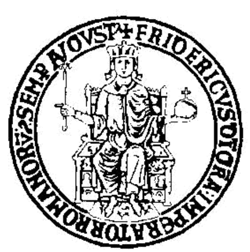
UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II



DIPARTIMENTO DI SANITA' PUBBLICA

DOTTORATO DI RICERCA IN SANITA' PUBBLICA E MEDICINA PREVENTIVA - XXXI CICLO

DEVELOPMENT, VALIDATION, COMPARISON AND CLINICAL IMPLEMENTATION OF DIFFERENT MULTIGENE ASSAYS FOR THE PRE-SURGICAL RISK STRATIFICATION OF INDETERMINATE THYROID FINE NEEDLE ASPIRATIONS.

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Abstract

Background: The aim of this PhD dissertation is to show the development, validation, comparison and preliminary clinical implementation of different multigene-based assays for the pre-surgical risk stratification of indeterminate thyroid fine-needle aspirates (FNA). In particular, this work was focused on the validation and comparison of two commercially multigene assays available for the study of the molecular alterations occurring in thyroid neoplasms, and on the development of a custom next-generation sequencing genomic panel. **Methods**: The two commercially available assays, one based on a real-time PCR (RT-PCR) technology and one based on a next-generation sequencing (NGS) platform were tested on a series of indeterminate thyroid FNA. Moreover, a custom NGS gene panel was designed and tested on a different series of retrospective and prospective FNA samples. **Results**: The commercial NGS panel showed parametric output data that were not sufficient to reliably use this panel on our clinical routine samples. Thus, the RT-PCR assay, which asses BRAF, N-H-KRAS, RET/PTC and PAX8/PPARG genomic alterations, were chosen for the subsequent clinical validation on a prospective series of n=1172 thyroid FNAs. In order to calculate the pre- and post- test risk of malignancy (ROM), only FNA with available histological follow-up (207/1006 adequate FNA, 20.6%) were included in the final study. The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) was adopted for the microscopic diagnosis. FNA classified as atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) showed a 25.9% pre-test ROM whereas post-test ROM was 42.6% in mutation-positives (MT-pos) and 14.5% in mutation-negatives (MT-neg) cases, respectively. Considering the MT-positive cases, the cases harbouring BRAF-like mutations (*BRAFV600E, RET/PTC1, RET/PTC3*) showed a statistically significant higher ROM (80%) than those with RAS-like mutations (*N-H-KRAS, PAX8/PPARγ*) (32.4%, p=0.010). Follicular neoplasm/suspicious for follicular neoplasm (FN/SFN) FNA showed a 44.1% pre-test ROM. Conversely, FN/SFN post-test ROM was 80% in MT-pos and 29.1% in MT-neg

cases. Although BRAF- and RAS-like mutations were associated to different ROM even in FN/SFN cases (100% vs 71.4%), this difference did not reach a statistical significance (p=1,0). Suspicious for malignancy (SFM) FNA showed a 93% pre-test ROM; this latter figure almost overlapped to post-test ROM of both MT-pos (100%) and MT-neg (84.6%) FNAs. In particular, BRAFV600E-mutated FNAs were consistently associated with a papillary carcinoma on histology, irrespective of TBSRTC categories. Moreover, in order to switch from a RT-PCR based technology, that allows the study of alteration in only 7 genes, to a more comprehensive NGS-based multigene assay, we designed and analytically tested the performance of a custom panel. This latter, include beyond the 7-genes, additional genes which may give an additional predictive value when performed on indeterminate thyroid FNAs.

Conclusions: Our preliminary data show that the 7-gene RT-PCR based test may contribute to the risk stratification in AUS/FLUS and FN/SFN categories, thanks to the significant difference in post-test ROM between MT-pos and MT-neg FNAs, confirming the high positive predictive value of BRAFV600E and BRAF-like mutations over the RAS-like genomic alterations. Moreover, the preliminary results of the custom NGS panel were satisfactory enough to expect its effective future adoption on our routine clinical samples.

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Introduction

Thyroid carcinoma represents about 1% of all human malignancies (1). On the overall, 4% to 7% of adults show a palpable thyroid nodules, while in 70% of cases subclinical nodules are present (1-2). The main challenge in a patient with a thyroid nodule remains the exclusion of malignancy because benign nodular thyroid disease represent the highly prevalent condition. In this setting, Fine-Needle Aspiration (FNA) of thyroid nodules is the most accurate and cost-effective tool with which to evaluate thyroid nodules, coupled with the introduction of the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC), a 6-tiered classification system which assign different malignancy risk to the morphological features observed in thyroid FNA, improving the standardization of cytological diagnosis (Table 1) (3). However, the malignancy risk rates associated with the Bethesda diagnostic classes that fall within the "indeterminate" categories (namely atypia of undetermined significance/follicular lesion of undetermined significance, follicular neoplasm/suspicious for follicular neoplasm, and suspicious for malignancy) significantly differ among institutions, thereby leading to ambiguity regarding the correct patients management. In this setting, molecular biology may help to improve the diagnostic accuracy of indeterminate samples avoiding the patients overtreatment when a benign nodules is present, increasing the pre-surgical detection of malignancies (4). Patients management guidelines for thyroid nodules suggest that in the case of an indeterminate lesion a surgical excision should be necessary, because only histological examination is able to make a clear-cut discrimination between benign and malignant lesions (5). To date, none of the clinical, imaging, and cytological parameters proved to be able to identify preoperatively the patients with "indeterminate" FNAB reports who really may benefit of a surgical approach. In order to address and overcome the limitations of cytological examination, many molecular biology techniques (e.g., sanger sequencing, pyrosequencing, Real-Time PCR and next generation sequencing) evaluating the genetic alteration in the relevant signalling pathway associated with the development of thyroid neoplasms (Figure 1) have been implemented or are

under evaluation (4-6).

Diagnostic category	Risk of malignancy (%)	Usual Management
Non-diagnostic or Unsatisfactory	5-10%	Repeat FNA
Benign	0-3%	Clinical and sonographic follow-up
Atypia of Undetermined Significance or Follicular Lesion of Undetermined Significance (AUS/FLUS)	10-30%	Repeat FNA, molecular testing, or lobectomy
Follicular Neoplasm or Suspicious for a Follicular neoplasm (FN/SFN)	25-40%	Molecular testing, lobectomy
Suspicious for Malignancy (SFM)	50-75%	Near-total thyroidectomy or lobectomy
Malignant	97-99%	Near-total thyroidectomy or lobectomy

Table 1. The Bethesda System 2017 for reporting Thyroid Cytophatology.

In this setting many laboratory developed tests (LDT) or commercial available kits designed on signalling pathways related to the development of thyroid lesions were evaluated. However, the increasing understanding of the genetic events involved in thyroid tumorigenesis and progression to more aggressive forms, may lead to the identification of more reliable tumour-specific markers (7). Currently, it is thought that papillary carcinoma and follicular carcinoma arise independently of one another, whereas there is limited evidence to suggest a progression from follicular adenoma to follicular carcinoma. Figure 1 depicts a summary of the known or suspected molecular events that lead to the oncogenesis of papillary carcinoma, follicular adenoma and follicular carcinoma, and finally poorly differentiated and anaplastic carcinoma. Several molecular mutational alterations have been discovered in thyroid cancer during the last three decades (Table 2) (8-9).

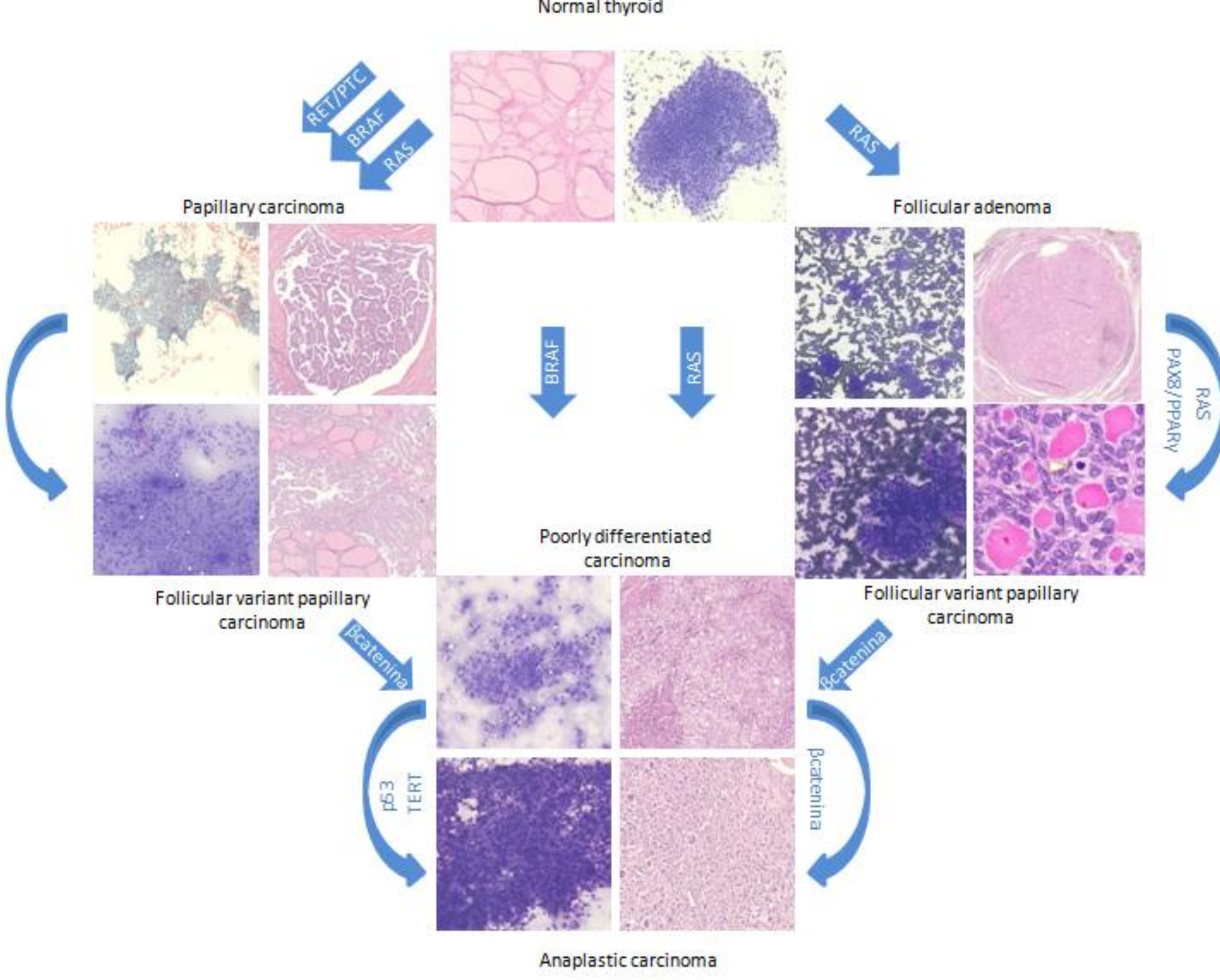


Figure 1. Molecular alterations in thyroid tumors.

Tumor Type	Prevalence %
<u>Papillary Carcinoma</u>	
• BRAF	• 40-45
• RET/PTC	• 10-20
•RAS	• 10-20
<u>Follicular Carcinoma</u>	
• RAS	• 40-50
• PAX8/PPARγ	• 30-35
•PIK3CA	• 10
<u>Poorly differentiated Carcinoma</u>	
• RAS	• 25-30
• β-catenina	• 10-20
• p53	• 20-30
•BRAF	• 10-15
<u>Anaplastic Carcinoma</u>	
• p53	• 70-80
• β-catenina	• 60-70
•RAS	• 40-50
•BRAF	• 20-30

TABLE 2. Average prevalence of mutations in various types of thyroid cancer.

Several groups have determined if genetic mutation analysis can be an useful adjunct technique in "indeterminate" cytology diagnoses. Differences in the utility of tests designed to “rule out” and/or “rule in” malignancy for indeterminate thyroid fine-needle aspirations (FNAs) (10). A simplified management algorithm is shown for illustrative purposes, in which patients with cytologically benign nodules are followed clinically, those with cytologically malignant nodules undergo total thyroidectomy, and patients with cytologically indeterminate nodules undergo further testing (11). Nikiforov et al. recently conducted a prospective study to assess the significance of testing a panel of tumor-specific mutations to improve the preoperative diagnosis of a thyroid nodule (1). Residual material from 1056 consecutive thyroid FNAB samples with indeterminate cytology was used for prospective molecular analysis consisted of *BRAF V600E, RAS, RET/PTC*, and *PAX8/PPARγ* rearrangements. The authors reported that these events were highly specific for detecting malignancy but less sensitive, mainly in nodules classified as "indeterminate". In this category, in fact, the detection of any mutation conferred the risk of malignancy of 88%, while the risk of cancer in mutation-negative nodules was 6% (13).

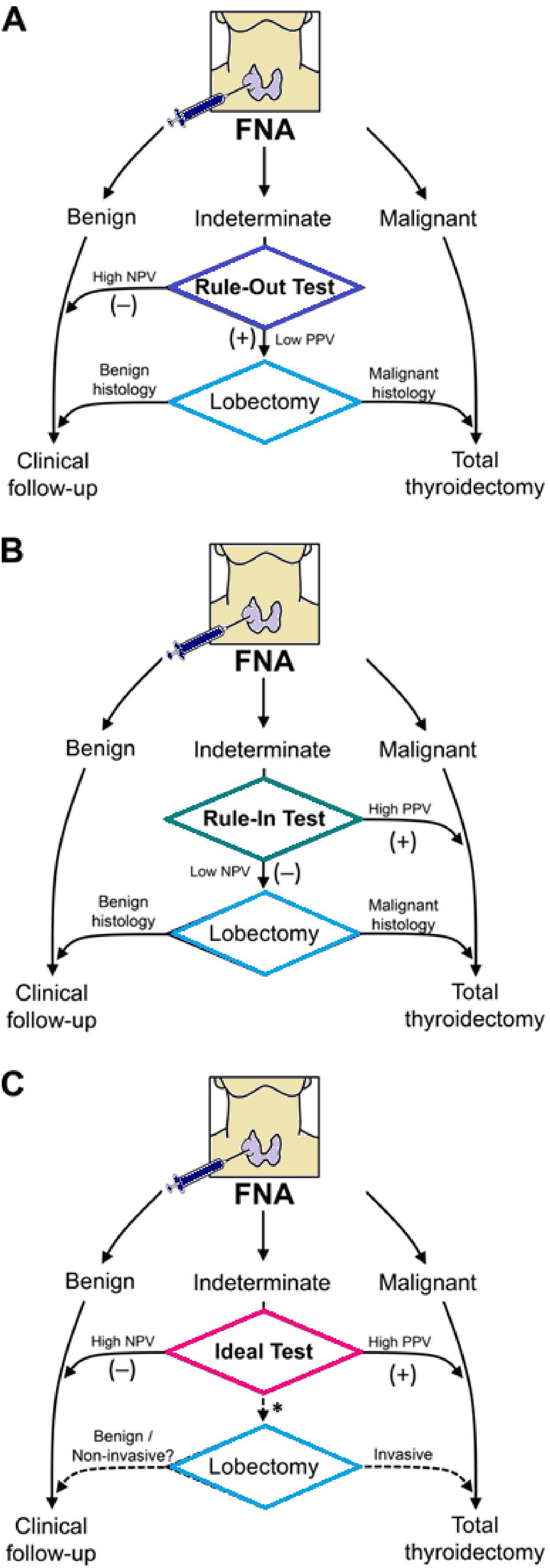

A
FNA
Benign
Indeterminate
Malignant
High NPV
Rule-Out Test
(–)
(+)
Low PPV
Benign histology
Lobectomy
Malignant histology
Clinical follow-up
Total thyroidectomy
B
FNA
Benign
Indeterminate
Malignant
Rule-In Test
High PPV
(+)
Low NPV
(–)
Benign histology
Lobectomy
Malignant histology
Clinical follow-up
Total thyroidectomy
C
FNA
Benign
Indeterminate
Malignant
High NPV
Ideal Test
High PPV
(–)
(+)
*
Benign / Non-invasive?
Lobectomy
Invasive
Clinical follow-up
Total thyroidectomy

Figure 2. Molecular cytopathology for thyroid nodules: (A) Tests optimized to “rule out” malignancy have a high negative predictive value (NPV) a negative (defined as “benign”) result indicates a low cancer risk, triaging a patient to active surveillance and reducing the rate of lobectomies performed for benign nodules. For tests with a low positive predictive value (PPV), a positive (defined as“suspicious”) result indicates an indeterminate risk of cancer and the consideration of diagnostic lobectomy; patients with nodules with benign histology require only clinical follow-up, whereas those with malignant histology may require a completion thyroidectomy. (B) Tests optimized to “rule in” malignancy such as the 7-gene mutation/fusion panel have a high PPV; a positive test result can direct patients to undergo total thyroidectomy upfront, sparing patients the need to return to the operating room for a completion thyroidectomy. A negative test result can not exclude malignancy due to the low NPV, and a diagnostic lobectomy would be advised. (C) The ideal molecular test for patients with cytologically indeterminate thyroid nodules should have a sufficiently high NPV and PPV so that a negative result can safely direct patients to watchful waiting and a positive result can direct patients to a total thyroidectomy, thereby reducing the need for a diagnostic lobectomy (indicated by the dotted lines). In the future, management algorithms may consider lobectomy to be appropriate and sufficient management for patients with nodules with molecular signatures suggestive of a noninvasive follicular variant of papillary carcinoma (indicated by the asterisk). Modified from Nishino et.al (29).

In a literature review by Filicori et al. was established that a mutation analysis model using the prevalence of *BRAF, RET, RAS, and PAX8/PPARγ* mutations to refine "indeterminate" lesions diagnosis. This model was applied retrospectively to a study cohort of 466 consecutive "indeterminate" lesions that underwent hemi- or total thyroidectomy for definitive diagnosis, to evaluate its accuracy for identifying malignancy. Out of 466 "indeterminate lesions" in the study, 30% were malignant. These included 66 cases of papillary carcinoma, 45 cases of follicular variant of papillary carcinoma, 18 cases of follicular carcinoma, and 10 others. The mutation analysis model would correctly identify only 48 of 139 (34%) of malignant "indeterminate" lesions. Therefore, when mutation analysis is negative, the overall risk of malignancy would be 23%. "Indeterminate" lesions with a negative mutation analysis still carry a significant risk of malignancy, requiring surgery for definitive diagnosis (14). Although these data seem to suggest that genetic approach has low sensitivity for "indeterminate" nodules, the preoperative detection of these mutations, particularly for BRAF mutation, may help to identify patients at high risk of recurrence and death (14).

In the past, in relation to the narrow spectrum of genetic alterations analyzed, single gene techniques, such as Sanger Sequencing or Real - Time Based (RT-PCR) approach were largely used in many molecular biology laboratories (15). However, after data with the Human Genome Project were obtained, the need to obtain ore genetic information in clinical practice increase tremendously, leading to the introduction in routine setting of multiplex molecular biology

techniques, e.g. Matrix Adsorption Laser Diode Mass Spectrometry (MALDI) evaluation Time of Fly (TOF) of different nucleic acids and, most important, the Massive and Parallel sequencing approach, also known as Next Generation Sequencing (NGS) (16). Several NGS panels evaluating different genes regions are commercially available but these panels often contain genes or hotspots that are not of particular interest for molecular diagnostics of thyroid neoplasms (17).

Considering all together the data discussed, an important research hot – topic to improve the classification of thyroid lesions is represented by the application of new molecular biology techniques to analyse the clinical relevant gene alterations, starting from cytological samples derived nucleic acids, to develop an integrated morpho – molecular approach leading to better define the patients clinical management.

Aims of this study

The aim of this PhD thesis is to show the development, validation, comparison and clinical implementation of different multigene assays for the pre-surgical risk stratification of indeterminate thyroid fine needle aspirations. In particular, during these three PhD course years, I was actively involved in the validation and implementation in clinical practice of a commercial RT – PCR based assay to study the molecular alterations in 7 different genes (*BRAF, KRAS, NRAS, HRAS, RET/PTC1, RET/PTC3 and PAX8/PPARγ*) in order to further stratify the risk of malignancy (ROM) on a prospective series of n=1156 thyroid FNA.

The second aim of this PhD thesis is to show the preliminary results obtained from the NGS testing of thyroid cytological samples, by using both a commercial (Comprehensive Thyroid and Lung - Archer) and in-house developed custom NGS panel, specifically targeting the genes involved in thyroid oncogenesis.

Chapter 1:
Validation and implementation in clinical practice of a commercial 7 genes RT – PCR based assay.

1.1 Material and Methods:

On the overall, n=1172 FNA were analyzed. In particular n= 755 cases were classified as Atypia of Undetermined Significance or Follicular Lesion of Undetermined Significance (AUS/FLUS), n=140 cases were classified as Follicular Neoplasm or Suspicious for a Follicular neoplasm (FN/SFN), n=111 cases were classified as Suspicious for Malignancy (SM), n=166 were classified as Malignant (M).

Total cases	**1172**
AUS/FLUS	755
FN/SFN	140
SM	111
M	166

Table 3. TBSRTC of analyzed FNA.

From all the selected sample DNA and RNA was simultaneously extracted from each selected slide by using the DNA/RNA All Prep Mini Kit (Qiagen, Hilden, Germany) with a modified protocol, adapted for cytological specimens. The evaluation of nucleic acids concentration was obtained for each sample by using the TapeStation System (Agilent Technologies, Santa Clara, California, USA) with a dedicated software (TapeStation 4200 suite).

1.1.1 DNA and RNA Extraction.

The nucleic acids were extracted using ALL PREP DNA / RNA mini kits (Qiagen GmbH, Hilden, Germany) according to a modified version of the manufacturer's protocol. In particular, the

protocol developed consist of the following steps:

1. add 350 µl of RLT in the sample;
2. Pipette 5 times and vortex for 1 m and spin;
3. Transfer everything to a DNA column for ALL Prep (white cap with lilac ring);
4. Centrifuge for 30 seconds x 13000 rpm and then transfer the column into a new 2 ml tube and store at 4 °;
5. the eluate for RNA extraction and proteins was stored at – 80°C;
6. Add 250 µl of 100% ethanol directly and pipette 5 times (do not centrifuge and do not flicker);
7. Transfer 700 µl to the RNAsi Spin Column and centrifuge 30 seconds x 13000 rpm; take the eluate and transfer it to a 1.5 tube;
8. Store at 4 ° (it will contain the PROTEINS);

For RNA purification:

1. By using the dedicated column a 2 ml tube was mounted again (it will contain the column with the membrane with the RNA);
2. Add 700 µl of RW1 to the spin column and centrifuge for 30 seconds at 13,000 rpm.
3. Discard the sub-matrix ONLY NOW, because now contain nothing.
4. Add 500 µl of RPE to wash and purify RNA, centrifuge for 30 seconds at 13,000 rpm and discard the sub-matrix and reassemble the column;
5. Repeat the same step with RPE and then transfer the tube in a new 1.5ml tube for the elution in 35 µl of RNasi Free Water.
6. Store the samples at – 20°C until a processing.

For DNA purification:

1. Proceed with the sample purification by using two different buffer: AW1 and AW2;
2. Transfer the tube in a new 1.5 ml tube for the elution in 35 µl of DNasi Free Water.

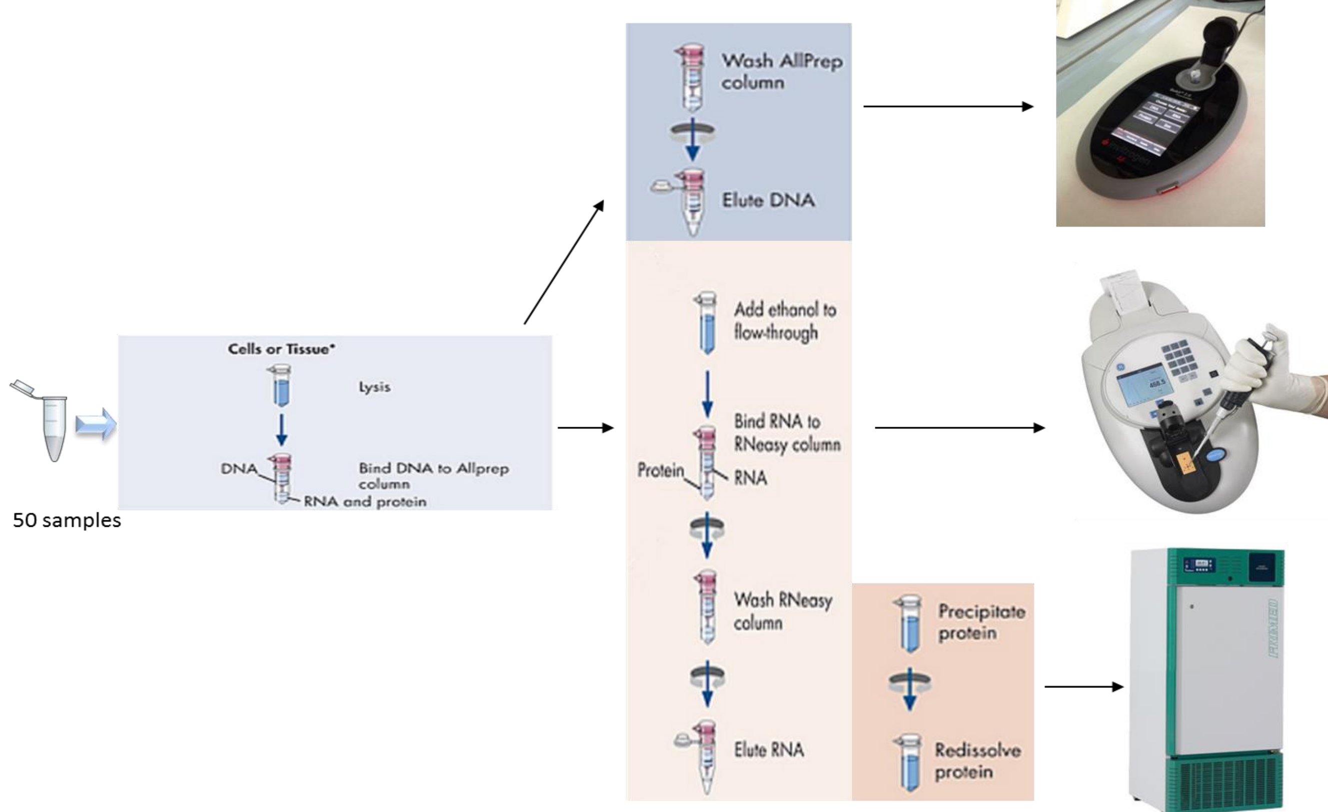


Figure 3. Schematic representation of DNA/RNA extraction protocol from the sample to the storage at -20°C.

The quantity and quality of DNA was evaluated using a capillary electrophoresis TapeStation (Agilent Technologies, Santa Clara, California, USA). In addition, the amount of RNA was also evaluated using a spectrophotometer (NanoDrop, Thermo Scientific, Milan, Italy) and the absorbance values at 260/280 were used to evaluate the purity, in terms of protein contamination, while the values 260/230 absorbance materials have been used to verify the purity.

1.1.2 Real-Time PCR.

The amplification reaction for DNA and RNA was performed using the Thyroid Cancer Mutation Analysis and Fusion Gene Detection kit (Entrogen Woodland Hills, CA 91367, USA) (18). The kit includes the use of TaqMan probes and 8 different mixes to detect different mutations in 7 genes that play a fundamental role in thyroid classification. The Kit reference range is as follows: *BRAF (V600E); KRAS (G12A, G12D, G12R, G12V, G13D, G12C, G12S); NRAS (Q61H: CAC,*

CAT), Q61L, Q61K, Q61R; HRAS (G12V, G13R, Q61R); RET / PTC1; RET / PTC3; PAX8 / PPARɣ.

Following the tests for the technical validation of the kit, the reaction mix was optimized in a final volume of 15 µl for the DNA and 12.5 µl for the RNA. In particular, the DNA mix consists of 2X PCR reaction mix, Primer Mix, nuclease free water and 2 µl sample at a concentration of 2.5 ng/µl. As for the RNA mix, this is composed of 5X enzyme mix, Primer Mix, Manganese Acetate, nuclease free water and 2.5 µl sample at a concentration of 2 ng/µl. The amplification reactions were set up in duplicate on 384-well plates and ran on Quant Studio 5 Real-Time (Life Technologies, Massachusetts, USA). For DNA the following thermal protocol was used: 1 cycle at 95 ° C for 10 min; 40 cycles at 95 ° C for 15 seconds and 40 cycles at 60 ° C for 1 min; the temperature increase from 60 to 95 ° C is 0.1 ° C per second. For RNA, the kit provides a single step of reverse transcription and amplification and the reaction is subject to the following thermal protocol: 1 cycle at 55 ° C for 5 min; 1 cycle at 60 ° C for 5 minutes; 1 cycle at 65 ° C for 5 minutes; 40 cycles at 95 ° C for 10 seconds and 40 cycles at 60 ° C for 45 seconds; the temperature increase from 60 to 95 ° C is 0.1 ° C per second. The results obtained were analyzed using the QuantStudio Design & Analysis software (Lifetechnologies, Massachusetts, USA).

1.1.3 Analysis of sensitivity and specificity.

Before the implementation of the Thyroid Cancer Mutation Analysis and Fusion Gene Detection kit (Entrogen Woodland Hills, CA 91367, USA) in clinical practice, we evaluated the analytical sensitivity and specificity by using a sample set previously tested by a validated Next Generation Sequencing panel. In particular, n = 1 *BRAF V600E*; = 1 *KRAS G12D*; n = 1 *NRAS Q61H* and n = 1 wild - type samples were selected basing on the reference range reported by the manufacturer of Thyroid Cancer Mutation Analysis and Fusion Gene Detection kit. In particular, for each sample, three dilution points of the mutated allele were prepared: 10%, 5% and 1%, to evaluate the limit of detection (LOD) for the selected mutations. Furthermore, to evaluate the reproducibility, the

reported experiment was repeated twice in two different time from two different operators.

1.2 Data Interpretation

RT-PCR was feasible in the majority of cases (1069 of 1172 cases; 91.2%); only n=103 cases (8.8%) were failed. In particular, n=10 (0.8%) were completely failed samples, conversely n=2 cases are failed in the amplification for DNA while, in majority of cases, n=91 were failed on amplification for RNA. The mean and median DNA yields of the successfully processed samples were 8.75 ng/µl and for RNA 2.92 ng/µl, respectively (range, 0.0281-60 ng/µl for DNA, while for RNA range were 0.1-36.3 ng/µl). It is interesting to note that in the majority of processed samples (75.7%) featured suboptimal DNA and RNA material was classified as AUS/FLUS.

1.3 Results

1.3.1 Molecular Analysis

BRAF, *RAS*, *RET* and *PAX8/PPARγ* somatic variants were identified in 88 of 1069 samples. In particular in the category of AUS/FLUS (n=116/1069 we have found n=47 MT and n=58 WT. In the FN/SFN category (n=34/1069) we have found n=10 MT and 22 WT, while in the SFM category (n=57/1069), the mutation rate was n=31 MT and n=21 WT (Table 4).

	BRAF	HRAS	NRAS	KRAS	RET/PTC	PAX8/PPARγ	WT	INADEQUATE
AUS/FLUS	10	14	18	1	1	3	58	11
FN/SFN	3	2	5	0	0	0	22	2
SFM	28	0	3	0	0	0	21	5

Table 4. Distribution of gene mutations in relation to samples classification.

1.3.2 Clinical interpretation

Our preliminary data show that the 7-gene RT-PCR based test may contribute to the risk stratification in AUS/FLUS and FN/SFN categories, thanks to the significant difference in post-test ROM between MT-pos and MT-neg FNAs, confirming the high positive predictive value of *BRAFV600E* and *BRAF-like* mutations over the *RAS-like* genomic alterations.

Chapter 2:
Performance evaluation of Archer- Comprehensive Thyroid and Lung (CTL) NGS Panel.

2.1 Materials and Methods:

Touch imprint cytology is a well known rapid histopathological method of intraoperative analysis of biopsy specimens and allows the extraction of good quality and quantity DNA and RNA respect to conventional cytological and histological samples. To preliminary investigate the performance of Archer- Comprehensive Thyroid and Lung (CTL) Panel a serie of n. 8 touch imprint was produced from eight different fresh thyroid tissues. In particular n.6 samples showed an histological diagnosis of Papillary Thyroid Carcinoma (PTC), n. 2 samples were diagnosed as follicular variant of PTC. (Table 5)

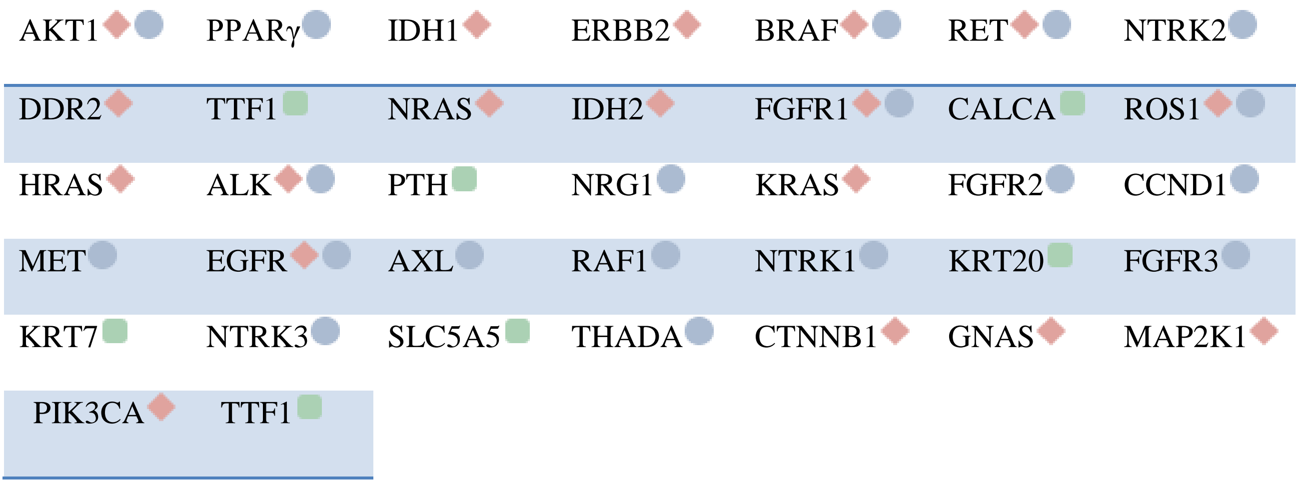
#	ID	Bethesda System	Histology
1	18/9598	Malignant	PTC
2	18/3561	Suspicious for Malignancy	PTC
3	18/10633	Suspicious for Malignancy	fvPTC
4	18/8786	Suspicious for Malignancy	fvPTC
5	18/6951	Malignant	PTC
6	18/7691	Malignant	PTC
7	18/7887	Follicular Neoplasm or Suspicious for a Follicular Neoplasm	PTC
8	18/19142	Malignant	PTC

Table 5. Sample set selected to produce the touch imprints.

DNA and RNA were simultaneously extracted from each touch imprint following the previously reported adapted protocol and the quantity and quality of extracted nucleic acids were assessed by using TapeStation 4200 platform (Agilent Technologies, Santa Clara, California, USA) as previously described (section 1.1.1). In addition, each DNA and RNA was analyzed by our standard RT - PCR procedure by using the Thyroid Cancer Mutation Analysis and Fusion Gene Detection kit.

2.1.1 Archer FusionPlex Comprehensive Thyroid and Lung NGS Kit (CTL).

The Archer® FusionPlex® Comprehensive Thyroid and Lung (CTL) Kit is a targeted NGS panel designed to detect gene fusions, SNV, indels, splicing and gene expression in 36 genes (Table 6) associated with lung and thyroid cancers (19).



AKT1◆●	PPARγ●	IDH1◆	ERBB2◆	BRAF◆●	RET◆●	NTRK2●
DDR2◆	TTF1■	NRAS◆	IDH2◆	FGFR1◆●	CALCA■	ROS1◆●
HRAS◆	ALK◆●	PTH■	NRG1●	KRAS◆	FGFR2●	CCND1●
MET●	EGFR◆●	AXL●	RAF1●	NTRK1●	KRT20■	FGFR3●
KRT7■	NTRK3●	SLC5A5■	THADA●	CTNNB1◆	GNAS◆	MAP2K1◆
PIK3CA◆	TTF1■					

Legend:

- ◆ SNV/Indel
- ■ Expression
- ● Fusion, splicing or exon-skipping

Table 6. Gene list covered by Archer® FusionPlex® Comprehensive Thyroid and Lung

2.1.2 cDNA preparation

cDNA was produced by using a random examer primer mix starting from 20 ng of extracted RNA. in a 20 µL mix for each sample. The retrotrascription of RNA into cDNA was obtained by using an incubation at 65°C for 5 min followed by a storage at 4°C. To obtain a second strand of cDNA produced in the first reaction, a second 20 µL mix was prepared for each sample adding a new set of random examer primers. the thermal condition to obtain a second cDNA strand were as follow: 25°C for 10 min; 42°C for 30 min; 80°C for 20, with a final step at 4°C. The ends of synthesized cDNA were repaired by using the End Repair mix (Archer) following the

manufacturer's instructions with an incubation of 25°C for 30 min. After the end repair process, the full constructed cDNA were cleanup with the Agencourt AMPure Beads (Beckman Coulter, Brea, California, USA) by using a standard protocol. The prepared full constructed and purified cDNAs were assessed by using PreSeq RNA QC assay protocol.

2.1.3 PreSeq RNA QC assay.

To evaluate the amplifiability of produced cDNA, we prepare qPCR reaction mix for duplicate analysis of each diluted cDNA sample and one No Template Control (NTC) made from 10 µL Ultra-Pure Water, for quantified and control the RNA that then following the Archer protocol according to the manufacturer's instructions. Six microliters of the reaction mix were added into each assigned well of a qPCR plate/tube, then 4µL of the diluted cDNA samples or NTC into assigned wells/tubes containing reaction mix were added. The prepared mix was slowly vortexed to avoid introducing bubbles. Prepared reactions were amplified following the reported thermal conditions (Table 7) :

	Step	Temperature °C	Time- Fast (or standard) Mode	Cycle
PreSeq RNA QC Assay (Real-Time PCR Thermal Cycler Program)	Activation	95	20 (20*) seconds	1
	Denaturation	95	3 (15*) seconds	35
	Primer Annealing and Extension	60	30 (60*) seconds	
	Melt-curve gradient	60-95	0.5°C/sec increment	1

Table 7. Thermal conditions for libraries preparation for the Archer FusionPlex Comprehensive Thyroid and Lung panel.

The samples showing a Cycle Threshold (Ct) within 28 were considered adequate for library preparation.

2.1.4 Library Preparation and Sequencing

The libraries were prepared following the Archer Fusion Plex Protocol adapted for Ion Torrent platforms, by using specific Archer barcode set (MBC Adapter*)* with a two step PCR amplification process (one conventional PCR and one emulsion PCR). In particular, barcoded libraries were combined in a pooled library for manual template preparation by emulsion PCR on the Ion OneTouch 2 system (Thermo Fisher Scientific). The pooled library concentration was increased from the 2 µl suggested by the manufacturer to 6 µl to obtain a higher number of charged ion sphere particles without having an unacceptable rate of polyclonal reads. The template ion sphere particles were enriched and the sequencing primers and polymerase were added, loaded on 316 chips, and sequenced on the PGM sequencer with the Ion PGM Sequencing 200 Kit v2 (Thermo Fisher Scientific), according to the manufacturer's instructions.

2.2 Data interpretation.

Data analysis and interpretation were based on the Ion Torrent Suite Software V.5.0.3 (ThermoFisher Scientific) workflow by using the hg19 human as a reference genome, the RNAseq plugin (version 5.0.3.5) was applied for the automatic variant annotation. In addition, BAM files were used for visual inspection by using the Golden Helix Genome Browser (version 2.0.7;Golden Helix Inc, Bozeman, Mont), and only the mutations reported in the COSMIC database (http://cancer.san-er.ac.uk/) were taken into account. Post sequencing metrics (number of mapped reads, the average base depth, the percentage of on-target reads, and uniformity were recorded for each sample. No minimal parametric thresholds were considered and all variant calls occurring in the relevant thyroid genes underwent verification by Real-Time.

2.3 Results.

RNA quantity and Quality:

TapeStation 4200 platform was used to assess the quantity and quality of RNA extracted from touch imprints. The results are reported in the following table (Table 8):

ID	Quantity ng/µl	RIN
1	38.7	3.8
2	12.6	4.0
3	41.5	3.8
4	10.2	4.2
5	34.8	4.6
6	58	4.0
7	2.5	3.8
8	1.35	3.8

Table 8. Quantity and quality evaluation of sample by TapeStation.
The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. Using intact RNA is a key element for successful RT-PCR analyses or other technologies .

2.3.1 PreSeq RNA QC assay.

RT - PCR Sybr Green (Biorad) based approach on QuantStudio 5 instrument (Thermofisher) was used to evaluate the amplifiability of cDNA obtained starting from 20ng of extracted RNA. A Ct value within 28 cycle was considered as adequate to proceed with library preparation. The obtained results were reported in the following Table 9 and Figure 5:

#	ID	[ng/µl]	Ct value	bp	[Library pg/µl]
1	9598	38,7	26,62	276	4770
2	3561	12,6	29,952	222	4800
3	10633	41,5	31,639	157	9230
4	8766	10,2	28,583	228	4130
5	6951	34,8	29,618	230	4220
6	7691	58	29,313	244	5580
7	7887	2,5	19,293	X	X
8	19142	1,35	21,111	X	X

Table 9. Ct value obtained by using RT - PCR Syber Green based assay to evaluate the quality of cDNA obtained from the reverse transcription of touch imprints derived RNA.

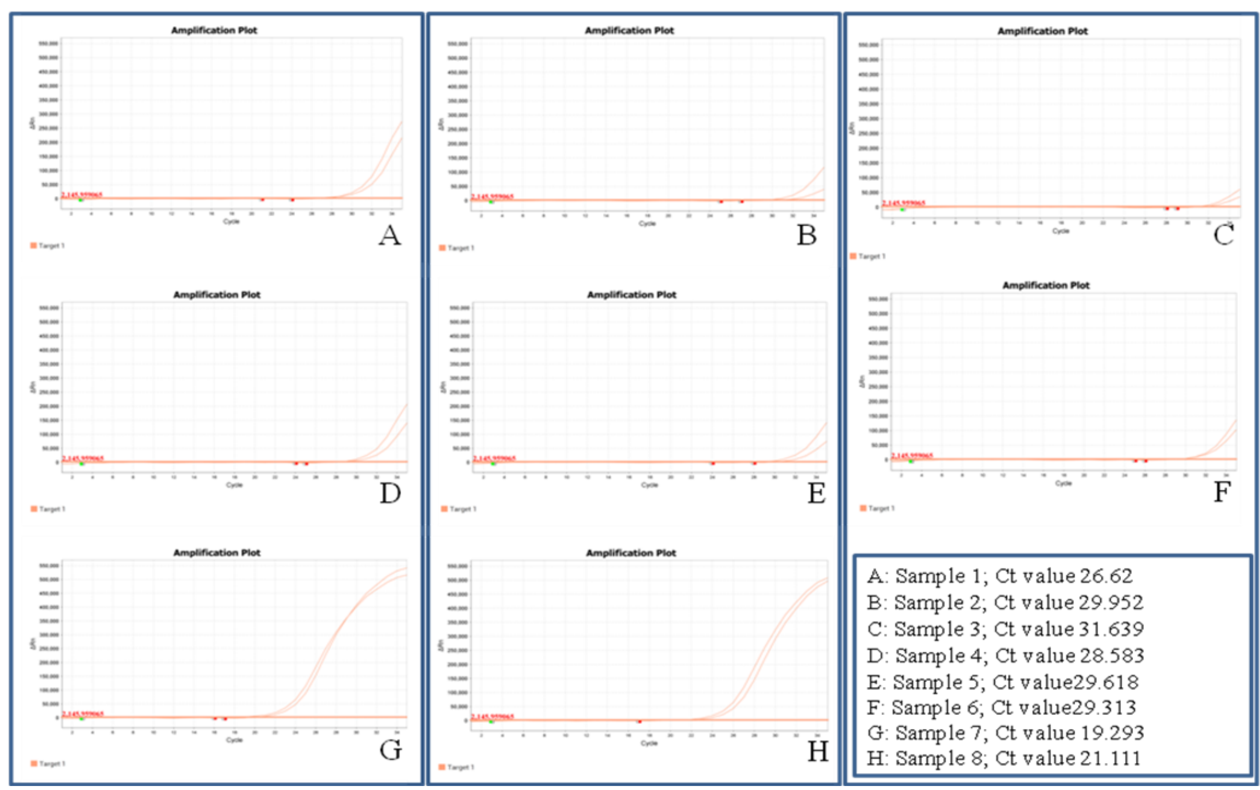


Figure 4. the amplification plots (A - H) obtained from cDNA evaluation by using SyberGreen on QuantStudio 5 instrument and analyzed with QuantStudio Cloud Software are reported. For each amplification plot, on y axis are reported the Relative Fluorescence Unit (Rfu) reached during the amplification process and on x axis are reported the PCR cycles.

2.3.2 Library preparation and Sequencing.

Basing on the data obtained by PreSeq RNA QC assay, only n. 6 samples were able to proceed with the library preparation step. In accordance with the aim of our project, we decide to proceed with all samples to the libraries preparation to evaluate the performance of the Archer® FusionPlex® Comprehensive Thyroid and Lung (CTL) Kit also on suboptimal RNA samples that represent a more closed samples respect to cytological one. All the libraries were quantified and qualified by using High Sensitivity DNA assay on Tape Station 4200 platform and the results were reported in the following Figure 6:

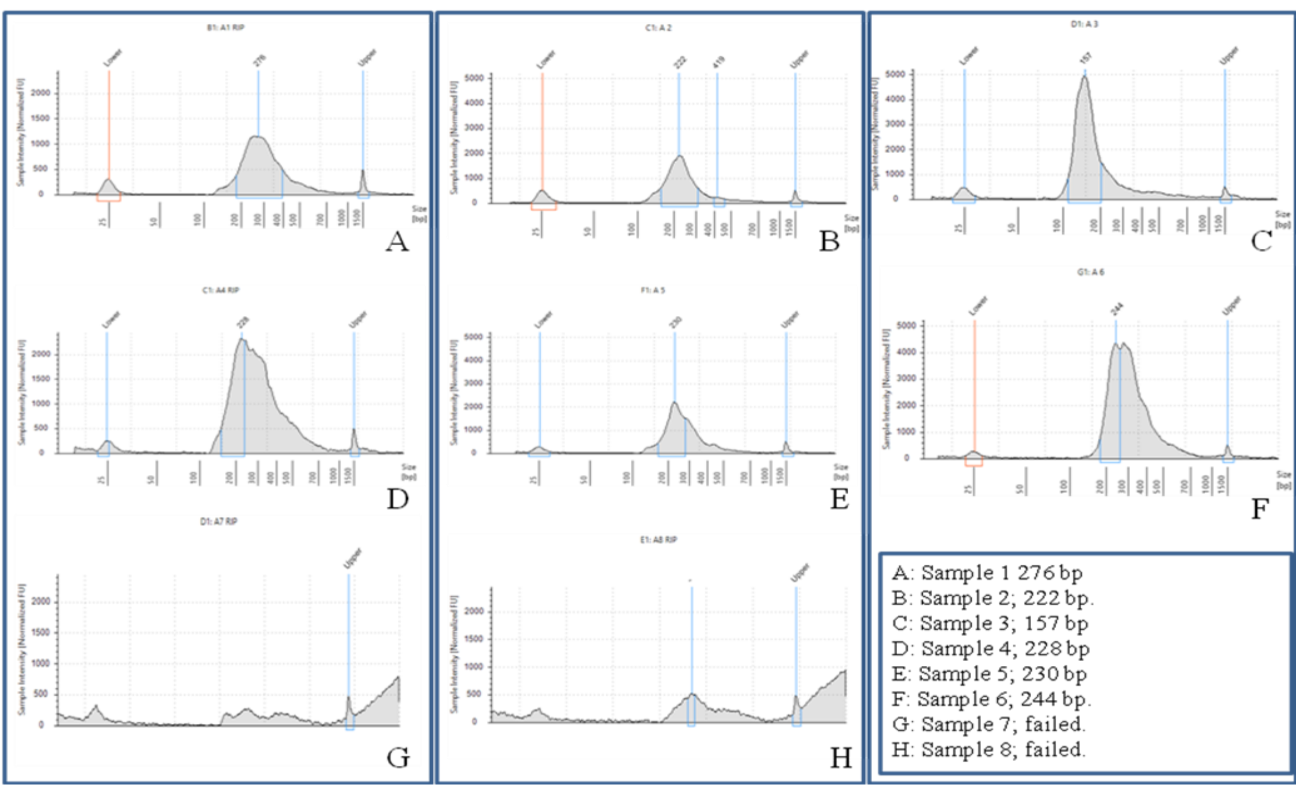


Figure 5. Quantification and Qualification results obtained for each libraries produced starting from cDNA constructed by using 20 ng touch imprint derived RNA. The electropherogram (A - H) report the length spectrum of cDNA libraries fragments and relative quantity. The electropherogram from A to F represents adequate library spectrum, while the results report in electropherogram G and H show an absence of an adequate libraries production.

Basing on the results obtained from Tape Station 4200 analysis, sample number 7 (Panel G - Figure 5) and sample number 8 (Panel H - Figure H) were excluded from pooled library to

proceed with the emulsion PCR for the clonal amplification of cDNA fragments. The pellet recovered form OneTouch recovery tube (thermofisher) was prepared for sequencing, as described in the materials and methods section (section 2.3.2) and the result obtained are summarized in Table 10 and Figure 7.

ISP Summury **708.588 Total reads**	
82% Loading 5,192,328	**18% Empty Wells**
100% Enrichment 5,169,418	0% No Template
54% Clonal 2,815,335	46% Polyclonal
25% Final Library 708,588	0% Test Fragments 0% Adapter Dimer 75% Low Quality

Table 10. Ion Sphere Particles Summary Data

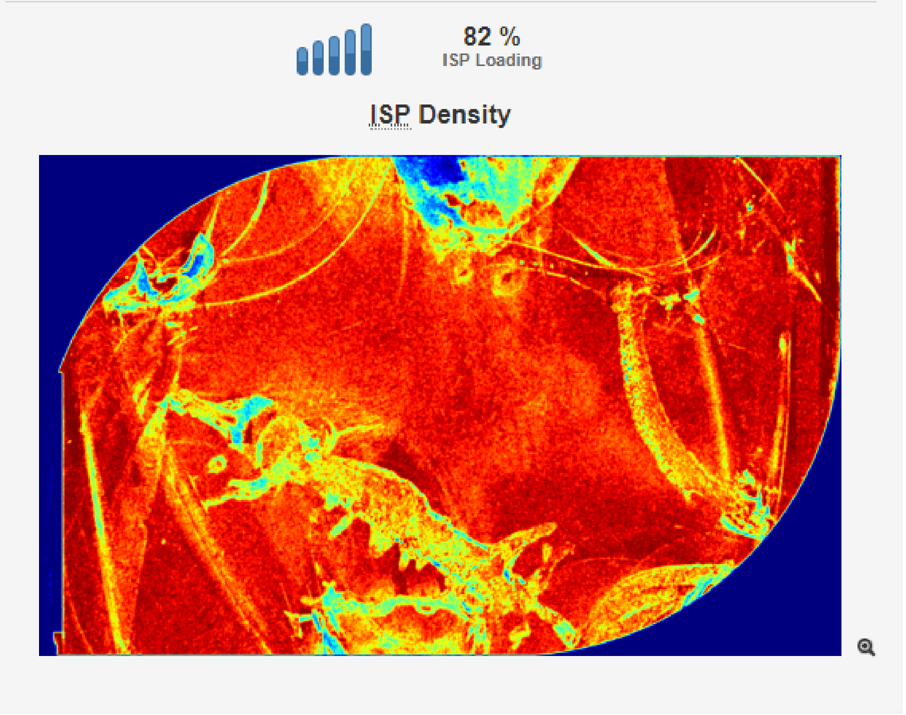


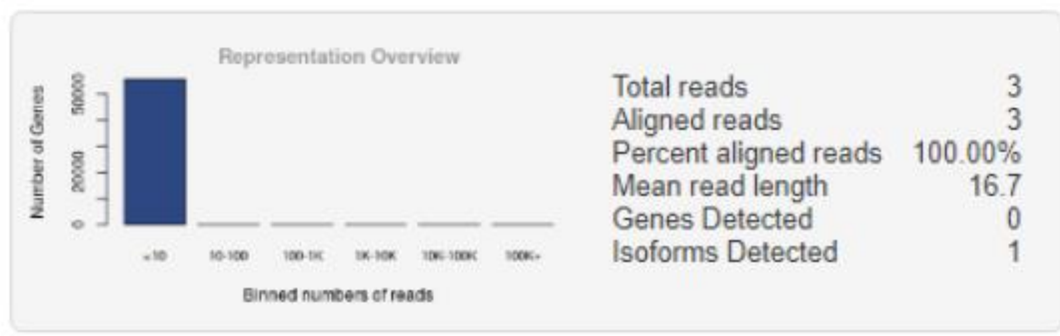
Figure 6.This are the run summary for the PGM.

On the overall, only 2 library were adequately produced and analyzed by using *RNASeqAnalysis plugin*. The obtained report, contained analytical performance parameters and detected variants, was reported below:

RNASeq Analysis Report

Sample Name: 6951

Reference: hg19



Gene Mapping Summary		Base Mapping Summary	
Reference genes	55,765	Total base reads	50
Reads mapped to genes	0	Total aligned bases	44
Genes with 1+ reads	0	Percent aligned bases	88.00%
Genes with 10+ reads	0	Percent coding bases	0.00%
Genes with 100+ reads	0	Percent UTR bases	0.00%
Genes with 10,00+ reads	0	Percent ribosomal bases	0.00%
Genes with 10,000+ reads	0	Percent intronic bases	65.91%
Isoforms Annotated	230,756	Percent intergenic bases	34.09%
Isoforms Detected	1	Strand balance	1.0000

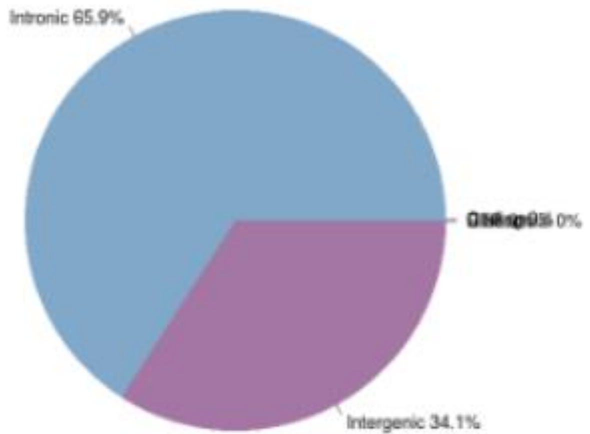


Figure 7. RNA seq analyses by using Torrent Suite with RNA seq Plug-in v.5.0 (Thermofisher).

Chapter 3:
Design, development and validation of custom NGS panel for our routine setting: NexThyro.

3.1 Materials and Methods:

We developed a custom panel to screen hotspots in 9 genes (7 gene and 2 translocations) for clinically relevant mutations in thyroid nodules, as well is in other solid tumor types. Our selection was based on information retrieved from COSMIC and by literature. Key factors determining our gene selection were 1) the present or likely future clinical significance in terms of therapeutic, prognostic or diagnostic value, both from clinical research and pre-clinical data; 2) the frequency of known hotspot mutations within the types of tumors predominantly screened in our laboratory; and 3) the cost per sample, taking into account the number of samples weekly screened and the desired depth of coverage to allow for sufficient sensitivity. We found that the resulting total size of 8 kb is a feasible compromise, offering a sufficiently extensive and clinically relevant mutational profiling in a cost-efficient way.

Here, we describe the design, development and performance evaluation of a custom NGS targeted panel for thyroid cancer.

3.1.1 Design NexThyro panel.

The Ion AmpliSeq Designer suite v5.3.1 with hg19 was used as reference genome to develop a customized fusion panel targeting 9 gene (*BRAF*, *RET*, *NRAS*, *HRAS*, KRAS, *TERT and GNAS*, *RET/PTC and PAX8/PPARγ)* (Tables 11 - 12) that are associated with prognosis of patients.

Gene	chr
BRAF	chr7 [ex.11;ex.15]
RET	chr10 [ex10; 11; 13; 15; 16]
NRAS	chr1 [ex2; 3; 4]
HRAS	chr11 [ex2;3]
KRAS	chr12 [ex2; 3; 4]
TERT	chr5 [ex2;3;4;5;6;7;8;9;10;11;12;13;14;15]
GNAS	chr20 [ex8;9]

Table 11. The table reported the gene that are investigated with the our custom panel "*NexThyro*". This genes have an importance in the carcinogenesis of thyroid tumor.

Fusion Pair
AFAP1-RET
AKAP13-RET
CCDC6-RET
CUX1-RET
ERC1-RET
ERC1_ELKS-RET
FKBP15-RET
GOLGA5_PTC5-RET
HOOK3-RET
KIAA1468-RET
KIF5B-RET
KTN1-RET
NCOA4-RET
PARG-RET
PAX8-PPARG
PCM1-RET
PCM1_PTC4-RET
PRKAR1A-RET
RET-NCOA4
RET-TRIM33
TBL1XR1-RET
TRIM24-RET
TRIM24_PTC6-RET
TRIM27-RET
TRIM33-RET
TRIM33_PTC7-RET

Table 12. In this table are reported the thyroid translocation that are important in the tumor pathway.

A double primer pool leading to the selection of, for the first, 31 amplicons (ranging from 125 to 175 bp) and for the second, 30 amplicons for DNA, and for the RNA, always a double pool of primers was selected for the panel, the first 17 transcripts, and the second pool, 49 transcripts, enabled us to cover all COSMIC annotated mutations (*https://cancer.sanger.ac.uk/cosmic*) in the selected exons of the target genes. The amplicon design covering 8kb of genomic DNA was optimized for the simultaneous analysis of 8 samples (simultaneously DNA and RNA of the same patients) with the 316v2 chip (Thermofisher, Foster

City, CA, USA) on a Personal Genome Machine Torrent (Thermofisher). The panel performance was evaluated in different steps: the analytical sensitivity of the assay was assessed on DNA from two cell lines and by using an artificial reference standard with multiple mutations in different genes. Second, clinical sensitivity and specificity was determined using archival DNA and RNA from 16 patients (2 set of 8 patients), previously genotyped with a RT-PCR.

3.1.2 Library Preparation.

The library was prepared using the Ion AmpliSeq Library Kit 2.0-96LV (Life Technologies) and NexThyro primer pools. Any modifications of the manufacturer's protocol were previously assessed on a pre-study sample set of 16 thyroid FNA specimens with characteristics similar to those of the FNA samples included in the final study. Barcoded libraries were prepared as indicated in the manual kit procedure (ie, with a maximum requirement of 6 µl of DNA extracted from any FNA sample). Thus, the minimum DNA concentration required to obtain 10 ng of total DNA input was 1.6 ng/µl. In the case of samples containing a low amount of DNA (DNA<1.6 ng/µl), the DNA was not diluted further and the quantity of nuclease-free water was reduced accordingly. The number of multiplex polymerase chain reactions (PCRs) was increased from the suggested 20 cycles to 26 cycles, similarly to previous studies (20). Each library was barcoded with the Ion Xpress Barcode Adapters 1-16 Kit (Thermo Fisher Scientific Inc), quantified using the Qubit photometer and the Qubit dsDNA HS (High Sensitivity) Assay Kit (both Life Technologies), and diluted in nuclease-free water to obtain a final concentration of 100 µM.

3.1.3 Emulsion PCR.

Sixteen barcoded libraries were combined in a pooled library for manual template preparation by emulsion PCR (emPCR) on the Ion OneTouch 2 system (Life Technologies). The pooled library concentration was increased from the manufacturer suggested 2 µl to 6 µl to obtain a higher

number of charged ion sphere particles (ISP) without having an unacceptable rate of polyclonal reads (data not shown). As previously described (21), the template ISPs were enriched, and the sequencing primers and polymerase were added, loaded on 316 chips and sequenced on the PGM sequencer with the Ion PGM Sequencing 200 Kit (Life Technologies), according to the manufacturer's instructions.

3.2 Data Analysis.

Signal processing and base calling were carried out using the default base-caller parameters on Torrent Suite [v.5.0.2] and coverage analysis was performed using *NexThyro* designed bed files with coverage plug-in (v.5.0.2.0). BAM files were visually inspected with the Golden Helix Genome Browser v.2.0.7 (Bozeman,MT,USA). Variants were automatically annotated using variant caller plug-in (v.5.0.2.1) at specific optimized parameters of the *NexThyro* panel. In particular, only variants with 5X allele coverage and a quality score 20X, within an amplicon that covered at least 500X alleles, were called.

3.3 Results.

3.3.1 Sequencing Performance.

The experiments carried out on serial dilutions (10%; 5%; 1%) of mutated (BRAF V600E and RET – PTC1) cell lines derived nucleic acid showed an a limit of detection of 1% (data not reported) for both DNA and RNA *NexThyro* primers pool.

We have analyze n= 16 samples for the DNA and n=16 samples for the RNA, in two different NGS run. As reported in the Material and Methods section, the NGS analysed samples were previously tested on the Real-Time and, in particular, n= 7 samples classified as AUS/FLUS, n= 2 samples classified as FN/SFN and n= 6 samples classified as Malignant showing a specific mutation pattern (reported in Table 13) were processed by using *NexThyro* panel.

The mean and median library yields of the successfully processed samples were 34.4 ng/µl respectively (range, < 0.5 - 109 ng/µl). Only n=4 samples were suboptimal to proceed at sequencing. The samples characteristics and the analytical results obtained were reported (Table 13):

ID	Code	Library	Diluition		Bethesda System	Molecular diagnosis in RT-PCR	NGS *NextThyro*
			Sample	Water			
1	Dna17/1296	29.1	5.15	194.85	FN/SFN	MT NRAS	OK
2	Dna 17/12309	69.3	5.41	494.59	AUS/FLUS	MT HRAS	OK
3	Dna 17/12437	10.2	7.35	92.65	Malignant	MT BRAF	OK
4	Dna 17/5797	1.41	7.98	7.02	Malignant	WT	OK
5	Dna 17/6059_1	62.7	5.98	494.02	AUS/FLUS	WT	OK
6	Dna 17/8923_1	54.7	6.86	493.14	Malignant	WT	OK
7	Dna 16/13176	109	4.82	695.18	AUS/FLUS	WT	OK
8	Ras 34487	20.2	7.43	192.57	Colon	G13D	OK
9	Rna 17/1296	<0.50			FN/SFN	WT	
10	Rna 17/12309	<0.50			AUS/FLUS	WT	
11	Rna 17/12437	42.2	3.55	196.45	Malignant	WT	OK
12	Rna 17/5797	<0.50			Malignant	WT	
13	Rna 17/6059_1	8.04	4.66	45.34	AUS/FLUS	RET/PTC3	OK
14	Rna 17/8923_1	36.2	4.14	195.86	Malignant	RET/PTC1	OK
15	Rna 16/13176	74.3	5.05	494.95	AUS/FLUS	Pax8/PPARγ	OK
16	Rna 17/5733	<0.50			AUS/FLUS	WT	

Table 13: The table reported the quantity of the library quantified with the fluorometer Qubit (Thermo Fisher Scientific Waltham, Massachusetts, Stati Uniti). The Bethesda Classification for each samples and the molecular diagnosis after the sequencing was also reported.

Regarding the second NGS run, we analyzed n= 2 samples classified as FN/SFN, n=4 samples that were classified as SFM and n= 8 samples classified as Malignant (Table 14) previously assessed by using our validated RT – PCR approach (RT – PCR mutational status was reported in Table 14).

The mean and median library yields of the successfully processed samples were 33.3 ng/µl

respectively (range, < 0.5 - 106 ng/µl). Only n=4 samples were suboptimal to proceed at sequencing. The results obtained were reported in the following Table (Table 14):

ID	Code	Library	Diluition		Bethesda System	Molecular diagnosis in RT-PCR	NGS *NextThyro*
			Sample	Water			
1	Dna 18/5297	81.7	4.59	495.41	SFM	MT BRAF	OK
2	Dna 18/5338	61.3	6.12	493.88	SFM	MT BRAF <?	MT K601E
3	Dna 18/5316	58.6	6.40	493.60	Malignant	MT BRAF	OK
4	Dna 18/5378	<0.50			Malignant	WT	
5	Dna 18/5580	24.8	6.05	193.95	Malignant	MT NRAS	OK
6	Dna 18/2897	<0.50			Malignant	WT	OK
7	TERT 19887	106	3.54	496.46	?	?	OK
8	Ras 18/1052	79.9	4.69	495.31	Colon	G13C	failed
9	Rna 18/5297	13.3	5.64	94.36	SFM	WT	OK
10	Rna 18/5338	<0.50			SFM	WT	
11	Rna 18/5316	<0.50			Malignant	WT	
12	Rna 18/5378	49.1	7.64	492.36	Malignant	WT	OK
13	Rna 18/5580	0.55			Malignant	WT	OK
14	Rna 18/2897	51.2	7.32	492.68	Malignant	MT RET/PTC1	OK
15	Rna 17/5733	4.03	9.31	40.69	FN/SFN	WT	OK
16	Rna 17/1296	0.85			FN/SFN	WT	OK

Table 14: The table reported the quantity of the library quantified with the fluorometer Qubit (Thermo Fisher Scientific Waltham, Massachusetts, Stati Uniti).
The Bethesda Classification for each samples and the molecular diagnosis after the sequencing.

Discussion and Conclusions:

The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) lead to an improvement in the reproducibility of the FNA diagnosis of thyroid nodules enabling the cytologists to recommend a clinical follow-up or a surgical approach according to the ROM of each TBSRTC class (22). However, basing only on morphology, up to 30% of thyroid FNA samples are classified as indeterminate (1-2). In relation to different experiences, various diagnoses are included in the indeterminate category: AUS/FLUS and follicular neoplasm/ suspicious for follicular neoplasm (FN/SFN) only or AUS/ FLUS and FN/SFN plus the suspicious for malignancy (SM) diagnostic category. The FN/SFN and SM diagnostic classes have intermediate (15%-30%) to high ROMs (60%-75%) that warrant diagnostic lobectomy in the former case and oncologic thyroidectomy in the latter case (3-4). In particular, AUS/FLUS includes low-risk cases that could be managed with follow-up plus FNA repetition as recommended by TBSRTC (23). The implementation of molecular techniques could be useful to further stratify the malignancy risk of the indeterminate FNAs. As showed by our results, the 7-gene test may be useful to refine at least FNA diagnoses as AUS/FLUS and FN/SFN (7). However, despite the high specificity of *BRAFV600E* and *RET/PTC* as markers of malignancy, the clinical usefulness of the 7-gene test is limited by the low specificity and low positive predictive value of RAS and RAS-like mutations (*PAX8/PPARγ* and *BRAFK601E*) because these mutations are also detected in such benign thyroid nodules as follicular adenomas (24). In addition, the indolent encapsulated PTC follicular variant recently reclassified as a low-risk neoplasm (non-invasive follicular thyroid neoplasm with papillary-like nuclear features [NIFTP]) accounts for a large proportion of RAS-mutated nodules (25). However, it remains to be established whether mutation-positive adenomas and NIFTP are follicular-patterned in situ carcinomas and should be considered as true-positives in the evaluation of the positive predictive value of the 7-gene test. To evaluate the potential impact of NIFTP on this series, we reviewed the available histology of benign samples originally classified as

follicular adenomas, but none of them met the criteria for an NIFTP diagnosis (26). Unfortunately, surgical follow-up was available only for a limited number of patients. In fact, at our institution, we have a facility dedicated to the ultrasound-guided FNA of thyroid nodules that serves patients referred from a different part of South Italy (27). However, not all patients with a cytological or molecular diagnosis (e.g. *BRAFV600E*) that recommends a surgical approach choose to be treated at our institution (eg, patients may prefer private/community hospitals near them) (28). We argue that these patients underwent surgery outside our institution, and we were not able to correlate the molecular findings with the histological outcomes. This represents a limitation of our study, although it reflects our local practice.

To overcome the limitations of the 7-gene test to improve the clinical management of these patients a pilot study aiming to evaluate the performance of NGS to extend the number of genes investigated simultaneously for each patients starting from cytological samples derived nucleic acids was conducted. In particular, as a first step, a commercially available NGS panel (Archer- Comprehensive Thyroid and Lung Panel) designed to cover 37 genes (Table 6) starting only from RNA was evaluated on a series of n. 8 touch imprint produced from eight different fresh thyroid tissues. In particular n.6 samples showed an histological diagnosis of Papillary Thyroid Carcinoma (PTC), n. 2 samples were diagnosed as follicular variant of PTC. Touch imprint cytology is a well known rapid histopathological method of intraoperative analysis of biopsy specimens and allows the extraction of good quality and quantity DNA and RNA respect to conventional cytological and histological samples. However, by using this approach, only 2 samples (2/8; 25%) results adequate for sequencing data analysis. Considering the low performance obtained by using this Archer- Comprehensive Thyroid and Lung Panel, a custom NGS panel (*NexThyro*) covering 9 ((*BRAF*, *RET*, *NRAS*, *HRAS*, KRAS*, TERT and GNAS*, *RET/PTC and PAX8/PPARγ)* (Tables 11 - 12) was designed and developed in combination with dedicated bed files to analyse the sequencing data obtained starting from low DNA and RNA

quantity. In particular, a double primer pool leading to the selection of, for the first, 31 amplicons (ranging from 125 to 175 bp) and for the second, 30 amplicons for DNA, and for the RNA, always a double pool of primers was selected for the panel, the first 17 transcripts, and the second pool, 49 transcripts, enabled us to cover all COSMIC annotated mutations in the selected exons of the target genes. The amplicon design covering 8 kb of genomic DNA was optimized for the simultaneous analysis of 8 samples (simultaneously DNA and RNA of the same patients) with the 316v2 chip (Thermofisher, Foster City, CA, USA) on a Personal Genome Machine Torrent (Thermofisher). The analytical sensitivity of the assay was assessed on DNA from cell lines. The experiments carried out on serial dilutions (10%; 5%; 1%) of mutated (BRAF V600E and RET – PTC1) cell lines derived nucleic acid showed an a limit of detection of 1% (data not reported) for both DNA and RNA *NexThyro* primers pool. As an explorative analysis, the clinical performance of *NexThyro* were assessed by using archival DNA and RNA from 16 patients, previously genotyped with a RT-PCR. The preliminary results obtained by using our custom NGS panel were satisfactory enough to expect, after an extensive clinical validation, its effective future adoption on our routine samples.

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