**SUPPLEMENTARY INFORMATION**

***@ 3.5 Single/limited target analysis***

***Multiplex ligation-dependent probe amplification (MLPA)*** is a semi-quantitative technique to detect DNA copy number variations such as hemi- and homozygous deletions, duplications and gene amplifications, in one or more genes. It uses a set of predefined nucleotide probes that hybridize to their corresponding target sequences. Hybridized probes are subsequently ligated to each other, followed by PCR amplification of ligated probes and separation of the amplified DNA fragments by capillary electrophoresis. Copy numbers for the respective target genes is then compared to those in a normal diploid reference sample. MLPA works well on DNA extracted from frozen or FFPE specimens. The main advantage is the simultaneous detection of several copy number variations, such as 1p/19q codeletion, +7 /-10, *CDKN2A/B* homozygou*s* deletion and *EGFR* amplification*,* in a relatively simple and rapid workflow, and the use of multiple probes make this assay more robust and accurate than FISH [1-3]. Detection of the *EGFR*vIII rearrangement in *EGFR-*amplified IDH-wildtype glioblastoma is also possible but less sensitive than RT-PCR or immunohistochemistry because *EGFR*vIII can be present in only a subpopulation of *EGFR*-amplified cells [4, 5]. Using mutation-specific probes, MLPA can also detect point mutations, such as hotspot mutations in *IDH1, IDH2, H3-3A*, *BRAF* or the *TERT* promoter. A few studies published over 10 years ago suggested methylation specific MLPA (MS-MLPA) as a useful assay for assessment of the *MGMT* promoter methylation status [6, 7]. However, in the kit that was used at that time, 2 of the 3 analyzed CpGs were located outside DRM1 and 2, and a good dataset on the performance of an updated MS-MLPA assay (including information on the CpGs that are interrogated) is currently lacking.

***Reverse transcription PCR (RT-PCR)*** assays are used in clinical diagnostics for detection of RNA transcripts, for example those of *KIAA1549::BRAF* fusions [8]. It is also a sensitive method for detection of intragenic rearrangements such as the EGFRvIII deletion variant [4].

***Quantitative PCR (qPCR)*** assays are used for the assessment of DNA copy number variations. qPCR can make use of commercial copy number assays (e.g., TaqManTM assays) or of gene-specific PCR primers with intercalating fluorescent dyes to quantify the amplified PCR products. qPCR-based assays usually provide information on relative DNA copy numbers of the target gene in relation to one or more reference genes with presumably normal DNA copy number. This can cause problems in tumors with grossly rearranged (aneuploid) genomes due to lack of balanced reference loci. At the mRNA level, quantitative RT-PCR assays are commonly used to assess changes in gene expression by measuring target mRNA expression levels in relation to house-keeping genes such as ß-actin and others [9-11]. This approach might also serve to validate RNA sequencing outputs. At present, qRT-PCR analysis has little relevance in the molecular diagnostics of CNS tumors.

***Methylation-specific PCR (MSP)*:** See section on *MGMT* promoter methylation analysis.

***Microsatellite analysis for detection of loss of heterozygosity (LOH).*** Microsatellites are highly polymorphic DNA loci composed of repeated sequences of 2-10 nucleotides, with the number of repeats often varying between the maternal and paternal alleles of an individual. Thus, PCR-based amplification of microsatellite loci from selected chromosomal regions can detect LOH at these regions in tumor cells. Microsatellite-based LOH analysis has been and is still used for the assessment of chromosomal losses in CNS tumors, in particular 1p/19q codeletion in oligodendrogliomas. Typically, five or more microsatellite loci are analyzed on both 1p and 19q, to ensure detection of informative loci across each chromosomal arm and reducing the risk of false positive findings due to partial deletions. Disadvantages of this method are that ideally non-tumor DNA is available (e.g., extracted from a blood or buccal swab sample) as the constitutional reference, and the limited sensitivity in samples with low tumor cell percentage [12].

@ **3.6 *MGMT* promoter analysis**

Assays designed to predict the *MGMT* methylation status interrogate 2 to 9 CpGs that are typically located in DMR2. The methylation of CpGs within and between DMR1 and DMR2 is highly correlated; therefore, the interrogation of different sets of CpGs yields similar results with few discrepancies. For the discrimination of methylated versus non-methylated CpGs, most assays use bisulfite conversion-based technologies, such as methylation–specific PCR (MSP), with the exception of restriction-based technologies such as methylation‐specific multiplex ligation‐dependent probe amplification (MS‐MLPA). Bisulfite chemically transforms unmethylated cytosines into uracil that in the amplification step by PCR are translated to thymidine, while methylated cytosines are not transformed. This step is followed by a method that allows discrimination of sequence differences.

Differences in the location of the CpGs interrogated by the various assays can be appreciated in Figure 2. The MSP methods commonly target CpG sites 76 to 80 and 84 to 87 that correspond to the original MSP assay by Esteller et al. [13]. PSQ variants commonly target CpGs 74 to 78, but may range from CpG 72 to 95 [14]. The most commonly used technologies comprise qMSP-based assays and PSQ. The result of the BeadChip based assay (MGMT-STP27) is included in the report issued by the methylation-based classifier of CNS tumors [15], and comparisons with other assays have become available [16-18]. The limitations of the publicly available data do not allow conclusion of “the most” prognostic CpG set. The pros and cons of the different technologies have been reviewed in detail elsewhere [19, 20] [14, 21]. Guidance on optimal *MGMT* testing is challenging due to a lack of robust comparative studies and lack of evaluation of cut-offs (+/- grey zones) in clinical trials [14]. Immunohistochemistry is not recommended [22].

**@ 3.7 Immunohistochemistry as an alternative approach**

**IDH1 p.R132H**

The anti-IDH1 p.R132H antibody specifically detects the mutant protein corresponding to the canonical *IDH1* R132H mutation; detection of less common (non-canonical) *IDH1* and *IDH2* mutations is typically performed by DNA sequencing, although mutation-specific antibodies for certain of these mutations are also available. The anti-IDH1 R132H antibody can even detect single cell infiltration in e.g., the periphery of a tumor or in post-treatment lesions and thus can exceed the sensitivity of other assays.

**H3 p.K28M (H3 K27M*)***

The mutation specific antibody H3 p.K28M detects the *K27M* mutation in DMGs which may be present in different genes encoding the three different histone H3 variants. Over 70% of these mutations are encoded by *H3-3A*, one of the two genes encoding the histone variant H3.3, less commonly mutations are located in *H3C2* or *H3C3* encoding H3.1, and rarely in *H314* encoding H3.2. This antibody does not detect the *H3 K28I* (K27I) mutation, which is rarely present in DMGs, H3K27-altered, and it cannot discriminate in which particular H3 encoding gene the mutation is present.

**H3 p.G35R/V (H3 G34R/V)**

Diffuse hemispheric glioma, H3 G34-mutant is defined by missense somatic mutations of the histone *H3-3A* gene, resulting either in a p.G35R (frequency 94%) or a p.G35V protein. For both types of mutant protein, specific antibodies are available [23]. However, as false negative immunoreactivity has been described, absence of the mutation should be confirmed in especially high-grade, diffuse, hemispheric gliomas in younger patients lacking nuclear OLIG2 and ATRX expression upon immunohistochemistry.

**BRAF p.V600E**

The *BRAF V600E* mutation is present in various low- and high-grade gliomas and glioneuronal tumors. Immunohistochemistry for BRAF p.V600E mutant protein is often relatively weak and then more equivocal compared to staining for IDH1 p.R132H, H3 p.K27M and H3 p.G34R. Also, expression of BRAF p.V600E protein can be limited to parts of the tumor. For example, in gangliogliomas it can be limited to either the ganglion cells or may be only focally present in glial tumour parts. A strong widespread expression is rare and the presence of a mutation should ideally be confirmed by sequencing.

Loss of nuclear ATRX and H3 p.K28me3 protein, and p53 overexpression per se are not specific for a particular tumor type but provide diagnostic and prognostic information in the context of some glioma types.

**ATRX**

In IDH-mutant gliomas, loss of nuclear ATRX expression is diagnostic for astrocytoma, IDH-mutant, whereas preserved nuclear ATRX positivity should prompt the analysis for 1p/19q codeletion. ATRX loss is also a characteristic finding in diffuse hemispheric gliomas, H3 G34-mutant, and can be present in diffuse midline glioma, H3-altered and high-grade astrocytoma with piloid features. Furthermore, it can be lost in high-grade gliomas in neurofibromatosis type 1 (NF1) patients [24].

**p53**

Strong and widespread (>50% of the tumor cell nuclei) p53 protein expression serves as a surrogate marker for presence of a *TP53* mutation. However, in a small subset of *TP53* mutated tumors nuclear p53 staining is completely absent [25]. Strong and widespread p53 staining is present in the majority of astrocytomas, IDH-mutant and is usually absent in oligodendrogliomas, IDH-mutant and 1p/19q-codeleted. Relatively high p53 expression in tumor cell nuclei is present in approximately 30% of glioblastomas, IDH-wildtype and is also relatively frequent in high-grade gliomas in NF1 patients [24].

**H3 p.K28me3**

Loss of nuclear H3 p.K28me3 staining is an essential diagnostic criterion for DMG, H3-altered (including the subtypes DMG, H3.3 p.K28-mutant; DMG, H3.1 or H3.2 p.K28-mutant; DMG, H3-wildtype with EZHIP overexpression; and DMG, *EGFR*-mutant). Furthermore, reduced H3 p.K28me3 staining is a diagnostic finding in posterior fossa group A (PFA) ependymomas, and has also been reported in oligodendrogliomas, IDH-mutant and 1p/19q-codeleted, particularly in *IDH1 R132H*-mutant tumors [26-28].

**S-methyl-5'-thioadenosine phosphorylase**

Recent data suggests that immunohistochemistry for S-methyl-5'-thioadenosine phosphorylase (MTAP) may be a suitable surrogate for *CDKN2A/B* status [29]. There is not yet sufficient evidence to suggest this as a reliable surrogate marker.

***Supplementary Figure 1***

***Workflow for determining need for Lab-developed test validation under the in-vitro diagnostics regulation***

Ein Bild, das Diagramm enthält.

Automatisch generierte Beschreibung

Diagnostic assays fall under the in-vitro diagnostics regulation of the EU and thus, diagnostic labs have to review their procedures for compliance. This workflow supports determining if a given test requires the validation as a lab-developed test (LDT) and states the respective requirements.

***Supplementary Table 1***

***European Federation of Neurological Societies (EFNS) Evidence classification scheme for a diagnostic measure ([30])***

**Class I**: A prospective study in a broad spectrum of persons with the suspected condition, using a ‘gold standard’ for case deﬁnition, where the test is applied in a blinded evaluation, and enabling the assessment of appropriate tests of diagnostic accuracy. Amended in the present guideline to also apply for common practices that are required by national guidelines and/or other certification/accreditation bodies.

**Class II**: A prospective study of a narrow spectrum of persons with the suspected condition, or a well-designed retrospective study of a broad spectrum of persons with an established condition (by ‘gold standard’) compared to a broad spectrum of controls, where test is applied in a blinded evaluation, and enabling the assessment of appropriate tests of diagnostic accuracy

**Class III**: Evidence provided by a retrospective study where either persons with the established condition or controls are of a narrow spectrum, and where test is applied in a blinded evaluation

**Class IV**: Any design where test is not applied in blinded evaluation OR evidence provided by expert opinion alone or in descriptive case series (without controls) Rating of recommendations

**Level A** rating (established as useful/predictive or not useful/predictive) requires at least one convincing class I study or at least two consistent, convincing class II studies

**Level B** rating (established as probably useful/predictive or not useful/predictive) requires at least one convincing class II study or overwhelming class III evidence

**Level C** rating (established as possibly useful/predictive or not useful/predictive) requires at least two convincing class III studies

1. Schouten, J.P., et al., *Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification.* Nucleic Acids Res, 2002. **30**(12): p. e57.

2. Natte, R., et al., *Multiplex ligation-dependent probe amplification for the detection of 1p and 19q chromosomal loss in oligodendroglial tumors.* Brain Pathol, 2005. **15**(3): p. 192-7.

3. Jeuken, J., et al., *Multiplex ligation-dependent probe amplification: a diagnostic tool for simultaneous identification of different genetic markers in glial tumors.* J Mol Diagn, 2006. **8**(4): p. 433-43.

4. Weller, M., et al., *Assessment and prognostic significance of the epidermal growth factor receptor vIII mutation in glioblastoma patients treated with concurrent and adjuvant temozolomide radiochemotherapy.* Int J Cancer, 2014. **134**(10): p. 2437-47.

5. Jeuken, J., et al., *Robust detection of EGFR copy number changes and EGFR variant III: technical aspects and relevance for glioma diagnostics.* Brain Pathol, 2009. **19**(4): p. 661-71.

6. Jeuken, J.W., et al., *MS-MLPA: an attractive alternative laboratory assay for robust, reliable, and semiquantitative detection of MGMT promoter hypermethylation in gliomas.* Lab Invest, 2007. **87**(10): p. 1055-65.

7. van den Bent, M.J., et al., *A hypermethylated phenotype is a better predictor of survival than MGMT methylation in anaplastic oligodendroglial brain tumors: a report from EORTC study 26951.* Clin Cancer Res, 2011. **17**(22): p. 7148-55.

8. Tian, Y., et al., *Detection of KIAA1549-BRAF fusion transcripts in formalin-fixed paraffin-embedded pediatric low-grade gliomas.* J Mol Diagn, 2011. **13**(6): p. 669-77.

9. Ferre, F., *Quantitative or semi-quantitative PCR: reality versus myth.* PCR Methods Appl, 1992. **2**(1): p. 1-9.

10. Freeman, W.M., S.J. Walker, and K.E. Vrana, *Quantitative RT-PCR: pitfalls and potential.* Biotechniques, 1999. **26**(1): p. 112-22, 124-5.

11. Coulibaly, B., et al., *Epidermal growth factor receptor in glioblastomas: correlation between gene copy number and protein expression.* Hum Pathol, 2010. **41**(6): p. 815-23.

12. Priesterbach-Ackley, L.P., et al., *Molecular tools for the pathologic diagnosis of central nervous system tumors.* Neurooncol Pract, 2019. **6**(1): p. 4-16.

13. Esteller, M., et al., *Inactivation of the DNA repair Gene O6-Methylguanine-DNA Methyltransferase by promoter hypermethylation Is associated with G to A mutations in K-ras in colorectal tumorigenesis.* Cancer Research, 2000. **60**(9): p. 2368-2371.

14. McAleenan, A., et al., *Prognostic value of test(s) for O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation for predicting overall survival in people with glioblastoma treated with temozolomide.* Cochrane Database Syst Rev, 2021. **3**: p. CD013316.

15. Capper, D., et al., *DNA methylation-based classification of central nervous system tumours.* Nature, 2018. **555**(7697): p. 469-474.

16. Tzaridis, T., et al., *MGMT promoter methylation analysis for allocating combined CCNU/TMZ chemotherapy: Lessons learned from the CeTeG/NOA-09 trial.* Int J Cancer, 2021. **148**(7): p. 1695-1707.

17. Hench, I.B., et al., *Fast routine assessment of MGMT promoter methylation.* Neurooncol Adv, 2021. **3**(1): p. vdaa170.

18. Bady, P., et al., *MGMT methylation analysis of glioblastoma on the Infinium methylation BeadChip identifies two distinct CpG regions associated with gene silencing and outcome, yielding a prediction model for comparisons across datasets, tumor grades, and CIMP-status.* Acta Neuropathol, 2012. **124**(4): p. 547-60.

19. Mansouri, A., et al., *MGMT promoter methylation status testing to guide therapy for glioblastoma: refining the approach based on emerging evidence and current challenges.* Neuro Oncol, 2019. **21**(2): p. 167-178.

20. Wick, W., et al., *MGMT testing--the challenges for biomarker-based glioma treatment.* Nat Rev Neurol, 2014. **10**(7): p. 372-85.

21. Brandner, S., et al., *MGMT promoter methylation testing to predict overall survival in people with glioblastoma treated with temozolomide: a comprehensive meta-analysis based on a Cochrane Review.* Neuro Oncol, 2021. **23**(9): p. 1457-1469.

22. Preusser, M., et al., *Anti-O6-methylguanine-methyltransferase (MGMT) immunohistochemistry in glioblastoma multiforme: observer variability and lack of association with patient survival impede its use as clinical biomarker.* Brain Pathol, 2008. **18**(4): p. 520-32.

23. Haque, F., et al., *Evaluation of a novel antibody to define histone 3.3 G34R mutant brain tumours.* Acta Neuropathol Commun, 2017. **5**(1): p. 45.

24. D'Angelo, F., et al., *The molecular landscape of glioma in patients with Neurofibromatosis 1.* Nat Med, 2019. **25**(1): p. 176-187.

25. Suzuki, H., et al., *Mutational landscape and clonal architecture in grade II and III gliomas.* Nat Genet, 2015. **47**(5): p. 458-68.

26. Habiba, U., et al., *Loss of H3K27 trimethylation is frequent in IDH1-R132H but not in non-canonical IDH1/2 mutated and 1p/19q codeleted oligodendroglioma: a Japanese cohort study.* Acta Neuropathol Commun, 2021. **9**(1): p. 95.

27. Feller, C., et al., *Histone epiproteomic profiling distinguishes oligodendroglioma, IDH-mutant and 1p/19q co-deleted from IDH-mutant astrocytoma and reveals less tri-methylation of H3K27 in oligodendrogliomas.* Acta Neuropathol, 2020. **139**(1): p. 211-213.

28. Pekmezci, M., et al., *Loss of H3K27 trimethylation by immunohistochemistry is frequent in oligodendroglioma, IDH-mutant and 1p/19q-codeleted, but is neither a sensitive nor a specific marker.* Acta Neuropathol, 2020. **139**(3): p. 597-600.

29. Maragkou, T., et al., *Evaluation of MTAP and p16 immunohistochemical deficiency as surrogate marker for CDKN2A/B homozygous deletion in gliomas.* Pathology, 2023.

30. Leone, M.A., et al., *Guidance for the preparation of neurological management guidelines by EFNS scientific task forces - revised recommendations 2012.* Eur J Neurol, 2013. **20**(3): p. 410-419.