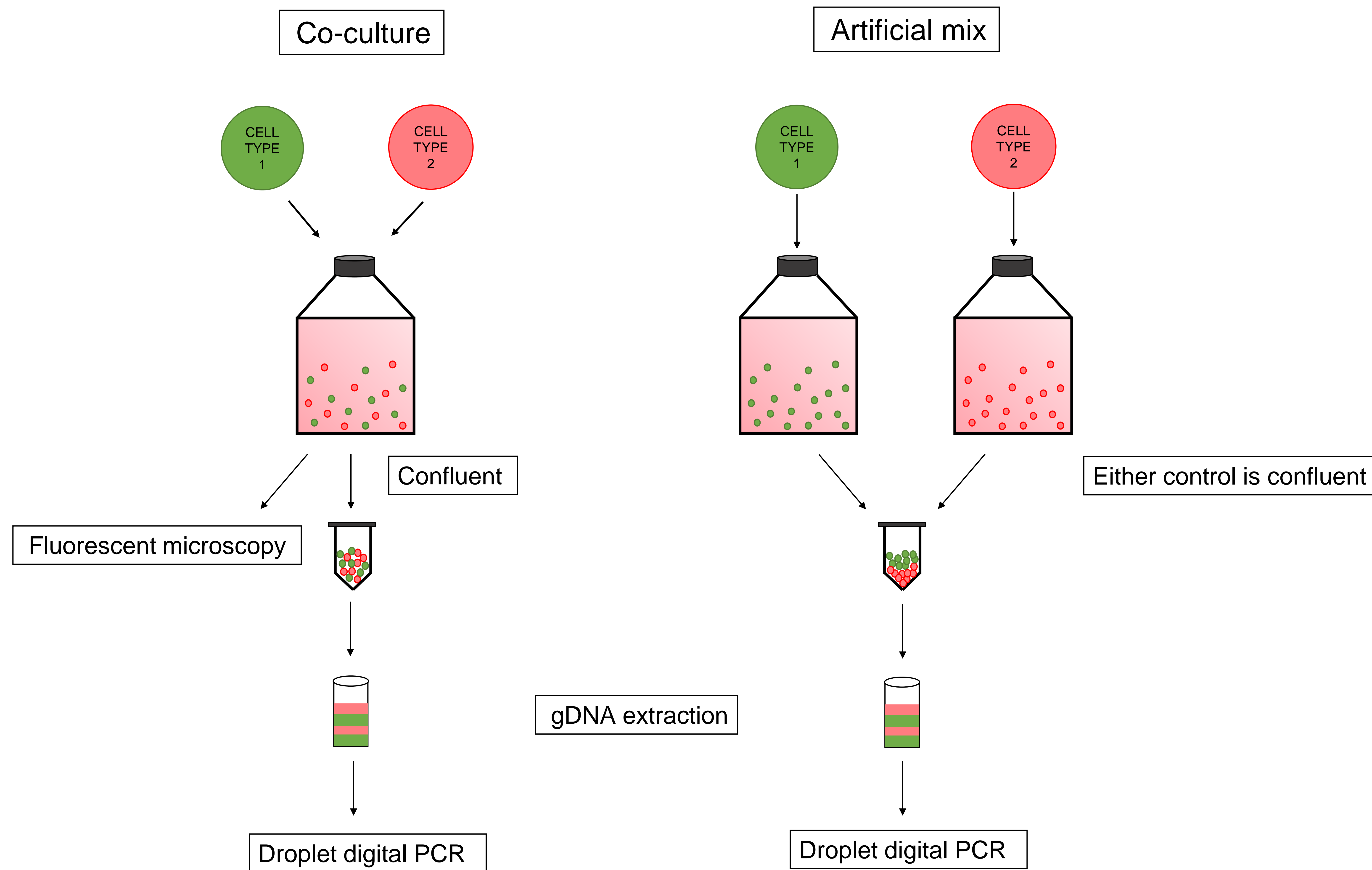
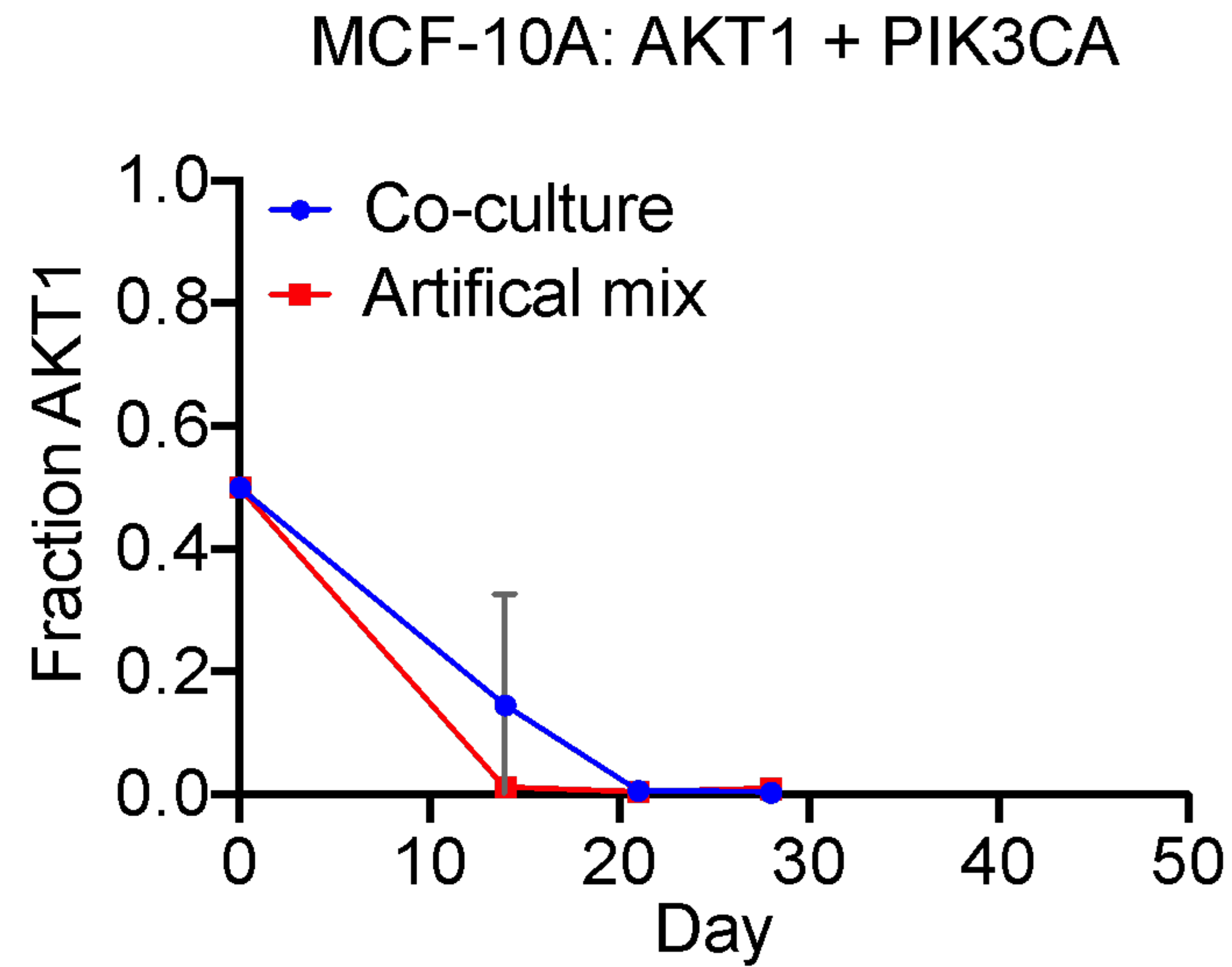
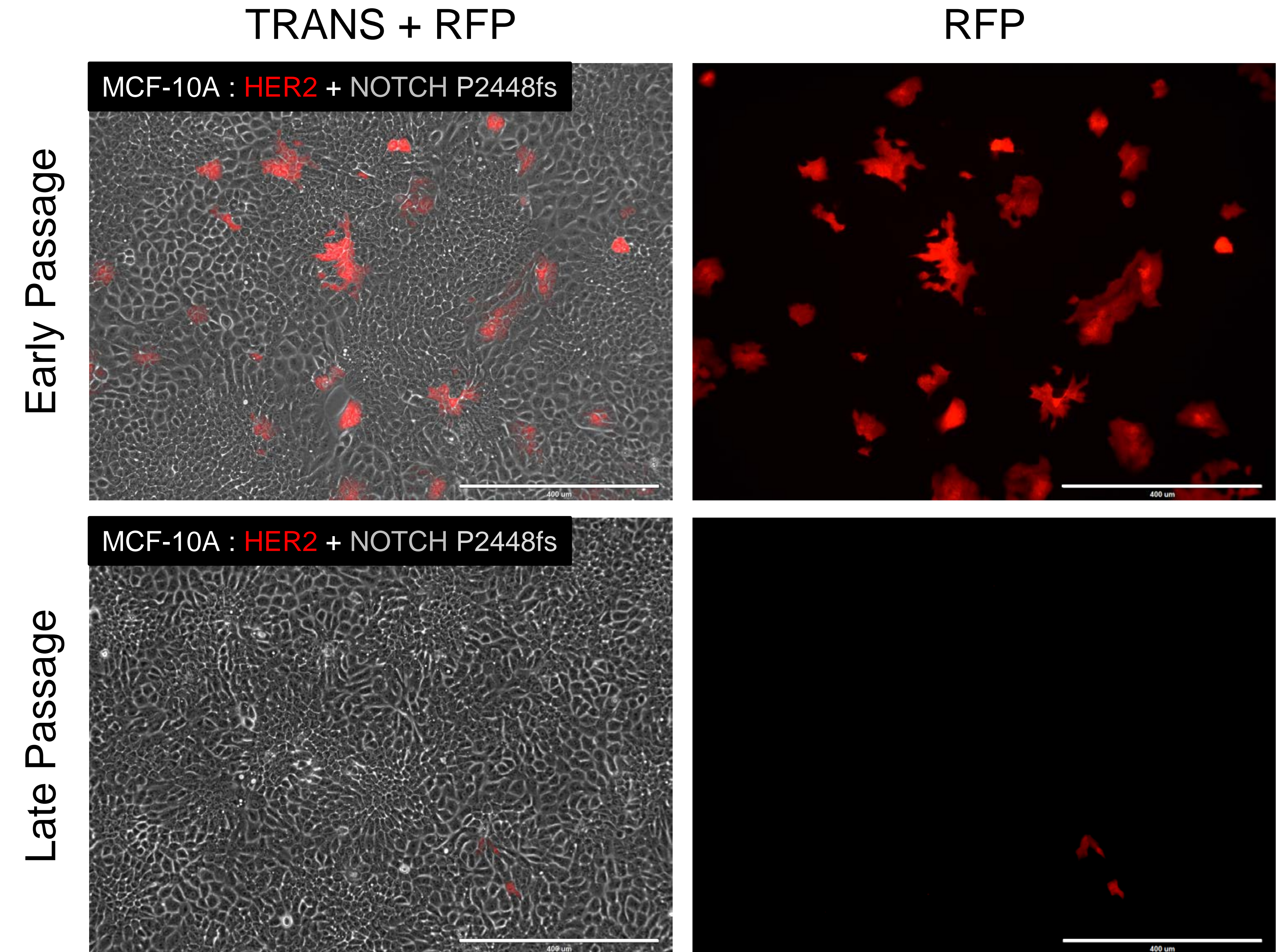


Supplementary Fig. S1: Experimental set up and workflow for co-cultures

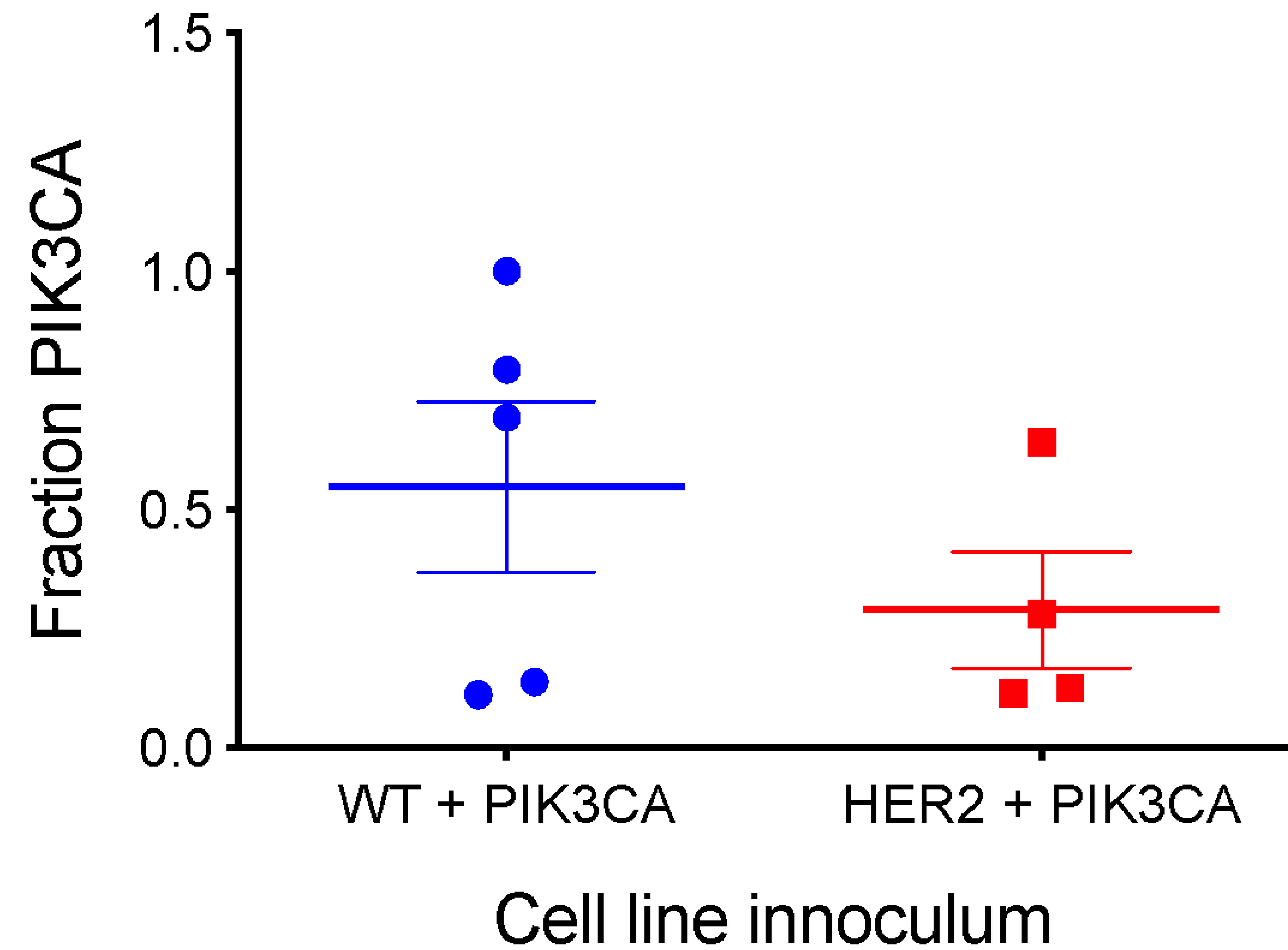
Schematic representation of experimental set up and workflow for the co-culture system utilized in this study. The co-cultures (left), were seeded and grown with both cell types in a single flask. The artificial mixtures (right), were seeded and grown with each cell type separately. A mixture was created after cell collection with equal volume (but often different concentrations, due to differences in growth rates) of each cell type. This artificial mixture acted as a control for ddPCR. gDNA was extracted from the co-cultures and the artificial mixtures and processed for analysis by ddPCR.





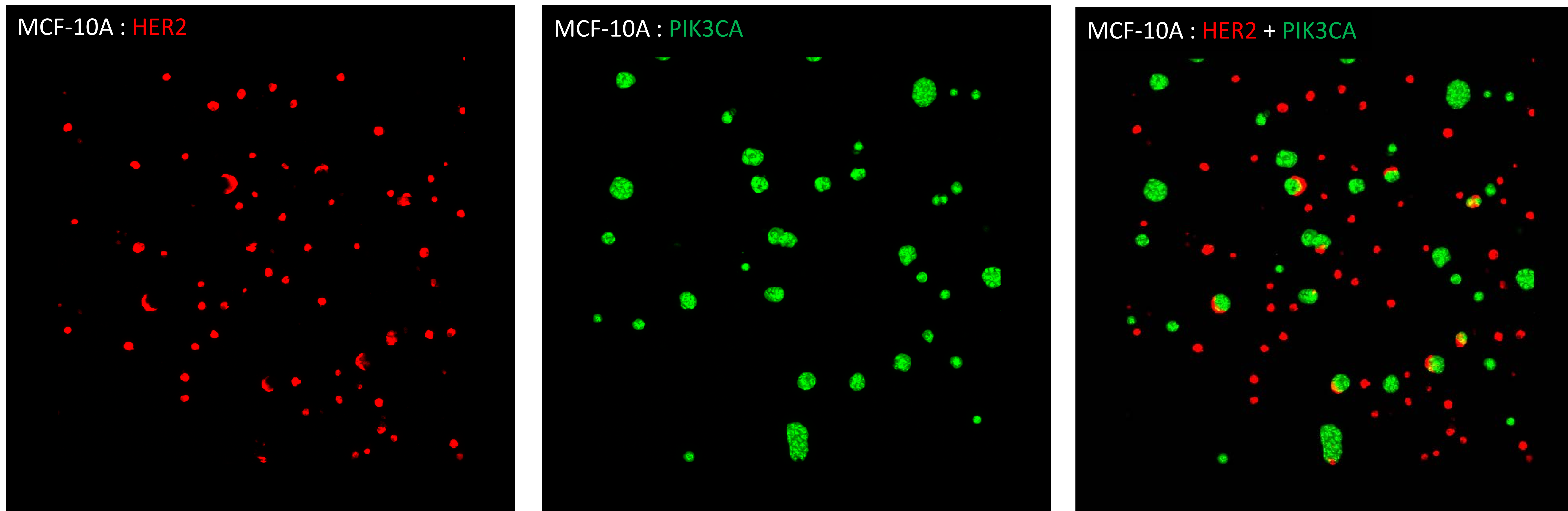
Supplementary Fig. S2: *AKT1* mutant cells do not gain EGF-independence in co-culture with *PIK3CA* mutant cells and *NOTCH1* mutant cells do not impart EGF independence to *HER2* mutant cells.

a) Droplet digital PCR (ddPCR) showing the fraction of MCF-10A *AKT1* E17K mutants in a 1:1 co-culture with *PIK3CA* mutants (red) and in an artificial mixture with *PIK3CA* mutants (blue). b) Representative microscopic images showing the growth of *HER2* mutants (red) and *NOTCH1* mutants (untagged) in co-culture. The early and late passage indicate day 27 and day 46 respectively. Scale = 400uM



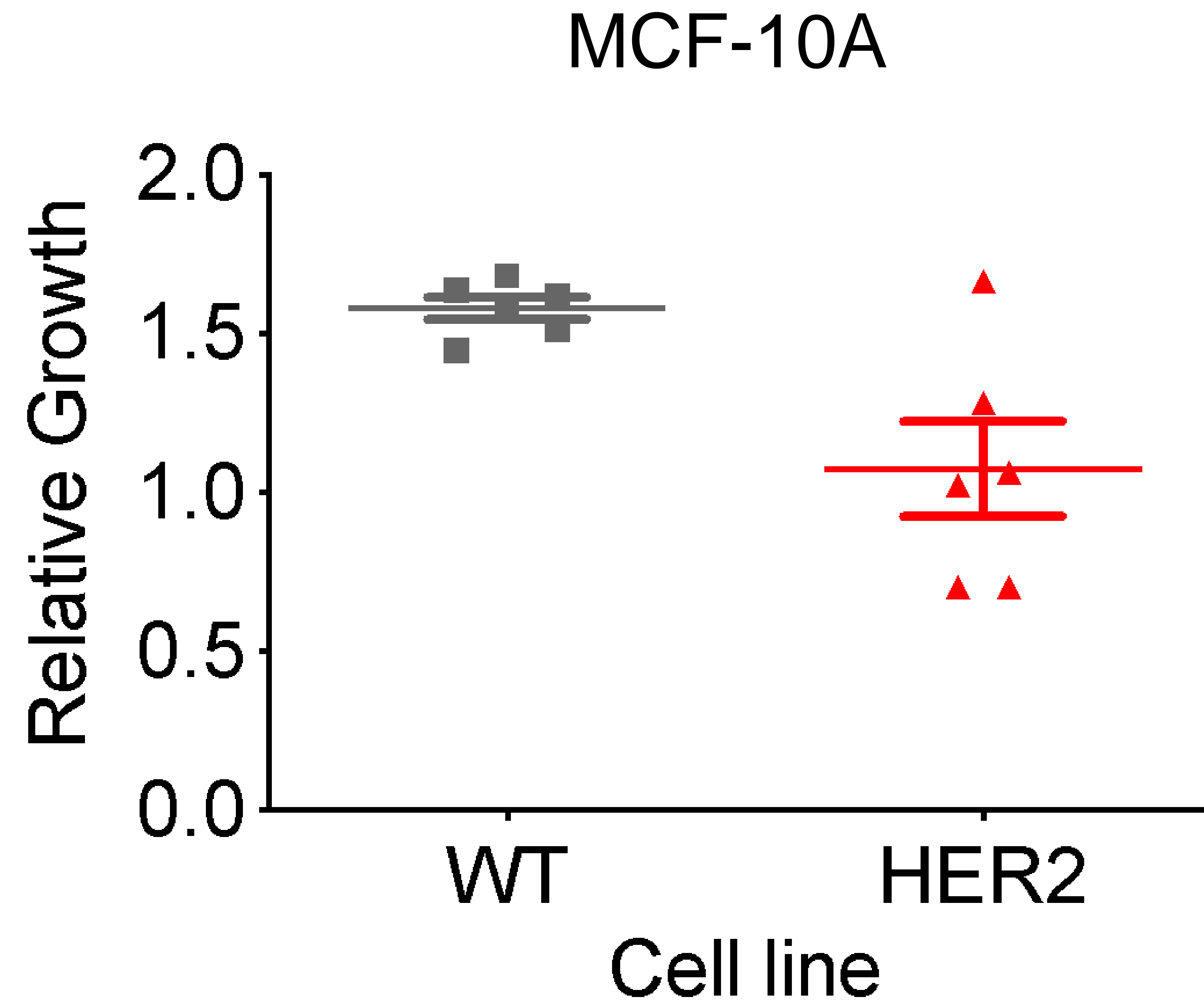
Supplementary Fig. S3: Analysis of cellular composition of co-inoculated tumors *in vivo*

The panel of MCF-7 modified cells were injected subcutaneously into athymic nude mice with estrogen supplementation. Tumors were homogenized and processed for analysis by ddPCR. Results are shown as the fraction of *PIK3CA* mutant cells in the tumor. n=5 for WT:*PIK3CA* and n=4 for *HER2*:*PIK3CA*.



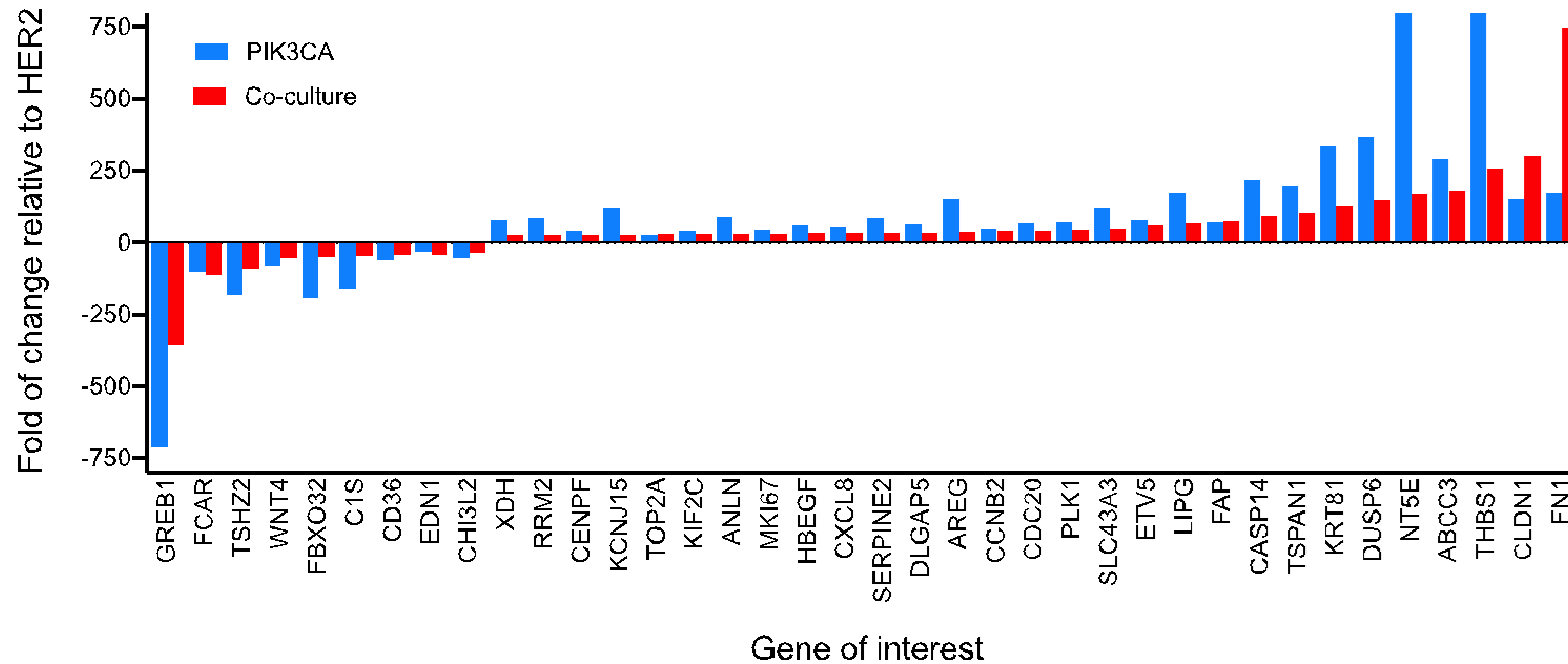
Supplementary Fig. S4: Growth dynamics of *HER2* and *PIK3CA* mutant cells in 3D basement membrane matrix.

Representative confocal microscopy images of colonies formed in co-cultures of *HER2* and *PIK3CA* mutant cells in the MCF-10A background. Individual images of *HER2* mutant cells (red) and *PIK3CA* mutant cells (green) are shown, in addition to the overlay. For animation, please see Supp. Video 2.



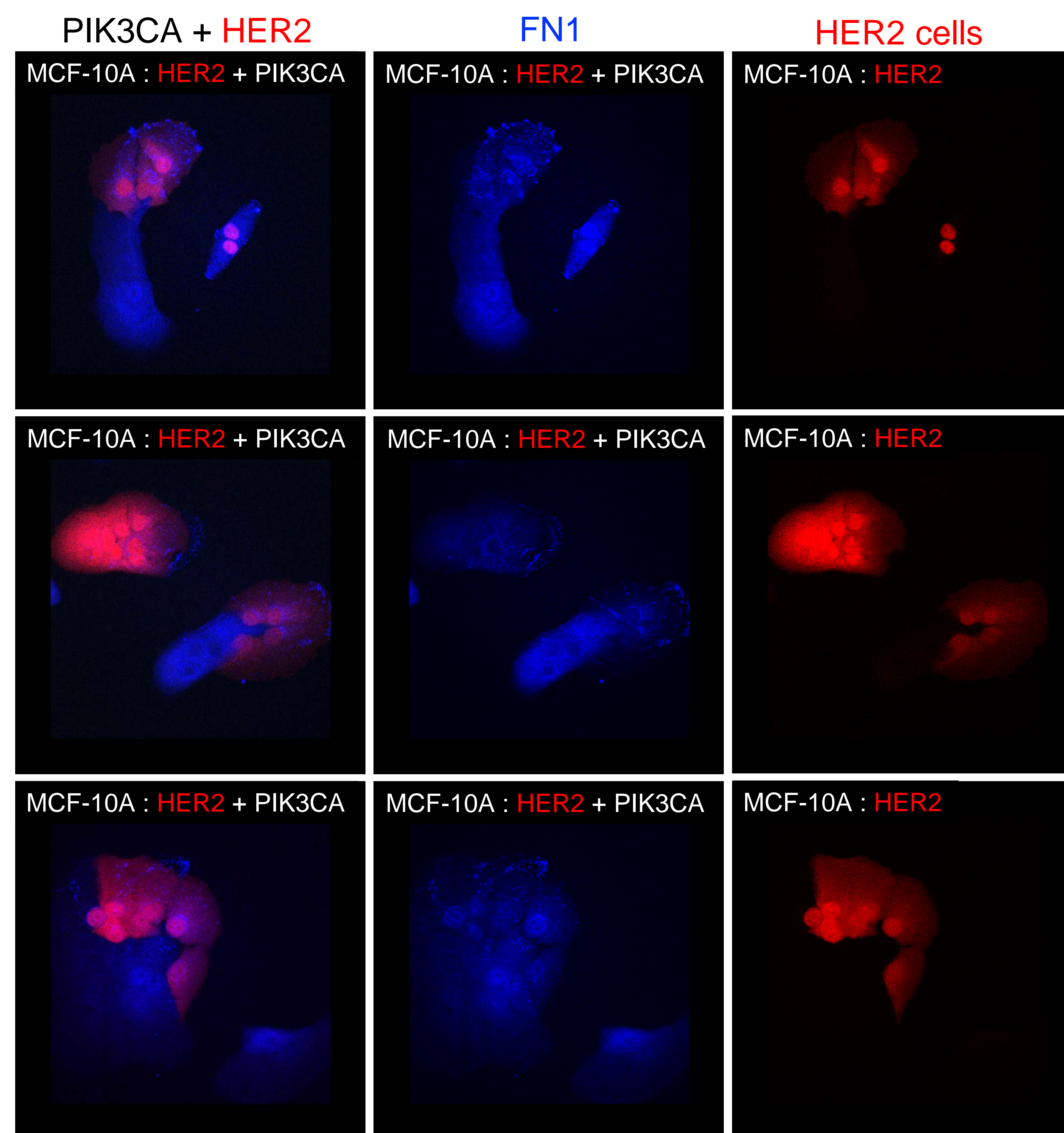
Supplementary Fig. S5: Effect of *PIK3CA* mutant cell conditioned media on wild-type and *HER2* mutant cell growth

Transwell assays in which *PIK3CA* mutant MCF-10As were plated into transwell plates and WT or *HER2* mutant cells were seeded onto transwell inserts. The WT:*PIK3CA* and *HER2:PIK3CA* pairs were grown with shared media for 7-10 days and then cell counts were performed (n=6).



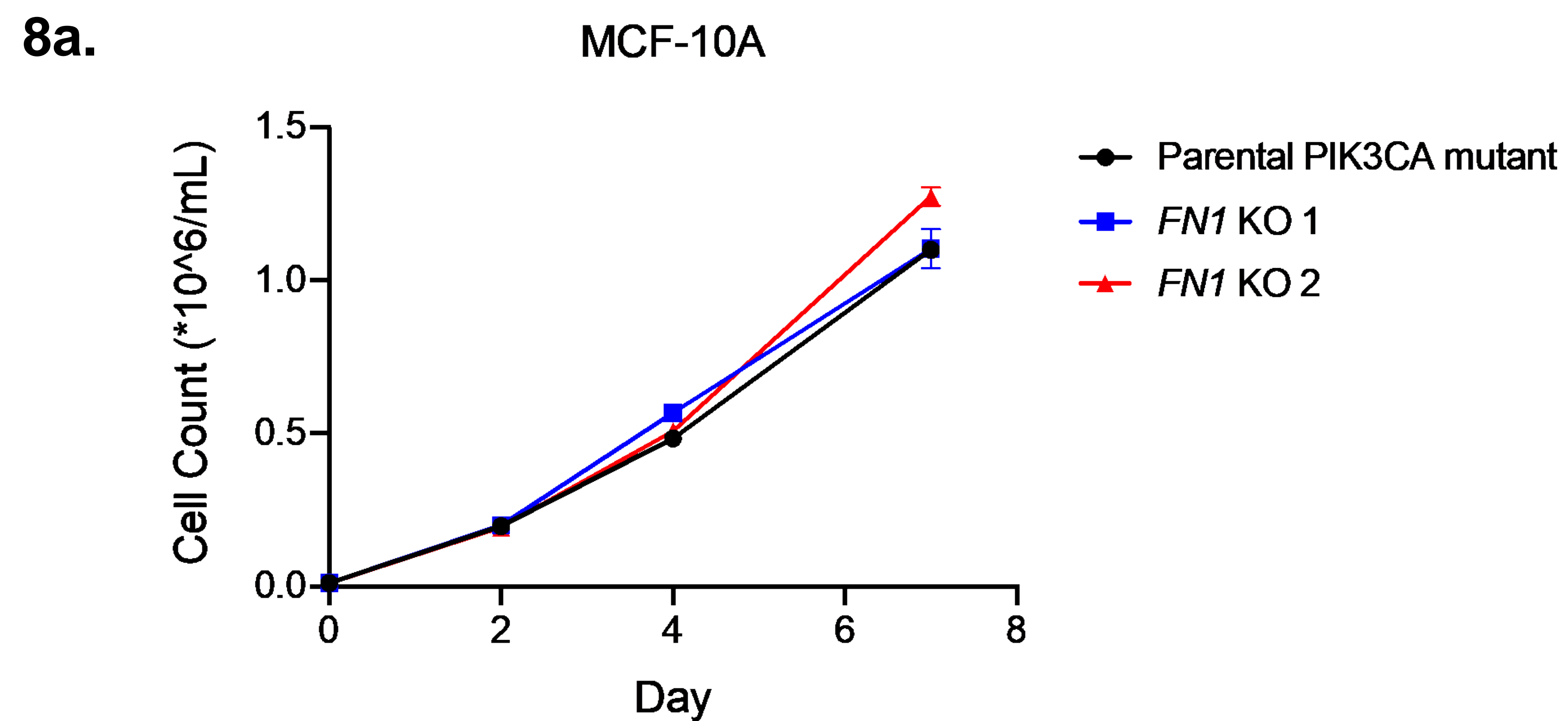
Supplementary Fig. S6: Changes in gene regulation of *PIK3CA* and *HER2* co-cultures.

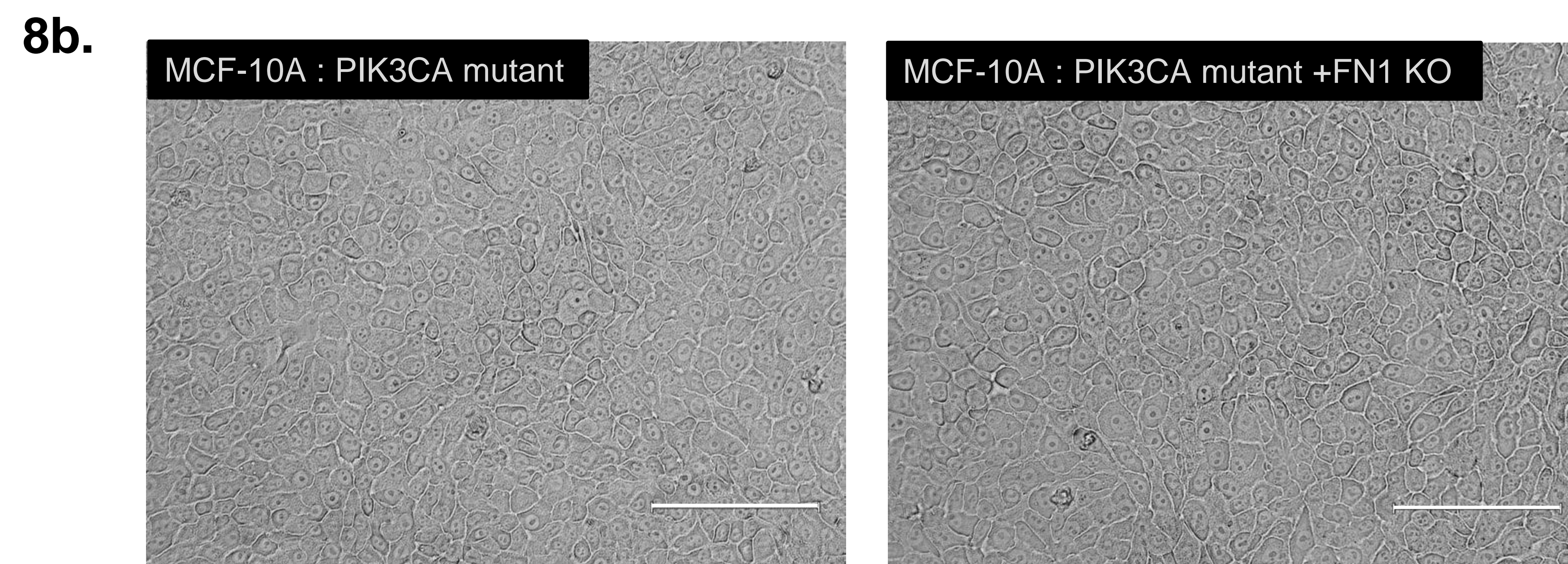
Transcriptome-wide analysis of MCF-10A *PIK3CA* and *HER2* mutant cells grown individually and in co-culture. Fold change in gene expression of individually-grown *HER2* mutant cells was compared to the individually-grown *PIK3CA* mutant cells (blue) and to the co-cultured cells (red). The Clariom S Array was used to assess gene expression differences. Analysis was performed using TAC software. <-30 and >30-fold cutoffs were used.

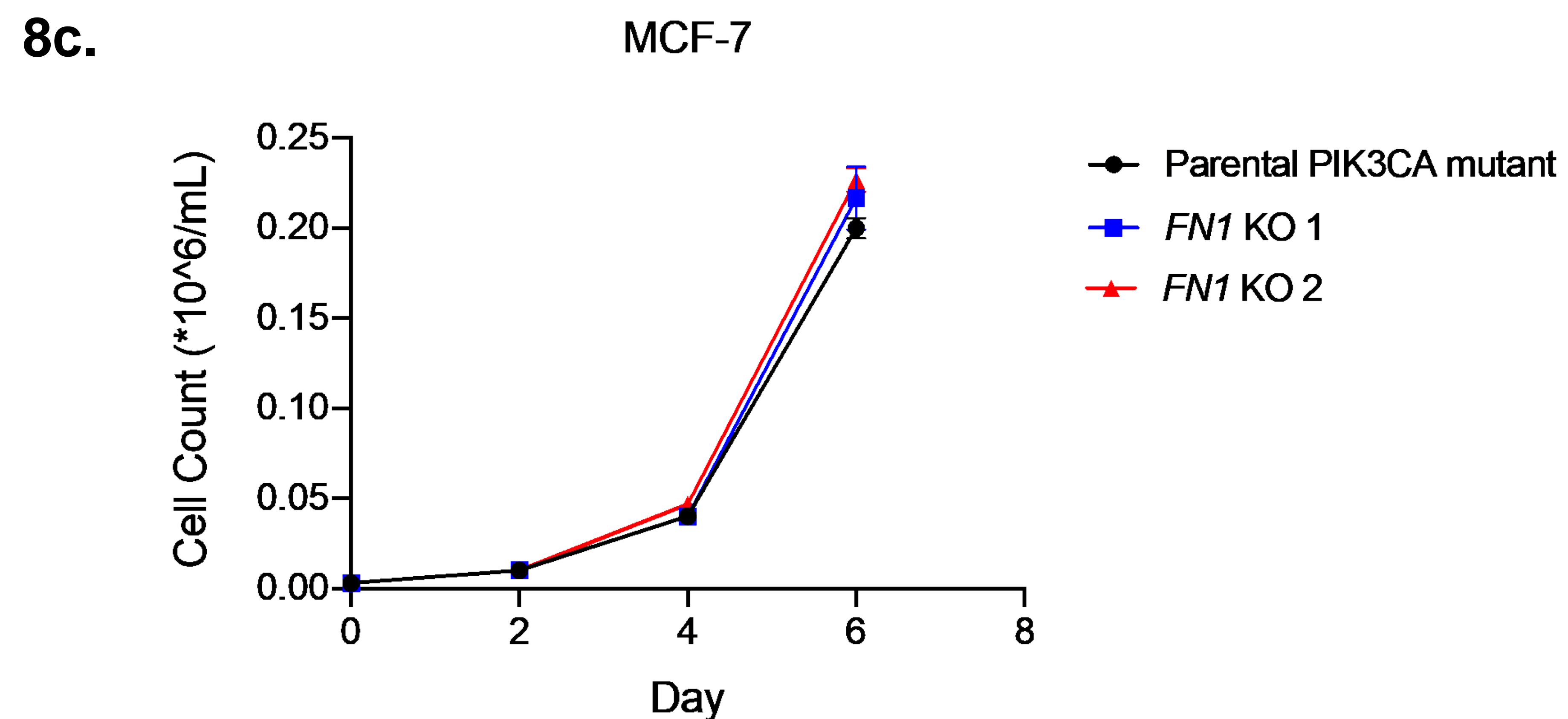


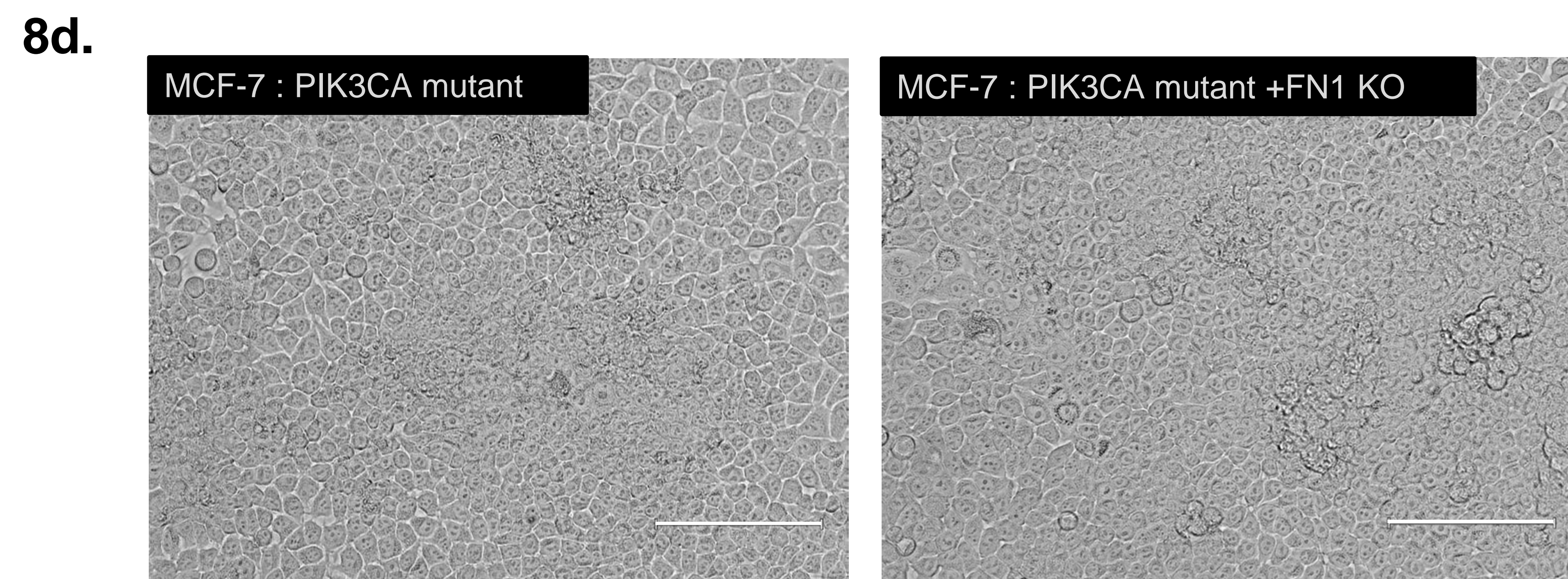
Supplementary Fig. S7: Immunofluorescence of fibronectin expression in *in vitro* *HER2:PIK3CA* co-cultures

Representative images of fibronectin expression in co-cultures of MCF-10A *HER2* and *PIK3CA* mutant cells using IHC and confocal microscopy after 5-7 days of growth. Fibronectin is shown in blue, *HER2* mutant cells in red, and *PIK3CA* mutant cells are untagged.



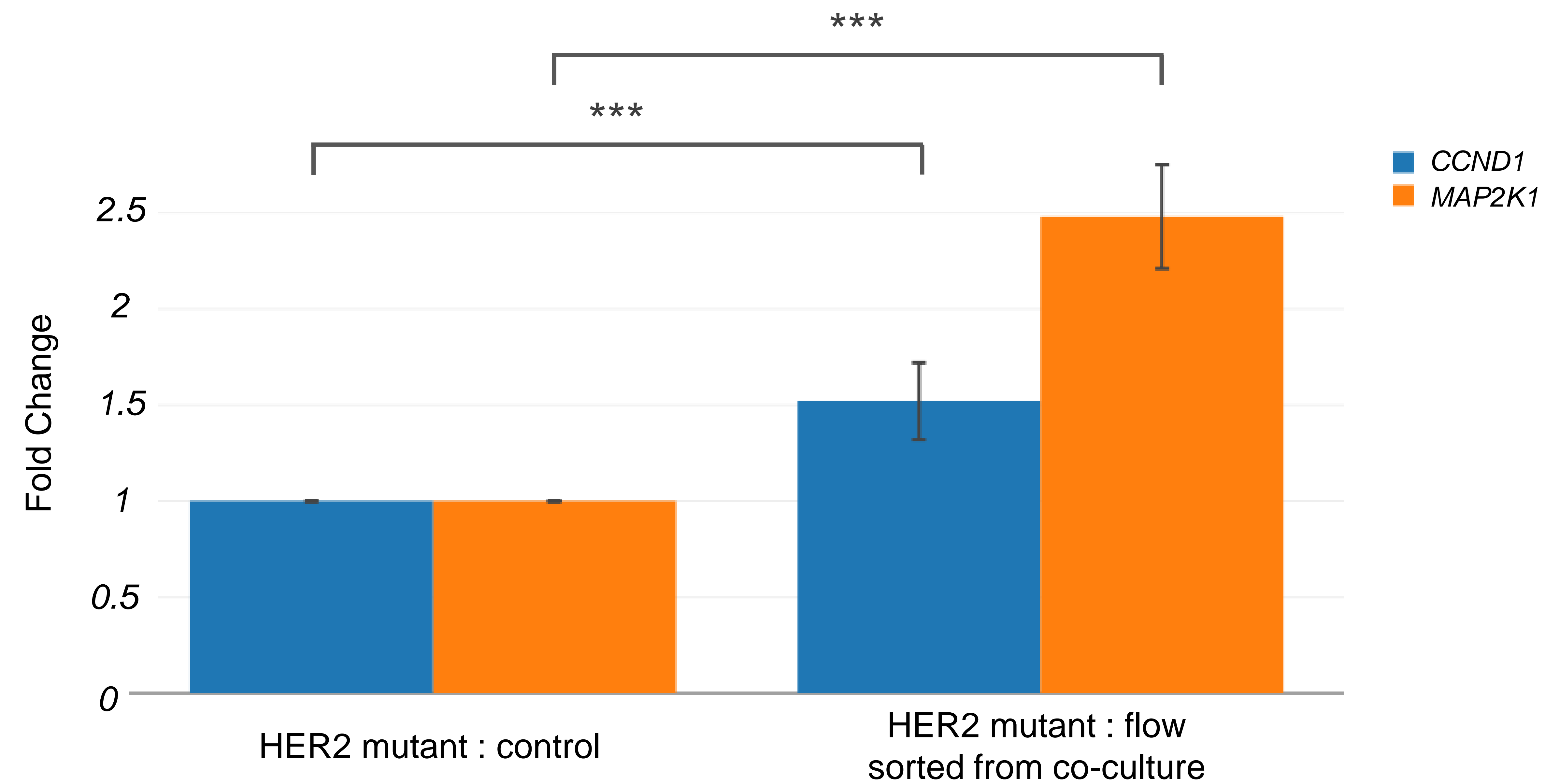






Supplementary Fig. S8: Characterization of *FN1* knockouts

a) Proliferation curves comparing the growth of the the *FN1* knockouts in MCF-10A background to their parental *PIK3CA* mutants. Cells were seeded at a density of 10000 cells per well. b) Representative microscopic images comparing the morphology of the MCF-10A *FN1* knockouts to the *PIK3CA* mutants. Scale = 150uM. C) proliferation curves and d) representative microscopic images comparing the growth and morphology of the MCF-7 *FN1* knockouts to their parental controls. The cells were seeded at a density of 3000 cells per well for the growth assay. Scale = 150uM



Supplementary Fig. S9: Changes in gene expression in HER2 mutants grown in co-culture
Flow sorted microarray data analysis showing an upregulation in *CCND1* and *MAP2K1* expression in the *HER2* mutants grown in co-culture when compared to those grown in isolation. n=4, $p>0.001$, calculated using a student's t-test.

Supplementary Table S1: The mutational profile and growth factor dependence of the cell lines used in this study.

CELL-LINE	PARENTAL	MUTATION OF INTEREST	GROWTH FACTOR/HORMONE DEPENDENCE
PIK3CA	MCF-10A	Heterozygous PIK3CA E545K mutation knocked into MCF-10A	EGF independent
HER2	MCF-10A	Heterozygous HER2 L755S mutation knocked into MCF-10A	EGF dependent
WT	MCF-10A	Heterozygous SF3B1 R702R (silent mutation) knocked into MCF-10A	EGF dependent
AKT1	MCF-10A	Heterozygous AKT1 E17K mutation knocked into MCF-10A	EGF dependent
PIK3CA	MCF-7	Parental MCF-7 with 3 copies of the PIK3CA gene. 2 copies with E545K mutation, 1 WT copy	Estrogen dependent
WT	MCF-7	Corrected MCF-7 where the two PIK3CA E545K mutant alleles have been reverted to wildtype	Estrogen dependent
HER2	MCF-7	WT corrected MCF-7 with a single HER2 L755S mutation knocked in as a heterozygous mutation	Estrogen dependent

Supplementary Table S2: Separate table included

Supplementary Table S3: A list of primers and probes used for ddPCR assays and the guide RNAs and primers used for creating FN1 knockouts using the CRISPR-Cas 9 system

GENE DETAILS	**TYPE**	**SEQUENCE**
DROPLET DIGITAL PCR - Dual Mutant Assay		
PIK3CA E545K	Mut Probe	CTCTGAAATCACTAAGCAGGAGAAAGATTT
	Dual Mut Forward Primer	TCAAAGCAATTTCTACACGAGAT
	Dual Mut Reverse Primer	ATTTTAGCACTTACCTGTGACT
HER2 L755S	Dual Mut Probe	CCATCAAAGTGTCGAGGGAAAACA
	Dual Mut Forward Primer	CTGATGGGGAGAATGTGAAA
	Dual Mut Reverse Primer	TCTAAGATTTCTTTGTTGGCTTTG
SF3B1 R702R	Dual Mut Probe	AGCAGCAGAAAGTTAGGACC
	Dual Mut Forward Primer	TTTTGTAGGTCTTGTGGATGAG
	Dual Mut Reverse Primer	CAATGGCCAAAGCACTGA
DROPLET DIGITAL PCR – Traditional Assay		
PIK3CA E545K	WT Probe	CTCTGAAATCACTGAGCAGGAGAAAGATT
	Mut Probe [b]	CTCTGAAATCACTAAGCAGGAGAAAGATTT
	Forward Primer	TCAAAGCAATTTCTACACGAGATCCT
	Reverse Primer	CTCCATTTTAGCACTTACCTGTGACT
CRISPR-Cas9 knockouts		
FN1 (MCF-10A)	Guide RNA	GAATGGACCTGCAAGCCCAT
FN1 (MCF-7)	Guide RNA	TCACACACCTATGGGCTTGC
FN1 (MCF-10A and MCF-7[a])	Screening: Forward Primer	CCTGATGTGGCCTTTTCACT
	Screening: Reverse Primer	AGACCTGAATTCCAGTGAAAACC
	Screening: Sequencing Primer	AGACCTGAATTCCAGTGAAAACC

a – The same screening primers were used for both the MCF-10A and MCF-7 knock outs
b – The same PIK3CA E545k mutant probe was used for traditional and dual mutant ddPCR assays

Supplementary Methods:

Cell fraction calculations for droplet digital PCR

1) For MCF-10A background *HER2* L755s and *SF3B1* R702R co-cultures with *PIK3CA* E545K mutant cells. Each cell line contains a single-copy heterozygous mutation, allowing for the quantification of one cell per mutant allele using the Dual Mutant Assay, assuming no cell fusions.

$y = a/(a+b)$
Where,
y = fraction of cell line A
a = # of positive “A” genotype mutant droplets = # of cell line A cells
b = # of positive *PIK3CA* E545K mutant droplets = # *PIK3CA* mutant cells

2) For MCF-7 background WT *PIK3CA* cells co-cultured with parental MCF-7s. The WT cells contain 3 WT alleles of *PIK3CA* and the parental MCF7s contain 2 mutant copies of *PIK3CA* to 1 WT. Cellular percentages were calculated using the traditional single-locus WT/Mut assay and according to the equation 2. The number of MCF7 cells was calculated by dividing the number of *PIK3CA* mutant droplets by 2. The number of WT cells was then calculated by subtracting the number of MCF7 cells that contain a single WT copy of *PIK3CA* from the number of WT droplets and then dividing that number by 3.

$y = (a/2)/((a/2)+(b-(a/2))/3))$
Where,
y = % of MCF-7s
a = # of positive *PIK3CA* E545K mutant droplets b = # of positive *PIK3CA* E545K WT droplets (a/2) = # of MCF-7 cells
(b-(a/2))/3 = # of WT cells

3)For MCF-7 background *HER2* mutant cells co-cultured with parental MCF-7s. The *HER2* mutant MCF-7s are derived from the *PIK3CA* WT MCF-7s and have 3 WT copies of *PIK3CA* and 1 mutant copy of *HER2* L755S to 1 WT copy. Cellular percentages were calculated using a Dual Mutant assay and equation 3.

$y = (a/2)/((a/2)+(b))$
Where, y = % of MCF-7s
a = # of positive *PIK3CA* E545K mutant droplets
b = # of positive *HER2* L755S mutant droplets = # of HER2 mutant cells (a/2) = # of MCF7 cells