Appendix 2. Functional Studies

The following functional studies are approved by the Haemoglobinopathy VCEP. The panel will be constantly assessing available evidence for potential approval of additional assays.

EP Gene	*HBA2*
Name of Assay #1	**Haemoglobin stability test**
Measured Parameter	Hb stability
Readout description	Reduced stability, precipitation in isopropanol or after heating at 50°C.
Readout type	Qualitative
Expected Deleterious Result Range (***PS3_Strength***)	Presence of precipitate, visible to the naked eye.
Expected Deleterious Result Strength (***PS3_Strength***)	PS3_Supporting
Expected Benign Result Range ***(BS3)***	Clear; possibly <5% precipitate at 50 °C/30 min.
Expected Benign Result Strength (***BS3_Strength***)	BS3_Supporting
Notes	False-positive or doubtful results if HbF levels >3%, and by prolonged storage due to methaemoglobin formation. Hyper-unstable Hb variants are rapidly destroyed, hence not readily detected by stability tests. In these cases, no functional evidence should be applied.
References	Dacie and Lewis Practical Haematology, 9th Ed.

Name of Assay #2	**Biosynthesis assay**
Measured Parameter	Globin synthesis
Readout description	Change in biosynthetic ratio of globins (Thalassaemia)
Readout type	Quantitative
Expected Deleterious Result Range (***PS3_Strength***)	α0 thal trait: β/α 1.44 (1.22-1.82) HbH: β/α 2.30 (1.80-2.95)
Expected Deleterious Result Strength (***PS3_Strength***)	PS3_Supporting
Expected Benign Result Range ***(BS3)***	β/α 0.96 (0.78-1.14)

Expected Benign Result Strength (***BS3_Strength***)	BS3_Supporting
Notes	β/α values apply for subjects ≥2 years of age.
References	Old J. et al Prevention of Thalassaemias and Other Haemoglobin Disorders: Vol. 2: Laboratory Protocols, 2nd Ed.

Name of Assay #3	**Haemoglobin electrophoresis, HPLC**
Measured Parameter	Detection and quantification of variant haemoglobins
Readout description	Change in electrophoretic mobilities, or Change in relative peak area and rate of elution (retention time) AND quantification
Readout type	Quantitative
Expected Deleterious Result Range (***PS3_Strength***)	Not concordant with normal chromatogram or readout. Hb X <15%
Expected Deleterious Result Strength (***PS3_Strength***)	PS3_Supporting
Expected Benign Result Range ***(BS3)***	Concordant with normal chromatogram or readout.
Expected Benign Result Strength (***BS3_Strength***)	BS3_Supporting
Notes	Do not apply only for the detection of variant haemoglobins, or for the quantification of normal haemoglobins A, F and A2.
References	Dacie and Lewis Practical Haematology, 9th Ed.

Name of Assay #4	**In vitro splicing assay**
Measured Parameter	Alternative RNA splicing
Readout description	Splicing pattern with i) autoradiograms of radiolabeled minigene constructs, ii) long-read RNA sequencing
Readout type	Quantitative
Expected Deleterious Result Range (***PS3_Strength***)	Abnormal splice product detected (wild-type and aberrant transcripts are present)
Expected Deleterious Result Strength (***PS3_Strength***)	PS3_Supporting
Expected Benign Result Range ***(BS3)***	No abnormal splice product detected (only wild-type RNA transcript is present)

Expected Benign Result Strength (***BS3_Strength***)	BS3_Supporting
Notes	Beware of abnormal transcripts that lead to truncated proteins (PP4) without functional consequences, and cell systems where NMD is not active.
References	PMID: 24549662

Name of Assay #5	In vitro cell-based assay
Measured Parameter	Gene expression (luciferase/fluorescence, RNA, protein)
Readout description	Transfection of erythroid cell cultures (e.g., K562, HEL, HUDEP-2) with constructs bearing i) reporter cassettes with mutated promoter, 5'UTR and enhancer sequences or ii) a cloned mutant human globin gene. Show changes in gene expression using appropriate methods.
Readout type	Qualitative / Quantitative
Expected Deleterious Result Range (***PS3_Strength***)	Changes in the expression level of the reporter gene or transgene in comparison to normal levels.
Expected Deleterious Result Strength (***PS3_Strength***)	PS3_Supporting
Expected Benign Result Range *(**BS3**)*	No changes in the expression level of the reporter gene or transgene in comparison to normal levels.
Expected Benign Result Strength (***BS3_Strength***)	BS3_Supporting
Notes	Also applies to RNA studies with cells from heterozygous or homozygous probands (wild-type, aberrant transcript detection)
References	