UK NEQAS
Haematology and Transfusion

UK NEQAS Haematology
DNA diagnostics for the Haemoglobinopathies: Performance Assessment

Survey no: 2302DN Distribution date: 12th June 2023

PRN:

Category	Specimen 2302DN1	Specimen 2302DN2	Truncated score for this survey	Cumulative performance score for 3 consecutive surveys
Participation	0	0	0	0
Alpha mutation analysis	0	0	0	0
Beta mutation analysis	0	0	0	0
Interpretation Interpretation of diagnostic results using case details	0	0	0	0

Note: The figures given denote the performance score given for each category, assessed against the model answer. The scores for Reporting and Nomenclature shown below are advisory only.

Reporting Required recommendations	0	0		
Nomenclature alpha and beta genotypes	0	0		
Nomenclature HGVS beta globin mutation	0	0		

Reasons for adverse performance scores:
2302DN1:

2302DN2:

Assessor 1: Dr Barbara De la Salle Signature: BDeLaSalle. Date:30/08/2023

Assessor 2: Dr Cornelis Harteveld Signature: Date:30/08/2023

For information on data analysis and performance assessment see the UK NEQAS (H) Participants' Manual (www.ukneqash.org)

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UK NEQAS
Haematology and Transfusion

UK NEQAS Haematology: 2302DN

EQA for DNA Diagnostics in the Haemoglobinopathies Scheme

Survey 2302DN (12th June 2023)

Date of issue 18th October 2023

DNA Diagnostics in the Haemoglobinopathies Scheme

Organisation

This programme is operated by the UK National External Quality Assessment Scheme for Haematology (UK NEQAS Haematology). The Scheme Director is Barbara De la Salle.

The programme is operated under the expert guidance of the UK NEQAS Haematology Special Scientific Advisory Group and Steering Committee.

Objectives

The objectives of this programme are to assess:

- The interpretation of haematological data (with salient clinical information) to direct appropriate DNA tests.
- The use of DNA investigations to achieve the correct results. The exercise was not intended to assess the techniques, methodology or approach to analysis used, except in their ability to achieve the expected results.

Distribution schedule

Survey 2302DN was dispatched on 12th June 2023. The next distribution (2303DN) is planned for October 2023.

Survey 2302DN (distributed 12th June 2022)

Survey material

Two specimens (2302DN1 and 2302DN2) were distributed. The specimens were accompanied by background information that included gender, ethnic origin, age, full blood count and haemoglobinopathy screen data.

The survey material for 2302DN1 was sourced from surplus patient material provided by the Leiden University Medical Centre. 2302DN2 was DNA that has been extracted from cultured cells and was therefore of a higher concentration and greater purity (as indicated by the 260/230 ratio of a Nanodrop spectrophotometer) than 2302DN1. There is a plan to move to cultured cells as a source of DNA permanently in the future.

Specimen distribution and return of results

Specimens were distributed to 49 laboratories registered for the scheme and 47 (96%) returned results. One participant received a non-participation penalty for this survey. The other could not participate due to unforeseen circumstances which they notified us of in advance and therefore had their non-participation penalty waived.

7 UK laboratories were registered for this survey and 6 returned results.

ANALYSIS OF RESULTS FOR SPECIMEN 2302DN1

Expected results for specimen 2302DN1

Female, 24 years old, Turkish origin.
The patient is pregnant and undergoing antenatal screening.

Laboratory Results for 2302DN1

Parameter	Result
Hb (g/L)	77
RBC ($x10^{12}$/L)	4.32
MCV (fl)	56
MCH (pg)	18
Haemoglobinopathy screen	Hb variant detected in the S window on HPLC
Hb A2 (%)	3.8
Hb F (%)	2.2
Hb X (%)	24.5
DNA Analysis	Alpha genotype: $--^{MED2}/\alpha\alpha$ Beta genotype: β^A/β^S

Specimen quality 2302DN1

Of the 47 returns received, 44 participants (94%) reported specimen 2302DN1 as satisfactory. Of the 3 participants that reported an unsatisfactory sample, none requested a repeat set of specimens. The reasons given for reporting the sample as unsatisfactory were “DNA not optimal for MLPA”, “poor DNA quality” and “quality issues with this DNA sample”.

Methods used in 2302DN1 (some laboratories use more than one method)

Alpha genotype	Beta genotype
Sanger Sequencing (23)	Sanger Sequencing (31)
MLPA (25)	MLPA (12)
Multiplex Gap PCR (18)	Vienna labs strip assay (3)
Vienna labs strip assay (8)	NGS (9)
Gap PCR (2)	Reverse Dot Blot (3)
NGS (9)	ARMS-PCR (1)
Reverse Dot Blot (2)	Multiplex Gap PCR (1)
PCR + Reverse Hybridisation (1)	PCR + Reverse Hybridisation (1)
Multiplex Triple PCR (1)	Real-time PCR

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Alpha genotype results for specimen 2302DN1

46/47 participants undertook alpha thalassaemia genotyping. One participant did not do alpha genotyping because they are registered for beta genotyping only.

30/46 participants (65%) returned the expected result: $--^{MED2}/\alpha\alpha$.

The patient is a carrier of the Mediterranean 2 type deletion of 26.5kb involving the two alpha and zeta globin genes.

Of the remaining 16 participants:

- 3 reported the Calabrian deletion ($--^{CAL}/\alpha\alpha$). This deletion is 32.2Kb in size which is very similar to the MED2 deletion. As they have very similar breakpoints, the two are not distinguishable by some assays.
- 13 participants reported a normal alpha genotype ($\alpha\alpha/\alpha\alpha$).

No participants received adverse scores for the alpha genotype. The three laboratories that reported the Calabrian deletion reported the correct phenotype and made appropriate recommendations. All three also mentioned the limitation of their assays (including being unable to distinguish between the --CAL and the --MED2 deletions) in the technical limitations sections. The 13 laboratories that reported a normal genotype were not penalised as it was recognised that the incorrect genotype was due to the limitations of the assays used.

Laboratories using targeted assays such as Vienna labs strip assay and GAP PCR were unable to detect this less common deletion but those that used methods such as MLPA which scans the entire region for deletions were more successful.

The laboratory methods used by the 30 labs that reported the expected result were MLPA and NGS. The labs that reported the Calabrian deletion used Reverse Dot Blot and PCR+ Reverse Hybridisation.

There were two laboratories that reported the --MED2 deletion using MLPA but mentioned in their technical limitations or as a miscellaneous comment that they could not distinguish between the MED2 and the Dutch1 deletions using this assay.

Two of the 30 participants that reported the expected genotype received adverse scores for incorrect annotation (legacy nomenclature):

- One wrote "$--^{MED}/\alpha\alpha$" and did not specify that it was the "MED2" deletion in the annotation. It is important to distinguish which Mediterranean deletion has been detected as they are very different in size.
- The other wrote "α^{o}MED2/$\alpha\alpha$" which is incorrect annotation.

Beta genotype results for specimen 2302DN1

46/47 participants undertook beta thalassaemia genotyping. One participant did not do beta genotyping because they are registered for alpha genotyping only.

46/46 participants (100%) returned the expected result: β^{A}/β^{S}.

The patient is a carrier of the sickle cell mutation in Codon 6 (GAG>GTG) of the beta globin gene which gives rise to the variant "Hb S".

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3/46 participants returned the expected result but received adverse scores for incorrect annotation (legacy nomenclature) of the beta genotype in their report.

- One participant wrote HGVS nomenclature within the legacy annotation
- One participant did not write the genotype in traditional annotation but reported the correct genotype/phenotype in the interpretation section
- One participant wrote "CD20A>T" which is incorrect annotation but reported the correct phenotype

Two participants received adverse scores for HGVS nomenclature. One participant did not provide any HGVS annotation for the beta genotype and one incorrectly wrote the HGVS annotation ("c." was missing from the annotation provided).

Interpretation of results for 2302DN1

45/47 participants were assessed on the interpretation of their results. Two participants did not provide interpretation of results or recommendations because they are manufacturing companies that do not offer a clinical service to patients.

44/45 (98%) participants correctly interpreted the results using the case details provided.

One participant received an adverse score for their interpretation of the results because they did not specify that the alpha thalassaemia deletion is "alpha zero". It is essential to classify the severity of alpha globin gene deletions and/or mutations as "plus" or "zero" because they have such significant phenotypic differences.

Recommendations for 2302DN1

45/47 participants were assessed on the recommendations made. Two participants did not provide interpretation of results or recommendations because they are manufacturing companies that do not offer a clinical service to patients.

35/45 participants (71%) returned the expected recommendation of "Testing the baby's biological father" using comment code 911. As this patient is a pregnant lady undergoing antenatal screening, it is essential that the baby's biological father is tested for haemoglobinopathies in order to ascertain the risk of their child being born with a clinically significant haemoglobinopathy.

12 participants received adverse scores for the recommendations made:

- 10 did not recommend "Testing of the baby's biological father" using comment code 911.
- 2 did not select code 915 (Further molecular testing is recommended due to the method limitations of this laboratory) and were penalised because they had limitations of their assays which meant they did not detect the --MED2 deletion and therefore should have recommended further testing.

Refer to the graph below for a breakdown of all the recommendations made (some participants used more than one code).

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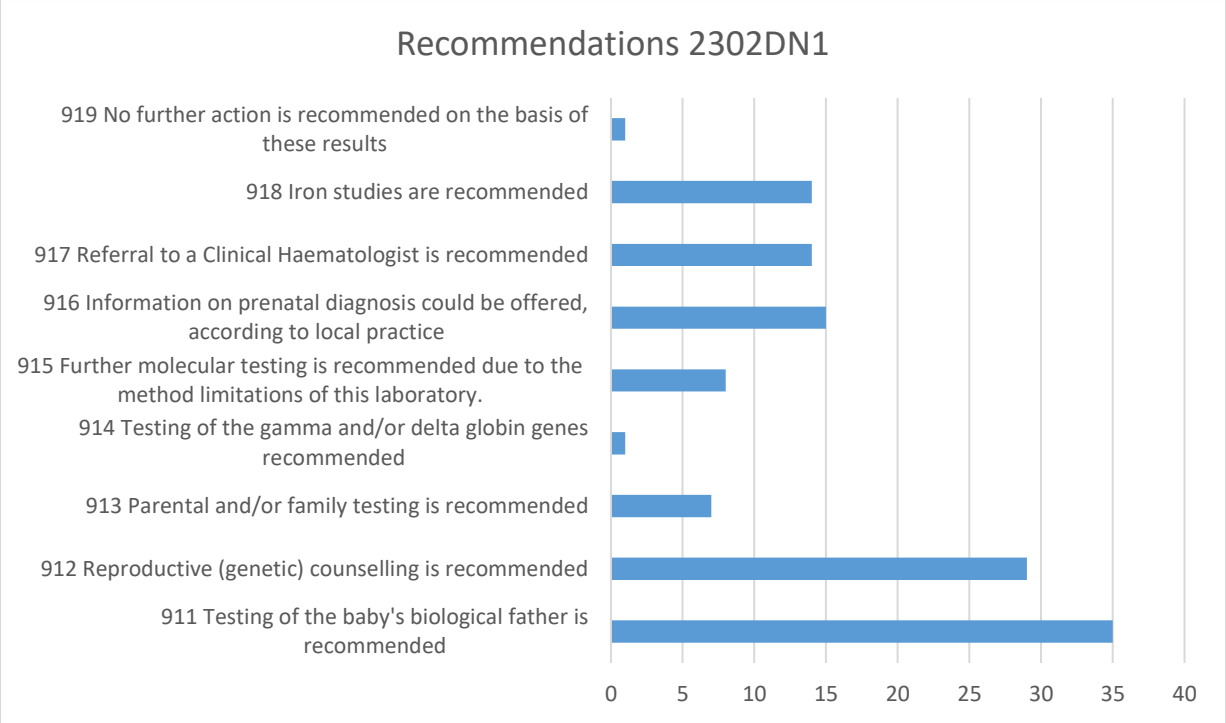

Appendix 1 shows an exemplary report of 2302DN1. *This is provided for participants to use as guidance if they wish to improve their reporting practice but they are not expected to formally adopt this format.*

Model answer for 2302DN1

Criteria for the model answer:	Sample 2302DN1
Analysis	
Mutation analysis:	Alpha globin genotype: $--^{MED2}/\alpha\alpha$
	Beta globin genotype: β^A/β^S
Interpretation using case details:	Sickle cell (or Hb S) carrier or trait
	AND Alpha zero thalassaemia carrier or trait
Reporting	
Recommendations made on report:	911: Testing of the baby's biological father recommended.
Nomenclature	
HGVS nomenclature	HBB: c.20A>T

Expert comments for 2302DN1

This case involves an antenatal mother who is a sickle cell carrier and also a carrier of an alpha zero thalassaemia deletion. Haemoglobinopathy mutations are common in Turkey; common enough that a prevention program involving premarital screening was introduced in 2003 and has reduced the number of affected births by 90%. The prevalence of haemoglobinopathy mutations vary dramatically by region (from 0.3% to 13% of the population being sickle trait, for example). In addition to Hb S, beta thalassaemia and alpha thalassaemia mutations are also frequently identified (including alpha zero deletions). Therefore the combination of sickle cell with alpha zero thalassaemia is not as surprising as might originally be thought.

It was interesting to see the variability in alpha zero deletion calls by the different labs. Most reported the MED2 deletion, with a subset of labs commenting that their assay could not distinguish the MED2 deletion from the Dutch1 deletion. A small number of labs reported it as the CAL deletion but again stated that their assay could not distinguish between the CAL and the MED2 deletion. It is not surprising that assays were not able to clearly distinguish these deletions because both their sizes and breakpoints are similar (for example the breakpoints of the MED2 and the Dutch1 deletion are only around 340bp apart, and both breakpoints are within highly homologous Alu elements so even assays that probe across the breakpoints might not be able to distinguish them. As many labs commented, it is important to acknowledge the fact that it is not always possible to determine the exact deletion present, particularly when using dosage type tests such as MLPA that do not provide breakpoint information.

Presumably where the assay results were not definitive, labs were sometimes selecting the deletion that the kit/assay indicated, or selecting the deletion that they were more familiar with, whereas others were trying to match to the ethnic origin of the person under test. In the Turkish population, the most common alpha thal deletion is the 20.5, followed by the MED deletions. Therefore it does seem more likely that this person from Turkey would have the MED2 deletion rather than the Calabrian or the Dutch1. However, ultimately determining the precise deletion and naming it is not the most important aspect of this case; the correct diagnosis of alpha zero thalassaemia carrier status was more important, as it was strongly suggested by three pieces of evidence in the referral details; the low MCH coupled with the significantly reduced variant % suggested with presence of either alpha thalassaemia or iron deficiency, and the Turkish family origin suggested that possibility of alpha zero. This was well interpreted by all participants, with those that failed to detect a deletion due to using targeted assays almost all recommending that further testing was required to confirm whether alpha thalassaemia was present. This demonstrated that the labs undertaking the genetic testing also have a good grasp of relevant haematological phenotypes, which is critical to ensure that all mutations are identified because, as this lady demonstrates, it is not uncommon to have more than one globin gene mutation.

The recommendation selected by the majority of laboratories was testing of the baby's biological father. As this antenatal mother carries two clinically significant mutations, there is a high chance that the baby will inherit one of her mutations (3/4 chance) so testing of the baby's father would be critical to allow the couple to have accurate information on their reproductive risks. In some countries, prenatal diagnosis would be offered to the mother even if the baby's biological father is not available for testing, because due to the relatively high worldwide prevalence of haemoglobinopathy mutations there is a reasonable chance that an untested father could carry a globin mutation. Therefore (after testing the father and offering reproductive counselling) providing information on prenatal diagnosis was the most common option selected. Testing of other family members to establish their reproductive risks could also be valuable given the possibility of inheriting two independently assorting pathogenic variants. Other recommendations, both selected by fourteen labs, were to suggest iron studies or to refer to a Consultant Haematologist – presumably both of these were due to the degree of anaemia in this case (Hb 77) which is lower than typically seen in antenatal alpha zero thalassaemia carriers, and is a reminder than even in an antenatal screening context when the primary purpose is to identify carriers, the health and clinical care of the antenatal mother should also be considered.

Comment kindly provided by Dr Melanie Proven, Principal Clinical Scientist, National Haemoglobinopathies Reference Laboratory, Oxford.

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ANALYSIS OF RESULTS FOR SPECIMEN 2302DN2

Expected results for specimen 2302DN2

Female, 1 year old, Pakistani origin.
Infant with severe anaemia. The family recently moved to the UK from Pakistan.

Laboratory Results for 2302DN2

Parameter	Result
Hb (g/L)	37
RBC (x10^{12}/L)	1.64
MCV (fl)	68.9
MCH (pg)	22.6
Haemoglobinopathy screen	No abnormal Hb variants detected. No Hb A detected.
Hb A2 (%)	1.1
Hb F (%)	84.1
Hb X (%)	~
DNA Analysis	Alpha genotype: αα/αα Beta genotype: $\beta^{IVS1-1(G>T)}/\beta^{IVS1-1(G>T)}$

Specimen quality 2302DN2

Of the 47 returns received, 46 participants (98%) reported specimen 2302DN2 as satisfactory. The one participant that reported an unsatisfactory sample, did not request a repeat set of specimens. The reason given for reporting the sample as unsatisfactory was “DNA not optimal for MLPA”.

Methods used 2302DN2 (some laboratories use more than one method)

Alpha genotype	Beta genotype
Sanger Sequencing (22)	Sanger Sequencing (31)
MLPA (20)	MLPA (19)
Multiplex GAP PCR (18)	Vienna labs strip assay (3)
Vienna labs strip assay (8)	NGS (9)
GAP PCR (1)	Reverse Dot Blot (3)
NGS (7)	ARMS PCR (1)
Reverse Dot Blot (2)	Multiplex GAP PCR (2)
PCR + Reverse Hybridisation (1)	PCR + Reverse Hybridisation (1)
Multiplex Triple PCR (1)	Real time PCR (1)

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Alpha genotype results for specimen 2302DN2

46/47 participants undertook alpha thalassaemia genotyping. One participant did not do alpha genotyping because they are registered for beta genotyping only.

46/46 participants (100%) returned the expected result: αα/αα.

The patient has a normal alpha globin genotype (no common mutations or deletions detected).

Beta genotype results for specimen 2302DN2

46/47 participants undertook beta thalassaemia genotyping. One participant did not do beta genotyping because they are registered for alpha genotyping only.

39/46 participants (85%) returned the expected result: $\beta^{IVS1\text{-}1(G>T)}/\beta^{IVS1\text{-}1(G>T)}$

This patient is homozygous for the beta zero thalassaemia mutation IVS1-1(G>T). This genotype alongside the clinical information provided fits with a diagnosis of beta thalassaemia major in this child.

7 participants returned an incorrect genotype.

- One participant reported homozygous IVS1-1(G>A).
- One participant reported homozygous Cd30 (likely G>C as this mutation was mentioned in their list of "mutations analysed" using reverse dot blot in their submitted laboratory report).
- The remaining 5 participants did not report a normal genotype but indicated that they could not identify the mutation present using their laboratory methods.

Only one participant received an adverse score for reporting an incorrect beta genotype.

Five participants returned the expected result but received adverse scores for incorrect annotation (legacy nomenclature) of the beta genotype in their report. 4/5 used HGVS nomenclature in the legacy annotation and one did not describe the full mutation in the annotation but only wrote β^0/β^0.

Interpretation of results for 2302DN2

45/47 participants were assessed on the interpretation of their results. Two participants did not provide interpretation of results or recommendations because they are manufacturing companies that do not offer a clinical service to patients.

43/45 (89%) participants correctly interpreted the results using the case details provided. Two participants received adverse scores for their interpretation. One described the phenotype as "homozygous beta thalassaemia" but did not specify "beta zero" or "beta thalassaemia major" in the report. The other described the patient as having "beta thalassaemia based on a variant in both of the two beta globin genes" but similarly did not specify that this is a beta zero mutation or that patient has a "beta thalassaemia major" phenotype. It is important to relay the clinical severity of the phenotype based on the genotyping results by using the right terminology.

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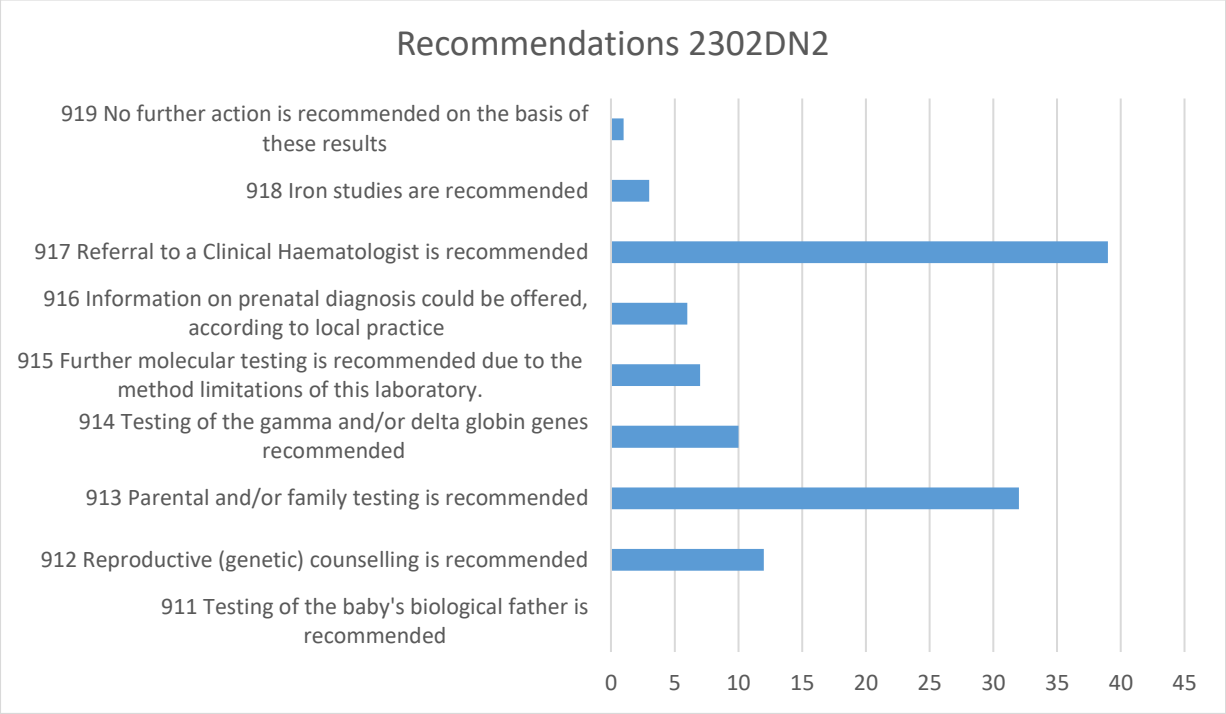
Recommendations for 2302DN2

45/47 participants were assessed on the recommendations made. Two participants did not provide interpretation of results or recommendations because they are manufacturing companies that do not offer a clinical service to patients.

39/45 participants (87%) returned the expected comment of "Referral to a clinical haematologist is recommended using comment code 917. 32/45 (71%) participants also recommended parental/family testing using comment code 913.

6 participants received adverse scores for their recommendations. 5/6 failed to recommend referral to a clinical haematologist. One of these 5 used comment code 919 (No further action is recommended on the basis of these results) which is inappropriate. 1/6 did use code 917, however they did not detect the correct beta mutations using their targeted assay and therefore should have recommended further molecular testing using comment code 915 or written as a free text comment.

Refer to the graph below for a breakdown of all the recommendations made (some participants used more than one code).



Appendix 2 shows an exemplary report of 2302DN2. *This is provided for participants to use as guidance if they wish to improve their reporting practice but they are not expected to formally adopt this format.*

Model answer for 2302DN2

Criteria for the model answer:	Sample 2302DN2
Analysis	
Mutation analysis:	Alpha globin genotype: αα/αα
	Beta globin genotype: $\beta^{IVS1\text{-}1(G>T)}/\beta^{IVS1\text{-}1(G>T)}$
Interpretation using case details:	Beta thalassaemia major
	or homozygous beta zero thalassaemia
	or transfusion-dependent beta thalassaemia
Reporting	
Recommendations made on report:	917: Referral to a clinical haematologist is recommended
	Also acceptable:
	913: Parental testing indicated
Nomenclature	
HGVS nomenclature	HBB: c.[92+1G>T];[92+1G>T]
	or HBB: c.[92+1G>T] homozygous

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Expert comments for 2302DN2

Case 2302DN2 concerns a one year old baby girl who had recently moved from Pakistan to the UK and was found to have severe anaemia. She was found to be homozygous for the beta globin IVS1-1(G>T) mutation. This substitution of a highly conserved nucleotide abolishes the endogenous splice donor site and results in use of two exonic cryptic splice sites (16 and 38 nucleotides upstream), both of which cause a frameshift in the spliced transcript, introducing an early stop codon and triggering nonsense-mediated decay of the RNA transcript. Therefore this is classed as a beta zero thalassaemia mutation because no beta globin chains are produced. It is important to clarify the type of beta thalassaemia mutations during diagnosis, because some clinical trials for gene therapies are not open to patients with two beta zero mutations, due to the higher risk of immune response against the normal beta globin gene chain.

Whenever a homozygous mutation is observed it is important to consider whether this result could actually represent a heterozygous mutation with a deletion on the other allele, or whether it could be a false homozygous result due to allele dropout of the normal sequence. If there are heterozygous polymorphisms present in the region of the homozygous base call, this reduces the likelihood of both possibilities. However, there are only a few common polymorphisms in the beta globin gene and it is quite common for individuals with a homozygous mutation to have the same haplotype (and thus the same polys) so this analysis is not always helpful (as was the case in this sample). Labs that performed CNV analysis such as MLPA will have had assurance than no deletions were present. If evidence that allele dropout is not causing a false homozygous result is required, the best way to rule this out is with a second assay using non-overlapping primers/probes). In this case there was good concordance between the genotype and the phenotype provided, so there was no reason to suspect a false result.

As this result concerned a baby/toddler, it was important to provide information predicting (as far as possible) the implications for this child in later life. Homozygous beta zero thalassaemia mutations are expected to result in beta thalassaemia major (also known as transfusion-dependent beta thalassaemia). However, it is well established that outcomes are not always as straightforward as this. There are multiple possible modifying factors that could ameliorate the phenotype resulting in a beta thalassaemia intermedia phenotype, such as alpha thalassaemia or factors increasing the production of Hb F. Some have been ruled out by this testing (e.g. alpha thalassaemia) whereas others could be established by additional testing (e.g. gamma gene promoter sequencing, which is not covered by this scheme). This may have been the reason that some labs recommended further testing. However, as the genotype identified was a good fit for the severe phenotype described in this patient (Hb 37) there was not a particular requirement to go looking for ameliorating factors in this case.

The most common recommendation was referral to a haematologist, which was very important to initiate appropriate treatment for this extremely anaemic child. The second

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most popular recommendation was to test the parents; the reason for this may have been two-fold; some labs would automatically recommend this to demonstrate true homozygosity in the child and confirm the diagnosis, whereas others may have had the reproductive implications for the parents in mind, where confirming the parental mutations would be useful for PND or PGD workup. Some parents are particularly interested in the possibility of having a healthy sibling due to the option of sibling donation of stem cells to provide treatment options for the affected sibling.

In the UK, cases of beta thalassaemia major are detected by newborn screening prior to the onset of symptoms so it is unusual to see an HPLC result of an untransfused beta thalassaemia major case after haemoglobin switching is complete. Children who come into the country within the first year of life will also be offered neonatal-style screening, but this child was slightly older so would not have been provided with screening on arrival.

Comment kindly provided by Dr Melanie Proven, Principal Clinical Scientist, National Haemoglobinopathies Reference Laboratory, Oxford.

APPENDIX 1
Exemplary Report for 2302DN1

ALPHA GLOBIN GENOTYPE:
Heterozygous for the Mediterranean 2 alpha zero thalassaemia deletion ($--^{MED2}/\alpha\alpha$)

BETA GLOBIN GENOTYPE:
Heterozygous for a mutation in Codon 6 (GAG>GTG) which gives rise to Hb S (β^A/β^S).

CONCLUSION
This patient is a sickle cell carrier and also a carrier of alpha zero thalassaemia.

CLINICAL IMPLICATIONS
Heterozygous carriers of the sickle cell mutation are typically asymptomatic.
Heterozygous carriers of the alpha zero thalassaemia deletion mutation are typically asymptomatic, although some may have mild anaemia.

REPRODUCTIVE IMPLICATIONS
The sickle cell mutation can result in sickle cell disease when inherited alongside certain other mutations affecting the beta globin gene.
If the alpha zero thalassaemia deletion mutation is inherited alongside another alpha zero thalassaemia deletion mutation it results in severe alpha thalassaemia and Hb Bart's hydrops fetalis syndrome.
If the alpha zero thalassaemia deletion mutation is inherited alongside an alpha plus thalassaemia deletion it results in Hb H disease. Classical Hb H disease is associated with mild to moderate anaemia and most individuals do not require clinical intervention.

RECOMMENDATIONS
As this lady is currently pregnant, haemoglobinopathy screening should be offered to the baby's biological father and reproductive counselling may need to be provided to the couple. This result also has implications for family members and so haemoglobinopathy testing could be offered to other members of this patient's family.

APPENDIX 2
Exemplary Report for 2302DN2

ALPHA GLOBIN GENOTYPE:
No abnormalities detected ($\alpha\alpha/\alpha\alpha$).

BETA GLOBIN GENOTYPE:
Homozygous beta zero thalassaemia. ($\beta^{IVS1\text{-}1(G>T)}/\beta^{IVS1\text{-}1(G>T)}$)

CONCLUSION
This patient has beta thalassaemia major.

CLINICAL IMPLICATIONS
This phenotype is typically associated with a transfusion-dependent severe anaemia.

REPRODUCTIVE IMPLICATIONS
This beta thalassaemia mutation can result in severe haemoglobin disorders, such as transfusion dependant beta thalassaemia (beta thalassaemia major) or sickle cell disease, if inherited alongside another beta globin gene mutation.

RECOMMENDATIONS
As this is a young child, new to the country, referral to a clinical haematologist is highly recommended. Parental and other family member testing should be offered.

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UKAS PROFICIENCY TESTING
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