

Rapid and ultrasensitive digital PCR (dPCR) profiling of EGFRvIII in tumor cells and tissues

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Abstract

Background. Amplification of the epidermal growth factor receptor (*EGFR*) gene is commonly found in glioblastoma (GBM). About 57% GBM overexpresses EGFR and are associated with tumor progression, poor prognosis, and shorter life expectancy. Molecular profiling of solid tumors usually takes several weeks and may be biased by intrinsic tumor heterogeneity.

Methods. The unique sequence created by the fusion of exon 1 and exon 8 in EGFRvIII was used to guide the design of primers and a Minor Groove Binder (MGB) probe. Extracted total RNA was reverse transcribed and pre-amplified by PCR, followed by detection of the EGFRvIII mutation by dPCR.

Results. The lowest limit of quantification of our EGFRvIII assay was 0.003%. The EGFRvIII variant was identified in patient-derived glioma neurosphere cell lines, xenograft mouse model, and patient-derived tumor specimens. The overall workflow can be accomplished within 24 hours. In certain samples, EGFRvIII was detected when next-generation sequencing was unable to identify the variant. This finding highlights the ability of the dPCR assay to identify EGFRvIII mutations in heterogeneous solid tumors such as GBM in a rapid fashion by profiling samples from spatially distinct areas of tumors from the same patient.

Conclusions. In this study, we developed a highly sensitive digital PCR (dPCR) platform and leveraged our assay to detect the variant III alteration of EGFR (EGFRvIII) and amplified EGFR in patient-derived glioma neurosphere cell lines, orthotopic xenograft GBM mouse models, and patient-derived tumor specimens in less than 24 hours from minute quantities of starting material.

Key Points

- EGFRvIII dPCR assay is rapid and ultrasensitive for the detection of EGFRvIII and EGFR amplified in patient tumors.
- The unique sequence generated due to fusion of exon 1 and 8 is utilized to design primer and probe specific to EGFRvIII.
- The lowest limit of quantification of EGFRvIII detection using dPCR is 0.003%.

Alterations in the epidermal growth factor receptor (*EGFR*) gene are a common finding in glioblastoma (GBM).^{1,2} Variant III of *EGFR* occurs when an in-frame deletion of exon 2–7 leads to a deletion of 267 amino acids from the extracellular domain of

the EGFR receptor.³ EGFR receptors bearing variant III are unable to bind the canonical EGFR ligands, resulting in a constitutively active state, post-treatment tumor progression, radiation and chemotherapeutic resistance, and a poor prognosis.^{1,4}

Importance of the Study

The dPCR assay we have developed is able to detect the EGFRvIII variant in tumor samples within 24 hours of surgery with a high degree of sensitivity. Furthermore, this assay is better able to capture the heterogeneous distribution of mutations in complex solid tumors than NGS due

to the requirement for only small amounts of starting material. To the best of our knowledge, this is a novel assay of dPCR for the detection of EGFRvIII in human cells and tissues, which may potentially fulfill the clinical demands for a rapid and accurate diagnostic assay.

This novel domain stems from the union of normally distant parts of the protein and creates an epitope that is not present in any normal tissues.⁵ Both amplified EGFR and EGFRvIII positive tumors have been linked to the invasive behavior of GBMs through several mechanisms.⁶

Despite the best available therapies, including surgical resection, radiotherapy, and chemotherapy, the median survival is less than 2 years for glioblastoma patients.⁷ Identification of patients that benefit from targeted therapeutic agents has been challenging. Agents targeting the EGFRvIII mutation have failed for multiple reasons including identifying a patient population that will benefit the most from these therapies.^{8,9} For instance, the novel peptide vaccine, Rindopepimut, which targets the unique epitope resulting from EGFRvIII, was well tolerated and produced an immune response.¹⁰ However, antigen escape variants were observed, indicating that tumor heterogeneity may play an important role in guiding the appropriate selection of therapy following initial treatment.^{8,11} Another approach to targeting the EGFRvIII mutation is the use of Chimeric antigen receptor (CAR) T cells, which are genetically modified T cells engineered for enhanced reactivity against tumor antigens.¹² However, this approach has shown limited ability to address GBM recurrence.¹³ One strategy to improve the efficacy of CAR T cells maybe institution of therapy shortly after tumor resection.

Current methods to detect EGFRvIII from tumor specimens include whole genome profiling using next-generation sequencing (NGS). Limitations to this approach include cost, availability of sufficient tissue, and the time required to execute the assay.¹⁴ For example, whole-genome sequencing, comparative genomic hybridization (CGH), and single nucleotide polymorphism (SNP) arrays may be subject to high error rate when trying to identify specific subclonal populations.¹⁴ Serial monitoring of tumor genotype is also unfeasible with genome-wide sequencing techniques.¹⁴ Alternatively, quantitative PCR may be used when small amounts of sample are available, but the low abundance of mutant DNA from human samples limits its quantitative impact.¹⁵ Detection of EGFRvIII through immunohistochemistry (IHC) has become more feasible with the advent of EGFRvIII-specific antibodies. However, the intricacies of IHC protocols and spatial heterogeneity of the EGFRvIII mutation limit the diagnostic potential of IHC.

The limitations of each of these assays warrant the development of an approach that can detect the EGFRvIII

mutation from small amounts of spatial heterogeneous tissue in a rapid fashion. Such an assay may become an invaluable tool to determine which patients will benefit from early intervention with targeted therapeutics agents and to monitor treatment response. Recently, a digital PCR (dPCR) assay has been approved by the FDA for diagnostic testing for BCR-ABL in chronic myeloid leukemia (CML). This assay can monitor and directly quantitate the molecular response of patients with chronic myeloid leukemia under tyrosine kinase inhibitor therapy.¹⁶

In this study, we developed a sensitive dPCR assay that is capable of detecting the variant III of EGFR in patient-derived glioma neurospheres, orthotopic xenografts, and ultimately patient-derived tissue specimens. Our assay utilizes a minor groove binding (MGB) probe and primers that recognize the unique sequence generated by the fusion of exon 1 and exon 8. Our EGFRvIII MGB probe, labeled with either VIC or fluorescein amidite (FAM), recognizes the unique fusion sequence at the junction of exon 1 and exon 8. The assay leverages around approximately 20,000 individualized reactions of dPCR to identify rare mutations from background DNA. Furthermore, the assay is able to detect the variant III of EGFR in a wide variety of specimens in less than 24 hours. The assay duplicates the findings of NGS and IHC in patient-derived tumor specimens, and in certain cases detects EGFRvIII when NGS and IHC cannot. Thus, the assay we describe herein represents a novel diagnostic approach to accurately guide new targeted therapies against tumors harboring EGFRvIII.

Materials and Methods

Cell Culture

U87 and U87 EGFRvIII were provided by Dr. Laura Johnson (University of Pennsylvania, Philadelphia, PA), and maintained in improved MEM supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 1× HEPES, and 1.0% penicillin–streptomycin. Patient-derived glioma stem cells lines NS039, HK296, HK248, and HK301 were obtained from Dr. Harley Kornblum (UCLA, Los Angeles, CA, USA) and maintained in DMEM/F12 1:1 containing 1.0% penicillin–streptomycin, 1× B-27 with vitamin A, 1 mM pyruvate, 50 ng/mL epidermal growth factor (EGF, Peprotech, Rocky Hill, NJ), 20 ng/mL fibroblast growth factor (FGF, Peprotech), 5 µg/mL heparin sulfate (Sigma Aldrich). The patient derived glioma stem cell line T4213 was obtained

from Dr. Yi Fan (UPenn) and maintained in Neurobasal A media supplemented with 1.0% penicillin–streptomycin, 0.5× B-27 without vitamin A (Invitrogen), 1 mM sodium pyruvate, 1× glutamate, 20 ng/mL EGF (PeproTech) and 20 ng/mL FGF (PeproTech). All cells were cultured in a humidified incubator maintained at 37°C and 5% CO₂. Cell lines were certified mycoplasma free by the MycoAlert® assay (Cambrex) on a regular basis.

Isolation of Cells by Microcapillary Aspiration

The isolation of selected cells was performed using a capillary-based vacuum-assisted cell acquisition system (Kuiqpick™, NeuroInDx, Signal Hill, CA).¹⁷ Calibration was initially performed under bright-field conditions, followed by the collection of individual fluorescent cells via microcapillary aspiration under fluorescence microscopy. Single cells (U87 glioma cells or patient derived GSCs) were collected in cell lysis buffer (0.2% Triton X-100 and 2 units of RNase OUT).

Control Blood Spike Experiment

All animal experiments were approved by the Institute for Animal Care and Use Committee at the University of Pennsylvania. Mouse blood was collected via terminal cardiac puncture into a 3 mL syringe containing 250 μL of acid citrate dextrose. One thousand cells of patient-derived GSCs were then spiked into the collected mouse blood. Spiked cells were processed through a CelSee microfluidic chip (CelSee, Plymouth, MI, USA) following the manufacturer's instructions, and then plated into single wells of an eight-well chamber slide using DMEM media. Cells were incubated with an adenovirus probe (Oncolys BioPharma, Fort Lee, NJ, USA) for 24 hours at 37°C. This adenovirus probe utilizes telomerase expression to drive replication of an adenovirus that contains a GFP reporter. The chamber wells were then imaged via a computer-driven semi-automated fluorescence microscope, with subsequent analysis performed via Image Pro Plus (Media Cybernetics, Rockville, MD, USA). Analysis included sorting and filtering based on reproducible parameters such as fluorescent intensity (two and a half standard deviations above background mean), cell diameter (between 7 and 70 μm), and absence of clumping or debris.¹⁸

Flank Tumor Implantation

NS039 cells were resuspended in PBS, and then 2.0 × 10⁶ cells were injected into the right flank of 6-week-old female athymic nude mice (Charles River Labs). Mice were humanely euthanized, and tumor tissue was collected following 7 weeks of growth.

Intracranial Tumor Implantation

U87 overexpressing EGFRvIII were modified to stably express mCherry and firefly luciferase (Genecopoeia, Rockville, MD, USA). Patient-derived T4213 GSCs were

modified to stably express green fluorescent protein and firefly luciferase (Genecopoeia). Cells were dissociated into single cell suspensions using TrypLE Express (Life Technologies), and 5 × 10⁵ cells were resuspended in PBS and stereotactically implanted into the right striatum of the brains of 6- to 8-week-old athymic nude mice (Charles River Laboratories). Tumor growth was monitored weekly by bio-luminescence (BLI), and mice were humanely sacrificed after 4–6 weeks post implantation or when neurological defects became significant. The brain was removed and tumor tissue was dissected for histological or molecular analysis.

Patient Samples

Minute portions of human patient glioblastoma were obtained fresh immediately following the intra-operative diagnosis of high-grade glioma. The diagnosis of glioblastoma was confirmed by a board-certified neuropathologist (MPN). The use of human tumor tissue was approved by an independent institutional review board at the Hospital of the University of Pennsylvania (HUP IRB protocol 827290).

Patients samples were also obtained from the Penn Neurosurgery Tumor Tissue Bank under a University of Pennsylvania Institutional Review Board approved the protocol. Samples were de-identified and associated with sequencing results obtained from the University of Pennsylvania's Center for Personalized Diagnostics.

Sample Preparation and Workflow

The entire workflow is illustrated in Figure 1, with each step described further below.

Total RNA Extraction from Tumor Materials

Mouse and patient-derived tumor tissue were snap-frozen in liquid nitrogen and stored at –80°C. Tumor samples from 21 patients were also collected in PreservCyt® to compare the stability of tissue for RNA extraction in PreservCyt media. Tumor tissue was ground with a chilled mortar and pestle to a fine powder and transferred to a collection tube. Total RNA was collected using the Nucleospin RNA isolation kit (Macherey-Nagel Germany) according to the manufacturer's instruction.

RNA was extracted from fresh-frozen paraffin-embedded (FFPE) patient samples as well as from frozen sections using RecoverALL™ total nucleic acid isolation kit (Invitrogen, Waltham, MA, USA) following the manufacturer's protocol.

cDNA Extraction from Tumor Materials

Total RNA from tumor material was extracted and reversed transcribed to cDNA, which was subsequently amplified by PCR as described by Picelli et al.¹⁹ The Poly(A) tail was hybridized with OligodT primer (5'-AAGCAGTGGTATCAACG CAGAGTACT₃₀VN-3') by incubating 2 μL of total RNA with 1 μL of dNTP mix and 1 μL of oligodt30VN (100 μM) at 72°C for 3 minutes. Reverse transcriptase mix was prepared

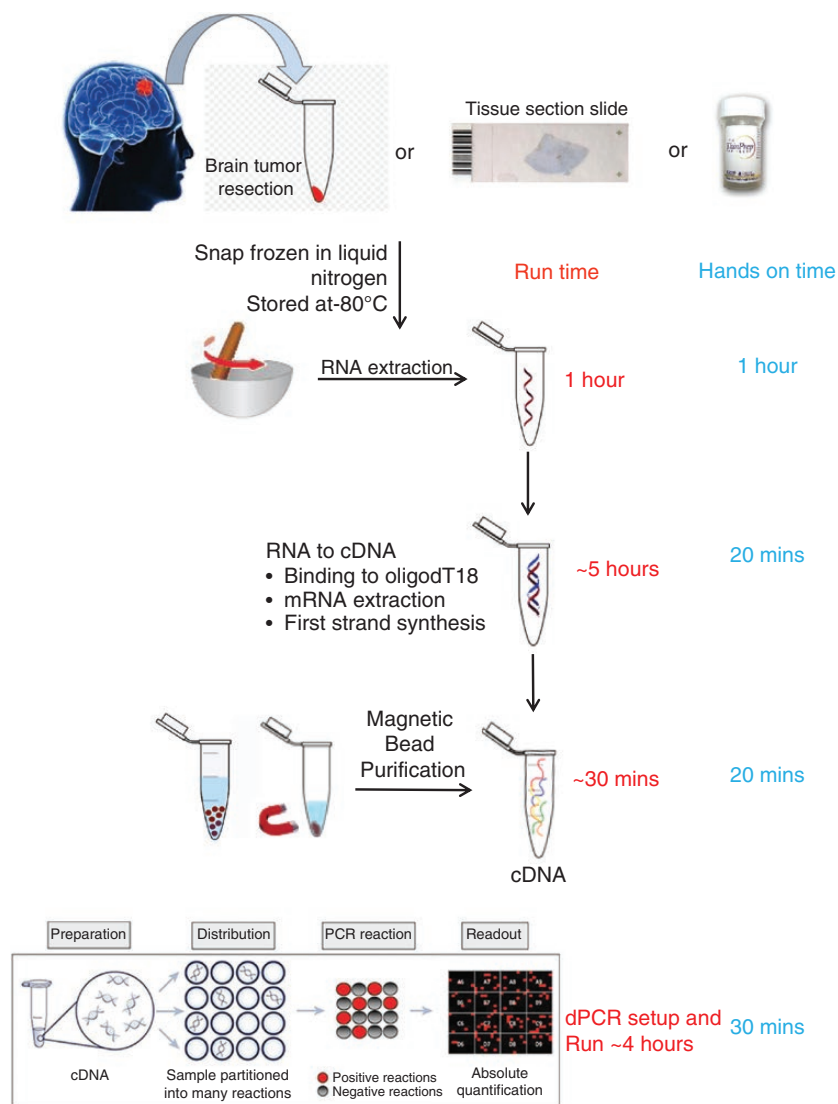


Fig. 1 Schematic representation of a potential diagnostic digital PCR assay. A tumor specimen is collected at the time of resection. This specimen is either snap-frozen in liquid nitrogen, stored at -20°C for immediate processing, or stored in PreservCyt® media. RNA is extracted from the sample mentioned as well as from FFPE slides and then undergoes first-strand cDNA synthesis followed by magnetic bead separation. cDNA is analyzed via a digital PCR platform using unique primer-probe combinations to detect EGFR WT and EGFRvIII. The highly multiplex nature of digital PCR allows for the quantification of EGFR WT and EGFRvIII copy numbers. Total assay time is less than 24 hours.

with 5 M betaine and locked nucleic acid–modified TSO (5′-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3′), and the reaction was carried out in a thermal cycler with a heated lid for 90 minutes at 42°C followed by 10 cycles at 50°C for 2 minutes and 42°C for 2 minutes. cDNA was further pre-amplified using 10 μM random IS PCR primer (5′-AAGCAGTGGTATCAACGCAGAGT-3′) with KAPA HiFi Hotstart Ready Mix (2X) (Kapa Biosystems, Wilmington, MA, USA) and thermocycled under following conditions: denaturation at 98°C for 3 minutes, followed by 18 cycles at 98°C for 20 seconds, 67°C for 15 seconds, extension at 72°C for 6 minutes, final extension at 72°C for 5 minutes. PCR products were purified using Agencourt® AMPure®XP kit (Beckman Coulter, Brea, CA, USA). cDNA concentration was determined using Qubit 3 fluorimeter utilizing

Qubit dsDNA high-sensitivity assay kit (Life Technologies Corporation, Eugene, OR, USA). cDNA was diluted further to a concentration of 10 ng/ μL before proceeding for dPCR assay.

Digital PCR Probe for EGFRvIII and EGFR WT Detection

Primers and MGB probe for EGFRvIII were designed such that the MGB probe recognizes the junction of exon 1 and exon 8. Forward and reverse primers are located on exon 1 and exon 8, respectively (Figure 2B). Forward and reverse primers and MGB probe for EGFR WT are located on exon 5, exon 6, and at the junction of exon 5 and 6,

respectively (Figure 2A). The EGFR WT MGB probe was fluorescently labeled with fluorescein amidite (FAM), and the EGFRvIII MGB probe was fluorescently labeled with either FAM or VIC. All dPCR experiments were run following the guidelines suggested by digital MIQE (Minimum Information for Publication of Quantitative Digital PCR Experiments).²⁰ Gene copies per microliter were normalized by running RNaseP (ThermoFisher, Foster City CA) on the same chip with either the EGFRWT or EGFRvIII reaction mix.

The digital PCR was carried out using the Quantstudio 3D digital PCR master mix V2 (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions using 900 nM each primer pair, and 250 nM the MGB probe. The Quantstudio 3D digital PCR chip was loaded and sealed, thus avoiding cross contamination between reaction wells. The chips were thermo-cycled using the ProFlex PCR system (Applied Biosystem, Life Technologies). Cycling conditions were as follows: one cycle of 96°C for 10 minutes, followed by 45 cycles of 54°C for 2 minutes, 98°C for 30 seconds, final extension at 54°C for 2 minutes, followed by an infinite hold at 10°C. Individual chips were read on a Quantstudio 3D Digital PCR chip reader. For each chip, the instrument generates a single .eds file that contains the processed image data, and results from a preliminary analysis. The data are then transferred onto the ThermoFisher cloud for further analysis by proprietary software.

Calculation of EGFRvIII in Tumor Samples

Tumor samples collected from GBM patients were analyzed for EGFR WT and EGFRvIII. For every tumor sample, two chips were run separately with the same concentration of cDNA containing EGFRvIII primers and probe and EGFRWT (FAM labeled) with RNaseP (VIC labeled), respectively.²¹ EGFRWT and EGFRvIII copies/ μ L were normalized to the RNaseP internal control. Amplified EGFR was considered positive if the ratio of EGFR WT to RNaseP is ≥ 1.5 .²¹ The percentage of EGFRvIII was calculated by $[(vIII \text{ copies} / \mu L) / (vIII \text{ copies} / \mu L + WT \text{ copies} / \mu L)]$ and multiplied by one hundred.

Western Blot

Cells were lysed in the presence of RIPA buffer (Thermo Scientific) supplemented with 1 mM PMSF, 1 mM sodium orthovanadate, 1x complete MINI (Roche). Protein concentration was determined using a BSA standard curve and Pierce® 660 nM reagent. Denatured protein was then loaded on 4–12% Bis–Tris gel (Invitrogen), and subsequently transferred onto PVDF membrane. Membranes were blocked in non-fat milk followed by incubation in primary antibody overnight at 4°C. Secondary antibody incubation was for 1 hour at room temperature. All antibodies were from Cell Signaling (Danvers, MA, USA). Details of antibodies and conditions are illustrated in Table S1. Immunoreactive bands were visualized via enhanced chemiluminescence by incubating membranes with the ECL™ Western Blotting Detection Reagent in accordance with the manufacturer's recommendations (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Immunofluorescence

Cells (1.5×10^3) were plated onto a chamber slide and allowed to grow overnight. Cells were then fixed using 1% formalin in PBS for 10 minutes, followed by permeabilization using 0.25% Triton X-100 for 10 minutes at room temperature. After blocking with 10% serum for 1 hour, slides were incubated with primary antibodies overnight at 4°C, followed by incubation with Alexa Fluor® 495 conjugated goat anti-rabbit IgG (H+L) (Invitrogen), for 1 hour at room temperature.

Semi-Quantitative PCR to Confirm the Presence of vIII Mutation

cDNA amplified from glioma cells (both U87 and GSC) was reverse transcribed using the following primers: 5'-ATGCGACCCTCCGGGACG (forward, exon 1) and 5'-ATTCCGTTACACACTTTGCGGC (reverse, exon 9). Expected amplicon sizes with these primers were ~1,000 bp for EGFR WT cDNA and ~200 bp for EGFRvIII cDNA. cDNA was then resolved on a TBE gel and stained with SYBR safe DNA stain (Invitrogen). Bands were visualized using the ChemiDoc imaging system (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical significance was performed in GraphPad Prism 7 using a one-way ANOVA followed by post-hoc Tukey's test. *P*-values less than 0.05 were considered significant.

Results

We developed a novel dPCR assay to detect the EGFRvIII mutation with exquisite sensitivity; the schematic workflow is shown in Figure 1. Our assay utilized a custom-designed MGB probe and primers that recognize the sequence between exon 5 and exon 6 for EGFR WT (Figure 2A), a unique fusion sequence between exon 1 and exon 8 in the variant III of EGFR (Figure 2B), sequence details are shown in Figure 2C. These newly developed probes and primers were optimized and validated using either plasmid DNA containing the wild-type EGFR sequence or EGFRvIII sequence (Figure 2D and E). Next, we determined the limit of detection (LOD) and limit of quantification (LOQ) of our assay. The LOD was defined as the lowest detectable concentration of EGFRvIII. The LOQ referred to the lowest detectable ratio of EGFRvIII to EGFR wild type. Utilizing EGFRvIII plasmid DNA, we were able to determine that the LOD of this assay was 0.625×10^{-5} ng/ μ L. In a mixture of EGFRvIII and wild-type plasmid DNA, the LOQ for our assay was 0.003% (Figure 2F). Copies per microliter of EGFRvIII detected in samples containing 0.003% vIII was found to be statistical significant ($P < .0001$) when compared with water only and 0% vIII samples.

We utilized U87 cells over-expressing the EGFRvIII (U87 vIII) to demonstrate the ability of our assay to detect the EGFRvIII mutation from cell lines. Immunoblot

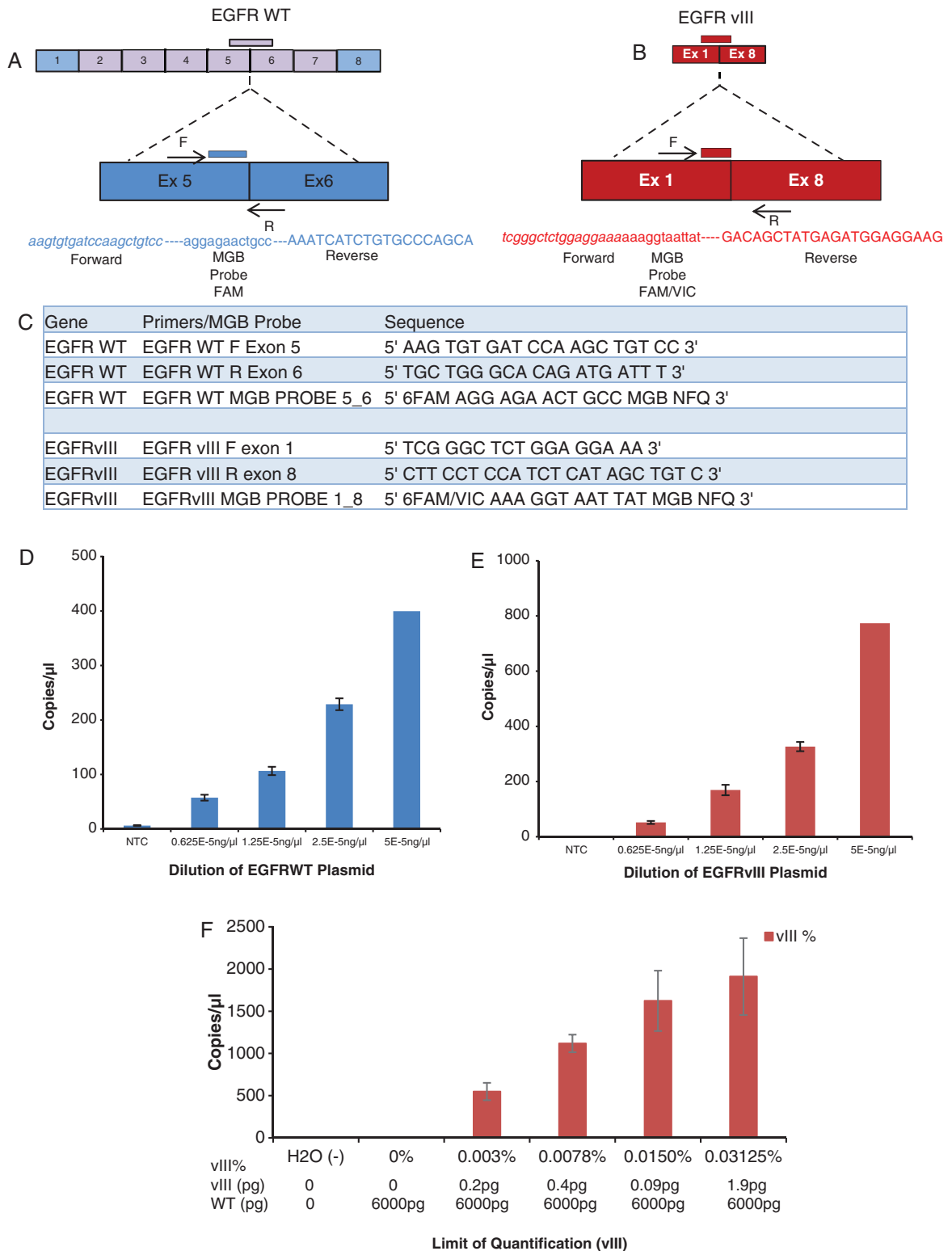


Fig. 2 Schematic representation of the location of the MGB probe and primers on EGFR. (A) The EGFR WT FAM-labeled MGB probe recognizes the junction of exon 5 and exon 6. Forward and reverse primers flank either side of the probe. (B) The EGFRvIII MGB probe is labeled with either FAM or VIC and recognizes the junction of exon 1 and exon 8. Forward and reverse primer pairs with nucleotide sequences on either side of the MGB probe. (C) MGB probe and primer pair nucleotide sequences for EGFR WT and EGFRvIII. (D) Digital PCR reaction conditions were optimized using EGFR WT primers and probe and a dilution series of EGFR WT plasmid DNA concentrations ranging from 0.625E-5 ng/µL to 5E-5 ng/µL. Our assay was able

and immunofluorescence analysis qualitatively confirmed the presence of the mutated EGFRvIII receptor in U87vIII when compared with wild-type control cells (Supplementary Figure S1 A and B). This confirmatory step allowed us to move forward with PCR analysis of these cell lines. We used conventional PCR to detect the vIII sequence in DNA extracted from U87vIII cell lines using gene-specific primers (Supplementary Figure S1C). Direct Sanger sequencing was used to detect the EGFRvIII-specific junction between exon 1 and exon 8 from isolated U87vIII cells. In order to perform Sanger sequencing, pure populations of U87 vIII cells were isolated from a mixed population of U87 WT and U87 vIII using the KUIQpick system (Supplementary Figure S1D). Supplementary Figure S1D, left panel, shows selected cell to be picked and Supplementary Figure S1D, right panel, shows empty space after the cell was picked. KUIQpick isolation allowed us to work with smaller numbers of clonally distinct cells, and extract cDNA at higher concentrations. Extracted cDNA was amplified using gene-specific primers for EGFR and then sequenced (Supplementary Figure S1E). The EGFR transcript variant 1 (accession NM_005228) sequence was acquired from NCBI's RefSeq database. Sequence readings were trimmed and aligned to the reference using Geneious version 7.1.8. Representative sequencing experiments for the single EGFRvIII clones are shown in Supplementary Figure S1F.

Following these confirmatory steps, our dPCR assay was run on cDNA extracted from both U87 WT and U87 vIII. The concentrations of cDNA tested ranged from 1.25×10^{-3} to 5×10^{-3} ng/ μ L. The EGFR WT probe and primers were able to detect expression of the wild-type isoform of EGFR in both U87 WT and U87vIII at all concentrations (Figure 3A). Similarly, the EGFRvIII probe and primer mixture were able to detect vIII in U87vIII cDNA, but not in U87WT cDNA, confirming the specificity of our probe and primer mixture in cDNA extracted from cells (Figure 3B). Figure 3C and D represents the scatter plots of vIII expression on U87 WT cDNA (Figure 3C) and U87 vIII cDNA (Figure 3D) at a concentration of 2.5×10^{-3} ng/ μ L. Non-amplified wells are denoted in yellow, and red dots represent amplification of EGFRvIII, detected by VIC signal.

The presence or absence of EGFRvIII in patient-derived glioma stem cells (GSCs) (NS039, HK301, HK296, HK248 and T4213) was initially confirmed by conventional PCR using gene-specific primers to EGFR (Figure 4A). Higher vIII was observed in NS039 and HK296 cell lines. HK301 was also found to have a small amount of EGFRvIII, but a higher abundance of EGFR WT. HK248 and T4213 did not contain any EGFRvIII. The above findings were confirmed by western blot (Figure 4B). Following validation by conventional PCR and western blot, our dPCR assay was run on cDNA extracted from our patient-derived GSCs. HK296

was found to have the highest copies per microliter of vIII followed by NS039 (Figure 4C). HK301 was also found to contain very low copies of vIII (43 copies/ μ L), but this is obscured by its abundance of EGFR wild type (Figure 4C). HK296 was also found to have high copy numbers of both the wild-type and vIII isoforms together. HK248 and T4213 were negative for both EGFR WT and vIII (Figure 4C). Given these results, we conclude that the dPCR assay is able to quantitatively detect the EGFRvIII mutation, as well as the wild-type isoform.

Next, we sought to determine the ability of our assay to detect EGFRvIII in the presence of other cell populations. One thousand NS039 and T4213 cells were spiked into mouse whole blood and then processed on CelSee microfluidic chips, as described in the Materials and Methods section. Cells isolated from CelSee chips underwent further processing to extract cDNA. One nanogram of cDNA was analyzed using our dPCR assay. EGFRvIII was detected in abundance in NS039 recovered from spiked blood (Figure 4D and E), but was not detected in T4213 (Figure 4D and F). This suggests that our assay is not influenced by other cell types and background genomic material.

Orthotopic GBM Mouse Xenografts

We validated our assay using both flank and orthotopic xenografts of glioma cells. For intracranial tumor implants, growth was monitored weekly by BLI (Figure 5A and B), and mice were humanely euthanized 4–6 weeks post implantation or when neurological defects became significant. Immediately following euthanasia, tumor tissue was dissected out of brains for histological and molecular analysis by dPCR. Six tumors were analyzed using our dPCR assay: animals 346, 347, and 348 were inoculated with T4213 and animals 349, 350, and 351 were inoculated with U87vIII cells. EGFRvIII was not detected in tumors from animals 346, 347, and 348, while tumors from animals 349, 350, and 351 demonstrated an abundance of vIII (Figure 5C). Isolated scatter plots confirm that tumors from mice inoculated with U87 vIII have a strong VIC signal, indicating that they are positive for vIII, whereas tumors from mice inoculated T4213 had no appreciable VIC signal (Figures 5D and E). Direct comparison of different xenografts highlights the ability of our assay to identify differences in vIII from distinct tumor types (Figure 5F). The vIII variant was found in both NS039 flank tumors as well as U87 vIII intracranial tumors, but was absent T4213 intracranial tumors (Figure 5F). Analysis of NS039 flank tumors also revealed increased copy numbers of both EGFR WT and EGFRvIII, demonstrating that our assay has the potential to identify the genotype of single cells from three-dimensional tumors (Figure 5F).

Fig. 2 Continued to produce a concentration-dependent increase in the copies per microliter of EGFR WT. The lowest quantity of EGFR WT DNA detected using our dPCR assay was 0.625E-5 ng/ μ L. (E) Different concentrations of EGFRvIII plasmid DNA ranging from 0.625E-5 ng/ μ L to 5E-5 ng/ μ L were assayed using our digital PCR assay and EGFRvIII primers and probe producing a concentration-dependent increase in the copies per microliter of EGFRvIII. The lowest quantity of EGFRvIII DNA detected was 0.625E-5 ng/ μ L (F) EGFRvIII plasmid DNA was assayed at concentrations ranging from 0.23 pg to 1.9 pg in a background of 6,000 pg EGFR WT plasmid DNA. The LoQ of our EGFRvIII assay is 0.23 pg of EGFRvIII DNA with sensitivity as low as 0.003%.

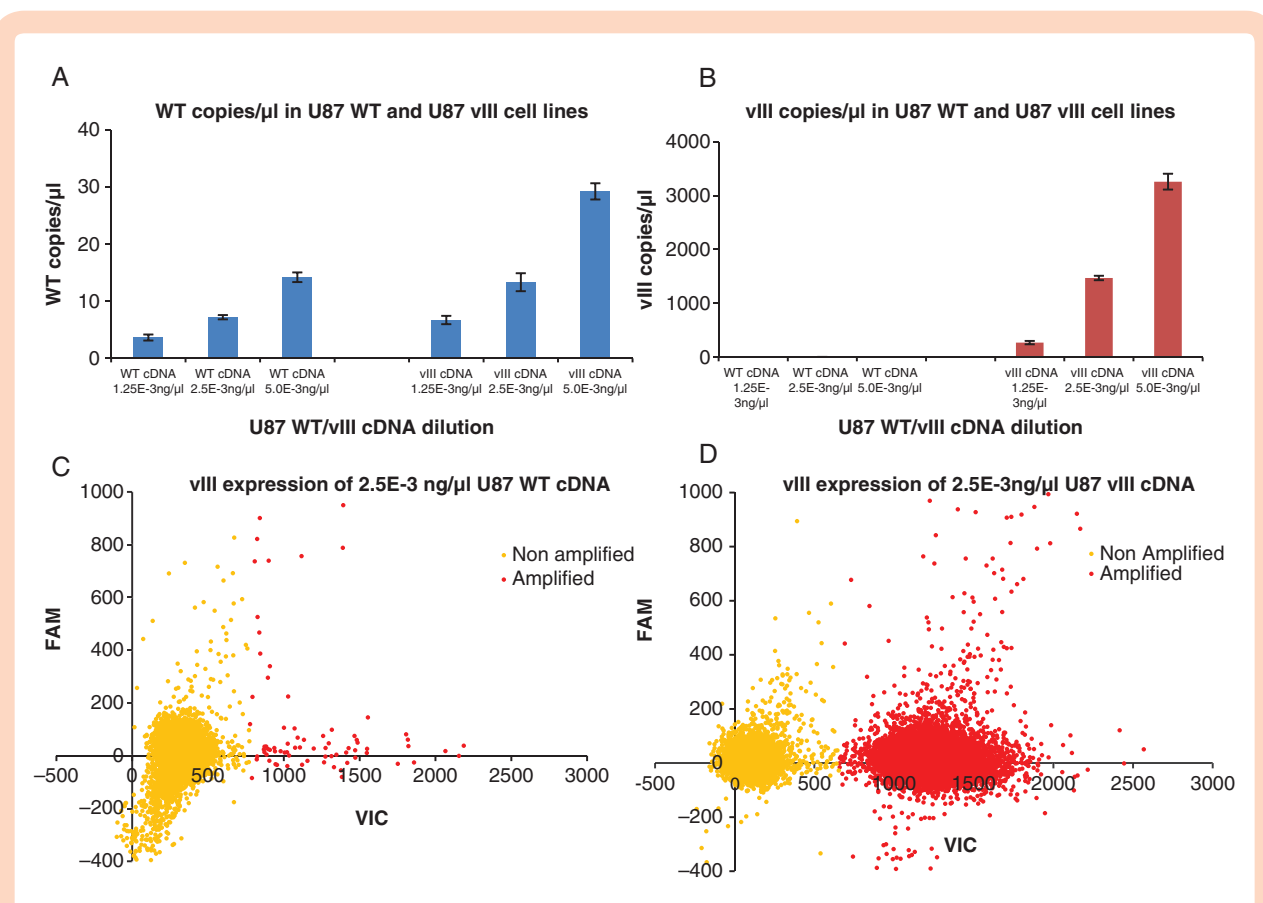


Fig. 3 Lower limit of detection of EGFRvIII digital PCR assay. (A) The digital PCR assay containing probe and primer pairs against EGFR wild type was able to detect wild-type copies in U87 WT as well as U87 vIII cDNA at concentrations as low as 1.25E-3 ng/ μ L. (B) Changing the probe and primer pairs allowed for the detection of EGFRvIII in U87 vIII cDNA at concentrations as low as 1.25E-3 ng/ μ L. EGFRvIII was not detected in U87 WT cDNA. Representative scatter plots of vIII expression (red) of U87 EGFR WT (C) and U87 EGFRvIII (D) cDNA at a concentration of 2.5E-3 ng/ μ L, respectively. Non-amplified wells are denoted in yellow.

cfRNA Quantification in Patient-derived GSCs Growth Media

To detect the EGFRvIII mutation in cell-free RNA (cfRNA), we collected growth media from different patient-derived GSC and human-derived glioma cell lines. [Supplementary Figure S2](#) represents vIII expression in cfRNA extracted from growth media of different patient-derived GSCs and human-derived glioma cell lines. cfRNA is extracted from 200 μ L of growth media as described in the Materials and Method section and spiked into whole mouse blood. Our dPCR assay was then run on cDNA extracted from total cfRNA. The expression pattern of vIII in cfRNA resembles vIII expression in actual GSC cell lines.

Patient Sample Analyses

We utilized our dPCR assay to analyze patient-derived tumor specimens in a blinded pilot study approved by the Institutional Review Board at the University of Pennsylvania. Our dPCR assay was used to analyze cDNA derived from close to 40 fresh frozen tissues, 14 FFPE

blocks, and 2 frozen tissue specimens ([Table 1](#)). EGFR WT was considered positive if the copies per microliter of amplified EGFR were greater or equal to one and a half times the copies per microliter of control or reference allele. The percentage of EGFRvIII was determined by finding the ratio of EGFRvIII to total EGFR (EGFR WT and EGFRvIII).

$$\frac{\text{vIII copies}/\mu\text{L}}{\text{vIII copies}/\mu\text{L} + \text{WT copies}/\mu\text{L}} \times 100.$$

In accordance with NGS conventions, EGFRvIII was considered positive if the percentage of vIII was greater than 10%. Patients were not enrolled in clinical trials if the percentage of EGFRvIII was less than 10% as determined by NGS.

RNA was extracted from FFPE samples that had been fixed and embedded approximately 2 to 5 years ago. Our assay was able to detect the vIII variant in tissue specimens known to carry vIII, but did not identify the mutation in tissue blocks that had been classified as negative ([Table 1](#)). Fourteen FFPE tissue blocks were analyzed for vIII via dPCR. We were able to detect vIII in three out of six blocks that had been classified as vIII positive by NGS and IHC. Interestingly, those three specimens were found to have higher expressions of

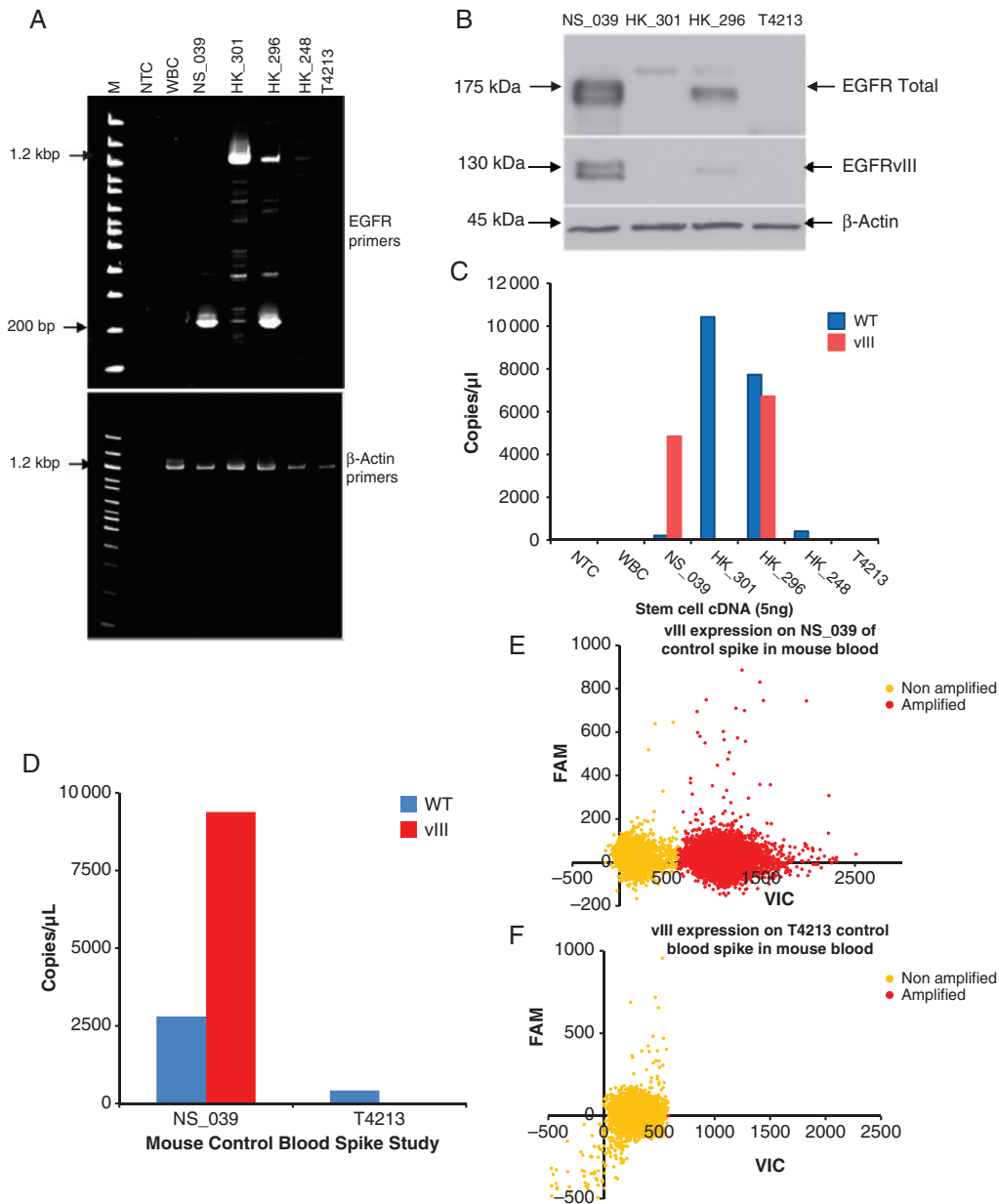


Fig. 4 Detection of EGFRvIII in patient-derived glioma neurospheres. (A) cDNA extracted from various patient-derived glioma neurosphere cell lines were amplified using EGFR ORF primers (*upper panel*), β -Actin primers (*lower panel*). (B) Western blot analysis confirms the expression of EGFR WT and EGFRvIII in various patient-derived glioma neurospheres. (C) Graphical representation of copies per microliter generated when dPCR assay was run on cDNA extracted from various patient-derived glioma neurospheres-EGFR WT (blue) and EGFRvIII (red). NS039 and HK296 both have abundant copies of vIII, whereas HK301 and HK296 have an abundance of EGFR WT. (D) Copies per microliter of EGFR WT and EGFRvIII generated when our dPCR assay was run on cDNA extracted from NS039 and T4213 cells spiked into mouse blood. These results emphasize that additional cell types do not affect the outcome of our assay. (E) Representative scatter plots of vIII expression (red) from NS039 cells (top) and T4213 (bottom) spiked into mouse blood following our digital PCR assay. Non-amplified wells are denoted in yellow.

vIII as determined by IHC (200-, 140-, and 200-fold, respectively) when compared with NGS percentages of 80%, 57%, and 95%, respectively. RNA extracted from FFPE samples undergoes fragmentation over time, which explains the detection of vIII by dPCR in higher vIII % samples.

Fresh primary tumor samples were tested in parallel for the presence of EGFRvIII with both our dPCR assay

and NGS. Our assay was able to duplicate the findings of NGS for the majority of the specimens. However, we encountered three discordant results (samples F5, F13, and F21 in [Table 1](#)), where the tumor specimen that had been characterized as vIII negative by NGS, but was found to be vIII positive by our dPCR assay. To investigate this further, the corresponding FFPE tissue block for the F5 sample

was re-sequenced and also examined by RT-PCR. Results of the repeat NGS sequencing and the RT-PCR profiling confirmed that the F5 sample is indeed EGFRvIII positive. Similarly, vIII expression in sample F21 was also confirmed by RT-PCR. Expression of vIII in all of these samples (F5, F13, and F21) was also confirmed by semi-quantitative PCR (results not shown). This suggests that the dPCR assay we have developed is able to detect the EGFRvIII variant with a high degree of accuracy, and may be able to identify the variant in specimens that were initially misclassified as negative for vIII. Furthermore, we were able to detect vIII in fresh tumor specimens down to as little as 100 μ g (data not shown). Storage of tumor specimens in a preservative such as PreservCyt[®] did not affect the quality or readout of our assay ([Supplementary Table S2](#)). A scatter plot of patient samples is shown [Supplementary Figure S3](#). Red dots represent EGFRvIII amplified with VIC signal in [Supplementary Figure S3A](#) and [B](#), blue dots represent FAM signal showing EGFR WT amplified. [Supplementary Figure S3C](#) and [D](#) represents EGFRvIII-negative patient sample, as no VIC signal was detected. The turnaround time for the dPCR assay is less than 24 hours, allowing for rapid detection of the EGFRvIII variant and thus far earlier enrollment of patients on trials for vIII-targeted therapies.

Discussion

Glioblastoma is the most common and clinically aggressive form of primary brain tumor. Mutations in EGFR are a common finding. The variant III version of EGFR is found in up to 30% of GBM patients, and is produced by an in-frame deletion of 801 bp from exon 2 to exon 7 (EGFRvIII).^{2,3} Loss of the EGFR extracellular domain in EGFRvIII is associated with ligand-independent constitutive activation, and elevated activation of signaling pathways implicated in proliferation, motility, and metastatic dissemination.^{22,23} Antibodies against EGFR and small molecules targeting EGFR have been used to treat patients with GBM, but were not clinically efficacious.²⁴ CAR-T cells targeting EGFRvIII can selectively eliminate EGFRvIII tumor cells.^{24–26} Although CAR-T efficiently eliminates EGFRvIII tumor cells in some patients, it did not produce a survival advantage in recurrent disease.¹³ Early targeting of GBM cells harboring the EGFRvIII mutation may circumvent issues involved in recurrence. Targeting the vIII mutant oncoprotein with a peptide vaccine strategy (rindopepimut) is another approach. In phase 2 studies, rindopepimut was well-tolerated and produced an immune response.¹⁰ However, antigen escape variants were noted, indicating that tumor cells suppress mutant EGFR expression in order to reach an optimal equilibrium for growth. The reemergence of the clonal EGFR mutation is observed after drug withdrawal, suggesting a highly specific, dynamic, and adaptive route by which cancers can evade therapies that target oncogenes maintained on extra chromosomal DNA.²⁷

Faster diagnosis of disease will allow for earlier initiation of novel therapeutic agents, allowing us to fully capitalize on our molecular understanding of glioblastoma. Furthermore, it is expected that the development of a

therapeutic strategy targeting EGFRvIII could have a major impact on the survival of patients carrying this alteration, thus establishing methods to measure EGFRvIII for biomarker purposes remains a major goal.

Efforts have been made to validate biomarkers that reflect the genetic profile of GBM, which would facilitate outcome prediction as well as help design targeted therapies.^{25,26} It is also conceivable that the molecular characterization of tumor tissue, circulating tumor cells, microvesicles, and cfRNA would help to elucidate genomic variations that occur during tumor evolution and disease recurrence, including the accumulation of late chromosomal deletions, amplifications, and mutations. Presently, NGS is utilized to identify *EGFR* mutations from patient-derived tissue specimens. However, NGS can take up to 3 weeks to identify a mutation due to clinical workflows. Studies have attempted to map EGFRvIII mutation via qPCR and southern blot using a series of primers corresponding to introns 1 to 6, and exon 7 and exon 8; however, this process is time intensive.^{15,27,28} Furthermore, qPCR relies on reference controls, which may be problematic since expression levels of endogenous controls and their transcripts differ between samples. Also, qPCR does not offer adequate sensitivity for the detection of a low copy number mutation.

Digital PCR potentially delivers a better solution. Digital PCR allows for the direct quantification of nucleic acids by partitioning the reaction mixture into 20,000 wells.^{29–32} A positive well indicates the presence of a single molecule in a given reaction.^{29–32} The digital PCR platform then applies a Poisson distribution to the reaction wells to produce a high-confidence measurement of the original target concentration.³³ Thus digital PCR has a higher degree of sensitivity and precision than quantitative PCR. Our assay is able to quantify the presence of vIII at the level of 1 pg in a background of 6,000 pg of EGFR wild type ([Figure 2F](#)).

We have utilized this assay to detect EGFRvIII in a small cohort of GBM tumor samples. We detected the presence of vIII in 5 out of 40 total patients. Original FFPE tissue blocks were sent for clinical next-generation sequencing at our institution, as well as in some cases to NeoGenomics Laboratories, Inc. Parallel testing was able to confirm the presence of EGFRvIII in the primary tumor, thus highlighting the sensitivity of our assay for detecting EGFRvIII in patient-derived tissue specimens. Our assay is able to rapidly and accurately characterize the vIII variant in brain tumors and other solid tumors when NGS is unable to detect the presence of the mutation.

This study focuses on the development of a very sensitive method for identifying EGFRvIII and amplified EGFR in tumors by dPCR. Our assay utilizes the unique sequence generated due to the fusion of exon 2 and exon 8. Under our established experimental conditions, the dPCR assay for EGFRvIII is able to identify EGFRvIII mutation in cell lines ([Figure 3](#)), patient-derived stem cells ([Figure 4](#)), and in xenograft tumors ([Figure 5](#)). With this dPCR assay, EGFRvIII can be detected in patients within a day of resection ([Figure 1](#)).

The results described in this report suggest that a dPCR strategy can be successful in detecting EGFRvIII deletion without the need to sequence the whole genome of a patient. Inclusion of a digital PCR assay to detect the presence of EGFRvIII mutation following tumor resection will help

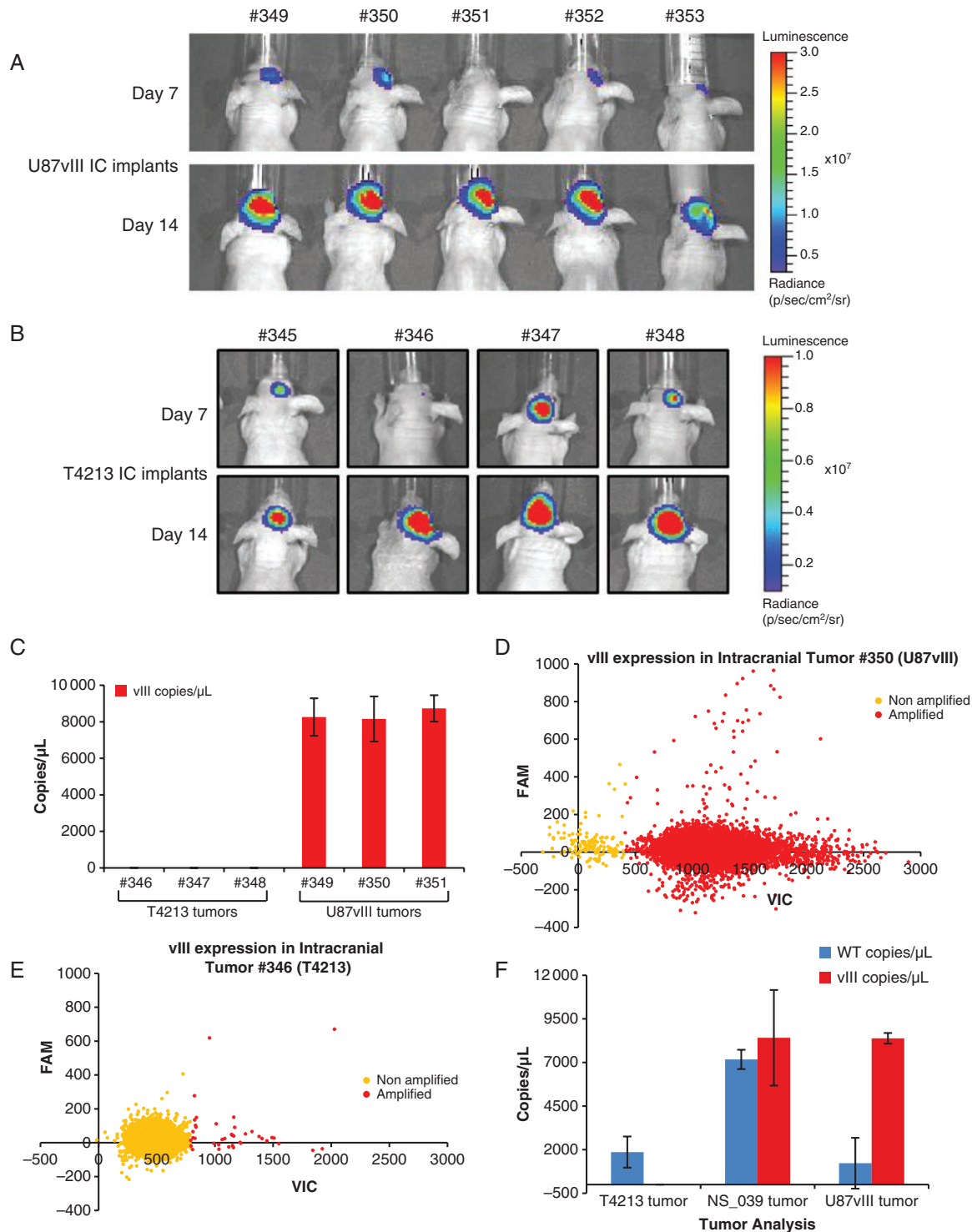


Fig. 5 EGFRvIII expression in orthotopic glioma xenografts. (A) Serial bioluminescent images over a period of a few weeks from mice that underwent orthotopic implantation of U87 EGFRvIII cells indicating tumor growth. (B) A similar set of bioluminescent images over a period of a few weeks from mice that underwent orthotopic implantation of EGFRvIII-negative T4213 cells confirming tumor growth. (C) Tumor tissue was collected from mice 4 to 6 weeks following orthotopic implantation of U87 vIII or T4213 cells. Digital PCR analysis identified EGFRvIII in U87 orthotopic tumors, but not in T4213 tumors. (D and E) Representative scatter plots of vIII copies from U87 vIII and T4213 orthotopic tumors. Non-amplified wells are denoted in yellow. (F) Comparison of vIII and WT expression across different tumor types including T4213 orthotopic, NS039 flank, and U87 vIII orthotopic tumors.

Table 1 Clinical diagnosis and demographic characteristics of patient samples. dPCR values represent the summary of expression of EGFRvIII and EGFR WT

Samples	Groups (NGS vIII result) (M: male, F: female) (age at surgery)	NeoGenomics	dPCR values			
			amp EGFR/control	EGFRvIII/control	amp EGFR	vIII%
F1A	Fresh, ND, M, 60yrs	Not tested	6.55	0.00		0%
F1B	Fresh, ND, M, 60yrs	Not tested	13.29	0.00		0%
F2	Fresh, ND, M, 68yrs	Not tested	192.59	0.02	+	0%
F3	Fresh, ND, M, 43yrs	Not tested	223.76	0.1	+	0%
F4	Fresh, ND, M, 48yrs	Not tested	284.67	3.17	+	1%
F5	Fresh, ND, M, 48 yrs	Detected	436.24	166.98	+	28%
F6	Fresh, ND, F, 54yrs	ND	93.52	0.00		0%
F7	Fresh, ND, F, 72yrs	Not tested	138.23	0.02		0%
F8	Fresh, ND, M 62 yrs	ND	98.31	0.00		0%
F9	Fresh, ND, M, 47yrs	Not tested	129.93	0.00		0%
F10	Fresh, ND, F, 89yrs	Not tested	75.25	0.02		0%
F11	Fresh, ND, M, 62yrs	ND	100	0.01		0%
F12	Fresh, ND, M, 61yrs	Not tested	102.45	0.00		0%
F13	Fresh, ND, M, 73yrs	Not tested	55.16	108.03	+	66%
F14	Fresh, ND, M, 46yrs	Not detected	101.31	0.03		0%
F15	Fresh, ND, M, 55yrs	Not detected	100	0.16		0%
F16	Fresh, ND, M, 50yrs	Not tested	104.82	0.03		0%
F17	Fresh, ND, M, 72yrs	Not tested	100	0.01		0%
F18	Fresh, ND, F, 84yrs	ND	97.58	0.00		0%
F 19	Fresh, ND, M, 55yrs	ND	100.32	0.37		0%
F 20	Fresh, ND, M, 64yrs	ND	34.12	0.01		0%
F21	Fresh, ND, M, 61yrs	Detected	100	124.66		56%
F22	Fresh, ND, F, 79yrs	ND	66.82	0.00		0%
F23	Fresh, QNS, F, 53yrs	ND	100	0.00		0%
F24	Fresh, QNS, F, 69yrs	Not tested	138.06	0.01		0%
F25	Fresh, Not tested, M, 85yrs	Not tested	132.81	0.01		0%
F26	Fresh, Not tested, M, 54yrs	Not tested	30.87	0.01		0%
F27	Fresh, ND, M, 67yrs	ND	99.94	0.01		0%
F28	Fresh, Not tested, F, 59yrs	ND	100.54	0.02		0%
F29	Fresh, Not tested, M, 67yrs	Detected	94.31	83.81		47%
F30	Fresh, Not tested,		120.13	0.003		0%
F31	Fresh, ND, M, 67yrs	Not tested	100	0.21		0%
F32	Fresh, ND, F, 54yrs	Not tested	82.19	0.00		0%
F33	Fresh, ND, F, 33yrs	Not tested	100	0.00		0%
F34	Fresh, QNS, M, 77yrs	ND	52.12	0.01		0%
F35	Fresh, Pending, F, 65yrs	ND	100.02	0.13		0%
F36	Fresh, Pending, F, 62yrs	ND	100.2	0.03		0%
F37	Fresh, Pending, M, 68yrs	Detected	97.06	83.01		46%
F38	Fresh, Pending, F, 73yrs	Pending	66.9	0.00		0%
F39	Fresh, Pending,	Pending	99.08	0.00		0%
F40	Fresh, Pending	Pending	98.25	0.00		0%
Fr 1	Frozen, 70%, F, 51yrs		87.59	109.54		56%
Fr 2	Frozen, 26%, M, 48yrs		113.67	63.22		36%
FFPE 1	FFPE, ND, F, 63yrs		49.6	0.04		0%

Table 1 Continued

Samples	Groups (NGS vIII result) (M: male, F: female) (age at surgery)	NeoGenomics	dPCR values		
			amp EGFR/ control	EGFRvIII/ control	amp EGFR vIII%
FFPE 2	FFPE, ND, M, 64yrs		2.22	0.00	0%
FFPE 3	FFPE, 95%, F, 52yrs		71.26	9.56	12%
FFPE 4	FFPE, ND, M, 56yrs		74.87	0.00	0%
FFPE 5	FFPE, 80%, M, 65yrs		85.55	94.85	53%
FFPE 6	FFPE, 57%, F, 52yrs		92.85	22.65	20%
FFPE 7	FFPE, ND, F, 46yrs		0.81	0.62	0%
FFPE 8	FFPE, ND, M, 53yrs		22.17	0.00	0%
FFPE 9	FFPE, ND, F, 54yrs		11.75	0.00	0%
FFPE 10	FFPE, 10%, M, 60yrs		96.97	0.00	0%
FFPE 11	FFPE, 41%, F, 54yrs		52.87	0.00	0%
FFPE 12	FFPE, 14%, F, 76yrs		147.9	0.00	+
FFPE 13	FFPE, ND, F, 72yrs		126.25	0.00	0%
FFPE 14	FFPE, ND, F, 22yrs		11.38	0.00	0%

F-Fresh, Fr-Frozen, FFPE- Formalin-fixed paraffin-embedded, ND-Not detected, QNS-Quantity not sufficient, Red denotes discordant results from NGS.

guide the appropriate treatment. Early detection of EGFRvIII may allow for institution of targeted treatments, such as CAR-T, allowing for earlier elimination of aggressive tumor populations. Detection of the EGFRvIII mutation via digital PCR represents a superior diagnostic assay in terms of speed and sensitivity.

Supplementary Material

Supplementary material is available at *Neuro-Oncology Advances* online.

Keywords

Glioma | dPCR | EGFRvIII | EGFR | GBM | diagnostic assay.

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Conflict of interest statement. The University of Pennsylvania has submitted a patent application based on the technology described in this manuscript. M. Alonso-Basanta has received speakers' bureau honoraria from Varian and speakers' bureau travel from IBA. D.M. O'Rourke reports receiving a commercial research grant from Novartis; has ownership interest (including stock, patents, etc.) in Isoma Diagnostics; is a consultant/advisory board member of Isoma Diagnostics; and has provided expert testimony for clinical expert testimony. G.D. Kao has ownership interest (including stock, patents, etc.) in Liquid Biotech USA. J.F. Dorsey ownership interest (including stock, patents, etc.) in Liquid Biotech USA, Inc.

Authorship statement

D.S. and J.F.D. designed the study. D.S. and S.S. performed experimental work. D.S. and S.S. performed data analyses. D.S. produced texts and figures. M.P.N., M.A.-B. and Z.A.B. provided patient material and data. D.S., S.S., G.K., Z.A.B., D.M.O., M.P.N., and J.F.D. contributed to the writing and revision of the manuscript.

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