## **Supplementary Material 1:** QuantStudio 3D Digital PCR System catalog number, PIK3CA posi-tive example by dPCR, calibration process for dPCR and cycling conditions of PI3KCA exon 9 and 20 for conventional PCR

**Supplementary Table 1.** Catalog number ofQuantStudio TM 3D Digital PCR Products

|  |  |
| --- | --- |
| **Product** | **Catalog Identification Code** |
| Assay E545K | Hs000000086\_rm |
| Assay H1047R | Hs000000088\_rm |
| AssayH1407L | Hs000000089\_rm |
| Master Mix v2 (5ml) | A26359 |
| QuantStudio TM 3D Digital PCR 20K Chip Kit v2 | A26316 |

**Supplementary Table 2.** Primers used for conventional PCR

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Gene** | **Exon** | **Size**  **(bp)** | **Forward Primer 5´-3´** | **Reverse Primer 5´-3´** |
| *PIK3CA* | 9 | 297 bp | CTGTGAATCCAGAGGGGAAA | CTCCATTTTAGCACTTACCTGTGACT |
|  | 20 | 245 bp | GATGACATTGCATACATTCG | CCTATGCAATCGGTCTTTGC |

bp base pairs

**Supplementary Table 3:** Cycling conditions PIK3CA primers exon 20

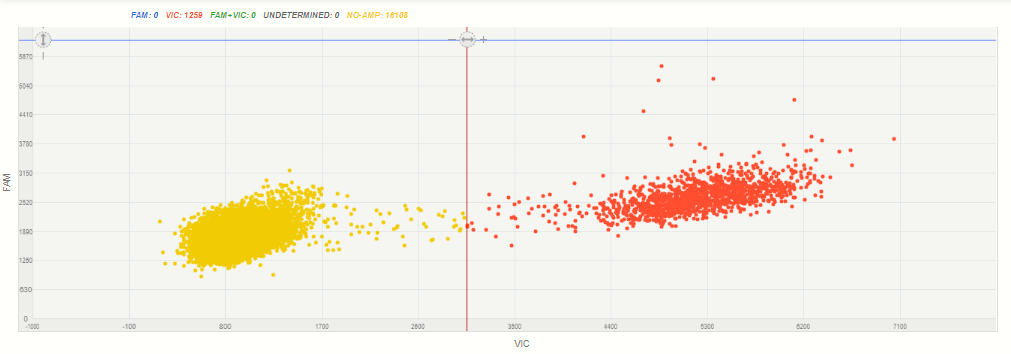
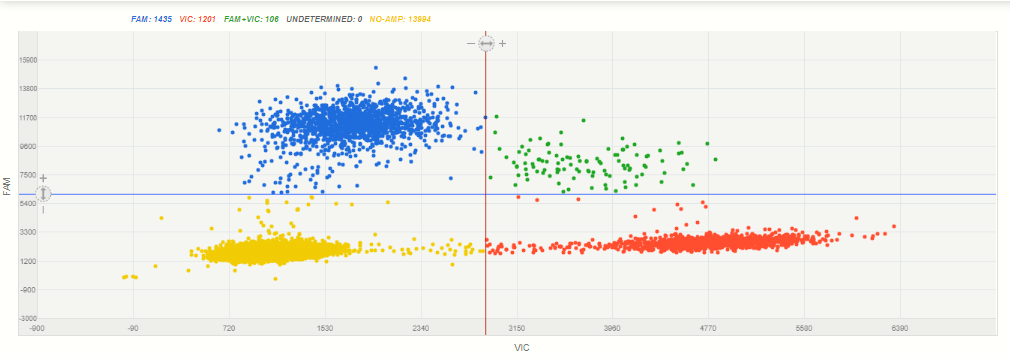
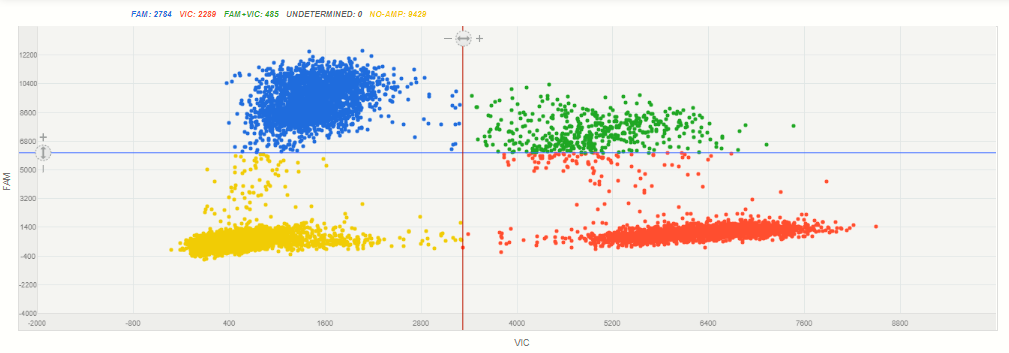
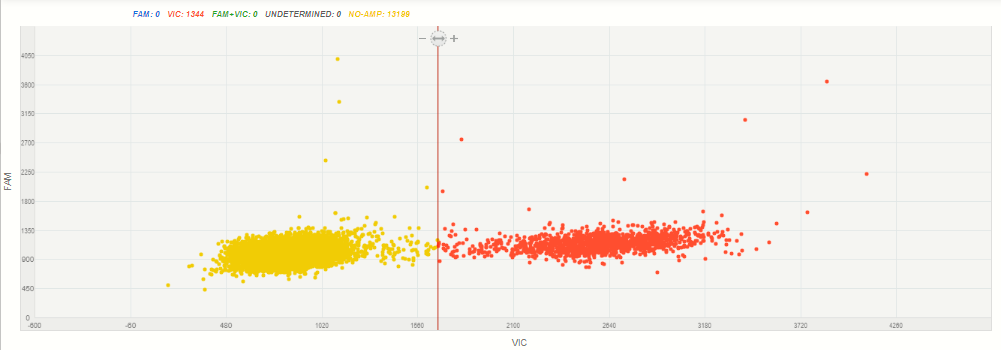
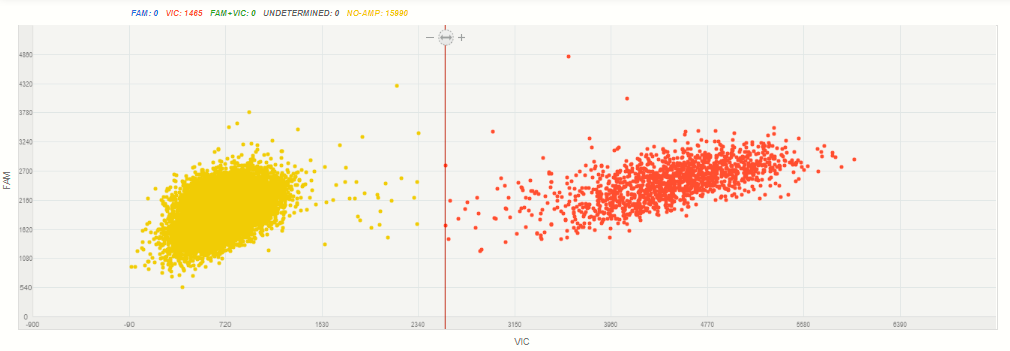
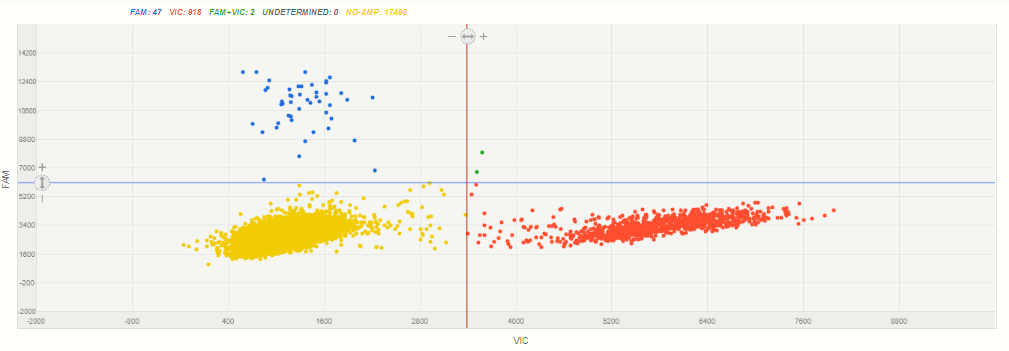
|  |  |  |  |
| --- | --- | --- | --- |
| **Phase** | **Temperature** | **Time** | **Cycles** |
| **Initial Denaturation** | 95°C | 5 minutes | 1 |
| **Denaturation** | 95°C | 15 seconds | 45 |
| **Annealing** | 55°C | 30 seconds |
| **Extension** | 72°C | 20 seconds |
| **Final Extension** | 72°C | 7 minutes | 1 |
| **Final stage** | 10°C | 5 minutes | 1 |

**Supplementary Table 4:** Cycling conditions PIK3CA primers exon 09

|  |  |  |  |
| --- | --- | --- | --- |
| **Phase** | **Temperature** | **Time** | **Cycles** |
| **Initial Denaturation** | 95°C | 1 minute | 1 |
| **Denaturation** | 95°C | 15 seconds | 40 |
| **Annealing** | 53°C | 15 seconds |
| **Extension** | 72°C | 30 seconds |
| **Final Extension** | 72°C | 7 minutes | 1 |
| **Final stage** | 4°C | 5 minutes | 1 |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Template** | **Mutation Essay** | **Mean copies per partition (**  **(Median)** | **Partition volume (nL)** | **Total partition number (median)** | **Total volume of partitions measure µL (median)** |
| Tumor DNA | H1047R | 0.039 | 0.755 | 17084 | 12.90 |
| E545K | 0.050 | 17065 | 12.88 |
| H1047L | 0.045 | 17080 | 12.90 |

**Supplementary Table 5:** Mean , partition volume, total partion number and total volume of part



A

B

E

D

C

F

**Figure S1.** Most representative positive cases by Digital PCR**.** Image A, C and E correspond to positive model cases from mutation H1047R, E545K and H1047L respectively. Image B, D and F correspond to negative cases from same mutations respectively. The blue dots (FAM) represent the mutant alleles, the red dots (VIC) are the wild type alleles, the yellow dots represent the wells without PCR reaction and the green dots are those with both wild and mutant alleles.

**Digital PCR Calibration Process**

The Total mutant allele frequency (TMAF) is the proportion of mutant alleles over the sum of wild and mutant alleles. We considered a patient to be positive for a mutation when she had at least one blue dot and a percentage of TMAF higher than 0.23%. This cutoff was established by a dilution assay, where we mixed a control positive case (per mutation) at different concentrations (1%, 0.1% and 0.01%) with DNA from a healthy control. Then Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated based on the standard deviation of the response and the slope (1). The standard error of the y-intercepts from the regression line was used as standard deviation for LOD and LOQ calculation.

**Results of the dilution and intra-rater assay**

In order to define the lowest mutant frequency to be detected by QuantStudio 3D digital PCR system, we performed a dilution assay. One highly positive case per mutation was selected as positive control and mixed at different concentrations with wild type DNA from peripheral blood from healthy controls. LOD for H1047R, E545K, H1047L and the three mutations together were: 0.04, 0.02, 0.11 and 0.08%, respectively. Meanwhile, LOQ for H1047R, E545K, H1047L and the three mutations together were: 0.11, 0.06, 0.34 and 0.23%, respectively. Further, to assess the reliability of the researcher performing dPCR, an intra-rater assay was carried out with 10 paired samples. The assay estimated an ICC of 0.998 (CI 95%: 0.992 – 0.999).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | Theorical Target/total (Y) | Target/Total (X) | CI Target/Total | Copies/microliter  (VIC) | CI Copies/microliter  (VIC) | Copies/microliter (FAM) | CI Copies/microliter (FAM) | Chips |
| d20R-1% | 1.00% | 0.82% | 0.602% -- 1.123% | 3692.6 | 3581.1 -- 3807.6 | 30.665 | 22.578 -- 41.649 | 1 |
| d20R-0.10% | 0.10% | 0.20% | 0.107% -- 0.367% | 3831.8 | 3719.3 -- 3947.7 | 7.62 | 4.1 -- 14.161 | 1 |
| d20R-0.01% | 0.01% | 0.12% | 5.23E-2% -- 0.253% | 4438.7 | 4306.5 -- 4574.9 | 5.138 | 2.308 -- 11.437 | 1 |

**Supplementary Tables 6:** *PIK3CA* H1047R mutations dilution assay by dPCR.

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Descripción generada automáticamente

|  |  |
| --- | --- |
| Linear Regression Output | |
| Observations | 3 |
| R square | 1.00 |
| Adjusted R square | 0.999 |
| Prob > F | 0.014 |
|  |  |
|  |  |
| LOD | 0.04 |
| lOQ | 0.11 |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | coefficients | standard error | t | p | 95% CI | |
| Variable (x) | 1.428 | 0.032 | 44.841 | 0.014 | 1.024 | 1.833 |
| Y intercept | -0.173 | 0.016 | -11.022 | 0.058 | -0.372 | 0.026 |

**Supplementary tables 7:** *PIK3CA* H1047L mutations dilution assay by dPCR.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | Theorical Target/total (Y) | Target/Total (X) | CI Target/Total | Copies/microliter (VIC) | CI Copies/microliter (VIC) | Copies/microliter (FAM) | CI Copies/microliter (FAM) | Chips |
| Dl-1 | 1% | 0.88% | 0.568% -- 1.371% | 1754.3 | 1680.7 -- 1831.2 | 15.641 | 10.09 -- 24.243 | 1 |
| dl-0.1 | 0.10% | 0.14% | 4.77E-2% -- 0.419% | 1727 | 1651.5 -- 1805.9 | 2.455 | 0.792 -- 7.612 | 1 |
| dl-0.01 | 0.01% | 0% | NA | 1631.3 | 1561.9 -- 1703.9 | 0 | NA | 1 |

|  |  |
| --- | --- |
| Linear Regression Output | |
| Observations | 3 |
| R square | 0.996 |
| Adjusted R square | 0.991 |
| P> F | 0.042 |
|  |  |
| LOD | 0.11 |
| lOQ | 0.34 |

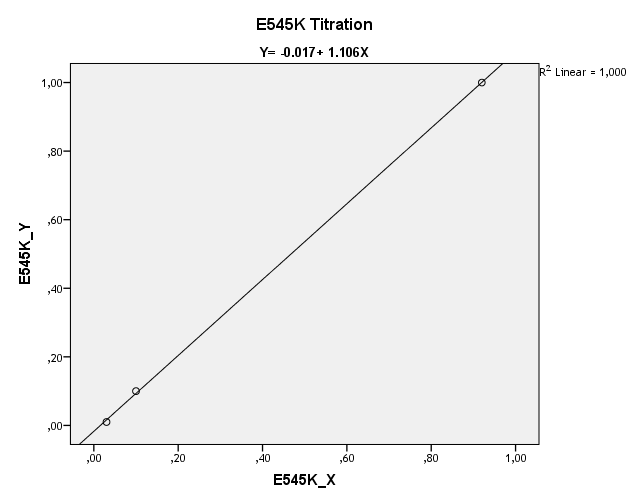
Captura de pantalla de un celular

Descripción generada automáticamente

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | coefficients | standard error | t | p | 95% CI | |
| Variable (x) | 1.155 | 0.077 | 15.064 | 0.042 | 0.181 | 2.130000 |
| Y intercept | -0.023 | 0.039 | -0.577 | 0.667 | -0.524 | 0.4790000 |

**Supplementary tables 8:** *PIK3CA* E545K mutations dilution assay by dPCR.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | Theorical Target/total (Y) | Target/Total (X) | CI Target/Total | Copies/microliter (VIC) | CI Copies/microliter (VIC) | Copies/microliter (FAM) | CI Copies/microliter (FAM) | Chips |
| d9-1 | 1.00% | 0.92% | 0.758% -- 1.118% | 9479.5 | 9268 -- 9695.9 | 88.261 | 73.395 -- 106.14 | 1 |
| d9-0.1 | 0.10% | 0.10% | 5.42E-2% -- 0.177% | 8792.3 | 8599.3 -- 8989.5 | 8.664 | 4.798 -- 15.646 | 1 |
| d9-0.01 | 0.01% | 0.03% | 1.23E-2% -- 8.37E-2% | 9634.7 | 9425.3 -- 9848.7 | 3.128 | 1.174 -- 8.335 | 1 |



|  |  |
| --- | --- |
| Linear Regression Output | |
| Observations | 3 |
| R square | 1.00 |
| Adjusted R square | 1.00 |
| Prob > F | 0.00891 |
|  |  |
| LOD | 0.02 |
| lOQ | 0.06 |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | coefficients | standard error | t | p | 95% CI | |
| Variable (x) | 1.106 | 0.013 | 86.891 | 0.007 | 0.944 | 1.268 |
| Y intercept | -0.017 | 0.007 | -2.521 | 0.240 | -0.104 | 0.069 |

**Supplementary tables 9:** *PIK3CA* mutations dilution assay by dPCR.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | Theorical Target/total (Y) | Target/Total (X) | CI Target/Total | Copies/microliter (VIC) | CI Copies/microliter (VIC) | Copies/microliter (FAM) | CI Copies/microliter (FAM) |
| d20R-1% | 1.00% | 0.82% | 0.602% -- 1.123% | 3692.6 | 3581.1 -- 3807.6 | 30.665 | 22.578 -- 41.649 |
| d20R-0.10% | 0.10% | 0.20% | 0.107% -- 0.367% | 3831.8 | 3719.3 -- 3947.7 | 7.62 | 4.1 -- 14.161 |
| d20R-0.01% | 0.01% | 0.12% | 5.23E-2% -- 0.253% | 4438.7 | 4306.5 -- 4574.9 | 5.138 | 2.308 -- 11.437 |
| d9-1 | 1.00% | 0.92% | 0.758% -- 1.118% | 9479.5 | 9268 -- 9695.9 | 88.261 | 73.395 -- 106.14 |
| d9-0.1 | 0.10% | 0.14% | 5.42E-2% -- 0.177% | 8792.3 | 8599.3 -- 8989.5 | 8.664 | 4.798 -- 15.646 |
| d9-0.01 | 0.01% | 0.03% | 1.23E-2% -- 8.37E-2% | 9634.7 | 9425.3 -- 9848.7 | 3.128 | 1.174 -- 8.335 |
| Dl-1 | 1.00% | 0.88% | 0.568% -- 1.371% | 1754.3 | 1680.7 -- 1831.2 | 15.641 | 10.09 -- 24.243 |
| dl-0.1 | 0.10% | 0.14% | 4.77E-2% -- 0.419% | 1727 | 1651.5 -- 1805.9 | 2.455 | 0.792 -- 7.612 |
| dl-0.01 | 0.01% | 0.00% | NA | 1631.3 | 1561.9 -- 1703.9 | 0 | NA |

|  |  |
| --- | --- |
| Linear Regression Output | |
| R square | 0.986 |
| Adjusted R square | 0.984 |
| P> F | 0 |
| observations | 9 |
|  |  |
| LOD | 0.08 |
| lOQ | 0.23 |

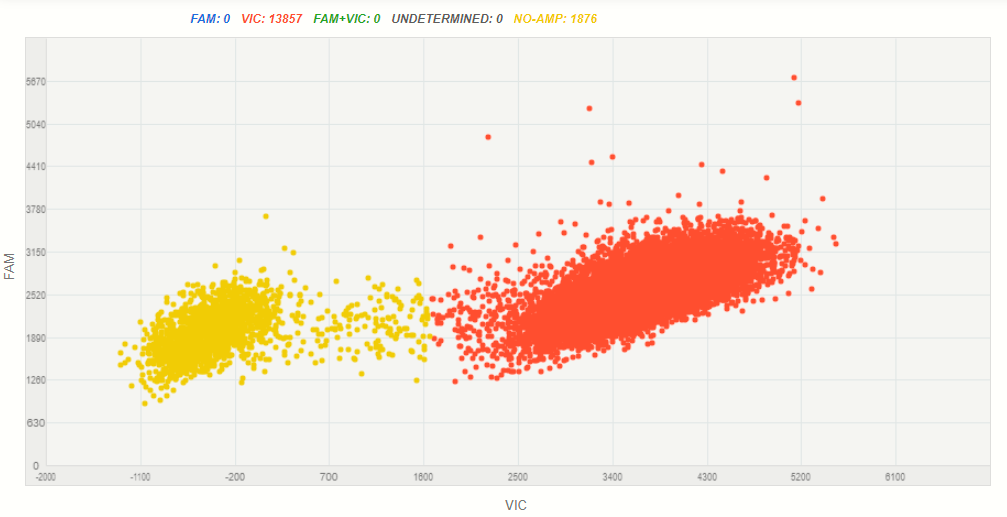
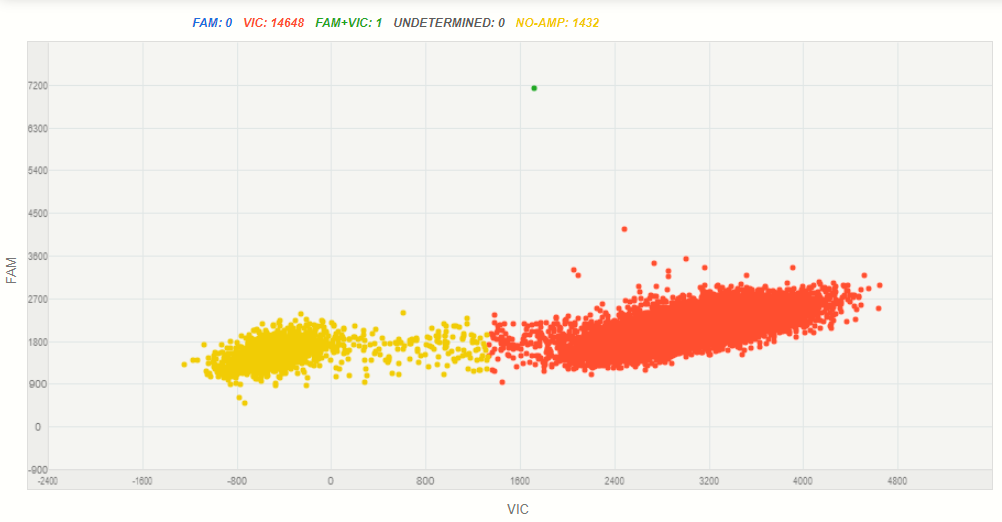
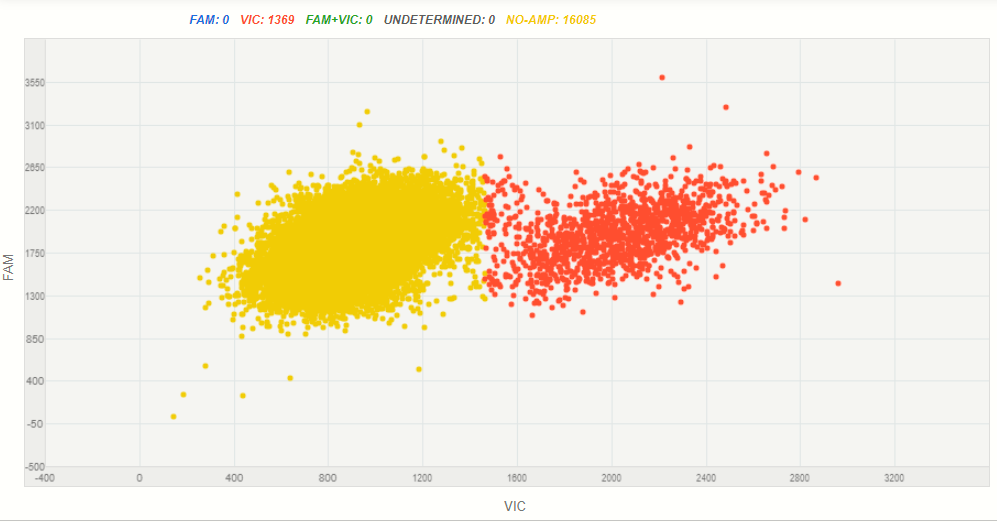
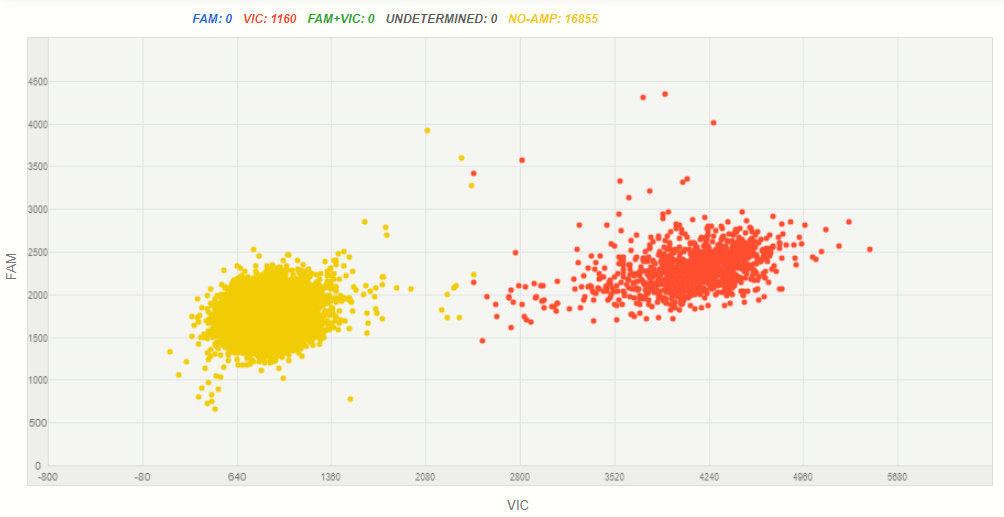
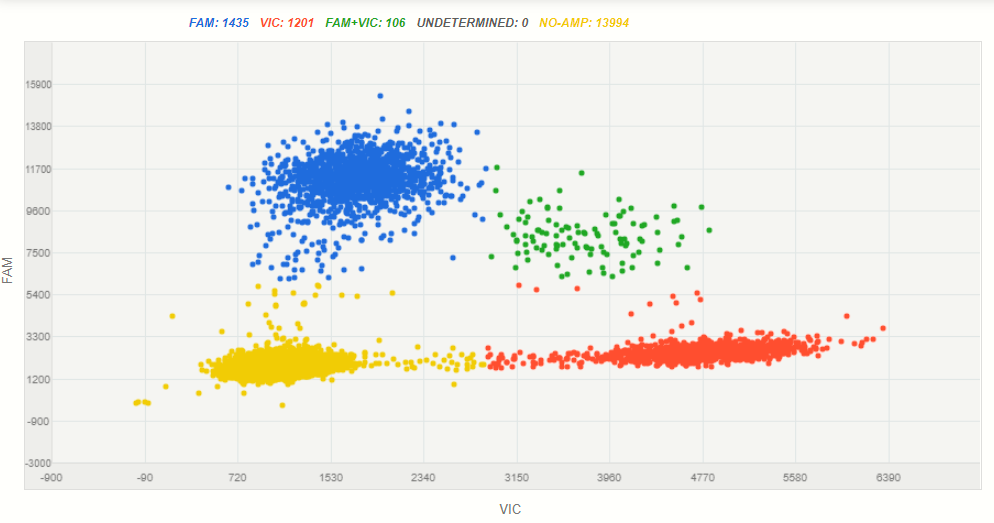
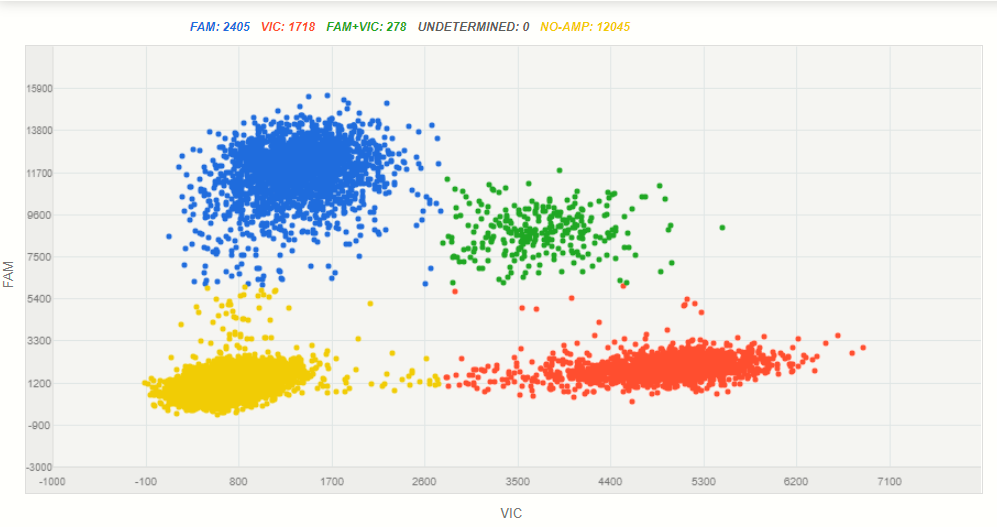
|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | coefficients | standard error | t | p | 95% CI | |
| Variable (x) | 1.208 | 0.055 | 22.014 | 0.000 | 1.079 | 1.338 |
| Y intercept | -0.066 | 0.028 | -2.346 | 0.051 | -0.133 | 0.001 |

Captura de pantalla de un celular

Descripción generada automáticamente

**Supplementary Table 10:** Inter-rater assay results from mutant allele frequency.

|  |  |  |
| --- | --- | --- |
| **Patient sample** | **First Assay** | **Second Assay** |
| I-02 (H1047R) | 57.940% | 54.300% |
| I-02 (H1047L) | 0.065% | 0.061% |
| I-24 (E545K) | 54.590% | 51.290% |
| I-28 (H1047L) | 0.300% | 0.000% |
| I-33 (H1047R) | 0.000% | 0.000% |
| I-35 (H1047L) | 0.853% | 0.807% |
| I-65 (H1047L) | 0.000% | 0.034% |
| I-68 (H1047R) | 0.020% | 0.000% |
| I-68 (H1047L) | 0.025% | 0.000% |
| I-70 (H1047L) | 5.131% | 3.402% |



**A-I**

**B-I**

**A-II**

**B-II**

**C-I**

**C-II**

**Figure S2:** Three examples from the dilution assays´s samples, figure A I and II correspond to sample I-02 H1047R, B I and II to I-33 H1047R, C I and II to I-68 H1047L.

**References:**

1. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Validation of Analytical Procedures: Text and Methodology Q2(R1). [Internet]. Available at: <http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html>

**Supplementary Material 2:** Digital MIQE checklist for authors, reviewers and editors

|  |  |  |  |
| --- | --- | --- | --- |
| **ITEM TO CHECK** | **IMPORTANCE** | **CHECKLIST** | |
| **EXPERIMENTAL DESIGN** |  |  |  |  |  |
| Definition of experimental and control groups | E | Materials & Methods | |
| Number within each group | E | Materials & Methods | |
| Assay carried out by core lab or investigator's lab? | D | Core Laboratory | |
| Power analysis | D | Not applicable | |
| **SAMPLE** |  |  |  |  |  |
| Description | E | Materials & Methods | |
| Volume/mass of sample processed | D | Not applicable | |
| Microdissection or macrodissection | E | Not applicable | |
| Processing procedure | E | Materials & Methods | |
| If frozen - how and how quickly? | E | Not applicable | |
| If fixed - with what, how quickly? | E | Not available | |
| Sample storage conditions and duration (especially for FFPE samples) | E | Materials & Methods | |
| **NUCLEIC ACID EXTRACTION** |  |  |  |  |  |
| Nucleic acid quantification | E | NanoDropTM Lite Spectrophotometer (ThermoFisher Scientific, Boston, MA, USA). | |
| DNA or RNA quantification | E | DNA | |
| Quality/Integrity, method/instrument, e.g. RNA integrity | E | Supplementary Material 1 | |
| Template structural information | E | Not applicable | |
| Template modification (digestion, sonication, preamplification etc) | E | Not applicable | |
| Template treatment | E | Not applicable | |
| Inhibition dilutions or spike | E | Not applicable | |
| DNA contamination assessment of RNA samples | E | Not applicable | |
| Details of DNase treatment where performed | E | Not applicable | |
| Manufacturer of reagents used and catalogue number | D | Materials & Methods/Additional Files | |
| Storage conditions (Nucleic acid): temperature, concentration, duration, buffer) | E | Materials & Methods | |
| **REVERSE TRANSCRIPTION (if necessary)** |  |  |  |  |  |
| cDNA priming method and concentration | E | Not applicable | |
| One or two-step protocol | E | Not applicable | |
| Amount of RNA used per reaction | E | Not applicable | |
| Detailed reaction components and conditions | E | Not applicable | |
| RT efficiency | D | Not applicable | |
| Estimated copies measured with and without addition of RT | D | Not applicable | |
| Manufacturer of reagents and catalogue numbers | D | Not applicable | |
| Reaction volume | D | Not applicable | |
| Storage conditions of cDNA | D | Not applicable | |
| **dPCR TARGET INFORMATION** |  | N/A |  |  |  |
| Sequence accession number | E | Included in article | |
| Location of amplicon | D | Not applicable | |
| Amplicon length | E | Not applicable | |
| In silico specificity screen (BLAST, etc) | E | Not applicable | |
| Pseudogenes, retropseudogenes or other homologs? | D | Not applicable | |
| Sequence alignment | D | Not applicable | |
| Secondary structure analysis of amplicon and GC content | D | Not applicable | |
| Location of each primer by exon or intron (if applicable) | E | Not applicable | |
| What splice variants are targeted? | E | Not applicable | |
| **dPCR OLIGONUCLEOTIDES** |  |  |  |  |  |
| Primer sequences | E | Not available | |
| RTPrimerDB Identification Number | D | Not available | |
| Probe sequences | D | Not available | |
| Location and identity of any modifications | E | Not available | |
| Manufacturer of oligonucleotides | D | Not available | |
| Purification method | D | Not available | |
| **dPCR PROTOCOL** |  | Supplementary Information 2, Table S1 |  |  |  |
| Complete reaction conditions | E | Materials & Methods | |
| Reaction volume and amount of cDNA/DNA | E | Materials & Methods | |
| Primer, (probe), Mg++ and dNTP concentrations | E | Materials & Methods | |
| Polymerase identity and concentration | E | Materials & Methods | |
| Buffer/kit identity and manufacturer | E | Materials & Methods/ Supplementary Material 1 | |
| Exact chemical constitution of the buffer | D | Not available | |
| Additives (SYBR Green I, DMSO, etc.) | E | Not applicable | |
| Plates/tubes catalogue number and manufacturer | D | Materials & Methods/ Supplementary Material 1 | |
| Complete thermocycling parameters | E | Materials & Methods | |
| Reaction setup (manual/robotic) | D | Manual | |
| Gravimetric or volumetric dilutions (manual/robotic) | D | Volumetric dilutions | |
| Total PCR volume prepared | D | Materials & Methods | |
| Partition number | E | Supplementary Material 1 | |
| Individual partition volume | E | Supplementary Material 1 | |
| Total volume of the partitions measured (effective reaction size) | E | Supplementary Material 1 | |
| Partition volume variance/SD | D | Not available | |
| Comprehensive details and appropriate use of controls | E | Materials & Methods | |
| Manufacturer of dPCR instrument | E | Materials & Methods | |
| **dPCR VALIDATION** |  |  |  |  |  |
| Optimisation data for the assay | D | Materials & Methods (dilution essay) | |
| Specificity (when measuring rare mutations, pathogen sequences etc) | E | Results: ROC curve (dPCR vs Sanger sequencing) | |
| Limit of detection of calibration control | D | Materials & Methods, Additional File | |
| If multiplexing, comparison with singleplex assays | E | Not applicable | |
| **DATA ANALYSIS** |  |  |  |  |  |
| Mean copies per partition (λ or equivalent) | E | Supplementary Material 1 | |
| dPCR analysis program (source, version) | E | Available upon request | |
| Outlier identification and disposition | E | QuantStudio 3D Analysis SuiteTM Cloud Software | |
| Results of NTCs | E | Available upon request | |
| Examples of positive(s) and negative experimental results as supplemental data | E | Supplementary Material 1 | |
| Where appropriate, justification of number and choice of reference genes | E | Included in article | |
| Where appropriate, description of normalization method | E | Not applicable | |
| Number and concordance of biological replicates | D | Not applicable | |
| Number and stage (RT or qPCR) of technical replicates | E | Not applicable | |
| Repeatability (intra-assay variation) | E | Materials & Methods, Results and Supplementary Material 1 | |
| Reproducibility (inter-assay/user/lab etc variation) | D | Not applicable | |
| Experimental variance or CI d | E | Materials & Methods | |
| Statistical methods for analysis | E | Materials & Methods | |
| Data submission using RDML (Real-time PCR Data Markup Language) | D | Not applicable | |
| E: Essential information; D: Desirable information. | | | |