Molecular diagnostics for monitoring insecticide resistance in the western flower thrips *Frankliniella occidentalis*

Short running title: Molecular diagnostics for resistance monitoring in *Frankliniella occidentalis*

Konstantinos Mavridis[1], Aris Ilias[1,2], Kyriaki Maria Papapostolou[1], Kyriaki Varikou[2], Kleita Michaelidou[3], Anastasia Tsagkarakou[2], John Vontas[1,4]*

[1]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, GR-70013 Heraklion, Greece

[2]Institute of Olive Tree, Subtropical Crops and Viticulture, Hellenic Agricultural Organization "DIMITRA", 70013 Heraklion, Greece.

[3]Laboratory of Translational Oncology, School of Medicine, University of Crete, Heraklion, Greece.

[4]Pesticide Science Laboratory, Department of Crop Science, Agricultural University of Athens, GR-11855 Athens, Greece.

*Correspondence to: konstantinos_mavridis@imbb.forth.gr

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ABSTRACT

BACKGROUND: Insecticide resistance has emerged in various western flower thrips (WFT) populations across the world, threatening the efficiency of chemical control applications. Elucidation of insecticide resistance mechanisms at the molecular level provides markers for the development of diagnostics, to monitor the trait and support evidence-based resistance management.

RESULTS: TaqMan and Droplet Digital PCR (ddPCR) diagnostics were developed and validated, against Sanger sequencing, in individual and pooled WFT samples, respectively, for the: G275E mutation (nicotinic acetylcholine receptor α6 gene- *nAChR α6*) associated with resistance to nAChR allosteric modulators - site I (spinosyns), L1014F, T929I, T929C and T292V mutations (voltage-gated sodium channel gene- *vgsc*) linked with pyrethroid resistance and I1017M (chitin synthase 1 gene- *chs1*) conferring resistance to growth inhibitors affecting CHS1 (benzoylureas). The detection limits of ddPCR assays for mutant allelic frequencies (MAFs) were in the range of 0.1%-0.2%. The assays were applied in nine WFT field populations from Crete, Greece. The G275E (MAF = 29.66%-100.0%), T929I and T929V (combined MAFs = 100%), L1014F (MAF = 11.01%-37.29%) and I1017M (MAF = 17.74%-51.07%) mutations, were present in all populations.

CONCLUSION: The molecular diagnostics panel that was developed in this study can facilitate the quick and sensitive pesticide resistance monitoring of WFT populations at the molecular level, to support evidence-based IRM strategies.

Keywords: ddPCR; TaqMan diagnostics; agricultural pests; resistance monitoring; target site resistance; thrips.

1. INTRODUCTION

The western flower thrips (WFT) *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is one of the most destructive sucking pests of economically important crops worldwide[1, 2]. Aside from causing direct feeding damage on leaves, flowers and fruits, they also transmit a set of plant viruses[3, 4]. *F. occidentalis* is a highly polyphagous species with at least 250 plant species from more than 65 families being listed as "hosts"[5]. Invasive populations of WFT spread globally as the international trade in horticultural products began to expand during the 1970s[6].

Although a wide number of biological control agents including parasitoids, predators, fungal pathogens and entomopathogenic nematodes have been developed and applied against WFT[7], chemical insecticide based control has been the most common control practice worldwide. In Greece, several insecticides belonging to different modes of action (MoA), such as sodium channel modulators (pyrethroids: deltamethrin, cypermehrin, acrinathrin), nicotinic acetylocholine receptor (nAChR) allosteric modulators (spinosyns: spinosad, spinetoram), glutamate-gated chloride channel (GluCl) allosteric modulators (avermectins: abamectin), inhibitors of acetyl-CoA carboxylase (spirotetramat), ryanodine receptor modulators (diamides: chlorantraniliprole) and azadiractin have been registered and used against WFT. The widespread use of insecticides has led to the development of WFT resistance against over 30 active ingredients[8].

Target site mutations have been primarily associated with insecticide resistance in *F. occidentalis*. Functionally validated target-side mutations in *F. occidentalis* has been described in the gene encoding the voltage-gated sodium channel (*vgsc*: T929I/C/V, L1014F), associated with pyrethroid resistance (MoA: Sodium channel modulators)[9], the chitin synthase (*chs1*: I1017M), associated with benzylorea resistance (MoA: Inhibitors of chitin biosynthesis affecting CHS1)[10] and the nicotinic acetylcholine receptor α6 subunit (*nAChR α6*: G275E) associated with resistance to spinosyns (MoA: nicotinic acetylcholine receptors allosteric modulators site I)[11].

Insecticide resistance monitoring is an important pre-requirement for the subsequent evidence-based insecticide resistance management (IRM), which can prolong and sustain the efficiency of chemical control. Bioassays have been employed for this purpose, but their usage has drawbacks such the inability to identify resistance mechanisms, the maintenance of live insect populations, and limited sensitivity in detecting resistance. Molecular diagnostics based on reliable markers can provide an added value to IRM strategies, when used alone or in conjunction with bioassays[12]. A prime example is target site mutations which, in most pest species are tested using conventional nucleic acid-based diagnostics like Sanger sequencing, allele specific PCR (PASA), PCR-RFLP and qPCR [12-15]. In thrips, in particular, a Kompetitive Allele-Specific PCR (KASP) [16] and a TaqMan qPCR assay[17] have been both described for determining the target site mutation G275E. Usually, such tests are used to genotype individual insects. However, as we have previously demonstrated for *Tetranychus urticae* (*T. urticae*) [18], *Bemisia tabaci* (*B. tabaci*)[19], but also for vector populations[20], using pooled samples can considerably boost the practicality and cost-effectiveness of the diagnostics, for insecticide resistance monitoring purposes. Droplet digital PCR (ddPCR) is the ideal technique for detecting target site mutations even in large pooled samples, because it accurately detects extremely small amounts of target DNA (mutated) in the presence of a substantial background of non-target DNA (wild type).

Here, we developed and validated TaqMan qPCR and ddPCR diagnostics for detecting insecticide resistance mutations in individual and bulk pooled samples of *F. occidentalis*. We then applied these diagnostics in field populations from Crete, Greece.

2. MATERIALS AND METHODS

2.1 *F. occidentalis* field populations and control samples

Nine field *F. occidentalis* populations were collected from greenhouse crops located in six distinct areas of Crete, Greece, during 2020. Fo1 was collected from cucumbers in Tympaki with an

application history of abamectin, sprirotetramat and flonicamid. Fo2 was collected from infested chrysanthemum in Rethimno; spinosad and cyromazine have been previously applied to control the infestation. Fo3 was collected from bean and pepper plants in Falasarna (pesticide application history: lufenuron and abamectin). Fo4 was collected from cucumbers in Sitia, with an application history of abamectin, lufenuron, spinosad, flonicamid and methiocarb. Fo5 was collected from cucumber in Ierapetra in Tympaki, with an application history of abamectin, deltamethrin, spinosad, acetamiprid, spinetoram, spirotetramat, and cyantraniliprole acibenzolar-S-methyl. Fo6 was collected from cucumbers in Tympaki with an application history of abamectin, spirotetramat, sufloxaflor, flonicamid and chlorpyrifos. Fo7 was collected from roses in Rethimno with an application history of sufloxaflor, cyantraniliprole, spinosad and abamectin. Fo8 was collected from chrysanthemum in Rethimno and abamectin, cyantraniliprole, spinosad and cyromazine were used to control the infestation. Fo9 was collected from roses in Gazi-Heraklion (insecticide application history not documented). The pesticides mentioned were used against thrips and other agricultural pests, such as mites and whiteflies. Application history refers to the last season prior to thrips collection. Collected WFT samples were stored in -80^{o}C until use for molecular analyses.

Individual *F. occidentalis* samples collected from the abovementioned populations (N =140 in total, Suppl. Table S1) were genotyped by Sanger Sequencing (described in 2.3) and used as control samples for the validation of TaqMan qPCR assays for individual samples, together with synthetic double stranded DNA control sequences (gBlocks™ gene fragments, IDT) of known wild type and mutant sequences.

2.2 Extraction of gDNA

Extraction of gDNA was performed from individuals and pools of 50 adult female WFT, using the CTAB method as previously described[21]. Concentrations of the extracted gDNAs were determined in pooled samples using the Qubit dsDNA BR assay on a Qubit Fluorometer 2.0 (Invitrogen, Carlsbad, CA). The mean ± SE double stranded DNA (dsDNA) concentration was 3.19 ± 0.39 ng/μL.

2.3 Genotyping of samples by Sanger sequencing

gDNA, derived from individuals and pools of approximately 50 adult females per population, was used as template for PCR amplification of target-side gene fragments encompassing insecticide resistance mutations G275E, I1017M, L1014F and T929I/C/V, associated with resistance to spinosyns, benzoylureas and pyrethroids respectively. Primers were designed based on available mRNA sequences (Accession numbers: XM_026422855.1 for *vgsc*, AB826436.1, KU557780.1, and HE965755.1 for *nAChR α6* and LC197806.1, XM_026430378.1, XM_026430377.1 for *chs1*) and genome[22] (Accession number: NW_020292540.1) on NCBI (Suppl. Table S2). PCR reactions (50µl) contained 1µL gDNA, 0.4 mM primers, 0.2 mM dNTPs, 5ulL of 10x buffer and 1U Kapa Taq DNA polymerase (Kapa Biosystems). The thermal conditions were: 95°C for 2 min, followed by 35 cycles of 95°C for 30sec, 54-60°C for 30 sec (depending on the primer set for the gene of interest in Suppl Table S2), 72°C for 30 sec, and final extension at 72°C for 2 min.

PCR fragments were purified using the Nucleospin Gel and PCR Clean-Up purification kit (Macherey-Nagel) according to the manufacturer's instructions and sequenced at GEWIZ (Leipzig, Germany) with the same primers that were used for PCR. The Sequencing data were analyzed using BioEdit 7.0.5.3 software. The mutant allelic frequency was calculated in pooled samples as previously described by Van Leeuwen et al[23] , i.e., by comparing the height of the wild type and mutant peaks in the sequencing chromatogram (proportional sequencing).

2.4 TaqMan qPCR assays

A set of four TaqMan assays were developed *de novo* and optimized for the purposes of this study (Suppl. Table S3). Their intended use is on individual WFT for genotyping target site mutations associated with resistance to nAChR allosteric modulators - site I (Spinosyns) (G275E), sodium channel modulators (Pyrethroids) (L1014F and T929I/C/V), and growth inhibitors affecting CHS1 (Benzoylureas) (I1017M). The qPCR was performed on a CFX Connect, Real-Time PCR System (Biorad), in 10 µL reactions consisting of 5.0 uL 2×TaqMan™ Universal PCR Master Mix (Applied

Biosystems), primers and probes as described in Suppl. Table S3, and were adjusted to the final volume with DEPC-treated water. The following thermal protocol was used: 95 ^{o}C for 10 min, and 50 cycles of 95 ^{o}C for 15 s, 60^{o}C for 1 min. Each run always included a non-template control, as well as wild type, mutant and heterozygote gBlock™ (IDT) control synthetic sequences. Genotyping was performed using the Allelic Discrimination module of the Bio-Rad CFX Maestro software (v4.0.2325.0418).

2.5 Droplet Digital PCR (ddPCR) assays

The QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA) was used for ddPCR reactions. These included ddPCR Supermix for probes at 1×, 5 U restriction enzyme EcoRI-HF® (New England Biolabs), 5 ng of dsDNA, and primers and probes specific to each assay (Suppl Table S3) in a total volume of 20 μL with droplets prepared and cycled, after optimisation, as previously described[18]. The thermal cycling protocol comprised: 95 ^{o}C for 10 min, and 50 cycles of 94 ^{o}C for 30 s, 54 or 58 ^{o}C for 1 min (Suppl. Table S4), and 98^{o}C for 10 min. Endpoint fluorescence was measured, raw data processed and percentage mutant allelic frequency (%MAF) was calculated as previously described[18].

2.6 Statistical Analyses

For the comparison and evaluation of ddPCR and proportional sequencing methods correlations were performed with the Passing & Bablok regression analysis that allows the estimation of any systematic, proportional or random differences between the two methods. The Cusum test was used to check for linearity of the regression model. The MedCalc (v12.5.0) statistical software was used for these analyses.

3. RESULTS

3.1 Development of TaqMan Diagnostics for individuals and validation versus Sanger sequencing.

TaqMan qPCR assays were developed for the detection of G275E, I1017M, L1014F, T929I, T929C and T929V mutations in individual WFT samples. In the G275E assay, the wild type probe was labelled with FAM and the mutant probe was labelled with HEX fluorescent dye. Contrariwise, in the I1017M, L1014F, T929I/C/V assays the wild type probes were labelled with HEX and the mutant probes were labelled with FAM fluorescent dye. In addition, the mutant probes for T929I, T929C and T929V mutations were all added in the same reaction together with the corresponding wild probe, allowing the detection of all mutations as one signal. In order to discriminate these mutations, the option of running three separate reactions can be followed.

After optimization in terms of primer and probe concentrations (Suppl. Table S3), using control synthetic sequences, TaqMan assays were applied in individual DNA samples (N = 140 in total), previously assessed and genotyped with Sanger sequencing, for validation purposes. The validation revealed that all potential genotypes (wild type, mutant and heterozygous) were assessed with total agreement between the Sanger sequencing and TaqMan assays. In the case of the T929I/C/V assay since all DNA samples available for validation were mutant either for T929V or T929I (no T929C mutation or wild type alleles were detected according to sequencing), we used the wild type and equimolar mix of wild type and mutant (artificial heterozygote) to cover these genotypes (Suppl. Table S1).

Representative examples for all assays, are presented in Figure 1 (parts A-D, respectively). The calling of samples was made automatically using the Allelic Discrimination module of the qPCR machine's Bio-Rad CFX Maestro software.

3.2 Development of ddPCR for pools and validation versus quantitative sequencing

The ddPCR assays were initially optimized in terms of the best droplet population separation (Suppl. Table S4).

Following assay optimisation, spiked samples were prepared for all assays by mixing control synthetic wild type and mutant sequences of known copy numbers resulting in the following

frequencies: 80.0%, 50.0%, 10.0%, 1.0%, 0.5%, 0.2% and 0.1% and were assessed by ddPCR (Figure 2). This allowed us to estimate the LoD (lowest MAF that reliably distinguishes from the wild type background and can be detected) for each assay, i.e., 0.10% for G275E, I1017M, T929I/C/V, and 0.20% for L1014F. These LoDs could permit the use of pools even larger than 50 thrips, which were used in this study, as they are translated to the accurate detection of 1 heterozygote thrips in a pool of 499 wild type individuals for the G275E, I1017M and T929I/C/V assays, and 1 heterozygote thrips in a background of 249 wild type ones for the L1014F assay.

Next, and in order to confirm that our assays perform reliably in pools of WFT samples, we compared the mutant allelic frequencies as estimated by proportional Sanger sequencing and ddPCR (Figure 3). The analysis revealed strong relationships ($R^2 > 0.9$ in all cases) between the measurements of two methods for assays G275E, I1017M, and L1014F and at the same time linearity of the models was confirmed (Cusum test for linearity P values > 0.05 in all cases) (Suppl. Table S5). Correlation analysis could not be performed for the T929I/C/V assay as all available samples were genotyped as mutant (MAF = 100%). Importantly, the Passing and Bablok regression for method comparison that was used showed that no systematic (all 95% CIs for the Intercept A contain the value 0), proportional (all 95% CIs for the Slope B contained the value 1.0) and random differences (as determined by ±1.96 RSD) between the two methods. (Suppl. Table S5).

3.3 Application of the ddPCR assays in pooled samples of *Frankliniella occidentalis* field populations.

The ddPCR assays were subsequently applied in pools of nine *F. occidentalis* field populations from Crete in order to calculate the mutant allelic frequency for each mutation, using the following formula: %MAF = (MUT copies/ (MU Tcopies + WTcopies)) × 100%. The MAFs of G275E, I1017M, L1014F and T929I/C/V as determined by ddPCR as presented in Table 1.

The nicotinic acetylcholine receptor G275E mutation associated with resistance to spinosad was detected in the all nine populations of the study. It was fixed or close to fixation (MAFs > 97.0%) in 5/9 populations (Fo5, Fo6, Fo7, Fo8, Fo9). Populations Fo2 and Fo4 presented high frequencies for

this mutation (87.83% and 62.31%, respectively), with the remaining two populations, i.e., Fo3 and Fo1, showing moderate frequencies (47.54% and 29.66% respectively).

The chitin synthase mutation I1017M associated with resistance to benzoylureas was detected in all populations at low to moderate frequencies. The populations that showed the highest MAFs were Fo2 (51.07%), Fo6 (45.63%), Fo4 (40.38%) and Fo7 (31.73%). The remaining five populations (Fo3, Fo1, Fo8, Fo5 and Fo9) presented low frequencies for the mutant I1017M allele, ranging from 17.74% to 20.48%.

Mutations that are implicated in sodium channel modulator (pyrethroid) resistance were also detected in all populations. The T929I/C/V ddPCR assay showed the exclusive presence of mutant alleles (MAF = 100%). According to sequencing only mutations T929I and T929V, were detected, whereas T929C was absent. The L1014F mutation reached a frequency of 37.29% in population Fo5 and detected at similar frequencies in populations Fo6 and Fo1 (34.68% and 34.44%, respectively). The Fo3 population presented an L1014F frequency of 28.16%, with the remaining five populations (Fo8, Fo9, Fo2, Fo7 and Fo4) displaying frequencies in the range of 11.01% to 18.80%.

4. DISCUSSION

Molecular diagnostics that assess target site resistance against three different MoAs (nAChR allosteric modulators-site I, sodium channel modulators, and growth inhibitors affecting CHS) in *F. occidentalis* were developed. The diagnostic panel includes four TaqMan-chemistry based assays that detect the following mutations: G275E (*nAChR α6*), L1014F, T929I, T929C and T929V (*vgsc*), and I1017M (*chs1)*. It is compatible for use both in conventional qPCR machines for individual genotyping (Figure 1) and in high-end ddPCR for genotyping in large pooled samples (Figure 2). The latter option can boost the practicality of monitoring in IRM programs by greatly decreasing time and resources. At the same time, it provides extremely high sensitivity suitable for detecting the early spread or re-emerging of resistance, through the detection of known resistance mutations at low

frequencies. To our knowledge, only two previous papers dealt with the development of target site diagnostics for *F. occidentalis*, a KASP[16] and a TaqMan qPCR[17] assay, both for the detection of G275E. These methods are highly reliable and robust, but require analysis at the level of individuals thus having lower throughput and requiring considerably more time and effort, compared to our method.

Studies regarding resistance of WFT populations from Greece are missing, although growers frequently report reduced efficacy of the majority of insecticides registered for its control lately. For this reason, we implemented our newly developed ddPCR assay panel in nine *F. occidentalis* populations from Crete, Greece, a region with intense agricultural activities largely affected by WFT.

The G275E mutation, was detected in all nine populations from Crete, Greece with frequencies ranging from 29.66 % to 100.0%. In the majority of the populations spinosad was previously applied against WFT in the field. G275E has been previously associated with phenotypic spinosyn resistance in some populations (e.g., in populations from Spain[11] and Australia[17]) whereas in others (populations from China[24], [16]) this association was not evident. Given also that additional resistance mechanisms may be in play, such as the expression of truncated nAChR α6 variants[25] or metabolic resistance, although the latter has not yet been confirmed[26, 27], further studies are needed in order to delineate the contribution of each mechanism to the phenotype. This is especially critical taking into account the extremely strong phenotypes that have been reported worldwide[11, 16, 17, 27, 28].

T929I, T929V and L1014F mutations, associated with pyrethroid resistance, were found in all tested populations. The T929I and T929V mutations were detected at a combined frequency of 100% in all of the populations tested and there was complete absence of any wild type or T929C mutated alleles. The role of T929I in pyrethroid resistance is considerably better documented in thrips species[9, 29-32], compared to T929V and T929C[9]. Given that there is no data suggesting that all three substitutions have the same role in resistance, future studies that would assess each mutation individually, by either running separate reaction or employing higher order of multiplexing, in phenotypically characterized thrips field populations are needed. The L1014F mutation was also present in frequencies ranging

from 11.01% to 37.29%. This mutation has been previously identified in *F. occidentalis*[9] and other thrips species[29].

The I1017M mutation, associated with resistance to benzoylureas, was detected in all populations in frequencies ranging from 17.74% to 51.07%. The benzoylurea lufenuron has been applied against WFT at a limited extend in Crete, however the high frequency of I1017M mutations may be due to application of inhibitors of chitin biosynthesis acaricides, applied often against *T. urticae*[33, 34]. This mutation was previously detected in novaluron-resistant WFT populations originating from Japan[10].

5. CONCLUSION

We present here the development of molecular diagnostics for *F. occidentalis* that can be applied either to individual or pooled samples of insects. We also report the successful application of the tool, in populations from Crete, allowing us to profile WFT resistance at the molecular level. The molecular diagnostics that we propose comprise a valuable tool for future studies aiming to delineate the exact diagnostic value of each mutation with respect to phenotypic resistance. They could also facilitate the quick and hypersensitive pesticide resistance monitoring of WFT populations and ultimately promote evidence-based IRM.

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CONFLICT OF INTEREST DECLARATION

The authors declare that there is no conflict of interest.

TABLES

Table 1 Mutant allele frequencies measured by ddPCR in pools of *F. occidentalis* field populations (N = 50 individuals per pool).

Pesticide MoA (class)	nAChR Allosteric Modulators - Site I (Spinosyns)	Growth inhibitors affecting CHS1 (Benzoylureas)	Sodium channel modulators (Pyrethroids)	
Mutation Gene	**G275E** *nAChR α6*	**I1017M** *Chs1*	**L1014F** *Vgsc*	**T929I/C/V** *Vgsc*
Population	**% Mutant Allelic Frequency**			
Fo1	29.66	19.82	34.44	100.0
Fo2	87.83	51.07	16.82	100.0
Fo3	47.54	20.48	28.16	100.0
Fo4	62.31	40.38	11.01	100.0
Fo5	98.16	17.91	37.29	100.0
Fo6	100.00	45.63	34.68	100.0
Fo7	97.35	31.73	12.74	100.0
Fo8	98.59	19.33	18.80	100.0
Fo9	100.00	17.74	17.68	100.0

nAChR: Nicotinic acetylcholine receptor; Chs: Chitin synthase; Vgsc: Voltage-Gated Sodium Channel; MoA; Mode of Action.

FIGURE LEGENDS

Figure 1 TaqMan assays for the detection of G275E (part A), I1017M (part B), L1014F (part C) and T929I/C/V (part D) mutations in individual thrips samples. In the G275E (part A) assay, the wild type probe was labelled with FAM and the mutant probes was labelled with HEX fluorescent dye, whereas for the remaining three assays probe labelling was done vice versa. In part D, samples TT 929 and TI 929 are wild type and artificial heterozygous control synthetic sequences that were in the absence of any wild type alleles in the control samples; mutation T929C was also not detected (data available from sequencing). Graphs and sample calling were made using the qPCR's machine software.

Figure 2 ddPCR assays for the detection of G275E (part A), I1017M (part B), L1014F (part C) and T929I/C/V (part D) mutations in pooled thrips samples and the quantification of their mutant allelic frequencies (MAFs). MAF samples were prepared by mixing control synthetic wild type (WT) and mutant (MUT) sequences of known copy numbers resulting in the following frequencies: 80.0%, 50.0%, 10.0%, 1.0%, 0.5%, 0.2% and 0.1%, in order to find the LoD for each assay. NTC: No Template Control; WT: Wild type control synthetic sequence (0.0% MAF); MUT: Mutant control synthetic sequence (100% MAF). In the right side the corresponding graphs for concentration (copy numbers/μL) vs MAF are presented.

Figure 3 Correlation analysis between allelic frequencies (G275E, I1017M, and L1014F) in the studied populations as determined by ddPCR versus proportional sequencing. Correlation was made using the Passing & Bablok regression for method comparison that additionally allows for the evaluation of systematic, proportional and random differences between methods. The full data from the regression analysis are presented in Suppl. Table S5.

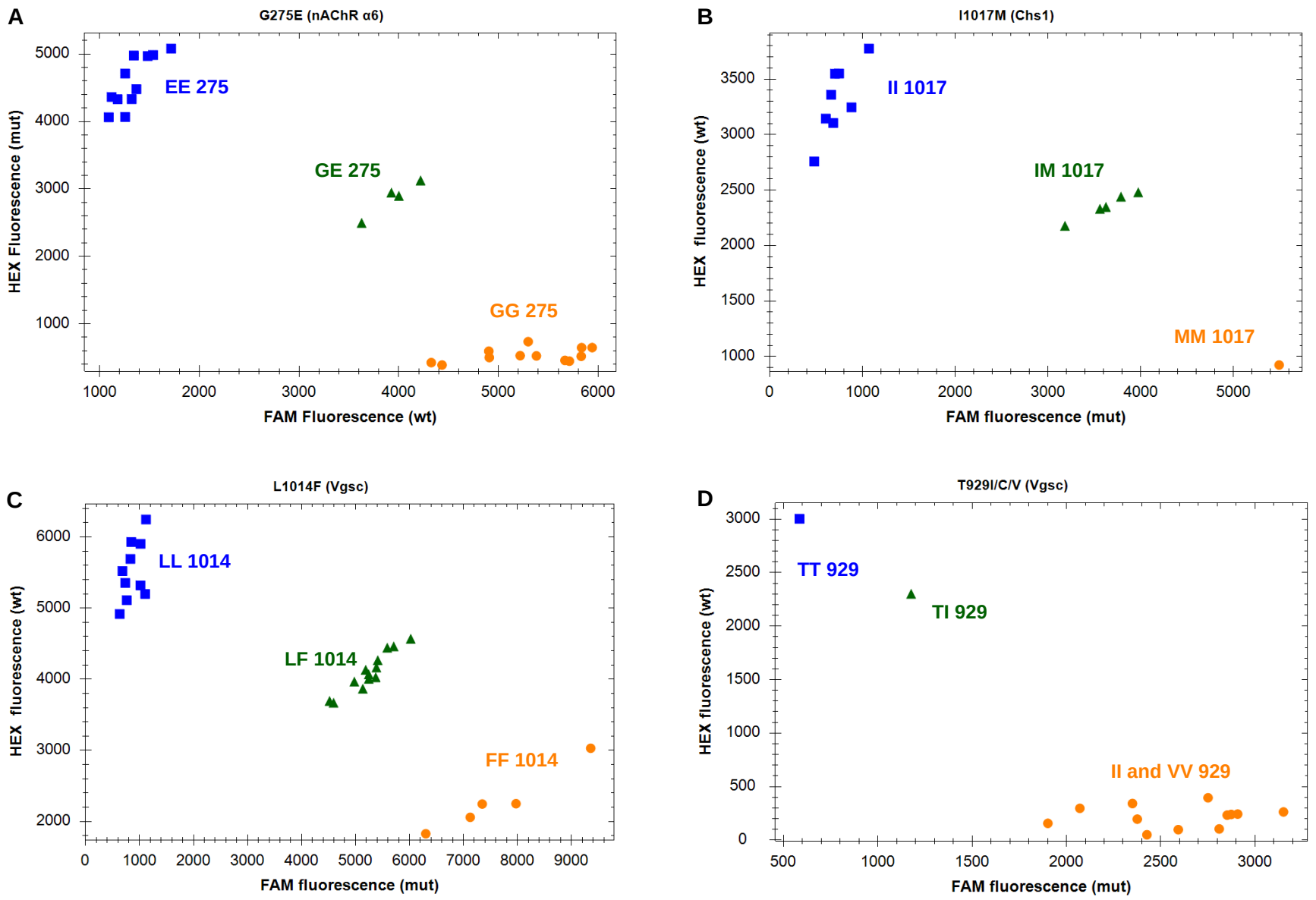
A
G275E (nAChR α6)
HEX Fluorescence (mut)
FAM Fluorescence (wt)
EE 275
GE 275
GG 275
B
I1017M (Chs1)
HEX fluorescence (wt)
FAM fluorescence (mut)
II 1017
IM 1017
MM 1017
C
L1014F (Vgsc)
HEX fluorescence (wt)
FAM fluorescence (mut)
LL 1014
LF 1014
FF 1014
D
T929I/C/V (Vgsc)
HEX fluorescence (wt)
FAM fluorescence (mut)
TT 929
TI 929
II and VV 929

Figure 1

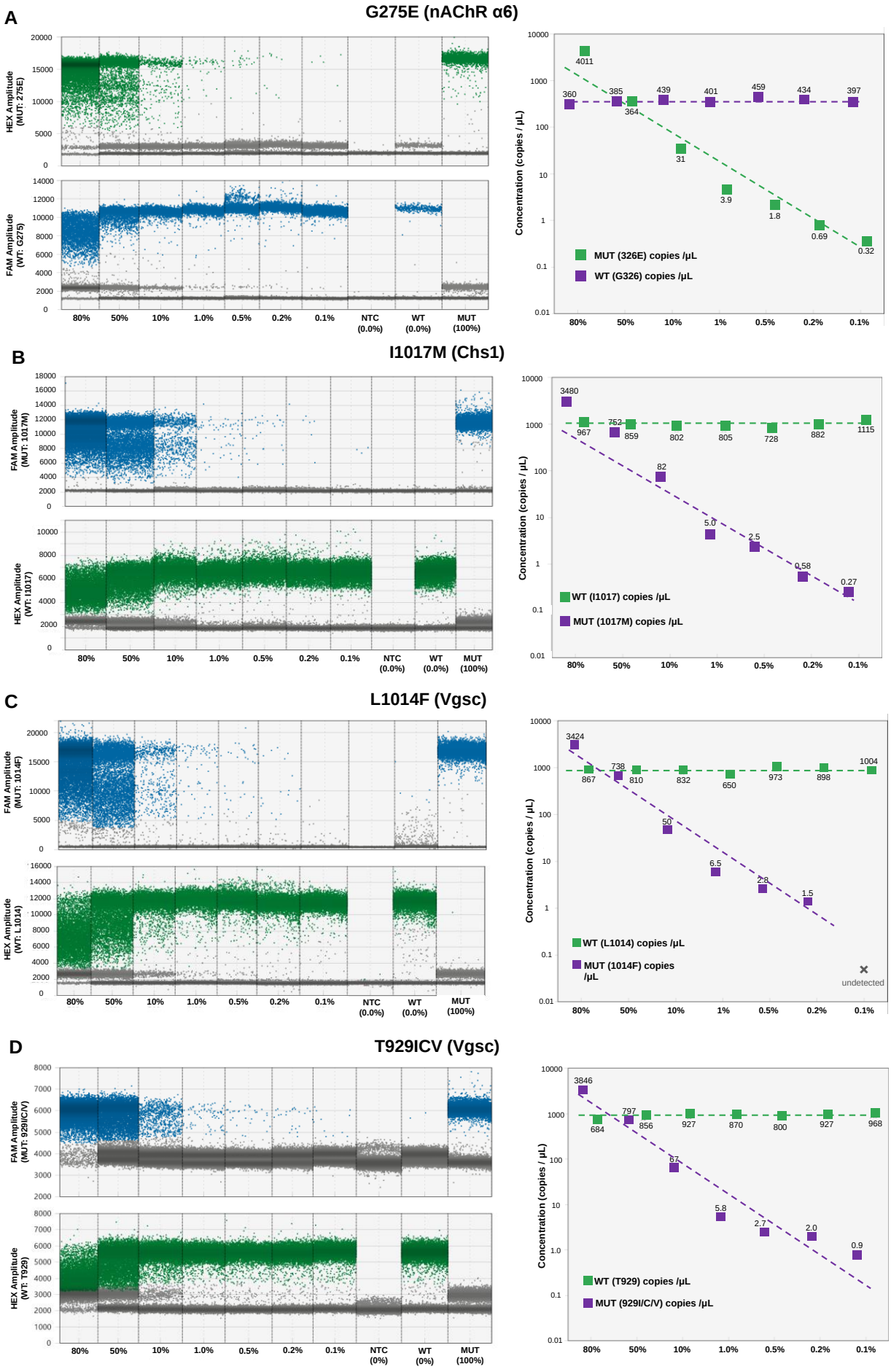
A
G275E (nAChR α6)
HEX Amplitude (MUT: 275E)
FAM Amplitude (WT: G275)
80% 50% 10% 1.0% 0.5% 0.2% 0.1% NTC (0.0%) WT (0.0%) MUT (100%)
Concentration (copies / µL)
4011 360 385 364 439 401 459 434 397 31 3.9 1.8 0.69 0.32
MUT (326E) copies /µL
WT (G326) copies /µL
80% 50% 10% 1% 0.5% 0.2% 0.1%
B
I1017M (Chs1)
FAM Amplitude (MUT: 1017M)
HEX Amplitude (WT: I1017)
80% 50% 10% 1.0% 0.5% 0.2% 0.1% NTC (0.0%) WT (0.0%) MUT (100%)
Concentration (copies / µL)
3480 967 752 859 802 805 728 882 1115 82 5.0 2.5 0.58 0.27
WT (I1017) copies /µL
MUT (1017M) copies /µL
80% 50% 10% 1% 0.5% 0.2% 0.1%
C
L1014F (Vgsc)
FAM Amplitude (MUT: 1014F)
HEX Amplitude (WT: L1014)
80% 50% 10% 1.0% 0.5% 0.2% 0.1% NTC (0.0%) WT (0.0%) MUT (100%)
Concentration (copies / µL)
3424 867 738 810 832 650 973 898 1004 50 6.5 2.8 1.5 undetected
WT (L1014) copies /µL
MUT (1014F) copies /µL
80% 50% 10% 1% 0.5% 0.2% 0.1%
D
T929ICV (Vgsc)
FAM Amplitude (MUT: 929I/C/V)
HEX Amplitude (WT: T929)
80% 50% 10% 1.0% 0.5% 0.2% 0.1% NTC (0%) WT (0%) MUT (100%)
Concentration (copies / µL)
3846 684 797 856 927 870 800 927 968 67 5.8 2.7 2.0 0.9
WT (T929) copies /µL
MUT (929I/C/V) copies /µL
80% 50% 10% 1.0% 0.5% 0.2% 0.1%

Figure 2

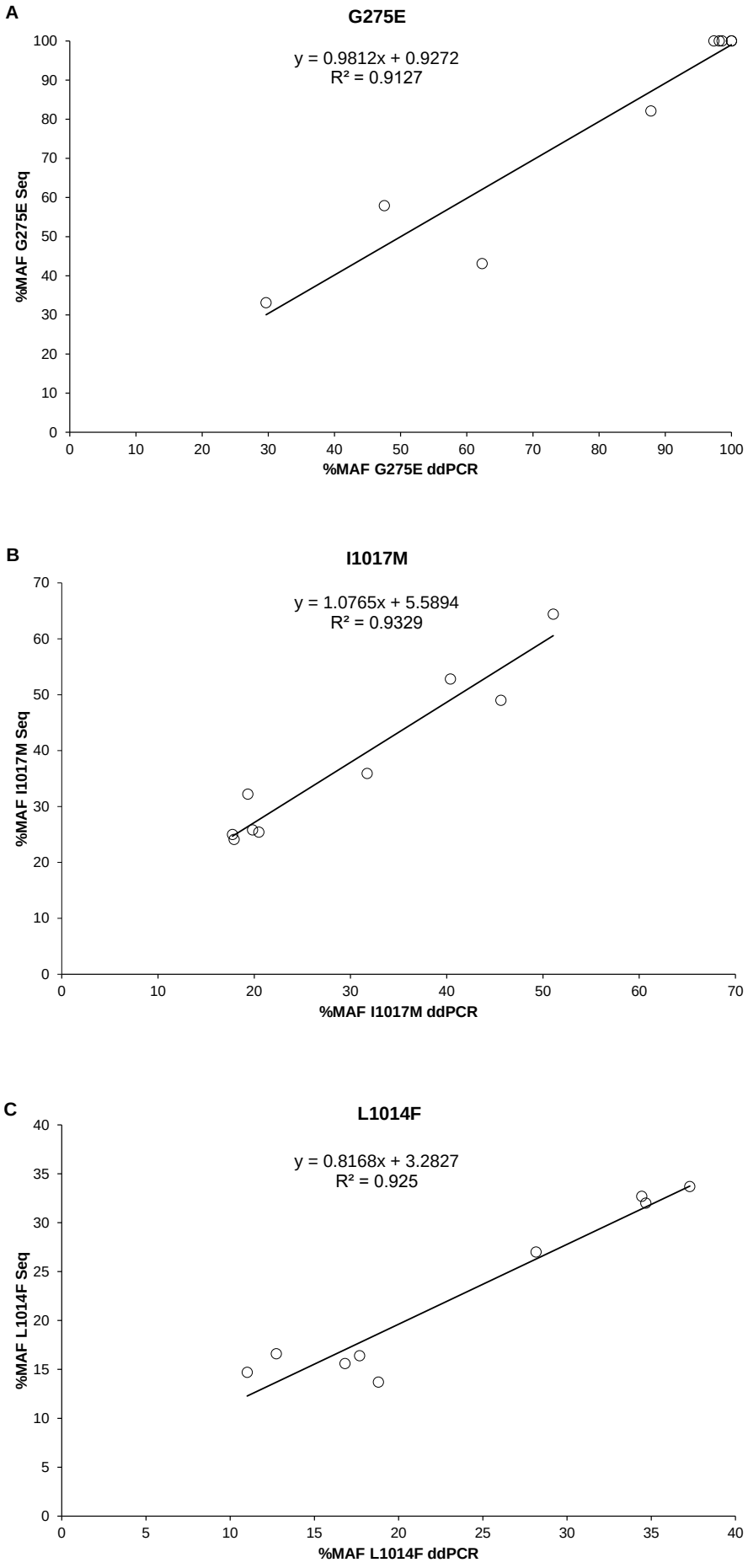
A
G275E
y = 0.9812x + 0.9272
R² = 0.9127
%MAF G275E Seq
%MAF G275E ddPCR
B
I1017M
y = 1.0765x + 5.5894
R² = 0.9329
%MAF I1017M Seq
%MAF I1017M ddPCR
C
L1014F
y = 0.8168x + 3.2827
R² = 0.925
%MAF L1014F Seq
%MAF L1014F ddPCR

Figure 3

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SUPPLEMENTAL INFORMATION

Supplementary Table S1 Results of the validation of the TaqMan assays.

Assay (mutation)	Sample Category (N analyzed)	Expected Genotype [based on Sequencing]	Observed Genotype [based on TaqMan qPCR assay]	Agreement
nAChR α6 (G275E)	Wild type (N = 13)	GG	GG	100%
	Heterozygous (N = 4)	GE	GE	
	Mutant (N = 12)	EE	EE	
Chs1 (I1017M)	Wild type (N = 16)	II	II	100%
	Heterozygous (N = 11)	IM	IM	
	Mutant (N = 2)	MM	MM	
Vgsc-A (L1014F)	Wild type (N = 10)	LL	LL	100%
	Heterozygous (N = 14)	LF	LF	
	Mutant (N = 5)	FF	FF	
Vgsc-B (T929I/C/V)*	Wild type (N = 1)	TT	TT	100%
	Heterozygous (N= 1)	TI	TI	
	Mutant (N = 53)	II or VV	II or VV	

*TT and TI samples correspond to synthetic control sequences (wild type and artificial heterozygous). Data from Sanger sequencing showed the absence of T929C mutation, as well as of any wild type alleles in the populations tested.

Supplementary Table S2 List of primers used for estimation of mutation frequency via Sanger sequencing.

Target genes	Resistance mutations	Oligo name	Sequence (5'–3')	PCR product size (bp)	Annealing T (°C)
nAChR_α6	G275E	a6_F2	ACGGTCAATAATTTCTCCGCTT	252	54°C
		a6_R2	CCATCCTGGCATTTCATGGATA		
VGSC	T929I/C/V and L1014F	vgsc_F1	CCGAGTGTTCAAGCTTGCCAA	544	58°C
		vgsc_R1	AATTGGACAGGAGCAAGGCA		
CHS1	I1017M	chs1_F1	CAGATGCTCTCCACGGGCTA	289	60°C
		chs1_R1	TGGTCTTCTTGGCCACCACT		

T: Temperature; bp: base pairs.

Supplementary Table S3 List of primers and probes used for qPCR and ddPCR.

Assay (mutation)	Oligo name	Sequence 5'→3' (including dyes)	Final concentration*
nAChR α6 (G275E)	275F	GCAACCTTAGAACTGGCACTACCT	800 nM
	275R	GAGGCCACCATGAACATGATG	800 nM
	275Pwt	FAM-TGTTTGTAGGAACTTAC-MGB	500 nM
	275Pmut	HEX-CTGTTTGTAGAAACTTAC-MGB	200 nM
Chs1 (I1017M)	1017F	CCACAAGAGTTCTGGTGCGT	800 nM
	1017R	ACAGGATGAGCAGCAGGTACA	800 nM
	1017Pwt	HEX-ATGGCMATCCCCTC-MGB	300 nM
	1017Pmut	FAM-ATGGCMATGCCCTC-MGB	300 nM
Vgsc-A (L1014F)	1014F	TGTGGGACTGTATGCTCGTTG	800 nM
	1014R	GAGCAAGGCAAGGAAAAGGT	800 nM
	1014Pwt	HEX-TCGGCAACTTGGTCG-MGB	300 nM
	1014Pmut	FAM-TCGGCAACTTTGTCG-MGB	300 nM
Vgsc-B (T929I/C/V)	929F	GCTCATATCTATCATGGGTCGG	800 nM
	929R	CAGGCGTTGTTGAATGACACG	800 nM
	929Pwt	AGGTAACTTRACCTTTG	450 nM
	929Pmut1(I)	AGGTAACTTRATCTTTG	150 nM
	929Pmut2(C)	AGGTAACTTRTGCTTTG	150 nM
	929Pmut3(V)	AGGTAACTTRGTCTTTG	150 nM

*Final concentration refers to TaqMan qPCR assays- for ddPCR see Suppl. Table S5.

F: Forward; R: Reverse; P: Probe; Wt: Wild-type; Mut: mutant; MGB: Minor Groove Binder.

Supplementary Table S4 Optimized ddPCR reaction conditions and analytical properties per assay

Assay (mutation)	Oligo*	Concentration	dsDNA quantity	Annealing Temperature	N of cycles	LoD for MAF
nAChR α6 (G275E)	F, R	1200 nM	10 ng	58ºC	50	0.10%
	Pwt	700 nM				
	Pmut	600 nM				
CHS1 (I1017M)	F, R	1200 nM	10 ng	54ºC	50	0.10%
	Pwt	600 nM				
	Pmut	600 nM				
Vgsc-A (L1014F)	F, R	1200 nM	10 ng	58ºC	50	0.20%
	Pwt	600 nM				
	Pmut	600 nM				
Vgsc-B (T929I/C/V)	F, R	1200 nM	10 ng	54ºC	50	0.10%
	Pwt	600 nM				
	929Pmut1(I)	250 nM				
	929Pmut2(C)	250 nM				
	929Pmut3(V)	250 nM				

*Sequence same as in Suppl. Table S4

F: Forward primer; R: Reverse primer; Pwt: Wild type probe; Pmut: Mutant probe from Suppl Table S4; LoD: Limit of Detection; MAF: Mutant allelic Frequency.

Suppl. Table S5 Passing-Bablok regression analysis for the comparison of ddPCR and proportional sequencing methods.

Regression analysis	Target site mutation		
	G275E	**I1017M**	**L1014F**
Systematic Differences [a]			
Intercept A	4.89	6.25	5.38
95%CI	-50.6 to 100.0	-2.79 to 9.95	-1.37 to 8.30
Proportional Differences [b]			
Slope B	0.951	0.996	0.767
95%CI	0.00 to 1.53	0.848 to 1.37	0.651 to 1.005
Random Differences [c]			
Residual Standard Deviation (RSD)	6.45	4.64	2.79
±1.96 RSD	-12.6 to 12.6	-9.10 to 9.10	-5.48 to 5.48
Linear model validity [d]			
Cusum test for linearity	P =0.36	P =1.00	P=0.63

[a] If the 95% CI for the Intercept A contains the value 0 no systematic differences are present.

[b] If the 95% CI for the Slope B contains the value 1 no proportional differences are present.

[c] 95% of random differences are expected to lie in the interval -1.96 RSD to +1.96 RSD

[d] If P>0.05 then the Passing-Bablok method is applicable