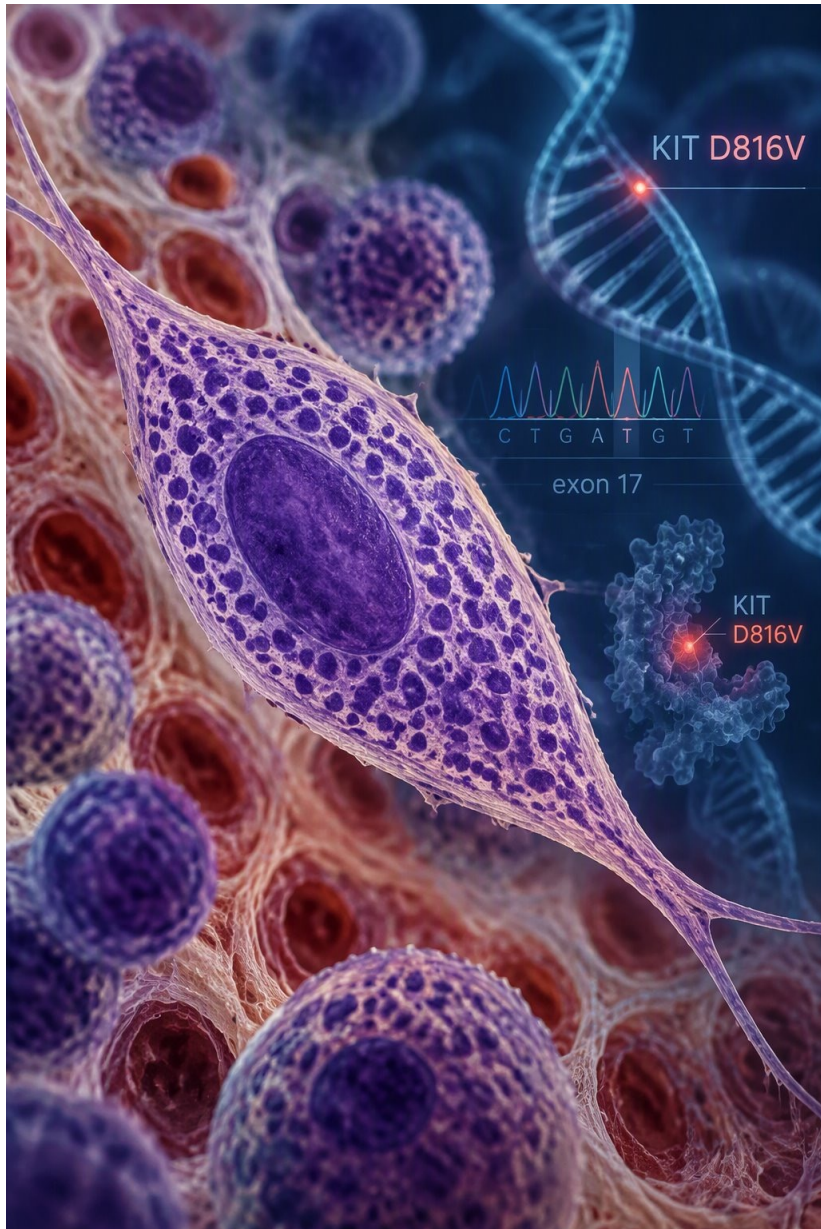




Pitfalls in the molecular diagnosis of SM

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Why Molecular Testing in SM Matters

DIAGNOSIS

An activating KIT mutation is a minor diagnostic criterion and provides evidence of mast-cell clonality.

PROGNOSIS

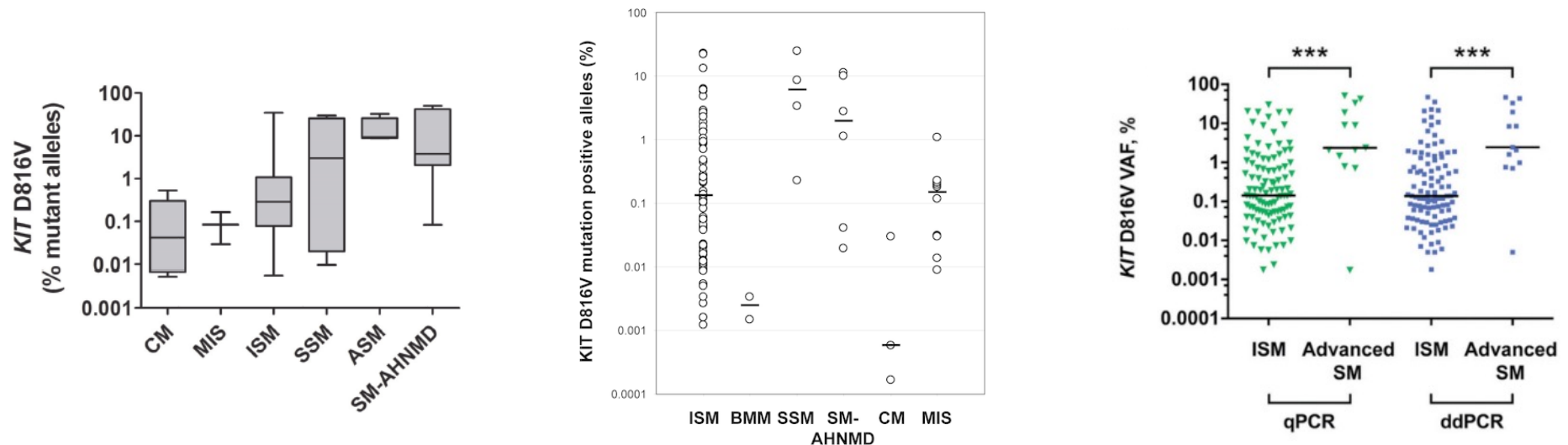
KIT D816V allele burden and additional myeloid mutations can reflect multilineage involvement and adverse biology.

THERAPY & MONITORING

The KIT molecular profile informs targeted treatment selection and may determine eligibility for mutation-directed therapies; quantitative testing can support longitudinal assessment.

Pitfall #1: Low Allele Burden of the Driver Mutation

KIT D816V is highly prevalent in systemic mastocytosis: KIT D816V is detected in >85% of all adult SM cases, including >95% of BMM, ISM and SSM patients and >80% of AdvSM, yet routine workflows may miss it because the neoplastic mast-cell fraction can be extremely low



**not detected
does not
mean absent!**



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Pitfall #2: Specimen Choice for KIT Testing

PERIPHERAL BLOOD vs BONE MARROW

- PB: minimally invasive screen
- BM: generally higher diagnostic yield
- Negative PB cannot rule out SM!

UNSORTED VS ENRICHED CELLS

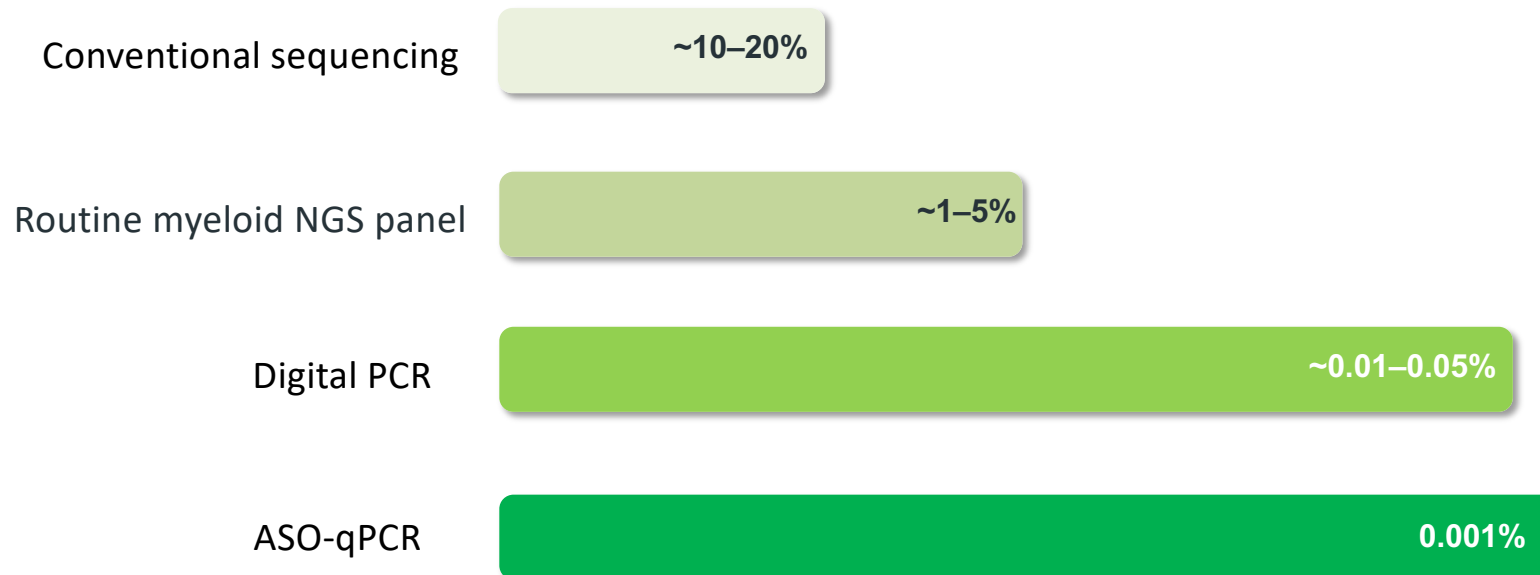
- Enrichment can increase sensitivity
- Not a routine approach, but useful in selected low-burden cases

WHEN BLOOD NEGATIVITY MISLEADS

- Escalate to BM when suspicion persists
- Check hemodilution and cellularity
- Document input and amplifiable copies

Interpret the molecular result in the context of specimen type and adequacy!

Pitfall #3: Analytical Sensitivity of Assays for KIT D816V



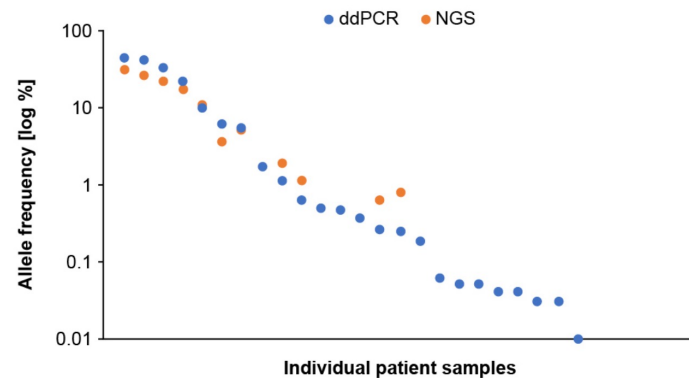
dPCR vs NGS

PIONEER STUDY

Table. Percent of PB samples testing positive for *KIT* D816V in PB samples from patients enrolled in part 1 of PIONEER

	Local assessment n (%)	TruSight NGS n (%)	ddPCR n (%)
<i>KIT</i> D816V detected	31 (80)	11 (28)	37 (95)
<i>KIT</i> D816V not detected	8 (20)	28 (72)	2 (5)
Patients analyzed	39	39	39

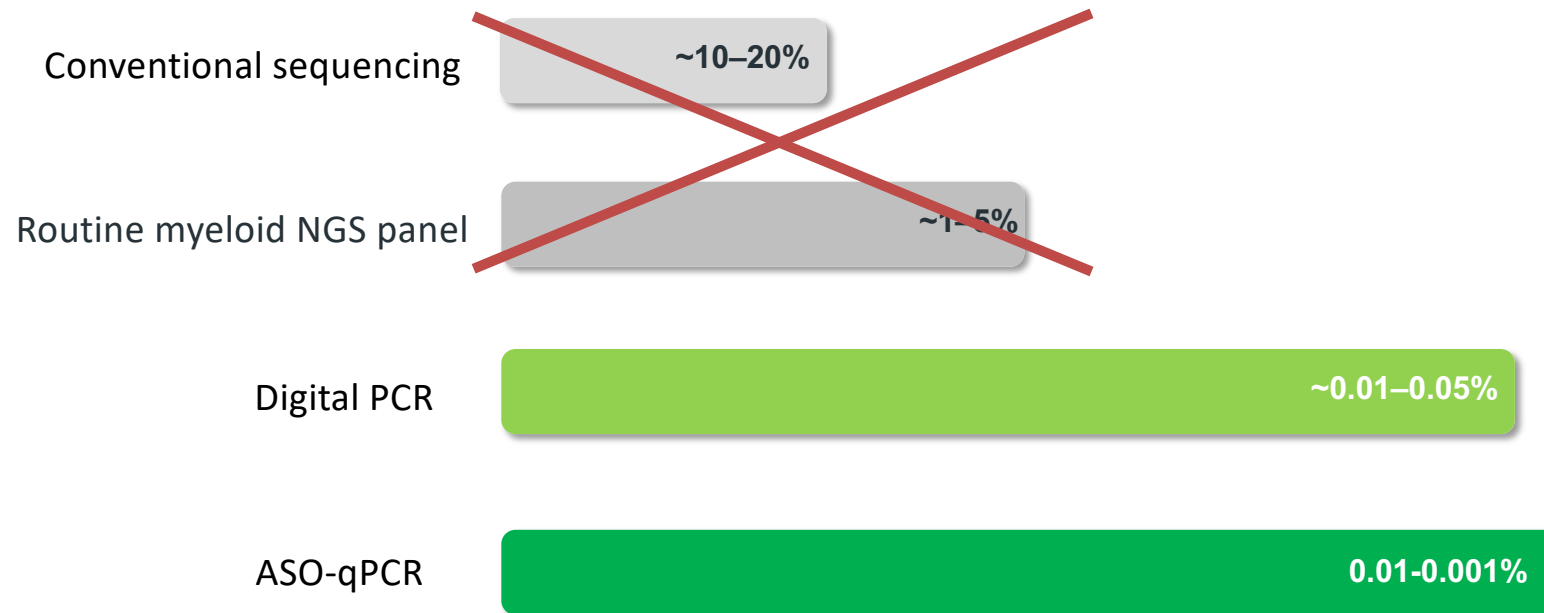
Figure. Performance of central ddPCR and NGS detection of *KIT* D816V VAF in PB samples from patients enrolled in part 1 of PIONEER



“REAL-WORLD”

- In one reference-laboratory series, of 181 samples tested for *KIT* D816V by both NGS and ddPCR, 37.6% (68/181) were positive by ddPCR, and 6.1% (11/181) were positive by NGS.
- NGS showed 16% sensitivity and 100% specificity for *KIT* D816V detection compared with ddPCR as the gold standard, which detected the variant in 84% more samples because of its lower limit of detection.

Pitfall #3: Analytical Sensitivity of Assays for KIT D816V

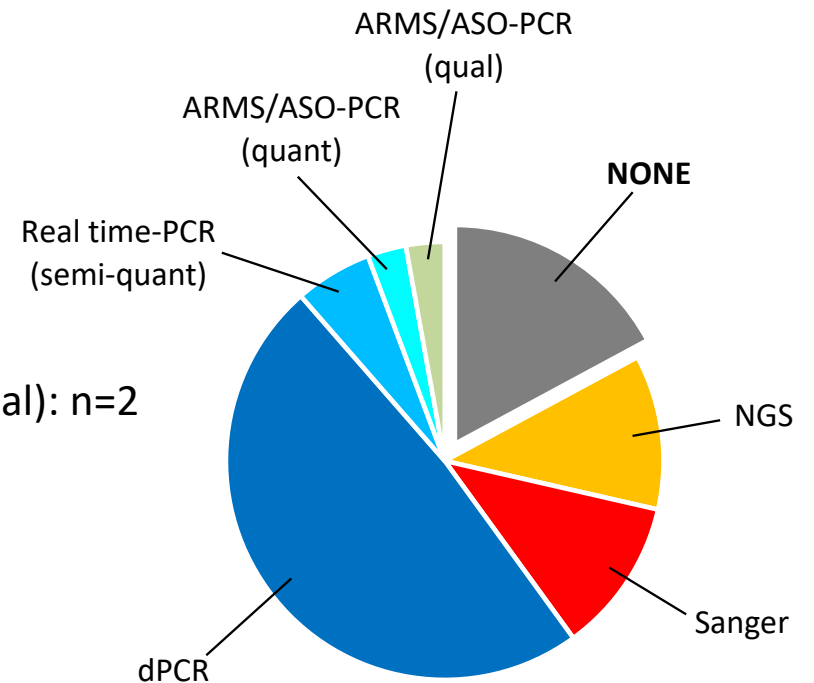


What if we could enhance sensitivity further?

Mapping KIT D816V Mutation Testing in Italy (2023)

SURVEY:

- 35 laboratories invited
- 29/35 (83%) were offering KIT D816V testing
 - Sanger sequencing: n=4
 - NGS (myeloid panel): n=4
 - digital PCR (home-brew or commercial): n=17
 - semi-quantitative real time PCR (home-brew or commercial): n=2
 - quantitative ARMS/ASO-PCR (home-brew): n=1
 - qualitative ARMS/ASO-PCR (home-brew): n=1



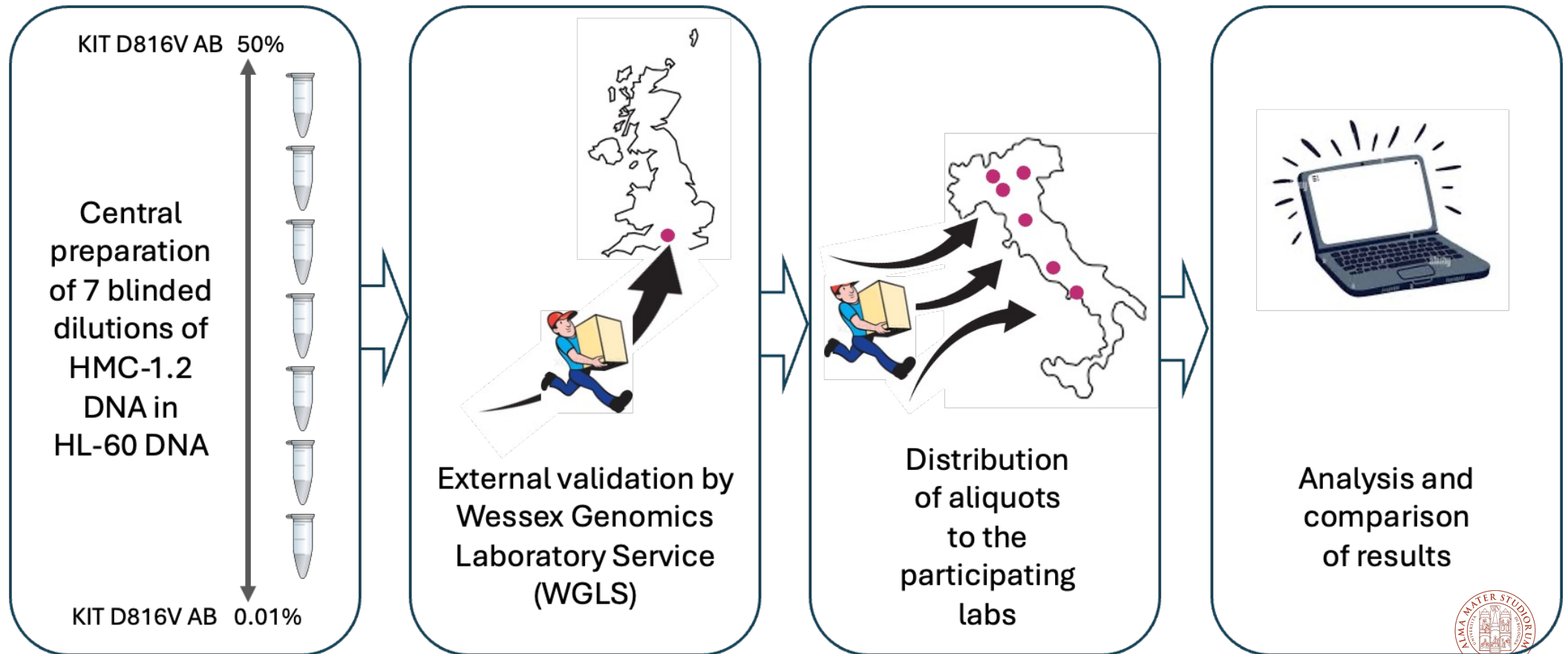
Standardizing KIT D816V Mutation Testing in Italy (2023-ongoing)

PILOT PHASE

- | | |
|---|---------------------|
| 1. Pavia (Dott.ssa Daniela Pietra) | ASO-qPCR, home brew |
| 2. Ospedale Maggiore Policlinico Milano (Prof. Niccolò Bolli) | ASO-PCR, commercial |
| 3. Verona (Dott.ssa Giovanna De Matteis) | dPCR, commercial |
| 4. Bologna (Prof.ssa Simona Soverini) | dPCR, commercial |
| 5. Firenze (Prof.ssa Paola Guglielmelli) | dPCR, commercial |
| 6. Policlinico Gemelli, Roma (Prof.ssa Patrizia Chiusolo) | dPCR, commercial |
| 7. Napoli (Prof.ssa Barbara Izzo) | dPCR, commercial |



Standardizing KIT D816V Mutation Testing in Italy (2023-ongoing)



Standardizing KIT D816V Mutation Testing in Italy (2023-ongoing)

SECOND PHASE (2024)

1. Bergamo (Dott.ssa Orietta Spinelli)
2. Milano Osp Niguarda (Dott. Silvio Veronese)
3. Monza (Dott.ssa Grazia Fazio)
4. Vicenza (Dott.ssa Elisabetta Novella)
5. Pisa (Dott.ssa Francesca Guerrini)
6. Roma Osp San Camillo (Dott. Giovanni Iaquina)
7. Pescara (Dott. Stefano Baldoni)
8. Bari (Prof. Francesco Albano)
9. Palermo Osp Buccheri-La Ferla (Dott.ssa Loredana Bruno)
10. Nuoro (Dott.ssa Giovanna Piras)

dPCR, commercial

dPCR, commercial

dPCR, commercial

dPCR, commercial

dPCR, commercial

dPCR, commercial

dPCR, commercial

dPCR, commercial

dPCR, commercial

dPCR, commercial



Standardizing KIT D816V Mutation Testing in Italy (2023-ongoing)

Third Phase (ongoing)

1. Torino Mauriziano (Prof.sse Daniela Cilloni/Carmen Fava)
2. Roma Tor Vergata (Dott.ssa Tiziana Ottone)
3. Udine (Dott.ssa Dora Fabbro/Dott.ssa Eleonora Toffoletti)
4. Ancona (Dott.ssa Silvia Svegliati)
5. Perugia (Dott.ssa Roberta La Starza)
6. Foggia (Dott. Maurizio Margaglione)

ddPCR, commercial

ddPCR, commercial

ddPCR, commercial

ddPCR, commercial

ddPCR, commercial

ddPCR, commercial



**Ulteriori laboratori interessati?
Scrivetemi! Simona.soverini@unibo.it**

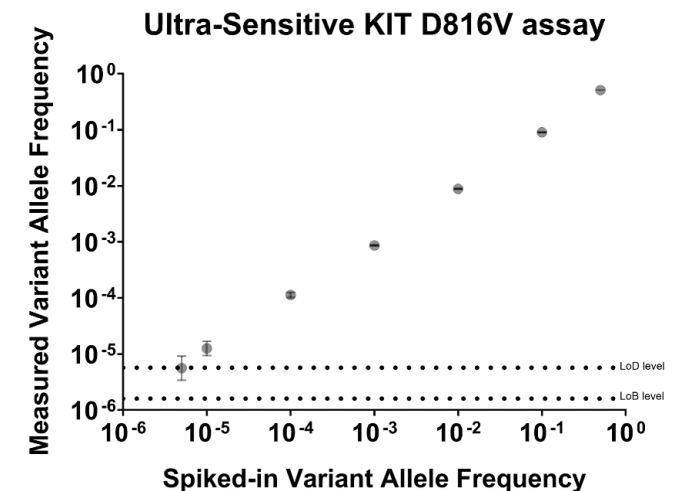
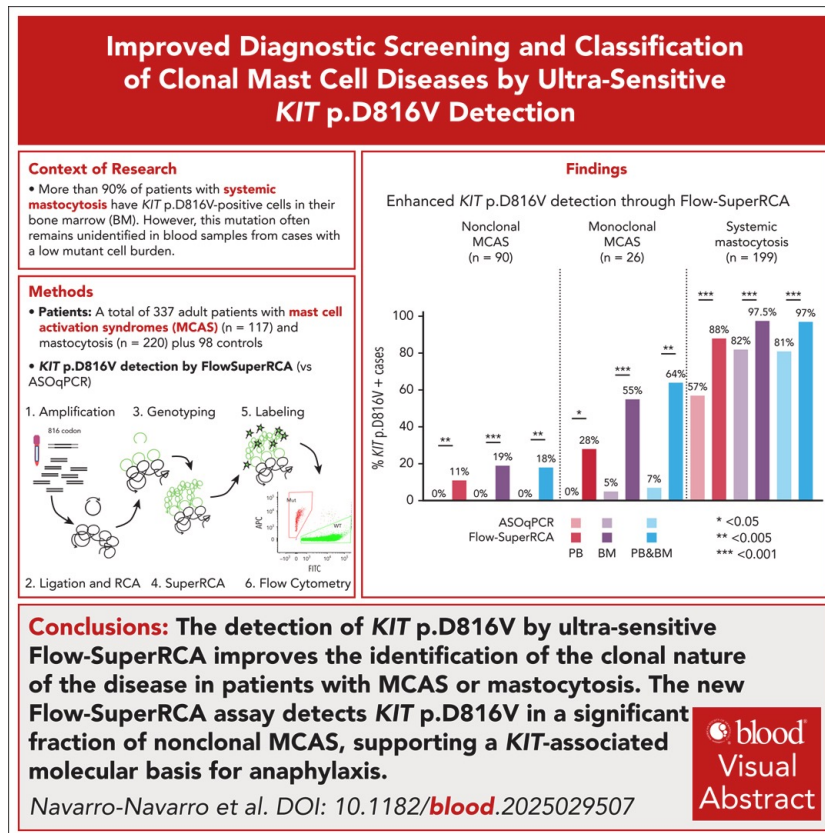
Novel Molecular Methods on the Horizon

superRCA KIT D816V Mutation Kit RUO (Rarity Bioscience)

Reaches a LOD of **0.0016% VAF**

superRCA Ultra-Sensitive KIT D816V Kit RUO

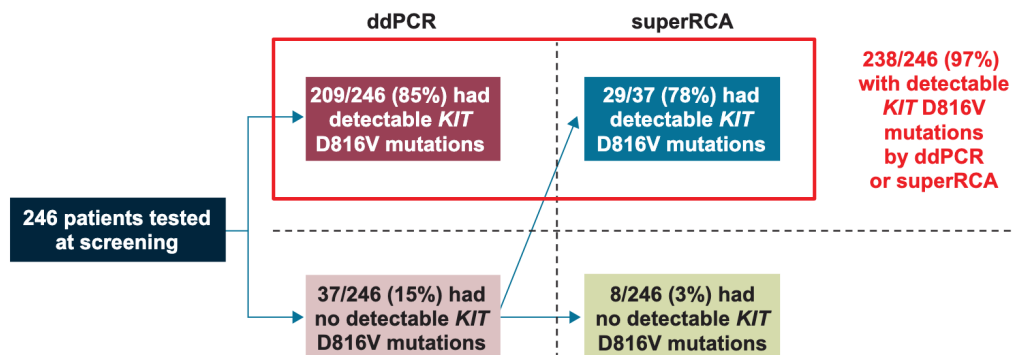
New assay utilizing more input DNA → more cytometric events



Sample Type	Purified DNA
Recommended DNA input	2640 ng (For lower input, see Instructions for Use)
Recommended Concentration	44 ng/μl
Limit of Blank	0.00016%
Limit of Detection/Quantitation	0.00057%
Linearity	0.00057-10% (R ² ≥ 0.9993)
Precision	CV < 5% at 0.1-50%

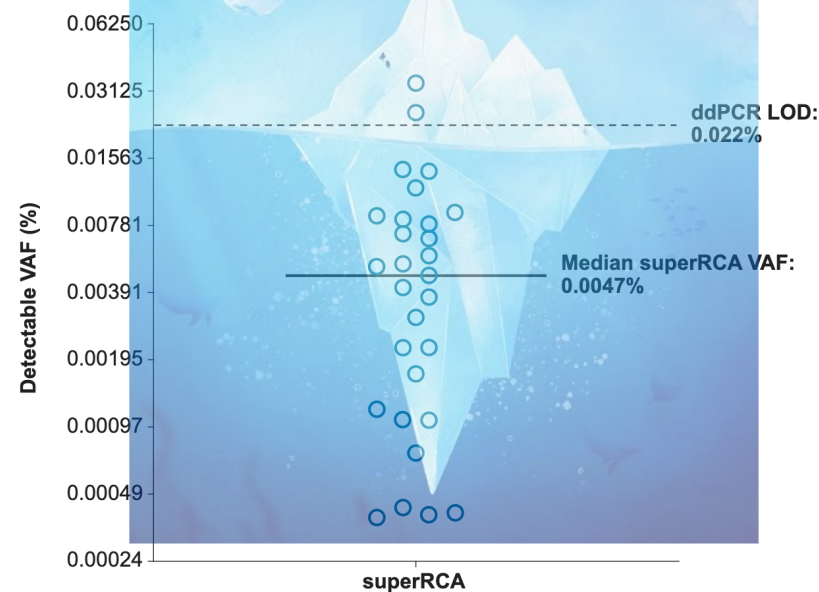
SuperRCA Ultra-Sensitive *KIT* D816V Kit RUO vs ddPCR

Figure 3. *KIT* D816V mutations detected in patients enrolled in PIONEER

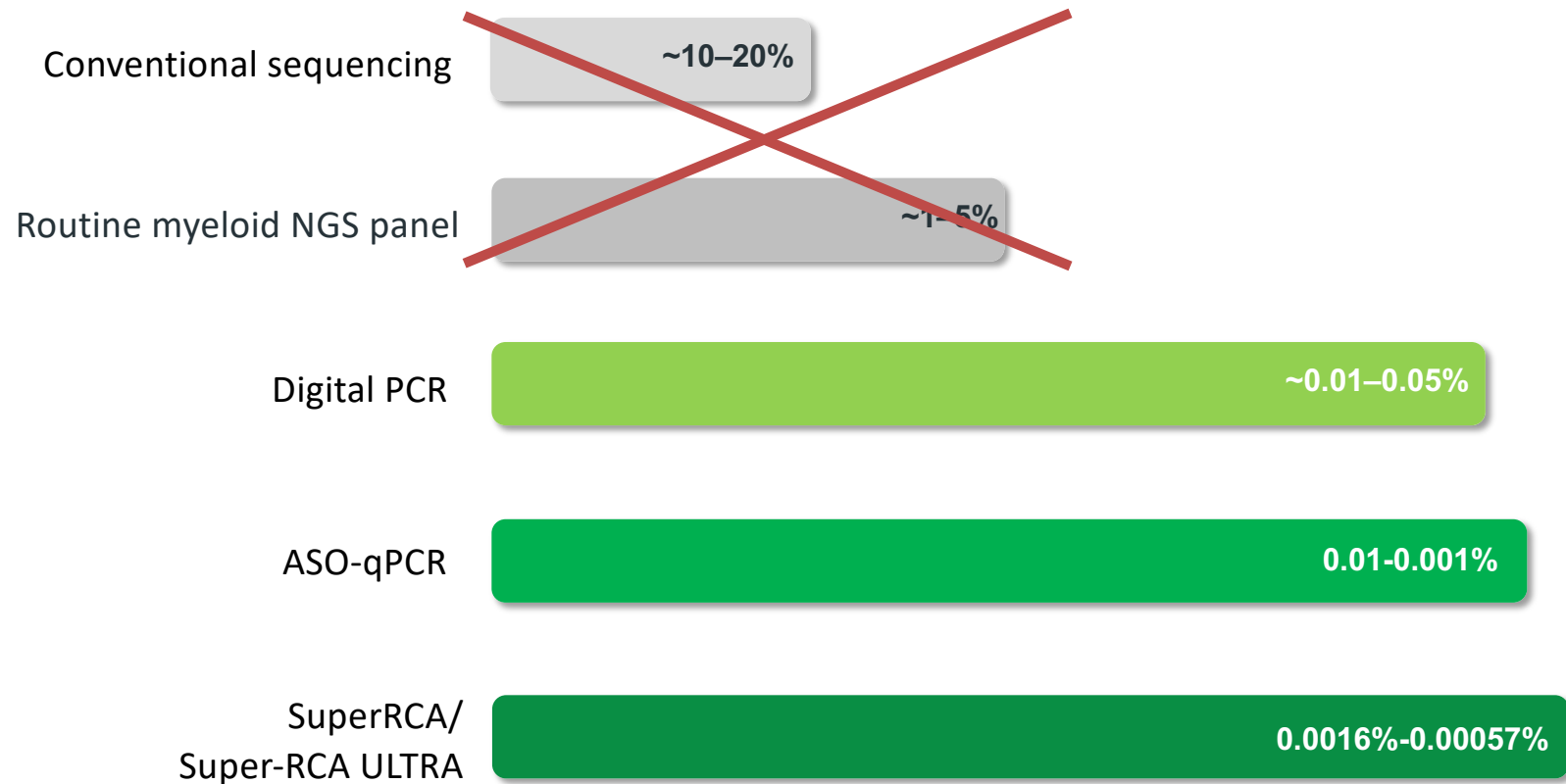


- In combination, superRCA and ddPCR detected *KIT* D816V mutations in the peripheral blood of 238/246 (97%) patients (**Figure 3**)
 - Using ddPCR, *KIT* D816V was detected in 209/246 (85%) peripheral blood samples
 - In patients with ISM who had undetectable *KIT* D816V by ddPCR, superRCA detected *KIT* D816V in 29/37 (78%) samples

Figure 4. *KIT* D816V VAF distribution by superRCA in ddPCR-undetectable patients



Pitfall #3: Analytical Sensitivity of Assays for KIT D816V

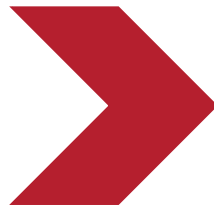


Pitfall #4: Looking Only for KIT D816V

D816V ASSAY

**Excellent
sensitivity
for one hotspot**

A targeted D816V assay
cannot detect atypical or
non-D816V variants!!



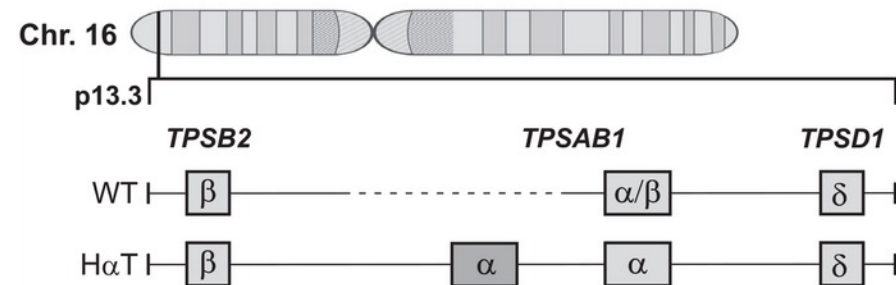
WHEN SUSPICION PERSISTS

- Consider other KIT activating mutations
- Review morphology, immunophenotype and specimen adequacy
- Use broader KIT testing to avoid missing true clonal disease (NGS)
- Consider mast-cell enrichment or repeat marrow sampling in selected cases

The reflex test should broaden coverage without sacrificing sensitivity, but this is actually not possible with NGS..

Pitfall #5: Tryptase Gene Copy Number Is Not a Routine Add-On

- Extra germline α -tryptase-encoding *TPSAB1* copies define hereditary α -tryptasemia (H α T), an autosomal dominant inherited genetic trait which usually leads to elevated bST levels
- Canonical α -tryptase copy number is 0–2, and β -tryptase copy number is 2–4, where total copy number is 4
- Individuals carrying up to 10 additional *TPSAB1* copies have been reported to date, and BST levels have been shown to follow a gene-dosage effect



Additional <i>TPSAB1</i> copy number	0	1	2	3	4	6	10
Tryptase Genotypes* (<i>TPSAB1</i> , <i>TPSB2</i>)	$\beta, \beta/\beta, \beta; \alpha, \beta/$ $\beta, \beta; \alpha, \beta/$ $\alpha, \beta; \alpha, \beta/$ $\beta, \beta, \beta; \beta, \beta/$ $\beta, \beta, \beta; \beta/\alpha, \beta;$ $\beta/\beta, \beta$	$\alpha\alpha, \beta/\beta, \beta;$ $\alpha\alpha, \beta/\alpha, \beta;$ $\alpha\alpha, \beta\beta/\beta, \beta;$ $\alpha\alpha, \beta\beta/$ $\alpha, \beta\beta$	$\alpha\alpha, \beta/$ $\alpha\alpha, \beta;$ $\alpha\alpha\alpha, \beta/\beta, \beta;$ $\alpha\alpha\alpha, \beta/\alpha, \beta$	$\alpha\alpha\alpha, \beta/$ $\alpha\alpha, \beta$	$\alpha\alpha\alpha\alpha, \beta/$ $\beta, \beta;$ $\alpha\alpha\alpha\alpha, \beta/$ α, β	$\alpha\alpha\alpha\alpha\alpha, \beta/$ $\alpha\alpha\alpha, \beta$	$\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha\alpha,$ $\beta/\alpha, \beta$
BST (ng/mL), median (range)	4.1 (0–10.4)	13.6 (6.5–33.9)	22.5 (10.5–39.5)	27.3 (23.4–40)	37 (25.5–62.7)	87 (NA)	133 (110–156)
Upper 99.5% PI for BST (ng/mL)	11.4	36.2	62.2	88.8	115.9	171.2	285.1
Upper limit using BST/(1+copy)[†]	20	40	60	80	100	140	220

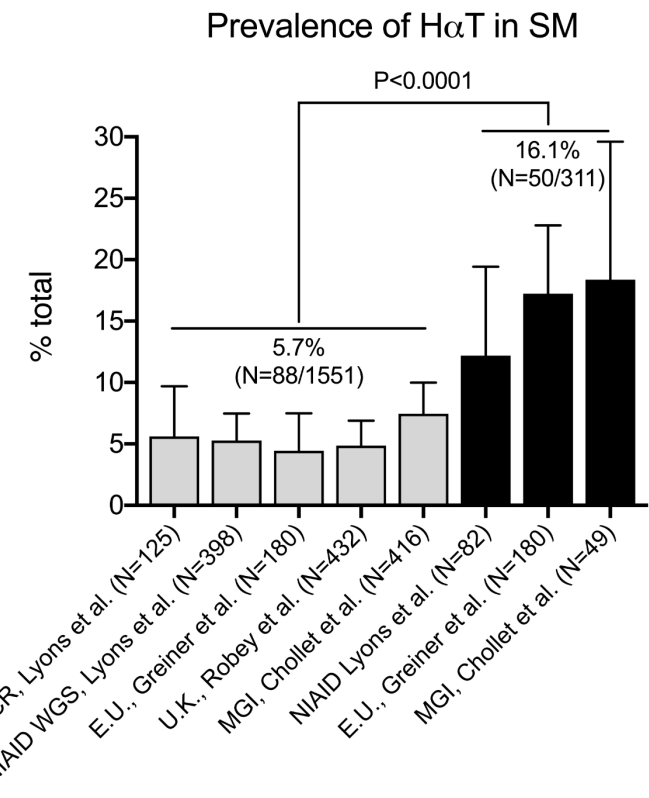
* Tryptase genotypes identified to date for this number of *TPSAB1* replications. In all cases *TPSAB1* replications associated with elevated BST identified encode α -tryptase.

[†] Upper limits were generated by inverting this formula such that the threshold was the current minor criterion of 20 ng/mL times one plus the replication number.

Pitfall #5: Tryptase Gene Copy Number Is Not a Routine Add-On

- HαT can coexist with systemic mastocytosis, affecting interpretation of the tryptase criterion
- Divide the basal tryptase level by 1 plus the extra copy numbers of the alpha tryptase gene or use the online calculator <https://bst-calculator.niaid.nih.gov/>

- Germline!
- Pre-test consent and expectations
- Family implications and cascade testing
- Interpretation of uncertain or uncommon genotypes
- Need for multidisciplinary evaluation and medical genetics counseling



Take-Home Messages

- A negative result does not exclude systemic mastocytosis.
- Sensitivity matters.
- Sample choice matters.
- KIT D816V is not the whole story.
- Integrated morphology, immunophenotype and molecular data prevent diagnostic failure.

Future developments

- More widespread availability and standardization of molecular testing
- Validation and standardization of super-RCA testing and definition of novel diagnostic algorithms incorporating this assay when most useful
- Evaluation of KIT D816V as MRD marker
- Implementation of more sensitive assays for non-D816V mutations (e.g., error-corrected sequencing?)