UK NEQAS
Leucocyte Immunophenotyping

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Pilot Myeloproliferative Neoplasms (MPN) Diagnostic Testing (Not Accredited)

Distribution – 222301 Participant -

Date Issued – 16 Jan 2023 Closing Date - 03 Mar 2023

Please note, this programme was previously titled Myeloproliferative Neoplasms Gene Panels (Pilot - Not accredited). It is designed for laboratories performing Myeloproliferative Neoplasms (MPN) testing using current algorithms[1] (Tefferi & Pardanani, 2014) to diagnose and subtype the disease. Participants are expected to test samples according to their current testing pathways, and from April 2022 this programme has been limited (according to WHO/NCCN guidance for the testing of MPN patients) to the core clinically significant MPN variants: *JAK2* p.Val617Phe and clinically significant variants within *JAK2* exon 12, *CALR* exon 9 and *MPL* exon 10. Testing of all four regions is not mandatory; testing should be performed according to laboratory strategy, as well as test repertoire. Extended next generation sequencing panel data is no longer included in this programme and cannot be submitted. External quality assessment of such testing is now encompassed by the Myeloid Gene Panels (Pilot – Not Accredited) programme).

Trial Comments

This trial was issued to 108 participants; 101 participants (93.5%) returned results. Of the seven participants that did not return results, three pre-notified us of their intended non-return.

Sample Comments

A single sample of DNA, MPN DT 109, was issued by UK NEQAS LI. Participants were informed that the sample was from an individual with a possible diagnosis of Essential Thrombocythaemia (ET). They were asked to analyse this sample according to their 'in house' strategy for testing patients with a suspected myeloproliferative neoplasm, limited to the core MPN variants: *JAK2* p.(Val617Phe), and clinically significant variants within *JAK2* exon 12, *CALR* exon 9, *MPL* exon 10.

Sample MPN DT 109

Did you detect the clinically significant ***JAK2* p.(Val617Phe)** variant in sample MPN DT 109: No

Did you detect a clinically significant ***JAK2* exon 12** variant in sample MPN DT 109: No

Did you detect a clinically significant ***CALR* exon 9** variant in sample MPN DT 109: Yes

Did you detect a clinically significant ***MPL* exon 10** variant in sample MPN DT 109: No

Your Results

Gene/Region	Your DNA sequence variant detected	Your protein variant	Other details
JAK2 p.(Val617Phe)			
JAK2 exon 12			
CALR exon 9	c.1154_1155insTTGTC	p.(Lys385AsnfsTer47)	
MPL exon 10			

All Participant Results

Gene region/marker	Participants detecting a variant/total number who tested the gene	Consensus DNA sequence variant*	Consensus protein variant*	Variant allele burden (%)+
JAK2 p.(Val617Phe)	1/97	No variant detected	No variant detected	n/a
JAK2 exon 12	0/78	No variant detected	No variant detected	n/a
CALR exon 9	100/100	c.1154_1155insTTGTC	p.(Lys385Asnfs*47)	27.0
MPL exon 10	0/85	No variant detected	No variant detected	n/a

*Results returned by participants (at both the DNA and protein level) may have been harmonised to the equivalent Human Genome Variation Society (HGVS) approved nomenclature (http://varnomen.hgvs.org/) during the compilation of 'All Participant Results' tables. Nomenclature is based on the MANE Select (v1.0)[4] reference transcript and genome build GRCh38. Protein nomenclature includes parentheses as it represents a prediction from analysis at the DNA level.
+Descriptive statistics calculated for any variant with >2 quantification data points. Percentage values quoted have been subjected to rounding up/down to 1 decimal place.

Your Performance

Performance	Performance status for this sample	Running performance	
		Satisfactory	Critical
n/a	n/a	n/a	n/a

Please note: this programme is not currently performance monitored. We will work towards a performance monitoring system as the programme develops.

Detailed breakdown of *CALR* exon 9 variant(s) detected

MANE Select transcript: NM_004343.4

DNA sequence change	n	Nomenclature guidance comments (as applicable)
c.1154_1155insTTGTC	75	Compliant with current HGVS recommendations[2,3]
c1154_1155insTTGTC	1	Symbol/syntax irregularity: omission of '.'
C.1154_1155INCttgtc	1	Symbol/syntax irregularity: 'INC' not compliant with HGVS and incorrect capitalisation
c.1,154_1,155 (insTTGTC)	1	Misleading symbol/syntax: incorrect use of parentheses and commas
c.1154_1155insTTGT	1	Out of consensus result
5 bp insertion	19	No HGVS based nomenclature provided, however result is in line with the consensus reported for this trial.
Not provided	2	

Colour coding reflects the level of compliance with current HGVS recommendations: **green** = fully compliant, **amber** = generally compliant with some omission(s) and **red** = nomenclature error(s)/ fails to comply with the recommendations.

Protein sequence change	n	Nomenclature guidance comments (as applicable)
p.(Lys385Asnfs*47)	35	Compliant with HGVS recommendations[2,3]. Parentheses appropriately reflect the analysis of DNA and the predicted status of the protein level description.
p.(Lys385AsnfsTer47)	14	
p.(Lys385fs)	3[a]	
p.(K385Nfs*47)	2	Compliant with HGVS recommendations[2,3] however, use of three letter amino acid codes is preferred. Parentheses appropriately reflect the analysis of DNA and the predicted status of the protein level description.
p.(Lys385Asnfs*)	1	Position of the termination codon has not been indicated / hybrid of standard and short format HGVS nomenclature is not advocated. Parentheses appropriately reflect the analysis of DNA and the predicted status of the protein level description.
p.Lys385Asnfs*47	4	Parentheses to indicate predicted status of the protein level description are absent and should be used when DNA is the input material.
p.Lys385AsnfsTer47	7	
p.Lys385fs	6[a]	
p.(K385fs*47)	1	Hybrid of standard and short format HGVS nomenclature is not advocated and use of three letter amino acid codes is preferred. Parentheses appropriately reflect the analysis of DNA and the predicted status of the protein level description.
p.K385fs*47	3	Hybrid of standard and short format HGVS nomenclature is not advocated and use of three letter amino acid codes is preferred. Parentheses to indicate predicted status of the protein level description are absent and should be used when DNA is the input material.
K385fs	1	Nomenclature error at the protein level. Please note current HGVS recommendations[2,3], which state that a letter prefix is mandatory to indicate the type of reference sequence used. Use of three letter amino acid codes is preferred. Parentheses to indicate predicted status of the protein level description are absent and should be used when DNA is the input material.
p.(Lys385Asnfs*?)	1	Nomenclature error at the protein level. Please note current HGVS recommendations[2,3], which state that 'fs*?' indicates that the new reading frame does not encounter a new termination codon.
Not provided	22[b]	

Colour coding reflects the level of compliance with current HGVS recommendations: **green** = fully compliant, **amber** = generally compliant with some omission(s) and **red** = nomenclature error(s)/ fails to comply with the recommendations.

[a] Participants utilising short format HGVS nomenclature.

[b] Comprises 19 participants descr bing a 5 bp insertion, two participants providing no description of the variant detected and one providing only the DNA sequence change.

MPN testing at diagnosis

	JAK2 p.(Val617Phe)	*JAK2* Exon 12	*CALR* Exon 9	*MPL* Exon 10
Tested at diagnosis	100	92	101	97
Not tested at diagnosis	1[a]	9[b]	0	4[c]

[a] Participant performs *CALR* testing only
[b] Includes two participants that clarified that *JAK2* ex 12 testing is performed only if requested
[c] Includes one participant that clarified that *MPL* ex 10 testing is performed only if requested

Testing strategy used by participants for patient samples

Participants were asked to describe their strategy for diagnostic testing of MPNs. The results are shown in the table below.

Strategy	n
Targeted next generation sequencing panel testing	47
Sequential single gene testing	40
Parallel single gene testing	13
Not given	1

Twenty participants also provided additional details regarding their testing strategy.

- Three participants describing their approach as targeted NGS panel testing qualified their response: one stated that *JAK2* V617F testing is performed first, and only proceeds to analysis of the remaining regions if the sample is *JAK2* V617F negative. The other two laboratories indicated that *JAK2* V617F testing is performed by qPCR, whilst testing of the remaining regions is performed utilising NGS.
- Similarly, 14 participants describing their approach as sequential single gene testing provided further information: seven laboratories clarified that they test for the *JAK2* V617F variant first and progress to further testing only if this result is negative; five laboratories described a combination of sequential and parallel testing.
- All three of the participants describing their approach as parallel single gene testing and providing additional information indicated that they actually perform a combination of parallel and sequential testing.
- Of all 20 participants providing further information, nine declared that testing is influenced by the clinical indication.

Assay Details

Participants were asked to provided details of the methodologies used for each of their MPN assays. Responses are summarised in the following tables. Please note that some participants did not respond to all questions and some provided more than one response for some elements of their testing.

PCR Type	*JAK2* p.(Val617Phe)	*JAK2* Exon 12	*CALR* Exon 9	*MPL* Exon 10
PCR for NGS	43	48	46	47
Allele Specific PCR	24	0	6	6
Droplet Digital PCR	10	1	0	2
Multiplex PCR	1	3	1	2
Real-Time PCR	14	0	3	7
Single PCR	2	9	31	5
Sanger sequencing	0	11	6	9
Melting curve analysis	0	3	2	4
Allele Specific Competitive blocker PCR	0	0	0	1
Cold PCR	0	0	1	0
Other	1	2	3	1
Not given	1	1	1	1

Analysis Type	*JAK2* p.(Val617Phe)	*JAK2* exon 12	*CALR* exon 9	*MPL* exon 10
NGS (Illumina)	31	32	33	32
NGS (ThermoFisher Ion Torrent)	11	14	11	13
NGS (Other)	2	5	4	4
Agarose Gel Electrophoresis	10	-	-	1
Capillary Electrophoresis	5	7	33	5
Digital PCR (Bio-Rad)	10	1	-	3
High Resolution Melt	-	3	3	6
Real-Time PCR Fluorescent Detection	25	-	4	7
Sanger Sequencing	-	15	10	11
Other	-	-	1	-
Not given	1	1	1	1

Protocol Type / Kit	*JAK2* p.(Val617Phe)	*JAK2* exon 12	*CALR* exon 9	*MPL* exon 10
Agilent Haloplex HS Panel	1	1	1	1
Agilent SureSelect Custom QXT Panel	1	1	1	1
Archer DX VariantPlex Myeloid Panel	2	2	2	2
Illumina AmpliSeq Panel	4	3	4	3
Illumina TruSight Myeloid Panel	2	3	3	3
Oncomine Myeloid Research Assay	5	6	5	6
Qiagen QiaSeq Custom Panel	6	7	7	7
Roche Kappa Capture	2	2	2	2
Sophia Genetics Myeloid Solution	2	2	2	2
In house Assay	36	40	60	40
BioRad PrimePCR ddPCR kit	10	-	-	-
Qiagen/Ipsogen MutaQuant Kit	10	-	-	1
Qiagen/Ipsogen MutaScreen Kit CE	-	-	-	5
Qiagen/Ipsogen MutaSearch Kit	3	-	-	-
Qiagen/Ipsogen RGQ PCR Kit	-	-	4	-
Rotor-Gene Q MDx	1	1	-	1
3B BlackBio Tru PCR kit	1	-	-	-
Other	6	8	7	7
Not given	1	1	1	1

Trial Comments

- Sample MPN DT 109 was comprised of DNA from a patient with a potential diagnosis of Essential Thrombocythaemia (ET).
- Of the 101 participants returning results for this trial, 100 tested this sample for clinically significant variants in exon 9 of the *CALR* gene. The single participant not providing a result for exon 9 of the *CALR* gene experienced repeated failure of their testing on this sample.
- All 100 participants reported a positive result, and overall, there was reasonable consensus in the variant detected and the nomenclature used to describe it, see below

- Ninety-seven participants (96.0%) tested this sample for the *JAK2* V617F variant. Of the four participants who did not provide a *JAK2* V617F result, only one indicated that they do not routinely test for this variant. Another indicated that *JAK2* V617F testing was not performed as they had already participated in our *JAK2* V617F programme. All four correctly identified the presence of a *CALR* exon 9 variant.
- Of the 97 participants submitting a result for *JAK2* V617F testing, 96 (99.0%) returned a negative result. The single participant returning an out-of-consensus positive result employed a parallel single gene testing approach and utilised the Qiagen/Ipsogen MutaQuant kit. The variant was detected at a level of 0.03%. Given that quantitative PCR is associated with a low level of background positivity, such low-level results should be interpreted with caution and within the clinical context.

- Seventy-eight participants (77.2%) tested this sample for clinically significant variants in exon 12 of the *JAK2* gene. In line with expectation, all 78 (100%) returned a negative result.

- Eighty-five participants (84.2%) tested this sample for clinically significant variants in exon 10 of the *MPL* gene. In line with expectation, all 85 (100%) returned a negative result.

NM_004343.4(*CALR*): c.1154_1155insTTGTC p.(Lys385Asnfs*47)

- In line with expectation, 100/100 (100.0%) participants analysing exon 9 of the *CALR* gene detected a clinically significant variant.
- Nineteen participants indicated that a 5bp insertion had been detected but did not describe the variant further; it is acknowledged that laboratories performing allele sizing or melt curve based assays to analyse *CALR* will lack sufficient sequence information from their results to provide HGVS based nomenclature.
- Two laboratories indicated that a clinically significant variant had been detected in exon 9 of the *CALR* gene but did not provide further information.
- For those 79 participants providing sequence information for this frame shift variant, application of the Human Genome Variation Society (HGVS) recommendations[2,3] for the description of sequence variants was generally good.
- Seventy-five participants (94.9%) correctly described the cDNA change as c.1154_1155insTTGTC. Three participants provided nomenclature with a minor irregularity, e.g. c1154_1155insTTGTC, or potentially misleading symbols/syntax, e.g. C.1154_1155INCttgtc or c.1,154_1,155 (insTTGTC). A single participant provided an out of consensus result: c.1154_1155insTTGT.
- Seventy-eight participants provided a description of the protein change, with 52 participants (66.7%) correctly describing the variant as p.(Lys385Asnfs*47), p.(Lys385AsnfsTer47) or the shorthand p.(Lys385fs). In addition, two participants (2.6%) also correctly described the protein change, but utilised single letter amino acid codes (3-letter codes are preferred). A further 22 participants (28.2%) were considered generally compliant with HGVS recommendations[2,3] but with some minor omissions. This included 20 submissions where parentheses were not used; please note, where gDNA represents the assay input material parenthesis should be included in the nomenclature description to indicate the amino acid change is predicted from DNA level data. Two participants (2.6%) were considered not compliant: one did not include 'p.' (current HGVS recommendations state that a letter prefix is mandatory to indicate the type of reference sequence used[2,3]); the other inappropriately included '?' implying that no termination codon is created.
- The *CALR* c.1154_1155insTTGTC p.(Lys385Asnfs*47) variant identified in this trial has been well characterised in the literature (COSMIC Genomic Mutation ID: COSV 57116551; Legacy Identifier COSM1738056[10]) where it is also referred to as a *CALR* type-2 mutation. Clinically significant variants in exon 9 of the *CALR* gene are observed in 25-30% of patients with ET and 15-35% of patients with primary myelofibrosis (PMF)[5,6]; in each cohort, type-1 (c.1092_1143del p.Leu367Thrfs*46) and type-2 variants together account for >80% of *CALR* variants detected[7-9]. Detection of a clinically significant variant in exon 9 of the *CALR* gene is included within the diagnostic criteria for both ET and PMF[6].

Quantitation of *CALR* variant

- Sixty-four participants provided a quantitative value for the *CALR* exon 9 variant: variant/(variant+wild type)x100. Values (rounded to a single decimal place) ranged from 7.6% to 50.0% with a median value of 27.0% and interquartile range of 3.6%.
- NGS was the most common methodology, used by 46 participants (71.9%) with a median variant level of 27.0% (range: 7.6% - 35.1%; IQR 3.9%).
- A further fourteen participants (21.9%) used capillary electrophoresis, with a median value of 26.8% (range: 23.0% - 50.0%; IQR 2.7%).
- The remaining four participants used Sanger sequencing (n = 3) or did not provide details of their methodology (n =1).

Methodology

- Of 100 participants returning information regarding their testing strategy, 47 (47%) stated that they employed targeted NGS panel testing. Whilst the other 53 participants (53%) stated a single gene testing approach (either sequentially or in parallel), some of these laboratories later state the use NGS assays within that strategy, for example seven perform NGS for *JAK2* exon 12 variants and of these, five also use it to analyse exon 10 of the *MPL* gene.
- Overall, the most commonly employed analysis methods were:
    - NGS (n=44, 46.3%), followed by Real-Time PCR with fluorescent detection (n=25, 26.3%) for *JAK2* p.(Val617Phe)
    - NGS (n=51, 65.4%), followed by Sanger sequencing (n=15, 19.2%) for *JAK2* exon 12 variants
    - NGS (n=48, 48.0%), followed by capillary electrophoresis (n=33, 33.0%) for *CALR* exon 9 variants
    - NGS (n=49, 59.0%), followed by Sanger sequencing (n=11, 13.3%) for *MPL* exon 10 variants.
- Thus, a wide range of methods were used to detect the core MPN associated genes, the majority of which have a theoretical limit of detection (LoD) adequate to detect them in the context of MPNs.
- Recent good practice guidelines advocate the ability to detect 1-3% VAF or lower for *JAK2* p.(Val617Phe) and 5% VAF for *JAK2* Exon 12, *CALR* exon 9 and *MPL* exon 10 variants[5]. Participants were asked to provide their LoD for each of their assays and the responses are summarised in the table below. This demonstrates that a subset of laboratories are still using techniques with an inadequate limit of detection; transition to more sensitive techniques is therefore highly recommended for those centres.

Limit of Detection	*JAK2* V617F	*JAK2* exon 12	*CALR* exon 9	*MPL* exon 10
<1%	36	4	5	4
1-3%	38	29	35	34
>3%/<5%	1	2	2	1
5%	14	25	40	29
>5%	0	14	8	8

Final comments

- We are aware that a minority of participants, most often those employing a single gene testing strategy, have found the amount of DNA issued in this programme too small for their testing requirements. Whilst we are constrained by the size of the donated patient sample, we are endeavouring to increase the amount DNA in each sample for future trials. Spare trial samples are generally available, and we encourage participants to request repeat samples when necessary (repeatsamples@uknegasli.co.uk).
- Please note that the number of trial issues for this programme has now increased from one to two per annum. This takes effect for the 2023-2024 registration period; the first trial of this registration year is scheduled for issue in June 2023.
- As panel testing of *JAK2*, *CALR* and *MPL* becomes more prevalent in the diagnosis of myeloproliferative neoplasms, UK NEQAS LI will soon begin the process of merging the current *JAK2* p.Val617Phe Mutation Status and MPN Diagnostic Testing Programmes to provide a more cost effective service whilst maintaining the rigour of the current programmes. Further information will be provided as this process proceeds.
- We would like to thank laboratories for their continued participation in the pilot MPN Diagnostic Testing (Not Accredited) programme.

References

1. Tefferi A *et* Pardanani A (2014) CALR mutations and a new diagnostic algorithm for MPN. *Nat Rev Clin Oncol* **11**: 125–126.
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7. Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, *et al*. (2013) Somatic mutations of calreticulin in myeloproliferative neo-plasms. *N Engl J Med* **369**(25): 2379–90.
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9. Tefferi A, Wassie EA, Guglielmelli P, Gangat N, Belachew AA, Lasho TL, *et al*. (2014) Type 1 versus Type 2 calreticulin mutations in essential thrombocythaemia: A collaborative study of 1027 patients. *Am J Hematol* **89**(8): E121-E124.
10. https://cancer.sanger.ac.uk/cosmic/mutation/overview?id=105238095

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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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/