UK NEQAS
Leucocyte Immunophenotyping

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

KIT p.Asp816Val (D816V) Mutation Status for Mast Cell Disease Programme

Distribution - 252601 Participant ID -

Date Issued - 27 May 2025 Closing Date - 27 June 2025

Trial Comments

In total, four vials of lyophilised cell line material (scored samples KIT 168 and KIT 169, with optional educational samples KIT Edu D and KIT Edu E) were distributed to 100 participants for KIT NM_000222.3:c.2447A>T p.Asp816Val (D816V) variant analysis. For this trial, 97 (97.0%) participants returned results for the scored samples (KIT 168 and KIT 169). Of the 3 laboratories not submitting results, two pre-notified us of extenuating circumstances.

Sample Comments

Samples KIT 168 and KIT 169 featured a population of 1% and 0.6% KIT NM_000222.3:c.2447A>T p.Asp816Val heterozygous positive cells in a non-mutated (wildtype) background, respectively. Educational sample KIT Edu D was manufactured to be negative for the c.2447A>T p.(Asp816Val) variant. Educational sample KIT Edu E was formulated to represent a clinically relevant low-level sample featuring 0.05% heterozygous KIT c.2447A>T p.(Asp816Val) positive cells in a non-mutated (wildtype) background. Please note, the lyophilised samples provided for this programme are not suitable for mast cell enrichment pre-processing.

Results and Performance

Your Results

KIT Mutation Status	Your Results	Consensus Result
Sample KIT 168		Mutation Detected
Sample KIT 169		Mutation Detected

All Participant Results

	Mutation Detected (Returns)	No Mutation Detected (Returns)
Sample KIT 168	94	3
Sample KIT 169	94	3

Your Performance

Performance	Performance Status for this Trial	Performance Status Classification Over 3 Trial Period	
		Satisfactory	Critical

N/A = Not Applicable

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Template Type

	Returns
DNA	95
cDNA	2

PCR Type

	Returns
Droplet Digital PCR	32
Allele Specific PCR	27
Real-Time PCR	21
Chip Digital PCR	5
Single PCR	5
Allele Specific Competitive Blocker PCR	4
Multiplex PCR	2

Protocol Type

	Returns
In-house Assay	57
BioRad PrimePCR ddPCR kit	29
LifeTechnologies TaqMan kit	6
Plentiplex Mastocytosis kit	5

Analysis Type

	Returns
Real-Time PCR Fluorescent Detection	45
Digital PCR	37
Agarose Gel Electrophoresis	4
Capillary Electrophoresis	4
NGS (Other)	4
Sanger Sequencing	3

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Journal Reference for Assay

	Returns
Kristensen T. et al (2011). JMD, 13:2, 180-188	36
In-house method	20
Schumacher J. et al (2008). JCP, 61, 109-114	6
Orfao A. et al (2007). Br J Haematol, 138:1, 12-30	2
Akin C et al. (2000) Exp Hematol 28:140-147	2
Hindson BJ et al, Anal Chem. 2011 Nov 15;83(22):8604-10	2
Lawley W. et al (2005). Mutat Res, 572, 1-13	1
Longley BJ. et al (1999). Proc Natl Acad Sci, 96:4, 1609-1614	1
Shimada A. et al (2006). Blood, 107, 1806-1809	1
Sotlar K. et al (2003). Am J Pathol, 162:3, 737-746	1
Nagata H. et al (1995). PNAS, 92:23, 10560-4	1
Sang Hyuk Park et al (2011) Leuk Res, 35:10, 1376-1383	1

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Comments

In line with sample formulation 96.9% (94/97) of laboratories detected the *KIT* c.2447A>T p.(Asp816Val) variant (mutation) in sample KIT 168. For those participants returning a false negative result, all stated an assay LoD>median VAF%. Methodologies included PCR with capillary electrophoresis (LoD=10%), Sanger sequencing (LoD=15%) and allele specific PCR with real-time fluorescent detection (LoD=1%). All three centres utilised genomic DNA as assay input material. Two of the laboratories noted they do not routinely perform mast cell purification for the enrichment of clinical samples. The allele specific PCR with real-time PCR fluorescent detection (TRUPCR c-KIT Mutation Detection kit) user did not provide any information regarding patient sample pre-processing.

In line with sample formulation 96.9% (94/97) of laboratories detected the *KIT* c.2447A>T p.(Asp816Val) variant (mutation) in sample KIT 169. The three participants that failed to detect the variant in this sample also submitted a false negative result for sample KIT 168. Again, stated assay LoD>median VAF% (see above for methodological information).

Detection of the *KIT* c.2447A>T p.(Asp816Val) variant (NM_000222.3) present at a very low level is clinically relevant in the context of mast cell disease[1]. A study by Kristensen *et al.*[2] in patients with mastocytosis found a range of variant positive cell fractions from 0.03% to 97%, with a median of 0.9% in bone marrow samples. In the same study, the variant level in skin biopsies ranged from 3% to 23% (median 8%).

Both the World Health Organisation (5th edition)[3] and International Consensus Classification (ICC) (2022)[4] include *KIT* p.(Asp816Val) (D816V) variant (or other activating *KIT* variants (mutations)) detection in bone marrow, peripheral blood or other extracutaneous organs as a diagnostic criterion for systemic mastocytosis. **Wang *et al.* advocate access to an assay with an analytical sensitivity down to 0.01-0.1% VAF in the clinical context of mastocytosis[5].**

Stated assay LoD	Returns
<0.01%	14
0.01%	16
>0.01 - <0.05%	13
0.05%	7
>0.05 - <0.1%	4
0.1%	13
>0.1 - <0.5%	2
0.5%	2
1%	2
>10%	3

Assay analytical sensitivity does not meet the current recommendations in the clinical context of systemic mastocytosis testing

Overall, just two centres extracted RNA and utilised cDNA as assay input material (to analyse expressed variant load at the transcript level) rather than genomic DNA for this trial (Sanger sequencing (n=1) and real time PCR with fluorescent detection with additional NGS (n=1)). EU-US Cooperative Group guidelines[6] highlight that *KIT* p.Asp816Val expressed allele burden should be considered a distinct biomarker and is not interchangeable with genomic DNA based results.

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In total 59.8% of returning participants provided quantitative information for sample(s) KIT 168 and/or KIT 169 (n=58). Results from those laboratories analysing genomic DNA are summarised in the table below. **Please note sample formulations for this programme focus on clinically relevant genomic *KIT* c.2447A>T p.(Asp816Val) variant levels.** Participants utilising RNA/cDNA as assay input material (n=2) should therefore not use the genomic DNA derived quantification statistics in this trial report to benchmark the performance of their assay which targets *KIT* transcripts to determine expressed variant load (expressed allele burden). We recognise the limitations of the EQA programme.

Percentage (%) VAF *KIT* NM_000222.3:c.2447A>T p.(Asp816Val)^		
	Sample KIT 168	**Sample KIT 169**
n*	57	57
Median	0.31	0.19
IQR	0.12	0.06

^ % variant allele frequency (VAF) = (variant/(wildtype+variant))x100.
* Includes only those laboratories using extracted gDNA as assay input material. Note the lyophilised samples provided for this trial are not suitable for mast cell enrichment pre-processing.
IQR = Interquartile range.

Fifteen returning laboratories stated the routine mast cell enrichment of clinical samples prior to extraction. If indicated, techniques included mononuclear cell fraction density gradient centrifugation (n=11), flow cytometric sorting (n=2) and magnetic bead separation (n=1). A further four laboratories noted they were looking to implement a mast cell enrichment method. The EU-US Cooperative Group[6] recognise the utility of enriched mast cell samples for cases of systemic mastocytosis with a low level of infiltrating aberrant neoplastic mast cells but do not consider it to be universally recommended as state-of-the-art outside of specialist centres.

Educational samples KIT Edu D and KIT Edu E

Optional educational samples KIT Edu D and KIT Edu E represented peripheral blood samples from patients with a suspected systemic mastocytosis diagnosis. Overall, 86.6% (84/97) of returning participants submitted results for these samples. Mindful of the application of increasingly sensitive assays (14 participants stated a LoD<0.01%), the aim of the educational samples was to facilitate the evaluation of achieved analytical specificity and sensitivity.

Sample KIT Edu D was formulated to be negative for the *KIT* c.2447A>T p.(Asp816Val) variant (mutation). In keeping with sample composition, at least 96.4% (81/84) laboratories reported no variant detected using a genomic DNA input (n=79) or RNA/cDNA input (n=2) assay. A further participant selected the 'equivocal' results option at data entry but provided an accompanying VAF=0% value (assay with genomic DNA input).

Two centres returned a false positive result for sample KIT Edu D. Both utilised an in-house real-time PCR assay with fluorescent detection (genomic DNA input) citing Kristensen *et al.* (2011) methodology[2]. The publication acknowledges a weak cross-reaction of the variant (mutation) specific assay (reverse primer) with the wildtype allele (single replicate). Appropriate real time qPCR quality parameters[7,8], including verification to establish the quantification cycle (Cq) (cycle threshold (Ct)) value limit and inclusion of a negative (normal) control are important to mitigate this issue and suitably define positivity.

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Comments participants noted they would routinely include in an applicable c.2447A>T p.(Asp816Val) variant (mutation) negative patient clinical report included:

- Further testing of a bone marrow sample to exclude a diagnosis of systemic mastocytosis (n=3)
- Further testing of a sample assessed for the presence of neoplastic mast cells (n=2)
- Consideration of a differential diagnosis (alpha tryptasemia) (n=1)

Sample KIT Edu E was formulated to be positive for the *KIT* c.2447A>T p.(Asp816Val) variant (mutation) at a very low-level. Overall, 56.1% (46/82) of laboratories (using genomic DNA input material) reported detection of the variant in concordance with sample composition.

Please note, of the two laboratories employing RNA/cDNA based assays only one successfully identified the variant in this educational sample (real time PCR with fluorescent detection plus additional NGS). We have excluded this laboratory from further discussion, as expressed allele burden should be considered a distinct biomarker[6].

For sample KIT Edu E, the median VAF calculated from genomic DNA based assays was 0.02% (IQR =0.03, n=41).

Eight (9.8%) participants returned an equivocal result for sample KIT Edu E. For half of these laboratories (n=4) the median VAF% calculated for this challenging sample was lower than their stated assay analytical sensitivity (LoD range=0.04-0.2%). A further centre failed to provide any LoD information. The three laboratories quoting a LoD<median VAF% utilised allele specific PCR with real-time PCR fluorescent detection (n=1, LoD=0.001%) or droplet digital PCR (n=2, LoD=0.01, 0.015). Of note, two of the centres stated the application of mononuclear cell fraction density gradient centrifugation for (some) clinical specimens.

In total, 28 (34.1%) laboratories failed to identify the variant in educational sample KIT Edu E. For 19 participants the median VAF% was lower than their stated assay analytical sensitivity (range LoD 0.03-10% LoD). However, four centres quoted an assay LoD<median VAF%; this included two participants both using an in-house real-time PCR assay with fluorescent detection (genomic DNA input) approach, quoting LoDs of 0.003 and 0.006%. Laboratories employing suitably sensitive techniques may have been on the cusp of detecting *KIT* c.2447A>T p.(Asp816Val) positivity in sample KIT Edu E. Indeed, one chip based digital PCR user submitted a result of variant not detected due to the ascertained VAF=0.01% falling just below their stated assay LoD=0.02% (equal to the trial median VAF%).

Final remarks

The ASH Education program has published a useful review, including a summary of the WHO and ICC criteria for the diagnosis and classification of mastocytosis[9].

The NCBI *KIT* gene webpage (http://www.ncbi.nlm.nih.gov/gene/3815) is a valuable resource for obtaining relevant reference sequences at the DNA and protein level. The Matched Annotation from NCBI and EMBL-EBI (MANE) project[10] states RefSeq NM_000222.3/Ensembl ENST00000288135.6 (isoform 1) as the MANE Select *KIT* transcript of choice for clinical reporting.

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The Human Genome Variation Society (HGVS) provides a series of recommendations (HGVS Nomenclature) with the aim of standardising the description of sequence variants[11-13]. Parentheses are used in this report comment section to denote predicted protein variant descriptions. However, we acknowledge that this approach to protein nomenclature would not be appropriate for the minority of participants extracting RNA and utilising cDNA as assay input material.

References

1 Cross, N. *et al*. The use of genetic tests to diagnose and manage patients with myeloproliferative and myeloproliferative/myelodysplastic neoplasms, and related disorders. *Br J Haem*. 195(3):338-351 (2021).

2 Kristensen, T. *et al*. Improved detection of the KIT D816V mutation in patients with systemic mastocytosis using a quantitative and highly sensitive real-time qPCR assay. *J Mol Diagn.* 13(2), 180-188 (2011).

3 WHO Classification of Tumours Editorial Board. Haematolymphoid tumours [Internet]. Lyon (France): International Agency for Research on Cancer; 2024 (WHO classification of tumours series, 5th ed.; vol. 11).

4 Arber, D. *et al.* International Consensus Classification of Myeloid Neoplasms and Acute Leukemias: integrating morphologic, clinical, and genomic data. *Blood* 140 (11): 1200-1228 (2022).

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6 Hoermann, G. *et al.* Standards of genetic testing in the diagnosis and prognostication of systemic mastocytosis in 2022: Recommendations of the EU-US Cooperative Group. *J Allergy Clin Immunol Pract*. 10(8):1953-1963 (2022).

7 Bustin, S.A. *et al*. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 55(4):611-22 (2009).

8 Bustin, S.A. *et al.* MIQE 2.0: Revision of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines. *Clin Chem*. 71(6):634-651 (2025).

9 Veitch, S. and Radia, D. Mastocytosis demystified. *Hematology Am Soc Hematol Educ Program*. 2023(1):396-406 (2023).

10 Morales, J. *et al.* A joint NCBI and EMBL-EBI transcript set for clinical genomics and research. *Nature* 604(7905):310-315 (2022).

11 HGVS Nomenclature (v21.1) https://hgvs-nomenclature.org/stable/).

12 Den Dunnen, J. *et al*. HGVS Recommendations for the description of sequence variants: 2016 Update. *Hum Mutat.* 37(6):564-569 (2016).

13 Hart, R.K. *et al.* HGVS Nomenclature 2024: improvements to community engagement, usability, and computability. *Genome Med.* 16(1):149 (2024).

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Information with respect to compliance with standards BS EN ISO/IEC 17043:2010

4.8.2 a) The proficiency testing provider for this programme is:
UK NEQAS for Leucocyte Immunophenotyping
Pegasus House, 4th Floor Suite
463A Glossop Road
Sheffield, S10 2QD
United Kingdom
Tel: +44 (0) 114 267 3600
e-mail: amanda.newbould@ukneqasli.co.uk

4.8.2 b) The coordinator(s) of UK NEQAS LI programmes: Mr Stuart Scott (acting Director).

4.8.2 c) Person(s) authorising this report: Mr Stuart Scott (acting Director) of UK NEQAS LI.

4.8.2 d) Administration and shipping for this programme is provided by EQA International Limited. Pre issue and post closure testing of samples for this programme is externally provided, although the final decision about sample suitability lies with the EQA provider; no other activities in relation to this EQA exercise were externally provided.

4.8.2 d) Where externally provided products or services are used in the delivery of EQA, a competent supplier is used, the EQA provider is responsible for this work and participants are informed accordingly.

4.8.2 g) The UK NEQAS LI Privacy Policy can be found at the following link: https://sheffield-ukneqas.ipassportqms.com/document_download/NjRlNTgxYzctMTI4ZS00MTg4LWI2ZDMtZDdkYzJhMTFlZTg3. Participant details, their results and their performance data remain confidential unless we are required by law to share this information. Where required by law or authorised by contractual arrangements to release confidential information, UK NEQAS LI will notify those concerned of the information released, unless prohibited by law. For UK participants, the relevant National Quality Assessment Advisory Panel is informed when a UK participant is identified as having performance issues.

4.8.2 i) All EQA samples are prepared in accordance with strict standard operating procedures by trained personnel proven to ensure homogeneity and stability. Where appropriate/possible EQA samples are tested prior to issue. Where the sample(s) issued is stabilised blood or platelets, pre and post stability testing will have proved sample suitability prior to issue.

4.8.2 l), n), o), r) & s) Please refer to the UK NEQAS LI website at www.ukneqasli.co.uk for detailed information on each programme including the scoring systems applied to assess performance (for BS EN ISO/IEC 17043:2010 accredited programmes only). Where a scoring system refers to the 'consensus result' this means the result reported by the majority of participants for that trial issue. Advice on the interpretation of statistical analyses and the criteria on which performance is measured is also given. Please note that where different methods/procedures are used by different groups of participants these may be displayed within your report, but the same scoring system is applied to all participants irrespective of method/procedure used.

4.8.2 m) We do not assign values against reference materials or calibrants.

4.8.2 q) Details of the programme designs as authorised by The Steering Committee and Specialist Advisory Group can be found on our website at www.ukneqasli.co.uk. The proposed trial issue schedule for each programme is also available.

4.8.2 t) If you would like to discuss the outcomes of this trial issue, please contact UK NEQAS LI using the contact details provided. Alternatively, if you are unhappy with your performance classification for this trial, please find the appeals procedure at www.ukneqasli.co.uk/contact-us/appeals-and-complaints/

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