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**Supplementary Table S2:** Patient characteristics. None of the differences between groups are statistically significant ( $p < 0.05$ ) using a two-tailed Fisher's exact test (categorical parameters) or a student's t-test (continuous parameters).

<b>Baseline characteristics</b>	<b>ESR1 Hotspot – (n=42)</b>	<b>ESR1 Hotspot + (n=16)</b>
Age	62 (39-90)	54 (38-75)
Ethnicity		
Hispanic	1 (2%)	0
Non-Hispanic White	37 (88%)	14 (88%)
Non-white	4 (10%)	2 (12%)
Smoking History		
Yes	17 (40%)	10 (63%)
No	25 (60%)	6 (37%)
Prior systemic therapy exposure		
Adjuvant endocrine therapy	42	16
Adjuvant tamoxifen	27	10
Adjuvant non-steroidal AI	14	6
Adjuvant exemestane	1	0
>1 line of endocrine therapy for stage IV disease	33 (79%)	13 (81%)
Prior chemotherapy	31 (74%)	14 (88%)
Systemic therapy at study enrollment		
Tamoxifen/non-steroidal AI	9 (21%)	4 (25%)
Exemestane	2 (5%)	2 (13%)
Exemestane + everolimus	4 (10%)	3 (19%)
Fulvestrant	7 (17%)	2 (13%)
Letrozole + Cdk4/6 inhibitor	9 (21%)	1 (6%)
Fulvestrant + Cdk4/6 inhibitor	5 (12%)	2 (13%)
Chemotherapy/other	6 (14%)	2 (13%)
Location of metastatic disease		
Bone only	5 (12%)	4 (25%)
Non-bone only	6 (14%)	3 (19%)
Bone and non-bone	31 (74%)	9 (56%)
Histology		
IDC	33 (79%)	13 (81%)
ILC	8 (19%)	1 (6%)
Unknown	1 (2%)	2 (13%)
Receptor status		
ER/PR+ HER2-	33 (79%)	9 (56%)
ER/PR+ HER2+	4 (10%)	4 (25%)
HER2 unknown	5 (12%)	3 (19%)
Median time from diagnosis to metastasis (range)	5.1 (0-21.4) years	3.3 (0-27.8) years
Median time from metastasis to enrollment (range)	1.5 (0.1-9.7) years	3.4 (0.4-8.7) years
Median time from diagnosis to enrollment (range)	7.1 (0.1-21.8) years	9.3 (2.4-36.2) years

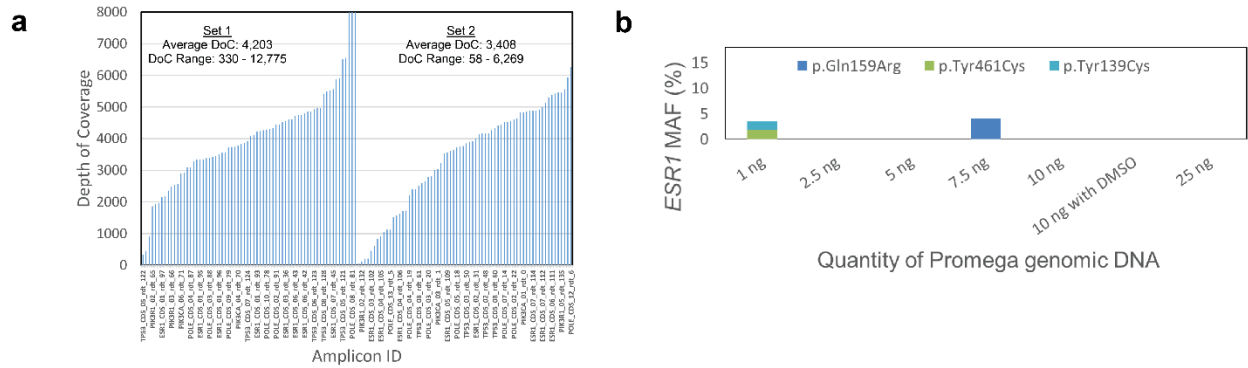
**Supplementary Table S3: *ESR1* gBlocks and LNA probes**

<b>gBlocks</b>	<b>Sequence (5'-gene-specific sequence-3')</b>	<b>bp</b>
<i>ESR1</i> -WT-gBlock	TAACAAAGGCATGGAGCATCTGTACAGCATGAAG TGCAAGAACGTGGTGCCCCTCTATGACCTGCTGCT GGAGATGCTGGACGCCACCGCCTACATGCGCCC ACTAGCCGTGGAGGGGCATCCG	125
<i>ESR1</i> -Y537C-gBlock	TAACAAAGGCATGGAGCATCTGTACAGCATGAAG TGCAAGAACGTGGTGCCCCTCTGTGACCTGCTGCT GGAGATGCTGGACGCCACCGCCTACATGCGCCC ACTAGCCGTGGAGGGGCATCCG	125
<i>ESR1</i> -Y537N-gBlock	TAACAAAGGCATGGAGCATCTGTACAGCATGAAG TGCAAGAACGTGGTGCCCCTCAATGACCTGCTGCT GGAGATGCTGGACGCCACCGCCTACATGCGCCC ACTAGCCGTGGAGGGGCATCCG	125
<i>ESR1</i> -Y537S-gBlock	TAACAAAGGCATGGAGCATCTGTACAGCATGAAG TGCAAGAACGTGGTGCCCCTCTCTGACCTGCTGCT GGAGATGCTGGACGCCACCGCCTACATGCGCCC ACTAGCCGTGGAGGGGCATCCG	125
<i>ESR1</i> -D538G-gBlock	TAACAAAGGCATGGAGCATCTGTACAGCATGAAG TGCAAGAACGTGGTGCCCCTCTATGGCCTGCTGCT GGAGATGCTGGACGCCACCGCCTACATGCGCCC ACTAGCCGTGGAGGGGCATCCG	125

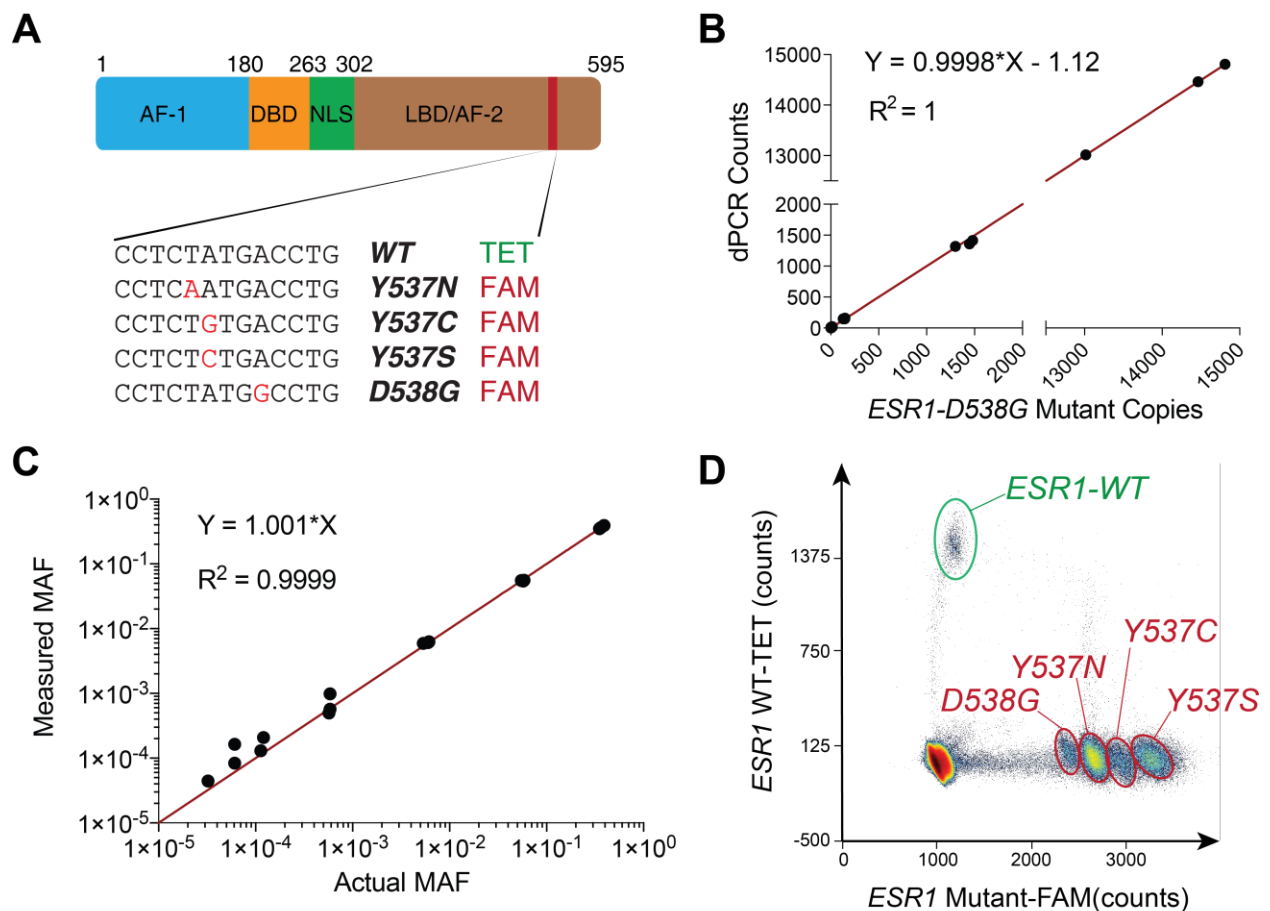
<b>Probe name</b>	<b>Probe sequence</b>	<b>T<sub>m</sub> (°C)<sup>a</sup></b>
<i>ESR1_TET_WT</i>	5'-/5TET/T+C+T+AT+G+A+CCTG/3IABkFQ/-3'	52.4
<i>ESR1_FAM_Y537C</i>	/56-FAM/CC+T+CT+G+T+GA+C/3IABkFQ/	52.9
<i>ESR1_FAM_Y537N</i>	/56-FAM/C+CCT+C+A+ATG+ACC/3IABkFQ/	54
<i>ESR1_FAM_Y537S</i>	/56-FAM/CCC+TC+T+C+TG+ACCT/3IABkFQ/	58.4
<i>ESR1_FAM_D538G</i>	/56-FAM/CTA+T+G+GCC+TGC/3IABkFQ/	56

<sup>a</sup>The melting temperatures (T<sub>m</sub>) were predicted using DNA Thermodynamics and Hybridization tool at IDT's website.

**Supplementary Fig. S1:** Sensitivity and limit of detection of dPCR-SEQ. (a) dPCR-SEQ was designed to amplify target regions with amplicon size <120bp. The multiplexed samples were sequenced by MiSeq. The observed mean read depth for 10 ng sheared genomic DNA was 3800 X. (b) Human genomic DNA from multiple anonymous donors (Promega, Madison, WI; Catalog No. G1521) was used as a reference sample to define the limit of detection. Promega control DNA was used at 1 ng, 2.5 ng, 5 ng, 7.5 ng and 10 ng. We have used 10 ng of ctDNA in dPCR-SEQ. Using a threshold of 1.6% MAF, we did not observe any false-positive mutations when analyzing control DNA with 10 ng input. With lower input quantities, we observed a higher background rate of mutations above the 1.6% MAF threshold.

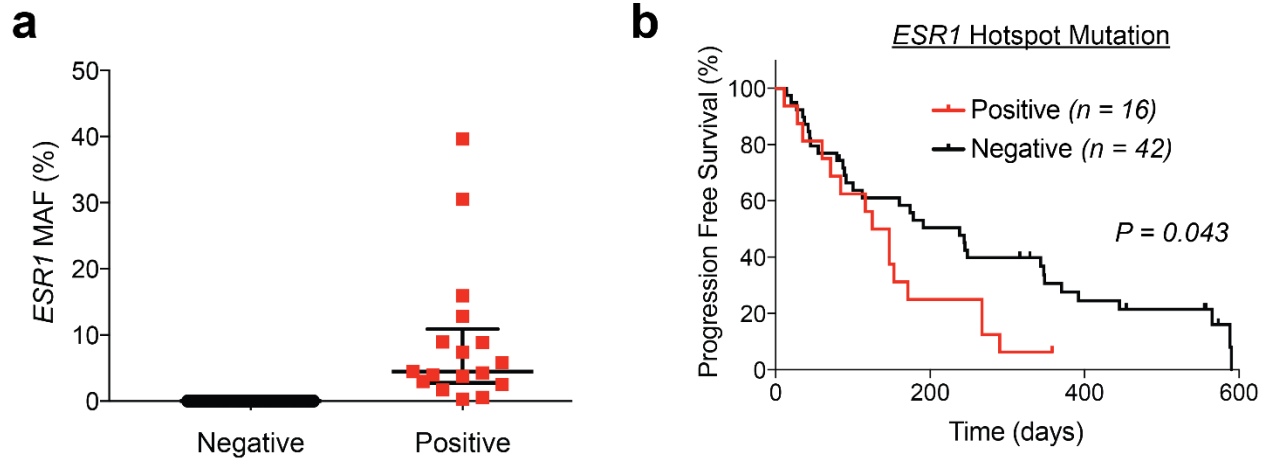


**Supplementary Fig. S2:** Multiplexed dPCR based *ESR1* assay. (a) A schematic for the multiplexed dPCR reaction. Highly specific FAM or TET conjugated Locked Nucleic Acid probes were developed to detect four hotspot *ESR1* mutations and the wild type (WT) allele. AF-1, activation function-1; DBD, DNA-binding domain; NLS, nuclear localization signal; LBD, ligand-binding domain; AF-2, activation function-2. (b) Titration of *ESR1*-D538G mutants was performed by mixing varying amount of mutants and keeping the WT copies constant. A plasmid containing a 125 bp fragment of the *ESR1* coding sequence containing the D538G mutation was used in this experiment. (c) Highly sensitive detection of D538G copies by dPCR in a titration analysis by spiking sheared genomic DNA from MCF-7 cells with varying amounts of mutant *ESR1*. (d) Two-dimensional dotplot of the 5-plex dPCR assay to detect D538G, Y537C, Y537N, and Y537S mutations and *WT ESR1* using the Raindrop Sense machine (Raindance Technologies).

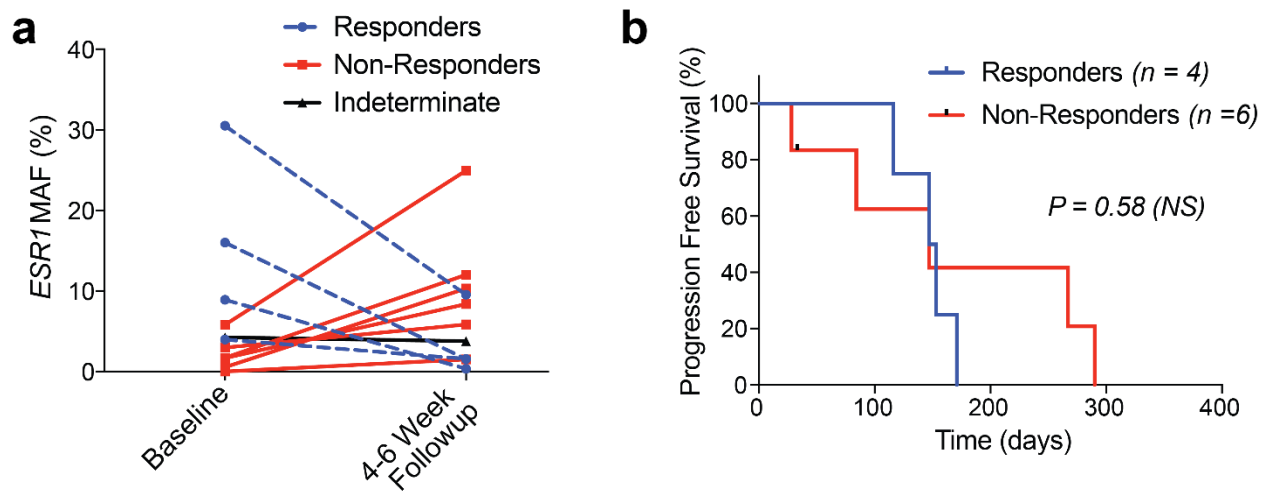




**Supplementary Fig. S3:** Detection mutations in plasma ctDNA from ER+ MBC patients. (a) *ESR1* mutant allele frequency (%) in 58 patients. (b) Kaplan-Meier progression-free survival plot for MBC patients stratified by detection of plasma *ESR1* hotspot mutations in the baseline blood sample collected for patients in the study. Two-tailed p value measured using the logrank test.



**Supplementary Fig. S4:** Hotspot *ESR1* mutant allele frequency in serial plasma samples from MBC patients. (a) Dynamic changes in *ESR1* mutant allele frequency in follow-up plasma ctDNA samples from *ESR1* mutation positive patients. Out of 11 patients with longitudinal blood samples available, 6 patients had an increase in the *ESR1* MAF (i.e., “Non-responders”), 4 patients had a reduction in the *ESR1* MAF (i.e., “Responders”), and one patient had unchanged *ESR1* MAF (i.e., “Indeterminate”) (b) Progression-free survival in “responder” and “non-responder” patients, stratified by changes in *ESR1* MAF in ctDNA. Survival time is calculated from collection of the first blood sample. Two-tailed p value measured using the logrank test.



**Supplementary Fig. S5:** Detection of *ESR1*, *PIK3CA*, and *TP53* mutation by dPCR-SEQ.

Lollipop plots showing the number (vertical axis) and position (horizontal axis) of mutations across the protein sequence of breast cancer relevant genes (*ESR1*, *PIK3CA* and *TP53*). Plot originally generated using the cBioPortal. Green circle indicates missense mutations. dPCR-SEQ was targeted to the complete coding region of *ESR1* and *TP53* whereas only hotspot regions in *PIK3CA* were targeted.

