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## 17.1 Introduction and Purpose

The level of degradation of nucleic acids, and especially of RNA, is a very important parameter to assess assay amenability when using fixed and paraffin-embedded (FPE) samples. The reason for this is that it's not a very well predictable phenomenon and its variable effects may false the results of qualitative and quantitative analysis on DNA and RNA. This variability depends on both biological and technical factors (type of tissue, fixation parameters, sample processing...). The degradation levels can be measured by different approaches and they are chosen according to the nature of the nucleic acid.

## 17.2 Agarose Gel Electrophoresis for DNA

Indicative integrity of genomic DNA can be checked on a 0.4% agarose gel with ethidium bromide and the level of degradation can be graded according to the electrophoretic migration of sample DNA in comparison to a known molecular weight marker (usually  $\lambda$ DNA/HindIII fragments). It can be run with or without previous enzymatic digestion depending on the expected level of degradation and the consequent length of DNA fragments present in the sample. In the first case, the degradation rate can be defined by comparing the band pattern between an intact DNA and the unknown sample; in the second case, the index of degradation is determined by the presence of a smearing pattern. For more details on the gel preparation, see also Appendix A.

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## 17.3 Denaturing Agarose Gel Electrophoresis for RNA

The quality of mRNA from fresh samples (cultured cell lines, fresh frozen tissue samples...) can be easily assessed by running total RNA in a denaturing gel stained with ethidium bromide. This method relies on the assumption that rRNA quality and quantity reflect those of the underlying mRNA population. RNA integrity evaluation is based on the intensity ratio between the 28S and 18S bands (2:1 ratio is considered an index for intact RNA) and on the observation of the rRNA degradation pattern. Anyway, it is not clear how rRNA degradation actually reflects the quality of the underlying mRNA population, since in certain conditions rRNA can turn over more rapidly than mRNA [1]. Besides, visual assessment of the 28S:18S rRNA ratio on agarose gels is not an objective parameter because the appearance of rRNA bands is affected by the electrophoretic conditions, the amount of loaded RNA, and the saturation of ethidium bromide fluorescence.

This approach is not suitable when RNA integrity assessment is performed on archival tissues because large amounts of input RNA are needed and the ribosomal bands are rarely detectable due to the high levels of RNA degradation.

### 17.3.1 Reagents

All reagents should be RNase free<sup>1</sup> and DEPC treated

- DEPC H<sub>2</sub>O
- 10% SDS or alternatively 1 M NaOH or H<sub>2</sub>O<sub>2</sub>
- Agarose powder electrophoresis grade
- 37% Formaldehyde
- 10X MOPS buffer: 0.2 M MOPS, 0.05 M sodium acetate pH 6, 0.01 M EDTA
- RNA loading buffer:<sup>2</sup> For 1.5 ml: 720 µl formaldehyde, 160 µl 10X MOPS buffer, 260 µl 37%

<sup>1</sup>Sterile, disposable plasticware should be preferably used because it is RNase free. If general laboratory glassware or plasticware is used, it should be presoaked in 0.1% DEPC-treated H<sub>2</sub>O for 2 h at 37°C. The DEPC treated-items should be rinsed thoroughly with DEPC H<sub>2</sub>O and then autoclaved. Alternatively, glassware can be put in an oven at 250°C for at least 2 h.

<sup>2</sup>It can be stored at 4°C for a maximum of 2 weeks or at -20°C for a maximum of 2 months.

formaldehyde, 80 µl 1% blue bromophenol, 100 µl 80% glycerol, 180 µl DEPC H<sub>2</sub>O

- Ethidium Bromide, 10 mg/ml (stock solution).<sup>3</sup> Stock solution of EtBr should be stored at 4°C in a dark bottle
- Running buffer: 1/10 of 37% formaldehyde in volume, 1X MOPS

### 17.3.2 Equipment

- Disinfected adjustable pipettes,<sup>4</sup> range: 2–20 µl, 20–200 µl, 100–1,000 µl
- Nuclease-free aerosol-resistant pipette tips
- 0.5 or 1.5 ml tubes (autoclaved)
- Centrifuge suitable for centrifugation/spinning at 13,200 or 14,000 rpm
- Thermoblock
- Electrophoretic apparatus with spacers
- Power supply
- UV transilluminator

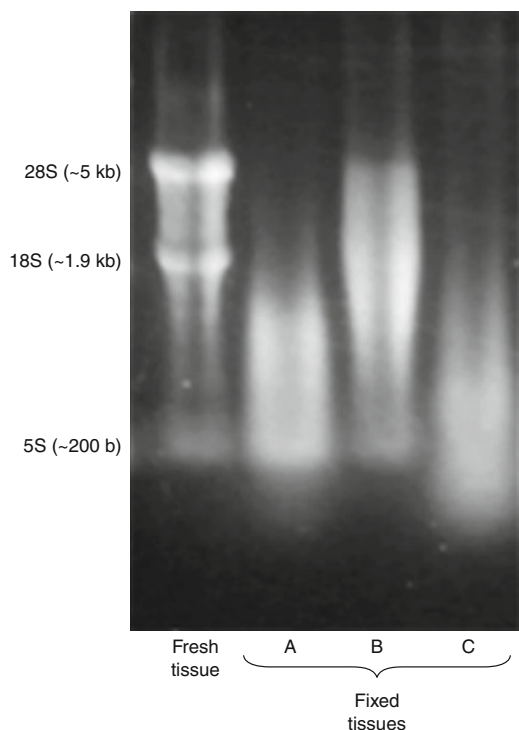
### 17.3.3 Method

- Wash the electrophoretic apparatus and spacers with 1% SDS (alternatively it is possible to use 1 M NaOH or 7% H<sub>2</sub>O<sub>2</sub>), cover and leave for 2 h under a fume hood. Wash with distilled H<sub>2</sub>O, followed by sterile H<sub>2</sub>O and finally DEPC H<sub>2</sub>O.
- Prepare the 1% agarose gel by melting 0.5 g agarose in 42.3 ml DEPC H<sub>2</sub>O. When cooled, add 5 ml 10X MOPS and 2.7 ml 37% formaldehyde. Pour the solution in the apparatus.<sup>5</sup>
- To prepare the RNA samples, use 5–50 µg total RNA. Mix 2–10 µl of RNA solution with 5 µl of RNA loading buffer. Adjust the volume with DEPC H<sub>2</sub>O to a final volume of 20 µl.

<sup>3</sup>EtBr is a potentially carcinogenic compound. Always wear gloves. Used EtBr solutions must be collected in containers for chemical waste and discharged according to the local hazardous chemical disposal procedures.

<sup>4</sup>Clean the pipettes with a disinfectant (e.g. Meliseptol@rapid) and leave them under the UV lamp for at least 10 min. Alternatively, it is possible to autoclave the pipette depending on the provider instructions.

<sup>5</sup>Formaldehyde is carcinogenic; for this reason, gels should be prepared and run under a fume hood.



**Fig. 17.1** Denaturing gel electrophoresis representing different rRNA degradation patterns. RNA from fresh frozen tissue is almost intact (ribosomal bands are clearly visible) while RNA from fixed tissues (formalin – A, methacarn – B and commercial FineFIX® – C) is highly degraded (only a smear is present)

- Denature RNA for 5' at 65°C and chill on ice.
- Add 1 µl ethidium bromide previously diluted to 200 µg/ml and load the samples into the gel.
- Separate the products at 70 V in 1X running buffer until the dye reaches three-fourths of the gel length.
- Check the gel under the UV light.<sup>6</sup> Two main bands corresponding to 28S and 18S rRNA should be detected. Acquire the intensity of the bands by a computer-based system. A 2:1 ratio between the two bands is considered an index of intact RNA. The presence of a smear and/or the absence of the ribosomal bands is an index of RNA degradation (Fig. 17.1).

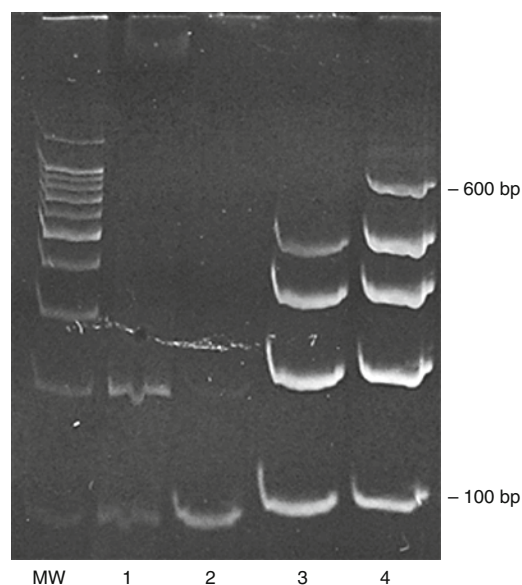
<sup>6</sup>If the gel must be submitted to further analysis (e.g., Northern blotting), deposit it on a clean plastic wrap before checking on the transilluminator.

## 17.4 PCR Amplification of DNA Fragments of Increasing Length

DNA quality from archival samples can be assessed by PCR amplification selecting fragments of increasing length. One commercial method that can be used for this purpose is the “Specimen Control Size Ladder” that is provided by InVivo Scribe Technologies. The kit consists of a master mix composed of a buffered magnesium solution, deoxynucleotides, and multiple primer pairs that allow multiplex amplification of five DNA fragments ranging from 100 to 600 base pairs. With the “Specimen Control Size Ladder” – Unlabeled both a conventional agarose and acrylamide gel can be run to visualize the results (Fig. 17.2).

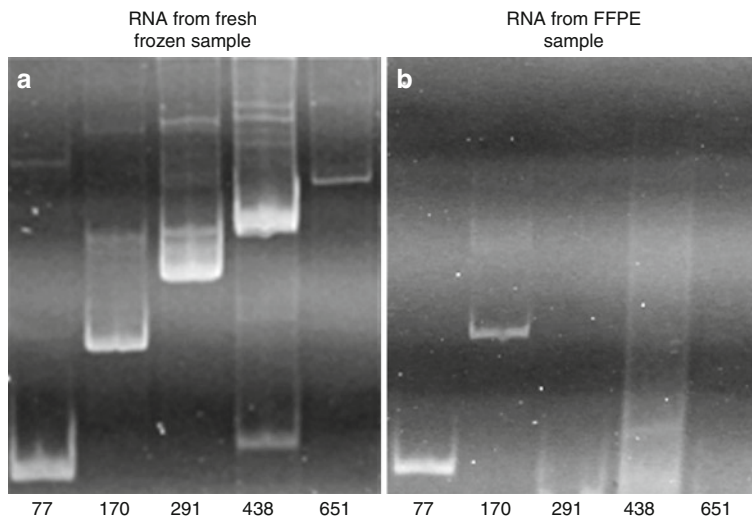
## 17.5 RT-PCR Amplification of mRNA Fragments of Increasing Length

The setting up of this method is very easy. Messenger RNA integrity is normally evaluated by RT-PCR amplification of a housekeeping gene selecting a ladder of mRNA fragments of increasing length. Reverse



**Fig. 17.2** Electrophoretic run of DNA samples at different degradation levels amplified using the “Specimen Control Size Ladder” kit. In this example, sample 2 is the most degraded, sample 4 is the best preserved

**Fig. 17.3** Polyacrylamide gel electrophoresis representing different mRNA degradation levels. (a) Intact RNA (isolated from a fresh frozen tissue) (b) Almost degraded RNA (extracted from a FFPE tissue). RT-PCR amplification has been performed using a ladder of mRNA fragments ranging from 77 to 651 bases



transcription is normally performed using MMLV enzyme combined with the random-hexamer priming strategy. The specific primer pairs used in the amplification step are designed for fragments of progressive length, preferably keeping either primer constant (Fig. 17.3). The length of the amplicons can be chosen arbitrarily, but is usually between 200 and 3,000 bp for DNA, and between 60 and 1,000 bp for RNA [2]. The major advantage of mRNA integrity determination is that the results are independent from rRNA integrity. This method can be useful in FFPE samples as it requires small amounts of starting material. (For the setting of this experiment follow the rules reported in Chaps. 19 and 25).

### 17.6 3'/5' mRNA Integrity Assay

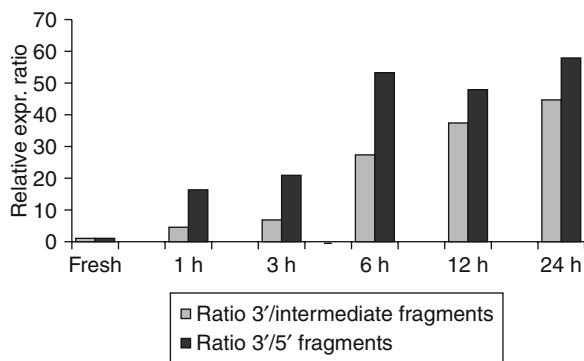
This method is specific to assess mRNA degradation levels and represents a variation of the previous approach (“RT-PCR amplification of mRNA fragments of increasing length”). It measures the integrity of the selected target gene (usually a highly expressed housekeeping gene such as GAPDH and beta-actin), which is expected to reflect the integrity of the entire mRNA subpopulation in the sample [3, 4]. In this assay reverse transcription is performed with oligo-dT and Superscript II/III. A real-time PCR reaction is carried out for three 60–80 bp-long amplicons along the transcript using three differently labeled TaqMan probes. Usually, amplicons are located at 100, 400–500, and 800–1,000 bases from the 3' end of the gene, corresponding to the so-called 3', intermediate and 5' fragment, respectively.

The quantitative ratios between the Ct values of the 3' and 5' amplicons reflect the degradation level and the efficiency of the RT enzyme to proceed along the transcript. The higher the RNA fragmentation, the lower the RT efficiency, and consequently, the PCR yield. A 3':5' ratio of about 1 indicates high RNA integrity, whereas progressive ratios indicate increasing degradation (Fig. 17.4). For details regarding the setting of the TaqMan-based experiments and interpretation of the results, see Chap. 25, Sect. 25.2.

### 17.7 Agilent Bioanalyzer 2100<sup>7</sup>

This microfluidic capillary electrophoresis system by Agilent Technologies is based on fluorescent dyes binding to nucleic acid and allows the checking of not only DNA and RNA integrity, but also nucleic acid quantity (as already reported in Chap. 16, Sect. 16.2.2.5). When RNA integrity is assessed, three types of data analysis can be performed by this instrument. The first is the visual analysis of the electropherogram (“visual method”). It assigns a categorization of the RNA degradation levels (e.g., from 1 to 5) [5], taking into account the ribosomal peak height, the baseline flatness, and the presence of additional peaks between the ribosomal peaks (Fig. 17.5). Alternatively, two automatically calculated parameters can be used. The first one is the 28S/18S ratio (“Ratio method”). A ribosomal

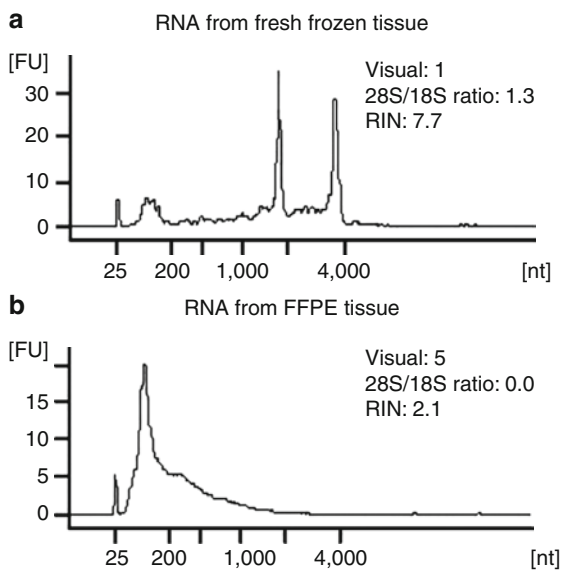
<sup>7</sup>See <http://www.chem.agilent.com/Scripts/PDS.asp?IPage=51> for more details



**Fig. 17.4** Quantitative ratios calculated from the Ct values associated with the 3' fragment and the two fragments located at increasing length from the 3' end of the gene. Ratios of RNA isolated from fresh frozen sample usually correspond to 1 and progressively increase in RNA extracted from formalin-fixed samples (in this example cultured cell samples were progressively formalin fixed from 1 to 24 h)

band ratio of (28S:18S) between 0.7 and 2.5 is considered to be typical of good-quality RNA. The second one is the RIN (RNA Integrity Number). It takes into account the whole electropherogram, including in the analysis the total RNA ratio (the fraction of the area in the region of 18S and 28S compared to the total area under the curve), the fast region (the area between 18S peak and the marker), and the marker height.<sup>8</sup> The RIN range is between 1 and 10 (RIN 1 corresponds to completely degraded RNA, whereas a RIN 10 is achieved for an intact RNA sample). Values equal or higher than 6 are associated with good-quality RNA [6]. The Agilent Bioanalyzer system offers several advantages. It requires a very small amount of starting material, allowing assaying DNA and RNA quality even in small samples. Furthermore, interpretation of integrity is standardized and for this reason it is comparable among different laboratories. However, this approach appears more suitable for fresh frozen samples than for archival specimens because of the high degradation levels of nucleic acids isolated from fixed and embedded material. When RNA integrity is assessed in these specimens, 28S/18S ratios are usually around 0 and RIN are usually between 1 and 3.5 [1]. These low values, however, do not preclude the use of nucleic acids isolated

<sup>8</sup>The marker is an internal standard that is used to align the ladder analysis with the individual sample analysis. The RNA ladder is a mixture of six RNA transcripts of well-defined size and total concentration that is run first from the ladder well. Both marker and ladder are provided in the kit.



**Fig. 17.5** Electropherograms representing different levels of RNA degradation. (a) Nearly intact RNA (isolated from fresh frozen tissue) (b) Highly degraded RNA (isolated from FFPE tissue). These traces can be used to evaluate RNA integrity by visual assessment (operator-dependent), 28S/18S ratio and RIN number (automatically calculated by the software)

from archival samples in PCR-based assays. For details about sample preparation and loading, follow the manufacturer's instructions.

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