DIGITAL PCR-PRINCIPLES

- ✓ Sample is partitioned so that individual nucleic acid molecules within the sample are localized and concentrated within many separate regions.
- ✓ The partitioning of the sample allows one to estimate the number of different molecules by assuming that the molecule population follows the Poisson distribution. As a result, each part will contain "0" or "1" molecules, or a negative or positive reaction, respectively.

DIGITAL PCR-PRINCIPLES

- ✓ After PCR amplification, nucleic acids may be quantified by counting the regions that contain PCR end-product, positive reactions.
- ✓ In conventional PCR, the number of PCR amplification cycles is proportional to the starting copy number.
- ✓ dPCR, however, is not dependent on the number of amplification cycles to determine the initial sample amount, eliminating the reliance on uncertain exponential data to quantify target nucleic acids and therefore provides absolute quantification.

DIGITAL PCR

- ✓ Digital PCR depends on the ability of PCR to detect a single molecule of a target locus. The sample is greatly diluted and divided into a large number of aliquots, so that some aliquots receive at least one molecule of the target (‘‘positive’’ aliquots), whilst others do not. The number of positive aliquots, as determined by PCR, then reflects the abundance of the target locus

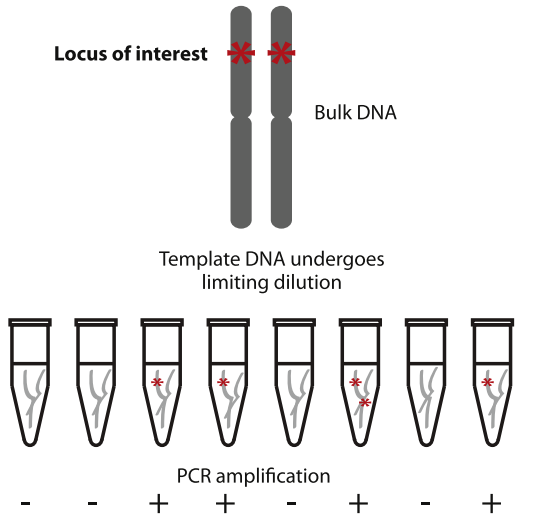


Fig. 1. Limiting dilution PCR. The principles underpinning digital PCR are very simple. DNA undergoes limiting dilution. PCR is then used to probe each aliquot for the presence (+) or absence (−) of a locus of interest.

DIGITAL PCR-QUANTIFICATION

- ✓ More often, the sample is not diluted quite so far.
- ✓ Then many (but not all) of the aliquots will be found to be positive, and some of these positive aliquots will probably (and unbeknownst to the experimenter) have contained two, three or more target molecules.
- ✓ Simply counting positive aliquots will underestimate the true number of molecules.
- ✓ This can be corrected by using the Poisson equation

$$A = -\log_e(1-P),$$

where A is the average number of molecules per aliquot, and P is the proportion of positive aliquots

DIGITAL PCR-QUANTIFICATION

- ✓ More commonly, the abundance of the target sequence is compared to that of a **reference sequence analysed** in the same way, to determine the target's **relative abundance**
- ✓ The reference sequence is usually chosen to be one whose abundance is known – for example, one which is present in two copies per diploid cell.

DIGITAL PCR-ADVANTAGES AND LIMITS

- ✓ Rare variants detection
- ✓ Minimal template requirements
- ✓ Estimating copy-number variation (assuming reference diploid loci have been validated and therefore have a relative copy-number of two, it is possible to distinguish between one (indicating allelic loss) and two copies, and also between higher integers, for example, five and six copies)

DIGITAL PCR-ADVANTAGES AND LIMITS

- ✓ The precision (reproducibility) of digital PCR-based quantitation and its capacity to detect very rare variants depends on the total number of aliquots that are interrogated – the precision and sensitivity increase as more aliquots are analysed.
- ✓ Contamination-the systems necessary to avoid contamination include a reliable supply of clean reagents, a dedicated PCR suite and controls on the concentration of template DNA permitted in the laboratory.

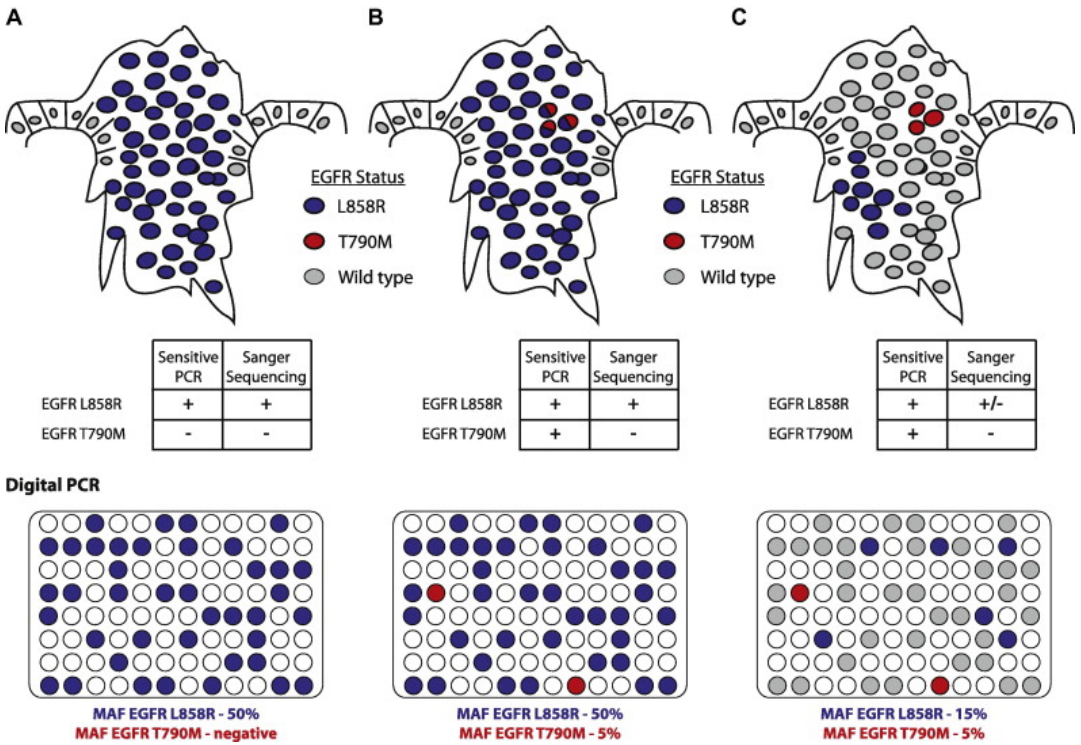


Fig. 2 Digital PCR, mutant allele frequency (MAF) and test sensitivity. The issue of sensitivity and mutant allele frequency in biopsy material has rarely been addressed but may have a significant impact on the interpretation of molecular biomarkers and th...

Elizabeth Day , Paul H. Dear , Frank McCaughan

Digital PCR strategies in the development and analysis of molecular biomarkers for personalized medicine

Methods, Volume 59, Issue 1, 2013, 101 - 107

http://dx.doi.org/10.1016/j.ymeth.2012.08.001

In **droplet digital PCR**, reaction chambers are separated not by the walls of a well but by carefully titrated emulsions of oil, water and stabilizing chemicals. First, samples are put into a machine where they are mixed with all the necessary reagents and dispersed into tiny droplets. The droplets for each sample are transferred into tubes that can be placed in a thermocycler for PCR. Afterward, the tubes are transferred to a droplet reading machine, which functions like a flow cytometer to analyze each droplet for whether or not a reaction has occurred.

Chip dPCR: two systems that mix samples with reagents, partition the reaction mixture, perform thermocycling and read each partition

Platform	Description	Number of reactions	Aliquot volume	Analysis	Published sensitivity for rare variants	Integrated thermocycling and analysis	Commercial availability	Published applications
Microdroplets								
ddPCR, BioRad	Microdroplets are generated in an emulsion and transferred to 96 well plates for cycling then to the custom analysis unit (QX100 droplet reader). The reader unpacks the emulsion to single droplets for analysis.	20,000 per 20 μl sample	1 nL	Automated droplet flow cytometer (two colors) with Taqman probes	0.001%	No	Yes QX100 Droplet digital PCR System	Genotyping absolute quantification [9,11]
RainDrop, raindance technologies	Microdroplets, are generated in an emulsion, collected and transferred for thermocycled. The emulsion is then injected onto a microfluidic device and each droplet is analysed	Continuous flow	9 pL	End point analysis with TaqMan probes	0.0005%	No	Yes RainDrop digital PCR system	Genotyping[65] Absolute quantification[63]
BEAMing (beads, emulsion, amplification, magnetics)	Microdroplets containing magnetic beads are generated in an emulsion and transferred to 96 well plates for thermocycling. The emulsion is dispersed and the beads separated. A circularizable probe is hybridized to the sequences on the beads and the changes of interest are labeled with fluorescently labeled dideoxynucleotide terminators	5×10^7 beads	9 μm diameter	Labeled beads are analyzed by flow cytometry	0.01%	No	No	Genotyping Absolute quantification [13]
Microfluidic chambers								
MegaPixel digital PCR	Surface tension based sample partitioning creates aliquots that are thermocycled and analyzed on the device. Fluorescent probes are annealing during thermocycling to enable analysis	1×10^6	10 pL	Microarray scanner	0.001%	Yes	No	Genotyping [20]
Spinning disk platform	Aliquots are generated by passive compartmentalization through centrifugation. These are thermocycled and analysed on the device	1,000	33 nL	CCD camera – end point melting curve analysis	–	Yes	No	Copy number variation and absolute quantification [61]
OpenArray Life technologies/ ABI	Microfluidc reaction chambers are loaded, thermocycled and analysed using the OpenArray system. Chambers may be preloaded with the assay of choice	3,072	33 nL	CCD camera – real time PCR end point melting curve analysis	–	Yes	Yes OpenArray Real-Time PCR platform	
Digital array chip, fluidigm	Microfluidic reaction chambers are loaded, thermocycled and analysed using the BioMark system	9,180 (12×765) Prototype $2 \times 100{,}893$	6 nL	CCD camera – real time PCR end point melting curve analysis		Yes	Yes BioMark HD system	microRNA expression [53] Single cell gene expression [66] Genotyping [35] Targeted resequencing [67] Copy number variation[19,21,60] Absolute quantification [25]

Performance characteristics of selected dPCR platforms currently on the market.

System	Reaction volume	Total no. of reactions	No. of reactions per sample	Maximum no. of samples	Melt curve capacity
Biomark Digital Array (12.765 or 48.770)	6 or 0.85 nL	9,180 or 36,960	765 or 770	12 or 48	Can use intercalating dye but no melt analysis
OpenArray	33 nL	3,072	3,072 (1 sample)	48 (64 reactions)	Yes
QX100	~1 nL	160,000	20,000	8	End-point analysis
RainDrop	~5 pL	80 million	1–10 million	8	End-point analysis

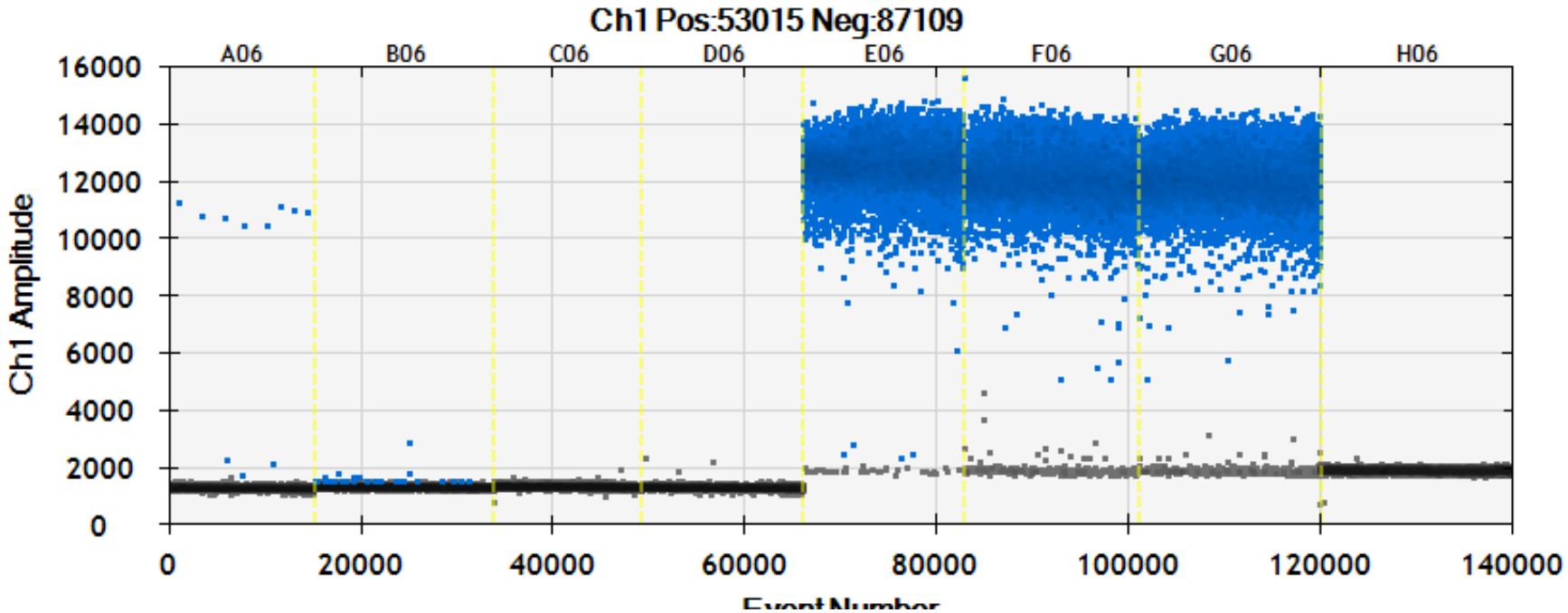
Table 1 | Commercial digital PCR offerings

Vendor	Instruments and list price	Consumables and list price	Number and volume of partitions	Volumes required	qPCR capacity	Multiplexing
Fluidigm Corporation	BioMark HD: $200,000–$250,000	12 arrays per chip[a] (765 wells per array): $400 per chip (works in both EP1 and BioMark)	12-inlet chip: 9,180 partitions, 6 nl per partition	12-inlet chip: 8 μl of mix, ~4 μl of sample; 57% analyzed[b]	Yes	Can use up to 5 colors to detect 5 targets (assumes 5th color is ultraviolet)
	EP1: $100,000–$150,000	48 arrays per chip[a] (770 wells per array): $800 per chip (works in both EP1 and BioMark)	48-inlet chip: 36,960 partitions, 0.85 nl per partition	48-inlet chip: 4 μl of mix, ~2 μl of sample[b]	No	Can use up to 5 colors to detect 5 targets
Life Technologies	OpenArray RealTime PCR System and QuantStudio 12K Flex instrument: $140,000 and $90,000–$190,000, respectively	OpenArray plates[a] (64 holes per subarray): $150 per plate	Varies; 3,072 partitions per plate, 48 subarrays per plate, 33 nl per partition (machines run 3–4 plates at once)	100 μl of sample per plate (across 48 arrays)	Yes	Uses 2 colors of probes to detect 2 targets
Bio-Rad Laboratories	QX100 ddPCR System (machines to generate and read droplets): $89,000	8 samples per chip (14,000–16,000 droplets per sample): $3 per sample	Up to 96 samples per run (assumes manual pipetting into PCR plate); 1,344,000 partitions per run (assuming separate thermocycler runs 12 chips at once), 1 nl per partition	Up to 9 μl per sample (20,000 droplets made); an average of 70% read	No	Uses 2 colors to detect 2 targets
RainDance[c]	RainDrop Digital PCR (machines to generate, collect and read droplets): $100,000	8 samples per chip (up to 10,000,000 droplets per sample): $10–$30 per sample	8 samples per run; up to 80,000,000 partitions per run, 5 pl per partition	5–50 μl per sample	No	Uses 2 colors, but can use varying concentrations of probes to detect up to 10 targets

[a]Arrays can hold separate samples, or the same sample can be spread over multiple arrays. [b]For rare allele analysis, protocols are available to eliminate the dead volume. [c]Plans full commercial launch later this year.

DIGITAL PCR-APPLICATIONS

- ✓ Mutation/rare variant detection
- ✓ Pharmacogenetics
- ✓ Gene Expression Analysis
- ✓ Methylation-specific digital PCR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A06
B06
C06
D06
E06
F06
G06
H06
Ch1 Amplitude
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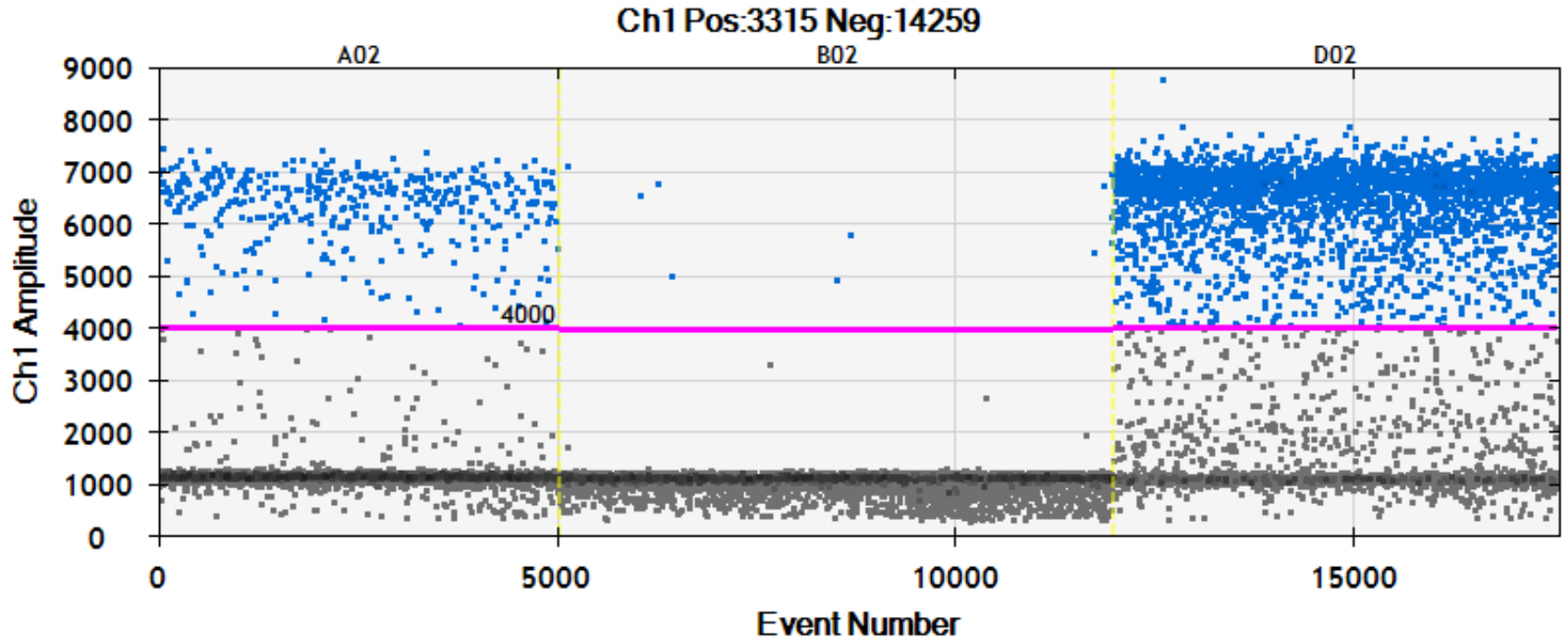
12000
10000
8000
6000
4000
2000
0
0
20000
40000
60000
80000
100000
120000
140000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A02
B02
D02
Ch1 Amplitude
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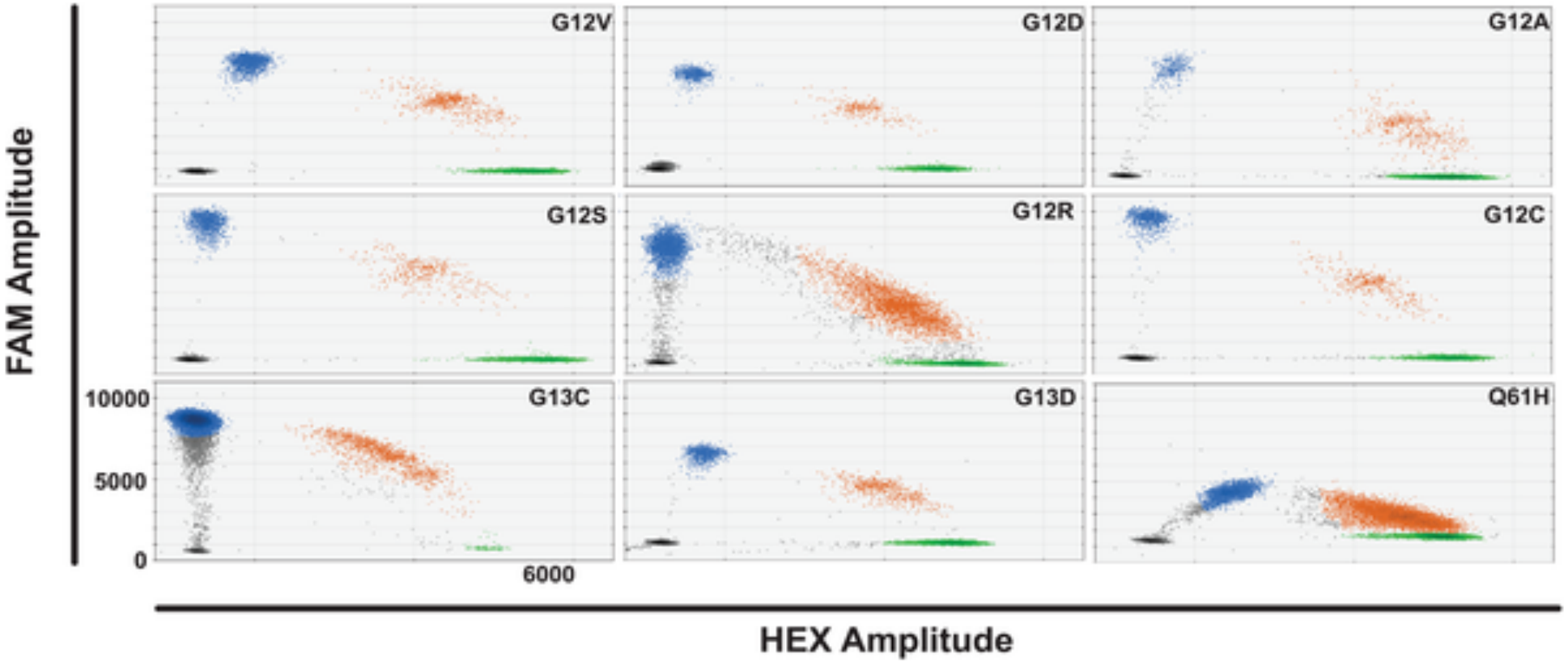
4000
0
5000
10000
15000
Event Number

Fig 1. KRAS duplex assays at optimal annealing temperature.

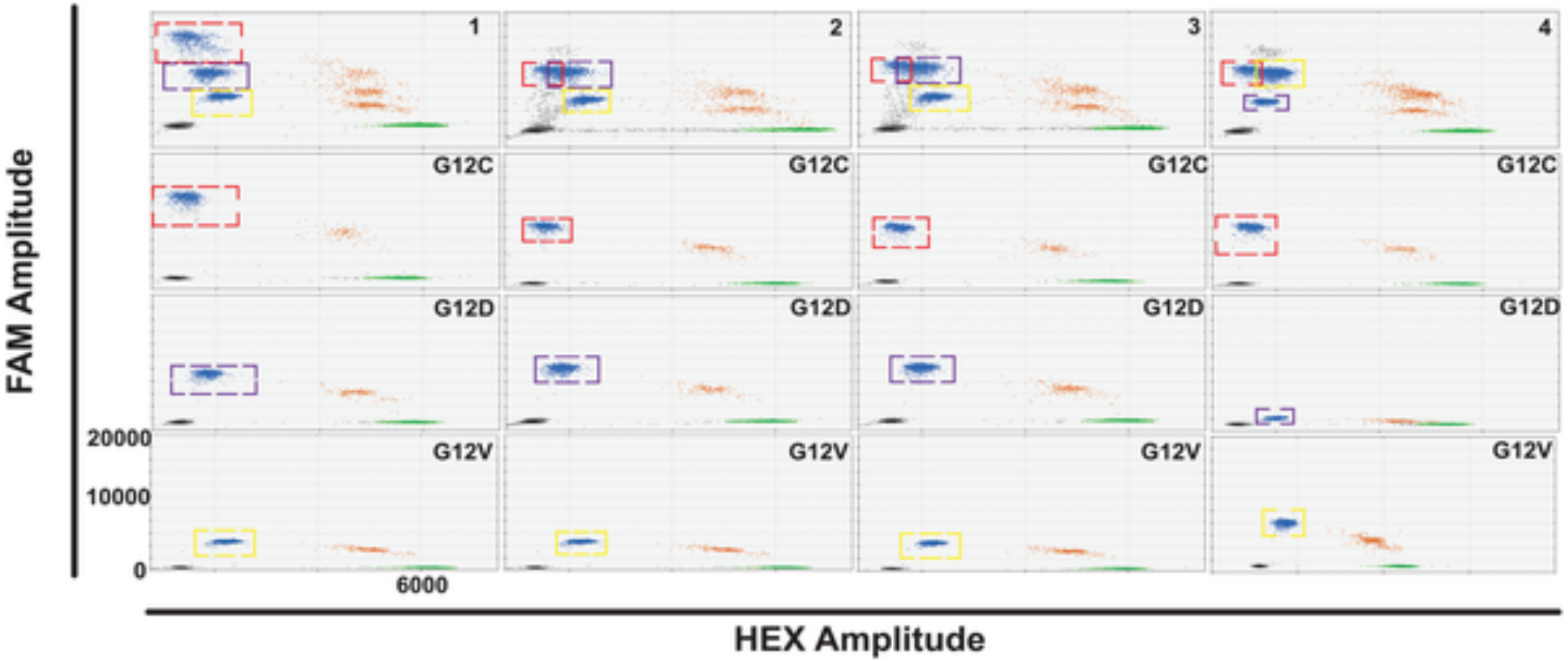


Black drops- empty droplets, blue- mutant DNA FAM positive droplets, green- wild-type DNA HEX positive droplets, brown—wild-type and mutant DNA double positive droplets.

Pender A, Garcia-Murillas I, Rana S, Cutts RJ, Kelly G, et al. (2015) Efficient Genotyping of KRAS Mutant Non-Small Cell Lung Cancer Using a Multiplexed Droplet Digital PCR Approach. PLoS ONE 10(9): e0139074. doi:10.1371/journal.pone.0139074
http://journals.plos.org/plosone/article?id=info:doi/10.1371/journal.pone.0139074

PLOS | ONE

Fig 2. Four different KRAS multiplex digital PCR assays combining G12C, G12V and G12D mutant assays and corresponding duplex assays.



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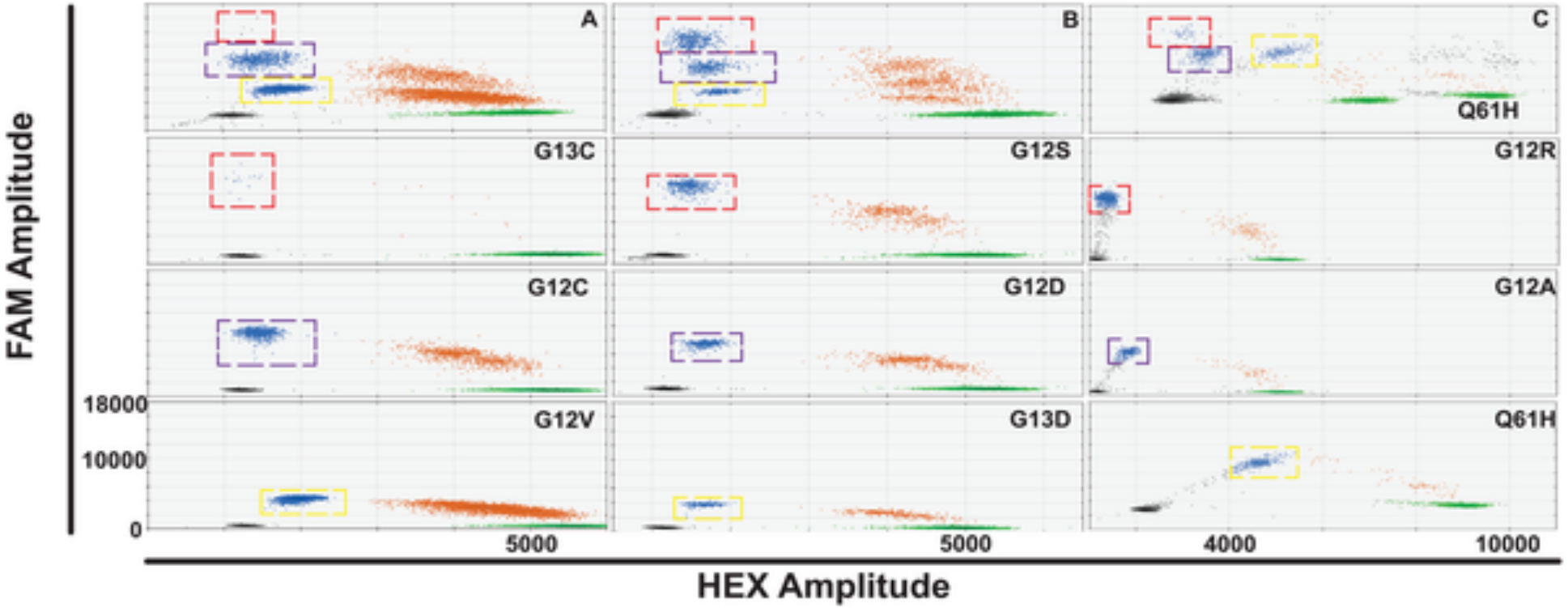
http://journals.plos.org/plosone/article?id=info:doi/10.1371/journal.pone.0139074

PLOS ONE

Fig 3. KRAS multiplex digital PCR assays A-C and corresponding duplex assays.



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http://journals.plos.org/plosone/article?id=info:doi/10.1371/journal.pone.0139074

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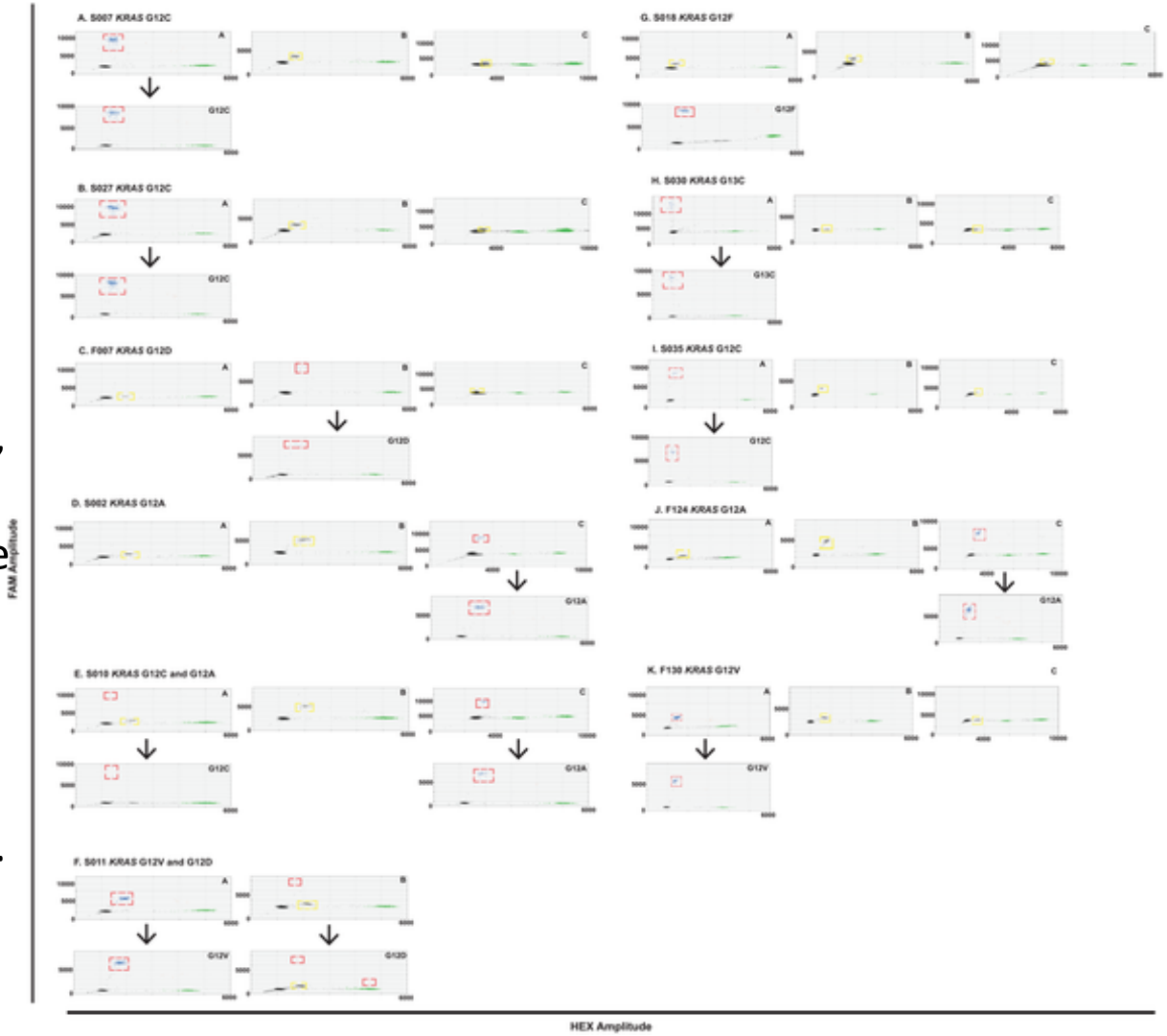
Fig 5. KRAS mutant FFPE tissue DNA analysis using multiplex and duplex assays to detect KRAS mutant clones.

Black drops- empty droplets, blue- mutant DNA FAM positive droplets, green- wild-type DNA HEX positive droplets, brown—wild-type and mutant DNA double positive droplets.



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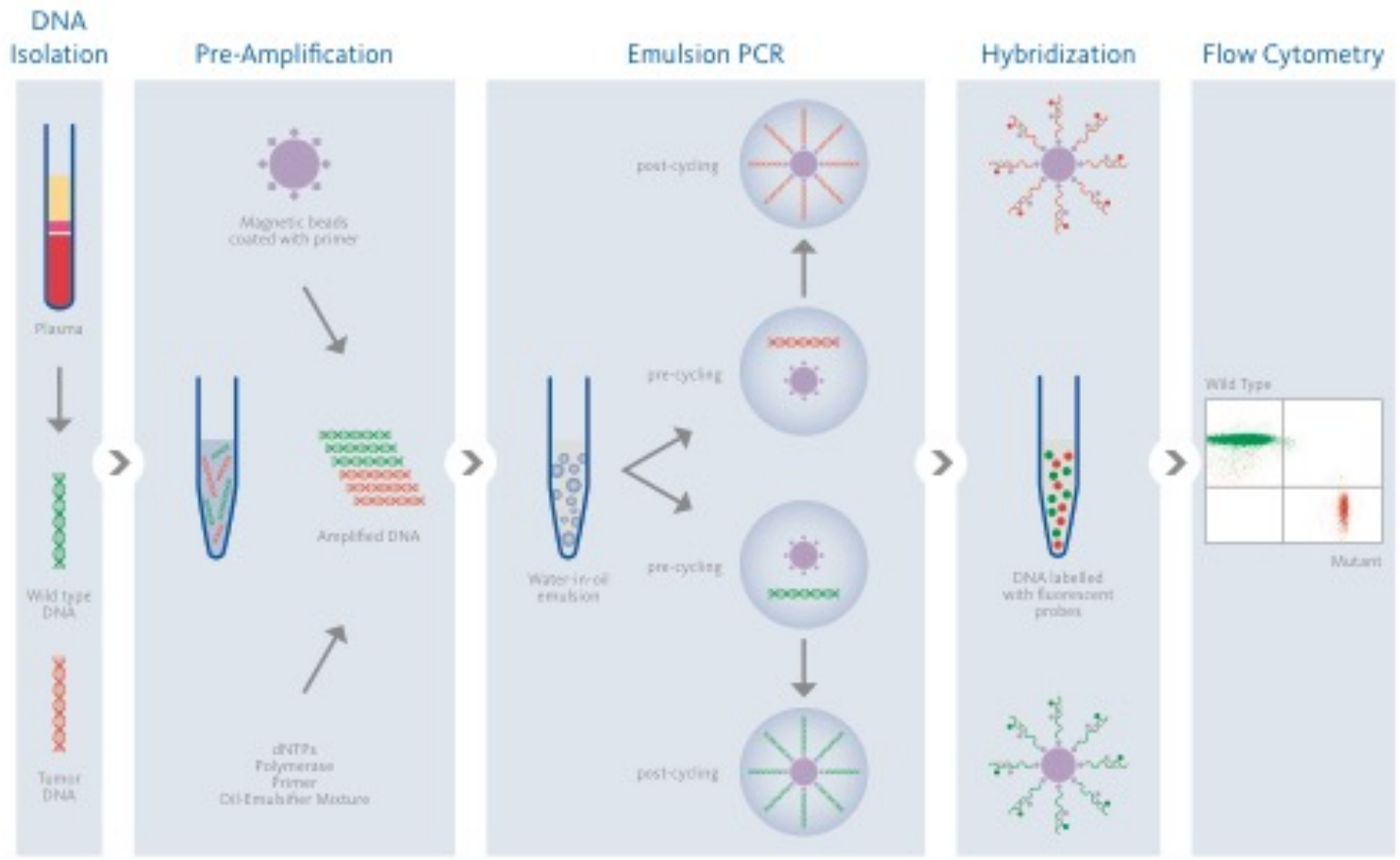
http://journals.plos.org/plosone/article?id=info:doi/10.1371/journal.pone.0139074

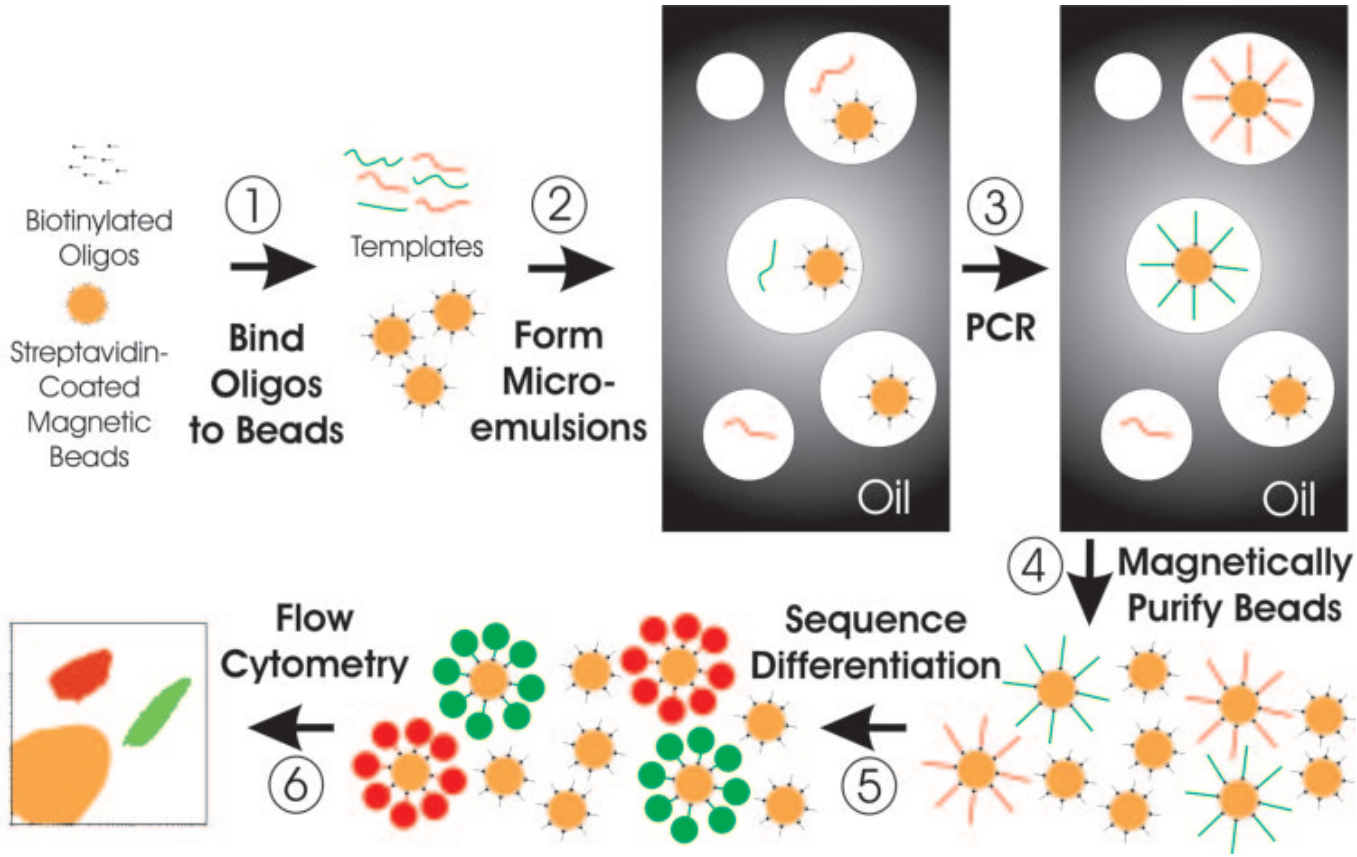
BEAMING ASSAYS

- ✓ Beads, Emulsification, Amplification, and Magnetics
- ✓ It combines emulsion PCR with magnetic beads and flow cytometry for highly sensitive detection and quantification.



BEAMING ASSAYS

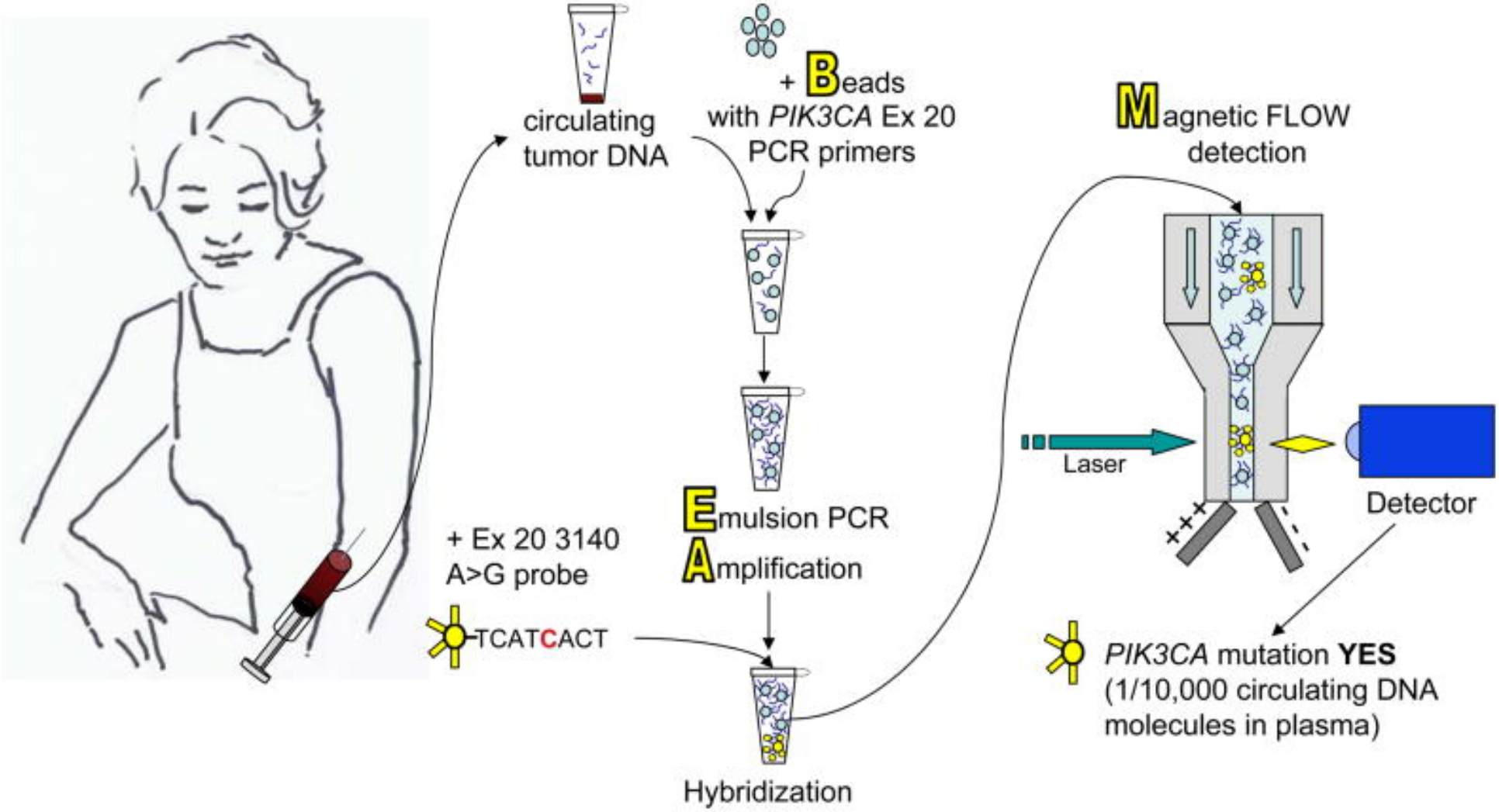
✓ Beads, Emulsification, Amplification, and Magnetics



1: Magnetic beads covalently coated with streptavidin are bound to biotinylated oligonucleotides (oligos).
2. An aqueous mix with PCR reagents plus primer-bound beads and template DNA are mixed with an oil detergent to create microemulsions. The aqueous compartments (white circles in the gray oil layer) contain an average of less than one template molecule and less than one bead. Red and green templates represent two template molecules, the sequences of which differ by one or many nucleotides.

3: The microemulsions are submitted to PCR. If a DNA template and a bead are present together in a single aqueous compartment, the bead-bound oligonucleotides act as primers for amplification. The straight red and green lines connected to the beads represent extension products from the two different kinds of templates. 4: The emulsions are broken, and the beads are purified with a magnet. 5: After denaturation, the beads are incubated with oligonucleotides that can distinguish between the sequences of the different kinds of templates. Fluorescently labeled antibodies then are used to label the bound hybridization probes, which renders the beads containing PCR product as red or green after appropriate laser excitation. 6: Flow cytometry is used to count the red and green beads.

BEAMING ASSAYS



Specific point mutations in targetable genes of interest such as PIK3CA can be detected in ctDNA using BEAMing technology. ctDNA molecules are loaded onto magnetic beads coated with specific PCR primers for the gene of interest. PCR is done on the beads in an oil and water emulsion (emulsion PCR) to amplify the DNA. Fluorescent-tagged probes specific for either the wild-type sequence or for particular common point mutations are added and hybridize to the amplified DNA. **Magnetic flow cytometry of the beads is done to detect the fluorescent tag and quantify the number of beads containing mutated DNA**.