Droplet-Digital PCR Provides a Rapid, Accurate and Cost-Effective Method for Identification of Biomarker FcyRIIIa-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 presents 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 identification 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 (FcγRIIib) which contains a homozygous 158V phenotype can complicate the FcγRIIIa-F158V genotyping result for PCR or sequencing based methods (Figure 1). Due to the FcγRIIib 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 FcγRIIa [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 FcγRIIib 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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Methods

ddPCR FcyRIIIa-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 FcyRIIIa 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, final extension of 72°C for 10' and a 4°C indefinite hold. FcγRIIIa-158 F/V genotyping was performed by calculating the ratio of FcyRIIIa-158F/V to FcyRIIIa-158V (559A) to FcyRIIIa-158V (559C) allele counts per sample. Expected 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-158F/V SNP genotyping.

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’ GTAAAACGACGGCCAGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
of 25 samples demonstrated median FcγRIIa-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 FcγRIIa-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 FcγRIIa-F158V ratios obtained for wild-type were 1.86±0.14 ± 0.0, 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 FcγRIIa-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

![Image](image1.png)

Figure 2: Categorization of FcγRIIa-F158V genotypes using a ddPCR assay. Droplet digital PCR is capable of consistently categorizing samples according to FcγRIIa-158F/V genotypes (n = 128). Blue bars represent total called copies of FcγRIIa-158F while orange bars represent the total called copies of FcγRIIa-158V. A FcγRIIa-158F homozygote (left) is represented by four copies of FcγRIIa-158F while a FcγRIIa-158V homozygote is represented by four copies of FcγRIIa-158V (right). Errors bars represent standard errors and categorical identifications of F/F, V/F and V/V.

![Image](image2.png)

Figure 3: Comparison of ddPCR and Illumina MiSeq sequence data medians for identification of FcγRIIa-158V genotypes. A, ddPCR results categorizing FcγRIIa-158F/V genotypes with differentiation between F/F (left), F/V (middle) and V/V (right) genotypes in a statistically significant manner (p < 10^-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 FcγRIIa-158V 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 FcγRIIa-158V 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 FcγRIIa-F158 V/V genotype | FcγRIIa-F158 I58F/58V genotype | FcγRIIa-F158 I58V/58V 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     | 0.9:1.1     | 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 FcγRIIa-158F/V genotypes agree with Illumina sequencing for all 25 samples processed indentiﬁng 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 day | Moderate |
| Flow cytometry | $10-15  | 1 day | High |
| ddPCR+Sequencing | $14-20  | >1 day | Moderate |
| Goldengate/Inﬁnium assay | >$174 | 3 days | Moderate |
| TaqMAN | $9-10  | <6 hours for 96 samples | Low |
| ddPCR | $9-10  | <6 hours for 96 samples | Low |

Table 2. Comparison of estimated ﬁnancial and time costs for different methods of FcγRIIa-158F/V 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.

Based 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).

Cost 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).

*Based 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).

*Estimated pricing generated using UC-Davis Genotyping services (http://dnatech.genomencenter.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

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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/A44556), 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/A533764F).

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/en/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/A533764F).

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% consistency 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 for ddPCR results were established at 1.8-2.2:1.8-2.2, 0.8-1.2:0.8-1.2 and 0:0-0.2:0-0.2 to categorize the results 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, 0.8-1.0:0.8-1.2 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 sequencening.

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 SNP using TaqMAN via ddPCR is provides a significant improvement over standard TaqMAN protocols, resulting in outperformance in replication and statistical power.

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References

1. Weiner GJ (2015) Building better monoclonal antibody-based therapeutics. Nat Rev Cancer 15: 361-370.

2. Alderson KL, Sondel PM (2011) Clinical cancer therapy by NK cells via antibody-dependent cell-mediated cytotoxicity. J Biomed Biotechnol 2011: 379123.

3. Ravetch JV, Perussia B (1989) Alternative membrane forms of Fc gamma RIII(CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. J Exp Med 170: 481-497.

4. Pillay V, Gan HK, Scott AM (2011) Antibodies in oncology. N Biotechnol 28: 518-529.

5. Wu J, Edberg JC, Reedea PB, Bansal V, Guyre PM, et al. (1997) A novel polymorphism of FcgammaRllla (CD16) alters receptor function and predisposes to autoimmune disease. J Clin Invest 100: 1059-1070.

6. Koene HR, Kleijer M, Algra J, Roos D, Von Dem Borne AE, et al. (1997) Fc gammaRllla-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRllla, independently of the Fc gammaRllla-48L/R phenotype. Blood 90: 1109-1114.

7. Mellor JD, Brown MP, Irving HR, Zalkberg JP, Dobrovic A (2013) A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. J Hematol Oncol 6:1

8. Musolino A, Naldi N, Bortesi B, Pezzuolo D, Capelletti M, et al. (2008) Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. J Clin Oncol 26: 1789-1796.

9. Cartron G, Daheux L, Salles G, Solal-Celigny P, Bardos P, et al. (2002) Therapeutic activity of humanized anti-CD20 monoclonal antibody and IgG Fc receptor FcgammaRllla gene. Blood 99: 754-758.

10. Bibeau F, Lopez-Crapez E, Di Fiore F, Thezanos S, Ychou M, et al. (2009) Impact of Fc(gamma)Rllla-Fc(gamma)Rlllb polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 27: 1122-1129.

11. Zhang W, Gordon M, Schultheiss AM, Yang DY, Nagashima F, et al. (2007) FCGR2a and FCGR3a polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. J Clin Oncol 25: 3712-3718.

12. Persky DO, Dorrman D, Goldman BH, Brazzel RM, Fisher RI, et al. (2012) Fc gamma receptor 3a genotype predicts overall survival in follicular lymphoma patients treated on SWOG trials with combined monoclonal antibody plus chemotherapy but not chemotherapy alone. Haematologica 97: 937-942.

13. Tamura K, Shimizu C, Hojo T, Akashi-Tanaka S, Kinoshita T, et al. (2011) FcgammaR2a and 3a polymorphisms predict clinical outcome of trastuzumab in both neoadjuvant and metastatic settings in patients with HER2-positive breast cancer. Ann Oncol 22: 1302-1307.

14. Hirvinen M, Heiskanen R, Oksanen M, Pesonen S, Likanen I, et al. (2013) Fc-gamma receptor polymorphisms as predictive and prognostic factors in patients receiving oncolytic adenovirus treatment. J Transl Med 11:193.

15. DallOzzo S, Andres C, Bardos P, Watier H, Thibault G (2003) Rapid single-step FCGR3A genotyping based on SYBR Green I fluorescence in real-time multiplex allele-specific PCR. J Immunol Methods 277: 185-192.

16. Leppers van de Straat FG, Van Der Pol WL, Janssen MD, Sugita N, Yoshie H, et al. (2000) A novel PCR-based method for direct Fc receptor FcgammaRllla allotyping. J Immunol Methods 242: 127-132.

17. Van Royen-Kerkhof A, Sanders EA, Wijnegarden S, Van Roon JA, Voorhors-Mogink M, et al. (2004) Flow cytometric determination of FcgammaRlla (CD32) polymorphism. J Immunol Methods 294: 135-144.

18. Jochems C, Hodge JW, Fantini M, Tsang KY, Vandeveer AJ, et al. (2017) ADCC employing an NK cell harboring expressing the high affinity CD16 allele with aveulamib, an anti-PD-L1 antibody. Int J Cancer 141: 583-593.

19. Bottcher S, Rigen M, Bruggemann M, Raat T, Luschen S, et al. (2005) Flow cytometric assay for determination of FcgammaRlla-158 V/F polymorphism. J Immunol Methods 306: 128-136.
20. Hindson CM, Chevillet JR, Briggs HA, Gallicotte EN, Ruf IK, et al. (2013) Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods 10: 1003-1005.

21. Erbe AK, Wang W, Gallenberger M, Hank JA, Sondel PM (2016) Genotyping single nucleotide polymorphisms and copy number variability of the FCGRs expressed on NK cells. Methods Mol Biol 1441: 43-56.

22. Kent WJ SC, Furey TS, Roskin KM, Pringle TH, Zahler AM, et al. (2002) The human genome browser at UCSC. Genome Res 12: 996-1006.

23. Wij K(2002) BLAT- the BLAST-like alignment tool. Genome Res 12: 656-664.