Pushing the boundaries of KIT p.D816V mutation detection with the Ultra-Sensitive superRCA assay

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BACKGROUND

KIT p.D816V is detected in up to 95% of patients with systemic mastocytosis (SM) and is an important molecular marker for disease diagnosis, classification, and monitoring.

KIT p.D816V variant allele frequencies (VAFs) are often very low, particularly in peripheral blood, creating a need for highly sensitive molecular methods.

Previous superRCA testing demonstrated detection down to 0.001% VAF using 660 ng DNA. Here, we evaluated a **superRCA Ultra-Sensitive KIT D816V assay** combining increased DNA input with further increased enumeration depth.

OBJECTIVE

Develop an even sensitive KIT p.D816V assay.

METHOD

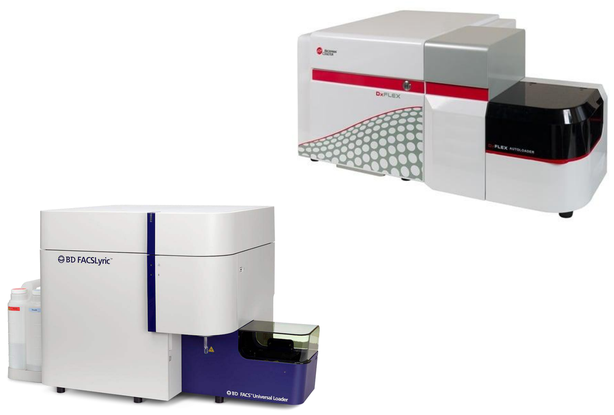
The superRCA technology is a novel method for mutation detection. DNA from lysed cells is genotyped and converted into large fluorescent particles analyzed by flow cytometry.

To further advance assay sensitivity, DNA input was increased **from 660 ng to 2,640 ng,** and the reaction volume was doubled to increase the average readout from 800,000 to 1,600,000 events by flow cytometry.

Based on Poisson statistics, an average of approximately **3 molecules is required for a 95% probability** of detecting at least one molecule. For quantitative detection, at least **4 target molecules are required to achieve a theoretical CV of 50%**, with higher molecule counts providing greater precision.

Assay validation following CLSI ep17 included

- A. Sensitivity - 32 donor replicates
  - Limit of Blank (LoB)
  - Limit of Detection (LoD)
  - Limit of Quantification (LoQ)
- B. Linearity, 24 replicates
- C. Precision – 96 replicates
- D. Trueness/bias – 7 samples with known VAF
- E. The assay was further evaluated between platforms, Beckman Coulter DxFLEX and the Waters (Becton Dickinson) FASCLyric



Probability that ≥1 mutant molecule is present

SENSITIVITY

$P(0) = e^{-\lambda}$
$P(\geq 1) = 1 - e^{-\lambda}$
For 95% detection: $\lambda = -\ln(0.05) \approx 3$

λ = 1	63.2%
λ = 2	86.5%
λ = 3	**95.0%**

PRECISION

λ	3	**4**	10	100
CV	57.7%	**50%**	31.6%	10.0%

$SD = \sqrt{\lambda}$

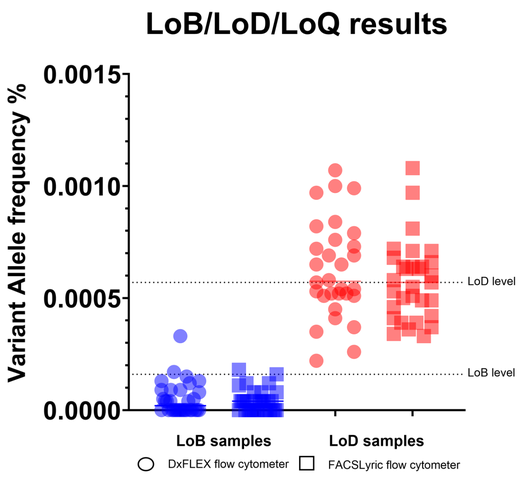
$CV = \frac{SD}{\lambda} = \frac{1}{\sqrt{\lambda}}$

λ – Mean no of captured mutant molecules

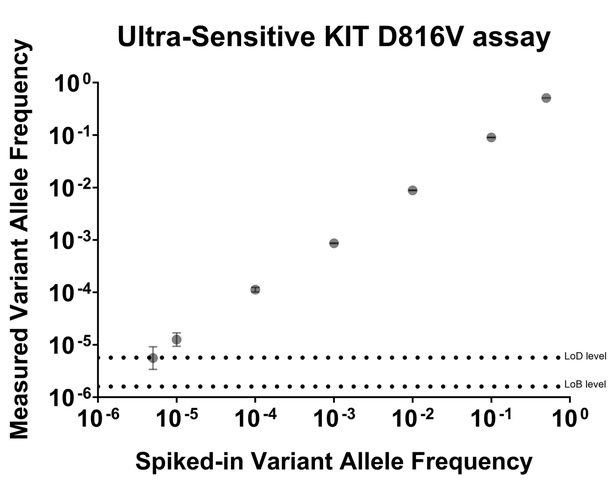
Theoretical detection & precision limits combining 95% confidence precision <50% CV based on molecular input

DNA input	Diploid cells	Gene copies (N)	>95% & <50% CV limit (4/N)
660 ng	100,000	200,000	**0.002% VAF**
1,320 ng	200,000	400,000	**0.001% VAF**
2,640 ng	400,000	800,000	**0.0005% VAF**

RESULTS



A. Sensitivity showed **LoB of 0.00016%**; **LoD and LoQ of 0.00057% VAF**



B. Linearity was assessed in triplicates between 50% to 0.01% and with six replicates from 0.001% to 0.0005%. Results showed an **R^2 of 0.9995 and CV of <32% at LoD**

C. Precision included four dilution levels and Lot-Lot, Between Runs, Between operators, and within run variation. All combined being total CV. **Total CV <5%**

Target VAF %	Mean VAF%	Lot-Lot CV%	Run-Run CV%	Oper. CV%	In-Run CV%	Total CV%
0.1	0.09	1.72	0*	1.85	3.7	**4.48**
1	0.87	1.37	1.69	0.74	1.68	**2.85**
10	8.93	0.99	0*	0*	1.86	**2.1**
50	50.84	0.74	0.17	0.07	0.76	**1.08**

D. Trueness/bias was compared against ddPCR from 0,03% to 50% VAF. Showing and **average bias of -0.026**

Sample	superRCA (mean VAF%)	ddPCR(mean VAF%)	Bias
1	0,023	0,025	**-0,076**
2	0,078	0,073	**0,066**
3	0,796	0,717	**0,105**
4	0,951	1,000	**-0,051**
5	8,31	10,33	**-0,218**
6	29,95	30,88	**-0,031**
7	48,94	50,47	**-0,031**

E. The two instruments were compared using matched samples, with an **average ratio of 0.991**

DxFLEX MAF%	FACSLyric MAF%	Ratio
0.00069	0.00062	**1.11**
0.00109	0.00130	**0.84**
0.01183	0.01127	**1.05**
0.08439	0.08622	**0.98**
0.8700	0.8840	**0.98**
8.8343	9.0217	**0.98**
50.7848	50.9566	**1.00**

CONCLUSION

Increasing DNA input and readout depth extends superRCA KIT D816V detection to **sub-0.001% VAF** without increasing background. The new ultra-sensitive assay demonstrated consistent and **quantitative detection down to 0.00057% VAF** levels across two different instrument platforms.