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MASSACHUSETTS GENERAL HOSPITAL PATHOLOGY

Tumours of the Central Nervous System (CNS) Molecular Information Reporting Guide

ICCR

Family/Last name

Given name(s)

Date of birth DD – MM – YYYY

Patient identifiers

Date of request DD – MM – YYYY

Accession/Laboratory number

All molecular elements are NON-CORE. DATASET SCOPE **OVERVIEW OF SELECTED MOLECULAR MARKERS** (Note 1)

ADEQUACY OF SPECIMEN FOR MOLECULAR ASSESSMENT (Note 2)

- ◯ Specimen is adequate for analysis
- ◯ Specimen is inadequate for analysis, *give reason, (select all that apply)*
  - ☐ Crush
  - ☐ Autolysis
  - ☐ Cautery
  - ☐ Necrosis
  - ☐ Decalcification
  - ☐ Tumour cell quantity
  - ☐ Fixation issues, *specify*
  - ☐ Other, *specify*

ATRX MUTATION (Note 3)

ATRX mutation

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ Sanger sequencing
- ☐ Next-generation sequencing
- ☐ PCR-based method
- ☐ Other, *specify*

ATRX expression (immunohistochemistry)

- ◯ Intact nuclear expression
- ◯ Loss of nuclear expression
- ◯ Cannot be determined

BRAF ALTERATIONS (Note 4)

BRAF mutation

- ◯ Absent
- ◯ BRAF V600E (c.1799T>A) mutation present
- ◯ Other BRAF mutation present, *specify*
- ◯ Cannot be determined

MUTATIONS ASSESSED (select all that apply)

- ☐ V600E
- ☐ Any mutation in exon 15
- ☐ Other, *specify*

TESTING METHOD (select all that apply)

- ☐ Sanger sequencing
- ☐ Next-generation sequencing
- ☐ PCR-based method
- ☐ Other, *specify*

BRAF V600E expression (immunohistochemistry)

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

BRAF rearrangement/duplication

- ◯ Absent
- ◯ Present, *specify*
- ◯ Cannot be determined

MUTATIONS ASSESSED (select all that apply)

- ☐ 7q34 tandem duplication
- ☐ KIAA-BRAF fusion
- ☐ BRAF-RAF1 fusion
- ☐ Other, *specify*

TESTING METHOD (select all that apply)

- ☐ In situ hybridization (FISH)
- ☐ RT-PCR
- ☐ Array-based method
- ☐ RNA-sequencing
- ☐ Other, *specify*

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CDKN2A/B HOMOZYGOUS DELETION (Note 5)

- ◯ Absent
- ◯ Cannot be determined
- ◯ Homozygous deletion
- ◯ Heterozygous deletion

TESTING METHOD (select all that apply)

- ☐ In situ hybridization (FISH, CISH)
- ☐ Array-based method
- ☐ Next-generation sequencing
- ☐ Other, *specify*

C19MC ALTERATION (Note 6)

- ◯ Absent
- ◯ Cannot be determined
- ◯ Absent with low level gain
- ◯ Present, *specify, including copy number*

TESTING METHOD (select all that apply)

- ☐ In situ hybridization (FISH, CISH)
- ☐ Array-based method
- ☐ Next-generation sequencing
- ☐ Other, *specify*

CHROMOSOMAL ARM 1p/19q CODELETION (Note 7)

- ◯ None detected
- ◯ Cannot be determined
- ◯ 1p/19q codeletion
- ◯ 1p only deletion
- ◯ 19q only deletion
- ◯ Polysomy, *specify*

TESTING METHOD (select all that apply)

- ☐ In situ hybridization (FISH, CISH)
- ☐ Array-based method
- ☐ PCR/Loss of heterozygosity assay
- ☐ Next-generation sequencing
- ☐ Other, *specify*

CHROMOSOME 7 GAIN (combined with chromosome 10 loss) (Note 8)

- ◯ Absent
- ◯ Cannot be determined
- ◯ Present

TESTING METHOD (select all that apply)

- ☐ In situ hybridization
- ☐ Array-based method
- ☐ Next-generation sequencing
- ☐ Other, *specify*

CHROMOSOME 10q23 (*PTEN* LOCUS) DELETION AND *PTEN* MUTATION (Note 9)

Chromosome 10q23 (*PTEN* Locus) deletion

- ◯ None detected
- ◯ Cannot be determined
- ◯ Interstitial deletion present
- ◯ Monosomy, *specify*
- ◯ Polysomy, *specify*

TESTING METHOD (select all that apply)

- ☐ In situ hybridization
- ☐ Array-based method
- ☐ PCR/Loss of heterozygosity assay
- ☐ Other, *specify*

PTEN mutation

- ◯ Absent
- ◯ Cannot be determined
- ◯ Present, *specify*

TESTING METHOD (select all that apply)

- ☐ Sanger sequencing
- ☐ Next-generation sequencing
- ☐ PCR-based method
- ☐ Other, *specify*

EGFR AMPLIFICATION AND EGFRvIII MUTATION (Note 10)

EGFR amplification

- ◯ Absent
- ◯ Cannot be determined
- ◯ Absent with low level gain
- ◯ Present, *specify, including copy number*

TESTING METHOD (select all that apply)

- ☐ In situ hybridization (FISH, CISH)
- ☐ Array-based method
- ☐ Next-generation sequencing
- ☐ Other, *specify*

EGFRvIII mutation

- ◯ Absent
- ◯ Present
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ Next-generation sequencing
- ☐ PCR-based method
- ☐ Immunohistochemistry
- ☐ Other, *specify*

HISTONE H3 MUTATION AND H3 K27 TRIMETHYLATION (me3) (Note 11)

Histone H3 gene family mutation

- ◯ Negative
- ◯ Positive for K27M
- ◯ Positive for G34R or G34V
- ◯ Positive, for other H3 mutation, *specify*
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ Sanger sequencing
- ☐ Next-generation sequencing
- ☐ PCR-based method
- ☐ Other, *specify*

Histone H3 K27M expression (immunohistochemistry)

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

Histone H3 G34R expression (immunohistochemistry)

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

Histone H3 K27me3 expression (immunohistochemistry)

- ◯ Intact expression
- ◯ Loss of expression
- ◯ Cannot be determined

IDH1/IDH2 MUTATION (Note 12)

***IDH1/IDH2* mutation**

- ◯ Absent
- ◯ Present, *specify*
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ Sanger sequencing
- ☐ Next-generation sequencing
- ☐ PCR-based method
- ☐ Other, *specify*

IDH1 R132H expression (immunohistochemistry)

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

Ki-67 IMMUNOHISTOCHEMISTRY (Note 13)

Percentage of positive nuclei ____ %

- ◯ Cannot be determined

L1CAM EXPRESSION (IMMUNOHISTOCHEMISTRY) (Note 14)

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

LIN28A EXPRESSION (IMMUNOHISTOCHEMISTRY) (Note 15)

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

MEDULLOBLASTOMA IMMUNOHISTOCHEMISTRY (Note 16)

ß-catenin expression (immunohistochemistry)

- ◯ Absence of nuclear expression
- ◯ Positive nuclear expression
- ◯ Cannot be determined

GAB1 expression (immunohistochemistry)

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

YAP1 expression (immunohistochemistry)

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

MGMT PROMOTER METHYLATION (Note 17)

- ◯ Absent
- ◯ Present
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ Methylation-specific PCR
- ☐ Other, *specify*

MONOSOMY 6 (Note 18)

- ◯ Absent
- ◯ Present, *specify*
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ In situ hybridization
- ☐ Multiplex ligation-dependend probe amplification (MLPA)
- ☐ Array-based method
- ☐ Microsatellite analysis

MYC GENE FAMILY AMPLIFICATION (*MYC* and/or *MYCN*) (Note 19)

- ◯ Absent
- ◯ Absent with low level gain
- ◯ Present, *specify, including copy number*
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ In situ hybridization (FISH, CISH)
- ☐ Array-based method
- ☐ Next-generation sequencing
- ☐ Other, *specify*

***NAB2-STAT6* FUSION** (Notes 20 & 25)

***NAB2-STAT6* fusion**

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ FISH
- ☐ Next generation sequencing
- ☐ Other, *specify*

STAT6 expression (immunohistochemistry)

- ◯ Absence of nuclear expression
- ◯ Positive nuclear expression
- ◯ Cannot be determined

PITUITARY HORMONES AND TRANSCRIPTION FACTORS IMMUNOHISTOCHEMISTRY (Note 21)

Tumour cells are reactive for (select all that apply)

- ☐ Prolactin
- ☐ Human growth hormone
- ☐ ß-TSH
- ☐ ß-FSH
- ☐ ß-LH
- ☐ Alpha subunit
- ☐ ACTH
- ☐ PIT1
- ☐ TPIT
- ☐ SF1
- ☐ Other, *specify*
- ◯ Cannot be determined

***RELA* FUSION** (Note 22)

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ FISH
- ☐ Next generation sequencing
- ☐ Other, *specify*

***SMARCA4/BRG1* ALTERATION** (Note 23)

***SMARCA4/BRG1* mutation**

- ◯ Absent
- ◯ Present, *specify*
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ Sanger sequencing
- ☐ Next-generation sequencing
- ☐ PCR-based method
- ☐ Other, *specify*

BRG1 loss of expression (immunohistochemistry)

- ◯ Intact nuclear expression
- ◯ Loss of nuclear expression
- ◯ Cannot be determined

***SMARCB1/INI1/HSNF5* ALTERATION** (Note 24)

***SMARCB1/INI1/HSNF5* mutation**

- ◯ Absent
- ◯ Present, *specify*
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ Sanger sequencing
- ☐ Next-generation sequencing
- ☐ PCR-based method
- ☐ Other, *specify*

INI1 (BAF47) loss of expression (immunohistochemistry)

- ◯ Intact nuclear expression
- ◯ Loss of nuclear expression
- ◯ Cannot be determined

***TERT* PROMOTER MUTATION** (Note 26)

- ◯ Absent
- ◯ Hotspot mutation (C228T or C250T)
- ◯ Other mutation, *specify*
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ Sanger sequencing
- ☐ Next-generation sequencing
- ☐ PCR-based method
- ☐ Other, *specify*

***TP53* MUTATION** (Note 27)

***TP53* mutation**

- ◯ Absent
- ◯ Cannot be determined
- ◯ Present, *specify*

EXONS ANALYSED

- ◯ Exons 5-8
- ◯ All exons
- ◯ Other, *specify*

TESTING METHOD (select all that apply)

- ☐ Sanger sequencing
- ☐ Next-generation sequencing
- ☐ PCR-based method
- ☐ Other, *specify*

p53 expression (immunohistochemistry)

- ◯ Negative or rare, lightly positive cells
- ◯ Intermediate (intermediate numbers of predominantly lightly positive cells)
- ◯ Positive (diffuse and strong nuclear positivity)
- ◯ Cannot be determined

***YAP1* FUSION** (Note 28)

- ◯ Negative
- ◯ Positive
- ◯ Cannot be determined

TESTING METHOD (select all that apply)

- ☐ FISH
- ☐ Next generation sequencing
- ☐ Other, *specify*

OTHER FINDINGS (Note 29)

Other immunohistochemical findings, *specify*

Other molecular findings, *specify test, testing method and findings*

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Scope

This dataset has been developed for the molecular assessment of central nervous system tumour samples (whether that molecular assessment is nucleic acid or protein-based). This dataset is not needed for those tumours in which molecular information is not captured for diagnostic purposes, but this dataset applies to a growing subset of Central Nervous System (CNS) tumours and it is anticipated that its use will increase over time.

It is intended that this dataset should be used in conjunction with the ”Histological assessment of CNS specimens” and the “Final integrated report/diagnosis for CNS specimens” datasets. A complete diagnosis of a CNS tumour should conform to the final integrated diagnoses in the 2016 World Health Organization (WHO) Classification of Tumours of the CNS[1], which requires integration of elements from histological and ancillary analyses.

Note 1 - Overview of selected molecular diagnostic markers for CNS tumours

The table below summarizes selected molecular diagnostic markers for CNS tumours; the list of tests is not exhaustive and other assays may be helpful in some diagnostic circumstances. In addition, the tests listed are those related to ruling in the corresponding diagnoses; however, it should be realized that the assays may also be used in particular diagnostic situations to rule out other diagnoses. An example of this would be ATRX immunohistochemistry, which is commonly used to support a diagnosis of IDH-mutant diffuse astrocytoma, but which is also used to evaluate a possible diagnosis of oligodendroglioma, IDH-mutant and 1p/19q-codeleted. Some specific tests recommended in the commentaries below represent one of several validated and equivalent approaches to the evaluation of the described molecular variable; for those tests that have multiple testing modalities (e.g., sequencing and immunohistochemistry for BRAF V600E), it is assumed that only one of these testing modalities would be used per case unless one test yields equivocal results (e.g., a result of weak immunohistochemical positivity versus nonspecific background staining should be followed by gene sequencing). For some tests, relevance may be related to the age of the patient (e.g., EGFR gene amplification in adult high-grade gliomas rather than paediatric ones) and *the reader is referred to the commentaries under each molecular parameter for further information.*

Summary of tests by tumour type

Note: this is a summary and the reader is referred to the specific notes for details on use of each test.

W = component of the 2016 CNS WHO diagnostic criteria and 2017 WHO diagnostic criteria for pituitary adenomas

D = commonly used to support or refine the diagnosis, or provide important ancillary information in the corresponding tumour type

D* = commonly used to rule out the diagnosis; see commentary for details

(D) = can be used to support or refine the diagnosis, or provide important ancillary information in specific tumour subtype(s); see commentary for details

DA = diffuse astrocytoma; AA = anaplastic astrocytoma; O = oligodendroglioma; AO = anaplastic oligodendroglioma; GBM = glioblastoma; PXA = pleomorphic xanthoastrocytoma; GG = ganglioglioma; AT/RT = atypical teratoid / rhabdoid tumour; ETMR = embryonal tumour with multilayered rosettes; SFT/HPC = solitary fibrous tumour / haemangiopericytoma; MPNST = malignant peripheral nerve sheath tumour

Test	**Gliomas**								**Embryonal tumours**			**Other**					
	DA, AA	**O, AO**	**Diffuse midline glioma**	**GBM**	**Pilocytic astrocytoma**	**PXA, GG**	**Ependymoma - supratentorial**	**Ependymoma – posterior fossa**	**Medulloblastoma**	**AT/RT**	**ETMR**	**Extraventricular neurocytoma**	**Meningioma**	**SFT/HPC**	**Craniopharyngioma**	**MPNST**	**Pituitary tumours**
***ATRX* mutation**																	
ATRX mutation	D			D													
ATRX loss of expression (immunohistochemistry)	D			D													
***BRAF* alterations**																	
BRAF mutation	(D)			(D)	D	D									D		
BRAF V600E expression (immunohistochemistry)	(D)			(D)	D	D									D		
BRAF rearrangement/duplication					D												
***CDKN2A/B* homozygous deletion**	(D)					(D)											
C19MC alteration											W						
Chromosomal arm 1p/19q codeletion		W															

Test	Gliomas								Embryonal tumours			Other					
	DA, AA	O, AO	Diffuse midline glioma	GBM	Pilocytic astrocytoma	PXA, GG	Ependymoma - supratentorial	Ependymoma – posterior fossa	Medulloblastoma	AT/RT	ETMR	Extraventricular neurocytoma	Meningioma	SFT/HPC	Craniopharyngioma	MPNST	Pituitary tumours
Chromosome 7 gain combined with chromosome 10 loss (see below)				D													
Chromosome 10q23 (PTEN locus) deletion and *PTEN* mutation																	
Chromosome 10q23 *(PTEN* locus) deletion or monosomy 10				D													
PTEN mutation				D													
***EGFR* amplification and EGFRvIII mutation**																	
EGFR amplification				D													
EGFRvIII mutation				D													
Histone H3 mutation and H3 K27 trimethylation (me3)																	
Histone H3 K27M mutation (sequencing) and expression (immunohistochemistry)	(D)		W	D													
Histone H3 G34 mutation (sequencing) and expression (immunohistochemistry)	(D)			D													
Histone H3 K27me3 expression (immunohistochemistry)			D					D								D	
***IDH1/IDH2* mutation**																	
IDH1/IDH2 mutation	W	W	D*	W	D*	D*						D*					
IDH1 R132H expression (immunohistochemistry)	W	W	D*	W	D*	D*						D*					
Ki-67 immunohistochemistry		D											D				D
L1CAM expression (immunohistochemistry)							D										
LIN28A expression (immunohistochemistry)											D						
Medulloblastoma immunohistochemistry																	
β-catenin nuclear expression (immunohistochemistry)									D						D		
GAB1 expression (immunohistochemistry)									D								

Test	Gliomas								Embryonal tumours			Other					
	DA, AA	O, AO	Diffuse midline glioma	GBM	Pilocytic astrocytoma	PXA, GG	Ependymoma - supratentorial	Ependymoma – posterior fossa	Medulloblastoma	AT/RT	ETMR	Extraventricular neurocytoma	Meningioma	SFT/HPC	Craniopharyngioma	MPNST	Pituitary tumours
YAP1 expression (immunohistochemistry)									D								
***MGMT* promoter methylation**				D													
Monosomy 6									D								
MYC gene family amplification																	
MYC amplification									D								
MYCN amplification									D								
***NAB2-STAT6* fusion**																	
NAB2-STAT6 fusion														D			
STAT6 nuclear expression (immunohistochemistry)														D			
Pituitary hormones and transcription factors immunohistochemistry																	W
***RELA* fusion**							W										
***SMARCA4/BRG1* alteration**																	
SMARCA4/BRG1 mutation									D	W							
BRG1 loss of expression (immunohistochemistry)									D	W							
***SMARCB1/INI1/HNSF5* alteration**																	
SMARCB1/INI1/HNSF5 mutation									D	W							
INI1 (BAF47) loss of expression (immunohistochemistry)									D*	W							
***TERT* promoter mutation**		D		D													
***TP53* mutation**																	
TP53 mutation	D								W								
p53 expression (immunohistochemistry)	D								W								
***YAP1* fusion**							D										

Note 2 - Adequacy of specimen for molecular assessment (Non-core)

Reason/Evidentiary Support

The 2016 CNS WHO uses histology and molecular parameters to define many tumour entities.[1] Procuring viable and adequate tumour tissue allows appropriate histological and molecular assessment. However, the requirements for an adequate specimen for molecular assessment are not always the same as those for histological assessment. For example, ischemic times are critical for the quality of nucleic acid in general; the sooner samples can be frozen or fixed, the better. If immediate freezing or immediate appropriate fixation is not possible, placement in refrigerator may reduce the degradation of nucleic acid.[2] Crush or freezing artefacts may affect adequacy for immunohistochemical or FISH testing, but do not often affect adequacy for molecular assays. Samples embedded in OCT compound for cryostat sectioning can be a good source, and an advantage of using such samples is that one can evaluate tumour cell quantity as well as quality by checking histological sections of each sample. Formalin-fixed, paraffin-embedded (FFPE) tissue samples also often provide a valuable source of information for molecular assessment.[3] FFPE samples, however, can sometimes be more difficult for molecular biology assays because of the fixation issues (such as overfixation and decalcification) that often cause nucleic acid degradation, resulting in fragmented DNA and RNA transcripts. Nonetheless, many laboratories have optimised molecular assays for FFPE tissue, given its commonplace nature. Histological examination of tissue specimens used for nucleic acid extraction and subsequent molecular testing is mandatory to assure that vital tumour tissue with sufficient neoplastic cell content is being analysed. In certain cases, microdissection of cellular tumour areas may be required to ensure sensitivity of molecular analysis.

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Note 3 - *ATRX* mutation (Non-core)

Reason/Evidentiary Support

In the setting of a diffuse glioma with an IDH mutation, the diagnosis of an IDH-mutant astrocytoma (including diffuse astrocytoma, anaplastic astrocytoma, and glioblastoma) is supported by the presence of a *TP53* mutation or alteration (mutation or deletion) of the α-thalassemia/mental retardation syndrome X-linked gene (*ATRX*; chromosome Xq21.1).[4-6] Evaluation for these two markers is also commonly used to rule out the possibility of an oligodendroglioma.

Among IDH-mutant tumours, inactivating mutations of *ATRX* appear restricted to those carrying *TP53* mutations and this combination is almost mutually exclusive with codeletion of 1p/19q.[5,7-9] Nearly all diffuse gliomas with IDH and *ATRX* mutations are associated with the alternative lengthening of telomeres (ALT) phenotype. Less commonly, *ATRX* mutations co-occur with H3.3 mutations in paediatric high-grade gliomas, most often in those with G34R/V-mutations.[10]

Documentation of *ATRX* loss/mutations can be achieved in a number of ways, with a practical and cost-effective manner being immunohistochemistry. The loss of nuclear ATRX immunostaining in neoplastic cells, with its maintained expression in non-neoplastic cells, such as endothelial cells or normal glia, is strongly associated with *ATRX* deletion or mutation and can be reliably used as a surrogate of genetic alteration.[9,11,12] Mosaic staining patterns have also been reported, but these are not always associated with ATRX mutation.[13] In combination with immunohistochemistry for IDH1 R132H and p53, ATRX immunohistochemistry provides definitive results in the majority of cases, with the added benefit of preserving cytoarchitecture for microscopic examination.[4,11]

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Note 4 - *BRAF* alterations (Non-core)

Reason/Evidentiary Support

BRAF Mutation

The *BRAF* V600E mutation in exon 15, which is the most common *BRAF* alteration, affects a large variety of CNS tumours. It has been reported in 96% of papillary craniopharyngiomas[14], 65-75% of pleomorphic xanthoastrocytomas (PXA) with and without anaplasia[15], 25-60% of gangliogliomas, 20–25% of dysembryoplastic neuroepithelial tumours (DNET), and 7% of pilocytic astrocytomas (PA), especially those in supratentorial locations.[14,15,16,17] *BRAF* mutation has been also detected in about one-half of epithelioid glioblastomas and, in up to 25% of diffuse astrocytic gliomas in children and young adults.[18] The detection of a *BRAF* mutation has diagnostic implications in specific tumours such as PXA, ganglioglioma, DNT, or epithelioid glioblastoma. Moreover, the detection of the mutation can help to distinguish a ganglioglioma from the cortical infiltration of a diffuse glioma. Besides its diagnostic value, *BRAF* mutation has therapeutic implications as targeted therapies against mutated BRAF V600 protein have been recently developed, including in settings such as *BRAF*-mutant craniopharyngioma.[19] In paediatric low-grade gliomas, *BRAF* V600E mutation has been linked to poor response to conventional cytotoxic therapy and poor prognosis.[20] In routine settings, BRAF V600E can be identified by IHC (see below) or by molecular approaches such as Sanger sequencing, high-resolution melting analysis, pyrosequencing, allele-specific quantitative polymerase chain reaction (ASQ-PCR), and next-generation sequencing (NGS).[21] Although Sanger sequencing is a well-established tool to detect *BRAF* V600E and other rarer *BRAF* mutations, it has a detection threshold of 20% (of mutated alleles). This high threshold reduces the relevance of this technique in samples that contain a minority of mutated cells. Molecular methods with much lower thresholds, such as ASQ-PCR, digital PCR, or NGS, are more sensitive although precise cut-offs for mutant allele frequency have not been defined.

BRAF V600E Expression (Immunohistochemistry)[22]

Immunohistochemistry is a commonly used method to detect the BRAF V600E protein in FFPE tissue in CNS tumours.[23,24] Two monoclonal antibodies (clone VE1 and clone V600E) against BRAF V600E are commercially available. Clone VE1 is the most widely used and is sensitive and specific.[25] The concordance between immunohistochemistry and detection of *BRAF* V600E mutation by molecular genetic techniques demonstrates variability between studies in different types of neoplasms, but the overall concordance is strong.[25] Immunohistochemistry plays a key role when FFPE material available is not sufficient for molecular genetic analysis and when low tumour cell content may lead to false-negative results. The presence of nonspecific staining is a potential pitfall, which could lead to false-positive results, and light staining can lead to false-negative interpretations.

BRAF Rearrangement/Duplication

Circumscribed duplication of the *BRAF* locus is a common copy number variation that occurs in PAs of the cerebellum, hypothalamus, or optic chiasm, but may occur in PAs from other sites as well. Chromosome 7q34 gain has been characterised as a *BRAF* duplication with a tandem insertion in the *KIAA1549* gene.[26] Fusion genes containing *BRAF* variants activate the MAPK signalling pathway, which appears to be the key signalling pathway in the development of PA. The major alterations leading to constitutive activation of MAPK in PAs are gene fusions and point mutations involving *BRAF*. Fusions between *KIAA1549* and *BRAF* are the most frequent genetic change in PAs (>70 %) and occur in almost all anatomical locations, although most frequently in the cerebellum and less frequently at other sites. The most common fusion is between *KIAA1549*-exon 16 and exon 9 of *BRAF*, followed by 15-9, and 16-11. Much rarer fusions involving *BRAF* or *RAF1* have also been found. Identification of the *KIAA1549-BRAF* fusions has been used as a diagnostic marker for PAs. It has been observed in pilomyxoid astrocytoma, ganglioglioma and in the recently described diffuse leptomeningeal glioneuronal tumour (DLGNT).[27] [28] *KIAA1549-BRAF* fusions, while all coding for a fusion protein that includes the activating *BRAF* kinase domain, can be derived from at least nine different fusion site combinations. This makes reverse transcriptase polymerase chain reaction (RT-PCR) a difficult method to identify or exclude all variants of the

fusion gene. Fluorescence in situ hybridisation (FISH) analysis, which demonstrates the tandem duplication at 7q34, is an indirect way to indicate the presence of a *KIAA1549-BRAF* fusion. However, *BRAF* copy number gains due to trisomy 7 or whole 7q gains are common in diffusely infiltrating astrocytomas including glioblastomas, and should not be mistaken as circumscribed *BRAF* duplication or *BRAF* fusion. A method that may identify all types of *BRAF* and *RAF1* fusion variants in a single experiment is RNA sequencing by NGS.

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Note 5 - *CDKN2A/B* homozygous deletion (Non-core)

Reason/Evidentiary Support

Homozygous deletion of the *CDKN2A/B* genes on the short arm of chromosome 9 is associated with higher-grade diffuse gliomas and has been suggested as a marker for assessing likely behaviour (and grading) of IDH-mutant diffuse astrocytic tumours, with those harbouring homozygous *CDKN2A/B* deletions following more aggressive courses.[29] On the other hand, *CDKN2A/B* deletions have been shown to be a characteristic genetic feature in pleomorphic xanthoastrocytomas, occurring in up to 87% of cases in one series; in this situation, along with *BRAF* V600E mutation, the *CDKN2A/B* deletions do not connote more aggressive behaviour.[30] In neuropathological practice, FISH or high-resolution cytogenetic techniques (e.g., array-CGH, SNP arrays, methylation arrays) can be used to detect homozygous *CDKN2A/B* deletions.

The *CDKN2A* gene encodes the p16 protein, which can be detected using immunohistochemistry. However, whether loss of p16 nuclear staining has similar prognostic information to homozygous *CDKN2A/B* deletion remains to be determined and, at the present time, p16 immunohistochemistry cannot be recommended as a substitute for assessing homozygous *CDKN2A/B* deletion.[29]

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Note 6 - C19MC alteration[31-37] (Non-core)

Reason/Evidentiary Support

Demonstration of C19MC alteration is required for the diagnosis of embryonal tumour with multilayered rosettes (ETMR), C19MC-altered. This alteration consists of C19MC amplification or fusion, typically a focal high-level amplicon of chromosome 19q13.42 covering a large, poorly characterised microRNA cluster (hence C19MC) and the miR-371-373 locus, which map about 100 kb apart. The width and the level of gains at this locus, as assessed by array-CGH, is variable but always encompasses the same miRNA cluster. Even in the absence of multilayered rosettes, a CNS embryonal tumour with C19MC-alteration is diagnosed as ETMR, C19MC-altered. In routine neuropathological practice, FISH or chromogenic in situ hybridisation (CISH), or high-resolution cytogenetic techniques (e.g. array-CGH, SNP arrays, methylation arrays) can be used to detect amplification of the C19MC region. ETMRs lacking C19MC alterations and those that are not tested for this alteration or in which the test results are inconclusive are designated as ETMR, NOS (not otherwise specified), or with a medulloepithelioma phenotype as medulloepithelioma. LIN28A immunohistochemistry (see **Note 15 - LIN28A expression (immunohistochemistry)**) has also been used in the diagnosis of ETMR.

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Note 7 - Chromosomal arm 1p/19q codeletion (Non-core)

Reason/Evidentiary Support

This cytogenetic alteration refers to whole-arm codeletion of chromosome arms 1p and 19q that together with IDH mutation constitutes the diagnostic molecular criteria for *oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade II*, as well as *anaplastic oligodendroglioma, IDH -mutant and 1p/19q-codeleted, WHO grade III*.[1] The whole-arm codeletion in oligodendroglial tumours is caused by an unbalanced t(1;19)(q10;p10) translocation.[38,39] Of note, only whole-arm 1p/19q codeletion combined with IDH mutation is the diagnostically relevant marker; partial deletions on either chromosome arm may be found in other types of diffuse gliomas, including IDH-wildtype glioblastomas, and are neither diagnostic for IDH-mutant and 1p/19q-codeleted oligodendroglial tumours[1] nor associated with favourable patient outcome.[40] Moreover, detection of 1p/19q codeletion in the absence of IDH mutation is suspicious of partial deletions, and by definition is not sufficient for a diagnosis of an IDH-mutant and 1p/19q-codeleted oligodendroglial tumour.

Various techniques are being used for the diagnostic assessment of 1p/19q codeletion. Commonly used methods include microsatellite analysis for loss of heterozygosity (LOH), FISH or CISH, and multiplex ligation-dependent probe amplification (MLPA). FISH/CISH can be applied on routine FFPE sections. However, analysis is often restricted to single loci on each chromosome arm, which may not reliably distinguish whole-arm losses from partial deletions. There is no standardized cut-off for determination of codeletion by FISH/CISH, with each laboratory needing to validate its assay. In addition, polysomies of chromosomes 1 or 19 may complicate diagnostic assessment and have been associated with less favourable outcome.[41-43] LOH analysis and MLPA assess multiple loci along each chromosome arm and thereby reduce the risk of false-positive findings due to partial deletions. However, extraction of tumour DNA (for MLPA) as well as tumour and leukocyte DNA (for LOH analysis) is required for these techniques. Microarray-based approaches may also be used for diagnostic purposes, including DNA methylation bead arrays that allow for simultaneous detection of 1p/19q codeletion, *MGMT* promoter methylation, and G-CIMP status indicative of IDH mutation.[44] Most recently, panel-based NGS approaches have been used for 1p/19q detection and simultaneous mutational analyses of *IDH1* and *IDH2*, a well as other alterations commonly associated with 1p/19q codeletion, such as *TERT* promoter mutation and *CIC* mutation.[45,46] Immunostaining for the proneural α-internexin protein[47,48] or NOGO-A[49] cannot substitute as a surrogate marker for 1p/19q codeletion.

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Note 8 - Chromosome 7 gain combined with chromosome 10 loss (Non-core)

Reason/Evidentiary Support

Partial or complete chromosome 7 gain and 10 loss are often found in glioblastoma, particularly glioblastoma, IDH-wildtype, but chromosome 7 or 7q gain can also be found in other glial brain tumours. In one study,[9] 81/136 glioblastomas and 123/136 glioblastomas had partial alterations or combined complete 7 gain and 10 loss, respectively. In contrast, no chromosome 7 gains or 10 losses were identified in only 11/136 glioblastomas. Chromosome 7 gain may be trisomy, tetrasomy or even higher polysomy. It is unclear whether extent of polysomy/ degree of gain impacts prognosis. Gain of chromosome 7 is more frequent than *EGFR* amplification in glioblastoma, IDH-wildtype.

Recent studies have advocated for testing of these markers as part of prognostic stratification.[50,51] The most likely significance of these changes, given their association with glioblastomas, is in the setting of an IDH-wildtype diffuse astrocytoma or anaplastic astrocytoma, in which 7 or 7q gain/10 or 10q loss may be associated with a course and outcome paralleling that of glioblastoma, IDH-wildtype, WHO grade IV. Of note, some subtypes of glioblastoma, such as gliosarcoma and giant cell glioblastoma, tend to have considerably less frequent *EGFR* amplification (5-6%) than IDH-wildtype glioblastoma, but still may show gains of chromosome 7 and losses of chromosome 10.

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Note 9 - Chromosome 10q23 (PTEN Locus) deletion and PTEN mutation (Non-core)

Reason/Evidentiary Support

Chromosome band 10q23 (*PTEN* Locus) Deletion

Hemizygous deletions affecting the *PTEN* gene locus at band 10q23 are detectable in the vast majority of glioblastomas, IDH-wildtype and IDH-mutant, due to monosomy 10 or deletion of 10q.[6,52] Losses of chromosome 10 or chromosome arm 10q have also been reported in smaller fractions of WHO grade II and III diffuse gliomas.[5,6,51] However, when detected in an IDH-wildtype astrocytic glioma of WHO grade II or III, monosomy 10 or 10q23 deletion may indicate a glioblastoma, IDH-wildtype, in particular when associated with gain of chromosome 7 and other glioblastoma-associated genetic alterations, like *EGFR* amplification and *TERT* promoter mutation.[5,51,53] Homozygous *PTEN* deletion is less common than hemizygous deletion, and mostly restricted to a small fraction of IDH-wildtype glioblastomas.[52] Detection of 10q23 (*PTEN* locus) deletion is commonly accomplished by FISH or CISH on routine FFPE tissue sections. Other diagnostically useful methods include MLPA, microarray-based DNA copy number profiling, and NGS-based analyses.

PTEN Mutation

Mutations in the *PTEN* tumour suppressor gene at 10q23 are found in approximately 30% of glioblastomas, IDH-wildtype.[52] *PTEN* mutation in IDH-wildtype glioblastomas is usually accompanied by loss of the second allele due to monosomy 10 or deletion of 10q. Mutations are distributed across the entire gene with the highest frequency of mutations seen in exons 5 and 6, which encode the catalytic domain of the PTEN protein.[54] Therefore, diagnostic investigation for *PTEN* mutations requires sequencing of all exons including the flanking intronic regions for detection of splice site mutations. NGS-based approaches represent the most convenient way to detect *PTEN* mutations, while Sanger sequencing is also possible but more laborious.[45,46,55] Immunohistochemical demonstration of loss of PTEN protein expression does not correlate well with *PTEN* mutation or *PTEN* promoter methylation in glioblastomas, and thus cannot serve as a surrogate marker.[56]

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Note 10 - *EGFR* amplification and EGFRvIII mutation[57] (Non-core)

Reason/Evidentiary Support

The epidermal growth factor receptor (*EGFR*) gene at 7p12 is the most commonly amplified proto-oncogene in gliomas.[58] *EGFR* amplification is detectable in approximately 40% of IDH-wildtype glioblastomas, WHO grade IV, and is particularly common in tumours from adult patients with the classic or receptor tyrosine kinase (RTK) type 2 molecular subtype of glioblastoma.[52,59] *EGFR* amplification is commonly associated with point mutations and other genetic rearrangements, the most common of which, EGFRvIII, being detectable in about 50% of *EGFR*-amplified glioblastomas.[60,61] EGFRvIII is caused by an 801-bp in-frame deletion of exons 2 to 7 that results in a constitutively active protein lacking major parts of the extracellular receptor domain including the ligand binding site.[61] Moreover, EGFRvIII carries a unique peptide encoded by the fusion site of exons 1 and 8 that has served as a tumour-specific epitope for anti-EGFRvIII immunotherapy.[62] As *EGFR* amplification and positivity for EGFRvIII are virtually restricted to glioblastoma, IDH-wildtype, their diagnostic detection in an IDH-wildtype diffuse astrocytic glioma may support a glioblastoma diagnosis even in the absence of characteristic histological features like microvascular proliferation and/or necrosis. Detection of *EGFR* amplification or EGFRvIII positivity also may be clinically relevant as a predictive marker of response to molecularly-guided therapies targeting *EGFR* and/or EGFRvIII.[63,64]

EGFR amplification is usually seen in the majority of neoplastic cells in a given tumour and can be readily detected by FISH or CISH on routine FFPE tissue sections, although amplification levels may be heterogeneous from cell to cell. Targeted molecular techniques based on extracted tumour DNA, such as quantitative real-time PCR and MLPA, are also suitable for diagnostic detection of *EGFR* amplification. More recently, microarray-based genomic or epigenetic analyses as well as NGS approaches are increasingly being used.[65] Gene amplification (defined by a circumscribed high-level copy number gain of the *EGFR* gene at 7p12) needs to be distinguished from low-level copy number gains of chromosome 7 caused by numerical chromosomal abnormalities, in particular trisomy 7, which are not restricted to IDH-wildtype glioblastoma but also common in diffuse and anaplastic astrocytomas[6] (see also **Note 8 Chromosome 7 Gain**). To date, there is no evidence that different levels of *EGFR* gene amplification (e.g., increases in copy number of 10-fold versus 100-fold) have distinct diagnostic or prognostic impact.

Detection of EGFRvIII in *EGFR*-amplified glioblastomas also can be performed at the DNA level, e.g., by MLPA, microarray-based techniques and NGS. However, detection at the mRNA or protein level using RT-PCR or immunohistochemistry with EGFRvIII-specific antibodies appears to be more sensitive.[60] This is due to the fact that EGFRvIII positivity usually shows regional heterogeneity and sometimes affects only a minor subset of the tumour cells.[60] Thus, representative sampling of tumour tissue is an important issue to avoid false-negative testing for EGFRvIII. Unfortunately, precise cut-off values for distinction between high- and low-level copy number gains have not been defined and may need to be adjusted for each testing method.

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Note 11 - Histone H3 mutations and H3 K27 trimethylation (me3) (Non-core)

Reason/Evidentiary Support

Any standard sequencing method can be used to detect the H3 K27M mutation, including pyrosequencing, Taq Man PCR, droplet-digital PCR, Sanger sequencing, and NGS. A similar array of sequencing methods can be used for H3 G34 mutations, however due to the GC rich nature of this region, targeted methods can be more difficult to set up. For detection of both mutations using targeted methods (and alignment of non-targeted methods), consideration needs to be given to the high degree of homology among the H3 genes (human H3 variants include H3.3, H3.1, H3.2, CENP-A, H3t, H3.X and H3.Y) and the number of genes encoding each protein (H3.3 is encoded by two genes, *H3F3A* and *H3F3B*, while H3.1 and H3.2 are each encoded by multiple genes found within gene clusters). The exact gene being tested and the method used should be provided in the report.

Histone H3 K27M Mutation (Sequencing) and Expression (Immunohistochemistry)

Recurrent mutations in *H3F3A* (H3.3) and *HIST1H3B/C/I* (H3.1) with lysine 27 substituted for methionine (H3 K27M) are characteristic of paediatric high-grade astrocytomas with a predilection for a midline location; less commonly, these mutations are found in adult midline diffuse gliomas.[10,66,67] These tumours have a poor prognosis. The H3.3 K27M mutation is found in approximately 70% of diffuse intrinsic pontine gliomas and H3.1 K27M in a further 15%. Furthermore, in the paediatric age group, H3.3 K27M is also found in approximately 50% of high-grade diffuse gliomas involving the thalamus and spinal cord. H3 K27M mutations also occur in a broader range of patient ages, morphologies, and locations; the median age to date is the third decade for spinal cord and thalamic tumours with patients as old as 65 years being reported with the alteration. Other locations include third ventricle, hypothalamus, pineal region and cerebellum.[68] H3 K27M mutation can also be found in diffuse astrocytomas without classic high-grade features that generally behave more aggressively than their wild type counterparts. In occasional cases, the mutation has been found in other tumour types, including gangliglioma,[69] pilocytic astrocytoma[70] and ependymoma.[71] Testing for this alteration should be considered, at a minimum, in all midline diffuse gliomas in patients under the age of 30. These alterations can be identified by sequencing or a mutation-specific antibody. Detection of the mutation by either immunohistochemistry or

sequencing is required for the diagnosis of *Diffuse midline glioma, H3 K27M mutant*. Lack of H3 K27-me3 is not a specific marker of H3 K27M status.

Immunohistochemistry with an antibody against the N-terminus of the mutant protein is highly sensitive and specific for detection of the H3K27M protein from either H3.3 or H3.1.[72,73] In practice, the antibody can produce a fair amount of background cytoplasmic staining in non-tumour cells and only diffuse strong nuclear staining in most (or all) tumour cells should be considered positive. Further, poorly fixed tissue or tissue from post-mortem or older blocks may be false negative. If equivocal, a sequencing-based method (see below) should be considered as the standard of care.

Histone H3 G34 Mutations (Sequencing) and Expression (Immunohistochemistry)

Recurrent mutations in *H3F3A* (H3.3) with glycine 34 substituted for arginine (H3 G34R) or infrequently valine (H3 G34V) are found most commonly in hemispheric high-grade gliomas of the adolescent and young adult population (median age 15 years; range 9-51 years).[74] The H3G34R mutation is found in ~15-20% of hemispheric high-grade glioma cases in the pediatric age group.[75] Outcome is slightly better than in H3K27M-mutant tumours in a midline location, with a median survival of approximately 18 months. Testing for this alteration should be considered, at a minimum, in hemispheric, IDH-wildtype, high-grade gliomas in patients under the age of 30, particularly if ATRX is lost and p53 is diffusely immunopositive. These alterations can be identified by sequencing or a mutation-specific (H3 G34R) antibody.

Immunohistochemistry with an antibody against the mutant protein is specific for detection of the H3G34R protein.[76] In practice, the antibody works well on FFPE tissue with specific nuclear staining but does not stain every tumour cell; as a result, sensitivity may prove to be an issue as more experience is gained with the antibody. If immunohistochemical results are equivocal or if suspicion for mutation is high, a sequencing-based method should be considered as the standard of care.

Histone H3 K27me3 Expression (Immunohistochemistry)

The presence of the H3 K27M mutant protein is associated with a fairly widespread (and thus detectable on Western blot or immunohistochemistry) loss of the repressive trimethyl (me3) mark on lysine 27 (K27me3). Tumour cells harbouring the H3 K27M mutation (either H3.1 or H3.3 K27M) will typically show loss of nuclear expression of this protein on immunohistochemistry with retention of staining in entrapped non-neoplastic cells, e.g., endothelial cells (similar to the pattern seen with ATRX or INI1). However, it should be noted, that while loss of H3K27me3 is sensitive for detection of H3 K27M mutant tumours, it is not specific. Other tumours, notably some posterior fossa ependymomas,[77] will also show loss of H3 K27me3; in ependymomas this lack of immunoreactivity aligns with the posterior fossa group A (PFA) tumours.[77,78] Similarly, in some H3-wildtype cases, partial loss may be seen. Thus, while helpful for confirmation when combined with an H3 K27M stain, loss of H3 K27me3 staining by itself should be considered a non-specific surrogate marker for identifying H3 K27M-mutant diffuse midline gliomas.

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Note 12 - *IDH1/IDH2* mutation (Non-core)

Reason/Evidentiary Support

IDH1/IDH2 Mutation and IDH1 R132H Expression (Immunohistochemistry)

Isocitrate dehydrogenase (IDH) is an enzyme that exists in five isoforms, each of which catalyses the reaction of isocitrate to α-ketoglutarate.[79] Mutations in *IDH1/IDH2* are frequent (greater than 80%) in WHO grades II and III astrocytomas but are found in only about 10% of the glioblastomas. Most glioblastomas that have progressed from lower-grade astrocytomas ('secondary glioblastomas) are IDH-mutant tumours.[4] The finding of IDH

mutations in an infiltrating astrocytoma is associated with better prognosis, grade for grade. The 2016 CNS WHO divides diffuse astrocytoma, anaplastic astrocytoma, and glioblastoma into classes that are IDH-mutant and IDH-wildtype. Oligodendrogliomas are now defined as diffuse gliomas with *IDH1/IDH2* mutations and whole arm deletions of chromosomes 1p and 19q. The mutant forms of *IDH1* and *IDH2* lead to the production of the oncometabolite 2-hydroxyglutarate, which inhibits the function of numerous α-ketoglutarate–dependent enzymes.[80] Inhibition of the family of histone demethylases and the ten-eleven translocation (TET) family of 5-methylcytosine hydroxylases has profound effects on the epigenetic status of mutated cells and leads directly to a hypermethylator phenotype that has been referred to as the glioma CpG island methylator phenotype (G-CIMP).[81]

IDH1 and *IDH2* mutations target the enzyme's active site and result in a substitution for a key arginine at codons R132 and R172, respectively.[4,82,83] The most frequent mutation, representing 92.7%, occurs at codon 132 of the IDH1 gene, and results in the substitution of arginine for histidine (R132H).[82] Less frequent *IDH1* mutations include R132C (4.2%), R132S (1.5%), R132G (1.4%), and R132L (0.2%).[82] Residue R172 in exon 4 of the *IDH2* gene is homologous to R132 in the *IDH1* gene, with R172K representing 64.5% of all *IDH2* mutations followed by R172M (19.3%), and R172W (16.2%).[82] *IDH2* mutations are much less frequent than *IDH1* mutations among diffuse gliomas (approximately 3%), but are slightly more common in oligodendrogliomas than astrocytomas.[82]

A monoclonal antibody has been developed to the mutant IDH1 R132H protein, allowing its use in FFPE specimens (mIDH1 R132H).[84] The ability of the antibody to detect a small number of cells as mutant makes this method more sensitive than sequencing for identifying R132H-mutant gliomas.[85,86] However, mutations in *IDH2* and other *IDH1* mutations will not be detected using immunohistochemistry with this antibody, and in the proper clinical setting, it may be necessary to test for other *IDH1* or *IDH2* mutations by sequencing analysis. It has been suggested that sequencing may not be warranted in the setting of a negative R132H immunostain in glioblastomas arising in patients older than 55 years due to the rarity of non-R132H *IDH1* and *IDH2* mutations in patients in this age group.[87,88] On the other hand, all diffusely infiltrating gliomas of WHO grade II and III that lack IDH1 R132H positivity by immunohistochemistry should be assessed for less common *IDH1* or *IDH2* mutations by sequencing or other appropriate methods.

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Note 13 - Ki-67 immunohistochemistry[1,89-91] (Non-core)

Reason/Evidentiary Support

The protein detected by the Ki-67 antibody is a marker of cell proliferation that is present in the nucleus during all active phases of the cell cycle (G_1, S, G_2, M), but absent in resting cells (G_0). In general, there is a progressive increase in Ki-67 labelling index associated with more aggressive behaviour of CNS tumours. Ki-67 immunohistochemistry can be useful for assessment of malignancy grade, especially when only small biopsies are available and for selection of areas for counting mitoses in large specimens. Moreover, Ki-67 labelling indices have been used to predict behaviour in lower-grade tumours such as WHO grade I meningiomas, pituitary adenomas, and WHO grade II oligodendrogliomas, among others—but are not universally used for these purposes.

Because of methodological variation, however, unequivocal Ki-67 labelling index cut-off levels for assigning WHO grade to CNS tumours are not available. For example, assessment of precise cut-off levels is difficult because of gradual increase in nuclear content of Ki-67 protein (marked increase in especially S phase of the cell cycle), staining of proliferating non-neoplastic cells in a tumour, considerable regional variation of the labelling index within a tumour, and substantial variability in staining results between institutions. In many centres, the MIB-1 antibody is used to determine the Ki-67 labelling index, one of its primary advantages over the original Ki-67 antibody being that it can be used on sections of FFPE tissue.

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Note 14 - L1CAM expression (immunohistochemistry)[92,93] (Non-core)

Reason/Evidentiary Support

Strong and diffuse cytoplasmic L1CAM (L1 Cell Adhesion Molecule) immunostaining of tumour cells is a sensitive surrogate marker for *RELA* fusion–positive ependymomas (see **Note 22 *RELA* fusion**); these tumours are the majority of paediatric ependymomas in the supratentorial compartment, generally present in children, and carry a *C11orf95-RELA* fusion. However, L1CAM immunopositivity is not a specific marker as it can also be expressed by other types of tumours. Nonetheless, L1CAM immunohistochemistry is recommended for indicating that a supratentorial ependymoma likely belongs to the *RELA* fusion–positive category when *RELA* fusion testing is not possible or yields equivocal results.

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Note 15 - LIN28A expression (immunohistochemistry)[33,37,94-96] (Non-core)

Reason/Evidentiary Support

Strong LIN28A cytoplasmic immunostaining of tumour cells is a highly sensitive marker for embryonal tumours with multilayered rosettes (ETMR), C19MC-altered (see **Note 6 C19MC alteration**). However, LIN28A immunostaining is not specific to these tumours as it can also be present in medulloepitheliomas lacking the C19MC alteration, as well as in some gliomas, atypical teratoid/rhabdoid tumours (AT/RT), germ cell tumours, and non-CNS neoplasms. LIN28A immunohistochemistry is recommended as a surrogate marker for ETMR, C19MC-altered when testing for C19MC -alteration is not available. In these tumours, LIN28A immunoreactivity is generally prominent in multilayered rosettes, in poorly differentiated small-cell areas, and in the papillary and tubular structures of the medulloepithelioma pattern. Nonetheless, molecular testing for C19MC status is required for the diagnosis of ETMR, C19MC-altered. Therefore, although LIN28 immunopositivity is a useful surrogate marker for recognition of ETMR, C19MC-altered, when no C19MC testing is done or the results of such testing are inconclusive, an ETMR should be diagnosed as ETMR, NOS.

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Note 16 - Medulloblastoma immunohistochemistry (Non-core)

Reason/Evidentiary Support

In the 2016 CNS WHO classification, medulloblastomas can be placed into one of four diagnostic molecular groups: WNT-activated, SHH-activated and *TP53*-wildtype, SHH-activated and *TP53*-mutant, and non-WNT/non-SHH (the latter encompassing group 3 and group 4 medulloblastoma as provisional diagnostic entities). These molecular groups are characterised by distinct clinical, pathological, and genetic attributes, and their use in integrated diagnoses alongside the histopathological variants of medulloblastoma provides information of prognostic and predictive utility. The groups of medulloblastomas were established by consensus from data in studies that had delineated molecular groups by gene expression profiling.[97] This approach remains the gold standard by which a medulloblastoma is assigned to a molecular group, but DNA methylation profiling is a reliable alternative.[98]

Some approaches that can be effectively applied to FFPE tissue use a restricted list of biomarkers to approximate molecular groups.[99,100] Included among these are immunohistochemical methods targeting surrogate markers of molecular groups, including nuclear β-catenin expression (WNT-activated), GAB1 (SHH-activated), YAP1 (WNT-activated or SHH-activated), and p53 (SHH, *TP53*-mutant), discussed in greater detail below.[101,102] While these immunohistochemical methods are relatively straightforward to develop in clinical

histopathology laboratories, they may be challenging to interpret when only small subsets of tumour cells are immunopositive. Additionally, sequencing techniques (including NGS) can be utilized to identify signature mutations associated with distinct molecular groups, some of which provide additional predictive information for targeted therapies (e.g., within the SHH family). Furthermore (see also **Note 18 Monosomy 6** and **Note 19 MYC gene family amplification**), detection of copy number alterations can further aid in molecular subtyping (e.g., monosomy 6 for WNT-activated tumours and isodicentric 17q for groups 3 or 4).

β-catenin Nuclear Expression (Immunohistochemistry)

Upon WNT activation, β-catenin, encoded by the *CTNNB1* gene, translocates to the nucleus, where it interacts with transcription factors. Thus, nuclear β-catenin immunopositivity reflects activation of the WNT signalling pathway.

In the clinically relevant WNT-activated group of medulloblastoma, immunohistochemistry for β-catenin reveals reactivity in tumour cell nuclei, although immunostaining is often patchy or focal. Scattered single β-catenin nucleopositive cells should not be interpreted as definitive evidence of WNT activation and requires further analysis to WNT status (see next section).

Immunohistochemistry with antibodies to β-catenin, GAB1, and YAP1 in the determination of medulloblastoma molecular groups

While medulloblastoma molecular groups have been defined on the basis of gene expression and DNA methylation profiling,[103] one immunohistochemical method uses antibodies to β-catenin, GAB1, and YAP1 to place a medulloblastoma into one of three groups: WNT, SHH, and 'non-WNT, non-SHH'.[101,104] This immunohistochemical approach is designed for medulloblastomas and should not be applied to other types of tumours. All three antibodies should be used in the determination of molecular group, providing increased confidence in the result when tissue is limited or processing is suboptimal. In addition, while the combination of β-catenin, GAB1, and YAP1 is a single, broadly implemented approach, different laboratories may use variations on this combination; for example, some centres substitute filamin-A for YAP1 and some use OXTC2 and ant-p75 NGR when GAB cannot be optimized.[105]

Nuclear immunoreactivity for β-catenin signifies WNT pathway activation (Table 1), and WNT-activated medulloblastomas often demonstrate this in most cells, although in some preparations nuclear immunoreactivity may be patchy. As mentioned above, scattered single β-catenin nucleopositive cells should not be interpreted as definitive evidence of WNT activation. In difficult cases with equivocal β-catenin immunoreactivity or a low proportion of nucleopositive cells, widespread immunoreactivity for YAP1 and an immunonegative GAB1 preparation (Table 1) help to classify a medulloblastoma as WNT-activated. In addition, confirmation of WNT status should be sought using molecular analysis to demonstrate monosomy 6 (see **Note 18 - Monosomy 6**) or a *CTNNB1* mutation. SHH and 'non-WNT, non-SHH' medulloblastomas demonstrate immunoreactivity for β-catenin in the cytoplasm, but not the nucleus, of tumour cells. Cytoplasmic GAB1 immunoreactivity is a surrogate marker for SHH medulloblastomas, but is often weak or absent in nodular regions of tumours classified as desmoplastic/nodular or medulloblastoma with extensive nodularity (MBEN). WNT and SHH medulloblastomas show nuclear and cytoplasmic immunoreactivity for YAP1, but YAP1 is immunonegative in 'non-WNT, non-SHH' tumours. YAP1 expression can also be attenuated in nodular regions of desmoplastic/nodular and MBEN variants.

Table 1

MB molecular groups – immunohistochemical markers (see text)			
Antibodies to:	**WNT**	**SHH**	**non-WNT/non-SHH**
β-catenin	cytoplasmic & nuclear	cytoplasmic	cytoplasmic
GAB1	negative	positive	negative
YAP1	positive	positive	negative

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Note 17 - *MGMT* promoter methylation (Non-core)

Reason/Evidentiary Support

O6 methylguanine-DNA methyl transferase (MGMT) is a DNA repair protein that facilitates repair of DNA damage induced by chemotherapeutic alkylating agents, and has therefore been associated with chemoresistance.[106] Epigenetic silencing of the *MGMT* gene by promoter methylation plays an important role in regulating MGMT expression in gliomas.[52,107,108] *MGMT* promoter methylation has been reported as a predictive marker for temozolomide sensitivity in clinical trials.[108-110] Promoter methylation correlates with better progression-free and overall survival in IDH-wildtype glioblastoma patients treated with temozolomide. In IDH-mutant anaplastic (WHO grade III) gliomas, MGMT status is a prognostic factor irrespective of treatment but is not predictive for outcome to alkylating chemotherapy versus radiotherapy.[111,112] The impact of *MGMT* promoter methylation on clinical care is still being established.

The optimal method to carry out MGMT analysis and interpretation of the results has yet to be determined. Pyrosequencing is a commonly used method[113-115] that has proved to be reproducible between different laboratories.[113-116] Methylation-specific PCR is semi-quantitative and has also been widely used including in pivotal clinical trials,[108,109] but may not be as reproducible as pyrosequencing.[115]

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Note 18 - Monosomy 6[117,118] (Non-core)

Reason/Evidentiary Support

Monosomy 6 is a chromosomal alteration present in approximately 85% of WNT-activated medulloblastomas. Its detection, together with the presence of β-catenin nuclear immunoreactivity and/or *CTNNB1* mutation, facilitates identification of this prognostically favourable molecular group.

Monosomy 6 can be detected by array CGH or microsatellite analysis using fresh-frozen material. MLPA (with probes covering the short and long arms of chromosome 6) can be a robust method to analyze even small amounts of FFPE-derived degraded DNA. Detection of monosomy 6 can also be undertaken by interphase FISH.

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Note 19 - *MYC* gene family amplification (Non-core)

Reason/Evidentiary Support

The c-Myc protein (MYC) has a fundamental role in cell proliferation, cell size, differentiation, stem cell self-renewal, and apoptosis. Its deregulation occurs in many cancers including a range of brain tumours. The MYC transcription factor family also includes its paralogues MYCN and MYCL.[119] *MYC*, *MYCN,* and *MYCL* amplifications are prognostically relevant in medulloblastomas.[120] *MYC* and *MYCN* gene amplification and fusions are seen in the SHH group, and non-WNT/non-SHH, but almost never in WNT-activated medulloblastomas.[120,121]

A commonly used laboratory method to detect MYC gene family amplifications is *in situ* hybridisation, either using FISH or CISH.[122] Other approaches include PCR-based methods such as real-time PCR, NGS, MLPA, or array technologies.[123,124,125]

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Note 20 - *NAB2-STAT6* fusion (Non-core)

Reason/Evidentiary Support

In-frame *NAB2-STAT6* gene fusions result from chromosome 12q13 inversions and represent highly sensitive and specific signature alterations of meningeal solitary fibrous tumour/haemangiopericytoma (SFT/HPC) of grade 1, 2, or 3; these fusions are also characteristic of the analogous soft tissue/extracranial counterparts, which are referred to as SFT or malignant SFT. Given the relative ease of detecting this genetic alteration using a STAT6 immunohistochemical surrogate (see **Note 25 - STAT6 expression (immunohistochemistry)**), diagnostic confirmation is highly recommended in the WHO 2016 classification scheme before a diagnosis of SFT/HPC is rendered.[1,87]

<u>*NAB2-STAT6* Gene Fusion</u>

NAB2-STAT6 gene fusions are detectable using RT-PCR or various other sequencing techniques, including NGS if designed appropriately.[126,127] Over 40 fusion variants have been detected to date, with the most common meningeal SFT/HPC subtypes fusing exon 6 of *NAB2* with exons 16, 17, or 18 of *STAT6* (roughly one-half of all cases).[127] Preliminary data also suggests that the *NAB2* exon 4-*STAT6* exon 2/3 fusions are more common in the lower grade and clinically less aggressive SFT/HPC, though larger studies are needed for further validation.[127,128]

STAT6 Nuclear Expression (Immunohistochemistry)

The STAT6 protein is normally expressed in the cytoplasm of cells, whereas NAB2 is expressed in nuclei; however, the *NAB2-STAT6* fusions cause the STAT6 protein to translocate to the nucleus. As such, STAT6 immunohistochemistry represents a highly reliable and practical surrogate for detecting this signature alteration, with nearly 100% sensitivity and specificity regardless of the fusion variant.[126,129] Nearly all meningeal SFT/HPC and extracranial SFTs display strong and extensive/diffuse nuclear positivity, whereas other diagnostic considerations, such as meningiomas, nerve sheath tumours, and various sarcomas, either lack expression or show only cytoplasmic staining. As such, the pathologist is cautioned against rendering a diagnosis of SFT/HPC in the absence of nuclear STAT6 immunoreactivity.

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Note 21 - Pituitary hormones and transcription factors immunohistochemistry[130] (Non-core)

Reason/Evidentiary Support

Standard immunohistochemical evaluation of pituitary adenomas includes specific anterior pituitary hormones (prolactin, growth hormone, follicle stimulating hormone, luteinizing hormone, thyroid stimulating hormone, adrenocorticotrophic hormone (PRL, GH, FSH, LH, TSH, ACTH, respectively) and/or pituitary transcription factors (PIT1, TPIT, steroidogenic factor 1/SF1).[131] Immunohistochemistry for these proteins, coupled with keratin (AE1/AE3 or CAM5.2) staining, for presence or absence of rounded cytoplasmic inclusions known as fibrous bodies, allows classification of adenomas for prognosis and medical treatment purposes. Antibodies directed against the pituitary transcription factor for corticotroph lineage adenoma (TPIT) are not as widely available as the other antibodies listed above.

For diagnostic purposes, some advocate first screening with three antibodies (PIT1, SF1, and ACTH) and then using the other anterior pituitary hormone assays based on initial results.[132] Others utilise the full panel initially and may variably supplement the panel with additional reticulin histochemical stain and/or a cell cycle labelling marker (MIB1). There appears to be little, if any, utility for p53 immunohistochemistry.

The new WHO 2017 Classification system[1] notes that: “Special adenoma subtypes that commonly show aggressive behaviour…include sparsely granulated somatotroph adenoma, lactotroph adenomas in men, Crooke cell adenoma and silent corticotroph adenoma, and plurihormonal PIT1-positive adenoma (previously called "silent subtype 3 adenoma").

For tumours of the posterior pituitary gland (granular cell tumour of the sellar region, pituicytoma, spindle cell oncocytoma), nuclear staining for the transcription factor TTF-1 is diagnostic.[1]

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Note 22 - *RELA* fusion (Non-core)

Reason/Evidentiary Support

Approximately two-thirds of supratentorial ependymomas in children are characterised by fusions between *C11orf95* and the *RELA* genes.[92,133] Detection of these fusions is essential for making the diagnosis of ependymoma, *RELA* fusion positive. These fusions can be identified clinically using RNA sequencing, RT-PCR based techniques, or FISH; whole genome sequencing can also detect the fusion. Targeted RNA sequencing and RT-PCR design should take into consideration the complex nature of the fusion events generated by

chromothripsis on chromosome 11. FISH probes overlying either *RELA* or *C11orf95* may be used to detect the rearrangements on chromosome 11.[92] These are designed using a break-apart strategy with red and green probes lying close to one another and producing a yellow signal in the wildtype situation; rearrangements will result in distancing of the probes from one another and distinct red and green signals. There are correlations between the presence of L1CAM positivity and RELA fusion in this type of this tumour (see **Note 14 L1CAM expression (immunohistochemistry)**). There may also be other surrogate markers for *RELA* fusion–positive tumours and therefore other validated equivalents can be used to guide diagnosis; however, to date none of these is specific for *RELA* fusion as defined by FISH or sequencing.

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Note 23 - *SMARCA4/BRG1* alteration (Non-core)

Reason/Evidentiary Support

AT/RT is defined as a CNS embryonal tumour that frequently (but not invariably) contains rhabdoid cells and demonstrates inactivation of *SMARCB1* (INI1) or *SMARCA4* (BRG1). AT/RTs with *SMARCA4* loss are extremely rare, but loss of BRG1 expression (and retention of INI1 expression) in these tumours can be readily demonstrated by immunohistochemistry.[134] Associated genetic alterations of *SMARCA4*, whether copy number alterations or mutations, can be detected by a variety of array or sequencing methods.

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Note 24 - *SMARCB1/INI1/HSNF5* alteration (Non-core)

Reason/Evidentiary Support

Inactivation of the *SMARCB1* (*INI1*, *BAF47*, *SNF5*) gene is present in almost all cases of AT/RT, resulting in nuclear loss of SMARCB1 protein which can be evaluated immunohistochemically. Genetic aberrations of the *SMARCB1* locus may include homozygous or heterozygous deletions and a variety of coding sequence mutations, leading to inactivation of both alleles. However, genetic testing is usually not required for making the diagnosis of AT/RT because immunohistochemistry is highly sensitive. SMARCB1 is a constitutively expressed protein, and therefore immunohistochemical staining for SMARCB1 is present in nuclei of non-neoplastic cells, such as vascular cells, residual brain cells, or inflammatory infiltrates, serving as internal positive control for neoplasms that have lost tumour cell staining. Some AT/RTs with nuclear loss of SMARCB1 exhibit cytoplasmic staining, possibly representing dysfunctional truncated protein. In tumours with histological features of AT/RTs but without demonstration of SMARCB1 inactivation (and without SMARCA4 inactivation), only a diagnosis of "CNS embryonal tumour with rhabdoid features" can be made.

A variety of other tumour types that may involve the nervous system exhibit loss of nuclear SMARCB1, including cribriform neuroepithelial tumour,[135] poorly differentiated chordoma,[136] rhabdoid tumour of the sellar region,[137] myxoid meningeal tumours,[138] and sinonasal carcinoma[135,136,137,138,139] The molecular and nosologic relationship of these tumours to AT/RT is unclear to date. Furthermore, complete or incomplete (reduced, mosaic) loss of SMARCB1 protein has been found in some cases of choroid plexus carcinoma, synovial sarcoma, epithelioid schwannoma, and schwannoma associated with schwannomatosis.[140]

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Note 25 - STAT6 expression (immunohistochemistry)

STAT6 staining is a highly reliable and practical surrogate for detecting NAB2-STAT6 fusion in (meningeal) SFT/HPC; see **Note 20 NAB2-STAT6 fusion**.

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Note 26 - *TERT* promoter mutation (Non-core)

Reason/Evidentiary Support

The *TERT* gene encodes telomerase reverse transcriptase, which is a major component of the protein complex telomerase and contributes to maintain telomere length. *TERT* promoter mutations create new binding sites for ETS transcription factors and subsequently increase expression and activity of telomerase. *TERT* promoter mutations occur in 55–80% of glioblastomas (far more commonly in IDH-wildtype glioblastomas), 70–80% of oligodendrogliomas, and 10–35% of diffuse astrocytomas.[141,142] They provide independent prognostic information for diffuse gliomas. Thus, in oligodendroglioma, IDH-mutant and 1p/19q-codeleted, *TERT*-mutant tumours are associated with better prognosis than *TERT*-wildtype tumours, while in diffuse astrocytoma, IDH-wildtype, *TERT*-mutant tumours are associated with worse prognosis than *TERT*-wildtype tumours.[143-145] About 20% of medulloblastomas carry *TERT* promoter mutations, and they are more common in adult patients and in the SHH-activated molecular type.[141] In meningiomas, *TERT* promoter mutations have been found in 6% of tumours where they represent a marker of poor prognosis independent of WHO grading.[146] About 50% of solitary fibrous tumours/hemangiopericytomas carry *TERT* promoter mutation while other tumours of the CNS only uncommonly exhibit these mutations.[141]

Two hotspot missense mutations (abbreviated as C228T and C250T) represent the vast majority of *TERT* promoter mutations. Other mutations have been rarely detected in brain tumours, such as C228A and C249T in gliomas.[141] Mutations can be detected by Sanger sequencing or by NGS.

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Note 27 - *TP53* mutation (Non-core)

Reason/Evidentiary Support

Mutations of the *TP53* gene, which encodes the p53 protein, are found in approximately two-thirds of all diffuse astrocytic gliomas[147] and in over 80% of IDH-mutant diffuse astrocytic gliomas.[5] *TP53* mutations are less common in IDH-wildtype glioblastomas (23-28%), and are notably uncommon in oligodendrogliomas, showing a strong inverse relationship with 1p/19q codeletion. *TP53* mutations are thus used as diagnostic markers for diffuse astrocytic gliomas, and have been used to distinguish low-cellularity diffuse astrocytic gliomas from reactive gliosis.[148] Evaluation of *TP53* mutation may also be used to rule out the possibility of oligodendroglial tumours among IDH-mutant gliomas. Furthermore, *TP53* mutations are important for subclassifying medulloblastomas with SHH pathway activation, dividing them into high-risk *TP53*-mutant cases in older children versus lower-risk *TP53*-wildtype cases in young children and adults. *TP53* mutations are common in some other types of brain tumours, but are not used diagnostically as in the above situations.

Different DNA sequencing techniques may be used for detecting *TP53* mutations. Screening can be accomplished via sequencing of all exons or just exons 5 through 8, where most mutations occur; the great majority of mutations are missense.

p53 Expression (Immunohistochemistry)

Immunohistochemistry is a useful screening tool, given that most missense *TP53* mutations result in increased p53 protein half-life that produces strong immunoreactivity in the majority of tumour cell nuclei (rather than scattered positivity and/or light nuclear staining). Strong p53 positivity in >10% of the tumour cell nuclei has been found to have a sensitivity of 77.4-78.8% and a specificity of 78.6-96.7% when compared to sequencing.[149,150] Positive nuclear p53 staining correlates well with missense mutations with a sensitivity of 92% and a specificity of 79.4%, whereas only 33% of tumours with truncating mutations show p53 positivity,[150] with such mutations typically leading to negative staining.[151]

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Note 28 - *YAP1* fusion (Non-core)

Reason/Evidentiary Support

Classifying ependymomas by molecular genetic alterations is beginning to find clinical utility. Currently, the *RELA* fusion-positive ependymoma is listed in the WHO classification, but any update would be expected to include other genetically defined entities, on the basis of recent studies describing the clinicopathological attributes of the varied molecular groups of ependymomas.[133,152] *RELA* fusions are found only in supratentorial ependymomas, not those in the posterior fossa or spinal compartments, and they are present in the majority of paediatric ependymomas at this site.[92] Among supratentorial ependymomas without a *RELA* fusion are those with a *YAP1* fusion, but these are rare and mostly restricted to young children.[133]

A *YAP1* fusion can be detected by a variety of methods, although an immunohistochemical approach is currently not available. Transcriptome sequencing can detect *YAP1* fused to several gene partners, such as *MAMLD1.*[133] This approach has some utility with derivatives from FFPE tissue, but methods using RT-PCR or interphase FISH are alternatives.[133]

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Note 29 - Other findings (Non-core)

Reason/Evidentiary Support

These sections should be used for documenting findings for other genetic alterations and/or for other tumour types, such as metastases and haematological lesions.

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