Chapter 2
The Cytology Specimen and Preparations: Advantages and Limitations

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Abbreviations

CB	Cell block
DNA	Deoxyribonucleic acid
FFPE	Formalin fixed paraffin embedded
FISH	Fluorescence *in situ* hybridization
FNA	Fine-needle aspiration
H&E	Hematoxylin and eosin
ICC	Immunocytochemistry
LBC	Liquid-based cytology
LCM	Laser capture microdissection
NGS	Next-generation sequencing
ROSE	Rapid on-site evaluation

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S. Roy-Chowdhuri et al. (eds.), *Molecular Diagnostics in Cytopathology*, https://doi.org/10.1007/978-3-319-97397-5_2

Key Terminology

Biomarker	According to the NIH biomarker definition working group, a biomarker is any objectively measurable characteristic that can be used as an indicator of normal biological processes, pathogenic processes, or a pharmacological response to a therapeutic intervention
Cell block	A cytologic preparation obtained by concentrating and fixing (most commonly in formalin) cytology specimens followed by paraffin embedding, thereby mimicking a histologic tissue block
Core-needle biopsy	A biopsy obtained by the use of a needle usually with caliber larger than 18 gauge
Cytospins	Preparations produced from cytocentrifugation of cytology specimens and concentration of cells onto a glass slide
Fresh samples	Samples obtained directly from patients without any fixatives
Liquid-based preparations	Automatically produced preparations from cytology specimens fixed in alcohol-based proprietary solutions, with automated machine-based processing, resulting in a thin-layer slide preparation
Microdissection	Dissection of specific areas of a slide with collection of the cells of interest. Can be performed manually or with the aid of laser (laser capture microdissection)

Smears	The preparation produced by the act of smearing or spreading the material obtained from fine-needle aspirations or exfoliative cytology onto a slide

Key Points

- Cytology samples should be used judiciously in order to maximize their use for molecular analysis
- FFPE cell blocks have an advantage for molecular testing due to their similarities to histological tissue blocks; however, they may be limited by DNA degradation caused by formalin fixation. Cytology specimens collected in alcohol-based fixatives may yield better-quality nucleic acids
- A variety of cytologic preparations can be routinely used for molecular studies, and this can augment the number of samples available for molecular testing
- Rapid on-site assessment is a useful tool to ensure adequate material is present and to check tumor fraction
- Cytologic smears and cytospins are well-suited for FISH studies, and unlike FFPE sections, are not subjected to nuclear truncation artifact
- The main limitation of wide adoption of cytology specimens in molecular analysis is due to the need for additional test validation

Molecular pathology has evolved in recent years and is now part of routine clinical laboratory analysis, with wide applications from detection of microorganisms to discovery of diagnostic biomarkers, with impact on personalized medicine and targeted therapy. In oncologic pathology in particular, it plays a vital role in guiding clinical management

and determining patient's overall response to therapy and prognosis.

Among the specimens available for molecular analysis, cytology samples provide a versatile option, with several advantages over histology specimens (core-needle/surgical biopsies) as outlined below. Therefore, it is extremely important for the practicing cytopathologist to be aware of the advantages (and limitations) of cytology specimens for molecular analysis, as well as optimize pre-analytical factors for achieving reliable results.

Overall molecular biomarkers are assessed using three main technologies: immunocytochemistry (ICC) for protein products, fluorescence *in situ* hybridization (FISH) for chromosomal abnormalities, and mutation analysis for gene alterations. Due to the fact that protocols for molecular analysis of histology specimens are usually validated using FFPE tissue blocks, FFPE cell blocks are usually preferred and most commonly used among cytologic preparations, due to seamless transition of the protocols originally designed for histology samples. However, all the other cytologic preparations have already been validated and are currently widely used. The current chapter envisions, therefore, to list the main advantages and limitations of cytology specimens for molecular tests as compared to surgical biopsies, discuss the appropriate handling of these samples, describe the main differences among the various specimen preparations, and discuss ways to minimize their limitations in order to achieve an optimal analysis using these types of preparations.

Advantages of Cytology Samples

Cytology and histology samples (small biopsies and surgical specimens) are received for processing either fresh or in a fixative solution. The main differences between these specimens for molecular testing are related to sample handling and processing and their effect on the nucleic acids and proteins.

For histology specimens, hematoxylin and eosin (H&E)-stained tissue sections obtained from formalin-fixed paraffin-embedded (FFPE) blocks are used for morphological evaluation during diagnostic work-up. Although other fixatives are available, 10% formalin has been widely adopted as a universal tissue fixative for producing paraffin blocks. FFPE tissue blocks have the advantage of yielding serial sections that can be used for ancillary studies, including a variety of molecular tests, and have been traditionally used for long-term storage. However, the detrimental effects of formalin fixation leading to DNA fragmentation and sequencing artifacts have been well described [1–6].

Cytology specimens, on the other hand, have the advantage of immediate and rapid fixation, as many of these samples are received fresh, frequently with a preliminary assessment performed by a cytopathologist or cytotechnologist at the time of the procedure, ensuring adequate material is obtained. Furthermore, due to their minimal volume, there is no delay for the penetration of the fixative solution. Since multiple cytologic preparations can be produced from fresh samples, usually more than one type is available for molecular testing.

For cytology samples, in addition to the H&E-stained slides from cell blocks, Papanicolaou- and Romanowsky-stained direct smears or cytospin slides or Papanicolaou-stained liquid-based cytology slides can be also produced from fresh or fixed material. Therefore, many non-formalin fixatives and multiple types of preparations are routinely used for diagnostic assessment and are often suitable for molecular tests and most frequently provide higher-quality nucleic acids than their formalin-fixed counterparts [2, 7].

Cytology samples are obtained by minimally invasive procedures, which are better tolerated and usually the method of choice for critically ill, advanced stage cancer patients [8]. In addition, fine-needle aspiration specimens show high proportion of neoplastic cells with lower numbers of nonneoplastic stromal and inflammatory cells.

For fluorescence *in situ* hybridization (FISH) assays in particular, cytologic preparations such as direct smears and

cytospin slides provide an advantage over FFPE sections as they are not subject to nuclear truncation artifact [9].

In summary, since for histology samples only FFPE tissue blocks are routinely available, judicious use of the tissue sections for morphological examination and ancillary diagnostic tests is required to save material for molecular tests. In contrast, for cytology, fresh samples are frequently available and can be utilized to produce a variety of substrates that offer therefore high versatility for molecular studies**.** Familiarity with handling and processing protocols for different cytologic preparations, their advantages and limitations, and the fixatives and transport media routinely used can help to overcome the self-imposed limited material of cytology samples and safeguard an accurate analysis (Table 2.1).

Limitations of Cytology Samples: What to Expect and how to Minimize Them

The main limitations of cytological samples are related to (1) the multitude of cytologic substrates and fixatives that require additional test validation as the majority of molecular assays are developed on FFPE tissue blocks, (2) the limited cellularity and the nucleic acid yield especially when the neoplastic cells are present on a single smear or concentrated in small areas of the slide, (3) when the specimen has low tumor content with large amount of nonneoplastic cells, and (4) medicolegal issues if smears or cytospin slides are used for testing and the slide has to be sacrificed without an archival slide for future morphological review.

Some of these limitations can, however, be solved using the following strategies: (1) samples can be enriched for tumor by microdissection of tumor-rich areas to optimize low tumor fraction samples for molecular testing (discussed further in Chap. 8); (2) digital images or scanned slides (whole slide imaging) can be used as archival records to circumvent

Table 2.1 Comparison of cytology and histology samples for molecular analysis

Overall features	Cytology samples	Histology samples
Influence from fixatives	Minimal; usually received fresh or fixed in ethanol Immediately fixed Fast fixation	Formalin causes severe DNA degradation and may hamper RNA yield Prolonged exposure to fixatives
Types of preparations	Multiple, often many can be obtained from the same sample	Limited, usually FFPE tissue or snap frozen
Quantity of tumor cells	Physical enrichment "per se" High tumor/stromal cell ratio	Depends on the area, may carry lots of stroma or "non-tumoral areas"
Nuclear truncation artifact for FISH assays	Avoided	Present
Validation	Except for FFPE cell blocks, non-formalin-fixed preparations need extensive validation	Widely validated, most platforms designed for FFPE material
Archived material	Need digital images or WSI if sacrificed, might be the only material available	Option to obtain extra sections for ancillary tests

FFPE formalin-fixed paraffin-embedded material, *WSI* whole slide imaging

legal requirements of slide retention; and (3) various cytologic preparations can be employed for different assays, such as cell blocks for ICC and direct smears for mutation analysis.

Transport Media and Fixatives

A multitude of transport media and fixatives are routinely used to preserve cytology specimens from the time of collection to sample processing and have been employed to achieve optimal morphological details for diagnostic purposes and prevent protein and nucleic acid degradation for molecular assays. Some transport media can also serve as fixatives. In overall, cytology samples are collected and transported until processing usually using one or more of the following: (1) fresh (no fixative), (2) ethanol, (3) air-dry fixation, (4) spray fixation, (5) alcohol-based preservative solutions, (6) formalin, (7) sterile saline, (8) phosphate-buffered saline (PBS), and (9) Roswell Park Memorial Institute (RPMI) medium. The choices might have effects on downstream analysis. For example, for biomarkers assessed by ICC, validation and protocol optimization must be performed when using alcohol-fixed cytology specimens since the results might differ from those obtained from FFPE samples [10].

The type of transport media or fixative is closely linked to the type of sampling method, the cytologic preparation produced and/or algorithm used for sample triage. Regardless of the differences in sample preparation, the different non-formalin fixatives used for cytology samples provide superior results in terms of DNA quality when compared to formalin-fixed material [11]. In general, cytologic preparations (except for cell blocks) usually preclude formalin fixation, thus avoiding fragmentation issues and base-pair changes associated with this fixative [6]. Alcohol-based preserving solutions used for LBC showed different results for DNA yield. Samples collected in CytoLyt (Hologic, Bedford, Massachusetts) gave fivefold higher DNA yield than those in CytoRich Red (Fisher Scientific UK Ltd., Loughborough, Leicestershire, England) [12]. Spray or ethanol-fixed Papanicolaou-stained slides provided the best results in terms of yield and fragment length compared to LBC and air-dried slides [12].

Cytologic Preparations

Routinely several cytologic preparations are produced from a single cytology specimen. Therefore, multiple options are available for the different molecular techniques with protocol optimization and validation required for each of the cytologic preparation employed (Fig. 2.1).

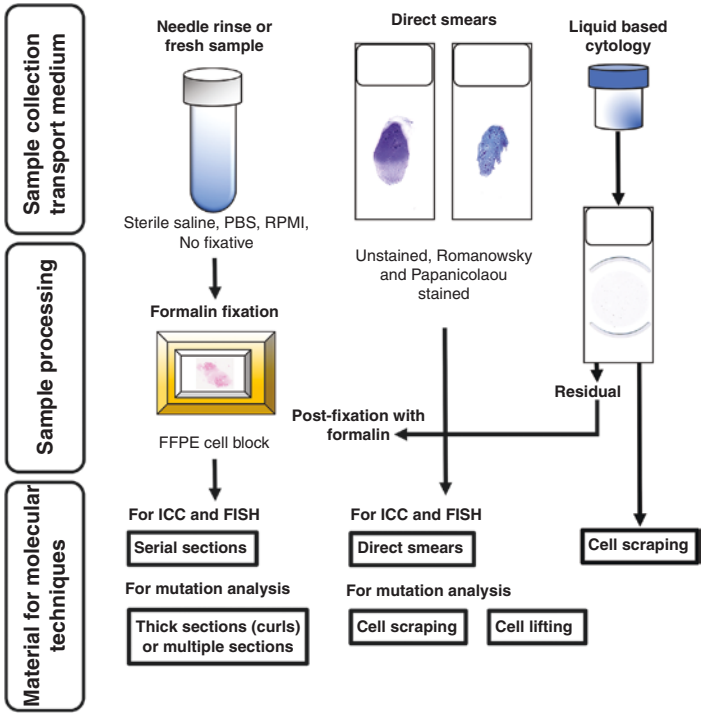

FIGURE 2.1 Diagram of main cytologic preparations with emphasis on sample collection and transport medium, sample processing, and the material required for molecular techniques. PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute; FFPE, formalin fixed paraffin embedded; ICC, immunocytochemistry; FISH, fluorescence *in situ* hybridization

Direct Smears

- Smears are the cornerstone of cytology, inexpensive and efficient for morphological analysis. Cost-effective method to address sample eligibility.
- Smears usually depict high tumor purity. This ensures a confident molecular analysis of the area of interest, even when there is low DNA yield.
- Romanowsky- or Papanicolaou-stained as well as unstained slides are adequate for molecular testing.
- For Romanowsky-stained smears, cellularity assessment can be performed on non-coverslipped slides for immediate selection and triage to molecular assays.
- Diff-Quik-stained slides are equivalent to cell block sections and Papanicolaou slides for NGS testing, without relevant variations in the total number of reads or in the percentage of reads aligning to the target region [13].
- Digital slides (whole slide scanning or digital images of specific areas) may be needed prior to slides being sacrificed for testing to circumvent medicolegal problems related to slide retention.
- For FISH analysis:
  - Nuclear truncation artifact avoided, a problem usually encountered when sections from paraffin blocks (cell blocks) are used.
  - Adhesive-coated or positive charged slides are recommended to prevent cell detachment.
- For gene mutation analysis:
  - Macrodissection (cell scraping) or the cell-lifting technique should be performed for DNA extraction. The latter employs the Pinpoint solution of the Pinpoint Slide DNA Isolation System (Zymo Research). For low cellularity smears, laser capture microdissection (LCM) can be used to enrich tumor cell content targeting specific areas with high tumor cellularity.

  - The enrichment of tumor cells is required for specimens with an estimated tumor fraction below a threshold dictated by the analytical sensitivity of the molecular assay employed.

- Nucleic acids extracted from smears show comparable or even superior results to those observed in formalin-fixed paraffin-embedded (FFPE) cell blocks due to non-formalin fixation.

Cell Blocks

- Most common source for molecular analysis among cytologic preparations.
- A variety of fixatives and preparation methods can be employed. Variation in acquisition, preparation, and processing of tumor material due to different clinical and laboratory practices might have minimal impact on test results [14].
- Generated from specimens fixed in formalin- or ethanol- and alcohol-based preserving solutions (CytoLyt and CytoRich red), with post-fixation in formalin.
- Similar processing protocol to histological specimens: Easily validated for clinical use.
- Long-term storage cell preservation.
- Multiple serial sections can be obtained from CBs for several different assays.
- Techniques for cell enrichment such as LCM can be used for samples with low cellularity.
- Special attention to formalin-induced errors that might limit an unbiased and exploratory sequencing analysis (higher chance of errors).
- For ICC:
  - Multiple external controls can be placed on the same tested section which cannot be performed for the other cytologic preparations.

- For FISH analysis:
  - A corresponding H&E-stained slide usually used to circle areas with high tumor cellularity or specific areas for scoring.
  - Non-fluorescence-based assays such as chromogenic *in situ* hybridization (CISH) and silver *in situ* hybridization (SISH) can be performed.
- For mutation analysis:
  - Microdissection of multiple unstained slides with regular thickness (4μ) or thick (>10μ) unstained sections ("curls") can be used for DNA extraction.
- For PCR-based assays, test failure is similar to that of histology specimens, regardless of the type of fixative used (alcohol or formalin) [15].

Liquid-Based Cytology (LBC)

- Valid alternative to conventional smears, limiting sampling artifacts as the automated process for producing LBC slides leads to minimal contamination by blood, inflammation, and cellular debris.
- Macrodissection (cell scraping) or the cell-lifting technique used for DNA extraction and digital slides (whole slide scanning or digital images of specific areas) required for slides to be sacrificed for testing, similar to smears.
- The residual cell suspension (cellularity can be macroscopically assessed by the cloudiness of the fluid) after LBC slides are produced can be submitted to formalin fixation to produce cell block slides that can be used for additional molecular tests or other ancillary techniques.

The residual LBC sample can also be sent directly for molecular testing although the residual solution may not be sufficient for testing and can be stored just for a short period of time [16]. However, multiple FNA passes and changes in workflow for sample processing can yield adequate material for analysis in the majority of cases [17, 18].

Cytospin Preparations

- Stained and unstained slides are adequate for testing.
- Underutilized preparation for mutation analysis. Most frequently used for FISH analysis.
- For FISH analysis:
  - Facilitates analysis due to the nature of the specimen: avoidance of nuclear truncation artifact.
  - Widely used and well validated in the literature, especially for study of lymphomas.
- Macrodissection (cell scraping) or the cell-lifting technique used for DNA extraction similarly to smears.
- Scalpel-blade cell scraping provide higher DNA yield than the cell lifting [19].
- Concentration of the cells in a small area in the center of the slide makes the analysis faster.
- Option to produce multiple slides from one sample and archive them for future studies.
- Archived cytospins could be used as a source of DNA, with results comparable to archived smears [7, 20].

Other Preparations for Storage and Future Molecular Analysis (Cryopreservation and FTA Cards)

Cryopreservation

- Biobanking of fresh cells has the advantage of not being fixed or processed.
- "In natura" DNA can be harvested from the cells, whenever needed.
- Lack of morphological assessment (exact percentage of tumor cells might not be accurately determined—A cytospin can be run with an aliquot of the material for cellularity assessment).
- Demands more sophisticated infrastructure, such as −70 °C freezers; might be costly and not readily available.

FTA Cards

- Cheap, convenient to extract and store. Fast turnaround time for targeted panels.
- Suitable for most PCR-based technologies and NGS. Easy to transport and a viable solution for remote or underdeveloped centers.
- Lack of morphological assessment (similar to frozen material, a cytospin can be produced at the time of collection for cellularity assessment).
- Not widely validated, and therefore, needs multicenter studies.
- Robust studies on RNA extraction are not yet available.

Conclusions

As the use of minimally invasive sampling procedures expands and medicine progresses to personalized therapies, more information will be needed from limited specimens for the management of patients who might require collection of tumor material for repetitive biomarker testing on resistant, recurrent, or metastatic tumors. A rationale use of different cytologic preparations and methods aiming to increase nucleic acid quality and yield, as described in this chapter, is a sine qua non condition for a steady incorporation of cytology as a valid source of molecular material.

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