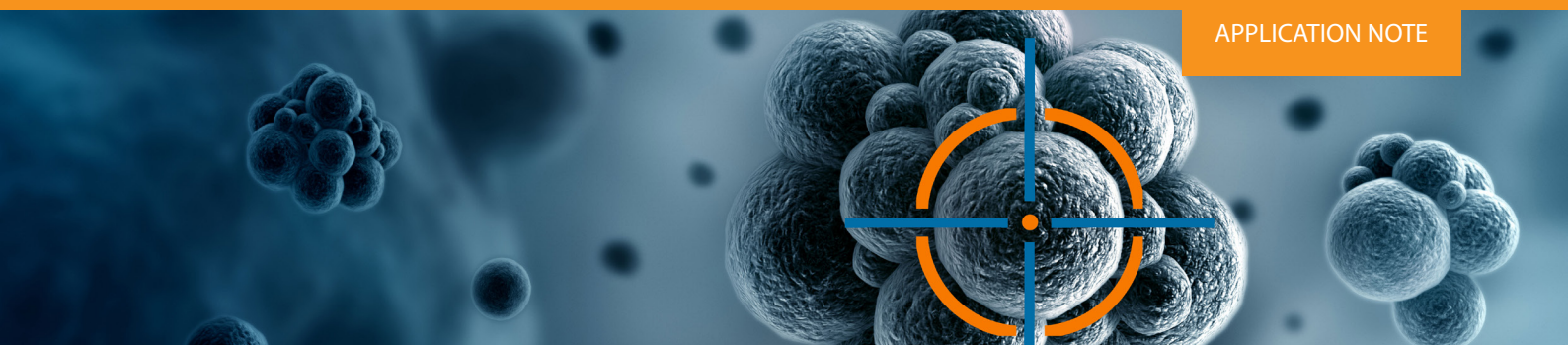
The analysis of EGFR p. L858R in FFPE material

Total workflow solution

APPLICATION NOTE



Mutations in the EGFR gene are important biomarkers in lung cancer. Therefore EGFR mutation detection can be used to determine the treatment strategy of a patient. The EGFR, p.L858R, c.2573T>G mutation is an example of such a variant and occurs with a frequency of approximately 43% in EGFR-mutated lung tumors. This mutation results in an amino acid substitution at position 858 in EGFR, from a leucine to an arginine. [1] Direct quantitative detection of such mutant DNA is not always feasible by current technologies because of the low ratio of mutant to wild type DNA. The RainDrop™ dPCR system from RainDance technologies is able to detect 1 mutant amongst 250,000 wild-type molecules with a potential lower limit of detection > 1:1,000,000 (0.0001%). Also the system is capable of generating 10 million individual droplets per sample providing for high linear dynamic range, minimal Poisson Correction and a reduced reagent consumption per sample. [2]

The analysis by RainDrop™ dPCR system within a single channel is particularly beneficial when samples are precious, low in DNA concentration or contain rare mutations. Furthermore, the hands off workflow is beneficial in a clinical environment when pipetting errors from other more complex workflows can potential misguide clinical decisions. The RainDrop™ dPCR system has the potential to be employed in the clinic, including diagnosis, cancer recurrence monitoring and treatment management. In a previously published validation project, in which EGFR was tested using a different dPCR method, samples were divided across multiple channels in order to determine mutant allele frequencies of 0.1% and below. [3] Here we present data showing an accurate and cost-effective total workflow solution for the quantification of EGFR p. L858R, using HDx™ Reference Standards from Horizon Discovery (Horizon). Horizon is a leading provider of human genomic reference standards, including Formalin-Fixed Paraffin-Embedded (FFPE) cell line sections, cell free DNA and purified genomic DNA. Drawing upon their proprietary genome editing platform, Horizon is able to engineer and extensively validate clinically relevant cancer genes in human cell lines with defined allelic frequencies. HDx Reference Standards are therefore ideal for validating molecular assay sensitivity and specificity.

DNA extraction

100% EGFR Wild Type FFPE Reference Standard (# HD141) and EGFR FFPE Gene -Specific Multiplex Reference Standard at 1% allelic frequency (# HD850) were used. FFPE DNA extractions were executed by the Research & Development department of MACHEREY-NAGEL GmbH & co using the NucleoSpin® DNA FFPE XS kit (#MN 740980). For the EGFR wild type Reference Standard and for the 1% EGFR p. L858R Reference Standard, 7 FFPE sections and 4 FFPE sections were used respectively, each containing a minimum of 400 ng of DNA. The DNA recovery and quality was determined by qPCR using the Quantifiler® Human DNA Quantification kit from Applied Biosystems.

All sample show Ct values < 26 indicating a moderate template quality. In figure 1 the yield after FFPE extraction is shown for each Reference Standard. The average yield found for the EGFR wild type standard is 578 ng and for the 1% mutant 589 ng, confirming a high DNA extraction performance of the kit.

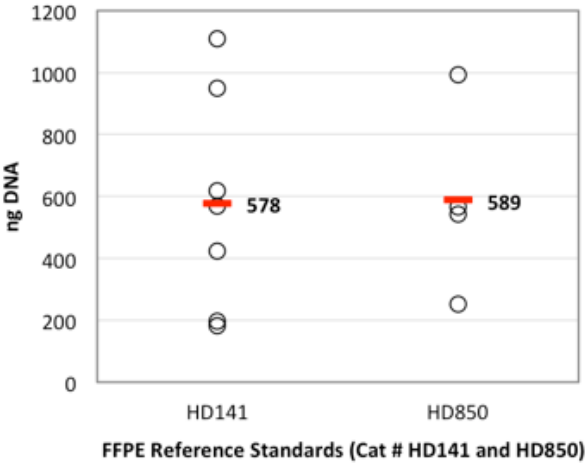


Figure 1 Total DNA yield from HDx™ FFPE standards with NucleoSpin® DNA FFPE XS kit according to qPCR (Quantifiler® Human DNA Quantification Kit)

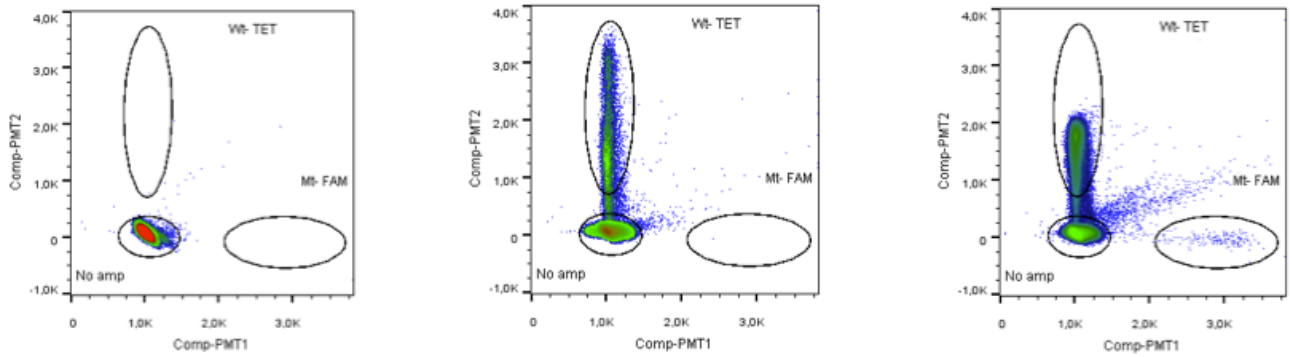
Analysis

The eluates for each Reference Standard were pooled. For the dPCR assay, 5 samples were included:

- Assay negative control (water),
- 100% wild type EGFR,
- 1% EGFR p.L858R,
- 0.1 % EGFR p.L858R and;
- 0.1 % EGFR p.L858R (replicated).

From each sample 10 µl of DNA was added to a total reaction mix of 50 µl. To obtain the 0.1% mutant L858R frequency sample, 1 µl of 1% EGFR, p.L858R was added to 9 µl wild type extracted DNA. The assay was obtained from RainDance technologies as a 10 x EGFR L858R assay mix [2] and the 2 x Universal Genotyping Master Mix from Life Technologies was used. The RainDrop™ Source instrument was used to generate the droplet emulsion from 50 µl reaction mixes. After target amplification within the emulsion the samples were analyzed on the RainDrop™ Sense instrument. The data analysis was performed using the RainDrop™ Analyst software.

Figure 2 shows representative cluster profiles for 3 of the samples: BLANK (A), wild type (B) and mutant (C). The normalized allele counts and allelic frequencies for all 5 samples are provided in table 1 with replicate samples (1 and 4) having an extremely consistent CV of 2.5 %, confirming a high reproducibility.



A BLANK **B** 100% wild type (EGFR) **C** Mutant (0.1% EGFR p.L858R)

Figure 2 Observed results on the RainDrop™ System for EGFR L858R. X-axis = MUT, FAM label; Y-axis = WT, TET label. Cluster A: Assay negative control (water); Cluster B: 100% EGFR wild type FFPE Reference Standard; Cluster C: 0.1% EGFR Quantitative Multiplex FFPE Reference Standard.

Table 1 Allele counts

Samples	Counts FAM (Wt)	Counts TET (Mt)	% EGFR L858R
Sample 1 (0.1% EGFR, p.L858R)	192314	208	0.1
Sample 2 Wt (0% EGFR, p.L858R)	33299	6	0.0
Sample 3 (1% EGFR, p.L858R)	102721	980	1.0
Sample 4 (0.1% EGFR, p.L858R)	185533	213	0.1
BLANK	NA	NA	NA

In summary, we present data that shows an accurate and cost-effective total workflow solution for the characterization of EGFR in FFPE material. The NucleoSpin® DNA FFPE XS kit results in Ct values below 26 with a high DNA recovery rate from FFPE, indicating a moderate template quality and high DNA extraction performance of the kit. Furthermore, the results from the RainDrop™ dPCR system, where only a single channel is needed, match the allelic frequencies that are found within the HDx Reference Standards confirming both its accuracy and reproducibility with further replicate samples having a CV of 2.5%.

Accompanied items

- 100% EGFR wild type FFPE Reference Standard # HD141
- 1% EGFR Quantitative Multiplex FFPE Reference Standard # HD850
- NucleoSpin® DNA FFPE XS kit # MN740980
- RainDrop™ Source instrument # RD20-04401
- RainDrop™ Sense instrument # RD20-04402
- SensoQuest Labcycler Gradient # SQ011-101

References

[1] Mitsudomi, T., Yatabe, Y. 2010. Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer. The FEBS Journal 277-301

[2] Milbury, C., Zhong, Q., Lin, J., Williams, M., Olson, J., Link, D., Hutchison, B. 2014. Determining lower limits of detection of digital PCR assays for cancer-related gene mutations. Biomolecular Detection and Quantification 1 8-22

[3] Application note: Genetically Defined B-Raf, K-Ras and EGFR Reference Standards used to validate the sensitivity of Droplet Digital PCRTM. 2015. https://www.horizondiscovery.com/media/resources/Application%20Notes/reference-standards/Genetically%20Defined%20B-Raf,%20K-Ras%20and%20EGFR%20Reference%20Standards%20used%20to%20validate%20the%20sensitivity%20of%20Droplet%20Digital%20PCR.pdf

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