Supplementary Appendix

Title: Cell-free DNA as a diagnostic analyte for molecular diagnosis of vascular malformations

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Supplemental Methods

Multiplex droplet digital PCR (ddPCR)

A multiplex ddPCR assay to detect the four most common *PIK3CA*-activating variants was previously developed.[1] Primers for exon 9 and 20 and probes containing locked nucleic acid (LNA) bases with either 5'-HEX™ or 5'-FAM™ reporter dye and 3' Iowa Black® fluorescent quencher were ordered (Integrated DNA Technologies (IDT), Coralville, IA, USA).[1] Variant locations are based on NM_006218.4. Each 22 µL reaction contained 10 µL ddPCR Supermix, template DNA (15 ng maximum), exon 9 primers 900 nM, exon 20 primers 900 nM, and probes in the following concentrations: 250 nM exon 9 WT, 250 nM exon 20 WT, 250 nM p.Glu542Lys (c.1624G>A), 125 nM p.Glu545Lys (c.1633G>A), 375 nM p.His1047Leu (c.3140A>T) and 250 nM p.His1047Arg (c.3140A>G). All wild-type (WT) probes had HEX fluorophores while all activating variant probes had FAM fluorophores with the exception of p.His1047Leu for which both FAM and HEX probes were designed and combined in a 1:1 ratio. Droplet generation was completed per protocol (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf, Bio-Rad, Hercules, CA) and PCR was performed using the following cycling conditions: 95 °C for 10 minutes, followed by 50 cycles of 94 °C for 30 seconds and 57.3 °C for 2 minutes with a ramp rate of 1 °C/sec, followed by a final extension at 98 °C for 10 minutes. ddPCR plates were incubated at 12 °C for at least 4 hours following droplet generation. Droplet fluorescence was detected with the Bio-Rad QX200 Droplet Reader within 24 hours. Data analysis was performed with Bio-Rad QuantaSoft software. Each run included a no template control, WT gDNA control, and a WT cfDNA control. For positive controls, gene blocks of 170 bp were designed for each variant and associated WT sequence (IDT). Runs included four positive controls which consisted of a 10-15% dilution of variant gene block in WT gene block for each of the four activating variants. Fifteen genomic DNA samples from patients with known somatic *PIK3CA* variants previously detected on singleplex ddPCR were run through the multiplex assay. The data was analyzed by three independent observers.

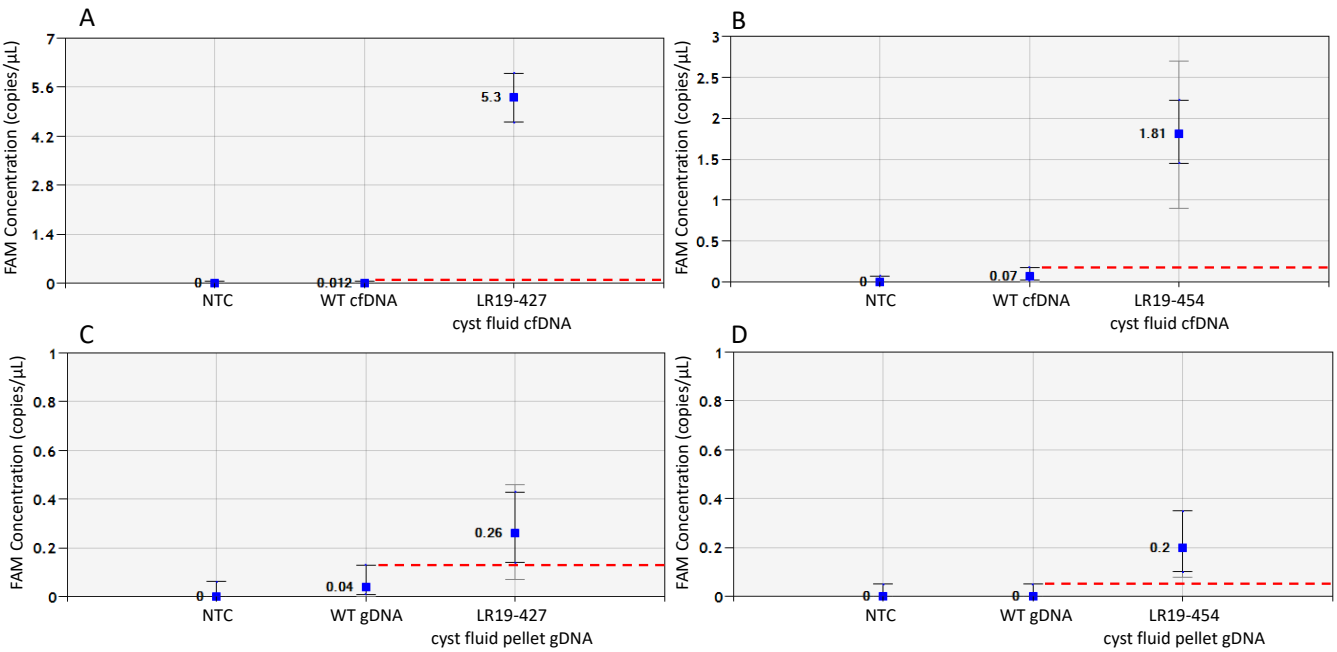

Figure S1: Determination of ddPCR positivity using total error.

FAM concentration for a sample of interest is compared to the corresponding wild-type (WT) and no template control (NTC) FAM concentration within QuantaSoft software. The FAM fluorophore was used to detect variant droplets. Samples are considered positive for the variant if the 95% confidence intervals for total error, defined as the greater of either the technical error (Poisson error, black error bars) or the empirical error (standard error of the mean, light gray error bars), do not overlap with the 95% confidence intervals for total error for the WT control. The maximum value for the WT control total error 95% confidence interval is shown as a dashed red line. A and B display clearly positive samples for cyst fluid cfDNA with no overlap between sample and WT cfDNA error bars. C and D display the results for the corresponding cyst fluid pellets. In C, the total error, as displayed by the light gray error bars, overlaps with the WT gDNA error bars, therefore this sample was considered negative for the variant despite having 12 variant droplets. In D, the total error for the sample approaches the WT gDNA error bars, but they do not overlap, therefore this cyst fluid pellet was considered positive for the variant.

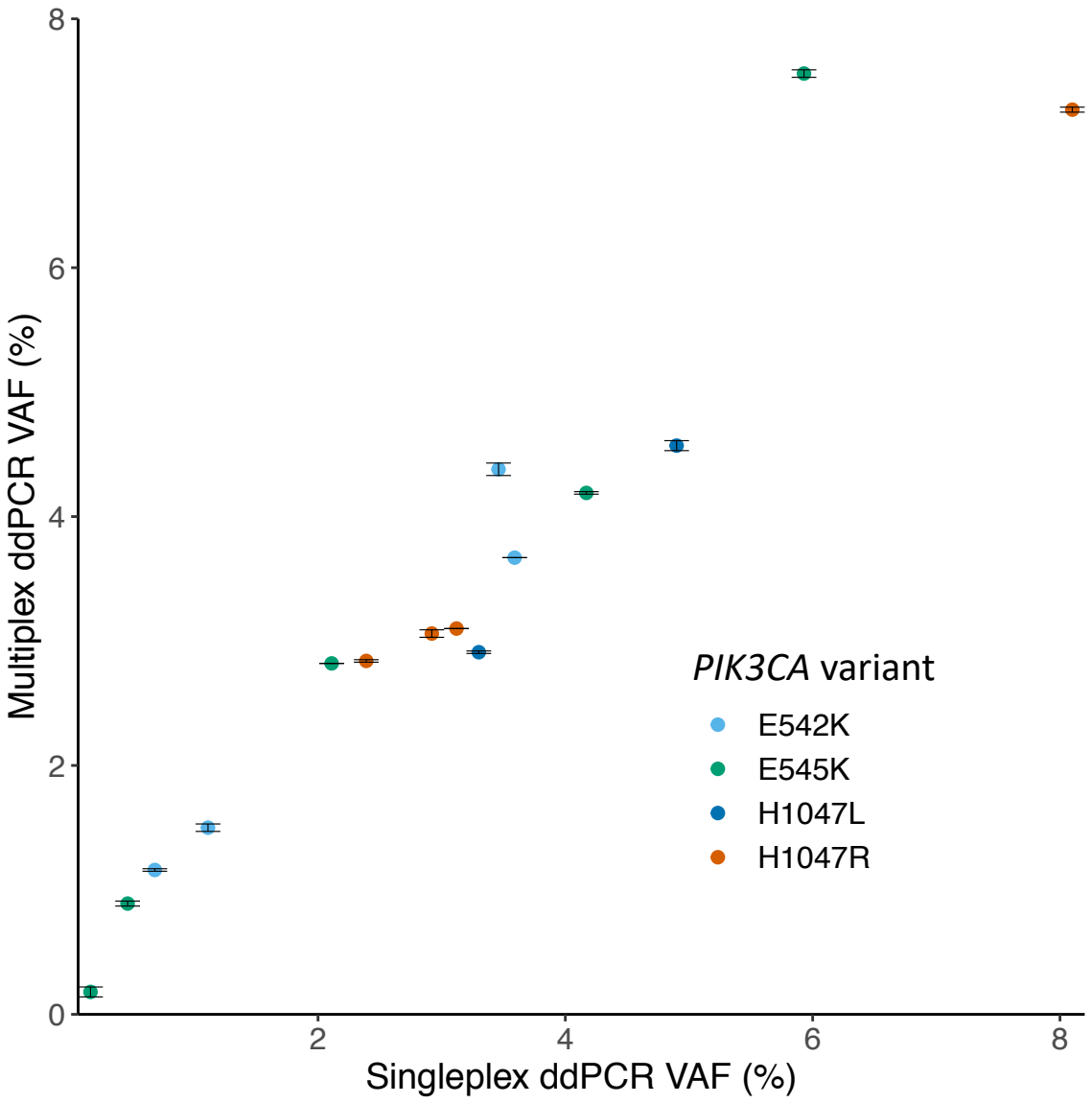

Figure S2. Multiplex droplet digital PCR (ddPCR) validation against singleplex ddPCR. Results of 15 genomic DNA samples assayed for *PIK3CA* variants on both singleplex ddPCR and multiplex ddPCR shows consistency in variant allele fraction (VAF) across all variants and across a range of VAF from 0-8%. Multiplex ddPCR VAF is a mean of three independent observers. Error bars denote standard deviation.

Table S1. Plasma cfDNA ddPCR results for patients with lymphatic malformations

Patient	Sex	Age[a]	Malformation Location	*PIK3CA* Variant	Tissue VAF (%)[b]	Plasma volume (mL)	Plasma cfDNA VAF (%)[b]	Plasma cfDNA droplets (Var/WT)
LR14-285	F	20	Abdomen + CLOVES	p.E545K (c.1633G>A)	1.8	8.0[d]	0.6	99/16176
LR16-033[c]	F	12 mo	Parotid	p.H1047R (c.3140A>G)	1.2	3.0	NEG	2/3191
LR16-145[c]	M	12 mo	Mediastinum	p.E542K (c.1624G>A)	0.1	0.9	NEG	0/800
LR16-263[c]	M	2 mo	Tongue/ FOM	p.Q546K (c.1636C>A)	9.5	0.6 0.3 2.0 2.0	NEG NEG NEG NEG	2/160 0/171 0/682 0/1205
LR16-266[c]	M	15 mo	Neck	p.H1047R (c.3140A>G)	0.5	1.4	NEG	3/1671
LR16-279[c]	F	5	Neck	p.E542K (c.1624G>A)	3.4	1.3	NEG	0/372
LR17-119[c]	F	21	Lip	p.H1047R (c.3140A>G)	2.8	2.5[d]	NEG	0/2040
LR17-120[c]	F	8	Parotid	p.E542K (c.1624G>A)	0.7	0.8	NEG	1/657
LR17-121[c]	M	2	Parotid	p.H1047R (c.3140A>G)	5.0	0.8 0.8	NEG NEG	0/451 1/1952

LR17-137[c]	F	3	Neck	p.E545K (c.1633G>A)	1.6	0.4	NEG	0/164
LR17-142[c]	M	4 mo	Hand	p.H1047R (c.3140A>G)	0.2	0.4	NEG	0/654
LR18-366	F	6	Lip	p.E545K (c.1633G>A)	4.0	0.4	NEG	0/391
LR18-533	M	3	Parotid	p.E542K (c.1624G>A)	8.3	1.4	NEG	0/945
LR18-535	F	14 mo	Neck	p.H1047R (c.3140A>G)	7.2	1.8	NEG	1/5478
LR18-537	M	23 mo	Forehead	p.E542K (c.1624G>A)	10.2	2.0[d]	NEG	3/2337
LR18-573	M	12	Lip	p.H1047R (c.3140A>G)	2.8	0.9	NEG	1/1196
LR19-011	F	14	Postauricular	p.H1047R (c.3140A>G)	6.8	0.8	NEG	0/269
LR19-345	M	21 mo	Parotid/FOM	p.E545K (c.1633G>A)	4.0	0.9	NEG	0/1359
LR19-347	M	2 mo	Neck	p.E545K (c.1633G>A)	6.2	0.4 0.4 0.5	NEG NEG NEG	0/201 0/651 1/1099
LR19-350	F	14	Tongue	p.H1047R (c.3140A>G)	2.0	0.8	NEG	0/468
LR19-351	M	11	Parotid	p.E545K (c.1633G>A)	0.7	0.8	NEG	1/1451

LR19-352	M	2	Buccal space	p.E545K (c.1633G>A)	2.5	0.4	NEG	0/513
LR19-404	M	17	Tongue	p.H1047R (c.3140A>G)	9.1	0.4 3.8[d] 3.6[d]	NEG NEG NEG	0/301 1/2743 0/2989
LR19-416	F	18 mo	Neck	p.E545K (c.1633G>A)	4.9	0.3	NEG	0/256
LR19-427	M	14	Parotid	p.E542K (c.1624G>A)	1.1	2.2[d]	NEG	0/483
LR19-454	F	2	Parotid/FOM	p.E542K (c.1624G>A)	9.4	4.0[d] 4.5[d]	NEG NEG	1/970 1/2256
LR19-474	F	2	Neck	p.E545K (c.1633G>A)	5.9	3.0[d]	NEG	2/3013

Abbreviations: cfDNA – cell free DNA, mo – months old, mL – milliliters, VAF – variant allele fraction, NEG – no variant detected, Var – variant, WT – wild-type

[a] Age at earliest plasma collection, in years unless otherwise stated.

[b] VAF calculated using ratio of droplet concentrations or ratio of smMIPs reads (variant reads/total reads). Only reported for samples in which sample variant concentration was statistically different from WT gDNA control variant concentration based on 95% total error confidence intervals.

[c] Patient previously reported.[2]

[d] Sample collected in Streck tube.

Table S2. Lymphatic malformation cyst fluid pellet gDNA multiplex ddPCR results

Patient	*PIK3CA* Variant by cfDNA multiplex	Multiplex ddPCR VAF (%)[a]	Multiplex ddPCR Droplets (Var/WT)	Singleplex ddPCR VAF (%)[a]	Singleplex ddPCR Droplets (Var/WT)
LR16-265[b]	p.H1047R (c.3140A>G)	0.9	11/1173	0.7	9/1355
LR19-442	p.E545K (c.1633G>A)	NEG NEG	6/7977 8/8714	NEG 0.1	6/12109 17/13259
LR19-443	N/A	N/A	-/20225	-	-
LR19-446	p.E542K (c.1624G>A)	No pellet available			
LR19-481[c]	p.E545K (c.1633G>A)	0.1	15/9596	NEG	9/12508

Abbreviations: cfDNA – cell free DNA, NEG – no variant detected, VAF – variant allele fraction, Var – variant, WT – wild-type

[a] VAF calculated using droplet concentrations and only reported for samples in which sample variant concentration was statistically different from WT gDNA control variant concentration based on 95% Poisson confidence intervals.

[b] Patient previously reported.[2] No variant was detected in previous publication.

[c] Patient's sample collected in Streck tube.

Supplementary Appendix Citations

1. Rowlands V, Rutkowski AJ, Meuser E, Carr TH, Harrington EA, Barrett JC. Optimisation of robust singleplex and multiplex droplet digital PCR assays for high confidence mutation detection in circulating tumour DNA. Sci Rep. 2019;9(1):12620.

2. Zenner K, Cheng CV, Jensen DM, Timms AE, Shivaram G, Bly R, et al. Genotype correlates with clinical severity in PIK3CA-associated lymphatic malformations. JCI insight. 2019;4(21).