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The Technique of Rapid on-Site Evaluation on Lung Cancer

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ABSTRACT
Lung cancer is the first leading cause of death and the second most common malignancy in the world. Most of the lung carcinoma diagnostic specimens were obtained by cytologic modalities, such as transbronchial needle aspiration (TBNA), transthoracic needle aspiration (TTNA), and bronchial brushing. These small specimens are also used for immunocytochemistry/immunohistochemistry and molecular analysis. It is therefore very important to ensure that adequate specimens are obtained to prevent repeated interventions or unnecessary invasive procedures. The technique for determining the diagnosis and treatment options specified in one procedure is rapid on site evaluation (ROSE). ROSE procedure was initiated by making a smear from the specimen obtained through TBNA, bronchial brushing, TTNA, and imprinting the core needle biopsy. The smear slides were fixed immediately with a rapid stain and assessed for adequacy of the sample. The rapid stains used were Diff-Quik, rapid Papanicolau, rapid Hematoxylin Eosin, and toluidine blue (TB). The choice of stain is determined by the hospital's infrastructure in service, budget allocation,and the availability of trained cytology staff. The use of ROSE in cytology specimens can improve sample adequacy, diagnostic accuracy and has higher sensitivity.

Keywords: *Lung cancer, rapid on site evaluation, cytology, Diff-Quik stain.*

1. INTRODUCTION

Lung cancer is the first leading cause of death in the world reaching 1.796.144 people in 2020. The global incidence is around 2.206.771 new cases and is the second most common cancer in the world. In Indonesia, lung cancer is the first cause of death around 25,943 people with 34.189 new cases in 2020. Lung cancer patients generally come at an advanced stage, only 30-40% can be operated on [1].

Diagnostic specimens of lung cancer are mostly obtained by cytologic modalities, such as transbronchial needle aspiration (TBNA), transthoracic needle aspiration (TTNA), and bronchial brushing. Minimally invasive sampling techniques via TBNA or TTNA have been used in the last decade and are preferred over invasive surgical procedures. These small specimens are not only used to observe cell morphology, but also for immunocytochemistry/ immunohistochemistry and molecular analysis. It is therefore very important to ensure that adequate specimens are obtained to prevent repeated interventions or unnecessary invasive procedures [2].

The technique for determining the diagnosis and treatment options specified in one procedure is rapid on site evaluation (ROSE). ROSE is a technique in which cytological samples from fine needle aspiration, bronchial brushing and imprinted core needle biopsy are rapidly stained and evaluated for adequacy of the sample. Using ROSE during TBNA was first studied by Davenport. Davenport got the positive results in terms of improved diagnostic yield have encouraged large centers to incorporate ROSE in their bronchoscopy units [3].

Cytological diagnosis of lung cancer without ROSE procedure has low sensitivity and specificity. The ROSE technique enhances lung cancer cytologic specimen collection procedures thereby improving diagnostic results. ROSE also significantly improved the number of specimens that were adequate for a diagnosis of central lung cancers adjacent to the airway or lymph node metastases. Baram et al. demonstrated that when

ROSE confirmed diagnostic material, this frequently spared the need for additional sampling and the overall cost of the procedure was lowered. ROSE can prevent repeated bronchoscopy or other invasive surgical procedures that can lead to complication [4].

2. RAPID ON-SITE EVALUATION ON LUNG CANCER

ROSE is a technique beneficial in determining the adequacy of the samples, thereby increasing the diagnostic yield, useful in triage of specimens for ancillary examinations and can also help determine a preliminary diagnosis [5]. ROSE is a rapid, real-time examination method at the bronchoscopy site [6]. This evaluation provides immediate feedback regarding the adequacy of the sample and guides the operators to modify the bronchoscopy technique, the site and depth of the sample [7].

2.1. Indication of ROSE

Rapid on-site evaluation is used in various procedures such as TBNA, TTNA, bronchial brushing and imprinted core needle biopsy in the lung. ROSE can also be used for endoscopic fine needle aspiration (EUS-FNA) in the pancreas, and fine needle aspiration in the thyroid [8].

2.2. Purpose of ROSE

The main purpose of ROSE in lung cancer is to confirm adequacy of sample. It is evidenced by the discovery of representative tumor cells, inflammatory cells and normal tissues. The number of samples is ensured to be sufficient for ancillary studies, including immunohistochemistry, microbiology studies, flow cytometry analysis, and molecular assays. A preliminary diagnosis can be made to evaluate whether the lesion is neoplastic or non-neoplastic. The final diagnosis is determined after the specimen was stained with Papanicolaou stain. Moreover, ROSE is similar to frozen section evaluation in that the preliminary diagnosis has a direct impact on patient management in deciding whether to proceed to mediastinoscopy [9].

2.3. Prosedur of ROSE

The cytopathologist is notified before the start of bronchoscopy procedure. The cytopathologist obtained a cytology worksheet containing patient data, brief clinical history, procedure type, anatomic site, and clinical indications. Slides are labeled prior to preparing smears. Specimens obtained by fine needle aspiration are sprayed onto the slide and made at least 2 smears. If the specimen is obtained from a bronchial brush, the brush is tapped against the slide and made smear. One slide was air-dried and stained with Diff-Quik and the second slide was fixed in 95% alcohol for subsequent Papanicolaou staining. Residual material from the needle was rinsed in 10% buffered formalin for making a cell block. Specimens can also be obtained by imprinting core needle biopsy. The specimens obtained are gently touched to two slides, and colored following the procedure above [10].

Assessment of adequacy based on cellularity and number of neoplastic cells consistent with clinical and radiological findings. If the sample is adequate the bronchoscopy procedure can be terminated. However, if the interpretation is inadequate, additional aspiration is performed until an adequate sample was obtained. The procedure also is terminated due to complications, the target lesion is difficult to access, or the lesion is too small (Figure 1). The number of cells and preliminary diagnosis are reported verbally to the clinician. The final diagnosis is determined after the cytopathologist viewed all the samples stained with the conventional Papanicolaou stain [10].

Specimen allocation for ancillary studies is decided at the time of ROSE depending on the preliminary findings. If the infection is suspected based on smear findings, a portion of the specimen is collected in a sterile container and sent for microbiological culture studies. For suspected lymphoma, a portion of the core or a dedicated pass in the case of FNA is collected in RPMI medium for flow cytometry studies [10].

2.4. Staining Method of ROSE

The ROSE procedure uses a rapid stain. Various kinds of cytological stains can be used such as Diff-Quik, rapid Papanicolau, rapid HE, and TB [5]. The choice of staining is determined by the hospital infrastructure in service, budget allocation, and the availability of trained cytology staff. Among the above stains the most commonly used is the Diff-Quik because of the speed of the procedure, the ability to perform slide stains on dry fixation, and is superior for evaluating lymphoid samples to Papanicolaou stains [8].

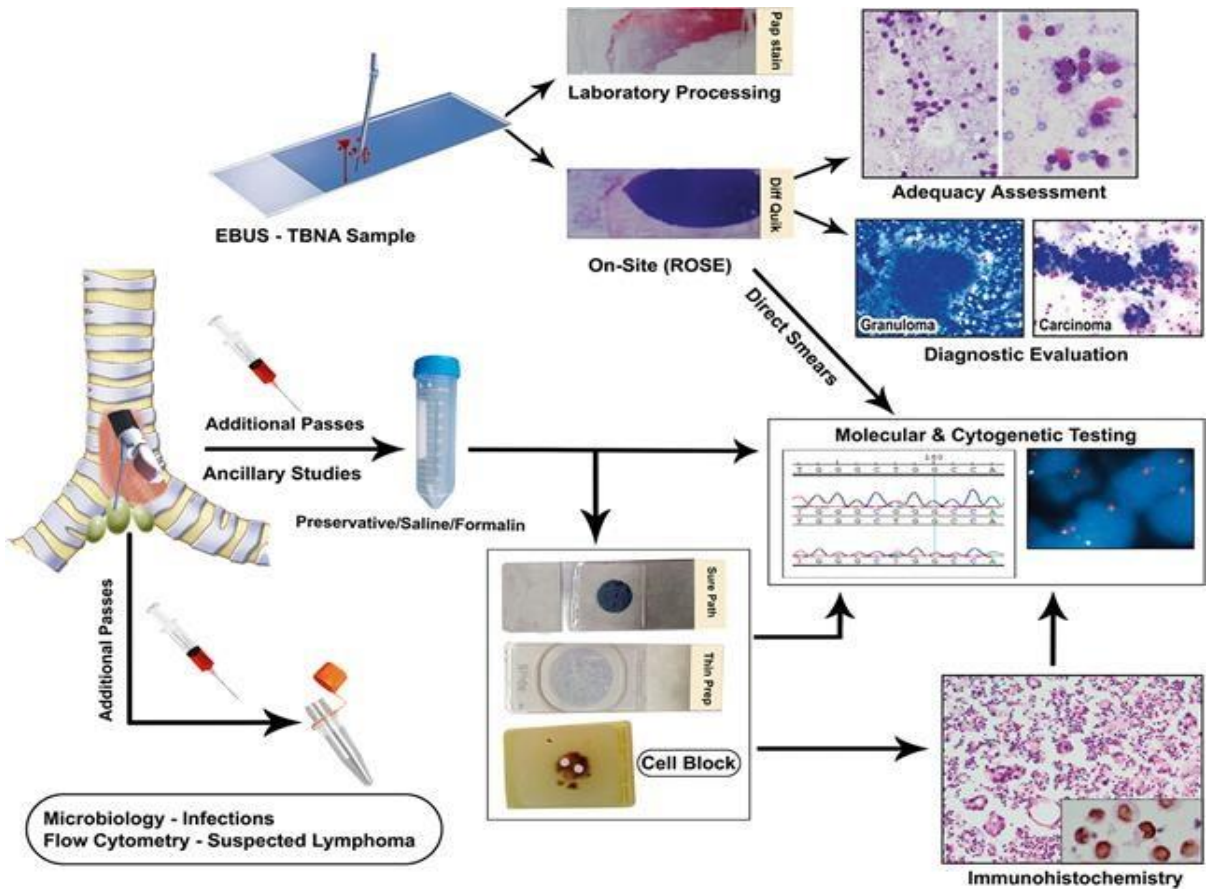


Figure 1. Schematic diagram to show purpose and algorithmic flow of rapid on-site evaluation (ROSE) for endobronchial ultrasound–guided transbronchial needle aspirate (EBUS-TBNA) specimens [9].

2.4.1. Diff-Quik stain

The Diff-Quik stain is a modification of the Wright Giemsa stain and is commercialized as a rapid staining kit. The Diff-Quik Staining Kit consists of three solutions, namely methanol solution for fixation, orange Eosin G solution (solution II), dark blue thiazine solution (solution III) [8,11]. The staining procedure only takes 20-30 seconds. This stain is proven to be easy and durable to maintain the color quality of slides for years [12]. One set of Diff-Quik solutions is used for only one specimen [11]. The staining procedure can be seen in table 1.

Table 1. Procedure of Diff-Quik stain

No.	Procedure	Duration
1	Prepare slide and air dry immediately	
2.	Dip slide in fixative solution	5-10 times
3.	Dip slide in solution II	10-20 dips
4.	Dip slide in solution III	10-20 dips,
5.	Rinse slide with tap water	
6.	Wipe back of slide	

The slide is checked directly without being covered by a glass deck. The examination uses objective lenses with 4x, 10x and 20x magnification. Cell size is doubled compared to the alcohol-fixed Papanicolaou stain, therefore 20x magnification showed a 40x magnification-like appearance [12].

The Diff-Quik stain has several technical advantages such as not requiring immediate fixation to alcohol because the smear is allowed to dry in air. Dry fixation minimizes cell loss compared to Papanicolaou staining with wet fixation. The staining procedure is quite simple and the processing time is very fast, about 20-30 seconds. The advantage of the cytological picture is that the cell size is larger due to the lack of cell size shrinkage with dry fixation so that the architectural picture of the cell is clearer (figure 2). In addition, it is easier to identify cytoplasmic granularity and cytoplasmic inclusions. Metachromatic staining materials such as mucin, colloids and stromal fragments can be seen better. Hematologic disturbances can be evaluated better than Papanicolaou staining. The picture of the lymphoid lesion looks clearly [12].

One limitation of Diff-Quik staining is that keratinized squamous cell and small cell malignancies cannot be assessed as well as the Papanicoloau stain. Cytoplasmic keratinization does not look good, nuclear and nucleoli do not show as well as Papanicolaou [12].

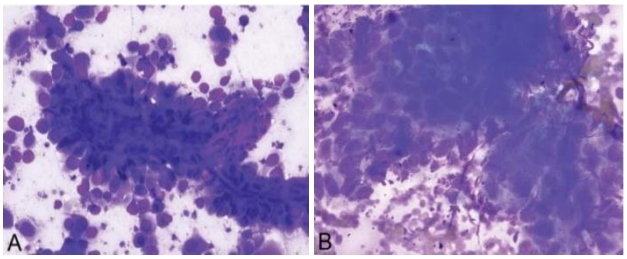


Figure 2. Diff-Quik stain A. Adenocarcinoma of the lung; B, Squamous cell carcinoma of the lung. (A–B, x600) [13].

2.4.2. Pewarnaan Toluidine Blue (TB)

Toluidine blue is a supravital stain that highlights nuclear details and a three-dimensional picture of cells [14]. Supravital staining is a method of staining nucleic acids such as ribosomes, rough endoplasmic reticulum, heterochromatin and nucleoli [11].

TB staining requires ingredients toluidine blue 2.8 g, Urea 4.0 g, ethanol 280 ml and distilled water 120 ml. TB staining is dissolving urea with water, slowly adding ethanol. Next, add toluidine blue, mix well and strain. The use of urea is useful for increasing contrast by reducing the attraction of non-ionized color molecules from the stained tissue. This dye has a shelf life of about 2 years [14]. A description of the TB stain can be seen in table 2.

Table 2. Toluidine blue staining procedure[11]

No.	Procedure	Duration
1.	Prepare slide and air dry immediately	
2.	Add in toluidine blue solution	1-2 minutes
3.	*Distilled water*	3 dips
4.	Dip in ethyl alcohol 95%	1 dip
5.	Dip in absolute ethyl alcohol	1 dip
6.	Dip in xylene	10 dips
7.	Cover with *deck glass* and wipe back of slide	

TB staining is easy to obtain, very cheap, effective and takes about 3 minutes. The study conducted by Chandra et.al stated that there was an increase in sample adequacy of 93% of the specimens obtained by fine needle aspiration or bronchial brushing. Cell morphology was shown to be moderate and adequate cellularity in fibrotic lesions that were less cellular (figure 3). Another plus is that the color can be removed and used for standard Papanicoloau stains [15].

The most important limitation of cytologic morphology, large reactive endobronchial cells can be misdiagnosed as malignant cells and give a false positive diagnosis. Plump spindle cell clusters or columnar cells can be misinterpreted as epithelioid cells or otherwise granulomas containing a small amount of epithelioid spread are not diagnosed [15]. Another limitation is 95% ethyl alcohol solution, absolute ethyl alcohol and xylene must be exchanged routinely. 95% ethyl alcohol is exchanged when it is light blue in color, while absolute ethyl alcohol and xylene are exchanged once a month or when the color turns dark blue [16].

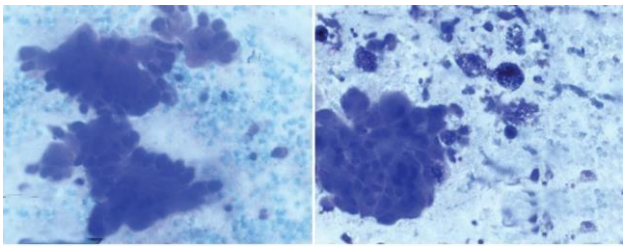


Figure 3. Toluidine blue staining A. Adenocarcinoma showing glandular structure (×400), B. Squamous cell carcinoma showing dirty, necrotic and keratinized background (×400) [17].

2.4.3. Rapid Papanicolaou

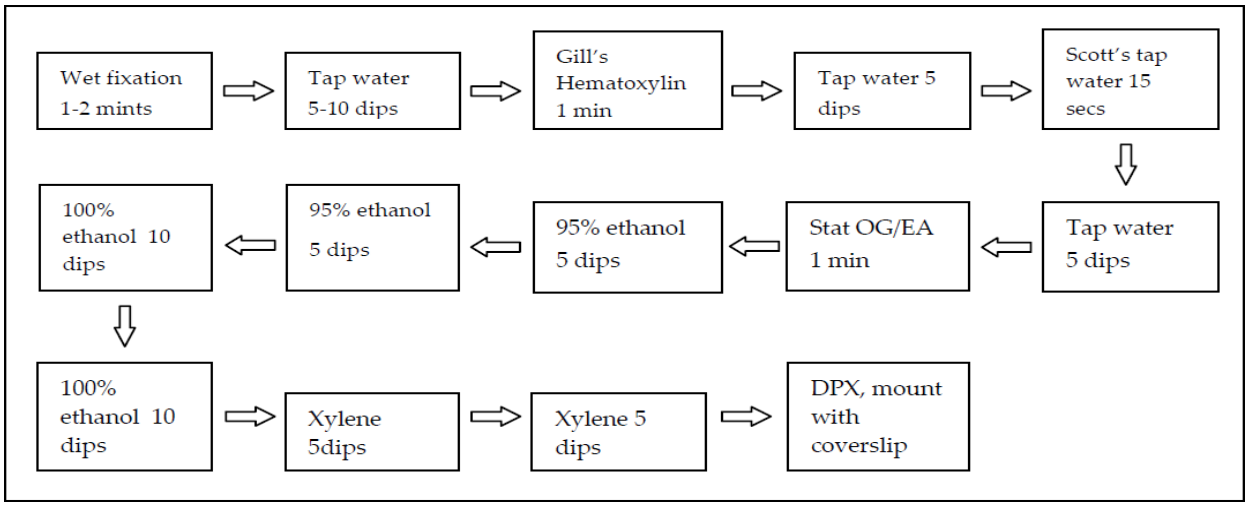
Rapid Papanicolaou developed by Kline, Tao and Sato, for staining takes 4 minutes, 5 minutes and 90 seconds, respectively, with a fixation time of 1-2 minutes [18]. The procedure for rapid Papanicolaau staining can be seen in table 3.

Papanicolaou's rapid stain has several disadvantages. Wet fixation takes a lot of time, and causes more cell loss. The preparation contains artifacts, especially at the edge of the smear and hemorrhagic background, so it takes more time to interpret the preparation. The volume of ethanol required is quite a lot so the costs required are more expensive. In addition, the color does not last long and the color quality is not satisfactory where the morphology of the stained cells is suboptimal/not visible well. Because of these shortcomings, a modification of the Papanicolaou coloring was made [18].

Young and Alvarez made a modification of the Papanicolaou stain in the form of Ultrafast Papanicolaou (UFP) in 1995. This stain is a combination of Romanowsky staining with conventional Papanicolaou which takes 90 seconds. The 90 second staining step proves the effectiveness of good quality stained smear and reduces or removes background blood from the smear [18].

The staining procedure is as follows: the smear slide is allowed to air dry. Slides were fixed in normal saline for 30 seconds and in alcoholic formalin for 10 seconds. Chemical reagents used for the manufacture of alcoholic formalin are 300 ml of 40% formalin added 2.053 ml of isoprofile alcohol and 647 ml of distilled water to obtain a total solution of 3,000 ml. Alcoholic formalin must be maintained at pH 5.0. This modification of alcoholic formalin fixation reduces the time required for fixation and staining from minutes to seconds [11,19].

Table 3. Papanicolaou rapid staining procedure [18].



Ultrafast Papanicolaou proved to be effective in staining smears with good quality (figure 4). However, morphology cell and nuclear features are not as good as conventional Papanicolaou staining. In addition, normal saline should be changed regularly after working the 5 slides. The pH of alcoholic formalin should be maintained at 5.0 for good smear quality. This solution is changed daily or after 5-6 slides. Soaking the slide for too long in an alcoholic formalin solution can affect cell morphology, the nucleus becomes blurred and the nuclear membrane wrinkles [19,20]. Another limitation is that dye solutions are not yet universally available. Richard-Allan hematoxylin and cytosine are manufactured by the Richard-Allan company [21]

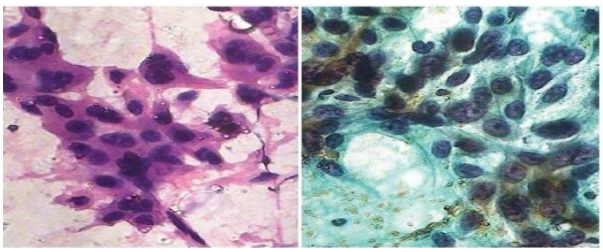


Figure 4. Comparison of rapid Pap (left) with ultrafast Pap stain (right) on metastatic squamous cells (x400) [18].

2.4.4. Rapid Hematoxylin Eosin (HE) Staining

Hematoxylin is a special dye for chromatin that makes it an alkaline dye. Hematoxylin stains the nucleus and chromosomes blue. Hematoxylin is a regressive stain and is extracted by highly acidic solutions or acidic alcohols. Eosin is an acidic color and the terms acidophilic, oxyphilic, and eosinophilic are often used interchangeably. Eosin can be used after fixation and is used as a counterstain in many staining combinations including hematoxylin [11]. The Rapid HE staining procedure can be seen in table 4.

Table 4. Procedure for rapid hematoxylin eosin staining [11].

No.	Procedure	Duration
1.	Absolute ethyl alcohol	1 minute
2.	Ethyl alcohol 95%	1 minute
3.	*Tap water*	Several dips
4.	Gill III hematoxylin	2 minutes
5.	*Tap water*	Several minutes
6.	Ammonium hydroxide	1-2 dips
7.	Eosin/floksin solution	30 seconds
8.	*Tap water*	Several dips
9.	*Tap water*	Several dips
10.	Ethyl alcohol 95%	Several dips
11.	Ethyl alcohol 95%	Several dips
12.	Absolute ethyl alcohol	Several dips
13.	*Acetone*	Several dips
14.	*Xylene*	1 minute

2.4.5. Conventional Papanicolaau Staining

The Papanicolaou stain is the most frequently used cytological stain for all Pap smears and non-gynecological exfoliative smears. This coloring was first discovered in 1942 and modified and then published by Dr. George Papanicolaou in 1954. The advantages of Papanicolaou staining are the nuclear chromatin is well stained so that nuclear details can be seen clearly, cytoplasmic differentiation and cytoplasmic transparency and shows intracytoplasmic keratin [22].

There are 3 kinds of dyes used in Papanicolaou staining. The first dye is hematoxylin to color the core. The hematoxylin used was regressed Harris hematoxylin. The second Orange G was OG6 for staining the cytoplasm and the third was Eosin Azure

(EA). Eosin Azure (EA) is a polychrome dye consisting of 3 dyes, namely Eosin Y, light green SF yellowish and Bismarck brown Y [22].

The principle of the Papanicolaou staining step begins with rehydration. The use of alcohol graded 50%, 70%, 80% and 95% can minimize cell damage. The core was then stained with hematoxylin. Subsequent differentiation was carried out to remove excess hematoxylin by acid alcohol. The excess color is cleaned with running water. Cytoplasm staining with orange G (OG), the smear was put back into alcohol because OG is a dye that is soluble in alcohol. Furthermore, staining the cytoplasm with EA. EA staining produces a blue-green color in the cytoplasm of the cells. Dehydration is done with absolute alcohol and finally use xylene for clearing. The limitations of Papanicolaou staining are that it takes a long time and loses more cells [22].

2.5. Assessment of Sample Adequacy on ROSE

Assessment of adequacy based on cellularity and cell number of the lesion. Cytopathologists can determine the diagnosis of the lesion is positive or negative a malignancy. This is because most ROSE procedures are performed for lesions with suspected malignancy and for staging in patients with known lung cancer [15].

An adequate smear is assessed by the presence of at least 200 neoplastic cells. Inadequate smears are low cellular, have few neoplastic cells, or only show bleeding, necrosis and mucoid material [15,23]. A cell count of 200 is considered adequate for further ancillary examination. Ancillary examinations are performed to assess tumor subtype and molecular analysis. In lung cancer, the immunocytohistochemistry panel examination of TTF1, Napsin A, p63, or CK5/6 was performed to determine the histologic subtype followed by molecular analysis such as EGFR, ALK and KRAS [10].

2.6. Advantages and Disadvantages of ROSE

2.6.1. Advantages of ROSE

The main advantage of ROSE is that it can improve sample adequacy and diagnostic accuracy and higher sensitivity. Immediate assessment of sample adequacy also reduces the number of false negative diagnoses by minimizing sampling error. Another advantage of ROSE is that an adequate sample can be used for other ancillary tests such as the preparation of cell blocks for immunohistochemistry, flow cytometry, microbiological-specific culture and staining, molecular and cytogenetic analysis. In addition, it reduces the possibility of repeating the procedure, reducing the risk of further invasive complications [24].

2.6.2. Disadvantages of ROSE

The main deficiency of ROSE is the need for an experienced cytopathologist during the ROSE examination. Only an experienced cytopathologist can decide that the sample obtained is consistent and correct. Inexperienced cytopathologists sometimes make an inaccurate initial diagnosis or determine suboptimal sample adequacy thereby ending the procedure early and increasing the need for additional procedures or a core needle biopsy [24].

3. CONCLUSION

The purpose of ROSE in lung cancer is to confirm sample adequacy. A preliminary diagnosis can be made of neoplastic and non-neoplastic lesions but is not a final diagnosis. The best staining for ROSE is Diff-Quik because the processing time is very fast about 20-30 seconds, the procedure is easy and the cell architecture is clearer.

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