

Assessing *HER2* Amplification in Plasma cfDNA

Isaac Garcia-Murillas and Nicholas C Turner

Summary

Digital PCR (dPCR) is a highly accurate method to determine DNA concentration. In dPCR DNA is portioned into many discrete single entities and these are analysed individually for the presence or absence of a target molecule of interest. Here we describe how digital PCR can be employed to determine the presence of oncogenic amplification through non-invasive analysis of circulating free DNA (cfDNA), and exemplify this approach by developing a plasma circulating free DNA dPCR assay for *HER2* copy number.

Key Words: Breast Cancer, *HER2*, circulating free DNA, plasma, digital PCR

1. Introduction

Genomic amplifications are important therapeutic targets. In routine clinical practice, the presence of amplifications is determined by analysis of a tumor biopsy at initial diagnosis. To optimally deliver targeted therapy, repeated sampling of a tumor is required to determine whether the genetic profile of a cancer has changed following prior therapy. This would require repeated biopsies of recurrent and metastatic cancers, yet this approach has limitations. Biopsy has associated risks, may be technically challenging depending on the site(s) of relapsed disease, and is often costly. A biopsy usually samples only a single area of tumor and in heterogeneous tumors may underestimate the array of genetic aberrations

present (1). Ideally, to overcome these limitations, and to allow repeated sampling, the presence of amplification could be diagnosed non-invasively.

DNA arising from tumor cells is found in the plasma of patients with cancer and this represents a potential source of non-invasively analysing tumor DNA (2). High sensitivity assays of coding mutations on cfDNA have reported high concordance with cancer mutational status (3, 4) Assays of cfDNA is non-invasive, can be repeated at multiple occasions throughout the disease course, and potentially may assess the full heterogeneity of mutations present. Analysis of cfDNA requires an assay of high sensitivity as DNA is frequently present at only low concentration in plasma and tumor cell derived DNA may be only a small fraction of the total plasma DNA (2, 5) with the remainder being derived from somatic cells.

Digital PCR has the potential to highly accurately quantify the concentration of nucleic acids in a sample, to a much greater degree than traditional quantitative PCR, through counting individual DNA molecules (6). To examine the potential of digital PCR for amplification detection we developed an assay to test for *HER* amplification on cfDNA extracted from plasma (7). This assay can also be used to test for *HER2* amplification on formalin fixed paraffin embedded (FFPE) tissue and Fresh Frozen Tissue (8). Chromosomal aneuploidy complicates copy number assessment in plasma, and the appropriate selection of a control probe. Here, we employ a copy number reference gene on the same chromosomal arm as *HER2* that is very rarely co-amplified with *HER2*, but is of stable neutral relative copy number in non-amplified cancers.

2. Materials

Ensure that the work is divided into clearly separated plasma processing, pre-dPCR and post-dPCR areas and that no reagents or equipment is shared between spaces. This includes lab coats, reagents, plastic ware, lab books, note books, computers and any other material.

2.1 Blood Collection, Plasma separation and Storage

Blood should be collected into Vacutainer Plastic K2 EDTA (BD Biosciences) blood collection tubes as per local guidelines, separated using a horizontal rotor swing out head centrifuge ideally within 2 hours of processing, aliquoted into cryogenic vials and stored at -80°C . Avoid repeated thaw-freeze cycles of plasma aliquots.

2.2 circulating free DNA (cfDNA) extraction from plasma

Circulating free DNA (cfDNA) should be extracted from plasma using Qiagen's QIAamp circulating nucleic acid kit on a dedicated plasma processing area using a QIAVac 24 Plus vacuum as instructed by the manufacturer. cfDNA should be eluted onto nuclease-free non-stick 1.5-ml microcentrifuge tubes. Extracted cfDNA can be stored short term at -20°C . For long term storage consider storing cfDNA at -80°C . Avoid repeated thaw-freeze cycles of extracted cfDNA and consider aliquoting it into smaller volumes.

2.3 Quantification and Quality control of cfDNA extracted from plasma

cfDNA should be quantified using a fluorescence-based method like Quant-iT PicoGreen dsDNA assay (Thermo Fisher Scientific) or Qubit dsDNA assays (Thermo Fisher Scientific) on 1 μl of eluate as per manufacturer instructions. Do not quantify cfDNA using a spectrophotometric method (260/280) as it will not be accurate enough. Alternatively cfDNA can be quantified using Agilent's Bioanalyzer/Tapestation (or similar) when checking its quality. cfDNA quality should be checked using Agilent's Bioanalyzer/Tapestation (or similar). These procedures should be carried out on dedicated pre-PCR area and equipment.

2.4 HER2 droplet digital PCR

Droplet digital PCR (ddPCR) should be performed on Bio-Rad's QX100/QX200, as described in the methods section, on a pre-PCR area. Reactions should be performed using 2x ddPCR Supermix for probes (Bio-Rad). Primers and probes (Table 1) should be used at a final concentration of 900nM and 250nM respectively on a final volume of 20 µl (Table 2).

3. Methods

3.1 Venous Blood collection, Isolation of Plasma from blood and Storage

1. Collect blood via vein puncture directly into an EDTA blood tube¹.
2. Centrifuge at 1600g for 20 min at room temperature on a horizontal rotor, swing out head centrifuge².
3. Remove plasma carefully without disturbing the buffy coat layer (white blood cells and platelets) and transfer into a clean conical centrifuge tube (Figure 1)³.
4. Centrifuge plasma at 16,000g for 10 minutes to pellet cellular debris.
5. Pipette off all the plasma into labelled Cryogenic vials⁴.
6. Place the cryogenic vials containing plasma upright into a freezer storage box in a -80 degree C freezer⁵.

3.2 Circulating free DNA extraction from Plasma using a QIAvac

Extract cfDNA from 2 ml of plasma using QIAamp circulating nucleic acid kit from Qiagen. Ensure that the plasma has been thaw on ice before starting procedure.

1. Pipet 200 µl QIAGEN Proteinase K into a 50 ml centrifuge tube (not provided)
2. Add 2 ml of plasma to the 50 ml tube.

3. Add 1.6 ml Buffer ACL (containing 1.0 µg carrier RNA). Close the cap and mix by pulse-vortexing for 30 sec¹.
4. Incubate at 60°C for 30 min.
5. Place the tube back on the lab bench and unscrew the cap.
6. Add 3.6 ml Buffer ACB to the lysate in the tube. Close the cap and mix thoroughly by pulse-vortexing for 15–30 sec.
7. Incubate the lysate–Buffer ACB mixture in the tube for 5 min on ice.
8. Insert the QIAamp Mini column into the VacConnector on the QIAvac 24 Plus. Insert a 20 ml tube extender into the open QIAamp Mini column².
9. Carefully apply the lysate–Buffer ACB mixture from step 7 into the tube extender of the QIAamp Mini column. Switch on the vacuum pump. When all lysates have been drawn through the columns completely, switch off the vacuum pump and release the pressure. Carefully remove and discard the tube extender³.
10. Apply 600 µl Buffer ACW1 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of Buffer ACW1 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure.
11. Apply 750 µl Buffer ACW2 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of Buffer ACW2 has been drawn through the QIAamp Mini column, switch off the vacuum pump and release the pressure.
12. Apply 750 µl of ethanol (96–100%) to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of ethanol has been drawn through the spin column, switch off the vacuum pump and release the pressure.

13. Close the lid of the QIAamp Mini column. Remove it from the vacuum manifold, and discard the VacConnector. Place the QIAamp Mini column in a clean 2 ml collection tube, and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

14. Place the QIAamp Mini Column into a new 2 ml collection tube. Open the lid, and incubate the assembly at 56°C for 10 min to dry the membrane completely.

15. Place the QIAamp Mini column in a clean 1.5 ml elution tube (provided) and discard the 2 ml collection tube from step 14. Carefully apply 25 µl of Buffer AVE to the centre of the QIAamp Mini membrane. Close the lid and incubate at room temperature for 3 min⁴.

16. Centrifuge in a microcentrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min to elute the nucleic acids.

17. Repeat steps 15 and 16 again eluting into the same tube. Final volume ~ 50 µl, labelled as eluate #1.

18. Repeat steps 15 and 16 (2 x 25 µl) eluting into a fresh 1.5 ml elution tube. Label as eluate #2

19. Store extracted DNA at -20°C

3.3 Quantification of extracted cfDNA

1. Take the Qubit dsDNA Assay Kit off the freezer and equilibrate at room temperature for 30 minutes

2. Make the Qubit working solution by diluting the Qubit dsDNA BR reagent 1:200 in Qubit dsDNA BR buffer. Do not mix the working solution in a glass container. Label two tubes for the standards

3. Load 190 µl of Qubit working solution into each of the labelled standard tubes

4. Add 10 μl of each Qubit standard to the appropriate tube and mix by vortexing 2–3 seconds. The final volume in each tube should be 200 μl
5. Load 199 μl of Qubit working solution into individual assay tubes
6. Add 1 μl of plasma eluate to the tubes containing Qubit working solution. The final volume in each tube should be 200 μl
7. Incubate tubes at room temperature for 2 minutes.
8. Read the standards and plasma samples on a Qubit. 2.0 Fluorometer as instructed by the apparatus
9. Calculate the concentration of the plasma samples taking into account the dilution step on (6)

3.4. Checking cfDNA quality using Agilent's Bioanalyzer

Run 1 μl of eluate on a High Sensitivity DNA chip as per manufacturer instructions. Assess the quality of the extracted cfDNA based on the presence of a peak at around 150-180 bp and the absence of a high molecular weight peaks of more than 1000 bp (Figure 2, blue trace). Genomic DNA (gDNA) contaminated cfDNA will exhibit high molecular weight peaks of more than 1000 bp (Figure 2, red trace).

3.5. droplet digital PCR (ddPCR) for *HER2* amplification

1. For each sample prepare a reaction mix as shown in Table 3 minus the DNA into nuclease-free non-stick 1.5-ml microcentrifuge tubes
2. Aliquot reaction mix into 0.2 ml PCR tubes, add DNA to be analysed to tube vortex and spin down

3. Assemble droplet generator cartridge into cartridge holder
4. Aliquot 20 µl of PCR reaction mix onto central row reservoirs of droplet generator cartridge¹.
5. Aliquot 70 µl of droplet generator oil (using same tip for all wells) onto bottom row reservoirs of droplet generator cartridge².
6. Cover cartridge with gasket making sure ends fit over plastic overhangs. Place into droplet generator³. When done, remove cartridge from generator, remove gasket and dispose and collect 40 µl of droplets from top row to dispense into a PCR plate (Fisher Scientific). Collect droplets using an 8 channel multichannel air displacement pipette by tipping pipette at 20-25⁰ angle and slowly (up to 15 sec) collect the droplets⁴. Dispense droplets on the PCR plate by tipping the pipette 20-25⁰ angle and touching the wall of the plate well, slowly (up to 15sec) release droplets by letting them slide along the wall of the well. Dispose of cartridge⁵.
7. Repeat as many times as needed, covering wells between droplet generations
8. Seal Plate and run samples on Thermal cycler as shown in Table 4
9. When PCR cycling is complete, read droplets on a QX100/QX200 Droplet Reader as per manufacturer instructions. Read the plate using Absolute Quantification
10. Samples that do not have at least 400 WT droplets should be bulk up by running more sample until at least 400 WT droplets are analysed

3.6. droplet digital PCR (ddPCR) Analysis

Data produced on the QX100/QX200 reader may be analysed using Bio-Rad QuantaSoft software. Gate the 4 distinct populations produced by the assay as shown in Figure 3 and use the in-built analysis tool to calculate the ratio of *HER2* to *EFTUD2* reference assay. Values

above ratio of 1.25 should be considered amplified, while ratios below that should be considered non-amplified.

For optimal results in samples, and in particular in samples with a copy number ratio near to the cut-off, the sequential probability ratio test (SPRT) (Figure 4A) should be used to determine whether the copy number is elevated, not-elevated, or indeterminate. The SPRT uses the maximum likelihood method to determine whether the observed ratio is above or below the threshold within acceptable bounds of error, or whether further digital PCR runs are required to determine the copy number ratio.

For data analysis using the SPRT test with a threshold likelihood ratio of 8, as previously reported with modifications (9, 10) should be used. Only informative droplets should be analysed, i.e., those droplets positive for either *HER2* alone or *EFTUD2* alone. The proportion of informative droplets positive for the *HER2* is calculated $P_{HER2} = N_{HER2}/N$ where N_{HER2} is the number of droplets positive for *HER2* alone, and N_{EFTUD2} is the number of droplets positive for *EFTUD2* alone, and $N=(N_{HER2}+N_{EFTUD2})$ the number of informative droplets.

The boundaries of the SPRT test are calculated as follows

$$\text{Upper} = ((\ln 8)/N - \ln d) / \ln g$$

$$\text{Lower} = ((\ln 1/8)/N - \ln d) / \ln g$$

where

$$d = (1-q_1)/(1-q_0)$$

$$g = q_1(1-q_0)/q_0(1-q_1)$$

and

q_1 = the proportion of informative droplets positive for HER2 if alternative hypothesis is accepted (plasma sample is from a patient with HER2 amplification).

q_0 = the proportion of informative droplets positive for HER2 if null hypothesis is accepted (plasma sample is from a patient without HER2 amplification).

q_1 is calculated from *HER2/EFTUD2* copy number ratio ($T_{AMP}=1.3$) for assigning a sample as *HER2* positive sample, and varies according to M_{EFTUD2} .

$$q_1 = (X_{AMP} - X_{AMP}n_{EFTUD2}/n) / (X_{AMP} + n_{EFTUD2}/n - 2X_{AMP}n_{EFTUD2}/n)$$

where $X_{AMP}=1-\exp(-T_{AMP}M_{EFTUD2})$ the expected proportion of informative droplets positive for *HER2* at the threshold ratio T_{AMP} .

q_0 is similarly calculated from *HER2/EFTUD2* copy number ratio ($T_{NONAMP}=1.2$) for assigning a sample as *HER2* positive negative, and also varies according to M_{EFTUD2} .

$$q_0 = (X_{NONAMP} - X_{NONAMP}n_{EFTUD2}/n) / (X_{NONAMP} + n_{EFTUD2}/n - 2X_{NONAMP}n_{EFTUD2}/n), \text{ where } X_{NONAMP}=1-\exp(-T_{NONAMP}M_{EFTUD2})$$

where $X_{NONAMP}=1-\exp(-T_{NONAMP}M_{EFTUD2})$

Using these equations for any given N, the upper and lower boundaries of the SPRT curve are calculated. If P_{HER2} is greater than the upper boundary then the test result is *HER2* positive. If P_{HER2} is less than the lower boundary then the test result is *HER2* negative. If P_{HER2} lies between the two boundaries, then further round(s) of digital PCR are required until a sample is above, or below, the boundaries.

The SPRT uses a likelihood ratio of 8, which corresponds roughly to two sided 95% confidence for differentiating between ratios of 1.30 and 1.20 (9). With these parameters a sample with an actual HER2:EFTUD2 ratio of, for example, 1.15 would have a very high probability (>99.9%) of being correctly called as negative, and likewise a sample with an actual ratio of 1.35 would have a very high probability of being correctly called as positive.

Figure 4B illustrates the use of the STR test on a cohort of 58 metastatic breast cancer patients (11 with *HER2*-amplified cancers and 47 with *HER2*-nonamplified cancers. Red triangles indicate patients with *HER2*-amplified tumors and black triangle *HER2*-nonamplified tumors. The displayed SPRT decision boundaries are for illustrative purposes only, as the exact level varies according to the *EFTUD2* control probe concentration (M_{EFTUD2}), with the displayed boundaries calculated with $M_{EFTUD2} = 0.025$. Cases with a number of informative droplets >5,000 are not displayed (7)

4. Notes

4.1 Venous Blood collection, Isolation of Plasma from blood and Storage

1. When collecting blood, haemolysis must be avoided.
2. EDTA BCTs should be stored in an upright position at room temperature and centrifuge within 2 hours of sample collection.
3. Invert the EDTA tube gently 8 to 10 times immediately prior to centrifugation.
4. Use a piston-driven air displacement pipette (avoid using a Pasteur pipette) to remove the plasma.
5. When freezing plasma do not use polystyrene boxes.

4.2 Circulating free DNA extraction from Plasma using a QIAvac

1. Make sure that a visible vortex forms in the tube. In order to ensure efficient lysis, it is essential that the sample and Buffer ACL are mixed thoroughly to yield a homogeneous solution. Do not interrupt the procedure at this time. Proceed immediately to the lysis incubation step.
2. Make sure that the tube extender is firmly inserted into the QIAamp Mini column in order to avoid leakage of sample. Keep the collection tube for the dry spin in step 13.
3. Note that large sample lysate volumes may need up to 10 min to pass through the QIAamp Mini membrane by vacuum force. To avoid cross-contamination, be careful not to move the tube extenders over neighbouring QIAamp Mini Columns.
4. Ensure that the elution buffer AVE has been warmed to 42°C before use. Elution volume is flexible and can be adapted according to the requirements of downstream applications. The recovered eluate volume will be up to 5 µl less than the elution volume applied to the QIAamp Mini column.

4.3. droplet digital PCR (ddPCR) for *HER2* amplification

1. Carefully load the cartridge and do not introduce bubbles onto the bottom of the reservoirs. Always fill all wells on a generator cartridge. If less than 8 samples then fill empty wells with 20 µl of 2 x Buffer Control kit.
2. Droplet generator oil contains EtOH so do not leave tube open for very long time to avoid evaporation. Always add the oil after having added the sample to the central row of the reservoir to avoid oil filling up the micro-channels connecting the reservoirs with oil.
3. When loading the cartridge onto the droplet generator, be careful as to not spill oil (hold by central part). Droplets will be generated in around 2 minutes.

4. Collecting droplets slowly will prevent droplets from breaking up and sticking together.
5. Once droplets are generated and deposited onto the PCR plate cover filled wells with tape to prevent evaporation.

5. Acknowledgements

Dr Nicholas Turner is a CRUK Clinician Scientist. We acknowledge NHS funding to the NIHR Biomedical Research Centre. Figure 4 is adapted from H. Gevensleben *et al.*, Noninvasive detection of HER2 amplification with plasma DNA digital PCR. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**, 3276-3284 (2013) with permission from The American Association of Cancer Research (AACR)

6. References

1. L. Ding *et al.*, Genome remodelling in a basal-like breast cancer metastasis and xenograft. *Nature* **464**, 999-1005 (2010).
2. P. J. Johnson, Y. M. Lo, Plasma nucleic acids in the diagnosis and management of malignant disease. *Clin Chem* **48**, 1186-1193 (2002).
3. M. Li, F. Diehl, D. Dressman, B. Vogelstein, K. W. Kinzler, BEAMing up for detection and quantification of rare sequence variants. *Nat Methods* **3**, 95-97 (2006).
4. R. E. Board *et al.*, Detection of PIK3CA mutations in circulating free DNA in patients with breast cancer. *Breast Cancer Res Treat* **120**, 461-467 (2010).
5. F. Diehl *et al.*, Circulating mutant DNA to assess tumor dynamics. *Nature medicine* **14**, 985-990 (2008).
6. B. Vogelstein, K. W. Kinzler, Digital PCR. *Proc Natl Acad Sci U S A* **96**, 9236-9241 (1999).
7. H. Gevensleben *et al.*, Noninvasive detection of HER2 amplification with plasma DNA digital PCR. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**, 3276-3284 (2013).
8. I. Garcia-Murillas, M. Lambros, N. C. Turner, Determination of HER2 amplification status on tumour DNA by digital PCR. *PloS one* **8**, e83409 (2013).
9. G. G. Wei Zhou, Steven N. Goodman, Katharine E. Romans, Kenneth W. Kinzler, Bert Vogelstein, Michael A. Choti, Elizabeth A. Montgomery, Counting alleles reveals a connection between chromosome 18q loss and vascular invasion. *Nature Biotechnology* **19**, (2001).
10. Y. M. Lo *et al.*, Digital PCR for the molecular detection of fetal chromosomal aneuploidy. *Proc Natl Acad Sci U S A* **104**, 13116-13121 (2007).

Figure Legends

Figure 1. When separating plasma for extraction of cfDNA, only the top layer should be taken. The buffy coat layer containing White Blood Cells should be left undisturbed

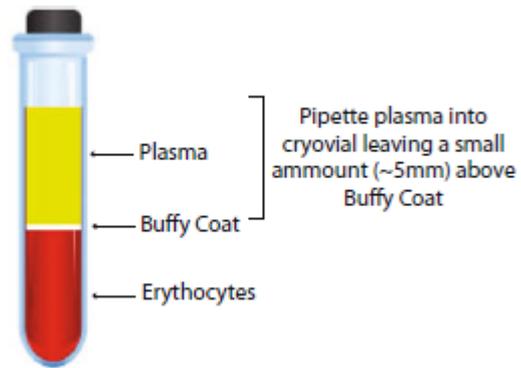
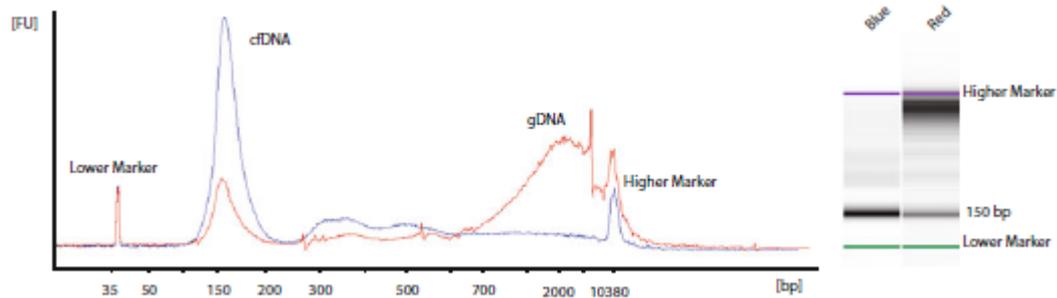


Figure 2. cfDNA extracted from plasma free from contaminant gDNA should exhibit a peak at around 150-300bp when analysed on the Bioanalyzer/Tapestation (blue trace), while cfDNA contaminated with gDNA will show an extra peak/s above 1000bp (red trace)



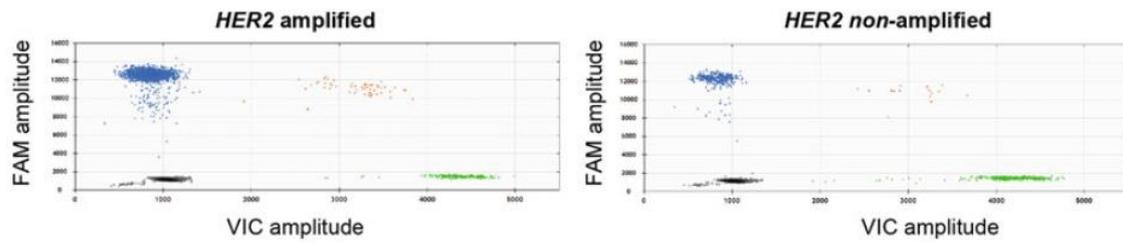


Figure 3. Representative droplet digital plots from a sample with high level amplification (A), and a non-amplified sample (B). The four quadrants represent top left – droplets with *HER2* DNA only, top right – droplets with both *HER2* and *EFTUD2* DNA, bottom right – droplets with *EFTUD2* DNA only, and bottom left – droplets with no DNA.

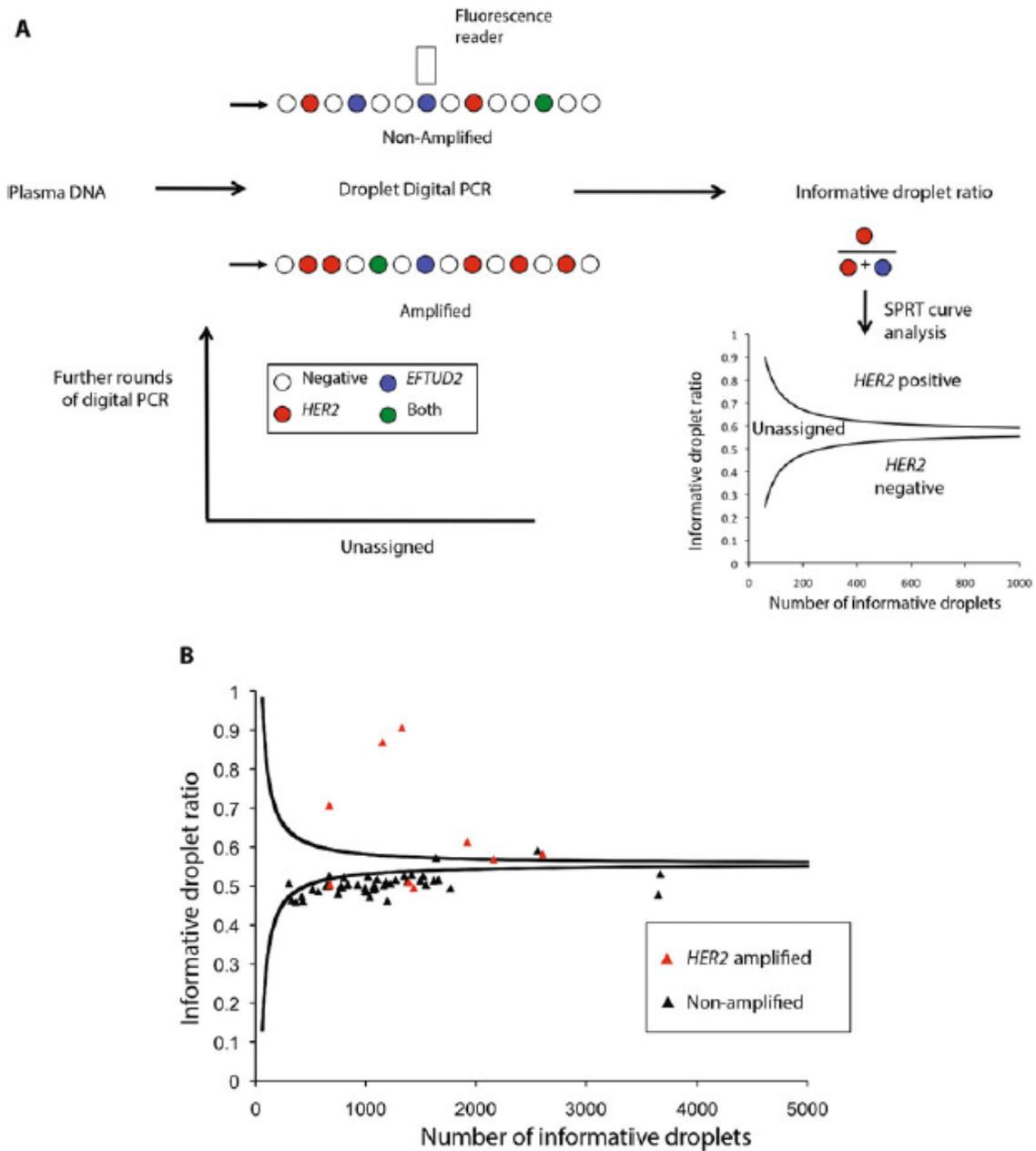


Figure 4. A. Droplet Digital PCR with a FAM labeled *HER2* probe and VIC labeled *EFTUD2* probe. DNA is partitioned into droplets, and after PCR, droplets are assessed by a fluorescent reader. The concentration of DNA in each sample can be quantified from the number of wells positive using the Poisson distribution. This is further analyzed with the SPRT using informative droplets, those droplets positive for *HER2* or *EFTUD2* alone, and not those positive for both or neither. The SPRT assesses whether the proportion of informative wells positive for *HER2*, informative wells ratio, is elevated as data accumulates.

SPRT defines 2 boundaries, with a ratio above the upper boundary being considered *HER2*-positive and below the lower boundary considered *HER2*-negative. A ratio between the 2 boundaries is considered as unassigned, and the sample is subjected to further rounds of digital PCR until the result is above or below the boundaries. B. Analysis of digital PCR with Sequential Probability Ratio Test (SPRT) using informative droplets on a cohort of 58 metastatic breast cancer patients, 11 patients with *HER2*-amplified cancers and 47 patients with *HER2*-nonamplified cancers. Red triangles indicate patients with *HER2*-amplified tumors and black triangle *HER2*-nonamplified tumors. Adapted from H. Gevensleben *et al.*, Noninvasive detection of *HER2* amplification with plasma DNA digital PCR. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**, 3276-3284 (2013) with permission from The American Association of Cancer Research (AACR)

TABLES

	Sequence 5'-3'
<i>HER2 assay</i>	
Forward primer sequence	ACAACCAAGTGAGGCAGGTC
Reverse primer sequence	GTATTGTT CAGCGGGTCTCC
Probe sequence	6-FAM/CCCAGCTCTTTGAGGACAAC/MGBNFQ
<i>EFTUD2 assay</i>	
Forward primer sequence	GGTCTTGCCAGACACCAAAG
Reverse primer sequence	TGAGAGGACACACGCAAAAC
Probe sequence	VIC/GGACATCCTTTGGCTTTTGA/MGBNFQ

Table 1. Sequences for the primers and probes used for the *HER2* amplification assay.

20× <i>HER2</i> cocktail	20× <i>EFTUD2</i> cocktail
90 µl 100 µM primer forward	90 µl 100 µM primer forward
90 µl 100 µM primer reverse	90 µl 100 µM primer reverse
25 µl 100 µM <i>HER2</i> FAM/MGBNFQ probe	25 µl 100 µM <i>EFTUD2</i> VIC/MGBNFQ probe
295 µl nuclease-free H ₂ O	295 µl nuclease-free H ₂ O

Table 2. Final concentration of primers and probe son the *HER2* amplifictaion assay.

	×1 (µl)
DNA	x
20× <i>HER2</i> cocktail	1
20× <i>EFTUD2</i> cocktail	1
Supermix for probes	10
Nuclease-free H ₂ O	to 20

Table 3. PCR master mix components and volume used for *HER2* amplification testinmg on cfDNA.

Step	Temperature (°C)	Time (min)	# Cycles	Ramp temp increment
Heated lid	105			
Denaturing	95	10:00	1	2.5°C/s
Denaturing	95	00:15	40	2.5°C/s
Anneling/ extension	60	01:00	1	2.5°C/s
Completion	98	10:00	1	2.5°C/s
Hold	10	Indefinite	1	

Table 4. PCR conditions for *HER2* amplification testing on cfDNA extractetd from plasma.