UK NEQAS
Leucocyte Immunophenotyping

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

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

Distribution - 232402

Date Issued - 12 September 2023 Closing Date - 13 October 2023

Trial Comments

Two vials of lyophilised cell line material (samples KIT 158 and KIT 159) were distributed to 83 participants for KIT NM_000222.3:c.2447A>T p.Asp816Val (D816V) variant analysis. For this trial, 82 (98.8%) participants returned results. One laboratory pre-notified us of their non return.

Sample Comments

Samples KIT 158 and KIT 159 (duplicate batch) both featured a population of 0.7 % KIT NM_000222.3:c.2447A>T p.Asp816Val heterozygous 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 158	Mutation Detected	Mutation Detected
Sample KIT 159	Mutation Detected	Mutation Detected

All Participant Results

	Mutation Detected (Returns)	No Mutation Detected (Returns)
Sample KIT 158	80	2
Sample KIT 159	77	5

Your Performance

Performance	Performance Status for this Trial	Performance Status Classification Over 3 Trial Period	
		Satisfactory	Critical
	Satisfactory	3	0

N/A = Not Applicable

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

Template Type

	Returns
DNA	79
cDNA	3

PCR Type

	Returns
Droplet Digital PCR	25
Allele Specific PCR	22
Real-Time PCR	22
Single PCR	4
Allele Specific Competitive Blocker PCR	3
Chip Digital PCR	3
Multiplex PCR	2

Protocol Type

	Returns
In-house Assay	52
BioRad PrimePCR ddPCR kit	19
LifeTechnologies TaqMan kit	6
Plentiplex Mastocytosis kit	5

Analysis Type

	Returns
Real-Time PCR Fluorescent Detection	40
Digital PCR	28
Agarose Gel Electrophoresis	5
NGS (Other)	5
Capillary Electrophoresis	3
Sanger Sequencing	1

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

Journal Reference for Assay

	Returns
Kristensen T. et al (2011). JMD, 13:2, 180-188	30
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
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
Hindson BJ et al, Anal Chem. 2011 Nov 15;83(22):8604-10	1

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

Comments

In line with sample formulation, 80 (97.6%) laboratories detected the *KIT* c.2447A>T p.(Asp816Val) variant (mutation) in sample KIT 158.

- Two participants, both utilising an in-house next generation sequencing assay (NGS) (gDNA input material), returned a false negative result. One of the laboratories noted an assay limit of detection (LoD) of 2% variant allele frequency (VAF), which is unsuitable (in the absence of mast cell enrichment) as a lone assay in the clinical context of mast cell disease (see further discussion below).
- The two out of consensus centres did not provide information regarding whether mast cell purification is routinely performed for standard clinical samples.

In line with sample formulation, 77 (93.9%) laboratories detected the *KIT* c.2447A>T p.(Asp816Val) variant (mutation) in sample KIT 159.

- Of the five participants failing to detect the variant (all utilising gDNA input material), four centres employed NGS.
    - This included the two laboratories also reporting a false negative result for sample KIT 158, previously discussed.
    - A further out of consensus laboratory utilising NGS stated an assay LoD of 2%, which is above the median reported VAF for sample KIT 159 (VAF=0.24%). Of note this laboratory was able to identify the variant in sample KIT 158 (duplicate batch) quoting a 0.5% VAF. However, this lack of robust sensitivity is problematic in the clinical context of mast cell disease (see further discussion below).
    - The remaining NGS user failing to detect the variant stated a LoD of 0.1%, which is just below that of median reported VAF and thus, may have been on the cusp of detection in sample KIT 159.
- The remaining participant reporting a false negative result used droplet digital PCR (BioRad PrimePCR ddPCR kit) with a quoted LoD of 0.04%.
- Three of the five centres stated mast cell enrichment of clinical samples is not routinely performed. The remaining two participants (both NGS users) did not provide this 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. 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) VAF is often too low for detection by conventional read-depth NGS gene panels**[2].

Both the World Health Organisation (5th edition)[3] and the International Consensus Classification (ICC) group[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. **Wang *et al.* advocate access to an assay with a sensitivity down to 0.01–0.1% VAF in the clinical context of mastocytosis[5].**

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

In the absence of mast cell enrichment, Sanger sequencing does not afford the required assay sensitivity in the clinical context of mast cell disease[6-7]. The single Sanger sequencing user participating in this trial was able to identify the variant in both sample KIT 158 and KIT 159. However, this centre extracted RNA and utilised cDNA as assay input material (to analyse expressed variant load at the transcript level) rather than gDNA. Overall, just three (3.7%) returning participants employed cDNA as assay input material for this trial (Real-Time PCR Fluorescent Detection (n=2) and Sanger sequencing (n=1)). The EU-US Cooperative Group guidelines[8] 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 at least one the samples distributed for this trial (n=40). 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=3) 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.

In total, 26 (54.2%) of participants responding to the question confirmed they routinely included quantification information (VAF) on mast cell disease patient clinical reports.

Percentage (%) VAF *KIT* NM_000222.3:c.2447A>T p.(Asp816Val)^		
	Sample KIT 158	**Sample KIT 159**
n*	39	36
Median	0.23	0.24
IQR	0.10	0.12

^ % 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.

Six returning laboratories noted 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 laboratory noted they were looking to implement a mast cell enrichment method. The EU-US Cooperative Group[8] 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.

Extended KIT gene analysis

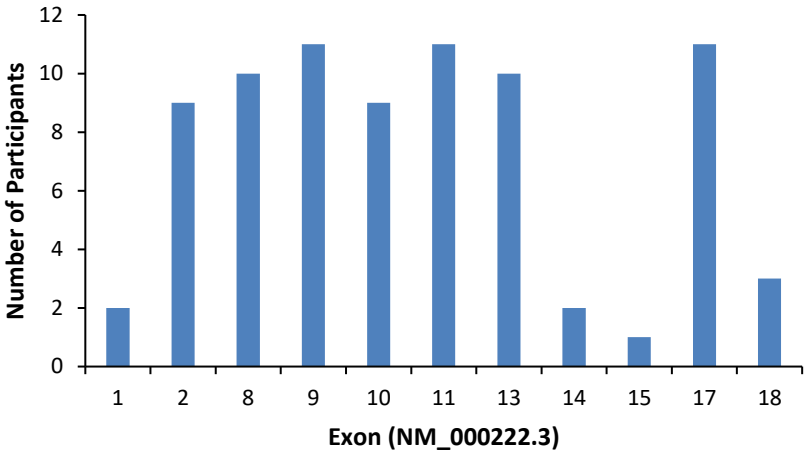
Over 90% adult systemic mastocytosis (SM) cases carry the *KIT* p.(Asp816Val) (D816V) variant. Nevertheless, many paediatric cases (mostly cutaneous disease) and adults with advanced forms (SM with an associated haematological neoplasm, aggressive SM and mast cell leukaemia) harbour other activating *KIT* variants[9].

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

As part of the previous trial distribution (KIT 232401), we surveyed participants regarding the provision of extended *KIT* gene analysis in the clinical context of mast cell disease. Thank you to the >75% of returning laboratories for providing this additional information (n=61).

- Sixteen (26.2%) laboratories informed us they offer a full *KIT* gene sequencing service.
- A further 13 centres (21.3%) provide analysis of *KIT* gene hotspots. Regions of interest are summarised in the chart below.
- At least five laboratories state using a NGS gene panel, two of the participants noted the limited sensitivity of their approach (approximately 5% LoD).

KIT gene extended analysis region of interest summary



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[10]. 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)[11] 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[12-13]. 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.

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

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 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).

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).

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

6 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).

7 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).

8 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).

9 Valent, P. *et al.* New Insights into the Pathogenesis of Mastocytosis: Emerging Concepts in Diagnosis and Therapy. *Annu Rev Pathol.* 18:(1):361-386 (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 http://ftp.ebi.ac.uk/pub/databases/lrgex/LRG_307.xml (accessed Dec 2023).

12 http://varnomen.hgvs.org/ (accessed Dec 2023).

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

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

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 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/

4.8.4) The UK NEQAS LI Policy for the Use of Reports by Individuals and Organisations states that all EQA reports are subject to copyright, and, as such, permission must be sought from UK NEQAS LI for the use of any data and/or reports in any media prior to use. See associated policy on the UK NEQAS LI website: http://www.ukneqasli.co.uk/eqa-pt-programmes/new-participant-information/