A picodroplet digital PCR approach for the absolute quantification of KRAS G12D using LNA probes

RainDance Technologies

APPLICATION NOTE

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Approximately 15–25% of patients with lung adenocarcinoma have tumour associated KRAS mutations. Missense KRAS mutations introduce an amino acid substitution at position 12, 13, or 61. KRAS mutations are negative predictors of radiographic response to the EGFR tyrosine kinase inhibitors, erlotinib and gefitinib.[1] Therefore KRAS detection can be used to determine the treatment strategy of a patient.

The G12D mutation results in an amino acid substitution at position 12 in KRAS, from a glycine (G) to an aspartic acid (D).[2] Direct quantitative detection of such mutant DNA is not always feasible by current technologies because the ratio of mutant to wild type DNA may be as low as 1/10,000, often beyond the capabilities of qPCR or digital PCR (dPCR) approaches with low reaction numbers.

The RainDrop™ system lower limit of detection (LLOD- determined by RainDance) for the KRAS p.G12D (c.35 G>A) mutation is 1/45,000 (3.5E+07 WT copies examined).[3] This highly sensitive and specific method has the potential to be employed in the clinic, including diagnosis, cancer recurrence monitoring, and treatment management.

Table 1 Primer and probe design

F primer	5'- CTG AAA ATG ACT GAA TAT AAA CTT GTG G -3'
R primer	5'- TAG CTG TAT CGT CAA GGC ACT –'3
WtProbe	5'-/56-JOEN/TTG GAG CT+G GTG GCG TA/3IABkFQ/-3
MtProbe	5'- /56-FAM/TTG GAG CT+G+A+TG GCG TA/3IABkFQ/ -3

Here we present data showing the minimum target input and the effects of sample input and fragment size using Locked Nucleic Acid (LNA) probes for the quantification of KRAS G12D. The assay was designed by BIOKÉ and performed on cell line and Formalin-Fixed Paraffin-Embedded (FFPE) material using the RainDrop system.

For this study primers and LNA probes from Integrated DNA technologies (table 1) and 2x Universal Genotyping Master mix from Life Technologies were used. Data analysis was performed using the RainDrop Analyst software. The positive intact droplet counts on channel photomultiplier tube (PMT) 1 (X-axis) indicate the number of target molecules for the mutation cluster (FAM) and accumulate in Q3. The wild type cluster (JOE) in Q1 is represented on the PMT 2 (Y axis). The cluster in the lower left corner (Q4) represents the negative cluster. A typical heterozygous cluster profile is shown in figure 1.

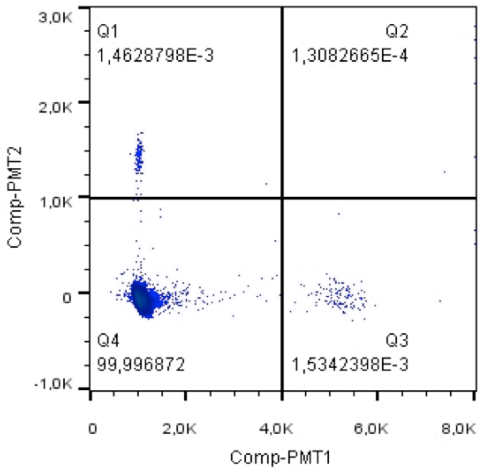

Figure 1 Duplex cluster profile of a Horizon Diagnostics heterozygous reference sample. Droplets containing the KRAS wild type (JOE cluster) accumulate in Q1, mutation KRAS G12D (FAM cluster) in Q3 and the droplets with no amplification in Q4.

Lower limit of input determination using Horizon Diagnostics reference material

The minimum target input was determined by using a Horizon Diagnostics (HDx) reference consisting of 50% heterozygous (KRAS Wt / G12D) gDNA. An eight step serial dilution was made. Input based on the NanoDrop ND-1000 spectrophotometer of 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 ng was used.

By plotting the allelic ratio of the biological replicates the accuracy of each dilution was analysed, in order to determine the lower limit of input. In figure 2 the log of total events is plotted showing a correlation coefficient of > 0.99, which indicates an accurate assay. The spreading of the allelic ratio of the replicates is presented in figure 3.

It shows the two lowest dilutions, 0.625 and 0.3125 ng as outliers. Without these 2 outliers the CV% score is 5.6%. The lower limit of input according to this graph is 1.25 ng, however the calculated lower limit is even lower. In total 129 positive droplets counts were found for this concentration, which corresponds with approximately 426 pg of amplifiable DNA (3.3 pg per haploid genome * 129 events).

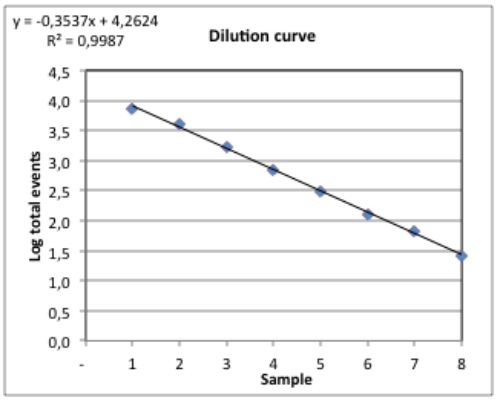

Figure 2 Dilution curve heterozygous HDx gDNA reference

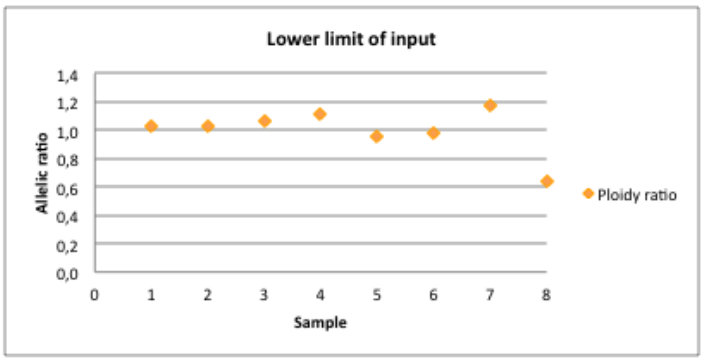

Figure 3 Spreading of allelic ratio of heterozygous HDx gDNA reference

Influence of sample input on the quantification

Genomic DNA (gDNA) from cell line LS480 was obtained from the Leiden University Medical Center (LUMC) pathology department. This was used to determine the influence of different sample input. Heterozygous (G12D) biological replicates of 30 ng (in duplicate), 20 ng and 10 ng were analysed. By correcting the total target counts to 30 ng the spreading between the replicates was found.

The CV% between the heterozygous replicates with different sample input is 1.4%, indicating that different amounts of sample input have no influence on the quantification performance.

Influence of fragment size on the quantification

To investigate the influence of fragment size, homozygous (KRAS wild type) biological replicates of intact and sheared gDNA (500 bp) from a Colo320 cell line (LUMC) were examined. The Covaris S2 focused ultrasonicator was used for the shearing. By comparing the total target counts between the intact and sheared replicates the spreading was determined.

The homozygous cell line replicates with intact and sheared (500 bp) gDNA score a CV% of 3.9%. This indicates that also with different fragment size the RainDrop system generates accurate data.

Reproducibility of FFPE material

DNA from FFPE sections taken from patient bowel tumours in 2013 were isolated and characterized by the LUMC.[4,5] To examine the reproducibility for FFPE material, duplicates were analysed with a known KRAS variant.

The target events of the FFPE samples confirmed the tumour characterization by the LUMC. The FFPE replicates from patient 1 (homozygous KRAS wild type biopsy) score a CV% of 5.2% and of patient 2 (heterozygous G12D biopsy) 7.2%. This shows that even with notorious FFPE material accurate quantification using the RainDrop system is possible.

In summary, here we show that the RainDrop dPCR system is capable to accurately perform absolute quantification on KRAS G12D in cell line and FFPE material with a lower limit of input 0.5 ng amplifiable DNA.
Fragment size and sample load don't have significant influence on the quantification giving a reproducibility of < CV 10%.

References

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