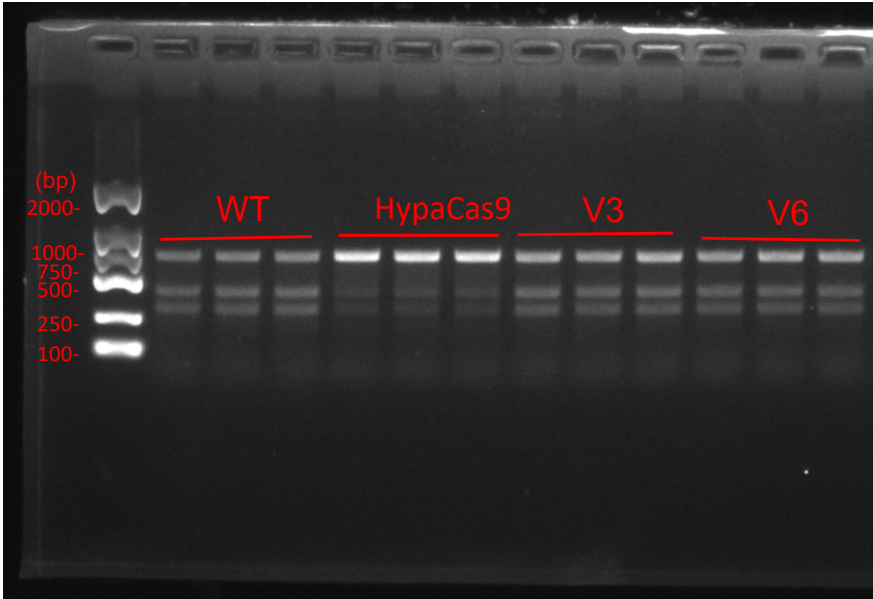
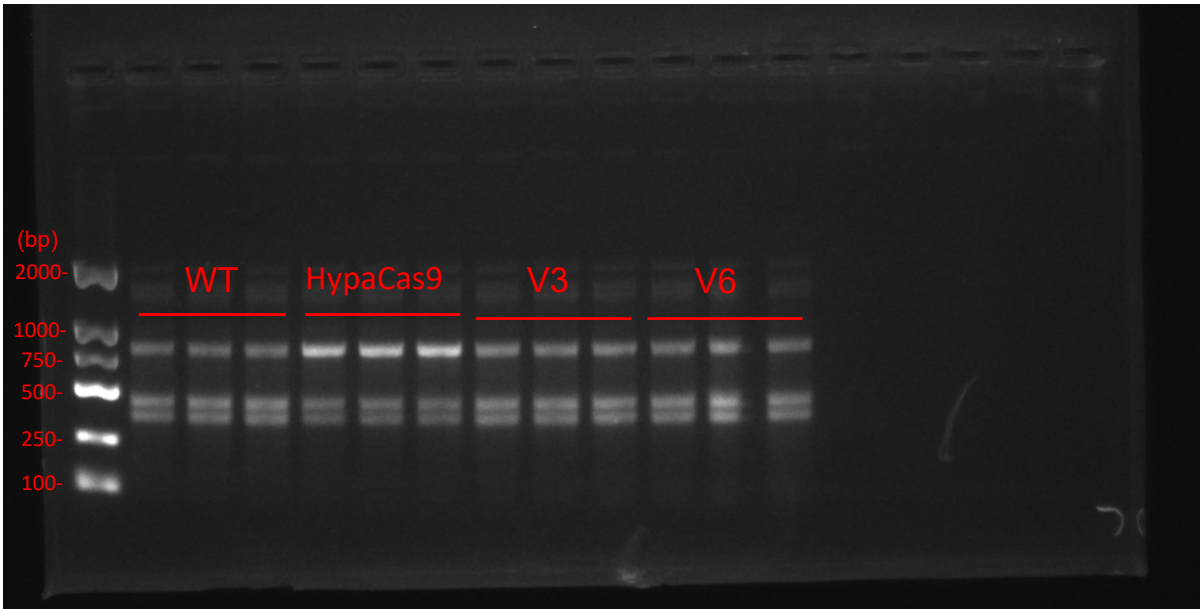


Figure S1, Uncropped gel images from PAGE experiments for T7EI.

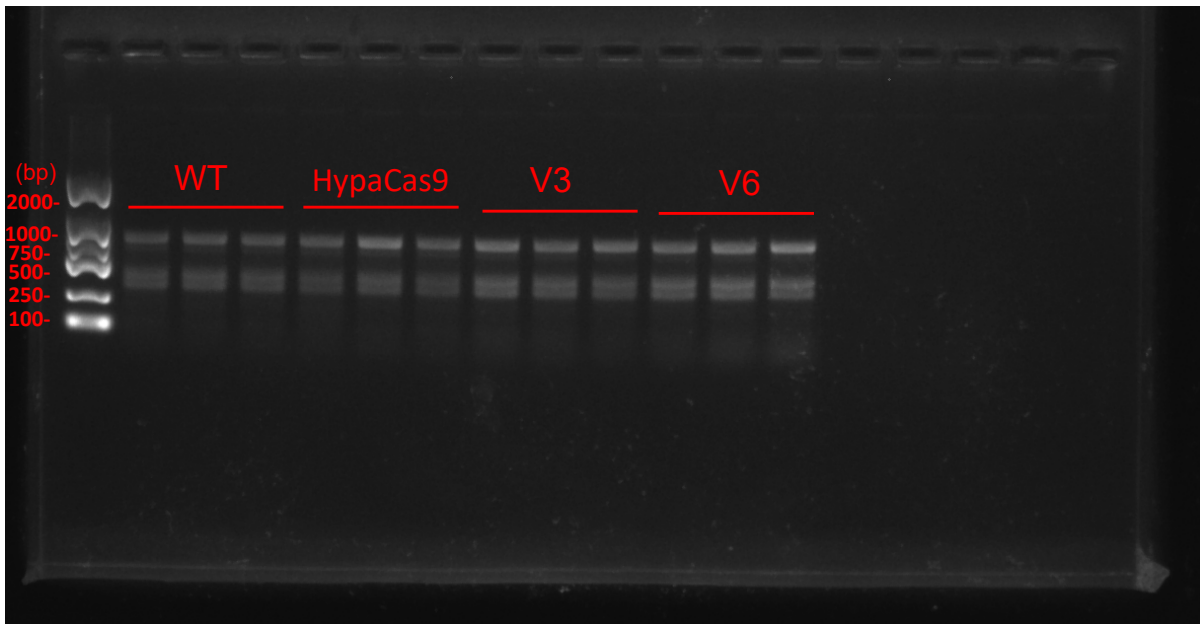
S1.1. EMX1-1 (DNA Marker (100~2000 bp), Sangon, #B500350)



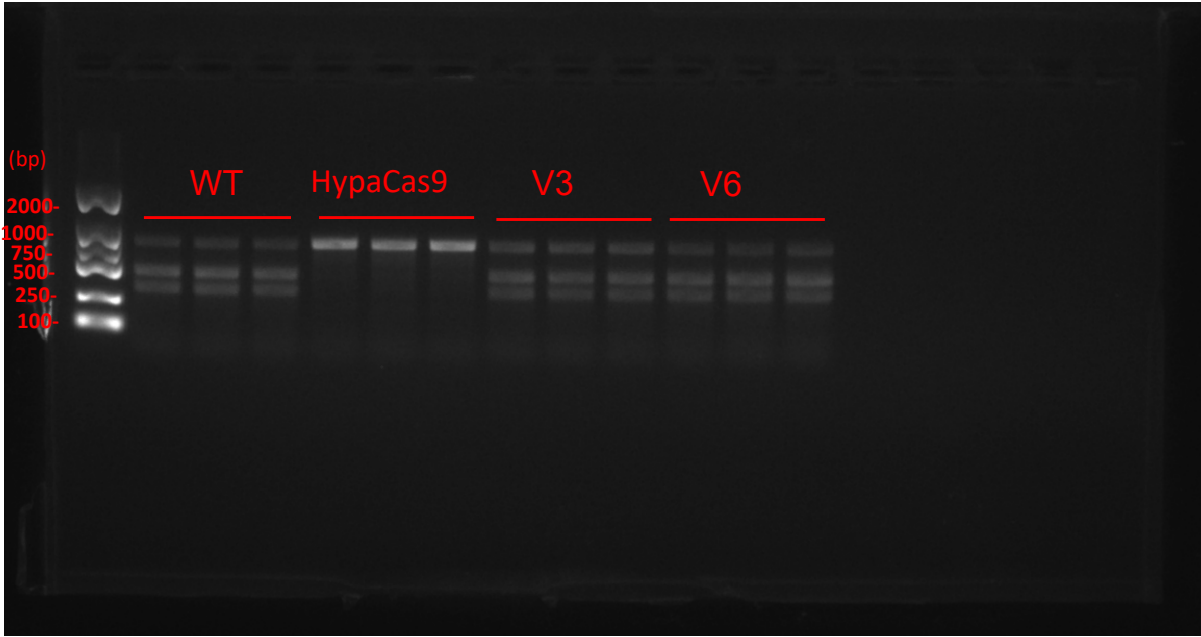
S1.2. EMX1-2 (DNA Marker (100~2000 bp), Sangon, #B500350)



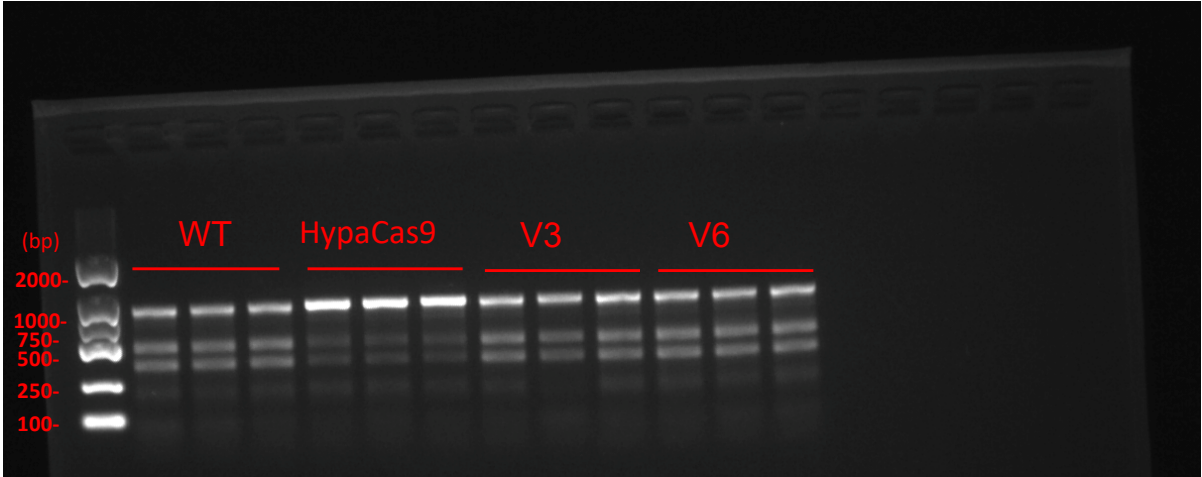
S1.3. FANCF-1 (DNA Marker (100~2000 bp), Sangon, #B500350)



S1.4. FANCF-3 (DNA Marker (100~2000 bp), Sangon, #B500350)



S1.5. VEGFA-1 (DNA Marker (100~2000 bp), Sangon, #B500350)



S1.6. VEGFA-2 (DNA Marker (100~2000 bp), Sangon, #B500350)



S1.7. VEGFA-5 (RealBand DNA Marker (100~1500 bp), #B600021)

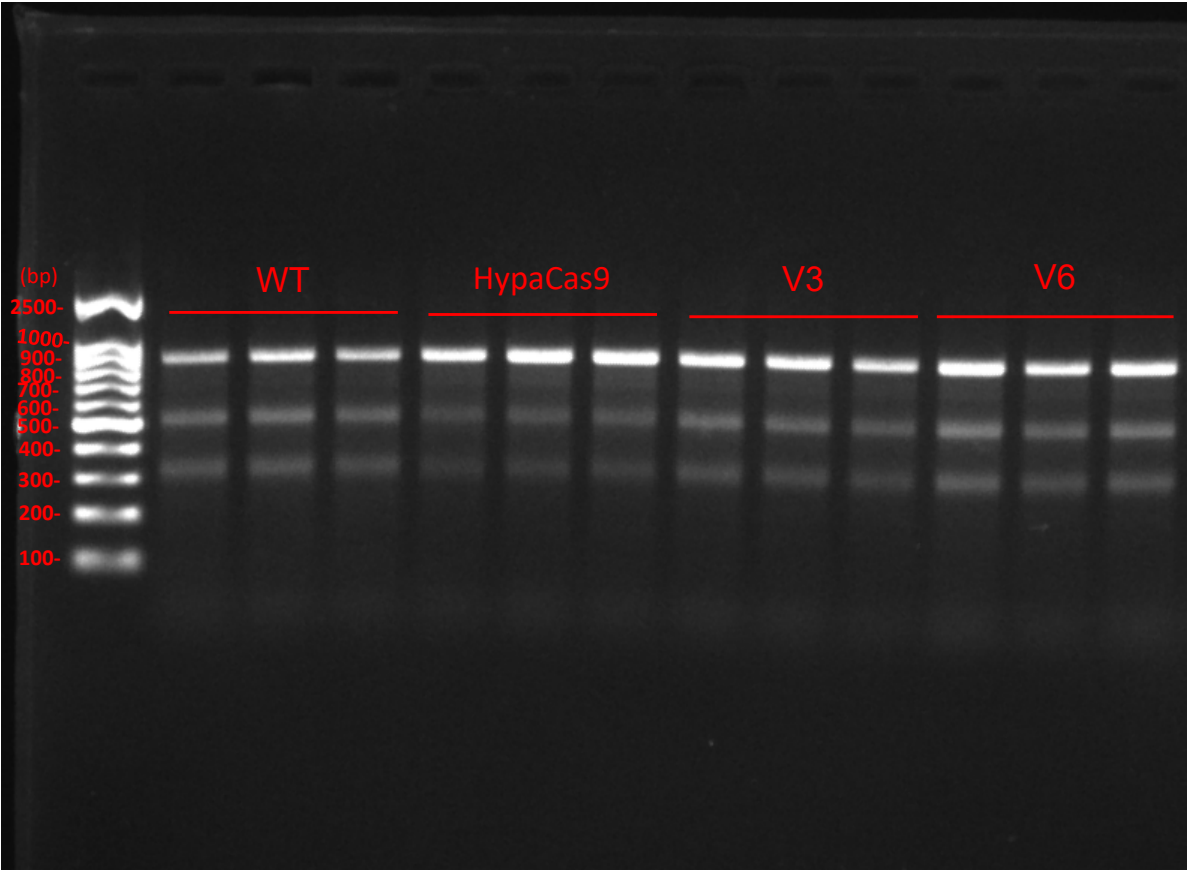
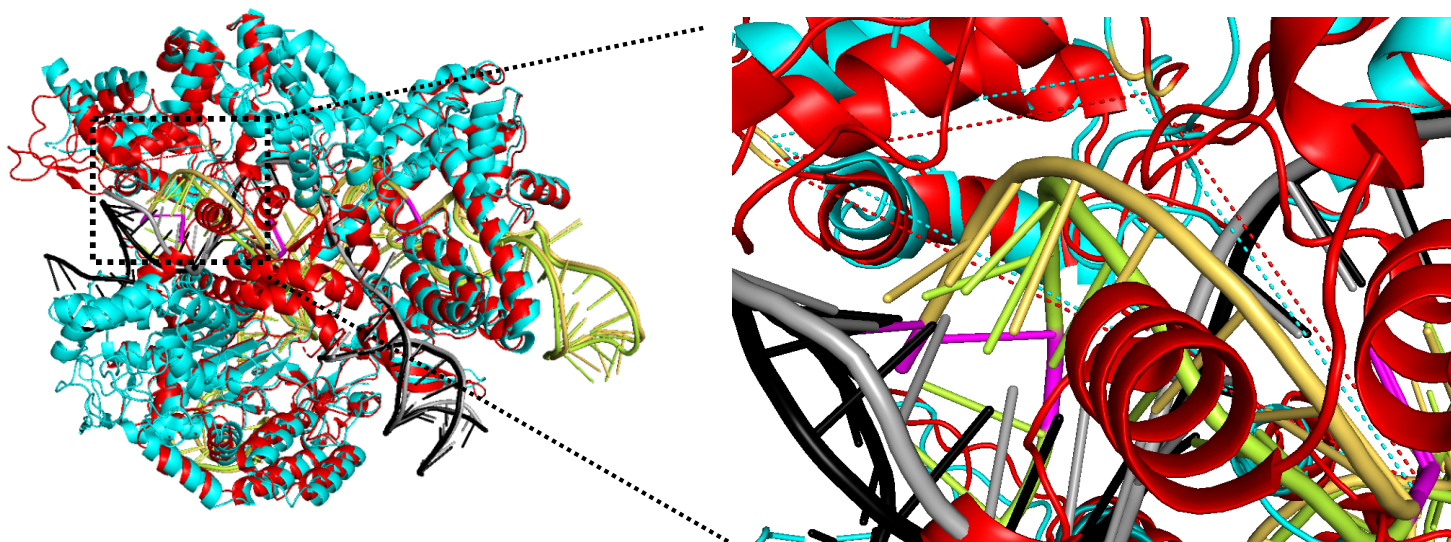
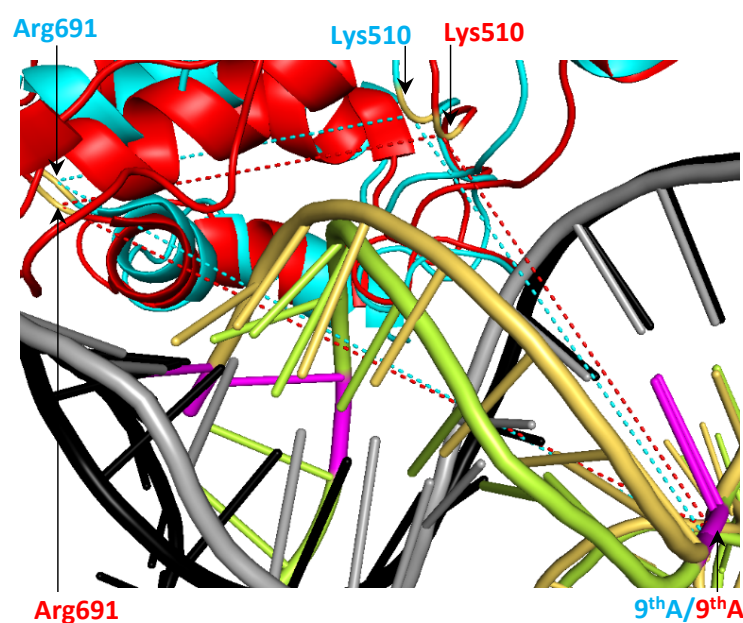


Figure S2, The comparison of the mutation sites of HiFi Cas9 and V6.

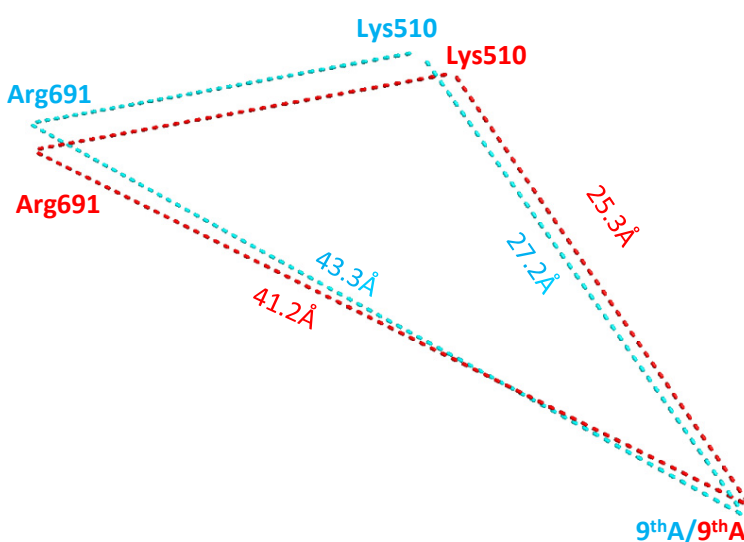
A



B



C



A. The alignment of the checking conformation (6O0Z, cyan) and cleaving conformation (6O0Y, red). Residues, sgRNA and DNA were marked with cyan, yellow and black in checking conformation (6O0Z); and were marked with red, yelloworange and gray in cleaving conformation (6O0Y).

B. Part of the REC3 and the hybrid alignment between the checking conformation and cleaving

C. The moved distance of Arg691 and Lys510 in checking and cleaving conformation. The Arg691 and 9<sup>th</sup> adenine as well as the distances between them were marked with cyan (6O0Z) and red (6O0Y) respectively.



Figure S3, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, and variants V1-V6 on EMX1-1 (3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The blue dots indicate mutation events while the brown (blue+ & green+) dots indicate wild-type events.)

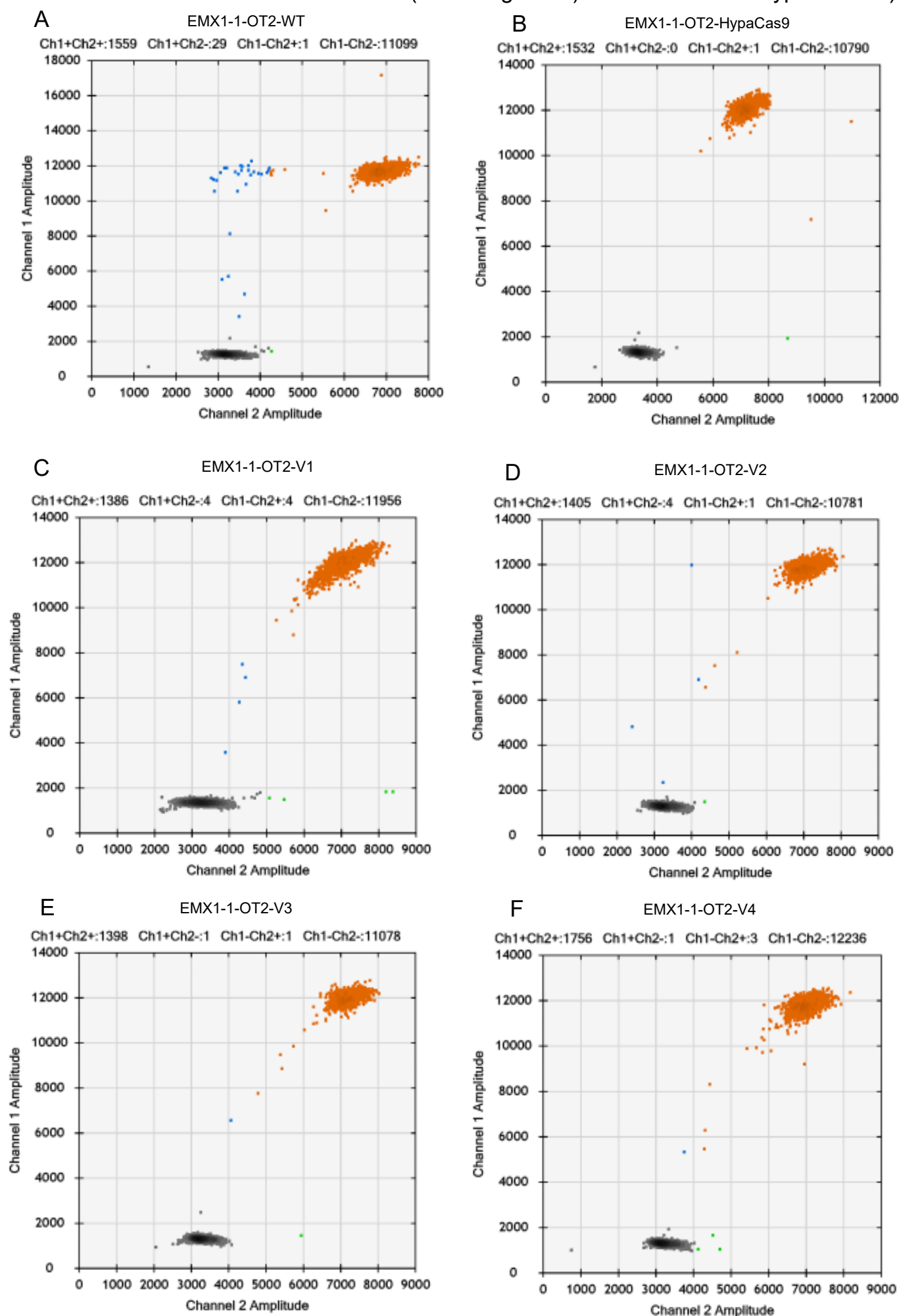


Figure S3, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, and variants V1-V6 on EMX1-1 (3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The blue dots indicate mutation events while the brown (blue+ & green+) dots indicate wild-type events.)

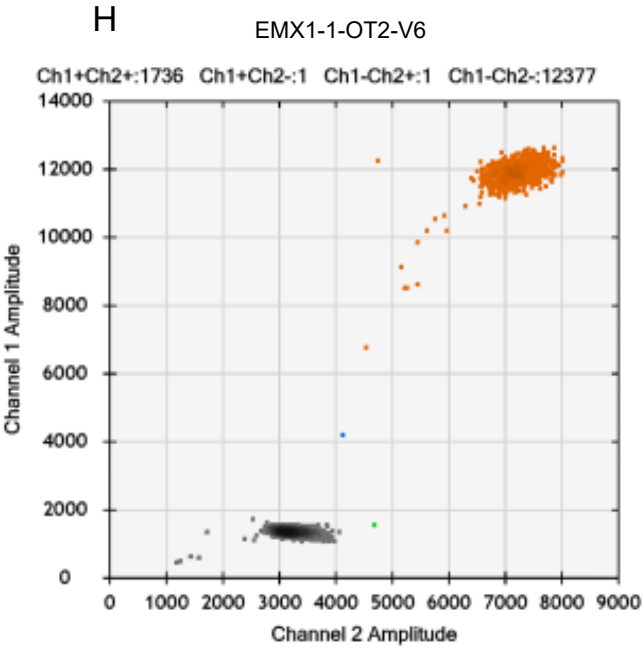
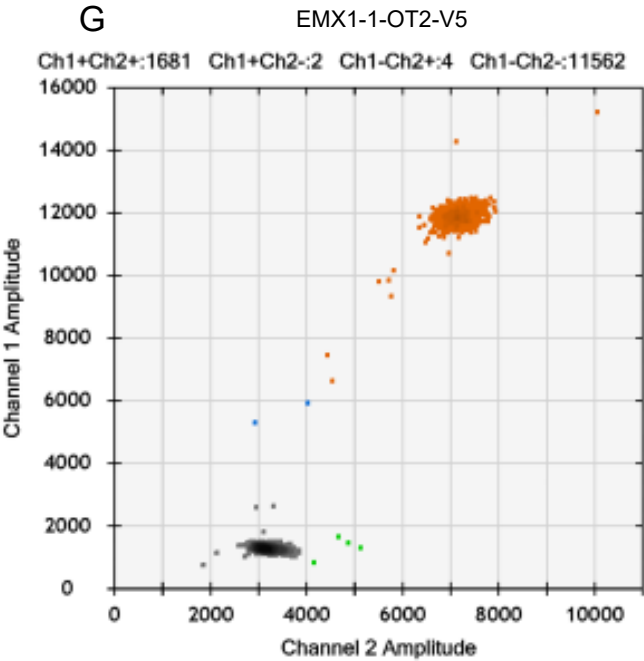


Figure S4.1, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on EMX1-2 (3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

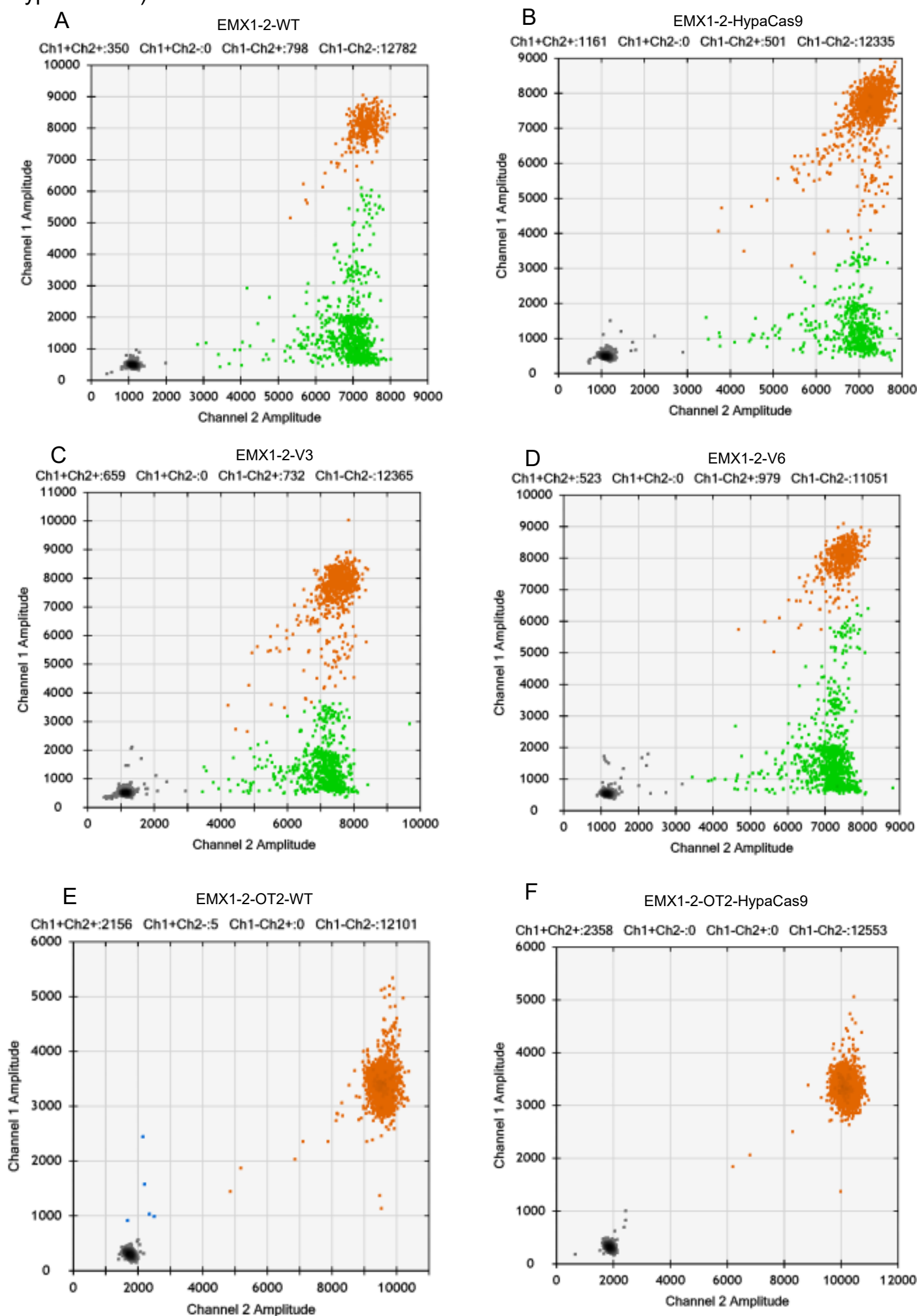


Figure S4.1, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on EMX1-2 (3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

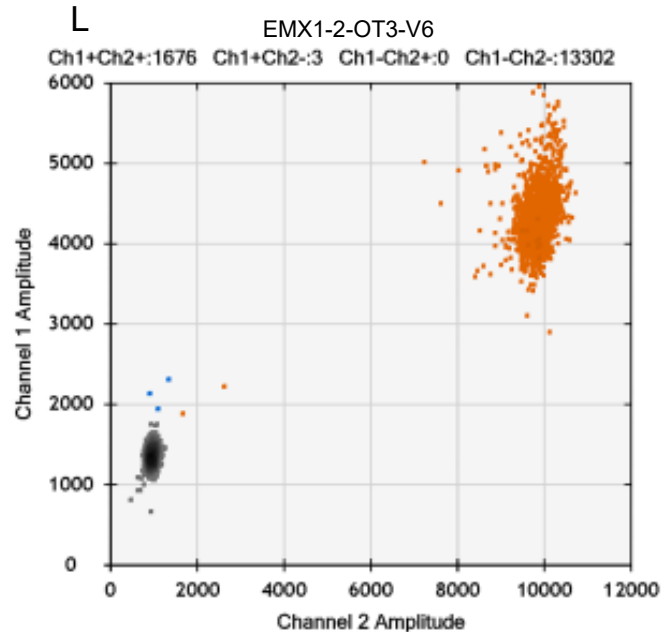
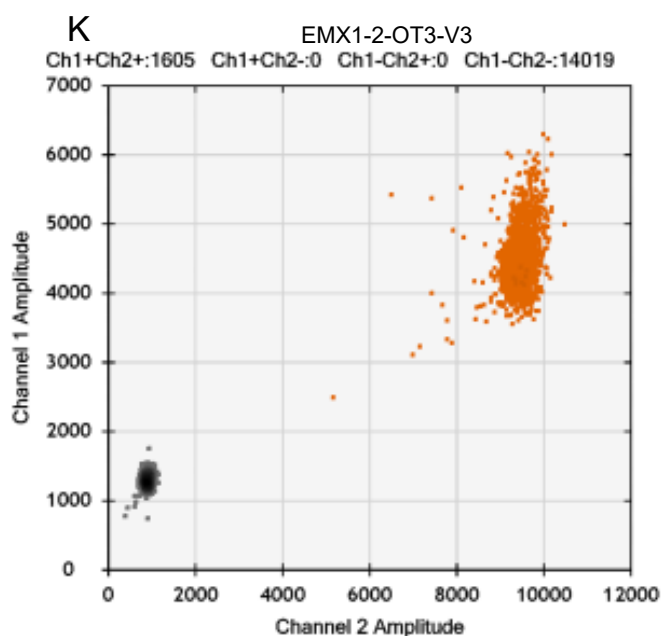
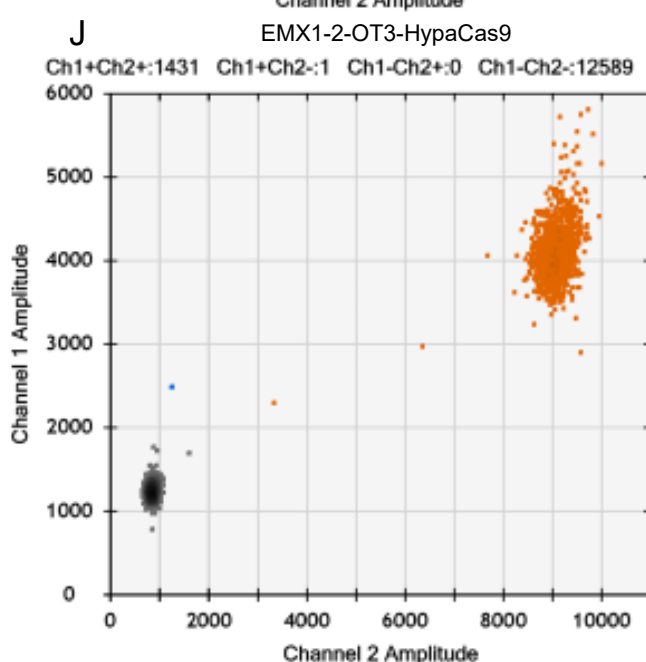
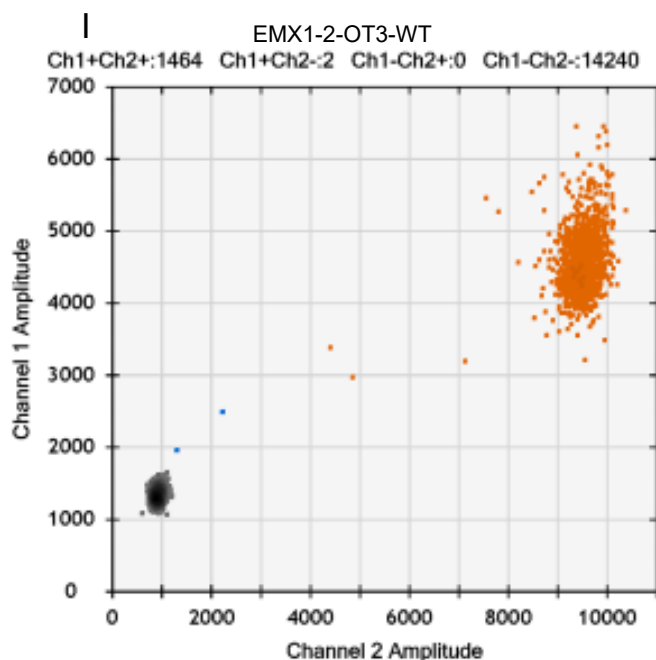
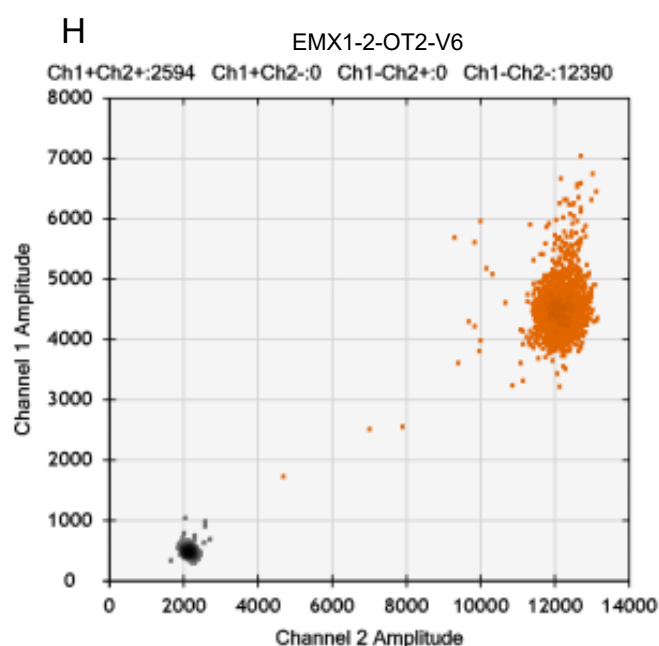
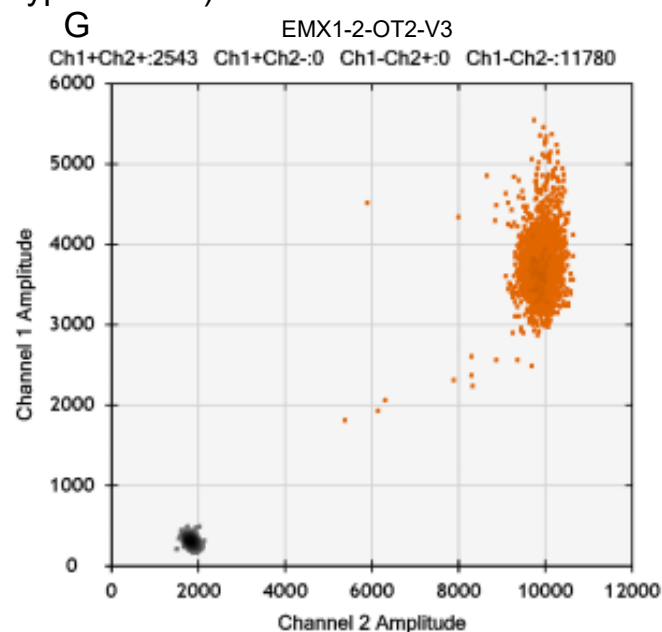




Figure S4.1, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on EMX1-2 (3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

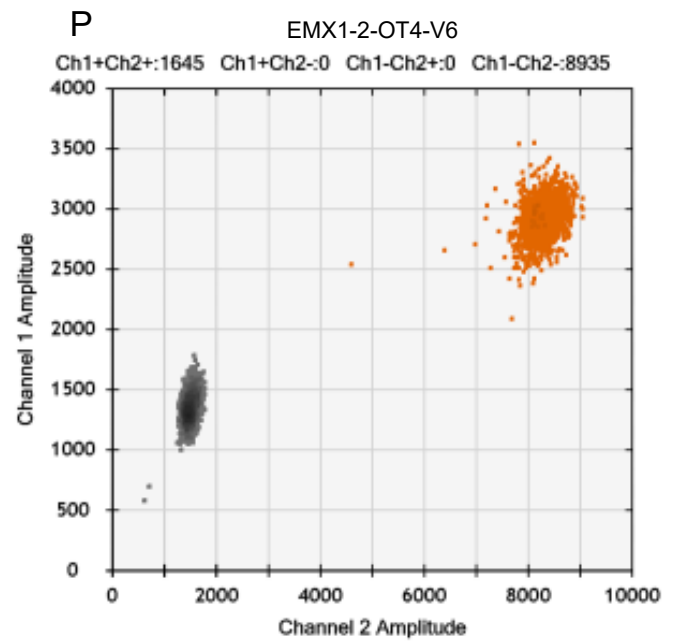
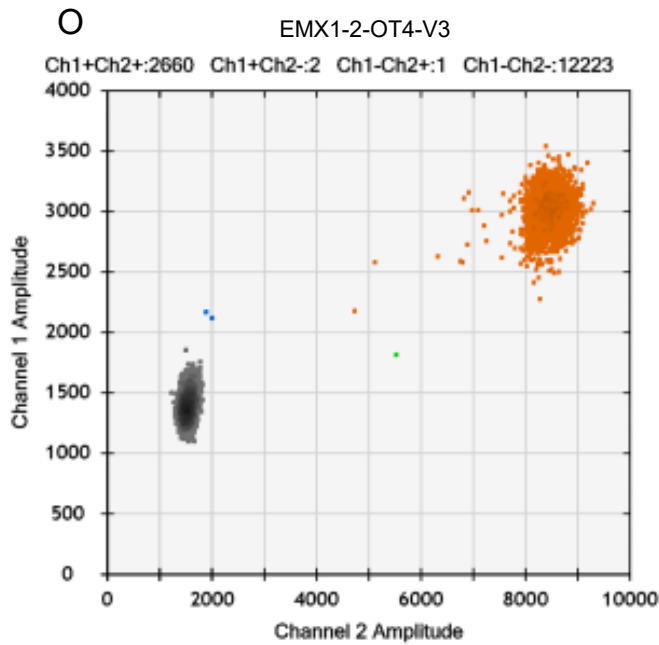
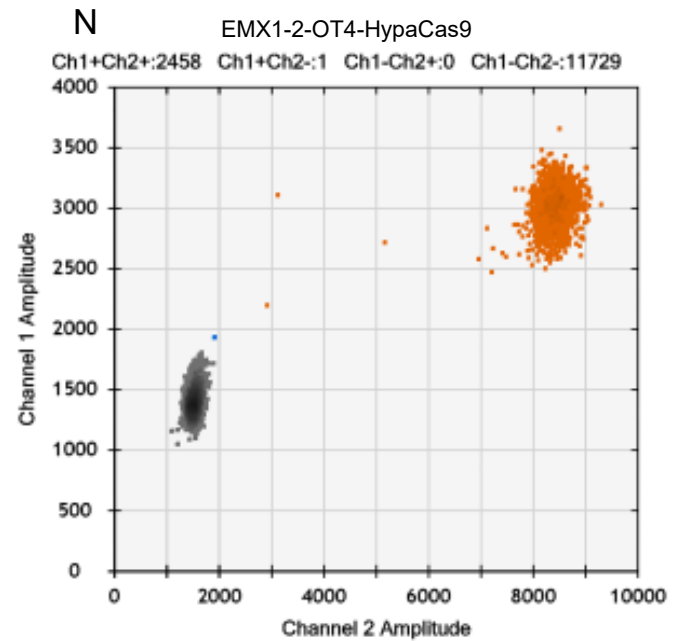
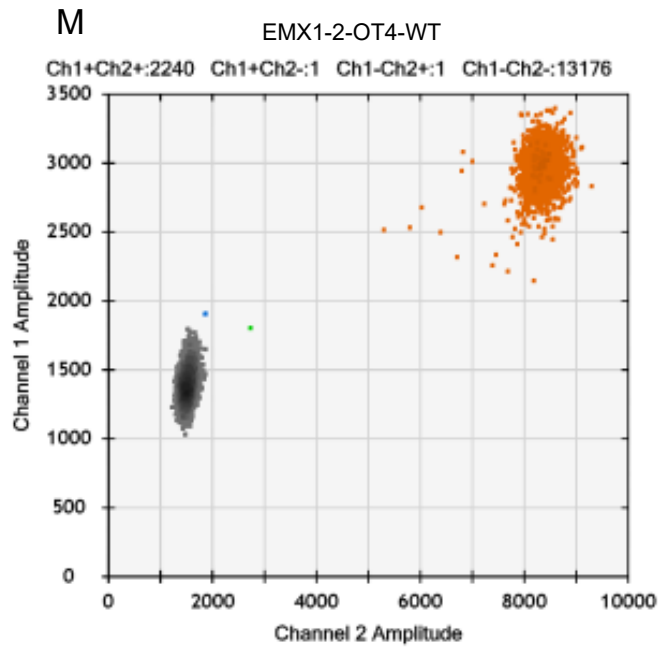


Figure S4.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-1 (3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

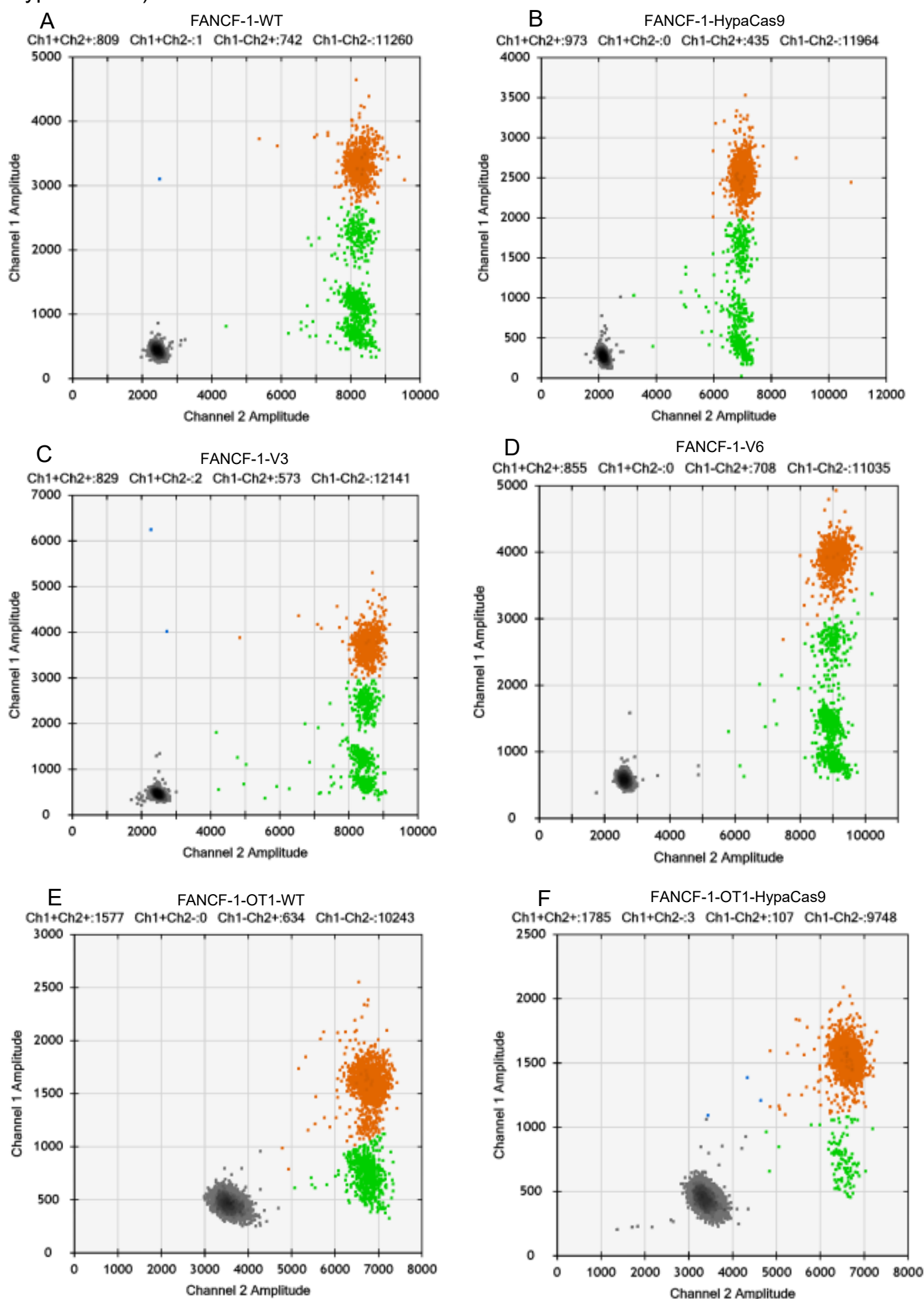


Figure S4.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-1 (3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

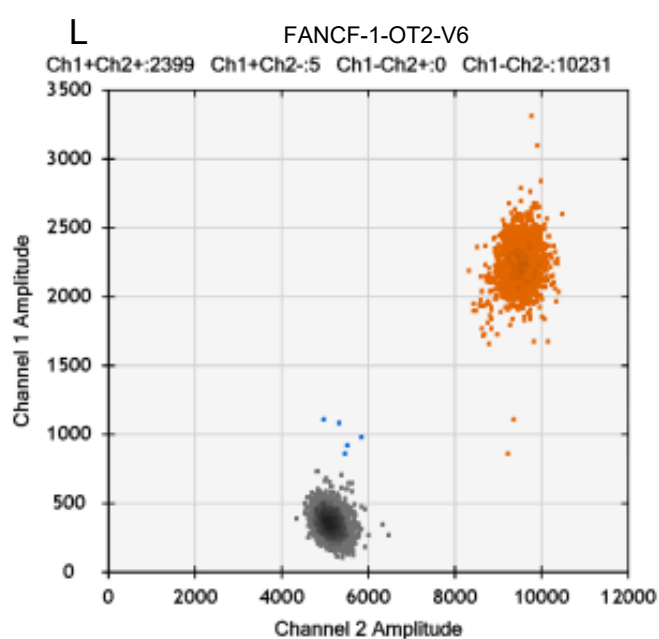
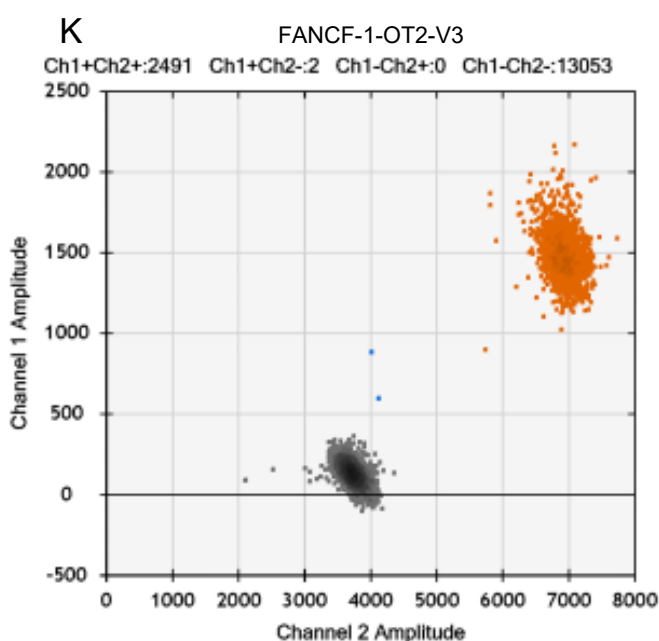
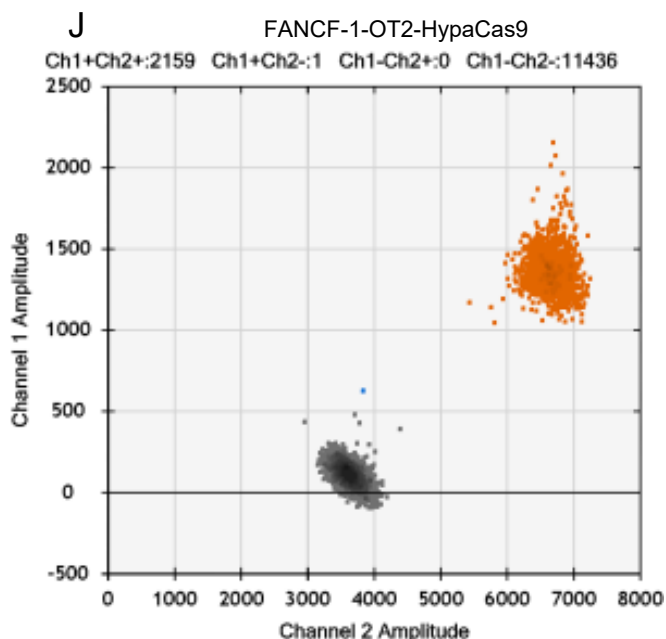
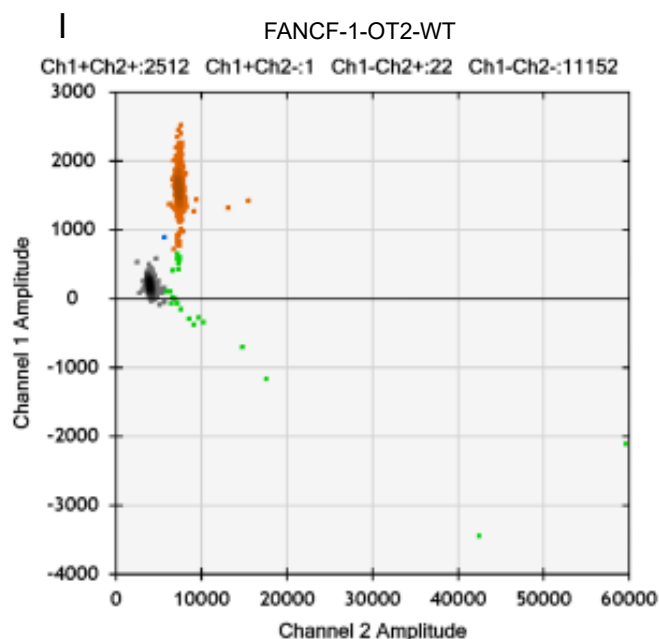
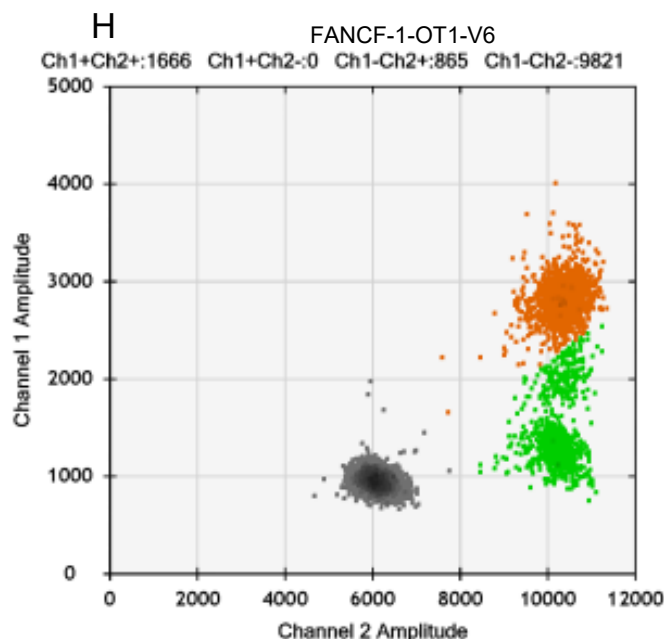
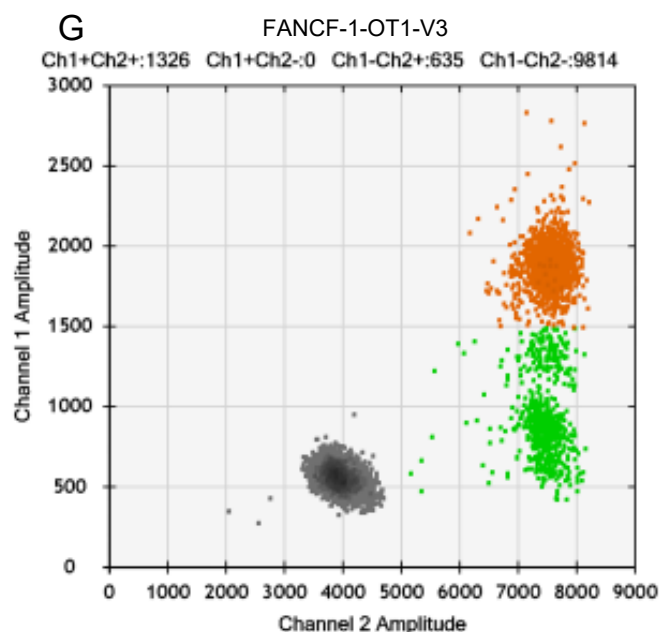


Figure S4.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-1 (3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

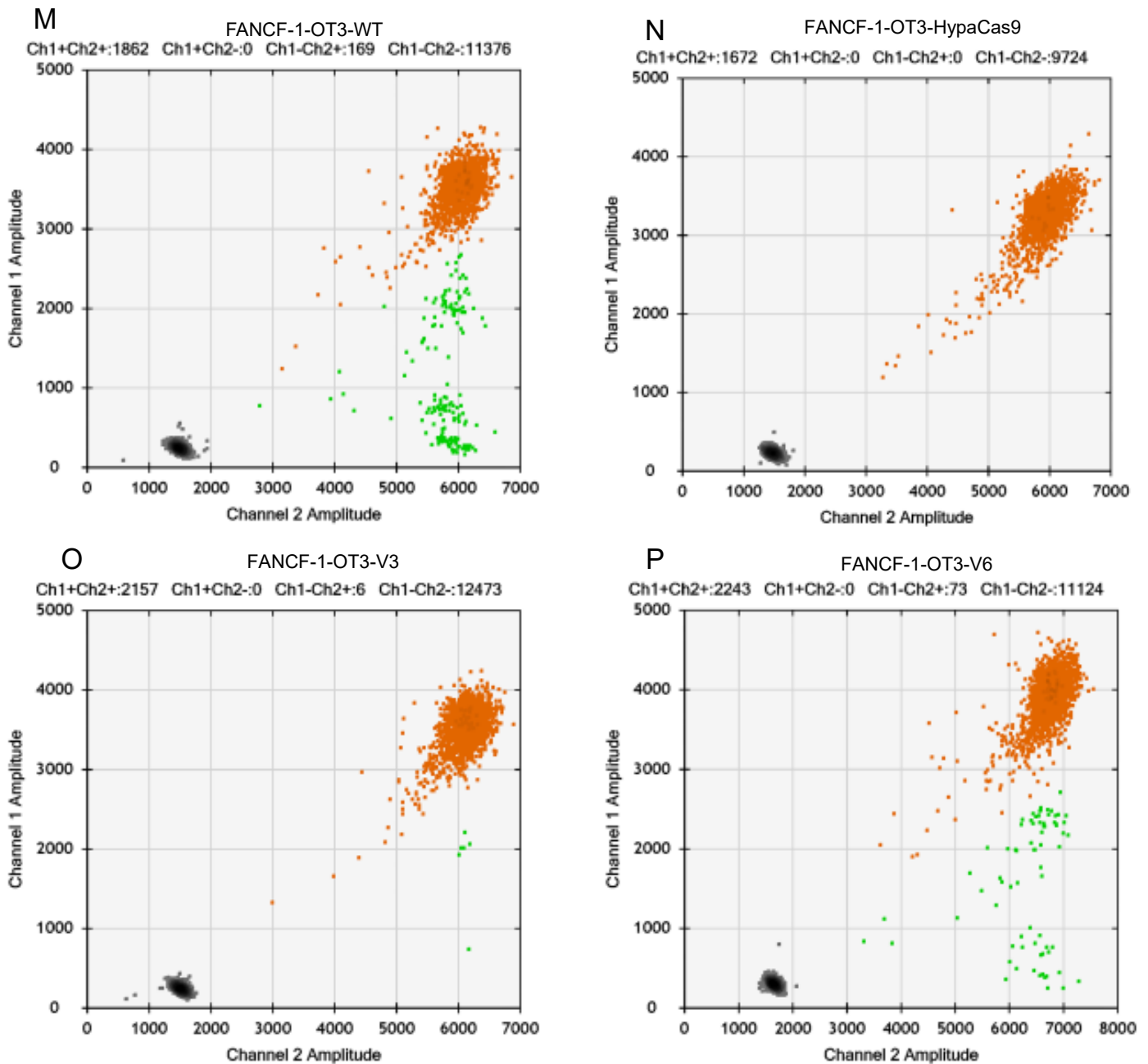


Figure S4.3, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on EMX1-2 (6day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

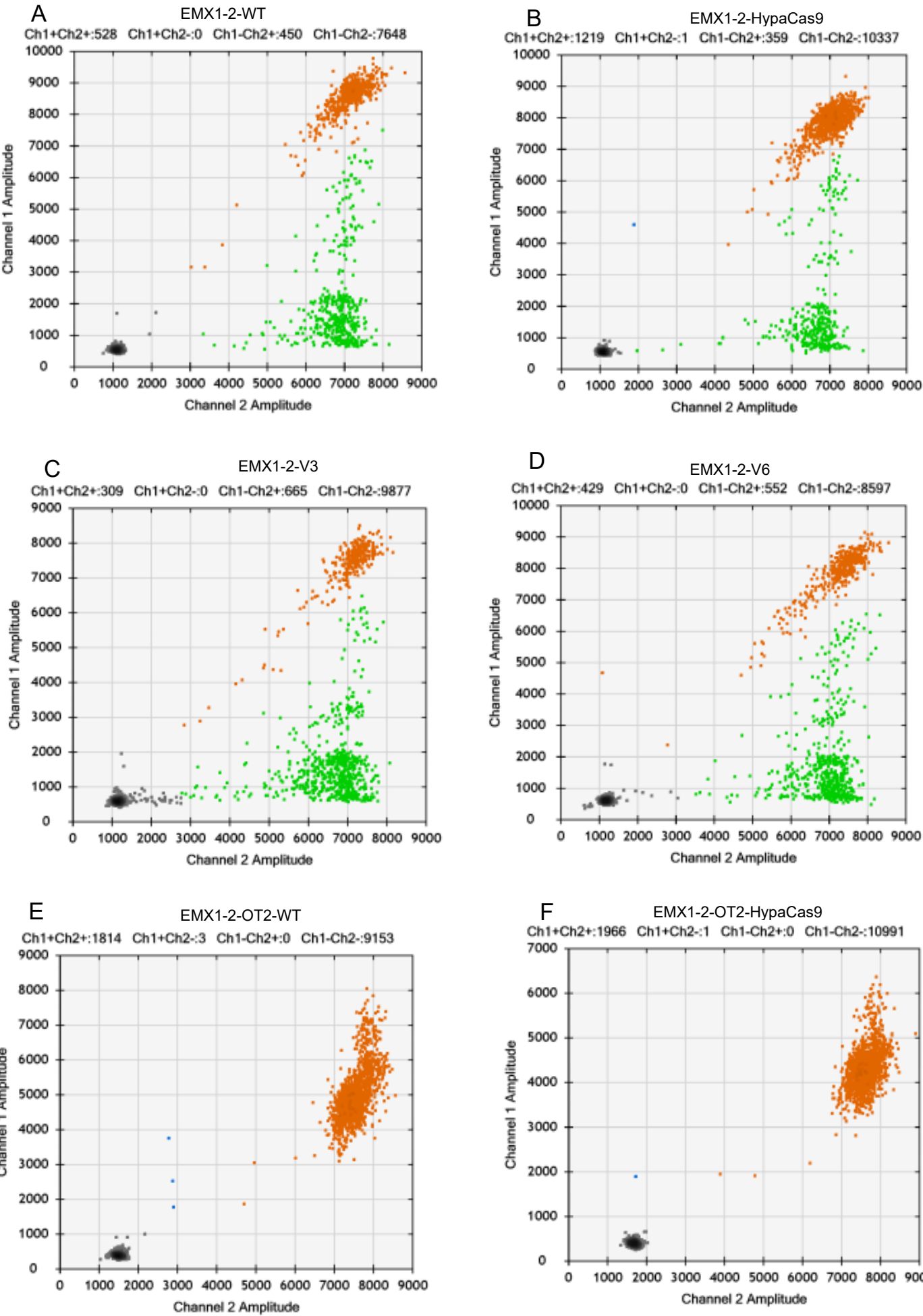




Figure S4.3, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on EMX1-2 (6day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

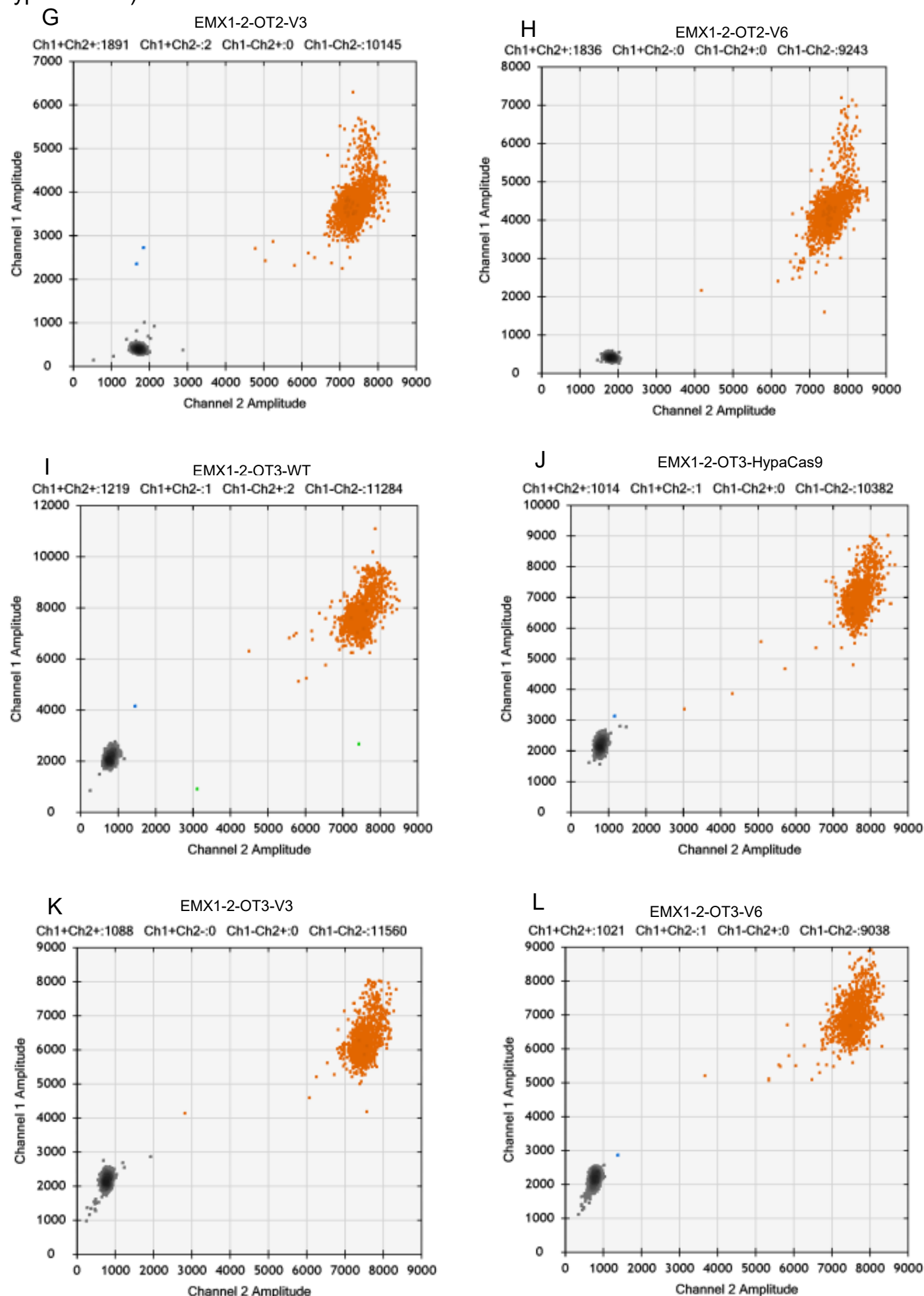


Figure S4.3, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on EMX1-2 (6day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

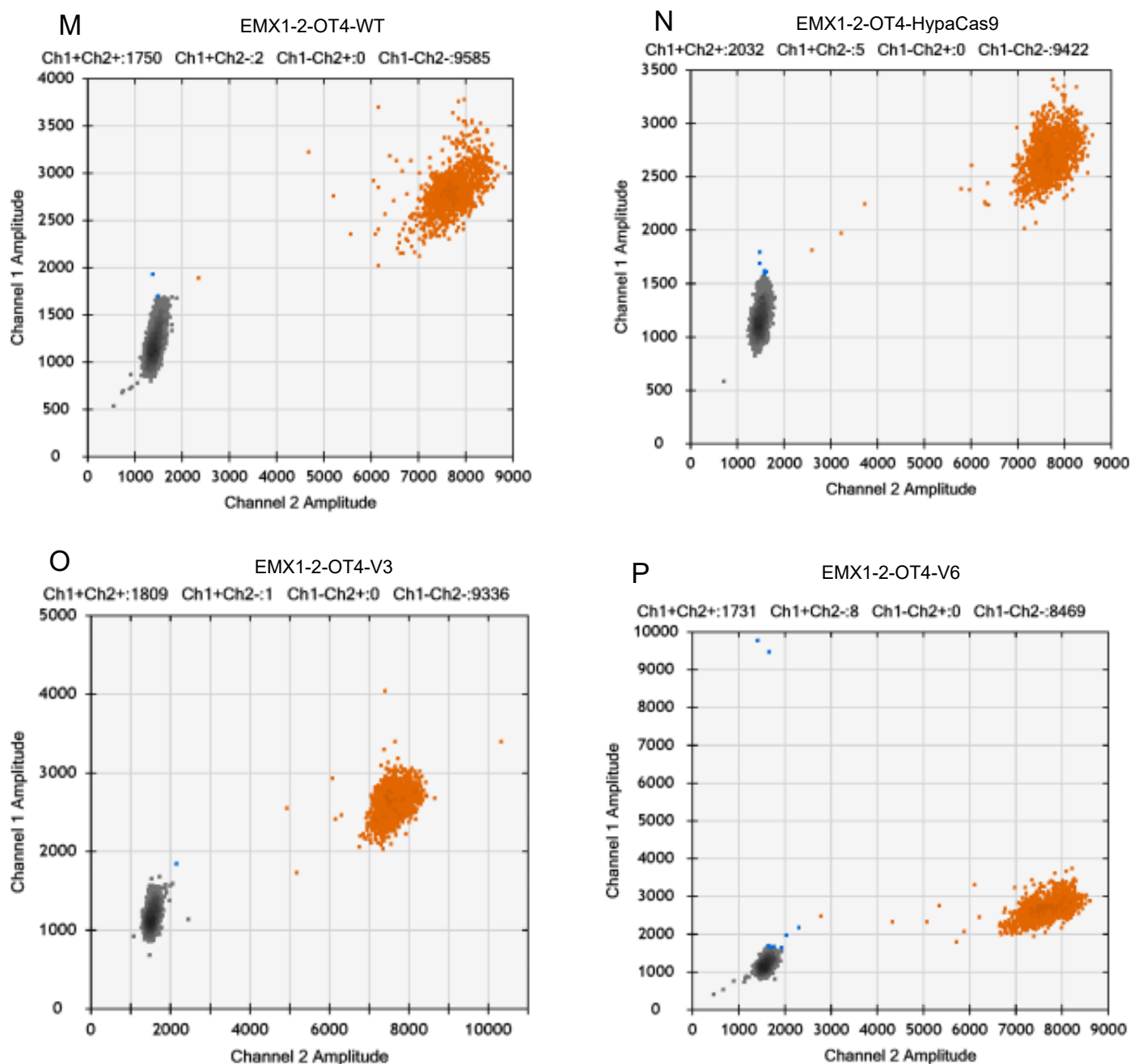


Figure S4.4, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-1 (6day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

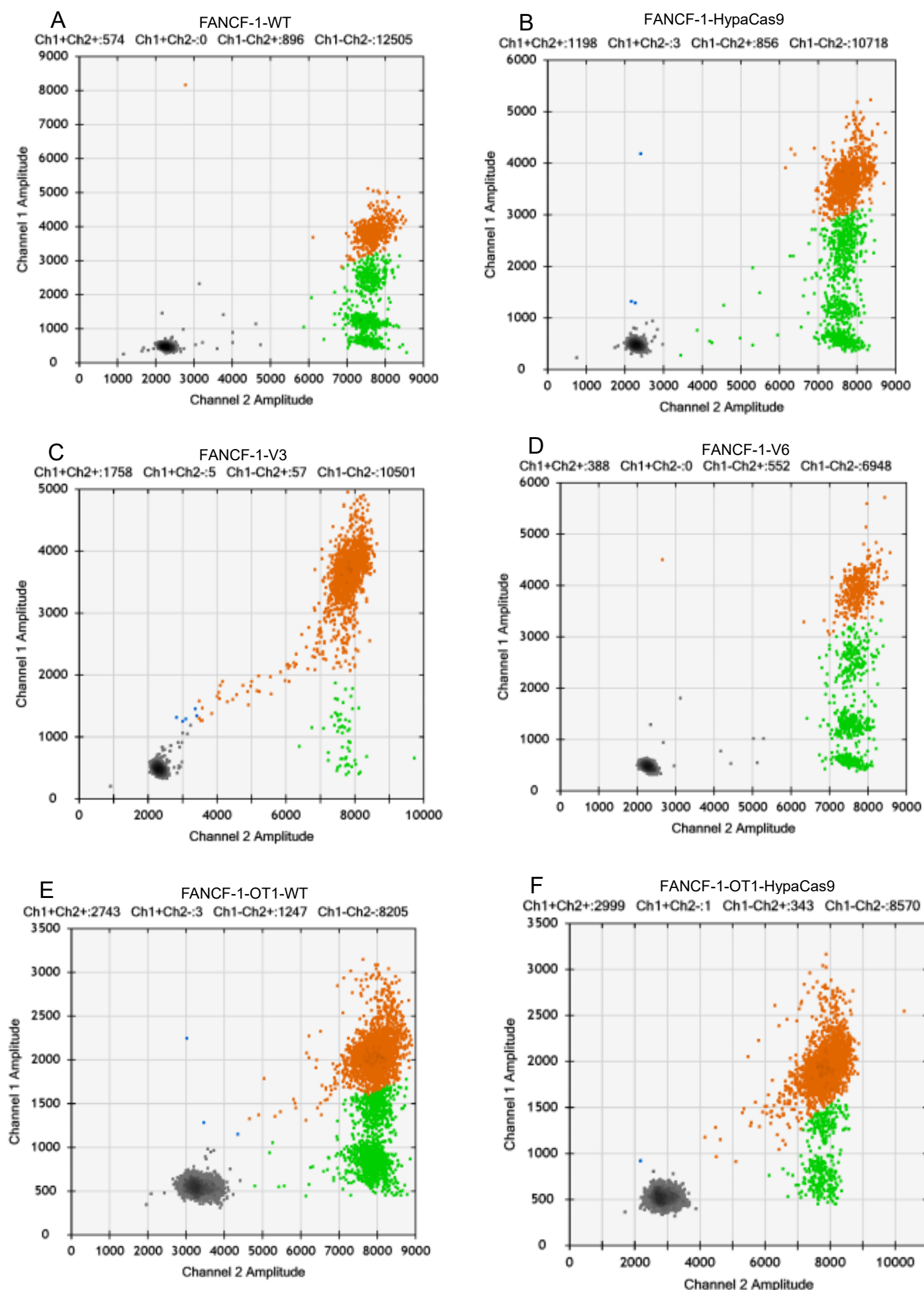


Figure S4.4, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-1 (6day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

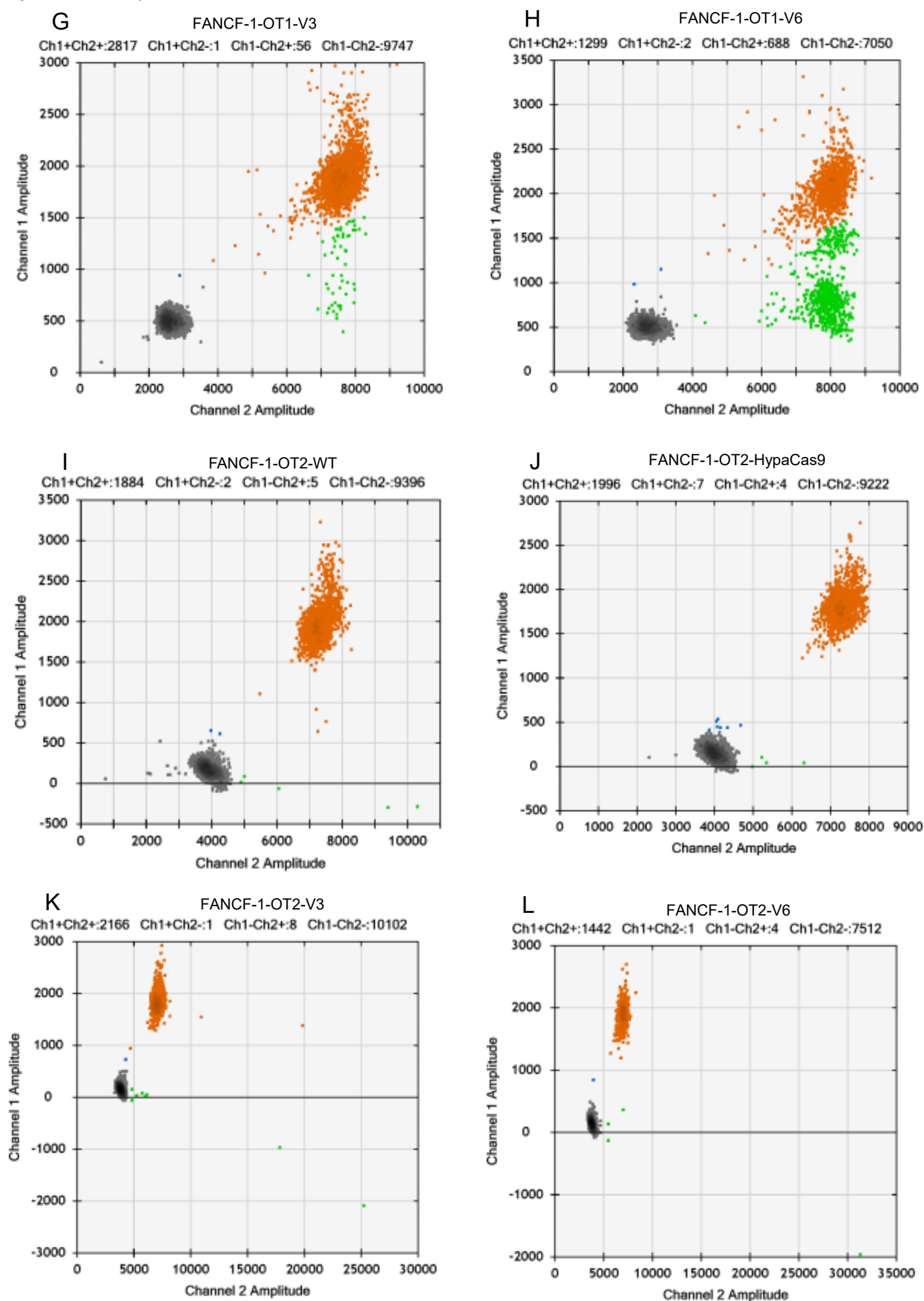


Figure S4.4, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-1 (6day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

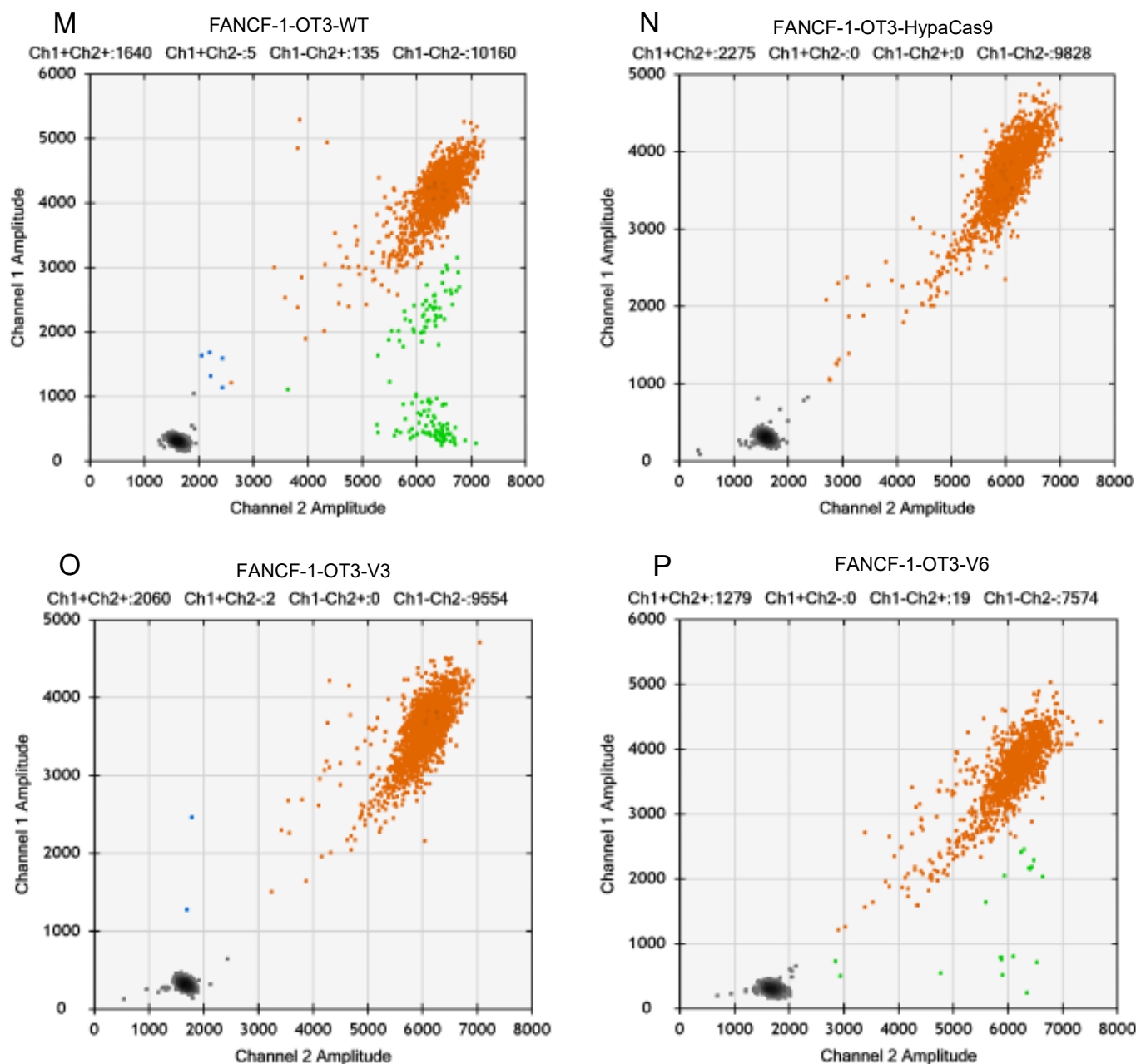




Figure S4.5, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-3 (6day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

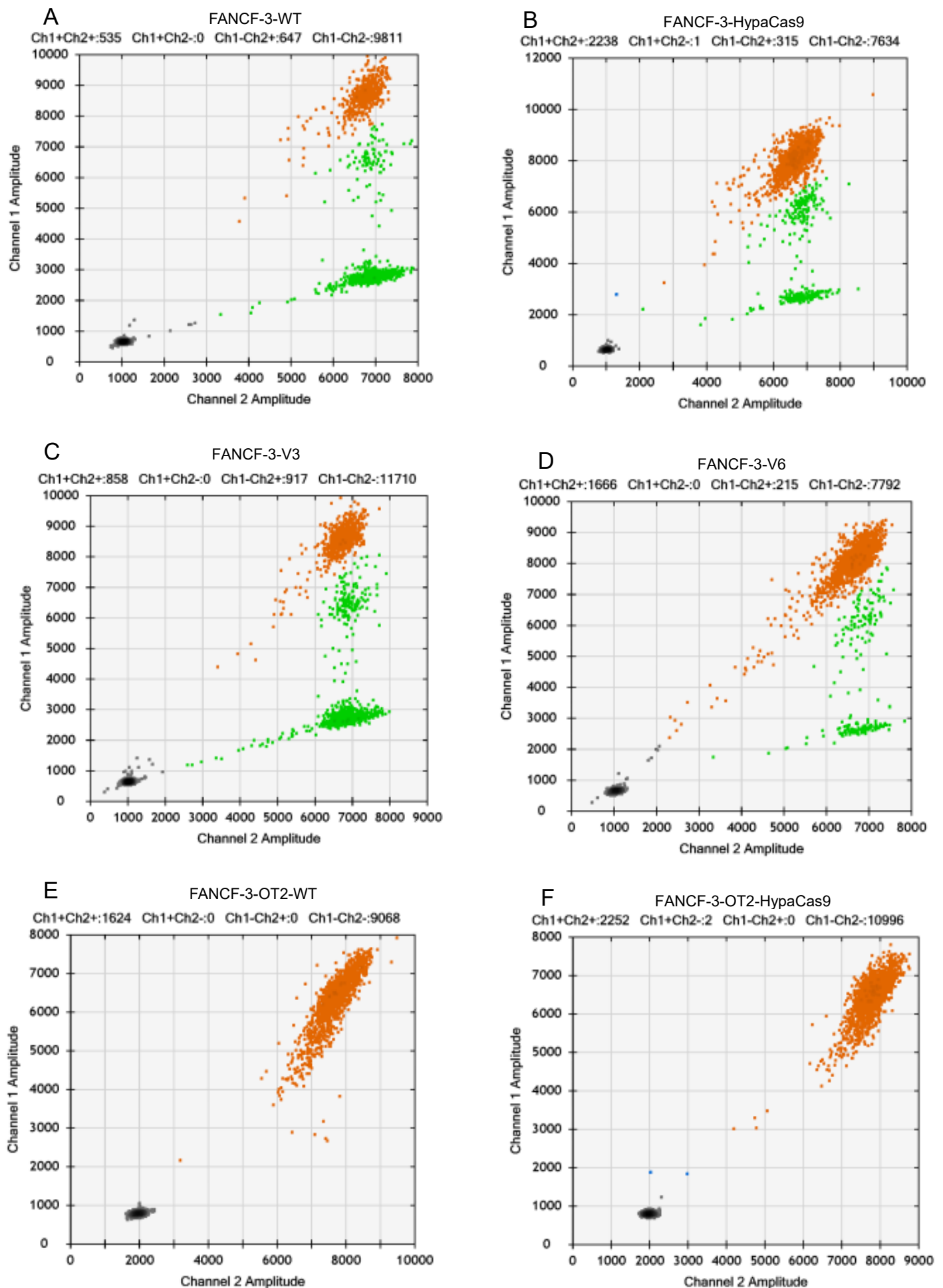


Figure S4.5, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-3 (6day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

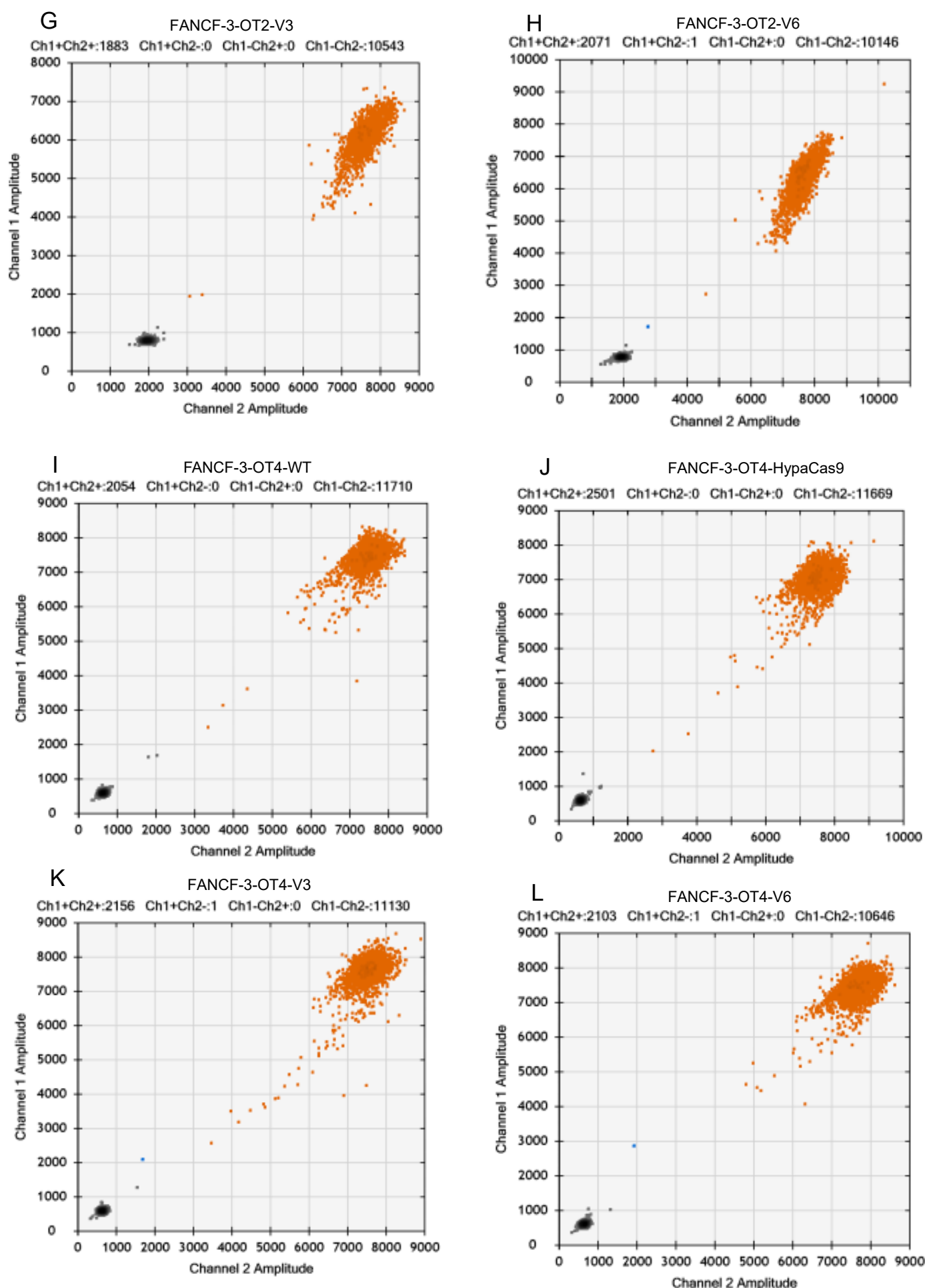


Figure S4.6, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on VEGFA-5 (6day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

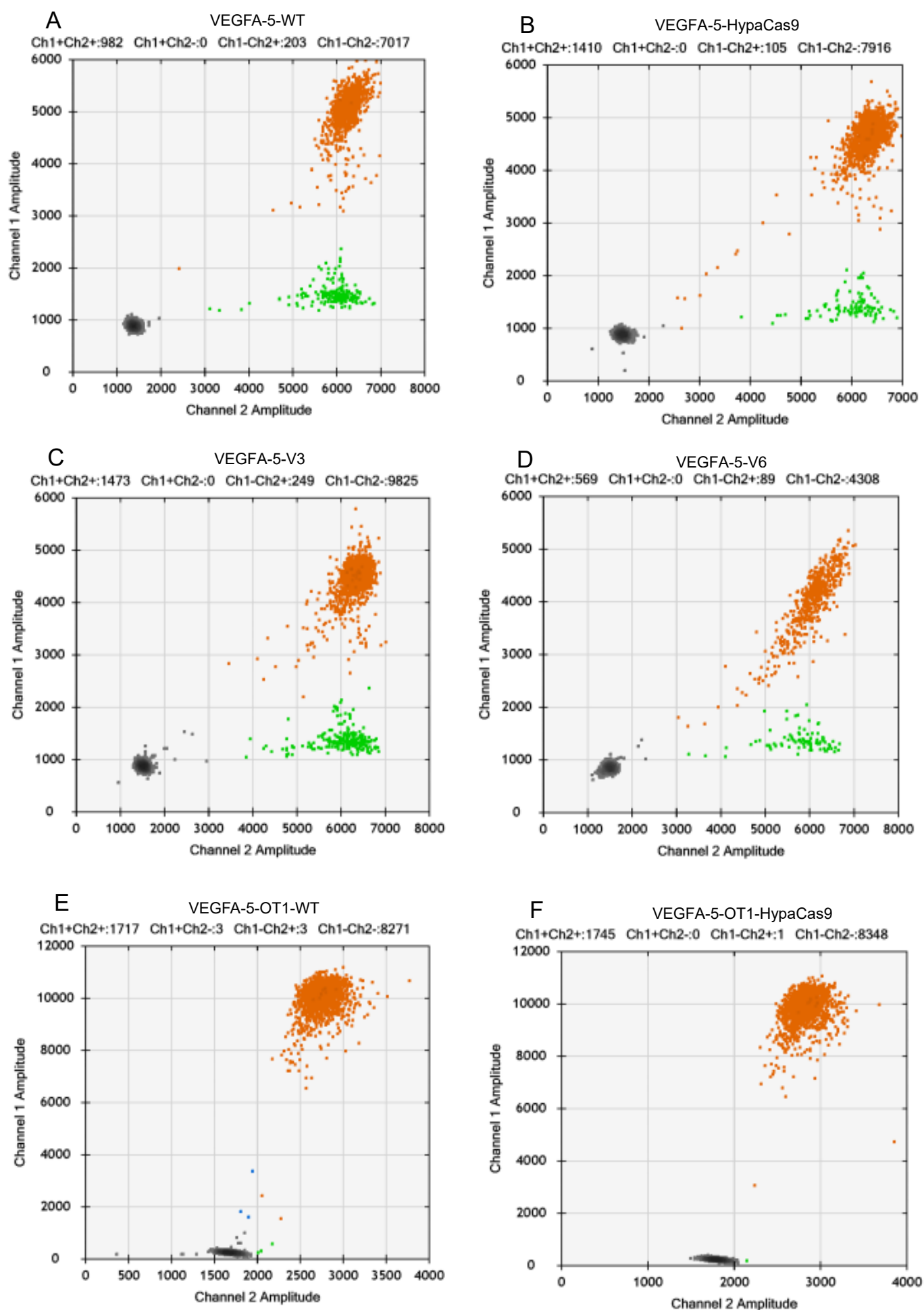


Figure S4.6, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on VEGFA-5 (6day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

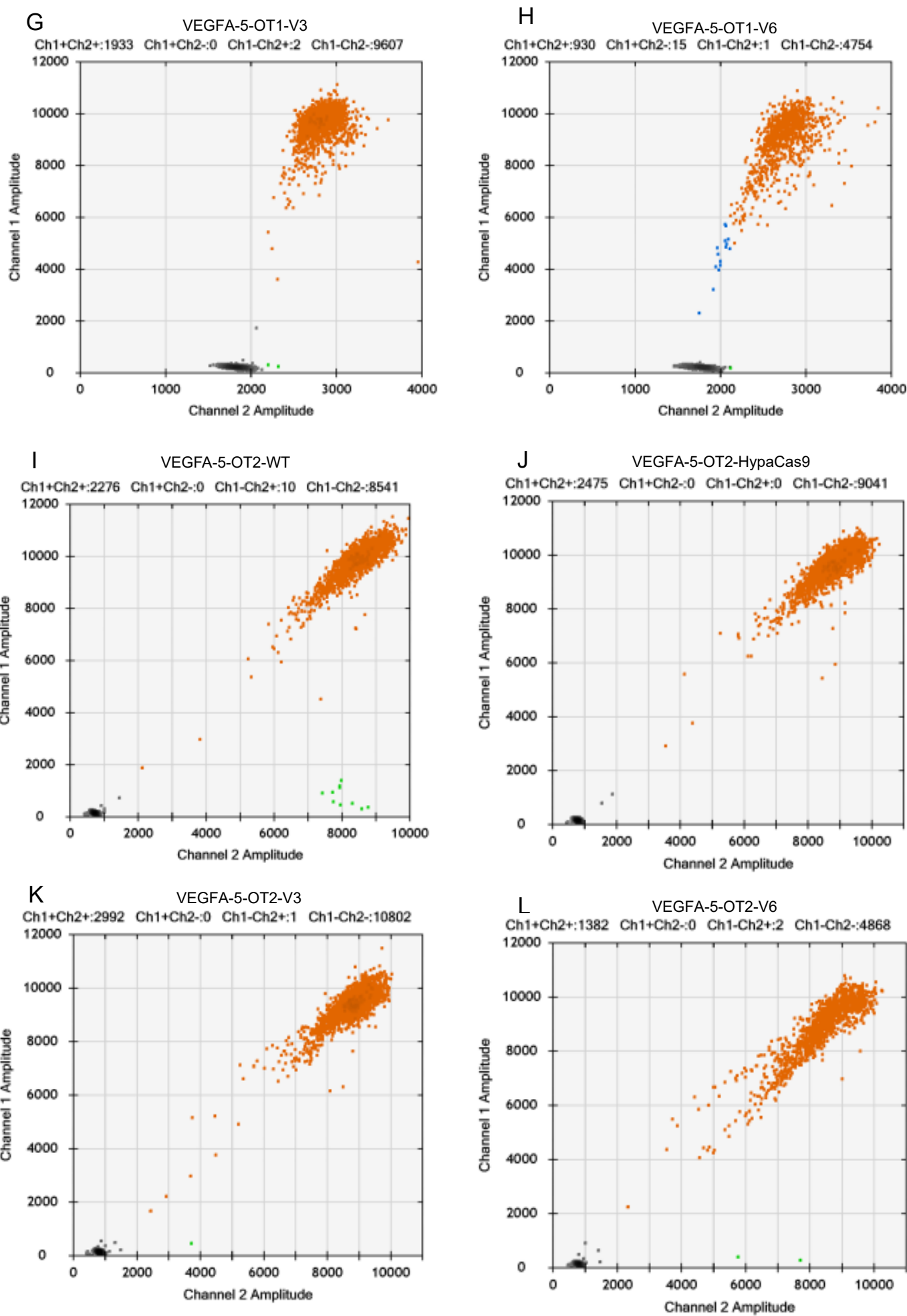


Figure S5.1, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, and variant V3 on EMX1-1 (U2OS, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. For A-C, the blue dots indicate mutation events while the brown (blue+ & green+) dots indicate wild-type events; for D-I, the blue dots indicate mutation events while the brown (blue+ & green+) dots indicate wild-type events.)

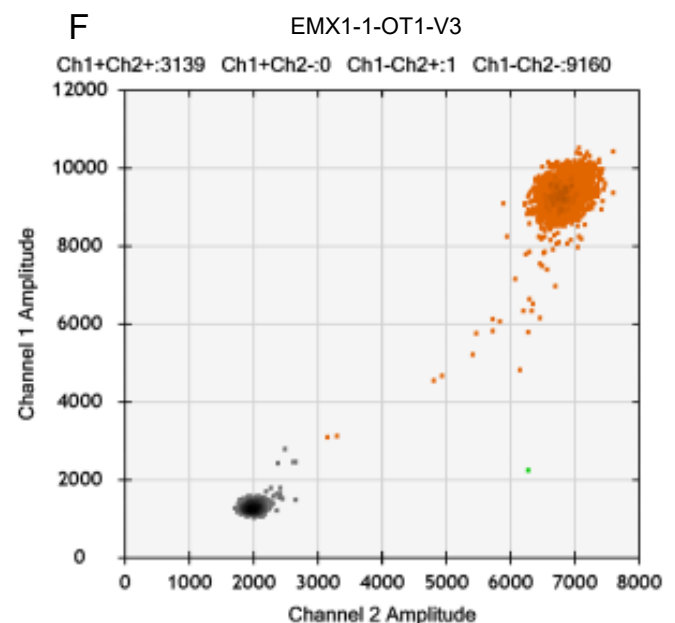
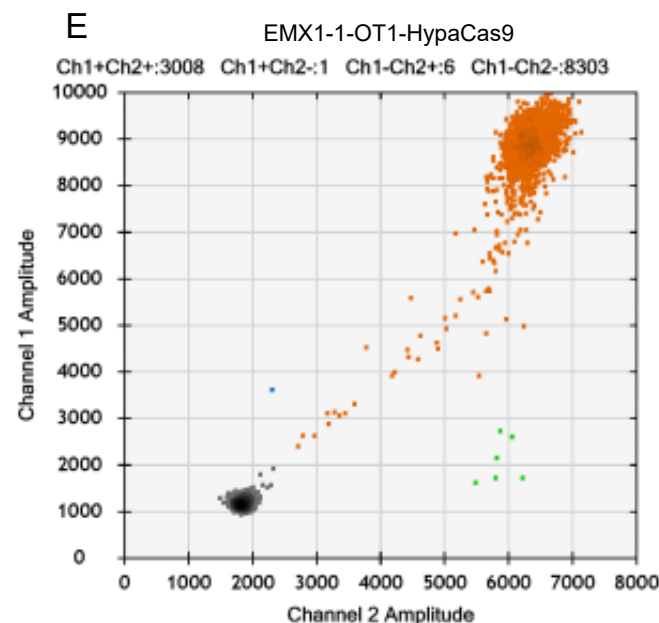
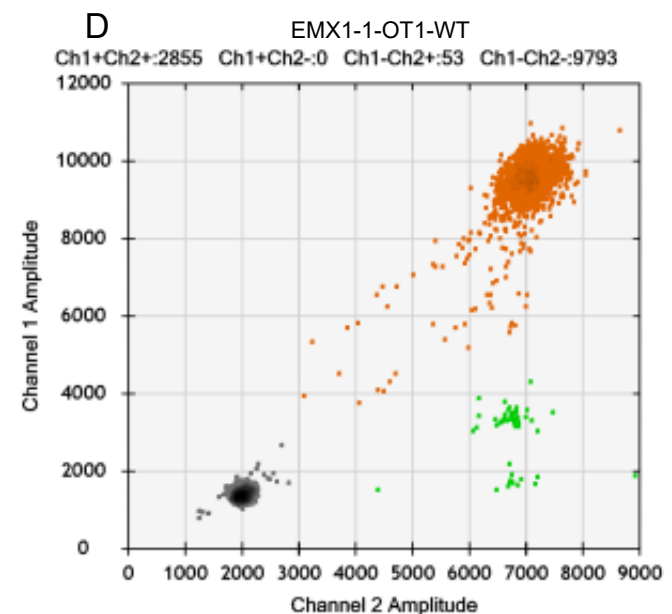
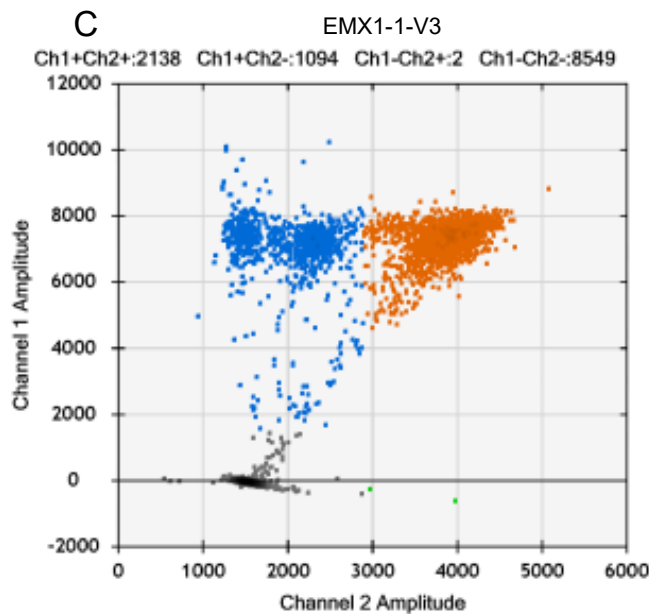
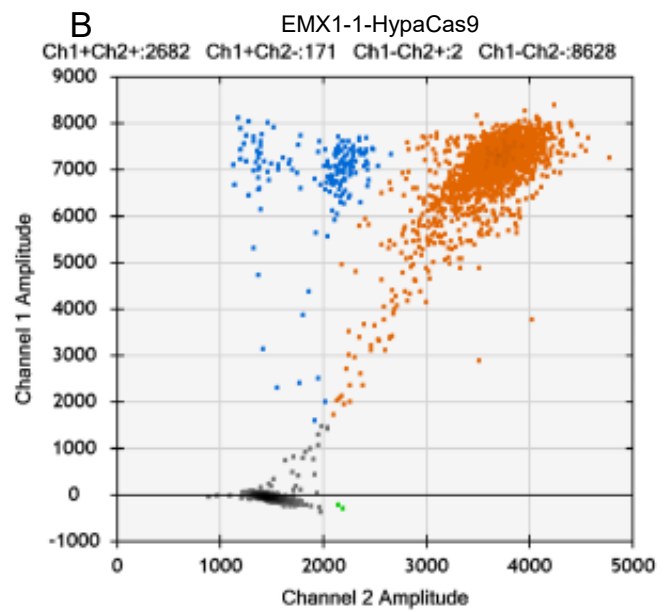
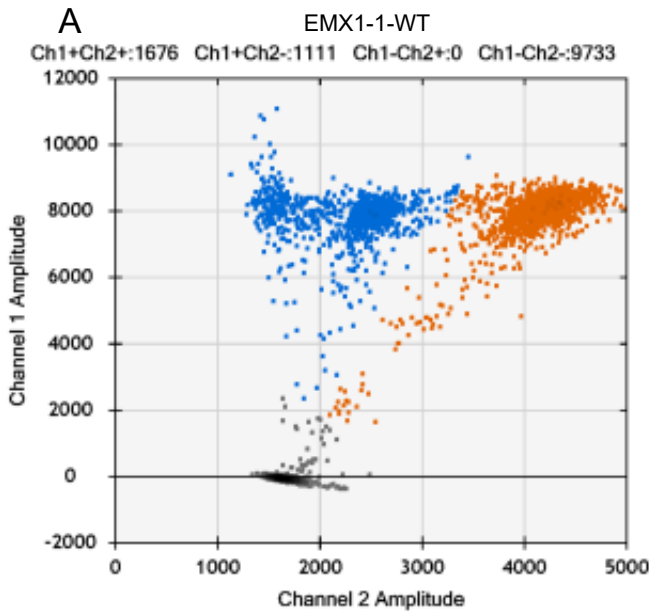




Figure S5.1, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, and variant V3 on EMX1-1 (U2OS, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. For A-C, the blue dots indicate mutation events while the brown (blue+ & green+) dots indicate wild-type events; for D-I, the blue dots indicate mutation events while the brown (blue+ & green+) dots indicate wild-type events.)

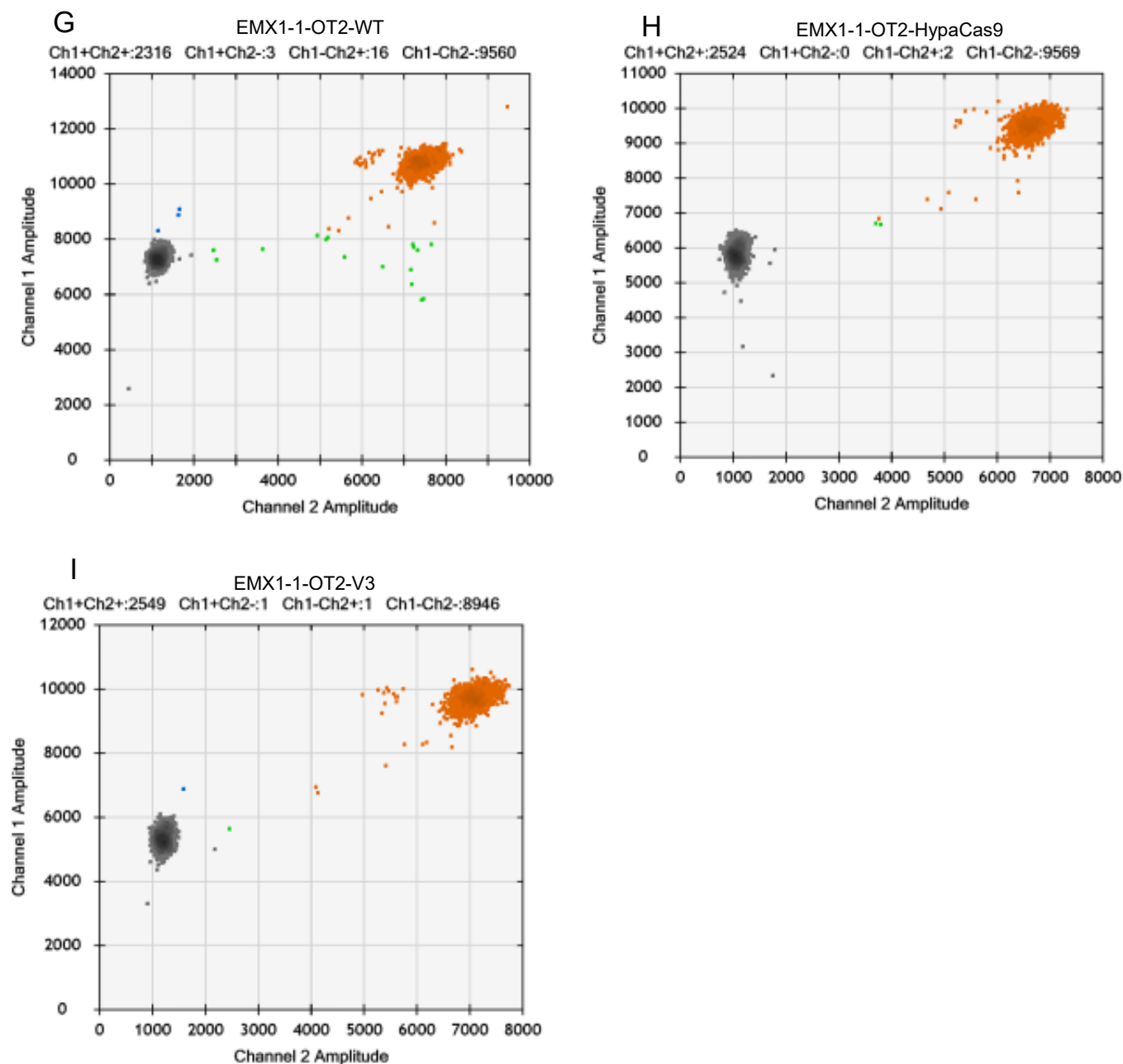


Figure S5.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-1 (U2OS, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

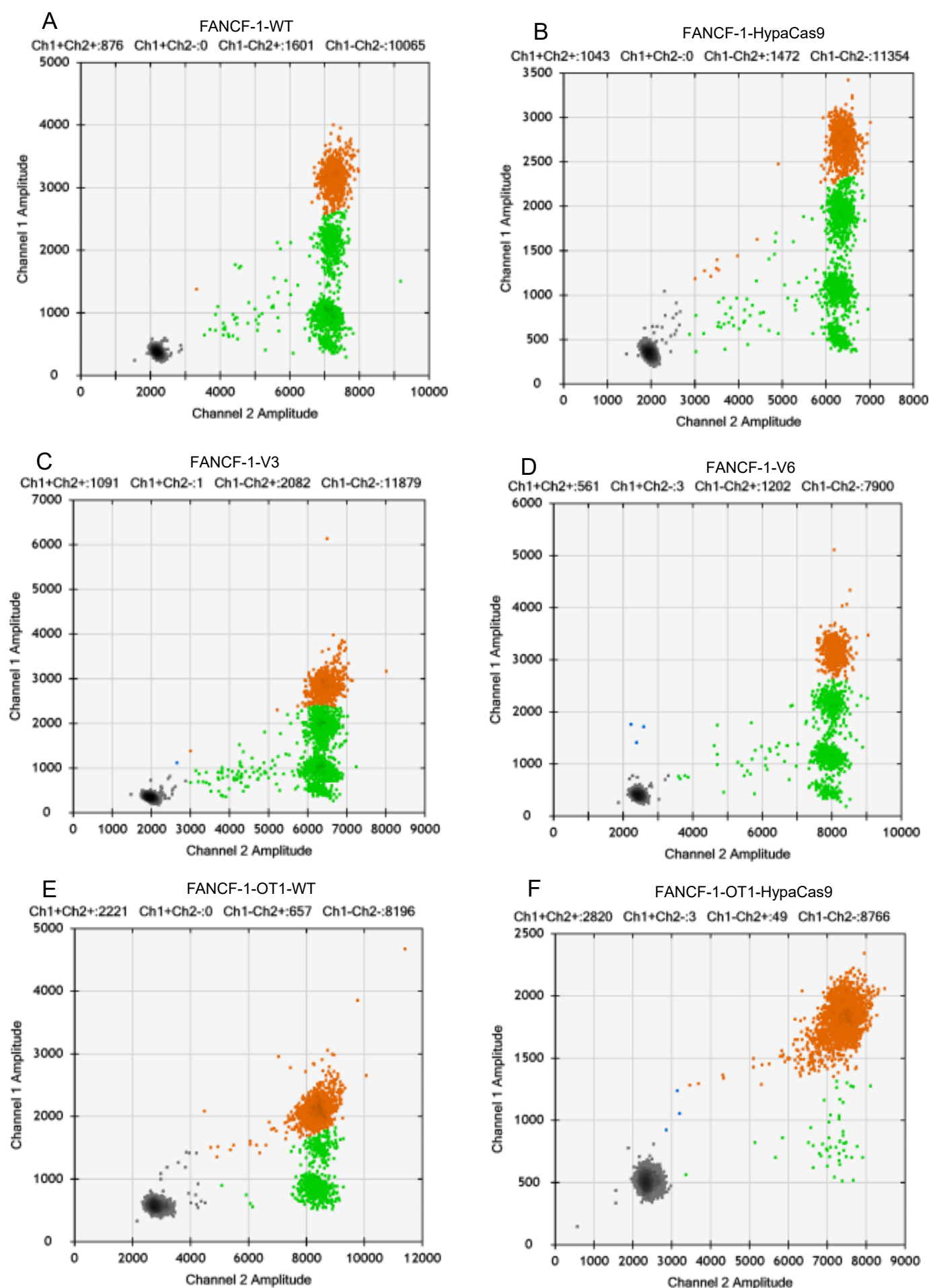


Figure S5.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-1 (U2OS, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

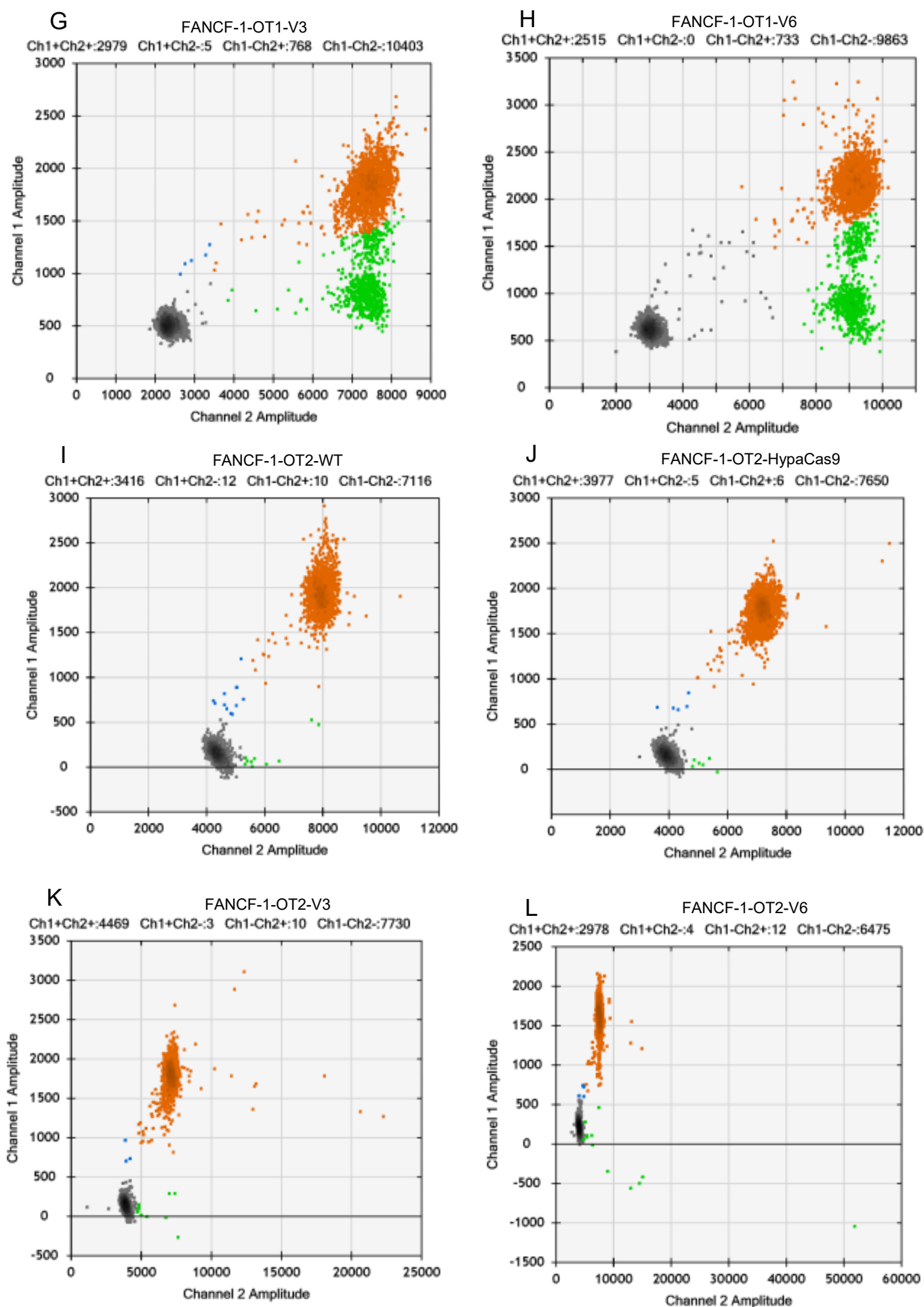


Figure S5.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, variant V3, and variant V6 on FANCF-1 (U2OS, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

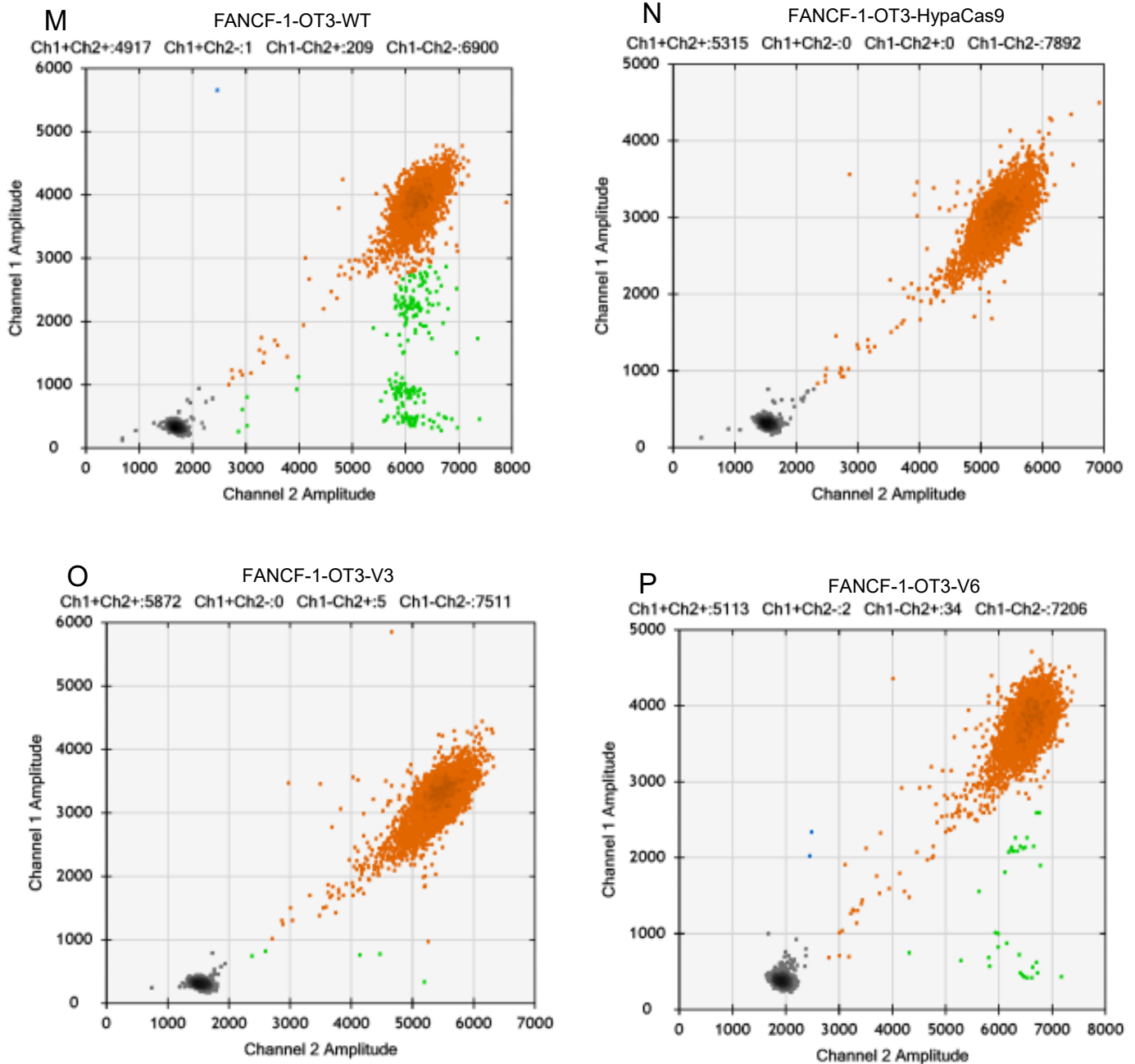


Figure S6.1, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, eSpCas9(1.1), variants V3, variant V6, variant V7, and variant V8 on EMX1-1-OT2 (293T, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The blue dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

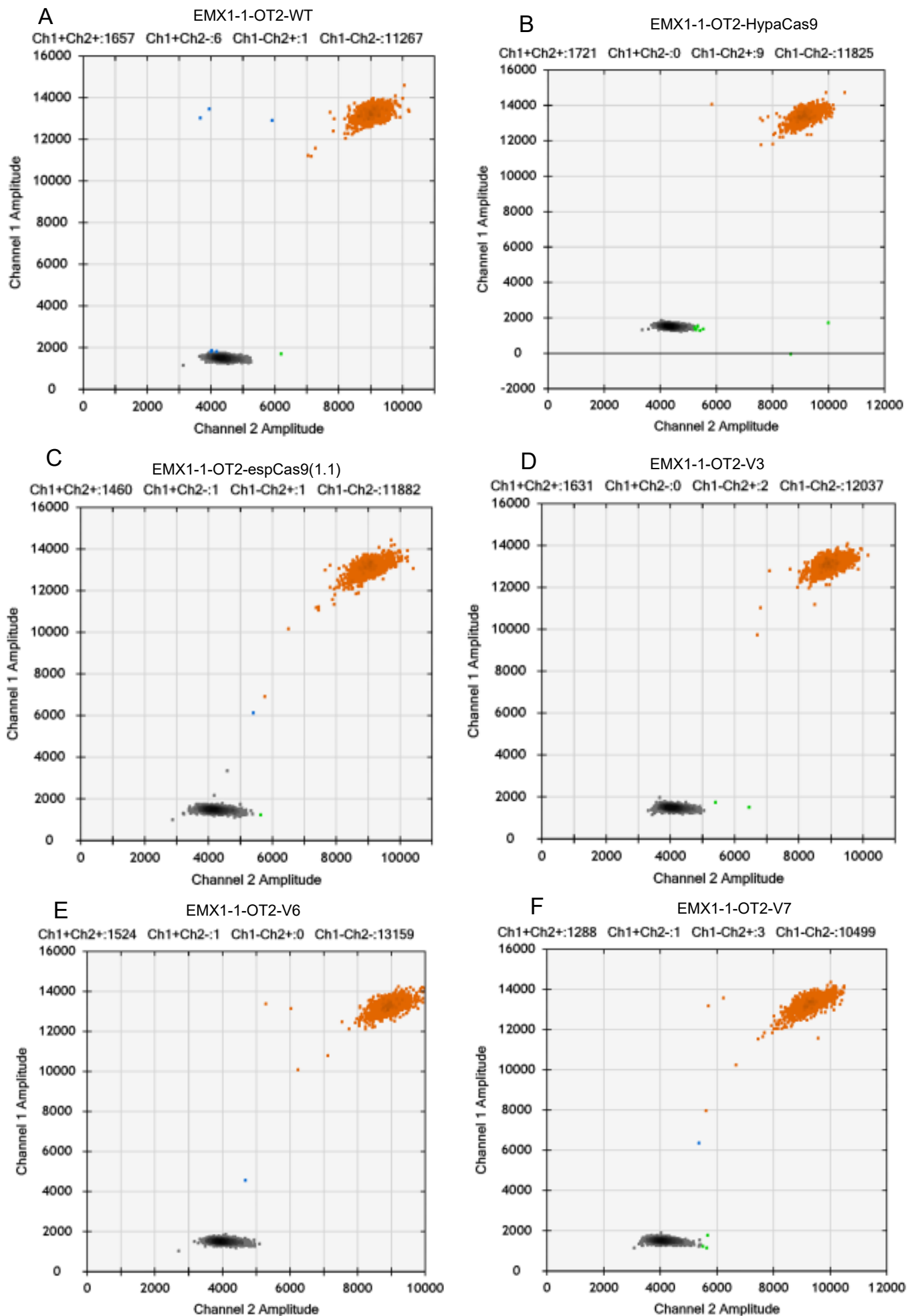




Figure S6.1, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, eSpCas9(1.1), variants V3, variant V6, variant V7, and variant V8 on EMX1-1-OT2 (293T, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The blue dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

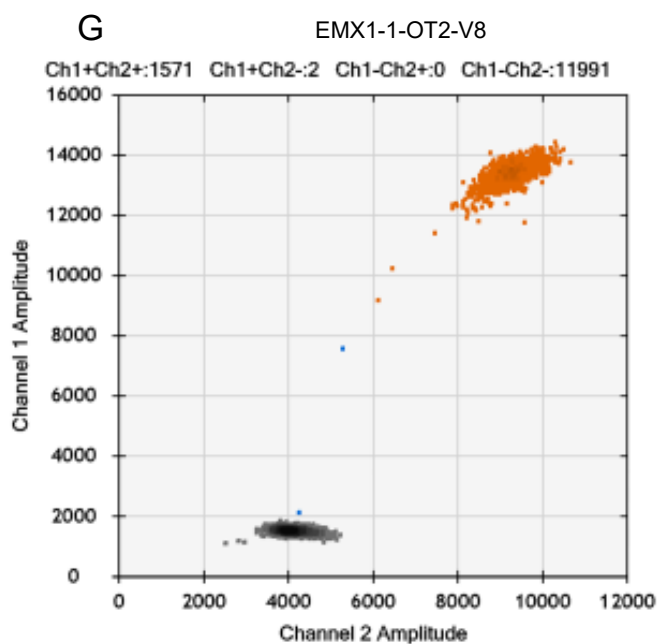


Figure S6.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, eSpCas9(1.1), variants V3, variant V6, variant V7, and variant V8 on FANCF-1 (293T, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

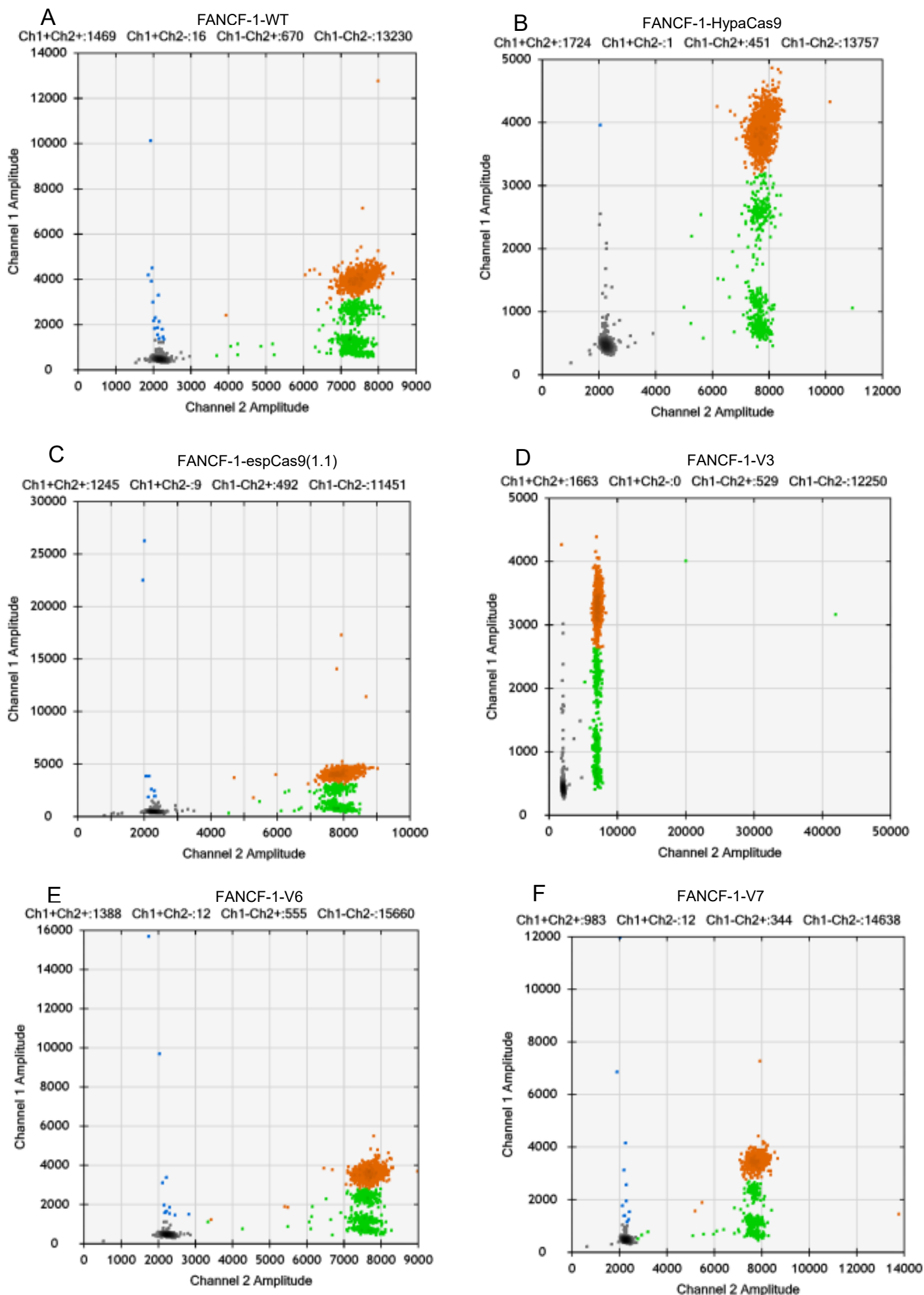


Figure S6.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, eSpCas9(1.1), variants V3, variant V6, variant V7, and variant V8 on FANCF-1 (293T, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

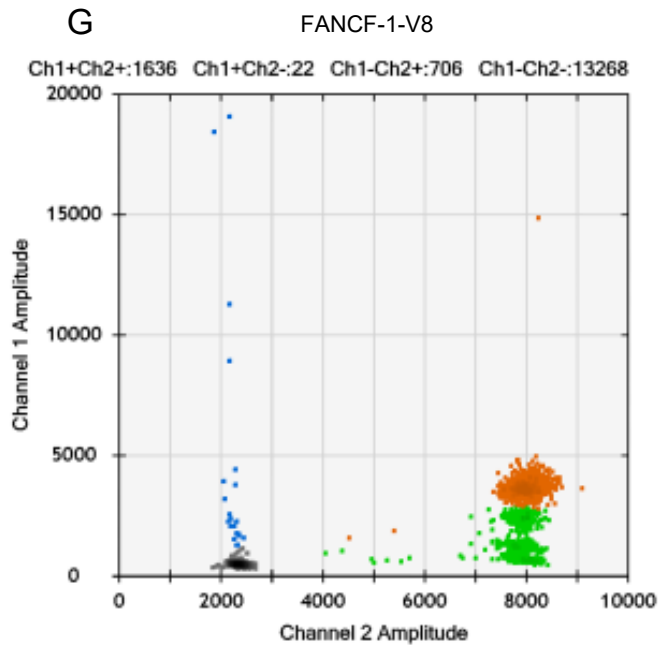


Figure S6.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, eSpCas9(1.1), variants V3, variant V6, variant V7, and variant V8 on FANCF-1 (293T, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

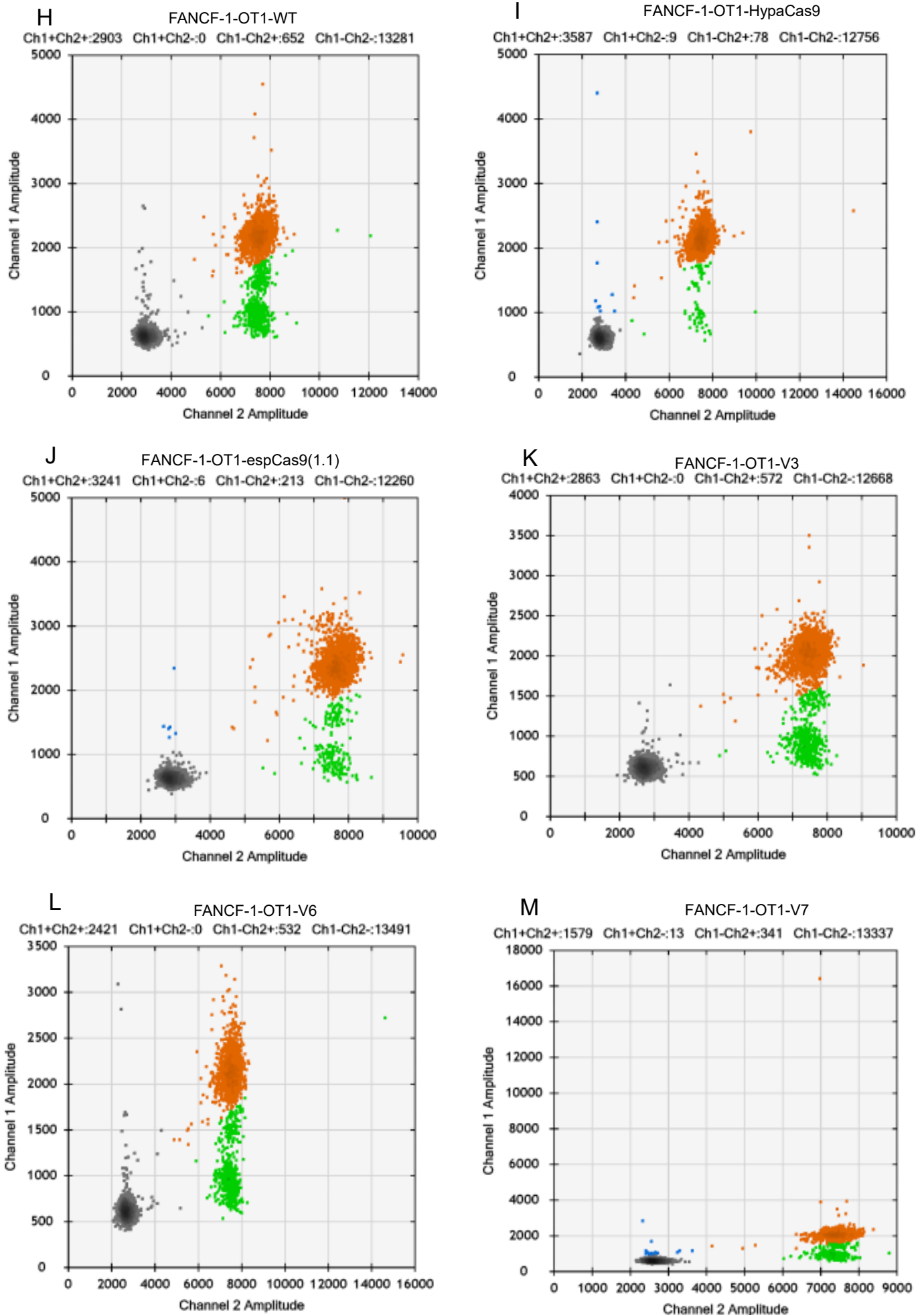


Figure S6.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, eSpCas9(1.1), variants V3, variant V6, variant V7, and variant V8 on FANCF-1 (293T, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

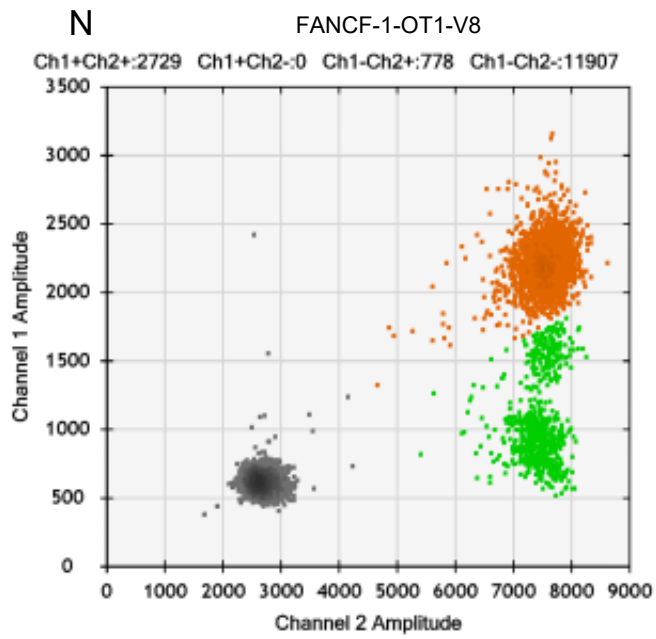




Figure S6.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, eSpCas9(1.1), variants V3, variant V6, variant V7, and variant V8 on FANCF-1 (293T, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

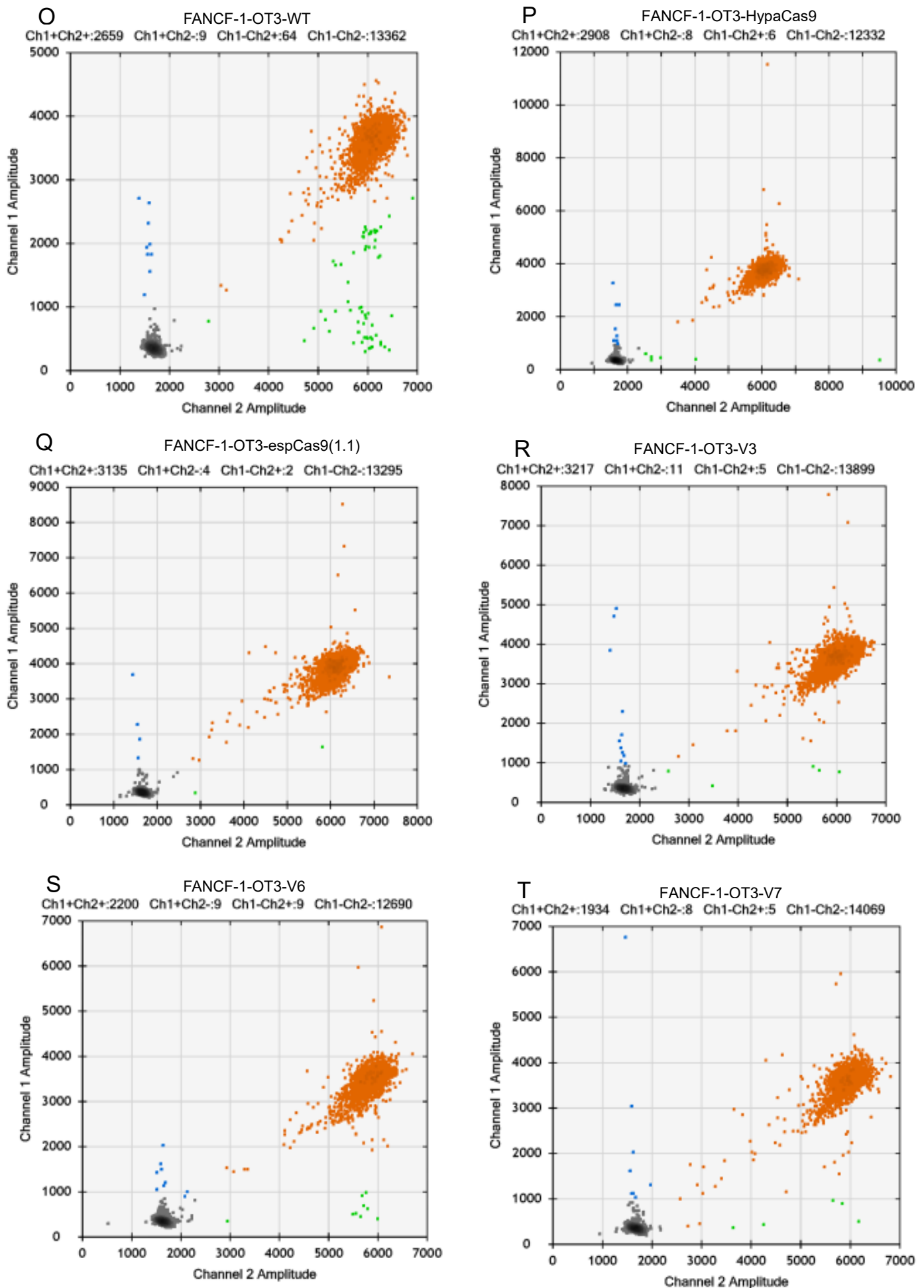


Figure S6.2, DdPCR 2D amplitude figure of WT-SpCas9, HypaCas9, eSpCas9(1.1), variants V3, variant V6, variant V7, and variant V8 on FANCF-1 (293T, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The green dots indicate mutation events, and the brown (blue+ & green+) dots indicate wild-type events.)

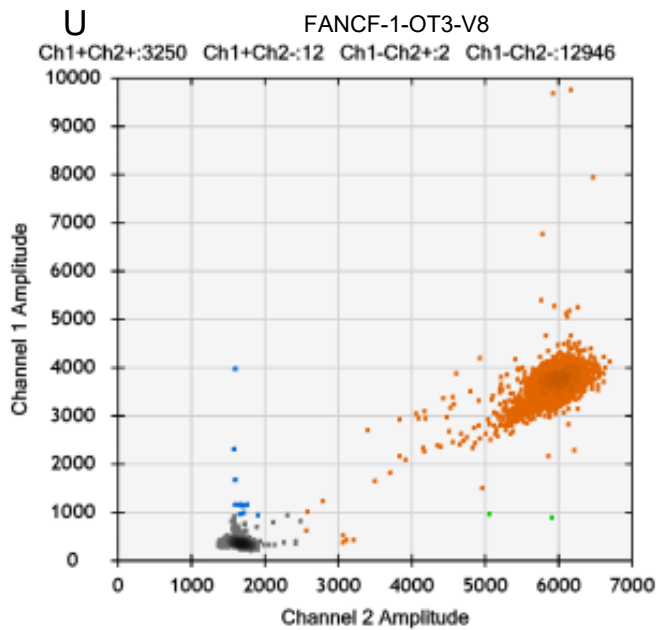


Figure S6.3, DdPCR 2D amplitude figure of WT-SpCas9, HiFi Cas9, and variant V6 on EMX1-1 (293T, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The blue dots indicate mutation events while the brown (blue+ & green+) dots indicate wild-type events.)

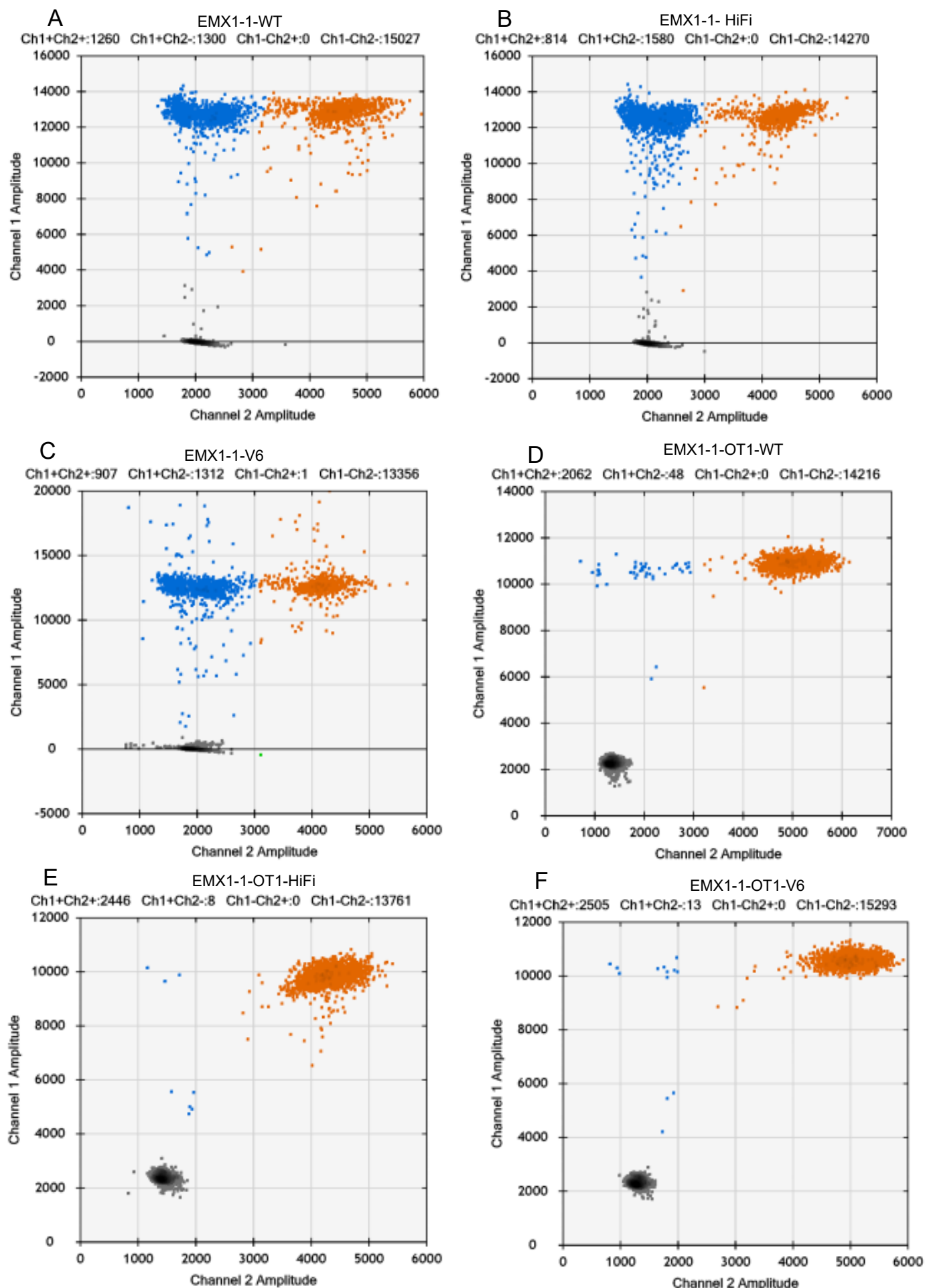


Figure S6.3, DdPCR 2D amplitude figure of WT-SpCas9, HiFi Cas9, and variant V6 on EMX1-1 (293T, 3day). (Channel 1 amplitude is the FAM amplitude, which shows blue fluorescence; Channel 2 amplitude is the VIC amplitude, which shows green fluorescence. The blue dots indicate mutation events while the brown (blue+ & green+) dots indicate wild-type events.)

