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COMPARISON OF DNA YIELD FROM STAINED AND UNSTAINED FNAC SMEARS BY MAGNETIC METHOD

Anesthesiology

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ABSTRACT

Introduction: A constant demand for quick delivery of reports often puts cytologists under a lot of pressure, so it is crucial to evaluate sample adequacy early. Cytologic samples provide a robust source of material for molecular studies. However, the optimal technique of DNA extraction is crucial to maximize the DNA yield. **Methods:** In this study, we outline our protocol for obtaining DNA from unstained and stained cytologic materials. A total of 50 paired samples were taken, for each sample 2 unstained dried slides were taken of which one slide was stained either with Giemsa or PAP and extraction done by magnetic method. The extracted DNA from stained and unstained FNAC smears was determined by using PCR, ABL ct differences and the purity of DNA. **Results:** Of the 2 sample types compared in this study, unstained FNAC samples had relatively higher DNA concentration and quality compared to stained FNAC samples. The method is simple, requires no destaining steps or extensive manipulation of the sample, and yields sufficient DNA for the standard performance of routine molecular studies. **Conclusions:** As a result, unstained smears are superior to stained smears in assessing sample adequacy, and further biomarker studies may thus be recommended to prove the unstained sample utility in routine practice of cytology clinics. This is the first study proving that magnetic method can be used for unstained FNAC samples.

KEYWORDS

FNAC, unstained slide, stained slide, DNA, RT-PCR

INTRODUCTION

In inpatient care, molecular technology is increasingly recognized for its role. Molecular laboratories are being asked to evaluate more genes at the same time, with smaller volumes of tissue. For molecular oncology testing formalin-fixed paraffin-embedded (FFPE) core needle biopsies, cell blocks from fine-needle aspirations (FNAs), and fresh FNA material in solution are common samples submitted (1).

The discipline of molecular diagnostic cytopathology is relatively new in and of itself. FNAC samples had also been used for clinical diagnosis, molecular profiling with impulsive results. Fine needle aspiration cytology is a rapid, safe, and relatively inexpensive procedure for predicting cancers and inflammatory lesions (2).The ability to use FNAC slides greatly increases the potential material available for molecular analysis (3). FNAC material for molecular testing is being used with nucleic acids extracted from stained slides for mutation or pathogen detection. In addition, the use of archival cytology slides greatly increases the possibility of selecting patients for targeted therapies based on molecular analysis (4), and this principle may be extended to tests on remotely obtained samples. The objective of this study was to present the use of DNA from unstained FNAC samples for real time pcr target amplification, as well as observations about their utility for molecular profiling.

METHODS AND MATERIALS

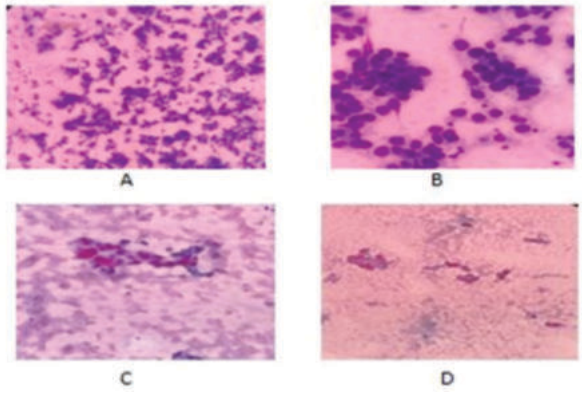


Fig 1 : Giemsa stained slides are A & B, A is 40x & B is 100x magnification. Pap stained slides are C&D, C is 40x & D is 100x magnification

Patients And Specimens:

This is a prospective study of three months from July –Sep, 2021 approved by the institutional ethics review committee (IEC/2021/146.1). FNAC slides of thyroid, breast, liver, lung and lymph nodes (Axillary and supraclavicular) were obtained from cytology department. The extra fnac slides which were kept aside (after taking required slides for reporting and storing) were taken for this study. A total of 50 paired samples were taken, for each sample 2 unstained dried slides were taken of which one slide was stained either with giemsa (5) or pap (6) as per the protocol. Giemsa and Pap staining were done on 25 slides each (Fig 1).

DNA Extraction from stained and unstained FNAC smears:

Cellularity and tumor percentage were determined by cytopathologist after review. Coverslips from stained slides were detached by soaking in xylene at 37°C for 24–48 hours and then rehydrated in 95% ethanol and air-dried and cells were scraped from slide with blade (from air dired unstained slides cells were scraped the same). With a loose pipette tip all the material was collected into a small clump on a butter paper and then transferred into the eppendorf tube for DNA extraction from Nucleomag Tissue Kit from Mancherey-Nagel (MN) company (Cat no.744300.4) as per the company protocol. As this kit is for ffpe samples, to use this on scraped cells deparffinization was avoided and procedure was started from lysis of cells. By measuring the absorbance ratio at 260/280nm & 260/230nm by spectrophotometer quantity and quality of DNA were determined.

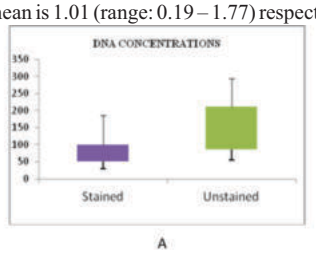
Real-Time PCR

FNAC DNA samples, from 10 Giemsa stained with their paired unstained slides and 10 PAP stained where paired unstained slides were taken for real time PCR amplification. ABL gene was used for real time taqman pcr as reference gene as per company's protocol (3B black biotech BCR ABL KIT, CAT NO), to check for the amplification efficiency of stained and unstained FNAC DNA samples. Real time pcr for EGR, BRAF and MTB detection were done from DNA of unstained FNAC slides of lung, thyroid and Axillary node as per kit protocol. RGQ EGFR Real time PCR kit (Cat.No : 870121, Qiagen) was used for EGFR mutation detection, TRUPCR BRAF kit (Cat.No : 3B1287) for BRAF mutation detection and TRUPCR® MTB/NTM nested real-time PCR Kit (Cat No: 3B268) for MTB pathogen detection were used

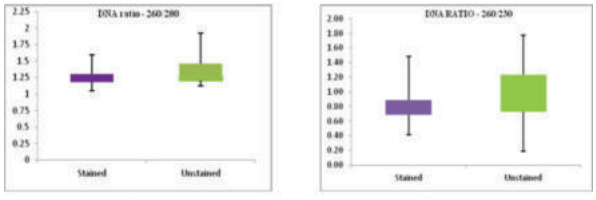
from 3B BLACK BIOTECH.

RESULTS

The results of statistical analysis for DNA concentrations and quality of FNAC samples for stained and unstained from smears on slides are presented by Box-plot diagram in the Fig-1. The quartile values of the DNA concentration were presented in Fig-1A, which is showing mean and range for stained samples as 99.08 ng/Ul (range: 29.2 – 485.5 ng/Ul) while for unstained samples it is 133.45 ng/Ul (range: 31.6 - 444.7 ng/Ul) respectively. DNA quality in 260/280 ratios the quartile values are represented in Fig 1B, showing mean and range for stained samples as 1.25 (range: 1.05 – 1.59) and for unstained samples, they are 1.34 (range: 1.12 – 1.92) respectively. The quartile values of DNA quality in 260/230 ratios was presented in Fig 1C, with showing mean of 0.81 (range: 0.41 – 1.48) for stained samples and while for unstained samples, mean is 1.01 (range: 0.19 – 1.77) respectively.

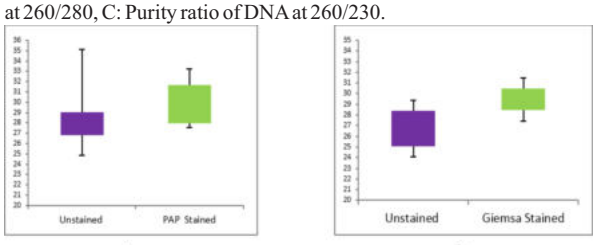


A



B C

Fig 1: Box-and-whiskerplots of stained versus unstained FNAC samples showing, A: for DNA Concentrations, B: Purity ratio of DNA at 260/280, C: Purity ratio of DNA at 260/230.

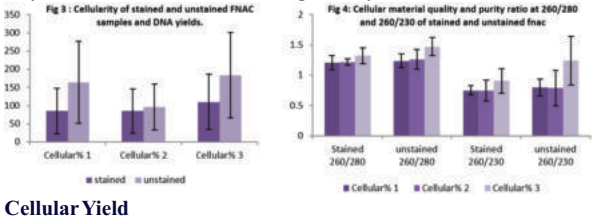


A B

Fig 2 : ABL CT value differences between unstained and stained (pap (A) & Giemsa (B))

ABL Gene CT Value Differences

Box-plot diagram (Fig 2) regarding ABL ct value differences showing mean and range for unstained sample as 25.71 and 22.32 – 32.60 while for PAP stained sample, they are 30.35 and 27.56 – 33.24 respectively. ABL ct value differences showing mean and range for unstained sample as 26.64 and 23.15 – 29.77 while for Giemsa stained sample, they are 29.11 and 25.74 – 32.25 respectively.



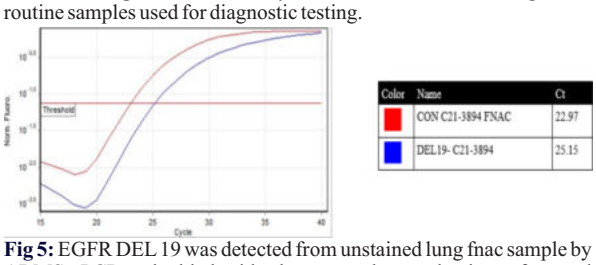
Cellular Yield

The results of the present study revealed that the DNA concentrations of unstained samples were significantly higher than the stained samples even when with low cellularity (Fig 3).

The purity of the cellular material was assessed as the ratio of the absorbance at 260/280 ($A_{260/280}$) and 260/230 ($A_{260/230}$) (Figure 4). Based on the A260/280 ratio, stained and unstained sample has produced relatively high purity, ranging between 1.321 and 1.473 ng/Ul and also A260/230 ratio produced 0.909 and 1.24 ng/Ul. There was no statistically difference significant difference between the groups of cellular percent 1 and 2.

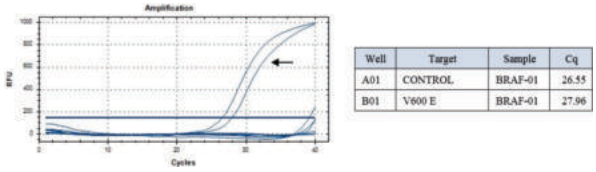
Real Time PCR Amplification Plots:

Amplification plots for biomarker EGFR and BRAF and MTB pathogen were shown in fig 5, 6 & 7. In Fig 5 EGFR detection by ARMS qPCR from unstained lung nodule for DEL 19 mutation is shown in blue curve along at ct 25.15, with internal control amplification in red colour curve at ct 22.97. From thyroid lesion of unstained fnac sample BRAF by real time pcr was done as shown in fig 6, where the control gene amplified at Ct-26.55 and BRAF V600E at Ct-27.96.For both EGFR and BRAF mutation detection was done from FFPE tissue blocks to confirm the results. DNA integrity by magnetic extraction method and amplification efficiency in both unstained samples were shown by control ct values, which are good as routine samples used for diagnostic testing.



Color	Name	Ct
	CON C21-3894 FNAC	22.97
	DEL19- C21-3894	25.15

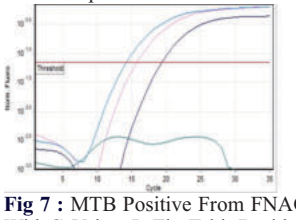
Fig 5: EGFR DEL 19 was detected from unstained lung fnac sample by ARMS qPCR and table beside shows ct values and colour of control and mutation curves.



Well	Target	Sample	Cq
A01	CONTROL	BRAF-01	26.55
B01	V600 E	BRAF-01	27.96

Fig 6 : BRAF V600E Detected As Indicated By Arrow From Unstained Thyroid Fnac Sample By Real Time PCR.

MTB pathogen detection by real time pcr was done simultaneously both on unstained fnac Axillary lymph node and lymph node cell block where the morphology was suspicious of koch's etiology and advised of TB detection by pcr. Fig 7 shows the amplification plot for MTB pathogen detection with controls and unstained fanc sample and cell block samples detected MTB at ct values of 15.73 and 14.22.



No.	Color	Name	Type	Ct
1		PC	PC	19.36
4		C21-2442 fnac slide	Unknown	15.73
5		C21-2442 cell block	Unknown	14.22
6		NTC	NTC	

Fig 7 : MTB Positive From FNAC Unstained Slide And Cell Block With Ct Values In The Table Beside

DISCUSSION

Cytologic preparations are increasingly being used for the molecular analysis of solid organ malignancies to direct targeted therapy. Recognition of the advantages and limitations of different cytologic specimen preparations, including the type of slide used for preparing smears and the method of DNA extraction, is extremely important for optimal utilization of cytologic material for molecular testing. Although the variety of cytologic preparations provides a wide selection of substrates for ancillary studies, the processing and preparation of cytologic materials and the methods of nucleic acids extraction vary considerably between laboratories (7). To optimize cytological material extraction for maximal DNA yields, the different techniques need to be compared for identical materials with strict criteria for quantitative and qualitative assessment.

To address this issue, in this study, the concentration and purity of extracted DNA from stained and unstained FNAC smears were determined spectrphtometer quantification by comparing at the absorbance ratio of 260/280 and 260/230 nm. Adequate amounts of DNA may be obtained from both stained and unstained slides. But, of the 2 methods compared in this study, unstained FNAC samples had relatively higher DNA concentration and quality compared to stained FNAC samples. The present study showed that the mean DNA concentration for unstained FNAC slides was 133.45 ng/Ul ng while

the average DNA concentration for the stained FNAC slides was 99.08 ng/Ul. Similarly, Aplenc et al., (8) reported that the mean DNA yield for unstained slides extracted with the Qiagen kit was 120 ng while it was 48 ng in stained slides of bone marrow samples. Also in accordance with our study, the Gari et al., (9) has shown the amplified DNA for human factor V gene in all of the 35 unstained bone marrow slides tested.

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA, ratio of ~1.8 and ~2.0 is generally accepted. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The ratio of 260/230 values is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm.

In the present study, the mean of DNA quality in 260/280 ratios for the unstained FNAC slide was 1.34, while it was 1.25 in stained FNAC slide and in 260/230 for the unstained FNAC slide was 1.01, while it was 0.81 in stained FNAC slide. This shows that the unstained FNAC slides has resulted in high yield of pure quality DNA as compared to the stained FNAC slides. Similarly, Asokkumar et al., (10) analyzed the purity of the DNA and RNA extracted by measuring the ratio of absorbance at 260 /280 nm (optimal threshold range,1.6 – 2.2) and 260 /230 nm (optimal threshold > 1) using spectrophotometer. They found that both EUS-FNB (95 %) and FNA (90 %) needles procured high-quality DNA in pancreatic lesions.

The mammalian ABL1 gene encodes the ubiquitously expressed nonreceptor tyrosine kinase ABL. In response to growth factors, cytokines, cell adhesion, DNA damage, oxidative stress, and other signals, ABL is activated to stimulate cell proliferation or differentiation, survival or death, retraction, or migration. In the present study, we first reported the distribution of ABL Ct values in stained (PAP and Giemsa) and unstained FNAC samples. The maximum ABL Ct distribution (late ct values) was registered in stained FNAC sample than unstained sample. This may be due to unstained sections may be automatically cut up front to prevent the cell loss that occurs with refacing into a block (11). Similarly, Oktay et al., (12) reported successful DNA and RNA isolation from both DQ and Papanicolaou-stained slides, enabling the detection of mutations and translocations in non-small cell carcinomas and thyroid lesions. BRAF V600E mutation detection from stained FNAC slide scrapes were shown by Gupta et al., (13) and Zhao et al.. (14). In a study by Awwad et al., (15), EGFR mutation detection from fnac samples were shown but not specified its from stained or unstained fnac samples. Gupta et al., (16) MTBC in pediatric patients by real time pcr from fnac was proven sensitive than culture. Till date most of the studies using fnac were from stained smears using column based extraction for DNA. In our study EGFR and BRAF mutation detection were shown as example in one sample each of lung and thyroid lesion and mycobacterium pathogen detection from lymph node as shown in Fig 5-7 directly from unstained fnac samples by magnetic extraction.

This is the first study, to the best of our knowledge illustrating that DNA by magnetic extraction from unstained smears yields higher DNA concentrations and purity than stained smears and lower ABL Ct value distribution was registered in unstained FNAC sample than stained sample. Proving that magnetic extraction method can be used over column method for DNA extraction for unstained smears. To use DNA from unstained slides for marker/pathogen detection studies should be done with good sample size to prove its sensitivity and specificity.

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