UK NEQAS
Leucocyte Immunophenotyping

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

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

Distribution - 232401 Participant ID -

Date Issued - 09 May 2023 Closing Date - 09 June 2023

Trial Comments

Three vials of lyophilised cell line material (scored samples KIT 156 and KIT 157, plus an optional educational sample KIT Edu B) were distributed to 86 participants for KIT NM_000222.3:c.2447A>T p.Asp816Val (D816V) variant analysis. For this trial, 80 (93.0%) participants returned results for the scored samples. Six laboratories failed to submit results; two laboratories pre-notified us of their non return.

Sample Comments

Sample KIT 156 was manufactured to be negative for the KIT NM_000222.3:c.2447A>T p.Asp816Val variant. Sample KIT 157 featured a population of 0.9% KIT NM_000222.3:c.2447A>T p.Asp816Val heterozygous positive cells in a non-mutated (wildtype) background. Educational sample KIT Edu B was formulated to represent a very low-level sample featuring 0.05% heterozygous 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 156		No Mutation Detected
Sample KIT 157		Mutation Detected

All Participant Results

	Mutation Detected (Returns)	No Mutation Detected (Returns)
Sample KIT 156	1	79
Sample KIT 157	79	1

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	76
cDNA	4

PCR Type

	Returns
Droplet Digital PCR	23
Real-Time PCR	22
Allele Specific PCR	21
Single PCR	6
Allele Specific Competitive Blocker PCR	3
Chip Digital PCR	3
Multiplex PCR	1

Protocol Type

	Returns
In-house Assay	51
BioRad PrimePCR ddPCR kit	18
LifeTechnologies TaqMan kit	6
Plentiplex Mastocytosis kit	5

Analysis Type

	Returns
Real-Time PCR Fluorescent Detection	41
Digital PCR	26
Agarose Gel Electrophoresis	4
Capillary Electrophoresis	4
NGS (Other)	3
Sanger Sequencing	2

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Journal Reference for Assay

	Returns
Kristensen T. et al (2011). JMD, 13:2, 180-188	31
In-house method	18
Schumacher J. et al (2008). JCP, 61, 109-114	7
Orfao A. et al (2007). Br J Haematol, 138:1, 12-30	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
Sotlar K. et al (2003). Am J Pathol, 162:3, 737-746	1
Nagata H. et al (1995). PNAS, 92:23, 10560-4	1

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Comments

In line with sample formulation, 79 (98.8%) laboratories reported sample KIT 156 as negative for the *KIT* c.2447A>T p.(Asp816Val) variant (mutation). A single participant returned a false positive result (VAF = 0.018%) using droplet digital PCR (gDNA template, BioRad Prime PCR ddPCR kit).

In line with sample formulation, 79 (98.8%) laboratories detected the *KIT* c.2447A>T p.(Asp816Val) variant (mutation) in sample KIT 157. A single participant returned a false negative result using a next generation sequencing (NGS) in house protocol (gDNA template, no further information available). No information was provided by the laboratory regarding whether the mast cell enrichment of clinical samples is routinely performed.

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. A study by Kristensen *et al*.[1] 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%). Due to the nature of systemic mastocytosis, *KIT* c.2447A>T p.(Asp816Val) variant allele frequency (VAF) is often too low for detection by conventional read-depth NGS gene panels[2].

In the absence of mast cell enrichment, Sanger sequencing does not afford the required assay sensitivity in the clinical context of mast cell disease[3-4]. The Sanger sequencing users participating in this trial (n=2) were able to identify the variant in sample KIT 157. However, both extracted RNA and utilised cDNA as assay input material (to analyse expressed variant load at the transcript level) rather than gDNA. The EU-US Cooperative Group guidelines[5] highlight that *KIT* p.Asp816Val expressed allele burden should be considered a distinct biomarker and is not interchangeable with gDNA based results.

Over 45% of returning participants provided quantitative information for sample KIT 157 (n=39). Results from those laboratories analysing gDNA 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=4) should therefore not use the gDNA 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 157
n*	38
Median	0.32
IQR	0.17

^ % 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.

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Overall, six returning laboratories reported the routine mast cell enrichment of clinical samples prior to extraction; techniques included mononuclear cell fraction density gradient centrifugation (n=5) and flow cytometric sorting (n=1). A further three laboratories noted they were looking to implement a mast cell enrichment method. The EU-US Cooperative Group[5] 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 Sample Summary - KIT Edu B

Sample KIT Edu B was formulated to represent a low-level sample featuring 0.05% heterozygous *KIT* c.2447A>T p.(Asp816Val) positive cells in a non-mutated (wildtype) background. The *KIT* p.(Asp816Val) variant has been reported at equivalent and lower VAF in the peripheral blood of systemic mastocytosis patients[6].

Sixty-one (76.3%) returning laboratories submitted a result for this optional educational sample.

- Overall, 55.7% (34/61) reported this low-level sample as positive for the *KIT* c.2447A>T p.(Asp816Val) variant.
- A further 18.0% (11/61) returned an equivocal result.
- The remaining participants reported no variant detected (n=16).

Sample KIT Edu B: Submitted result as per local reporting protocol						
	All methods	**Real Time PCR***	**Digital PCR**	**Allele Specific PCR****	**NGS**	**Sanger Sequencing**
n	61	31	19	7	2	2
Variant detected	34 (55.7%)	22 (71.0%)	6 (31.6%)	5 (71.4%)	1 (50.0%)	0
Equivocal	11 (18.0%)	5 (16.1%)	5 (26.3%)	1 (14.3%)	0	0
No variant detected	16 (26.2%)	4 (12.9%)	8 (42.1%)	1 (14.3%)	1 (50.0%)	2 (100.0%)

* Allele Specific PCR with real time fluorescence detection. ** Allele Specific PCR with gel or capillary electrophoresis.
NGS = Next Generation Sequencing (no further details known).

For this trial real time PCR with fluorescent detection-based allele specific approaches appeared most effective at detecting the *KIT* c.2447A>T p.(Asp816Val) variant in sample KIT Edu B.

Allele-specific oligonucleotide real time quantitative PCR (ASO-qPCR, including that with fluorescence detection) and digital PCR methods are reported to be capable of low-level variant detection[2-7] and recognised by the EU-US Cooperative Group as suitability sensitive techniques for detection and quantification of the *KIT* p.(Asp816Val) variant using genomic DNA[5]. A study by Greiner *et al*.[7] concluded both ASO-qPCR and digital PCR methods to be similarly sensitive at detecting the variant across peripheral blood and bone marrow samples from a range of systemic mastocytosis patients. Of note, neither of the Sanger sequencing users identified the low-level *KIT* c.2447A>T p.(Asp816Val) variant in sample KIT Edu B.

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Of the 34 laboratories reporting successful detection of the *KIT* c.2447A>T p.(Asp816Val) variant in sample KIT Edu B, seven laboratories actually stated an assay limit of detection (LoD) (range 0.05 - 1%) above the median VAF (0.02%). These included participants employing real time PCR (n=4), droplet digital PCR (n=1) and allele specific PCR with capillary electrophoresis (n=1) or agarose gel (n=1). Of all the participants reporting a positive result, 11 laboratories stated they would routinely offer to test a repeat sample to confirm this positive result in a clinical setting (nine centres specified the follow-up sample to be bone marrow).

The 11 laboratories reporting sample KIT Edu B as equivocal used a real time PCR based technique (n=5), droplet digital PCR (n=5) or allele specific PCR with capillary electrophoresis (n=1). For seven of the laboratories the median %VAF was lower than or equal to their stated assay LoD. Of the participants reporting an equivocal result, all centres stated they would routinely offer to test a repeat sample in a clinical setting (eight participants specified the follow-up sample to be bone marrow).

For the participants who tested sample KIT Edu B and submitted a result of no variant detected (n=16), only four laboratories stated they would request a repeat sample (three specified bone marrow). If there is a strong clinical suspicion of systemic mastocytosis in a patient testing negative for this actionable variant (particularly on a peripheral blood sample with a potentially very low number of circulating neoplastic mast cells) then the analysis of a repeat sample (bone marrow preferred) is advocated[2,3,5].

For 11/16 centres returning a negative result for sample KIT Edu B, the median VAF (0.02%) was known to fall below their stated assay LoD. However, for two laboratories the median %VAF was above their quoted assay LoD, this included a real time PCR (LoD = 0.0003%) and droplet digital PCR (LoD = 0.01%) user. Both centres stated they did not routinely conduct mast cell enrichment on clinical samples. Neither said they would routinely offer to test a repeat clinical sample in this scenario.

The quantification results for sample KIT Edu B (gDNA assay input material) are summarised in the table below. We acknowledge the limitations of this small dataset.

Sample KIT Edu B: Percentage (%) VAF *KIT* NM_000222.3:c.2447A>T p.(Asp816Val)^			
	All methods	**Real Time PCR**	**Digital PCR**
n*	25	12	10
Median	0.021	0.023	0.018
IQR	0.035	0.047	0.005

^ % variant = (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.

Forty-seven laboratories provided assay LoD information, as summarised in the table found on page 7.

- The median stated LoD for real time PCR with fluorescent detection and digital PCR based assays was 0.01% (n=23) and 0.05% (n=17), respectively.
- Only two NGS users provided LoD information (range 0.01-0.1%).
- The median quoted LoD for allele specific PCR with capillary electrophoresis was 1% (n=4), which is insufficient given the nature of mast cell disease[8].
- Five participants were noted as quoting an assay LoD <0.005% (real time PCR assays with fluorescent detection).

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Assay LoD (% VAF)	n
<0.005	5
0.005 -0.009	2
0.01	13
>0.01-0.04	7
0.05	5
>0.05-0.09	2
0.1	7
>0.1	6

Both the World Health Organisation (5th edition)[9] and the International Consensus Classification (ICC) group[10] 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. Wang *et al.* advocate access to an assay with a sensitivity down to 0.01–0.1% VAF in the clinical context of mastocytosis[8].

Extended KIT gene analysis

As part of this trial distribution, we surveyed participants regarding the provision of extended *KIT* gene analysis in the context of mast cell disease. Thank you to the >75% of returning laboratories for providing this additional information (n=61) we will summarise this in the next trial report.

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 release v1.0 is now available[11]. It states RefSeq NM_000222.3/Ensembl ENST00000288135.6 (isoform 1) as the MANE Select *KIT* transcript of choice for clinical reporting. Please note, Locus Reference Genomic (LRG)[12] reference sequences are no longer actively maintained and use of Ensembl/RefSeq transcripts specified by the MANE collaboration are preferred for all genes where available. The Human Genome Variation Society (HGVS) provides a series of recommendations with the aim of standardising nomenclature for the description of sequence variants[13-14]. Parentheses are used in this report 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.

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References

1 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).

2 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).

3 Arock, M., *et al.* KIT mutation analysis in mast cell neoplasms: recommendations of the European Competence Network on Mastocytosis. *Leukemia* 29(6):1223-32 (2015).

4 Martelli, M. *et al*. Recent advances in the molecular biology of systemic mastocytosis: Implications for diagnosis, prognosis, and therapy *Int J Mol Sci.* 21:(11):3987 (2020).

5 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).

6 Kristensen, T. *et al*. (2014) Sensitive KIT D816V mutation analysis of blood as a diagnostic test in mastocytosis. *Am J Hematol.* 89(5):493-8 (2014).

7 Greiner, G. et al. Digital PCR: A sensitive and precise method for KIT D816V quantification in mastocytosis. Clin Chem. 64(3):547-555 (2018).

8 Wang, S. *et al.* The international consensus classification of eosinophilic disorders and systemic mastocytosis. *Am J Hematol.* 98(8):1286-1306 (2023) - REVIEW

9 Khoury, J. *et al.* The 5th edition of the World Health Organization classification of haematolymphoid tumours: myeloid and histiocytic/dendritic neoplasms. *Leukemia* 36:1703-19 (2022).

10 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).

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

12 http://ftp.ebi.ac.uk/pub/databases/lrgex/LRG_307.xml (accessed August 2023).

13 http://varnomen.hgvs.org/ (accessed August 2023).

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

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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:
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Pegasus House, 4th Floor Suite
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4.8.2 b) The coordinators of UK NEQAS LI programmes are Mr Liam Whitby (Director) and Mr Stuart Scott (Centre Manager).

4.8.2 c) Person(s) authorizing this report:
Mr Liam Whitby (Director) or Mr Stuart Scott (Centre Manager) of UK NEQAS LI.

4.8.2 d) Pre issue testing of samples for this programme is subcontracted, although the final decision about sample suitability lies with the EQA provider; no other activities in relation to this EQA exercise were subcontracted. Where subcontracting occurs, it is placed with a competent subcontractor and the EQA provider is responsible for this work.

4.8.2 g) The UK NEQAS LI Confidentiality Policy can be found in the Quality Manual which is available by contacting the UK NEQAS LI office. Participant details, their results and their performance data remain confidential unless revealed to the relevant NQAAP when a UK participant is identified as having performance issues.

4.8.2 i) All EQA samples are prepared in accordance with strict Standard Operational 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 authorized 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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