Molecular diagnostics of cytologically indeterminate thyroid nodule fine-needle aspiration cytologies using the ThyroSPEC™ v1 panel

ThyroSPEC™ is a proprietary, highly accurate, cost-efficient MALDI-TOF mass spectrometry-based mutation detection panel that detects the most prevalent 116 point mutations and 21 gene fusions reported in thyroid cancer in the following genes:

<table>
<thead>
<tr>
<th>Point Mutations</th>
<th>Gene Rearrangements</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT1</td>
<td>AGK::BRAF</td>
</tr>
<tr>
<td>BRAF</td>
<td>AKAP::BRAF</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>CRTC1::MAML2</td>
</tr>
<tr>
<td>DICER1</td>
<td>EML4::ALK</td>
</tr>
<tr>
<td>EGFR</td>
<td>ETV6::NTRK3</td>
</tr>
<tr>
<td>EIF1AX</td>
<td>IGF2BP3::THADA</td>
</tr>
<tr>
<td>EZH1</td>
<td>PAX8::PPARG</td>
</tr>
<tr>
<td>HRAS</td>
<td>RET:::PTC</td>
</tr>
<tr>
<td>IDH1</td>
<td>RPS2P32::THADA</td>
</tr>
<tr>
<td>KRAS</td>
<td>SND1::BRAF</td>
</tr>
<tr>
<td>NRAS</td>
<td>SQSTM1::NTRK3</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>STRN::ALK</td>
</tr>
<tr>
<td>PTEN</td>
<td>TFG::NTRK1</td>
</tr>
<tr>
<td>RET</td>
<td>TMEM233::PRKAB1</td>
</tr>
<tr>
<td>SPOP</td>
<td>TPM3::NTRK1</td>
</tr>
<tr>
<td>TERT</td>
<td>TPR::NTRK1</td>
</tr>
<tr>
<td>TP53</td>
<td></td>
</tr>
<tr>
<td>TSHR</td>
<td></td>
</tr>
</tbody>
</table>

The cut-off value for point mutations is 10% variant allele frequency.

A prospective, observational validation study was conducted in the AHS Calgary Health Care Region, where a guidelines-based thyroid nodule pathway (PCN pathway) including thyroid nodule ultrasound malignancy risk stratification (EFN white paper) and determination of local malignancy risk for each Bethesda category¹ (Ghaznavi et al., Acta Cytologica, 2022) has recently been implemented.

All 615 patients in Southern Alberta with an AUS/FLUS or FN/SFN thyroid nodule diagnosed from July 30, 2020, until July 31, 2022, were included. Electronic health records were used for this study (IRB approval: HREBA.CHC-20-0068) to determine demographics, laboratory results, clinical history, surgical decision making, ultrasound findings, cytology diagnoses, molecular test results, outcomes, surgical procedure, histopathologic findings based on standardized synoptic reporting, and follow-up.

Test performance per Bethesda and US subcategories was determined in this study² (Stewardson et al., Thyroid, 2023), noting that in Calgary the pre-test risk of malignancy was 26% in the AUS/FLUS category and 43% in the FN/SFN category prior to implementation of molecular diagnostics¹. ThyroSPEC™ test performance ranges from resected nodules only, to all resected nodules and unresected nodules with more than 1 year follow-up². TR refers to ACR-TIRADS classification. The limit of detection threshold during validation was a 10% allelic frequency.
Prevalence of malignancy | Sensitivity | Specificity | NPV | PPV
--- | --- | --- | --- | ---
Bethesda III (AUS/FLUS) | 17-41% | 74% | 67-78% | 79-93% | 42-61%
Bethesda IV (FN/SFN) | 42-53% | 67% | 79-84% | 70-77% | 70-82%
ATA Low Suspicion, TR3 | 20-35% | 72% | 56-83% | 79-92% | 46-52%
ATA Intermediate Suspicion, TR4 | 22-49% | 80% | 69-79% | 78-83% | 52-72%
ATA High Suspicion, TR5 | 17-41% | 46% | 83-84% | 70-89% | 35-67%

Detected mutations are classified as follows:

### Benign molecular markers
- TSHR, EZH1, SPOP, PTEN

### Intermediate risk mutations
- NRAS, HRAS, KRAS, BRAF<sup>K601E</sup>, EIF1AX, IDH1, DICER1, TP53 or rearrangements in PPARG, THADA

### Malignant molecular markers
- BRAF<sup>V600E</sup>, TERT or rearrangements in BRAF, RET, NTRK1, NTRK3

### High-Risk mutations
- BRAF + TERT, RAS + TERT, RAS + EIF1AX, AKT1, PIK3CA, CTNNB1, EGFR, or rearrangements in ALK

### Medullary Thyroid Carcinoma markers
- RET mutations

**Mutation-specific malignancy risks**

- **TSHR, EZH1, SPOP, PTEN.** Mostly benign, very few malignant.<sup>2</sup>
- **No mutation detected.** Bethesda III (AUS/FLUS) post-test risk of malignancy up to 27%; Bethesda IV (FN/SFN) post-test risk of malignancy up to 35%.<sup>2</sup>
- **IDH1, DICER1, BRAF<sup>K601E</sup>, EIF1AX, TP53 or rearrangements in PPARG, THADA.** Post-test risk of malignancy is higher than pre-test risk of malignancy, not necessarily malignant as mutation specific risk remains unclear.<sup>2</sup>
- **NRAS, HRAS, KRAS.** 58% malignancy risk for resected Calgary RAS positive nodules.<sup>2</sup>
- **BRAF<sup>V600E</sup>, TERT, or rearrangements in BRAF, RET, NTRK1, NTRK3.** Indicate a malignant tumour (90% risk of malignancy).<sup>2</sup>
- **BRAF + TERT, RAS + TERT, RAS + EIF1AX, AKT1, PIK3CA, CTNNB1, EGFR, or rearrangements in ALK.** Indicate a malignant tumour (100% risk of malignancy).<sup>2</sup>
- **RET mutations.** Medullary thyroid carcinoma.

**Management options based on the mutation-specific malignancy risks**

- **TSHR, EZH1, SPOP, PTEN.** No molecular indication for malignancy.
- **Bethesda III (AUS/FLUS) no mutation detected.** Lobectomy or observation depending on further malignancy risk assessment including ultrasound, cytology and clinical assessment.<sup>3,4,5</sup> A ThyroSPEC-negative result does not rule out cancer, there is a residual risk of malignancy of up to 27% for ThyroSPEC negative Bethesda III (AUS/FLUS) nodules<sup>2</sup>.
- **Bethesda IV (FN/SFN) no mutation detected.** Molecular testing has not changed management recommendations based on ultrasound, cytology and clinical assessment.<sup>3,4,5</sup> A ThyroSPEC-negative result does not rule out cancer, there is a residual risk of malignancy of up to 35% for ThyroSPEC-negative Bethesda IV (FN/SFN) nodules<sup>2</sup>.
• IDH1, DICER1, NRAS, HRAS, KRAS, BRAFK601E, EIF1AX, TP53, or rearrangements in PPARG, THADA. Refer to Endocrinology to discuss lobectomy or observation depending on combined risk assessment ultrasound, cytology and clinical assessment3,4,5.

• BRAFV600E, TERT, RET, EGFR, BRAF + TERT, RAS + TERT, RAS + EIF1AX, AKT1, PIK3CA, CTNNB1 or rearrangements in BRAF, RET, NTRK1, NTRK3, ALK. Total thyroidectomy3,4,5. Refer to surgery.

• Less aggressive treatment is recommended for nodules 1cm or less according to current guidelines3.

Questions concerning the further clinical interpretation of ThyroSPEC results can be addressed to Dr. Ralf Paschke (ralf.paschke@albertahealthservices.ca).

Disclaimer: Interpret the above results within the context of other clinical data such as ultrasound, with clinical management decision making according to the independent medical judgement of the responsible physician and patient preferences. ThyroSPEC™ was not created to identify germline variants, nonetheless it is possible that ThyroSPEC™ will discover a germline mutation incidentally. If a germline variant is reported, referral to medical genetics may be advisable.


Referral Possibilities for Patients with Indeterminate FNA Cytology and Additional ThyroSPEC™ Testing

Intermediate Malignancy Risk Molecular Findings

- NRAS, HRAS, KRAS, BRAF<sup>V600E</sup>, EIF1AX, TP53, IDH1, DICER1, or rearrangements in PPARG, THADA<sup>1,3</sup>: post-test risk of malignancy higher than pre-test risk of malignancy. Variable malignancy risk, with accurate risk assessment depending on ultrasound, cytology and clinical assessment<sup>4</sup>

High Malignancy Risk Molecular Findings

- BRAF<sup>V600E</sup>, TERT, or rearrangements in BRAF, RET, NTRK1, NTRK3<sup>1,3</sup>: indicate a malignant tumour (>90% risk of malignancy)
- BRAF + TERT, RAS + TERT, RAS + EIF1AX, AKT1, PIK3CA, CTNNB1, EGFR, or rearrangements in ALK<sup>3</sup>: indicate a malignant tumour with poor prognosis (100% risk of malignancy)
- RET Mutations: indicate a medullary thyroid carcinoma

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