyzing 48

3   replicates of pure WT DNA and serial dilutions of the mutant synthetic oligonucleotides

4   recapitulating MAFs from 0.8% to 0.04% (see Methods section for more details). The *ESR1*-

5   ddPCR assay showed high specificity with a maximum of one false-positive event observed

6   per reaction (Figure 3A and Supplementary Table 2). The limit of blank (LOB) was estimated

7   at 0.004% for exon 5 mutations and 0.008% for exon 8 mutations. The limit of detection (LOD),

8   defined as the lowest MAF with all replicates presenting values above the LOB, was estimated

9   at 0.19% in mutant allele frequency for E380Q (Figure 3B and Supplementary Table 1) and

10   ranged from 0.07 to 0.13% depending on the exon 8 mutation tested (Figure 3C and

11   Supplementary Table 1). LOB and LOD were also tested in simplex conditions and similar

12   values were observed (LOB were 0.003% for the E380Q assay and 0.01% for the Drop-off Ex8

13   assay ; LOD were 0.09% for mutation E380Q and ranging from 0.06 to 0.1% depending on the

14   exon 8 mutation tested, see Supplementary Figure 1A).

15   **Validation in clinical samples**

16   To validate the performance of the *ESR1* multiplex ddPCR assay, we tested a series of 43

17   plasma samples from a prospective cohort of patients with HR+ HER2- MBC progressing

18   under hormone therapy. We successfully detected *ESR1* mutations in 11 out of the 42 (26%)

19   informative patient samples (Table 1). Four cases (P-05, P-17, P-37 and P-43) harbored an

20   E380Q mutation (36% of the mutant cases, Figure 4A) and 8 cases (P-08, P-18, P-20, P-25,

21   P-28, P35, P-39 and P-43) carried at least one mutation in exon 8 (73% of the mutant cases,

22   Figure 4B). Interestingly, based on the shape and the location of the MUT Ex8 clouds, we

23   detected that samples P-18 and P-28 harbored multiple mutations (Figure 4C). We also

24   observed that P-43 harbored *ESR1* mutations in both exons 5 and 8 (Figure 4C). In parallel,

25   targeted-NGS, including the *ESR1* gene, was performed on 32 plasma samples, for which we

26   had sufficient cfDNA, to allow head-to-head comparison with the *ESR1*-ddPCR assay.

27   Importantly, NGS analysis was performed in a blinded fashion, with no prior knowledge of

1   ddPCR results. The samples sequenced included 10 mutant and 22 WT cases according to

2   ddPCR analysis. All *ESR1* mutations detected by ddPCR were confirmed by NGS except for

3   2 cases (P-17 and P-20), in which the *ESR1* MAF was found to be <1% by ddPCR (Table 1,

4   Supplementary Figure 2A). P-25 was not tested with NGS due to lack of cfDNA (Table 1,

5   Supplementary Figure 2B). We also confirmed the WT status of the 22 remaining plasma

6   samples. In case P-18, NGS results indicated that the mutations were D538G and L536H, a

7   combination we did not test before (Table 1). In case P-28, we observed 3 mutations in exon

8   8: L536H, Y537N and Y537S. Interestingly, for case P-35, NGS showed that the mutation in

9   exon 8 was Y537N, which did not result from the usual C>T substitution in position 1609, but

10   from a small deletion/insertion at nucleotides 1608-1609 (c.1608_1609delinsTA). Overall, the

11   *ESR1* multiplex ddPCR performs better than standard targeted NGS in identifying *ESR1*

12   mutation carriers owing to its improved sensitivity.

13   **Monitoring of circulating *ESR1* mutant copies to predict response to palbociclib-**

14   **fulvestrant therapy**

15   We next analyzed the impact of the *ESR1* mutant status, detected with the *ESR1*-ddPCR, in

16   plasma samples collected at baseline and during treatment follow-up. To perform this analysis,

17   we extended the cohort to 60 patients. For each patient, four blood samples were collected:

18   before treatment (D0), after 15 days (D15) and 30 days (D30) of treatment and at the time of

19   progression (ToP). Among the 59 patients screened with contributive results, *ESR1* mutations

20   were detected in 17 (28.8%), which is in line with proportions of patients progressing under AI

21   treatment previously reported (8, 9, 11, 24). Out of the 17 patients carrying an *ESR1* mutation,

22   15 had an evaluable PFS (2 patients were withdrawn from the study shortly after the treatment

23   initiation, Figure 5A). First, we observed no impact of the *ESR1* mutational status (mutant vs

24   wild-type) detected at baseline on the PFS (Figure S3A). This is in agreement with previous

25   data from the PALOMA-3 trial reporting no improved PFS for patients carrying *ESR1* mutations

26   at baseline and treated with fulvestrant (11, 24). After 3 months of treatment, 6 patients

27   presented a disease progression according to RECIST criteria (Figure 5A). We further

1   analyzed the dynamics of *ESR1* mutant copy levels in regards to the 3-months disease

2   progression status (PD versus non-PD). At baseline, we observed a median number of ctDNA

3   copies of 54 per ml of plasma (mean = 188.5, range [3 - 1233]) and there was no apparent

4   association with disease progression at 3 months (Figure 5B). After 15 days of treatment, we

5   uncovered a dramatic decrease (median = 0, mean = 14.3, range [0 - 74]) with 58% (7/12) of

6   patients reaching undetectable *ESR1* mutant levels (Figure 5B). The evolution of the number

7   of ctDNA copies between D0 and D15, for each patient, demonstrates a decrease for all

8   (Figure 5C, Supplementary Table 3). To a minor extent, a significant decrease was also

9   observed on total cfDNA at D15 (Figure S3B-C), which was previously described and related

10   to the cytostatic growth arrest triggered by palbociclib (24). At D30, the majority of patients with

11   early disease progression had increased ctDNA levels whereas the majority of patients with

12   longer PFS displayed decreased or stable ctDNA levels (Figure 5C). Overall, a lower

13   proportion of patients displayed undetectable ctDNA levels (53% = 8/15, Figure 5B), the

14   majority of which (87.5% = 7/8) had no early disease progression. Patients with early disease

15   progression had significantly more ctDNA detected at D30 than patients with longer PFS (p <

16   0.0001; Figure 5D). Only one out of 6 patients with early disease progression had no ctDNA

17   detected whereas only 2 out of 9 patients without early disease progression showed detectable

18   ctDNA (Figure 5B). This ctDNA positivity at D30 correlates with PFS (p = 0.0063; Figure 5E-

19   F). Here, we show that the detection of ctDNA after 30 days of palbociclib-fulvestrant is a

20   promising dynamic biomarker associated with PFS.

21

22   **DISCUSSION**

23   We successfully developed a ddPCR assay detecting the most frequent activating *ESR1*

24   mutations at once that is compatible with liquid biopsies. By using an unconventional design,

25   which includes a drop-off assay, we targeted, in a single reaction, the E380Q mutation and all

26   the mutations occurring at codons 536 to 540. The multiplex *ESR1*-ddPCR covers >80% of

27   the currently described *ESR1* mutations and >90% of functionally characterized activating

28   *ESR1* mutations. Several teams have previously developed ddPCR assays which target only

1   the most frequent *ESR1* mutations found in exon 8: D538G, Y537S, Y537N and Y537C (6,21-

2   23). These assays were designed following the conventional ddPCR method containing

3   specific TaqMan probes complementary to each mutant or WT allele. This implies that each

4   mutation is tested in a separate reaction. Thus, multiplex assays were developed to reduce

5   the number of reactions (9, 11). However, these assays cannot identify more than 4 *ESR1*

6   mutations in a single reaction and the mutant samples were usually confirmed by singleplex

7   tests (24). To our knowledge, we are the first to have developed a ddPCR assay which can

8   detect, in a single reaction, at least eight different mutations in *ESR1*, namely: E380Q, L536H,

9   L536R, Y537C, Y537N (T>A), Y537N (delinsTA), Y537S and D538G. In addition, our system

10   can identify samples harboring multiple *ESR1* mutations (e.g., E380Q combined with one or

11   more mutations in exon 8). Polyclonal *ESR1* mutations are well-described events (9,10) and

12   the *ESR1*-ddPCR assay would be useful in monitoring the dynamics of each mutation during

13   treatment follow up as seen for P-43. The multiplex *ESR1*-ddPCR assay is highly sensitive,

14   detecting all tested mutations at frequencies lower than 0.19%, an improvement as compared

15   with NGS. We also demonstrated that the *ESR1*-ddPCR is highly specific by cross-validation

16   with NGS experiments. Lupini *et al.* recently developed an assay based on an 'enhanced-ice-

17   COLD-PCR followed by NGS' with a sensitivity reaching 0.01% (25). However, this ddPCR

18   assay targets specifically the Y537S mutation and involves an enrichment step of the mutant

19   copies preceding the ddPCR assay. Yet, in a context of patient monitoring by liquid biopsy,

20   biological samples are of limited quantity and must be tested rapidly at a low cost. The multiplex

21   *ESR1*-ddPCR can detect most *ESR1* mutations in a single reaction faster and at a lower cost

22   than NGS or any other currently available technology.

23   Interestingly, we observed that exons 5 and 8 mutations can be easily distinguished. Moreover,

24   any nucleotide change covered by the Drop-off Ex8 assay can be identified, as confirmed by

25   the detection of the previously unreported mutation Y537N (delinsTA). We observed that,

26   among exon 8 mutations, the shift in clouds is unique depending on the mutation, indicating if

27   the mutation is more likely to be a D538G or Y537C allele versus mutations in codon 536 or

28   other changes in codon 537. However, the fact that this assay does not recover the exact

15

1   nucleotide change in exon 8 constitutes its most prominent limitation. From a clinical

2   perspective, all mutations in codons 536-538 have been equally associated with resistance to

3   AI therapy. Nonetheless, preclinical data suggest that the Y537S mutation, which accounts for

4   about 10% of all *ESR1* mutations, may be less sensitive to fulvestrant than other mutations

5   (12). If this observation is confirmed to be clinically relevant, the *ESR1*-ddPCR could be used

6   as a first screening tool, since the shift associated to Y537S is distinguishable from the most

7   frequent mutation: D538G, followed by subsequent sequencing of exon 8, to distinguish Y537S

8   from other 536/537 mutations. A second theoretical limitation is that synonymous mutations

9   may also be detected by this assay. However, *ESR1* synonymous mutations, as well as

10 polymorphisms in codons 536-540, are extremely rare. According to the Exome Aggregation

11 Consortium, only two synonymous mutations, L536L and D538D were described at very low

12 frequencies, $6.303e^{-05}$ and $8.251e^{-06}$, respectively.

13 The improved analytical sensitivity of the *ESR1*-ddPCR is particularly useful to monitor ctDNA

14 during treatment follow-up. We demonstrated that *ESR1* mutations are good markers for

15 ctDNA dynamics exploration and prediction of treatment response. Indeed, we observed that

16 detection of ctDNA after 30 days of palbociclib-fulvestrant, using the *ESR1*-ddPCR, correlates

17 with the treatment response and has an impact on PFS.

18 In conclusion, this method presents the advantage to screen for at least 80% of the *ESR1*

19 mutations in a single reaction, as required by large screening studies involving plasma

20 samples. This assay is currently implemented in the randomized phase 3 PADA-1 trial

21 (NCT03079011) in which it allows for real-time monitoring of *ESR1* mutation rising in 1000

22 ER+ HER2- MBC patients treated with palbociclib and AI.

23

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TABLES

Table 1: Detection of *ESR1* mutations using ddPCR and NGS in plasma samples from patients with MBC. Samples with mutation identified by ddPCR are highlighted in grey. Corresponding ddPCR profiles are presented in Figure 4 and Supplemental Figure 2.

| Patient ID	ddPCR		NGS	
	ESR1 status	MAF(%)	*ESR1* status	MAF(%)
P-01	WT	-	WT	-
P-02	WT	-	WT	-
P-03	WT	-	WT	-
P-04	WT	-	WT	-
P-05	E380Q	16.6	c.G1138C, p.E380Q	17.0
P-06	WT	-	WT	-
P-07	WT	-	WT	-
P-08	MUT Ex8	2.5	c.A1613G, p.D538G	0.2 (7/4886 reads)
P-09	WT	-	WT	-
P-10	WT	-	Insufficient cfDNA	-
P-11	WT	-	Insufficient cfDNA	-
P-12	WT	-	WT	-
P-13	WT	-	Insufficient cfDNA	-
P-14	WT	-	Insufficient cfDNA	-
P-15	WT	-	WT	-
P-16	WT	-	Insufficient cfDNA	-
P-17	E380Q	0.3	WT	-
P-18	MUT Ex8	2.8	c.T1607A, p.L536H	1.7
		3.0	c.A1613G, p.D538G	6.7
P-19	WT	-	WT	-
P-20	MUT Ex8	0.6	WT	-
P-21	WT	-	WT	-
P-22	WT	-	WT	-
P-23	WT	-	WT	-
P-24	WT	-	WT	-
P-25	MUT Ex8	2.8	Insufficient cfDNA	-
P-26	WT	-	WT	-
P-27	WT	-	WT	-
P-28	MUT Ex8	13.6	c.T1607A, p.L536H	11.0
			c.T1609A, p.Y537N	1.5
			c.A1610C, p.Y537S	1.2
P-29	Not contributive	-	Insufficient cfDNA	-
P-30	WT	-	WT	-
P-31	WT	-	Insufficient cfDNA	-
P-32	WT	-	Insufficient cfDNA	-
P-33	WT	-	WT	-
P-34	WT	-	WT	-
P-35	MUT Ex8	6.4	c.1608_1609delinsTA, p.Y537N	11.9
P-36	WT	-	Insufficient cfDNA	-
P-37	E380Q	3.3	c.G1138C, p.E380Q	1.4
P-38	WT	-	WT	-
P-39	MUT Ex8	0.4	c.A1613G, pD538G	1.6
P-40	WT	-	WT	-
P-41	WT	-	Insufficient cfDNA	-
P-42	WT	-	WT	-
P-43	E380Q	0.2	c.G1138C, p.E380Q	0.3 (40/13029 reads)
	MUT Ex8	9.6	c.A1610G, p.Y537C	1.6
			c.A1613G, p.D538G	5.0

22

FIGURES LEGENDS
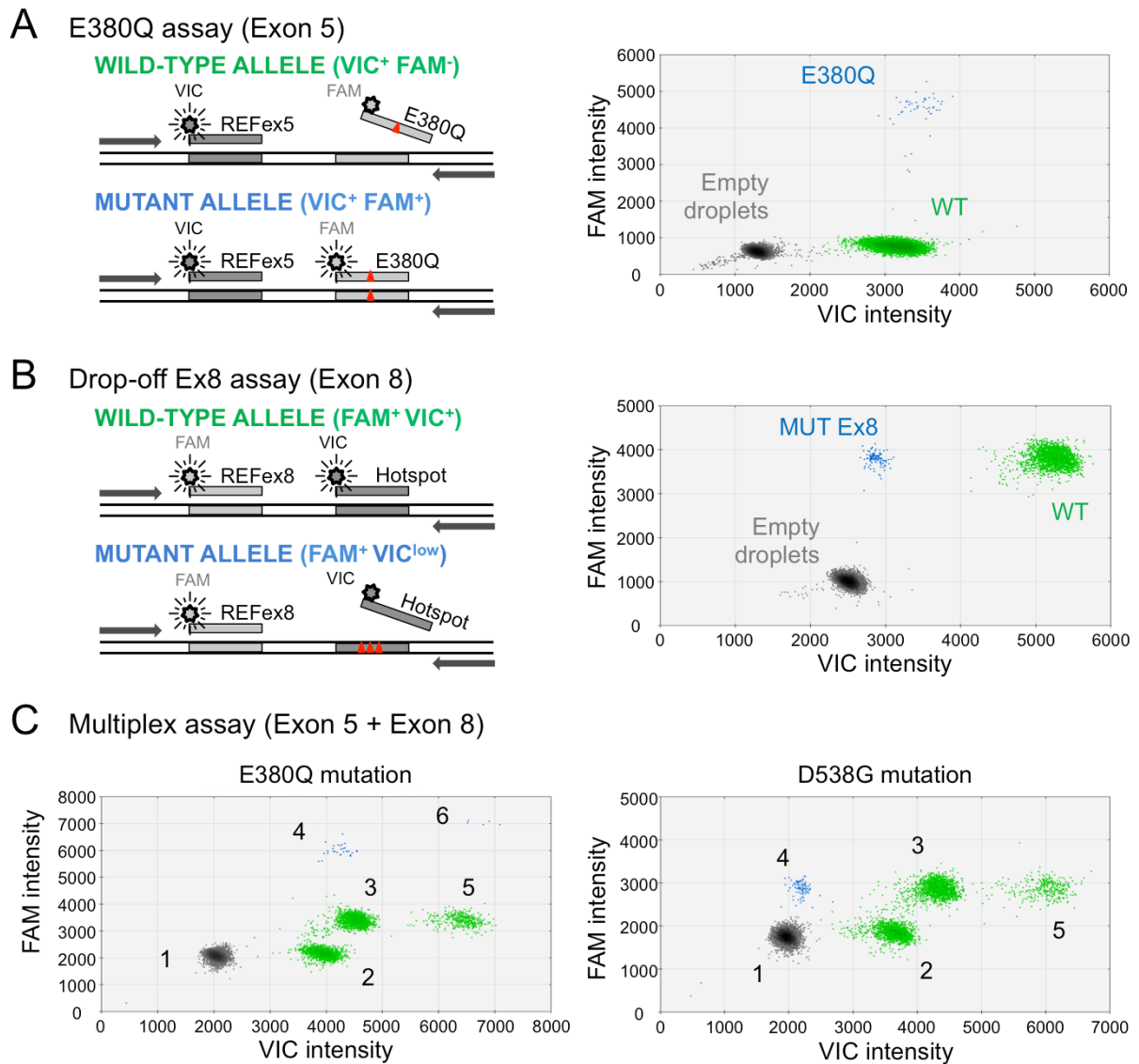
Figure 1. *ESR1* exons 5 and 8 assay design. A. Assay to detect the E380Q mutation in exon 5: wild-type alleles generate a signal from the reference probe only (VIC+, green cloud) whereas E380Q alleles generate a double positive signal (VIC+/FAM+, blue cloud). **B.** The Drop-off Ex8 assay detects the clustered hotspot mutations located in exon 8 (D538G in this example). Wild-type alleles generate a signal from both the reference and drop-off probes (FAM+/VIC+, green cloud) whereas alleles with alterations in codons 536-538 generate a signal from the reference probe only (FAM+, blue cloud). **C.** The multiplex assay detects, in a single reaction, the E380Q mutation (left panel) and mutations in exon 8 (right panel, D538G in this example). Cloud #1 = Empty droplets; Cloud #2 = WT Ex5; Cloud #3 = WT Ex8; Cloud

23

#4 = E380Q (left panel) or MUT Ex8 (right panel); Cloud #5 = WT Ex5 + WT Ex8; Cloud #6 =
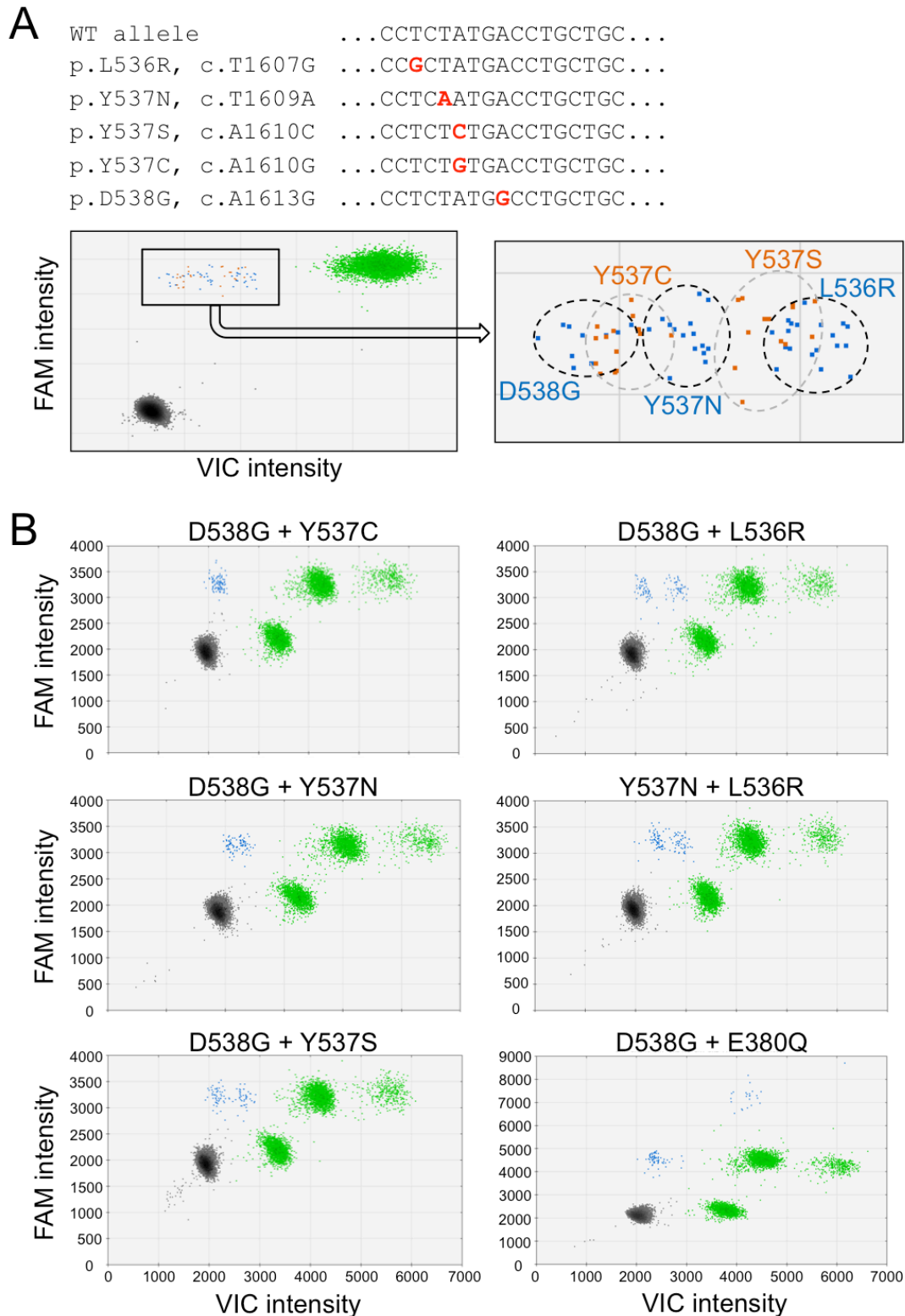
E380Q + WT Ex5 + WT Ex8.

Figure 2. Detection of *ESR1* polyclonal mutations. A. Most frequent mutations located in *ESR1* exon 8 targeted by the Drop-off Ex8 assay. **B.** Representative examples of double mutations (synthetic oligonucleotides combined) detected in multiplex condition.
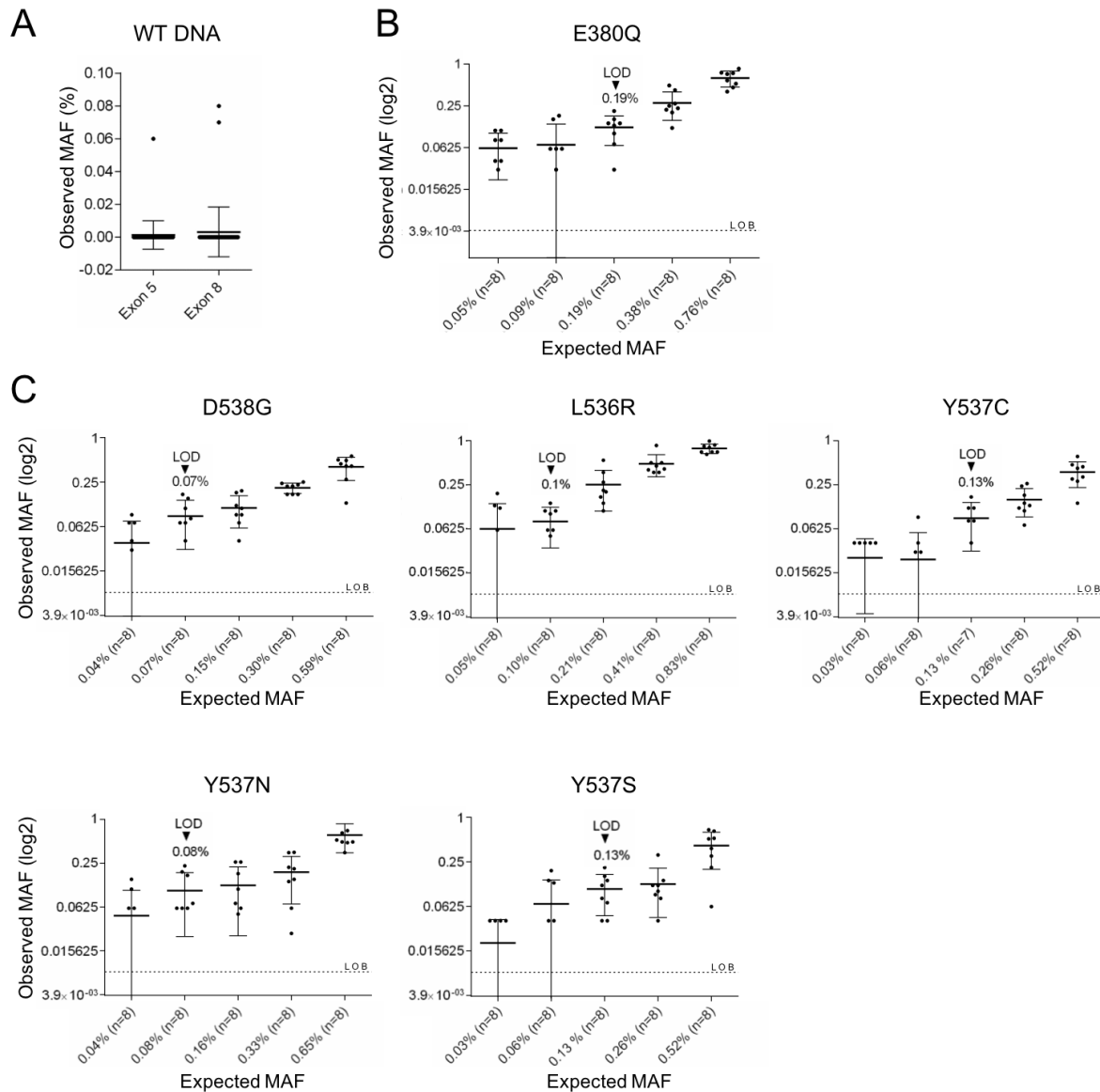
Figure 3. *In vitro* performance of the multiplex *ESR1*-ddPCR. A. False positive events for exons 5 or 8 mutations observed from pure WT DNA tested with the multiplex *ESR1*-ddPCR. **B.** LOD estimation for exon 5 E380Q mutation. **C.** LOD estimation for exon 8 mutations D538G, L536R, Y537C, Y537N or Y537S. See method section for more details and Supplementary Table 1. LOB: limit of blank, LOD: limit of detection, estimated as the 95% CI of the mean false-positive calls.
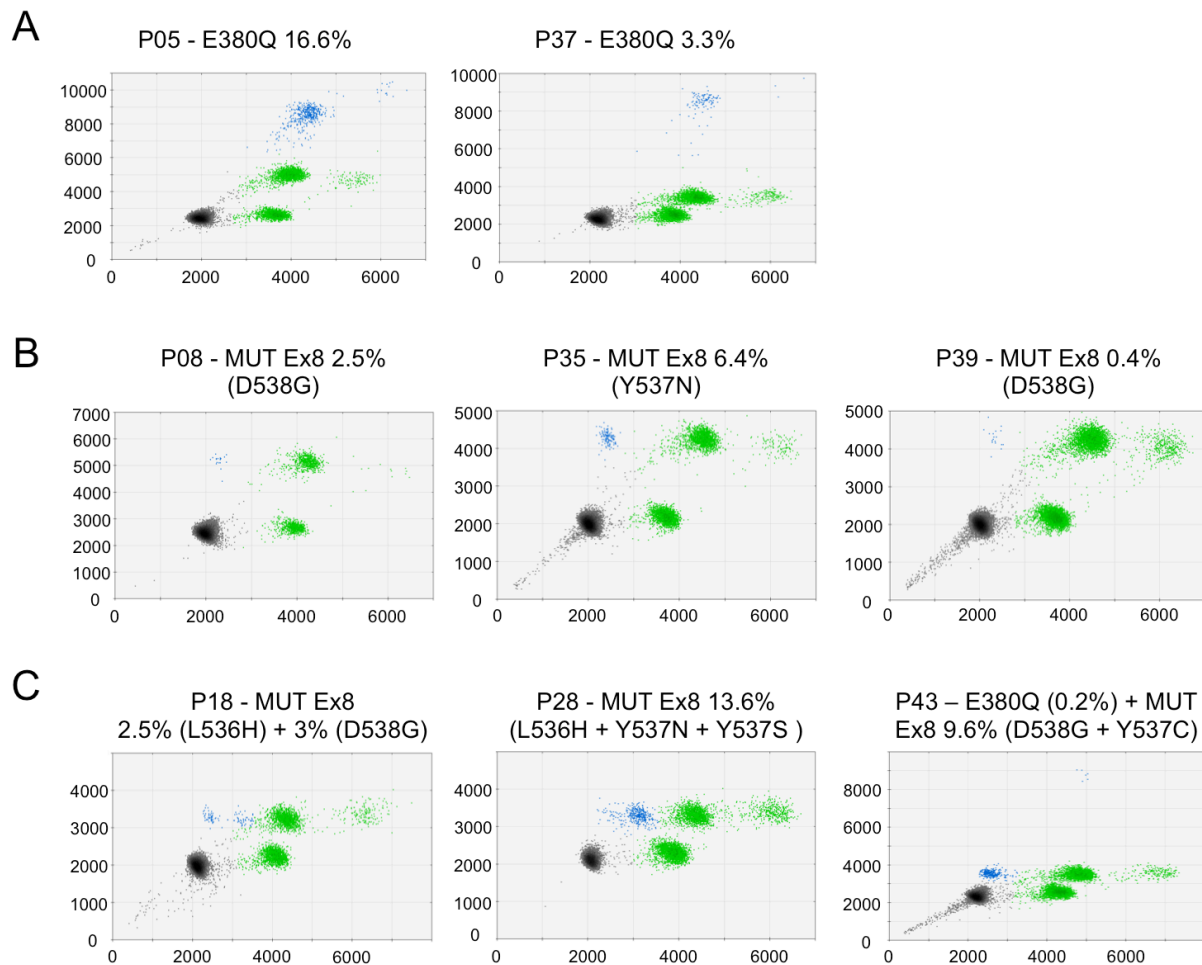
Figure 4. Validation of the *ESR1* ddPCR multiplex assay on patient plasma samples.
Representative ddPCR profiles for E380Q mutation **(A)**, exon 8 mutations (D538G or Y357N) **(B)** and polyclonal *ESR1* mutations **(C)**. Case P-18, double exon 8 mutations identified as L536H and D538G; case P-28, exon 8 mutations identified as L536H, Y537N and Y537S and case P-43, exon 5 + exon 8 mutations identified as D538G, Y537C and E380Q by NGS. FAM and VIC intensities are shown in arbitrary units. MAFs determined by ddPCR are indicated above each plot together with the mutation identified by NGS in brackets.
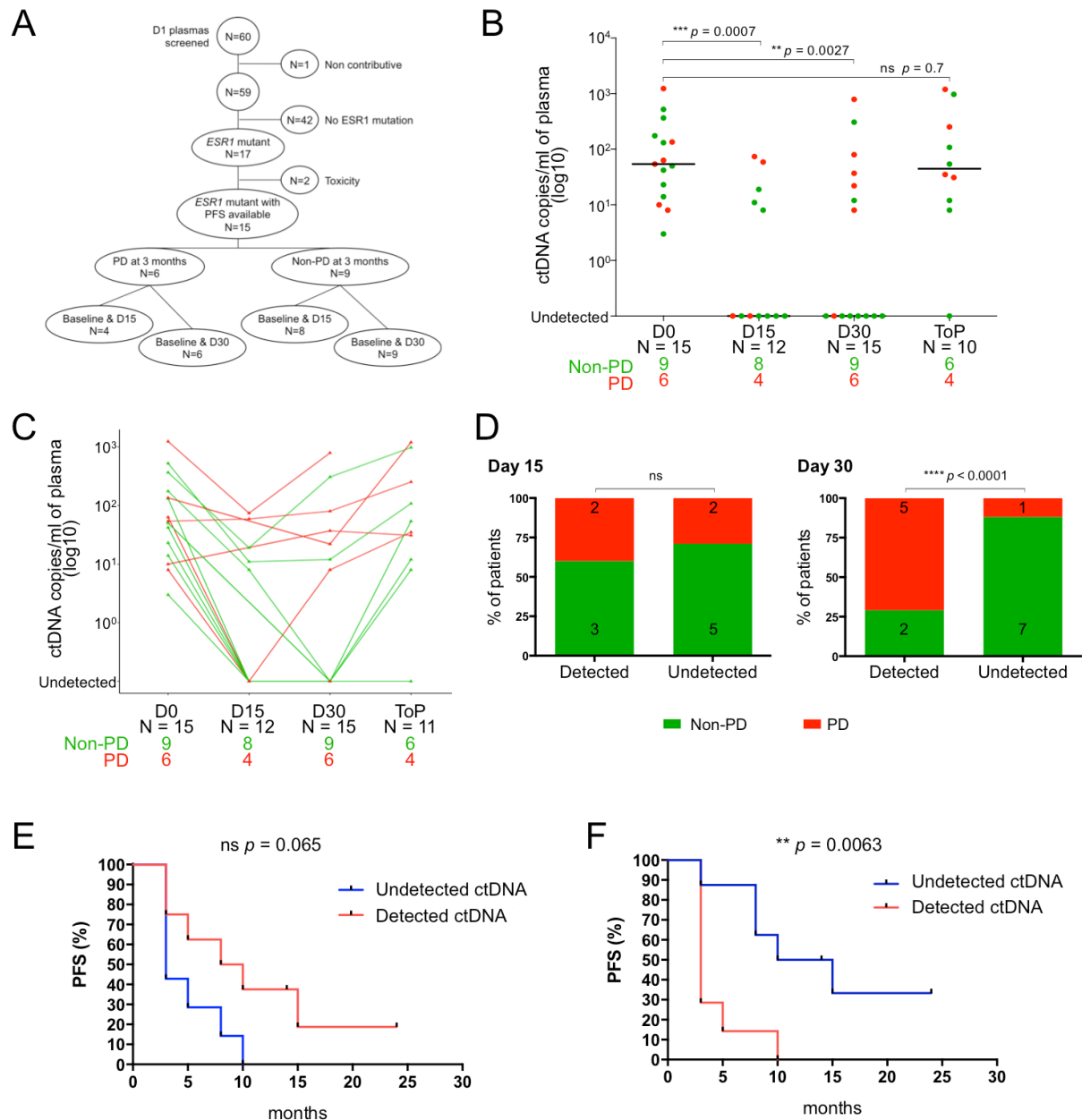
27

Figure 5. Monitoring of circulating *ESR1* mutant copies to predict response to palbociclib-fulvestrant therapy A. Flow chart of plasma samples analyzed from the ALCINA cohort 6 'Palbociclib' (NCT02866149) for *ESR1* mutations with the *ESR1*-ddPCR multiplex. PD: progressive disease, Non-PD: regressive or stable disease. **B.** Number of ctDNA copies detected per ml of plasma collected at the 4 time points during treatment follow-up (D0, N = 15; D15, N = 12; D30, N = 15; ToP, N = 10). **C.** ctDNA dynamics observed during treatment follow-up in patients experiencing progressive disease (PD, red) versus regressive or stable disease (non-PD, green) at 3 months according to RECIST criteria. (D0 : non-PD = 9, PD = 6;

D15 : non-PD = 8, PD = 4; D30 : non-PD = 9, PD = 6; ToP : non-PD = 6, PD = 4). **D.** Two

sample test proportions for detectable and undetectable ctDNA distribution at D15 and D30.

E, F. Survival (PFS) according to ctDNA detection at D15 **(E)** or D30 **(F)**.