Chapter 5 - Nucleic acid extraction

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5.1 MATCH TO SAMPLE TYPE AND COLLECTION MEDIA

Human papillomavirus (HPV) detection (mainly deoxyribonucleic acid (DNA) and ribonucleic acid RNA)) can be done from different types of samples. This includes smears, biopsies, surgical pieces, lavages, dry swabs, urine, or blood. Specific collection media and preservation procedures are needed for each type of sample to adequately preserve them for further molecular analyses. Basically, samples can be kept fresh on ice, frozen, fixed, or deposited in nucleic acid preservative media. Procedures used to store samples and extract nucleic acids for HPV testing must be adapted and validated to the type of sample and mode of preservation.

Fresh samples (biopsies, surgical pieces, swabs) are probably the most suitable samples for high-quality nucleic acid extraction. While DNA is a rather stable molecule, RNA has a very short half-life due to the ubiquitous presence of RNases.[1] Therefore, as soon as the sampling has been done, rapid transfer to the lab is required (15-30 min) to avoid nucleic acid degradation. Fresh-frozen samples in liquid nitrogen kept at -80°C can also be used as nucleic acids are well preserved. As an alternative, samples can be fixed in 10% neutral-buffered formalin (corresponding to a 4% (v/v) formaldehyde solution diluted in phosphate buffer at neutral pH) just after they have been taken. This buffer is commonly used as a histological fixative but is also compatible with techniques such as DNA amplification. Because aldehyde-based fixatives induce DNA crosslinking, pre-treatment steps using heat and/or proteinase K digestion are mandatory to purify DNA.

As for cervical cancer screening, manufacturers of HPV tests can provide specific collection devices (composed of a brush and a collection medium) adapted to molecular tests. They can also claim the use of different collection media, among which liquid-based cytology (LBC) media have been adequately validated for HPV detection, for example in VALGENT (VALidation of HPV GENotyping Tests) studies.[2] This validation step is of utmost importance since it ensures that the clinical performance of HPV tests is not affected by the preservative medium. Sampling cervical cells in LBC is convenient, because the same sample can be used for both HPV testing and cytological analysis. Analytical performance of different HPV platforms has been successfully studied and reported from cells harvested in numerous LBC media.[3-9] Two major LBC media are widely used in the world, namely the PreservCyt™ (ThinPrep™ Pap Test PreservCyt™) solution from Hologic and the SurePath® solution from Becton Dickinson. From an analytical point of view, numerous studies have been conducted to evaluate the stability of HPV DNA and RNA in these LBC media. A wide variety of methodologies has been used to measure the stability of residual cervical cells or HPV-infected cell lines deposited in LBC media and stored at different temperatures (from -80°C to room temperature) for days, weeks, or months. As for HPV DNA or RNA detection, either commercial HPV kits or in-house polymerase chain reaction (PCR) have been used. Even if the results of the different studies are difficult to compare, it appears that PreservCyt™ and SurePath® adequately preserve nucleic acids from two weeks to six months.[10-17] It is important to refer to the manufacturer's instruction for use to adapt the sample transport and storage conditions (time and temperature) to each HPV nucleic acid test.[16] Of note, because the SurePath medium contains formalin, pre-treatment steps to eliminate DNA crosslinking are necessary.

More recently, analysis of cell-free circulating HPV DNA from plasma (or serum for HPV) has been proposed to monitor patients with HPV-associated cancer.[18-21] For this purpose, whole blood samples can be collected either in Ethylaminediaminetetraacetic acid (EDTA) tubes or in collection tubes with a stabilization agent (e.g. Streck - Cell-Free DNA BCT®). While blood samples taken in EDTA tubes must be processed within 4 hours after phlebotomy,[22] those taken in cell-free DNA BCT tubes are stable for up to 14 days between 6°C to 37°C.

5.2 NUCLEIC ACID EXTRACTION

Before HPV detection and genotyping, nucleic acids need to be properly extracted from the specimen. Samples usually tested for HPV are LBC samples or tissue samples (fresh tissue or formalin-fixed paraffin-embedded tissue samples (FFPE)) from the site of infection.

Nucleic acid extraction includes the following steps: Cell lysis, binding of the nucleic acids to beads or columns, washing, and elution. Cell lysis is normally performed by adding a lysis buffer, containing detergent and denaturing substances (such as salts and specific pH conditions) to the sample. Lysis buffers are optimized to ensure that proteins and other potential PCR inhibitors are removed. Cell lysis leads to the disruption of cell membranes and the subsequent release of nucleic acids. Incubation with proteinase K is an important step for the proper extraction of nucleic acids from tissue samples. This is a proteolytic enzyme (serine protease) which is added to the sample for digestion of contaminating proteins and nucleases, which otherwise can damage nucleic acids. Normally, an overnight incubation at 56ºC is used, but if needed, both the volume and time of proteinase K digestion can be adjusted to increase the yield of extracted nucleic acids.[23]

For FFPE samples, an additional incubation step at a high temperature (normally 90°C) is needed, to remove the formalin-caused crosslinking between the nucleic acids and DNA/RNA-binding proteins, resulting in a higher yield of extracted nucleic acids and better performance in downstream applications.[24] Removal of DNA-protein crosslinking due to formalin is also required for LBC samples in Surepath® medium, as earlier mentioned in section 5.1. As for FFPE samples, the crosslinking can be removed using proteinase K and heat (e.g., 1 hour to overnight incubation at 56ºC, followed by 1 hour of incubation at 90ºC).

To collect nucleic acids after cell lysis, matrices such as silica are used for binding up the nucleic acids during the extraction process.[1,25] Depending on the preferred extraction method, either the sample can be transferred to a column containing a silica membrane, or (magnetic) silica beads can be added directly to the lysed sample. After silica binding, several washing steps are performed to remove cellular debris, denatured proteins, and potential PCR inhibitors. Finally, the extracted DNA can be eluted in an adequate elution buffer that is optimized to preserve the extracted nucleic acids. The volume of the elution buffer can be varied according to the desired final concentration of the extracted material.

Depending on the samples, optimizations of different extraction conditions to increase purity and yield may be necessary, such as extending the lysis period and proteinase K digestion. Many different extraction reagents are commercially available but in-house protocols are also available, and the laboratory should determine which method is best suitable for their samples and downstream HPV analyses.

General methods used for nucleic acid extraction yield both DNA and RNA (total nucleic acids), which should be kept in mind when planning downstream applications. As RNA would generally not affect the PCR reaction, the presence of RNA in the samples should therefore not interfere with HPV DNA detection. On the other hand, RNA present in the sample can bind to a variety of modulating enzymes and may thus potentially affect downstream enzymatic steps e.g. during cloning of DNA or preparation of DNA libraries for sequencing.[26,27] However, if the aim is to use RNA for downstream applications, one should consider using specific methods optimized for preserving RNA, as RNA is more unstable and more easily degraded than DNA. These considerations are further discussed in section 5.2.1.

If a pure DNA sample is required, contaminating RNA can be removed during the extraction process by incubating the samples with RNase A in the lysis step.

Although DNA is relatively stable, storage at 4°C is recommended only for short-term use. Long-term storage of DNA should be at -20°C or -80°C. For the best preservation of the DNA integrity, freeze/thaw cycles should be avoided by aliquoting the DNA eluate before freezing.[28]

Along with viral nucleic acids, also genomic DNA is released during the extraction process. The presence of genomic DNA allows for an internal control of sample adequacy for downstream applications since genomic DNA can be used e.g., for PCR analysis of human housekeeping genes (e.g. beta-globin, beta-actin) to verify that nucleic acids have been efficiently extracted from the sample. The concentration and purity of the extracted DNA/RNA can be further determined by measuring the absorbance at 260nm and 280 nm on a spectrophotometer. The reading at 260 nm allows for DNA quantification considering that an optical density of 1 corresponds to 50 ng/µL of single-stranded DNA. Ideally, the 260/280 nm absorbance ratio should be between 1.70 and 2.00. DNA concentration and integrity can also be measured with assays using fluorescent dyes.

When handling multiple samples during DNA extraction, caution should be taken to avoid contamination between the samples. Thus, different controls should be included. These include blank samples (e.g., only paraffin), to make sure that no contamination occurred during sectioning of the specimens, as well as extraction negative controls (ENCs), containing lysis buffer and deparaffinization buffer only. The latter is included to verify that no DNA contamination has occurred during extraction. The blank samples and the ENCs are analyzed together with the tissue samples during HPV detection and genotyping.

5.3 RNA EXTRACTION

Whatever the extraction method used, general requirements need to be fulfilled to obtain high quality RNA. First, biological material should be handled with precaution and processed immediately for RNA extraction. Otherwise, it should be properly stored either in liquid nitrogen or at -70°C or in a RNA preservative solution.[29] Second, RNA molecules are particularly unstable due to the ubiquitous presence of RNAses, some of them being particularly resistant to heat inactivation by autoclaving. Therefore, RNA extraction requires special attention to avoid the degradation of this nucleic acid. This includes environmental protection by defining a separate area to work on RNA. Furthermore, it is important to clean lab surfaces, wear gloves, and avoid aerosols from pipetting. The use of the histidine-specific alkylating agent diethylpyrocarbonate (DEPC) diluted at 0.1% in water or ethanol is helpful to inactivate RNAses and decontaminate glassware. 3% hydrogen peroxide is used to decontaminate polycarbonate or polystyrene materials. All used consumables, solutions, and materials must be RNAse-free.

Regarding RNA extraction, three groups of methods are available: isopycnic gradient centrifugation, organic solvent-based methods with phenol/chloroform, and solid phase-based methods allowing RNA binding to specific surfaces in the presence of chaotropic agents.

Isopycnic gradient centrifugation is rarely used since it demands ultracentrifugation and is time-consuming. However, it permits to obtain highly pure RNA molecules. Its principle is based on the ultracentrifugation of cellular extracts in caesium chloride (CsCl) density gradient to separate RNA

molecules according to their buoyant density. After RNA is captured in the appropriate density fraction, it is precipitated using ethanol.

Organic solvent-based methods were widely used since Chomczynski published 1987 a new method of RNA extraction using a mixture containing guanidium thiocyanate (a strong chaotropic agent, allowing RNAse denaturation), phenol, and chloroform.[1,29-32] This approach is based on the different solubility of constituents, RNA being solubilized in the aqueous phase whereas proteins are solubilized in the organic phase. Briefly, tissue samples are minced in small pieces on ice and homogenized with the denaturing solution. Then water-saturated phenol and sodium acetate (pH 4.1) is mixed with chloroform/isoamyl alcohol (49:1) and the mixture is stirred by inversion and cooled on ice. Samples are then centrifuged at 10,000g for 20 minutes at 4°C and the aqueous phase containing RNA is harvested. Isopropanol is added to the aqueous phase allowing RNA precipitation at -20°C for 1 hour. Tubes are then centrifuged at 10,000g for 20 minutes, leaving a pellet that is washed with 75% ethanol. The pellet is then dried and resuspended in the buffer of choice at 65°C. Numerous commercial kits based on the Chomczynski extraction method are available with all-in-one solutions permitting a simple and rapid RNA extraction. The main drawback of these techniques is the use of toxic organic solvents such as phenol.

Numerous commercial RNA extraction kits available in the market are solid phase-based methods. One of the main advantages of these techniques is the fact that they do not necessitate organic solvents. Solid phase-based methods are based on the ability of RNA molecules to adsorb to specific surfaces due to hydrophobic, polar, or ionic properties in the presence of chaotropic agents.[29-32] These surfaces include silica, polystyrene-latex, cellulose, or glass fibers that are generally immobilized on columns or beads. After a step of denaturation with chaotropic agents, the denatured biological material is applied to the solid phase. Once the RNA has adsorbed to the solid phase, contaminants are removed by several wash steps. Elimination of the washing solution can be achieved by centrifugation or by applying a vacuum until the solution passes through the column, depending on the platform used. In the case of silica-coated magnetic beads, a magnet is used to attract the beads to the side of the tube and the supernatant can be easily removed. The advantage of using magnetic beads is that no centrifugation, vacuum filtration, or column separation is needed.[1] After washing, a step of DNase incubation eliminates DNA to keep only RNA molecules. After the inactivation of DNase, RNA is eluted with nuclease-free water or Tris EDTA (TE) buffer.

Automated RNA extraction is also available and potentially beneficial since it allows high throughput testing, increases worker safety, and increases reproducibility.[1,29-32]

Quality control for RNA extraction consists of spectrophotometric analysis at 260 nm and 280 nm. The reading at 260 nm allows for RNA quantification considering that an optical density of 1 corresponds to 40 ng/µL of single-strand RNA. The ratio of absorbance at 260 and 280 (A260/A280) permits an estimation of RNA purity. A ratio between 1.8 and 2.0 indicates a pure RNA extract.

A commercial HPV testing platform widely used includes RNA extraction steps in the overall procedure. It relies on magnetic beads coated with specific oligomers that capture HPV E6/E7 messenger RNA from 14 high-risk HPV.

5.4 FFPE SPECIMENS

FFPE tissues represent very valuable materials for research, retrospective molecular studies, and diagnosis. It is well adapted for long-term storage at room temperature but presents some limits for molecular analyses. In the process of formalin fixation PCR inhibitors, DNA/RNA cross-linking, DNA fragmentation, or DNA modification (deamination of cytosine) may be generated. Finally, this can lead to poor DNA extraction yield and limited DNA amplification by PCR.[33-38] Fortunately, many formalin-induced modifications can be overcome thanks to adapted pre-analytical steps. Specific points are detailed below.

- Tissue fixation: 10% neutral-buffered formalin (corresponding to a 4% (v/v) formaldehyde solution diluted in phosphate buffer at neutral pH) fixation followed by paraffin embedding is widely used to process tissues for pathological examination. It is of utmost importance to use buffered formaldehyde to keep nucleic acids as intact as possible.[38] The duration of fixation is also important (between 18-72h) to avoid low fixation or over-fixation. In this latter case, over-fixation will lead to high levels of DNA cross-linking reducing nucleic acid extraction yield.

- Block storage: Over time, nucleic acids may be partially degraded due to oxidization of formalin into formic acid.[38] Nevertheless, long-term storage at room temperature has probably only minor effects on nucleic acid integrity even if the length of the amplifiable DNA decreases with long storage. However, the PCR amplicon size appears as a confounding factor as PCR success largely depends on the length of the PCR product. Thus, amplicons of less than 300 bp (ideally <150 bp) are reliably produced even after many years of block storage.[35] There is no advantage storing FFPE tissue blocks at 4°C.

- Block sectioning: It is recommended to use 5-10 µm FFPE sections for nucleic acid extraction. Increasing the thickness of the section leads generally to a low yield of extracted DNA probably linked to difficulties in completely digesting the specimen. The number of sections used to extract nucleic acids depends on the size of the specimen, from one up to 10-20 sections for the smallest specimen.[35] Nucleic acid extraction should be undertaken as soon as possible after sections have been done as PCR success decreases with time. Ideally, sections should be treated within hours after sectioning. To control for possible cross-contamination between block sectioning, it is recommended to clean thoroughly all surfaces and the microtome blade with DNA/RNA hydrolysis solutions.[38] Furthermore, alternately sectioning FFPE tissue known to be HPV negative is helpful to prevent possible cross-contamination between samples.

- Deparaffinization: Deparaffinization (dewaxing) is usually carried out with organic solvents like xylene or hexane to solubilize paraffin wax. After pelleting the sample, it is washed using ethanol. This process is however time-consuming and may lead to loss of material due to successive centrifugations; xylene is also a harmful chemical. To avoid the use of organic solvents, heating the specimen in lysis buffer (120°C, 20 min) represents a simple and advantageous alternative with a successful PCR amplification.[33,35,36,38] Heating the specimen helps reverse DNA cross-links allowing for the release of longer DNA fragments.[38]

- Proteinase K treatment: Proteinase K treatment permits the proteolytic degradation of cellular proteins, especially DNA-associated proteins. Manufacturers of commercial kits generally recommend incubating samples at 56°C for 16 h but increasing the temperature to 65°C improves DNA extraction and PCR success.[33,34] Extending the duration of proteinase K digestion does not seem to increase DNA yield.

- Nucleic acid purification: It is admitted that silica membrane-based extraction kits yield high-quality DNA compared to solvent-based, magnetic beads or resin filter-based procedures. Nevertheless, DNA purification seems not mandatory for HPV studies since numerous studies investigating HPV distribution in FFPE blocks were conducted successfully from crude tissue lysates with no DNA purification.[38] In our experiences, at the French NRL, we successfully genotyped FFPE samples to study HPV distribution from crude lysates using different versions of short PCR fragment (SPF10) primer-based assays.[39-42]

5.5 CELL-FREE DNA

Cell-free DNA or circulating cell-free DNA (ccfDNA) refers to extracellular DNA present in body fluids, e.g. blood or cerebrospinal fluid. ccfDNA was first identified in 1948 by Mendel and Metais in human blood.[43] Several years later, in 1977, Léon et al. showed that cancer patients had higher concentrations of ccfDNA than healthy controls,[44] paving the way for the use of ccfDNA in clinical practice.[43,45,46] The origin of ccfDNA is not fully established but it includes cell apoptosis, necrosis, and active secretion by cells.[43] In the blood of patients with solid tumours, it has been shown that a proportion of ccfDNA is of tumour origin and is called tumour circulating DNA.[47,48] Concerning HPV-related cancers, it has been shown that HPV sequences detected in the plasma are specific biomarkers of cancer lesions but not of precancerous lesions.[18] They have also been validated as predictive or prognostic biomarkers.[46,49-52]

ccfDNA has specific features that need to be taken into account in order to use it:

- It is highly fragmented (<200 base pairs) and consistent with an origin from apoptotic cells.[53]

- Its concentration in whole blood is extremely low, about 1-50 ng/mL [54] and up to 180 ng/mL in cancer patients. Factors such as physical activity,[55] autoimmune diseases[56] or cancers can increase the concentrations of ccfDNA. The amount of ccfDNA has been the subject of debate, presumably due to pre-analytical and analytical conditions that strongly influence its measurement.[22,57-59] In our routine practice, the average of ccfDNA is around 40 ng/mL (1.7 - 3150 ng/mL) of plasma in patients with HPV-associated cancers. Circulating tumour DNA represents less than 1% of total ccfDNA.[60]

- It has a short half-life, from a few minutes (for foetal DNA) to 1-2 hours for circulating tumour DNA.[61] Half-life strongly depends on the association of ccfDNA with molecular complexes, type and stage of tumours, as well as treatment modalities.[62]

To adequately study ccfDNA, the pre-analytical steps are of utmost importance and extraction methods have to be appropriate to retrieve high-quality ccfDNA.[22] Blood samples can be collected in EDTA tubes, and in this case, they must be processed within 4-6 hours to avoid the release of genomic DNA from blood cells. Alternatively, blood collection tubes that stabilize nucleated blood cells can be used (Streck - Cell-Free DNA BCT®). These collection tubes contain a preservative preventing the release of genomic DNA and the sample is stable for up to 14 days at 6°C-37°C. The plasma is then obtained following two successive centrifugations as described in **Table 5-1**.

Table 5-1 Plasma centrifugation protocol

Collection tube	EDTA	Streck
First centrifugation	1,200 g, 4°C, 10 min	1,600 g, RT, 10 min
Second centrifugation	16,000 g, 4°C or RT, 10 min	16,000 g, 4°C or RT, 10 min

RT: room temperature

Once isolated, plasma is stored at -20°C for further analysis. For long-term storage plasma aliquots should be placed at -80°C.

As for ccfDNA extraction various methods exist essentially based on solid-phase procedures (silica membrane, magnetic beads).[60] Methods consist first in lysis allowing digestion of proteins and inactivation of DNases. Then, DNA fragments are adsorbed on silica membranes or on magnetic beads that allow purification of ccfDNA by successive washes. Finally, the extracted ccfDNA is eluted in an elution buffer. The pH of this buffer should not be acid, so DNase/RNAse-free water is not recommended. In order to increase ccfDNA recovery, elution must be carried out in two steps.

5.6 URINE

Urine samples have many benefits, being cheap, non-invasive, and easy to collect. For many women, the use of urine as a liquid biopsy for HPV-DNA testing causes less physical and psychological stress than cervical samples.[63]

HPV testing from urine samples has been used in HPV vaccine surveillance programs to monitor the HPV prevalence in populations before and after HPV vaccine introduction.[64-67] Although urine samples are not specifically collected from the site of infection, several publications report a good correlation between HR-HPV detection in urine samples compared with cervical samples.[68-71] Interestingly, several reports show that HPV detection from urine samples also can be used for cervical screening purposes.[68,72,73] Nevertheless, it is worth keeping in mind that HPV DNA detected in urine may represent a transient infection and not the persistent infection necessary for causing lesions in the cervix.

Several methods for extracting nucleic acids from urine have been reported.[74,75] However, as no standardized method is established, each laboratory should select and optimize a suitable extraction method for their downstream applications. Some aspects should be considered for proper extraction of HPV-DNA from urine, such as usage of first-void urine, addition of DNA preservatives, and storage. Several studies show that significantly more HPV-DNA can be detected in first-void urine than in later fractions.[74,76,77] This is explained by the hypothesis that more mucus and exfoliated cell debris from the vagina, cervix, and uterus is lining the urethra opening.

Adding preservatives to the urine samples prevents degradation of nucleic acids due to DNases.[74,77,78] Moreover, preservative-fixed urine is necessary for the urine sample to be comparable with vaginal samples for HR-HPV detection.[71] An example of an efficient preservative is EDTA [78,79] or boric acid[80] and several collection devices already containing specific preservatives ensuring DNA stability are commercially available.

The time between urine collection and DNA extraction can affect the yield of extracted DNA.[78] If long-term storage is needed, the urine samples should be aliquoted and stored at -80ºC.

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