AWMGS
All Wales Medical Genomics Service

FAQs about the RNA-based NGS service at AWMGS

SAMPLE REQUIREMENTS

Sample requirements are included within the referral forms which can be found on the AWMGS website along with a service information sheet. You can also find information about other tumour specific tests provided by AWMGS.

QUESTIONS FROM PATHOLOGY

Are RNAse-free conditions essential for cutting slides for RNA extraction?

No. Use of RNAse-free conditions during slide preparation (and subsequent processing) aims to optimise the RNA yield from FFPE samples, and minimise the failure rate of the RNA-based NGS service. The AWMGS validation of the RNA-based NGS service was performed without additional RNAse-free measures during slide preparation.

AWMGS will be monitoring the failure rates of the RNA-based NGS service and will provide feedback to referrers/ pathologists as required.

Can I send less than the 5x10uM for RNA-based NGS?

Yes in some situations. Within AWMGS, we extract RNA from 10uM slides covering a maximum area of $2cm^2$ (1.4cm x 1.4cm) x 10uM in line with extraction protocol requirements. If the region of highest neoplastic cell content on a single 10uM slide is $2cm^2$ you could send only 1 x 10uM slide for RNA extraction.

However, a note of caution on sending less than 5x10uM slides for RNA-based NGS: the cellularity of the tissue will affect the RNA yield (although in-house investigations have not yet been performed regarding the exact relationship between RNA yield and cellularity). A $2cm^2$ area may have low cellularity (e.g. cytology sample), which could mean that insufficient RNA is obtained for RNA-based NGS.

Do I need to send material for the FISH salvage pathway at the point of requesting RNA-based NGS?

No, this is not a mandatory requirement. However, we advise that material for the FISH salvage pathway is sent upfront to avoid any delays in initiating testing should the RNA extraction or RNA-based NGS fail.

AWMGS appreciate that limited tumour material may mean that the sample requirements for RNA-based NGS (plus perhaps additional requirements for material for DNA-based NGS or other testing) cannot be met. In circumstances where tumour material is scarce it may be appropriate to cut FISH slides prior to material for RNA-based NGS, in order to ensure that all clinically relevant genetic testing can be carried out.

Can I request NTRK FISH testing in preference to RNA-based NGS?

Yes. However, the testing strategy within AWMGS is to use RNA-based NGS in the first instance to streamline testing and maximise the utility of precious FFPE material. Samples that fail RNA extraction or fail RNA-based NGS are processed via the FISH salvage pathway. In circumstances where limited tumour material is available, FISH testing can be requested as a first line testing option. Please note: the referral form would have to clearly state "FISH analysis required" to ensure the sample was processed correctly.

Please see above questions regarding potentially reducing the amount of material sent for testing.

If there is insufficient material to cut sections for both DNA-based NGS and RNA-based NGS, which gets priority?

This is a question for the referring clinician. Factors to consider:

- AWMGS have a circulating tumour DNA service which could be accessed instead of DNA-based NGS. AWMGS offer ctDNA service for the detection of EGFR variants in lung and RAS variants in CRC.
- The prevalence of variants within the given tumour type. The table overleaf provides relevant information relating to the frequency of clinically relevant genetic variants in tumour types that can currently access both DNA and RNA-based NGS testing at AWMGS:

Tumour Type	Prevalence of mutations in clinically relevant genes: DNA-based NGS panel	Prevalence of gene rearrangements in clinically relevant genes: RNA-based NGS panel	References
NSCLC	EGFR 10-15%[1]	ALK 3-7%[2]	1) Sharma, S.V. et al. (2007) Nat Rev Cancer 7: 169-181; 2) Sasaki, T., and Janne, P.A. (2011) Clin Cancer Res 17: 7213-7218.
	BRAF 1-3%[5]	ROS1 1-2%[3]	3) Davies, K. and Doebele, R. (2013) Clin Cancer Res 19(15): 4040-4045.
	KRAS G12C 13%[6]	NTRK1/2/3 up to 3%[4]	4) Vaishnavi, A. et al. (2013) Nat Med 19(11):1469–1472. 5) Cardarella S et al. (2013) Clin Cancer Res;19:4532-40. 6) Herbst RS and Schlessinger J (2019) Small molecule combats cancer causing KRAS protein at last. Nature 575(7782):294-295.
GIST	KIT 80-85%[5]	NTRK1/2/3 1.9%[6]	5) Heinrich et al 2003 J of Clin Onc 21(23): 4342-4349;

			6) Chen, Y., and Chi, P. (2018) J Hematol Oncol 11: 78
	PDGFRA 5-7%[5]		5) Heinrich et al 2003 J of Clin Onc 21(23): 4342-4349.
Glioma	IDH1 80% in astrocytomas and oligodendrogliomas[7], IDH1 15% in secondary glioblastomas[7]	EGFR 25-64%[8]	7) Vigneswaran et al (2015) Ann Transl Med 3(7); Horbinski, C. (2012) Surg Pathol Clin 5(4): 919-939; 8) Gan, H.K., et al (2013) The FEBS Journal 280(21): 5350-5370.
	BRAF 2-80%[9]	BRAF 80% in pilocytic astrocytomas and other low-grade gliomas[10]	9) Horbinski, C. (2013) J Neuropahol Exp Neurol 71(1):2-7; 10) Kumar-Sinha, C., et al (2015) Gen Med 7:129; Cocco, E., et al (2018) Nat Rev Clin Oncol 15(12): 731-747.
		NTRK1/2/3 up to 2% in adult gliomas and 5-40% in paediatric gliomas[11]	11) Chen, Y., and Chi, P. (2018) J Hematol Oncol 11: 78; Torre, M., et al (2020) Acta Neuropathologica Communications 8: 107.
Thyroid	BRAF 0.8-66.5% (66.5% in PTCs)[12]; RET 3.6-9.3 (7.3% in PTCs)[12]	RET 10-30% in PTCs[10]; NTRK1/2/3 5% in PTCs[10]	Notes: PTC represents 80% of thyroid cancers. 10) Kumar-Sinha et al (2015) Genome Medicine 7:129; 12) The AACR Project GENIE Consortium (2017) Cancer Discovery 7(8):818-831 (via https://www.mycancergenome.org/).

ABOUT RNA-BASED NGS TESTING

What is the difference between the RNA-based NGS panel and the DNA-based NGS panel?

Sample preparation for DNA and RNA samples differs, DNA samples are mechanically fragmented prior to processing and RNA samples undergo conversion to cDNA for processing. From this point the protocol used is the same, however the content of the DNA and RNA panels is different.

The DNA panel contains 523 genes, the RNA panel contains 55 genes. The full list of genes is available in the service information sheet.

Analysis is undertaken on subsets of clinically relevant genes, this varies depending on the tumour site.

DNA based NGS is used to identify single nucleotide variants and small insertions or deletions within coding regions (+/-5bp) of a gene.

RNA based NGS is used to identify gene fusions and certain structural rearrangements.

For example, DNA-NGS will identify a BRAF V600E variant, whereas RNA-NGS will identify an EML4-ALK gene fusion.

Dependent on the genetic analysis required for any particular sample/ tumour type, one or both panels may be required to provide all clinically relevant information.

Why was the RNA-based NGS panel introduced at AWMGS?

It was introduced for the following reasons:

- It is less labour intensive to identify gene rearrangements compared to Fluorescence In-Situ Hybridisation (FISH) analysis.
- The panel allows simultaneous analysis of up to 9 genes for the detection of gene rearrangements (including gene fusions). This helps AWMGS to streamline our testing pathways to improve efficiency of technical staff time and has the potential to use less tumour material than multiple FISH tests.
- The panel allows partner genes to be named in any gene rearrangements detected, which could have potential clinical implications in the future should therapies be designed against partner gene-specific fusions.
- Molecular diagnostics and precision medicine is rapidly advancing with new cancer therapies likely in the future, meaning that the number of genes requiring analysis for any given tumour type is likely to increase. Using RNA-based NGS panels ensures that AWMGS can offer the most comprehensive service to cancer patients as molecular diagnostics and precision medicine continue to rapidly evolve.

ABOUT AWMGS RNA-BASED NGS TEST

What genes are analysed on the RNA-based NGS panel?

There are 55 genes on the RNA panel, analysis across tumour sites is targeted to ALK, ROS1, NTRK1, NTRK2, NTRK3, RET, BRAF, MET, EGFR. It identifies rearrangements (including gene fusions) involving these 9 clinically relevant genes.

Tumour-specific analysis is used to target only clinically relevant genes along with other genes of potential interest (as determined via prior clinical consultation at NGS panel-design stage). The service information sheet details genes analysed for each tumour type.

Can all of the 9 genes on the RNA-based NGS panel be analysed?

Yes, by prior agreement with AWMGS through contacting helen.roberts20@wales.nhs.uk or Rhian.white@wales.nhs.uk. In this situation, any correspondence relating to this agreement **must** be sent

with the referral form and sample to AWMGS. The referral form **must** clearly state *"complete RNA panel analysis required"*.

The AWMGS referral form references lung, CRC, melanoma, glioma, GIST and thyroid for DNA-based NGS, and lung, glioma and thyroid for RNA-based NGS. What testing is available for 'other' tumours?

'Other' tumours (those not specified on the information sheet) can be tested using the DNA-based NGS panel and/or the RNA-based NGS panel. If the DNA-based panel was required, 23 genes within the panel would be analysed. If the RNA-based panel was required, the 9 genes listed above would be analysed.

Can I order RNA-based NGS analysis of NTRK and ROS1 analysis on an 'other' tumour?

No, bespoke genetic analysis is not available at AWMGS. The tumour specific panels utilised are described on the information sheet. If a patient required these genes analysed, AWMGS would use the complete RNA-based NGS panel. Prior agreement with AWMGS would have to be sought before sending the sample for testing. Contact helen.roberts20@wales.nhs.uk or Rhian.white@wales.nhs.uk for further details.

NTRK RELATED QUESTIONS

What tumour types is NTRK testing currently available for at AWMGS?

All tumour types have been validated for NTRK testing

NTRK testing was introduced on a phased approach as outlined in the NTRK Gene Fusion Testing Clinical Guidance. Validation was completed by the end of June 2022. Therefore, currently all tumour types can be tested for NTRK fusions.

LUNG RELATED QUESTIONS

For lung samples, does RNA-based NGS detect clinically relevant variants in the EGFR gene?

No. Clinically actionable EGFR variants (single nucleotide variants or small insertions or deletions) within lung tumours are detected by using of the below AWMGS tests:

- The DNA-based NGS panel for analysis of a solid tumour sample. The DNA-based NGS panel is requested using the same referral form as RNA-based NGS.
- The circulating tumour DNA service available at AWMGS, requires a patient blood sample and can be requested using this ctDNA referral form. Information regarding this service can be found here.

The lung-specific RNA-based NGS panel specifically identifies the presence of the EGFRvIII gene rearrangement, which is an in-frame deletion of exons 2-7 found in numerous tumour types. This is deemed an intragenic fusion which results in the EGFR (Epidermal Growth Factor Receptor) being constitutively active. EGFRvIII variants may be of interest in a research setting as could be a potential target for EGFRvIII-directed therapies in the future.

If testing for EGFR, ALK, and ROS1 is requested on a lung sample, what are the sample requirements?

- EGFR analysis requires DNA-based NGS: 1 x H&E plus 6x10uM sections.
- ALK and ROS1 analysis requires RNA-based NGS: 5x10uM sections. FISH salvage pathway requires: 4x3-4uM sections (no additional H&E needed).

These sample requirements are documented on the solid tumour lung referral form. Please note responses in *Questions from Pathology* regarding potentially reducing the amount of material sent for testing.

Can I request ALK, ROS1 and NTRK FISH testing in preference to RNA-based NGS?

Yes. However, the testing strategy within AWMGS is to use RNA-based NGS in the first instance to streamline testing and maximise the utility of precious FFPE material. Samples that fail RNA extraction or fail RNA-based NGS are processed via the FISH salvage pathway. In circumstances where limited tumour material is available, FISH testing can be requested as a first line testing option. Please note: the referral form would have to clearly state "FISH analysis required" to ensure the sample was processed correctly.

If ROS1/ALK genetic testing is required for confirmation of an equivocal IHC result, what samples should I send?

The sample requirements for RNA-based NGS should be followed as per the referral form. In circumstances where limited tumour material is available, FISH testing can be requested as a first line testing option; in this situation, the referral form would have to clearly state "FISH analysis required" to ensure the sample was processed correctly. Please note responses in *Questions from Pathology* regarding potentially reducing the amount of material sent for testing.