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High Definition PCR (HDPCR™) Detection of Molecular Biomarkers in Acute Myeloid Leukemia Contrived Samples for Measurable Residual Disease

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Introduction

Acute myeloid leukemia (AML), a malignant cancer starting in the bone marrow, accounts for 25% of all leukemias diagnosed in adults. Measurable residual disease (MRD) is a fundamental prognostic indicator for detecting early relapse of the disease and can be used to detect the presence of leukemic cells at low levels after treatment. AML-MRD detection requires biomarkers to have high specificity and sensitivity, with a general requirement of 0.1% for the Limit of Detection (LoD). Current testing methods include FISH and cytogenic testing for the detection of chromosomal abnormalities such as insertions, deletions, and fusions, but may take weeks to perform. Next-Generation Sequencing as an alternative testing option is available for some patients, but its high-cost and long turnaround times lead to the preference of FISH and single-plex testing. The need for a highly sensitive, fast turnaround, and low-cost testing option can be met by digital PCR (dPCR) testing. ChromaCode's High-Definition PCR (HDPCR) technology allows for dPCR multiplexing for a number of relevant biomarkers that exceeds the number of available color channels on an instrument. The assay leverages the analysis through ChromaCode Cloud, which quickly processes the data files and produces a report for each well.

Here we present a prototype HDPCR assay for the detection of 7 biomarkers and an internal control, including *PML-RARA*, *RUNX1-RUNX1T1*, *DEK-NUP214*, *KMT2A-MLLT3*, and *CBFB-MYH11* fusions, *NPM1* insertions, and *FLT3*-TKD point mutations, associated with acute myeloid leukemia. The assay was tested on the Qiagen QIAcuity digital PCR instrument, as well as the Roche Digital LightCycler, though it could be compatible with other dPCR instruments with minimal optimization required.

Methods

The ChromaCode HDPCR AML prototype assay is an assay designed to detect an internal control and seven (7) unique DNA mutations and RNA fusions in a single well[1,3]. The inclusivity of the assay is outlined in Table 1. The performance of the assay was characterized by testing the limit of detection (LoD) and limit of quantitation (LoQ) using contrived samples. The samples were built by spiking target plasmids into a negative hgRNA background, and then tested on the Qiagen QIAcuity and Roche Digital LightCycler with variant allele frequencies (VAF) down to 0.01%. The LoQ was performed over three (3) days by testing four (4) samples for each of the reportables in replicates of three (3) at 0.1% VAF.

The data was analyzed using proprietary algorithms and multi-spectral encoding[2] (Fig. 2), housed on the ChromaCode Cloud software (Fig. 2), capable of detecting and quantifying the eight (8) targets.

Table 1. Inclusivity of the 7 biomarkers in the AML-MRD prototype assay

SNP and Insertion Targets	Protein Change	Nucleic Acid Change
NPM1	p.W288Cfs*12	c.860_863dup
NPM1	p.W288Cfs*12	c.863_864insCATG
NPM1	p.W288Cfs*12	c.863_864insCCTG
NPM1	p.W288Cfs*12	c.863_864insCTTG
***FLT3*-TKD**	p.D835Y	c.2503G>T
Fusion Targets	**Translocation**	
CBFB-MYH11	ENST00000412916.7(CBFB):r.1_754::ENST00000300036.6(MYH11):r.4684_6880	
CBFB-MYH11	ENST00000412916.7(CBFB):r.1_754::ENST00000300036.6(MYH11):r.3964_6880	
CBFB-MYH11	ENST00000412916.7(CBFB):r.1_754::ENST00000300036.6(MYH11):r.3757_6880	
DEK-NUP214	ENST00000652689.1(DEK):r.1_1102::ENST00000359428.10(NUP214):r.2556_7568	
KMT2A-MLLT3	ENST00000534358.5(KMT2A):r.1_4355::ENST00000380338.8(MLLT3):r.1413_6772	
PML-RARA	ENST00000268058.7(PML):r.1_1753+?::ENST00000254066.9(RARA):r.634-?_2414	
PML-RARA	ENST00000268058.7(PML):r.1_1279+?::ENST00000254066.9(RARA):r.634-?_2414	
PML-RARA	ENST00000268058.7(PML):r.1_1595::ENST00000254066.9(RARA):r.634-?_2414	
RUNX1-RUNX1T1	ENST00000300305.7(RUNX1):r.1_1058::ENST00000360348.6(RUNX1T1):r.189_2370	

Results

The LoD for the HDPCR AML prototype assay was evaluated on the Qiagen QIAcuity and Roche Digital LightCycler by running contrived samples at an estimated 0.1% and 0.01% VAF. The samples were built by spiking plasmid containing the target sequences into Invitrogen Universal Human Reference RNA. The samples were then run on both instruments.

A positive call is made on ChromaCode Cloud when ten (10) or more positive partitions are detected. This threshold is determined by repeatably detecting these targets and differentiating them from noise in the presence of > 60,000 positive control (*ABL*) partitions. With this cutoff established, all seven (7) reportables in the AML assay were detected at < 0.1% VAF, with *PML-RARA* and *KMT2A-MLLT3* being detected below 0.01% VAF on the QIAcuity instrument. The individual LoD performance on each dPCR instrument for each target is reported in Table 2. A lower LoD may also be achieved with a higher sample input, though for this study each target had 40,000 – 50,000 positive control partitions.

The Limit of Quantitation (LoQ) was also evaluated on the QIAcuity, with LoQ determined to be < 0.1% for all targets with a 30% CV.

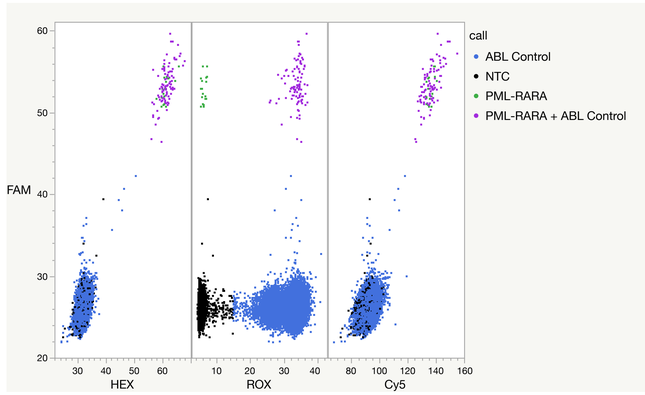

Figure 1. Example of resilient coding, where the *PML-RARA* target (green) amplifies in Channels FAM, HEX and Cy5, allowing for increased specificity. The software calculates the %VAF and counts detected by counting the number of *PML-RARA* positive partitions (green), as well as the number of positive partitions where both *PML-RARA* and the internal control *ABL* are detected (purple). The target counts are then compared to the internal control (blue) positive partitions present.

Table 2. Summary of the LoD and LoQ performance using the HDPCR AML prototype assay

Target	Qiagen QIAcuity LoD	Roche Digital LightCycler LoD	Qiagen QIAcuity LoQ based on 30% CV
NPM1	0.013%	0.074%	0.087%
***FLT3*-TKD**	0.018%	0.061%	0.095%
CBFB-MYH11	0.034%	0.069%	0.092%
DEK-NUP214	0.026%	0.163%	0.078%
KMT2A-MLLT3	0.004%	0.111%	0.074%
PML-RARA	0.006%	0.071%	0.069%
RUNX1-RUNX1T1	0.010%	0.047%	0.083%

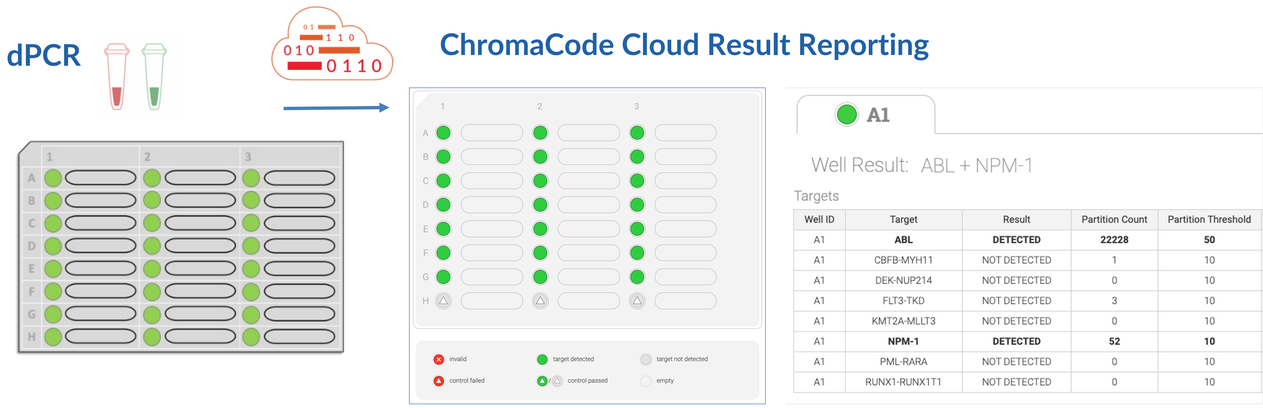

Figure 2. Representative workflow for the HDPCR AML-MRD assay. Sample is plated onto a digital instrument plate. After the run is complete, the file is uploaded to the ChromaCode Cloud, which processes the file and outputs a report for each individual well tested.

Conclusion

The HDPCR prototype assay includes 7 relevant AML-MRD biomarkers and shows the sensitivity of < 0.1% and specificity required to detect AML relapse in < 24 hours. Though the prototype assay has an inclusivity > 95% for most targets, the assay could be expanded further to include further variants of the targets present in the prototype.

Given the low background noise, the assay is capable of making a positive call with as few as 10 positive partitions present, meaning the %VAF of a detectable target could decrease if sample input is increased.

The targets can be reported using an easy cloud-based system, the ChromaCode Cloud, which rapidly and accurately reports the presence and VAF of relevant AML-MRD variants, making this prototype assay a potential solution to meet AML-MRD testing needs.

References

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