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Title of Presentation	Pushing the boundaries of KIT p.D816V mutation detection with the ultra-sensitive superRCA assay
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Abstract of Presentation	Background Activated KIT mutations, particularly KIT p.D816V, are central to systemic mastocytosis (SM) and other mast cell diseases. KIT p.D816V is found in up to 95% of SM patients and is an important molecular hallmark for diagnosis, classification and risk stratification. More than 80% of patients with SM present with variant allele frequencies (VAFs) below 0.1%, often approaching the lower limits of detection in peripheral blood (PB). KIT p.D816V allele-specific oligonucleotide quantitative PCR (ASO-qPCR) methods have reported analytical sensitivities of approximately 0.005% VAF using 400 ng DNA input, while droplet digital PCR (ddPCR) typically achieves sensitivities of approximately 0.02% VAF due to constraints in allele specificity, DNA input capacity and droplet partitioning. Consequently, more sensitive methods are needed to reduce false-negative findings in PB, decrease reliance on bone marrow sampling, and improve longitudinal disease monitoring. Navarra el al. demonstrated sensitivity down to 0.001% using the first commercially available superRCA KIT D816V assay with 660ng input. Here, we describe an ultra-sensitive superRCA KIT D816V assay developed to further improve low-level mutation detection through increased DNA input and enhanced readout depth. Method The superRCA workflow combines high-fidelity PCR pre-amplification, target

circularization, rolling circle amplification (RCA), allele-specific padlock probe genotyping, secondary RCA, and flow cytometric enumeration of fluorescent superRCA particles. In the ultra-sensitive assay, DNA input was increased four-fold from 660 to 2640 ng genomic DNA (approximately 800,000 haploid genome equivalents). Readout precision was improved by generating approximately twice the number of acquired events. Analytical performance was evaluated using KIT p.D816V-positive control DNA diluted into wildtype genomic DNA, covering a range of 0.0005% to 31.6% VAF, with detection of superRCA products by standard flow cytometry on the DxFLEX (Beckman Coulter) and FACSLyric (BD Biosciences) instruments. Background was assessed using 48 wildtype replicates from three independent genomic DNA sources. LoB and LoD were assessed using the nonparametric 95th-percentile rank method. Results Increasing DNA input four-fold significantly improved analytical sensitivity while preserving the assay's ultra-low background. The ultra-sensitive assay achieved an LoD <0.00075%, and importantly, all replicates at 0.0005% VAF were successfully detected corresponding to approximately one mutant allele among 200,000 wildtype alleles. The increased DNA input and enhanced readout depth did not increase background signal and reduced variability across low-VAF samples, resulting in improved measurement precision. Excellent quantitative performance was observed across the tested range (R^2 = 0.9997), demonstrating robust linearity from ultra-low to high mutation burdens. Conclusions By combining increased DNA input with enhanced readout depth, the ultra-sensitive superRCA assay extends the analytical sensitivity of KIT p.D816V detection to sub-0.001% VAF levels without compromising specificity. The ability to consistently detect variants at 0.0005% VAF supports more reliable analysis of peripheral blood samples, facilitating the possibility of less invasive sampling combined with improved assessment of low disease burden and longitudinal monitoring in patients with systemic mastocytosis. This finding sets a new benchmark for KIT p.D816V testing, pushing the boundaries of molecular testing.