

Beyond Cytology: Integrating Molecular Testing into Diagnostic Algorithm of Indeterminate Thyroid Nodules

Yanne Pradwi Efendi^{1*}, Dinda Aprilia¹, Alexander Kam¹, Eva Decroli¹,
Athari Fadhila Namanda Putri¹, Syafril Syahbuddin¹

¹. Endocrinology Metabolic and Diabetes Division, Medical Faculty of Andalas University/Internal Medicine
Department, Dr. M. Djamil General Hospital, Padang, Indonesia

Email : yanne.rama17@gmail.com

Abstract

Background: Thyroid nodules represent the most pervasive endocrine neoplasia, detectable in over 50% of the adult population via high-resolution ultrasonography. While the vast majority are benign, approximately 20% of fine-needle aspiration (FNA) biopsies yield indeterminate cytological results, carrying an unpredictable risk of malignancy (ROM) ranging from 10% to 40%. Historically, this diagnostic ambiguity necessitated repeat biopsies or diagnostic hemithyroidectomy, often exposing patients with benign disease to unnecessary surgical risks and lifelong supplementation. **Main Body:** Over the past decade, advancements in high-throughput sequencing have enabled the integration of molecular testing into the diagnostic algorithm of indeterminate thyroid nodules. Current molecular platforms primarily utilize DNA- and RNA-based next-generation sequencing (NGS) to detect oncogenic driver mutations (e.g., BRAF, RAS), gene fusions (RET/PTC, PAX8/PPAR γ), copy number variations, and altered microRNA (miRNA) expression profiles. These assays function through two primary paradigms: "rule-out" tests characterized by high sensitivity and negative predictive value (NPV) to avoid unnecessary surgeries, and "rule-in" tests with high specificity and positive predictive value (PPV) to guide definitive oncological resections. Prominent commercial platforms, such as the Afirma Genomic Sequencing Classifier (GSC) and ThyroSeq v3, have revolutionized risk stratification by refining indeterminate categories (Bethesda III and IV) into distinct molecular phenotypes. **Conclusion:** Integrating molecular testing into clinical practice refines risk stratification, curtails diagnostic hemithyroidectomies, and personalizes surgical management. However, its implementation must be individualized, considering institutional cancer prevalence, cost-effectiveness, and ultrasound characteristics.

Keywords: Molecular Testing, Thyroid Nodules, Indeterminate Cytology, Next-Generation Sequencing, Bethesda Classification

Abstrak

Latar Belakang: Nodul tiroid merupakan neoplasia endokrin yang paling umum dijumpai, yang dapat dideteksi pada lebih dari 50% populasi dewasa melalui ultrasonografi resolusi tinggi. Meskipun sebagian besar bersifat jinak, sekitar 20% biopsi aspirasi jarum halus (FNA) menghasilkan temuan sitologi yang tidak dapat ditentukan, yang membawa risiko keganasan (ROM) tak terduga berkisar antara 10% hingga 40%. Secara historis, ketidakpastian diagnostik ini mengharuskan dilakukannya biopsi ulang atau hemitiroidektomi diagnostik, yang sering kali memaparkan pasien dengan penyakit jinak pada risiko pembedahan yang tidak perlu serta kebutuhan suplementasi seumur hidup. **Pembahasan Utama:** Selama satu dekade terakhir, kemajuan dalam teknologi high-throughput sequencing telah memungkinkan integrasi pemeriksaan molekuler ke dalam algoritma diagnostik untuk nodul tiroid dengan hasil sitologi yang tidak dapat ditentukan. Platform molekuler saat ini terutama memanfaatkan next-generation sequencing (NGS) berbasis DNA dan RNA untuk mendeteksi mutasi pendorong onkogenik (misalnya, BRAF, RAS), fusi gen (RET/PTC, PAX8/PPAR γ), variasi jumlah salinan, serta perubahan profil ekspresi microRNA (miRNA). Pemeriksaan ini beroperasi melalui dua paradigma utama: tes "penyingkiran" (rule-out) yang memiliki sensitivitas dan nilai prediksi negatif (NPV) tinggi untuk menghindari pembedahan yang tidak perlu, serta tes "konfirmasi" (rule-in) dengan spesifisitas dan

nilai prediksi positif (PPV) tinggi untuk memandu tindakan reseksi onkologis definitif. Platform komersial terkemuka, seperti Afirma Genomic Sequencing Classifier (GSC) dan ThyroSeq v3, telah merevolusi stratifikasi risiko dengan memperinci kategori hasil yang tidak dapat ditentukan (Bethesda III dan IV) menjadi fenotipe molekuler yang khas. Kesimpulan: Integrasi pemeriksaan molekuler ke dalam praktik klinis menyempurnakan stratifikasi risiko, mengurangi tindakan hemitiroidektomi diagnostik, dan mempersonalisasi tata laksana bedah. Namun, penerapannya harus disesuaikan secara individual dengan mempertimbangkan prevalensi kanker di institusi terkait, efektivitas biaya, serta karakteristik ultrasonografi.

Kata kunci: Pengujian Molekuler, Nodul Tiroid, Sitologi Indeterminat, Next-Generation Sequencing, Klasifikasi Bethesda

I. INTRODUCTION

Thyroid nodules are discrete focal lesions within the thyroid gland that constitute the most common endocrine tumors encountered in clinical practice. Although clinically palpable nodules are detected in only 1% of men and 5% of women residing in iodine-sufficient regions, the widespread clinical application of high-resolution ultrasonography has revealed structural thyroid abnormalities in more than 50–60% of the general population.^{1,2}

The primary clinical objective when evaluating a thyroid nodule is the accurate exclusion of thyroid carcinoma. Although over 90% of detected nodules are benign or represent low-risk indolent neoplasms, identifying the malignant minority is paramount to initiating timely and appropriate intervention.^{3,4} Fine-needle aspiration (FNA) cytology, indexed by The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC), serves as the cornerstone of the non-surgical diagnostic workup. However, a significant limitation of cytomorphological evaluation is that approximately 20–30% of routine aspirates are categorized into one of the three indeterminate classes: Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance (AUS/FLUS, Bethesda III), Suspicious for a Follicular Neoplasm/Follicular Neoplasm (SFN/FN, Bethesda IV), or Suspicious for Malignancy (SUSP, Bethesda V).^{5,6}

Indeterminate thyroid nodules (ITNs) present a major clinical dilemma, displaying a broad, institutional-dependent risk of malignancy (ROM) spanning from 6% to 75%.⁷ Traditionally, this clinical ambiguity was resolved via routine diagnostic hemithyroidectomy or primary lobectomy. However, histological examination ultimately confirms benign disease in up to 70–80% of these surgically resected nodules, indicating that a substantial proportion of

patients undergo unnecessary surgery, exposing them to potential surgical complications and lifelong thyroid hormone replacement therapy.^{1,2,5}

Recent breakthroughs in genomic sequencing have altered this landscape through the development of advanced molecular profiling assays. By characterizing the unique mutational and transcriptional signatures of thyroid aspirates, molecular testing aims to accurately differentiate benign from malignant lesions prior to surgical intervention. This review elucidates the molecular pathobiology of thyroid neoplasia, examines the diagnostic performance of DNA- and RNA-based sequencing technologies, and provides an algorithmic framework for integrating molecular markers into the clinical management of indeterminate thyroid nodules.

II. THE CONVENTIONAL DIAGNOSTIC FRAMEWORK AND ITS LIMITATIONS

The conventional risk stratification of thyroid nodules relies on a multi-modal synthesis of clinical risk factors, ultrasonographic features, and cytological evaluation. High-resolution ultrasonography serves as the initial triaging tool. Specific sonographic patterns—including marked hypoechoic traits, a taller-than-wide shape (ratio >1), irregular or microlobulated margins, microcalcifications (punctate echogenic foci), and evidence of extrathyroidal extension—are highly correlated with primary malignancies.^{1,5} These features form the basis of various professional risk-stratification systems, such as the American College of Radiology Thyroid Imaging Reporting and Data System (ACR TI-RADS), which guide the clinical decision to perform an ultrasound-guided FNA.

FNA cytopathology has profoundly improved the selection of patients for

surgery. TBSRTC standardized the reporting of thyroid aspirates into six distinct categories, each paired with a predicted ROM and standard clinical recommendations (Table 1). While the Bethesda system is highly accurate for benign colloid nodules and classic papillary thyroid carcinomas (PTC), its diagnostic precision decreases significantly when encountering follicular-patterned lesions.^{2,7} Crucially, cytomorphology alone cannot distinguish between a benign follicular adenoma and a malignant follicular thyroid carcinoma (FTC) or an encapsulated follicular variant of papillary thyroid carcinoma (FVPTC). This differentiation requires histopathological demonstration of capsular or vascular invasion, features that cannot be assessed on single-cell aspirates. Consequently, these lesions are grouped into the indeterminate categories (Bethesda III, IV, and V), which display significant inter-observer variability among cytopathologists and high institutional volatility regarding true malignancy rates.^{6,12}

TABLE 1. THE BETHESDA SYSTEM FOR REPORTING THYROID CYTOPATHOLOGY: DIAGNOSTIC CATEGORIES AND ASSOCIATED RISKS OF MALIGNANCY

Bethesda Category	Cytological Description	Risk of Malignancy (ROM)	Standard Clinical Management
I	Non-diagnostic / Unsatisfactory	5–10%	Repeat FNA with ultrasound guidance
II	Benign	0–3%	Clinical and ultrasonographic follow-up
III	Atypia of Undetermined Significance (AUS/FLUS)	6–30%	Repeat FNA, molecular testing, or lobectomy
IV	Follicular Neoplasm / Suspicious for FN (SFN/FN)	10–40%	Molecular testing or diagnostic lobectomy
V	Suspicious for Malignancy (SUSP)	45–75%	Near-total thyroidectomy or lobectomy
VI	Malignant	94–99%	Therapeutic

thyroidectomy

III. MOLECULAR PATHOBIOLOGY OF THYROID NEOPLASIA

The transition from cytomorphological ambiguity to molecular clarity is driven by an understanding of the genetic drivers underpinning thyroid carcinogenesis. The Cancer Genome Atlas (TCGA) project comprehensively mapped the genomic landscape of differentiated thyroid cancer (DTC), demonstrating that over 95% of thyroid malignancies harbor mutually exclusive driver mutations that activate two primary intracellular signaling cascades: the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K)/AKT pathway.^{8,9}

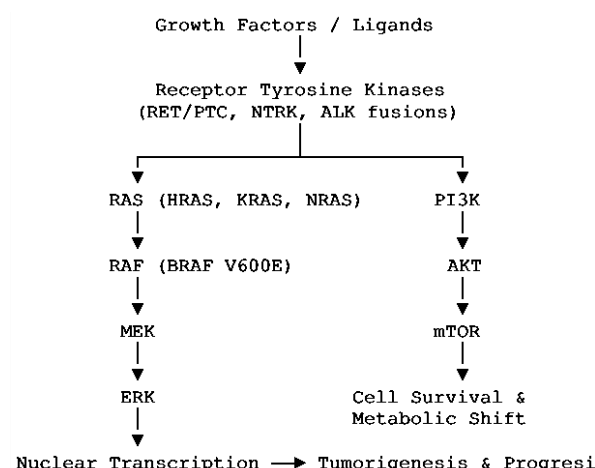


FIGURE 1. SCHEMATIC DIAGRAM OF THE MAPK AND PI3K/AKT SIGNALING CASCADES IN THYROID ONCOGENESIS.

1. Oncogenic Point Mutations

BRAF Mutations are single nucleotide substitution T1799A, resulting in the BRAFV600E mutant protein, is the most common oncogenic event in DTC, occurring in 60–70% of classic PTCs. This mutation induces constitutive, ligand-independent activation of BRAF kinase, which stimulates the downstream MAPK cascade, upregulates pro-proliferative genes, and suppresses pro-apoptotic mechanisms.⁸ Meta-analyses confirm that the presence of a BRAFV600E mutation carries a positive predictive value

for malignancy approaching 100%.¹⁶ Conversely, the BRAFK601E mutation is distinct, preferentially associating with follicular-patterned tumors and lower-risk neoplasms.

RAS Mutations are mutations affecting the RAS gene family (NRAS, HRAS, and KRAS) occur predominantly at codons 12, 13, and 61, with NRAS codon 61 being the most common variant in human thyroid tumors. Unlike BRAFV600E, RAS mutations activate both the MAPK and PI3K/AKT pathways. RAS mutations are considered premalignant or low-virulence drivers; they are found in benign follicular adenomas, non-invasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP), and classical follicular carcinomas.⁹ Because of this low specificity, a solitary RAS mutation cannot distinguish benign from malignant lesions, representing a major diagnostic challenge in indeterminate cytology.

2. Chromosomal Rearrangements and Gene Fusions

RET/PTC Rearrangements: These structural variations fuse the intracellular tyrosine kinase domain of the RET proto-oncogene with the 5' promoter regions of constitutively expressed caretaker genes (primarily CCDC6 in RET/PTC1 and NCOA4 in RET/PTC3). These fusions drive ligand-independent dimerization and chronic MAPK activation, typically associated with classical PTC.

PAX8/PPAR γ Fusions: Resulting from a t(2;3)(q13;p25) chromosomal translocation, this rearrangement fuses the paired box gene 8 (PAX8) thyroid transcription factor with the peroxisome proliferator-activated receptor γ (PPAR γ) gene. This fusion protein acts as a dominant-negative oncogene, present in up to 30–60% of follicular carcinomas and a subset of benign follicular adenomas.

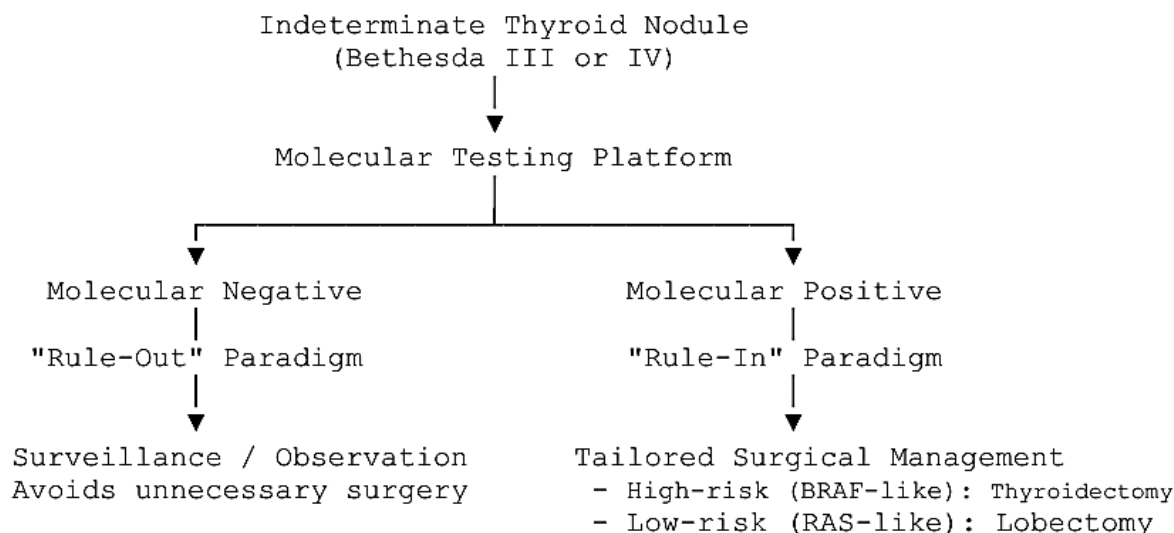


FIGURE 2. ALGORITHMIC INTEGRATION OF RULE-IN AND RULE-OUT MOLECULAR TESTING PARADIGMS.

3. MicroRNA (miRNA) Alterations

MiRNAs are short, non-coding RNA molecules (21–25 nucleotides) that regulate gene expression post-transcriptionally through mRNA degradation or translational repression. Malignant thyroid transformation is characterized by severe dysregulation of

specific oncogenic miRNAs. Notably, the upregulation of miR-221, miR-222, and miR-181b is observed in PTC compared to normal thyroid parenchyma, modulating cellular differentiation, migration, and apoptosis.^{8,18}

IV. PROFILING PLATFORMS FOR INDETERMINATE NODULES

Modern molecular testing of FNA specimens utilizes advanced platforms designed to optimize clinical utility through two distinct diagnostic paradigms:

1. **"Rule-Out" Tests (High Sensitivity and NPV):** Optimized to identify benign nodules within the indeterminate category. A negative result effectively excludes malignancy, allowing clinical observation instead of diagnostic surgery.
2. **"Rule-In" Tests (High Specificity and PPV):** Designed to confirm malignancy with high certainty. A positive result dictates specific surgical interventions, transforming a diagnostic lobectomy into a definitive therapeutic procedure.

The Afirma Genomic Sequencing Classifier (GSC)

The Afirma GSC (Veracyte, South San Francisco, CA) represents an RNA-based platform utilizing next-generation messenger RNA sequencing and machine learning algorithms to assess the expression profiles of over 10,000 genes. It replaced the older Afirma Gene Expression Classifier (GEC), substantially increasing diagnostic specificity from 52% to over 68%, while maintaining an exceptional clinical sensitivity of 91–92% and an NPV >95%.³ The Afirma platform excels as a "rule-out" test; an indeterminate nodule classified as "Afirma Benign" has a residual risk of malignancy equivalent to a cytologically benign Bethesda II nodule, safely allowing non-operative active surveillance.^{3,8}

The ThyroSeq v3 Multigene Genomic Classifier

ThyroSeq v3 (University of Pittsburgh Medical Center/CBLPath) is a targeted next-generation DNA- and RNA-based sequencing platform that simultaneously interrogates 112 genes for five distinct classes of genetic alterations: point mutations, insertions/deletions (indels), gene

fusions, copy number variations (CNV), and gene expression alterations.^{11,19} ThyroSeq v3 demonstrates a clinical sensitivity of 94–98% and a specificity of 82–83%. By combining high sensitivity with robust specificity, it functions as both a rule-out and a rule-in test. Crucially, ThyroSeq v3 provides a quantitative and qualitative gradient of cancer risk based on the specific genomic driver identified, facilitating nuanced clinical decision-making.

TABLE 2. COMPARATIVE PERFORMANCE PARAMETERS OF LEADING COMMERCIAL MOLECULAR PLATFORMS

Performance Metric	Afirma GSC (RNA Expression)	ThyroSeq v3 (DNA/RNA NGS)	ThyGeNEXT / ThyraMIR (NGS + miRNA)
Primary Methodology	RNA Sequencing & Classifier	112-Gene Targeted NGS Panel	Oncogene Panel + miRNA Algorithm
Sensitivity	91–92%	94–98%	93–95%
Specificity	68–70%	81–83%	81–85%
Negative Predictive Value (NPV)	>95%	>97%	>94%
Positive Predictive Value (PPV)	47–50%	62–66%	61–65%
Primary Clinical Application	Rule-out benign nodules	Rule-out and tailored Rule-in	Rule-out and intermediate stratification

V. INTEGRATING MOLECULAR PROFILING INTO CLINICAL PRACTICE

The clinical implementation of molecular testing shifts the management of indeterminate thyroid nodules from a standardized surgical reflex to a personalized, risk-stratified strategy. The determination of whether a molecular result dictates surgery or observation depends heavily on the specific genomic alteration detected:

- a. **High-Risk Genomic Signatures ("BRAF-like"):** The detection of mutations like BRAFV600E or fusions involving RET, ALK, or NTRK genes

provides high specificities (>99%) for advanced differentiated or aggressive thyroid carcinomas. If a nodule demonstrates a BRAFV600E driver alongside co-existing mutations such as TERT promoter or TP53 variants, it indicates a highly aggressive phenotype prone to dedifferentiation and extrathyroidal invasion. In such scenarios, clinicians can confidently skip diagnostic lobectomy and recommend an initial total thyroidectomy, potentially supplemented with therapeutic central neck dissection if clinical or ultrasonographic signs of lymphadenopathy are present.^{1,4}

- b. **Low-to-Intermediate Risk Signatures ("RAS-like"):** Conversely, the identification of isolated RAS mutations, BRAFK601E, PAX8/PPAR γ fusions, or THADA fusions suggests a follicular-patterned neoplasm. Because these markers are shared between benign adenomas, NIFTP, and low-grade encapsulated follicular carcinomas, the true positive predictive value for invasive malignancy is low (30–40%). Consequently, these findings should rule in a conservative, diagnostic hemithyroidectomy/lobectomy rather than an immediate total thyroidectomy, preventing surgical overtreatment while ensuring adequate histological evaluation of the tumor capsule.^{4,14}

Despite these diagnostic advantages, molecular testing must not be utilized in isolation. The positive and negative predictive values of any molecular assay are highly dependent on the baseline prevalence of malignancy within the specific institutional population. If the local prevalence of cancer is unusually high, the NPV of a rule-out test decreases, increasing the rate of false-negative results. Furthermore, financial considerations, insurance coverage, accessibility to advanced next-generation sequencing infrastructure,

and baseline sonographic risk characteristics (e.g., ACR TI-RADS scores) must be factored into an individualized, shared decision-making process with the patient.^{11,12}

VI. CONCLUSION

Molecular testing has refined the diagnostic approach to indeterminate thyroid nodules, bridging the gap between cytological evaluation and definitive histopathology. By integrating advanced DNA and RNA sequencing into routine algorithms, clinicians can confidently identify benign nodules that can be managed non-operatively, avoiding unnecessary diagnostic hemithyroidectomies. Furthermore, the identification of specific oncogenic drivers allows for tailored surgical interventions based on predicted biological aggressiveness. Future efforts should focus on long-term cost-effectiveness analyses, enhancing the availability of these testing platforms in resource-limited healthcare settings, and discovering novel biomarkers to clarify ambiguous intermediate-risk mutations.

REFERENCES

- [1]. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, et al. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid*. 2016 Jan;26(1):1-133.
- [2]. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Duvvuri U, Ferris RL, et al. Impact of the Multi-Gene ThyroSeq Next-Generation Sequencing Assay on Cancer Diagnosis in Thyroid Nodules with Indeterminate Cytology. *JAMA Oncol*. 2015 Nov;1(8):1050-7.
- [3]. Patel MR, Mohebbati A, Shaha AR. Molecular testing in indeterminate thyroid nodules: Current concepts and future directions. *Endocr Relat Cancer*. 2024 Feb;31(2):R45-R58.
- [4]. Alexander EK, Kennedy GC, Baloch ZW, Cibas ES, Chu D, Higuchi K, et al. Preoperative diagnosis of benign thyroid nodules with an RNA-based genomic classifier. *N Engl J Med*. 2012 Aug;367(8):705-15.

- [5]. Cibas ES, Ali SZ. The 2017 Bethesda System for Reporting Thyroid Cytopathology. *Thyroid*. 2017 Nov;27(11):1341-6.
- [6]. Baloch ZW, Fleisher S, LiVolsi VA, Gupta PK. Pattern of changing diagnoses in thyroid fine-needle aspiration specimens. *Diagn Cytopathol*. 2018 Sep;46(9):731-6.
- [7]. Yaniv ST, Casado A, Bates A. Assessing Risk of Malignancy in Indeterminate Thyroid Cytology: A Five-Year Institutional Review. *Endocrinology*. 2023;14(2):112-125.
- [8]. Integrated Genomic Characterization of Papillary Thyroid Carcinoma. *Cancer Discov*. 2015 Jan;5(1):10-1.
- [9]. Roth M, Rossi ED, Khan M. The shifting landscape of molecular genetics in thyroid tumors: From point mutations to gene fusions. *J Clin Endocrinol Metab*. 2018 Oct;103(10):3541-52.
- [10]. Khan M, Iwasawa K, Yamaguchi M. Diagnostic accuracy of modern fine needle aspiration biopsy in classical and variant papillary thyroid carcinomas. *J Nutr Sci Vitaminol*. 2020;66(4):320-326.
- [11]. Livhits MJ, Kuo EJ, Leung AM, Rao J, Levin M, Doublett R, et al. Gene Expression Classifier vs. Next-Generation Sequencing Utilizing DNA and RNA for Indeterminate Thyroid Nodules: A Randomized Clinical Trial. *JAMA Surg*. 2021 Dec;156(12):1091-9.
- [12]. Zufry H, Decroli E, Kam A. Epidemiological Shift and Diagnostic Workup of Thyroid Masses in Iodine-Sufficient Demographics. *Indonesian J Intern Med*. 2024 Mar;56(1):45-53.
- [13]. Steward DL, Carty SE, Sippel RS, Yang SP, Rodgers JA, Sipos JA, et al. Performance of a Multigene Genomic Classifier in Thyroid Nodules With Indeterminate Cytology: A Prospective Diagnostic Cohort Study. *JAMA Oncol*. 2019 Feb;5(2):204-12.
- [14]. Nikiforova MN, Wald AI, Roy S, Duran-Pena A, Santana-Santos L, Jaffe CC, et al. Targeted next-generation sequencing panel (ThyroSeq v3) for comprehensive genetic profiling of thyroid nodules and carcinomas. *Cancer*. 2018 May;124(9):1922-34.