

Droplet-Digital PCR Provides a Rapid, Accurate and Cost-Effective Method for Identification of Biomarker FcγRIIIa-F158V Genotypes

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Abstract

Development of novel monoclonal antibodies, vaccines and oncolytic virus therapies have relied on analysis of biomarkers as potential predictors of success. One well studied biomarker is the CD16/ FcγRIIIa receptor residue 158 F/V. Identifying variants through genotyping of the FcγRIIIa locus is widely practiced and highly varied with commonly used methods including: Sanger sequencing, flow-cytometry, PCR/RFLP, Goldengate (replaced by Infinium) and TaqMAN analysis. While each of these methods have considerable backing in publications related to CD16 FcγRIIIa 158 F/V, the majority present significant shortcomings in identifying both homozygotes (wild-type and mutant) and heterozygotes in a time and cost-efficient manner. Utilization of droplet-digital PCR with FcγRIIIa-F158V specific probes results in the accurate genotyping using direct recognition of sequence in genomic samples at a lower average cost and faster turnaround. Here we demonstrate the use of ddPCR to accurately identify FcγRIIIa-F158V genotypes with confirmation by Illumina sequencing in 128 patient samples.

Keywords: Biomarker; FcγRIIIa; FcγRIIIa-F158V; CD16; Droplet-digital PCR; ddPCR, Illumina

Introduction

Identification of novel biomarkers present an important medical progression in the cancer therapy, transplant rejection and autoimmune diseases utilizing newly developed monoclonal antibodies (mAbs) and oncolytic adenovirus treatments. Current cancer-related mAb therapies are predominated by IgG antibodies to promote identification and cytotoxic elimination of cancer cells through antibody-dependent cellular cytotoxicity (ADCC) [1,2]. One potential biomarker of significance for ADCC-based immunotherapies is CD16/FcγRIIIa, a key mediator of IgG related anti-tumor activity and is associated with increased binding of mAb therapeutics to NK cells [3,4]. FcγRIIIa possesses a well-studied amino acid variant in the hinge region of the protein caused by SNP 559 (T/G) resulting in the phenotype FcγRIIIa-F158V (Figure 1) [5,6]. The FcγRIIIa-F158V biomarker has been analyzed in studies of trastuzumab (HER2 breast cancer), rituximab (non-Hodgkin's lymphoma, large B-cell lymphoma, Burkitt lymphoma) and cetuximab (KRAS wild-type colorectal, head and neck cancer) for correlation of either the 158F or 158V phenotype with positive outcomes [7-10]. The presence of the FcγRIIIa-158V biomarker has been demonstrated to positively correlate with positive clinical outcomes in multiple mAb studies, suggesting that rapid analysis of this biomarker is important support to future treatment options [8-13]. Oncolytic viruses are another novel means for treatment of cancers which have found potentially critical information relating to treatment success in FcγRIIIa-F158V phenotypes [14].

Current methods for genotyping CD16-SNP559/ FcγRIIIa-F158V include allele specific PCR, nested PCR/RFLP, PCR/Sanger sequencing, Goldengate genotyping, allele-specific qPCR with SYBR green, flow-cytometry and TaqMAN SNP assay, all of which are commonly utilized to this day [5,6,12,13,15-17]. The breadth of methods produces complications when comparing studies due to shortcomings and provisos associated with each method [18]. A highly homologous gene on the same chromosome (FcγRIIb) which contains a homozygous 158V phenotype can complicate the FcγRIIIa-F158V genotyping result for PCR or sequencing based methods (Figure 1). Due to the FcγRIIb pseudogene, PCR analysis requires highly accurate primer design and sequencing is often a requirement for confidence in results which increases complexity, time requirements and costs

[16]. Goldengate genotyping is time intensive and designed for a large number of SNPs for individuals. Due to the breadth of information garnered from Goldengate arrays, usually designed for hundreds of SNPs, resulting data beyond the CD16 SNP559 of interest and adds to the complexity and cost for analysis. Allele specific qPCR using SYBR green does not rectify concerns related to PCR analysis and requires significant optimization and does not provide sequence data. Flow-cytometry has been widely used to identify FcγRIIIa-F158V via allele specific antibody interaction with FcγRIIIa [19]. This method provides an accurate, but expensive means for identification of 158V/V and 158F/F phenotypes that requires a significant investment in expensive equipment or access to a flow cytometry facility. In addition to its expense, flow cytometry for FcγRIIIa suffers from complications with resolving heterozygotes due to experimental shortcomings related to fluorescence and binding variations between antibodies for FcγRIIIa-158F and 158V alleles [14,15]. TaqMAN SNP assays are specific, highly sensitive and dependent on recognition of original (genomic) sequence for genotype calling which support their use for such analysis, however TaqMAN assays have complications related to reproducibility, statistical strength of outputs and are sensitive to PCR contaminants [15]. More importantly, the highly homologous FcγRIIb could interfere with TaqMAN assay for FcγRIIIa.

Combining TaqMAN qPCR assays with ddPCR will improve the quantitative aspect of the assay significantly. The primary differentiating factor between TaqMAN and ddPCR is that the latter encapsulates PCR reactions into ~20,000 droplets prior to PCR which allows for

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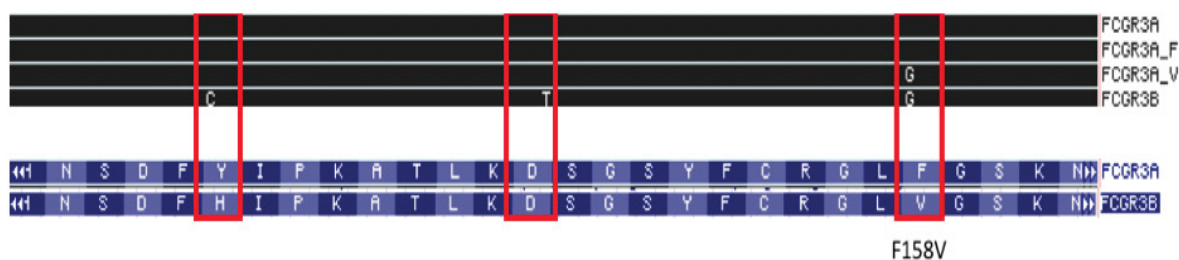


Figure 1. Illustration of the FcγRIIIa -F158V locus depicting the F158V associated SNP location and two other local SNPs. Alignment of FcγRIIIa (FCGR3A), its two amino acid 158 variants (FCGR3A-F and FCGR3A-V) and FcγRIIIb was done using the UCSC Genome Browser and BLAT tool (<https://genome.ucsc.edu>) [22, 23]. Three SNPs shared between FcγRIIIa and FcγRIIIb are boxed to draw attention to the sequence and associated amino acid, illustrating the difficulty in differentiating between the two genes using PCR and Sanger sequencing.

absolute quantification of template, providing the ability to calculate copy numbers of a target in a sample in a highly sensitive manner. The use of ddPCR over regular qPCR produces significant benefits which resolve TaqMAN related problems including significantly higher reproducibility, reduction in variability and more accurate classification of alleles [20]. In addition to the advantages over the traditional TaqMAN qPCR assays, ddPCR maintains significant accuracy, cost and time advantages over previously mentioned PCR and array-based assay types which support its use as a common and rapid means for FcγRIIIa-F158V SNP genotyping.

Methods

ddPCR FcγRIIIa-158F/V allele-specific assay

Droplet-digital PCR was performed using a Bio-Rad QX200 droplet generator, BioRad T-100 thermocycler for PCR and subsequent droplet analysis using a BioRad QX200 droplet reader and Quantasoft software. Primers and probes utilized were generated by ABI/Thermo-Fisher Scientific for SNP analysis (rs396991) targeting FcγRIIIa nucleotide 559 [3,21-23]. Final reaction mix consisted of: primers (900 nM each), probes (250 nM each) Bio-Rad ddPCR Supermix for Probes (no dUTP) (1X) and genomic DNA (10 to 50 ng) prior to loading into Bio-Rad QX200 droplet generator. Thermal cycling conditions utilized: 95°C for 10 minutes, (94°C for 30 seconds, 60°C for 1 minute) 40 cycles, 98°C for 10 minutes, 4°C hold with a ramp rate of 2°C/second and a heated lid at 105°C on a Bio-Rad T100 thermal cycler. FcγRIIIa-158F/V genotyping was performed by calculating the ratio of FcγRIIIa-158F (559A) to FcγRIIIa-158V (559C) allele counts per sample. Expected ratios for homozygous WT FcγRIIIa-158F is 2:2 158F/158V, resulting from identification of both the FcγRIIIa and closely related FcγRIIIb. Heterozygous FcγRIIIa-158F/V is identified by a 1:3 F:V ratio while homozygous FcγRIIIa-158V is identified by 0:4 F:V ratios. Ratios were generated by comparing the total copy number counts for FcγRIIIa-158F and FcγRIIIa-158V alleles generated by the QX200 droplet reader.

Confirmation of FcγRIIIa-158F/V genotype through Illumina sequencing

Illumina MiSeq generates millions of sequencing clusters, which is derived from a single DNA molecule. Sequencing the PCR amplicon on MiSeq will allow accurate quantification of FcγRIIIa-158 F/V and FcγRIIIb-158 V allele based on other two different base pairs. Following ddPCR, 25 samples were sequenced on Illumina MiSeq platform using either 60 second extension time for PCR or 180 seconds. CD16 specific primers were originally described in Al Tsang (rs396991): forward with a M13 sequence tag (5' GTAAAACGACGGCCAGTCT-GCTCTGCATAAGGTACATATTTACAGA 3') and reverse with a M13 sequence tag (5'CCTTTGTCGATACTGGTACGATGGTGAT-

GTTCACAGTCTCT 3'). PCR of exon 3 of CD16 was performed using Platinum[®] Taq DNA polymerase, 2 μL High Fidelity 10 X buffer, 0.6 μL 50 mM MgCL₂, 0.4 μL 10 mM dNTP, 1 μL primers (each), 1 μL DNA (10 ng) 0.8 μL polymerase and H₂O up to 20 μL. PCR reactions were performed at 95°C for 2 minutes followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 or 180 seconds with a final extension of 72°C for 10 minutes and a 4°C indefinite hold. PCR products were purified using Agencourt beads and eluted with 20 μL H₂O and yields confirmed using Nanodrop. Barcoding for Illumina sequencing was done with the forward primer consisting of P5, i5 barcode,

PE1SP (HP10), inline barcode and M13F (5'AATGATACGGC-GACCACCGAGATCTACACATTACTCGACTCTTCCCTA-CACGACGCTCTTCCGATCTATTACTCGGTAACGACGGC-CAGT 3') while the reverse primer consisted of the reverse CD16 specific primer, M13R, inline barcode, PE2, i7 barcode and P7 (5' GTTCGTTCTTCTGCCGTATGCTCTATTGAGAGCCACTGACCTCAAGTCTGCACACGAGAAGGCTAGATTGAGAGCCCTTTGTC-GATACTGGTAC 3'). Barcoded primers were added by PCR using 1 μL of M13 tagged CD16 as generated above as the template and cycle conditions: 95°C for 2 minutes followed by 10 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 or 180 seconds with a final extension of 72°C for 10' and a 4°C indefinite hold. FcγRIIIa-158 F/V alleles and FcγRIIIb-158 V were quantified and the total fraction of FcγRIIIa-158 F/V sequences produced and were expressed as a ratio with 2:0 representing expected 158 F/F wild-type, 1:1 denoting 158 F/V heterozygotes and 0:2 denoting FcγRIIIa-158 V homozygotes with all values rounded to the nearest tenth. Medians were used for analysis to decrease noise, use of averages demonstrated results consistent with presented analysis (data not shown). Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained from the NIH Clinical Center Blood Bank (NCT00001846). PBMCs were also obtained from cancer patients enrolled in three phase I studies at the National Cancer Institute: Avelumab (NCT01772004), M7824 (NCT02517398), and Talactoferrin (NCT00707304).

Results

Identification of FcγRIIIa-F158V genotype ratios by ddPCR confidently demonstrates homozygous wild-type (2:2), heterozygotes (1:3) or homozygous mutants (0:4) at the 158 residue. FcγRIIIa-158 F/V analysis resulted in median ratios for FcγRIIIa-158 F homozygotes of 2:2 ± 0.02 (n=49), FcγRIIIa-158 F/V heterozygotes of 1:3 ± 0.01 (n=64) while FcγRIIIa-158V homozygotes resulted in median ratios of 0:4 ± 0.0 (n=17) all p<0.1 × 10⁻⁹ (Figure 2). Subsequent Illumina sequencing

of 25 samples demonstrated median FcγRIIIa-F158V ratios of 2:2 ± 0.0 (n=9) for wild-type, 1:3 ± 0.03 (n=13) for heterozygotes and 0:4 ± 0.0 (n=2) for homozygous mutants, consistent with the overall sample population (Figures 3A-3C).

Identification of FcγRIIIa-F158V genotypes through sequencing analysis resulted in ratios based on a 2:0 ratio for wild-type, 1:1 ratio for heterozygotes and 0:2 ratio for homozygous mutants due to the ability to filter out results from FcγRIIIb that are present in ddPCR results. Illumina MiSeq analysis for PCR products using 60 second extension times resulted median FcγRIIIa-F158V ratios obtained for wild-type were 1.86:0.14 ± 0.01, heterozygotes produced a ratio of 0.94:1.06 ± 0.02 and homozygous mutant produced a ratio of 0:2 ± 0.0 all p<0.005. MiSeq analysis using PCR products with 180 second extension times resulted in median FcγRIIIa-F158V ratios for wild-type of 1.88:0.12 ± 0.02, for heterozygotes a ratio of 1:01:0.99 ± 0.02 and for homozygous

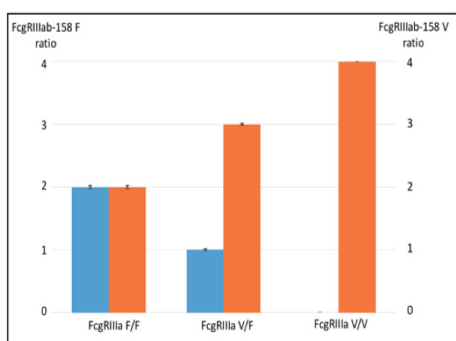


Figure 2: Categorization of FcγRIIIa-F158V genotypes using a ddPCR assay. Droplet digital PCR is capable of consistently categorizing samples according to FcγRIIIa-158 F/V genotypes (n = 128). Blue bars represent total called copies of FcγRIIIa-158F while orange bars represent the total called copies of FcγRIIIa-158V. A FcγRIIIa-158F homozygote (left) is represented by 2 copies of FcγRIIIa0158F and 2 copies of recognized FcγRIIIb-158V in expected ratios while a heterozygote (middle) is represented by 1 copy of FcγRIIIa-158F and 3 copies of FcγRIIIa-158V while a FcγRIIIa-158V homozygote is represented by four copies of FcγRIIIa-158V (right). Errors bars represent standard errors and categorical identifications of F/F, V/F and V/V are statistically differentiated by the Mann-Whitney test with p < 0.1 x 10⁻⁹.

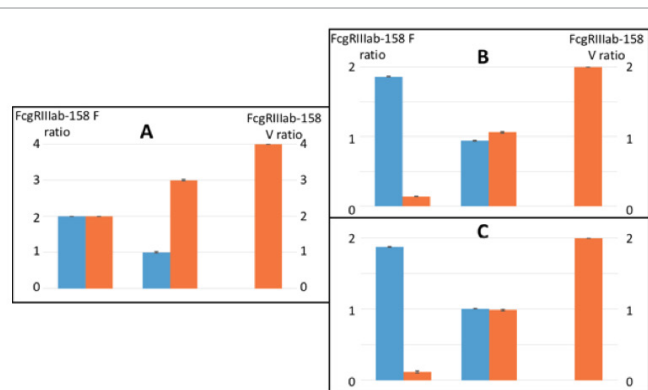


Figure 3: Comparison of ddPCR and Illumina MiSeq sequence data medians for identification of FcγRIIIa-F158V genotypes. A. ddPCR results categorizing FcγRIIIa-158 F/V genotypes with differentiation between F/F (left), F/V (middle) and V/V (right) genotypes in a statistically significant manner (p < 1x10⁻¹⁴). B. Illumina MiSeq 60'' PCR extension results illustrating differentiation between F/F (left), F/V (middle) and V/V (right) gene copy ratios at FcγRIIIa-158 with categorical differences being significant (p < 0.01). C. Illumina MiSeq 180'' PCR extension results illustrating differentiation between F/F, F/V and V/V ratios at FcγRIIIa-158 categorical differences being significant (p < 0.01). Errors bars represent standard error; P values were calculated using 2-tailed Student's T-test.

Sample	ddPCR Ratio	60'' Illumina Ratio	180'' Illumina Ratio	ddPCR FcγRIIIa-158 F/V genotype	60'' Illumina FcγRIIIa-158 F/V genotype	180'' Illumina FcγRIIIa-158 F/V genotype
1	1.0:3.0	1.0:1.0	1.0:1.0	F/V	F/V	F/V
2	0.8:3.2	0.8:1.1	1.0:1.0	F/V	F/V	F/V
3	1.0:3.0	0.9:1.1	1.0:1.0	F/V	F/V	F/V
4	0.0:4.0	0.0:2.0	0.0:2.0	V/V	V/V	V/V
5	1.0:3.0	0.9:1.1	1.0:1.0	F/V	F/V	F/V
6	2.0:2.0	1.9:0.1	1.9:0.1	F/F	F/F	F/F
7	1.0:3.0	0.9:1.1	1.0:1.0	F/V	F/V	F/V
8	1.1:2.9	0.9:1.1	1.0:1.0	F/V	F/V	F/V
9	1.1:2.9	0.9:1.1	1.0:1.0	F/V	F/V	F/V
10	1.0:3.0	0.9:1.1	1.0:1.0	F/V	F/V	F/V
11	2.0:2.0	1.9:0.1	1.9:0.1	F/F	F/F	F/F
12	2.0:2.0	1.9:0.1	1.9:0.1	F/F	F/F	F/F
13	2.0:2.0	1.9:0.1	1.9:0.1	F/F	F/F	F/F
14	2.0:2.0	1.9:0.1	1.9:0.1	F/F	F/F	F/F
15	1.0:3.0	0.9:1.1	1.0:1.0	F/V	F/V	F/V
16	2.7:1.3	1.9:0.1	1.9:0.1	F/F	F/F	F/F
17	1.0:3.0	1.0:1.0	1.0:1.0	F/V	F/V	F/V
18	0.9:3.1	0.9:1.1	1.0:1.0	F/V	F/V	F/V
19	1.0:3.0	0.9:1.1	1.0:1.0	F/V	F/V	F/V
20	1.0:3.0	1.0:1.0	1.0:1.0	F/V	F/V	F/V
21	1.0:3.0	1.0:1.0	1.0:1.0	F/V	F/V	F/V
22	2.0:2.0	1.9:0.1	1.9:0.1	F/F	F/F	F/F
23	0.0:4.0	0.0:2.0	0.0:2.0	V/V	V/V	V/V
24	2.0:2.0	1.9:0.1	1.9:0.1	F/F	F/F	F/F
25	2.0:2.0	1.9:0.1	1.9:0.1	F/F	F/F	F/F

Table 1. Comparison of ddPCR, Illumina MiSeq 60'' PCR extension and Illumina MiSeq 180'' PCR extension ratios and genotype interpretations for 25 samples. Results from ddPCR analysis of FcγRIIIa-158 F/V genotypes agree with Illumina sequencing for all 25 samples processed indenting homozygotes and heterozygotes without bias or issue. For values which did not result in standard ratios (0:4, 1:3, 2:2 for ddPCR and 1:1:0:2 for Illumina sequencing), they were rounded normally for genotype identification.

Assay type	Cost per sample	Time requirement	Complexity
PCR+Sequencing	\$12-17 ¹	>1 day	Moderate
Flow cytometry	\$10-15 ²	1 day	High
qPCR+Sequencing	\$14 -20 ³	>1 day	Moderate
Goldengate/Infinium assay	>\$174 ⁴	3 days	Moderate
TaqMAN	\$9-10 ⁵	<6 hours for 96 samples	Low
ddPCR	\$9-10 ⁶	<6 hours for 96 samples	Low

Table 2: Comparison of estimated financial and time costs for different methods of FcγRIIIa-1F58V identification based on widely-available pricing schemes. Cost do not include the associated equipment which varies between assays. Complexity is an assessment based on the number of skills, required training, equipment and variables involved.

¹Based on the per-sample use 373 of Taq 2X Master Mix from NEB(<https://www.neb.com/products/m0270-taq-2x-master-mix>) and low throughput full cycle sequencing through Frederick National Laboratory for Cancer Research Genomics Facility (<https://crtpfm1.ncifcrf.gov/fmi/webd#ATP%20Work%20Requests>)
²Based on data using University of Illinois Biotechnology Center pricing and a rate of 5 samples/hour, assistance increases cost beyond the estimate listed (<http://biotech.illinois.edu/flowcytometry/pricing>)
³Cost associated with triplicate* processing of samples using 25 μL reactions of SYBR Green PCR Master Mix from ThermoFisher (<https://www.thermoFisher.com/order/catalog/product/4334973>) and low throughput full cycle sequencing through Frederick National Laboratory for Cancer Research Genomics Facility(<https://crtpfm1.ncifcrf.gov/fmi/webd#ATP%20Work%20Requests>)
⁴Estimated pricing generated using UC-Davis Genotyping services (<http://dnatech.genomecenter.ucdavis.edu/genotyping/>) using the Infinium array estimation and sample requirements for the simpler iSelect assay. Each assay includes hundreds of SNPs for analysis, and actual cost will depend on on samples, SNPs analyzed, and cost sharing agreements. This value is expected to represent

the low side of a per-sample cost.

[§]Cost associated with triplicate* processing of samples using ThermoFisher TaqMAN Advanced Master Mix (<https://www.thermofisher.com/order/catalog/product/4444556>), two probes and primer combinations from Integrated DNA Technologies (estimated at \$150 for each set) and a reference set of primers/probes all combined in 25 µL reactions (<https://www.thermofisher.com/order/catalog/product/4333764F>).

[¶]Cost associated with processing of samples (single reaction per sample) includes an estimate for ddPCR master mix for probes (no dUTP), TaqMAN probes/primers, gaskets/droplet cartridges and droplet oil as published by Bio-Rad (<http://www.bio-rad.com/enus/category/digital-pcr>) in addition to a reference set of primers/probes (\$0.74/sample GAPDH) from ThermoFisher (<https://www.thermofisher.com/order/catalog/product/4333764F>).

*ThermoFisher recommends quadruplicate processing of samples for qPCR/Taqman assays, however, triplicate is considered a standard number of replicates.

mutants a ratio of 0.2 ± 0.0 all $p < 0.01$ (Figure 3). Direct comparison of the 25 samples analyzed through both ddPCR and MiSeq demonstrated 100% consistently of ddPCR identified FcγRIIIa-F158V genotypes with sequence data (Table 1).

Discussion

The proliferation of mAb, vaccine and oncolytic virus therapies for wide ranging disease applications has raised the need for a rapid and accurate means to identify the clinically important FcγRIIIa-158 F/V. Droplet digital PCR readily identified FcγRIIIa-F158V genotypes of 128 samples with a subset of 25 samples being sequenced using the Illumina MiSeq platform supported all of the ddPCR results analyzed.

Results from ddPCR demonstrate minor variations in the expected ratios obtained from FcγRIIIab-158 F/V analysis. Cutoff ranges for ddPCR results were established at 1.8-2.2:1.8-2.2, 0.8-1.2:2.8-3.2 and 0:4 to categorize genotypes as FF, FV or VV respectively, with sequence data confirming that these cutoffs produced accurate genotype calls (Table 1). MiSeq results utilized cutoffs of 1.8-2:0-0.2, 0.8-1.2:0.8-1.2 and 0-0.2:1.8-2 to categorize the results as FF, FV or VV respectively (Table 1). PCR products generated using a 60 second extension time were more sensitive to these variations which, using a 10% of expected ratio cutoff, produced easily interpreted results for FcγRIIIab-158 F/V genotyping. MiSeq analysis of PCR products using a 180 second extension were more consistent with expected ratios and should be utilized in the future for any required Illumina sequencing.

Conclusion

The analysis of FcγRIIIa-F158V is increasingly vital to the field of mAb, vaccine and oncolytic virus therapies as a potential biomarker and ddPCR provides the simplest and most accurate method for a rapid determination of the genotypes of a large sample set in direct contrast to currently employed methods of analysis. Direct genotyping analysis using TaqMAN via ddPCR is provides a significant improvement over standard TaqMAN protocols, resulting in outperformance in replication and statistical power. When attributing costs of determination for FcγRIIIa-F158V genotypes per sample ddPCR is further realized as a viable and desirable method, as it is among the cheapest method for SNP identification currently available (Table 2). Utilization of ddPCR for determination of FcγRIIIab-158 F/V genotypes is a more efficient and accurate method than all current standards.

The studies reported here were performed solely for academic purposes, and the results have not been used for prognostication.

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