RNA Extraction Workshop

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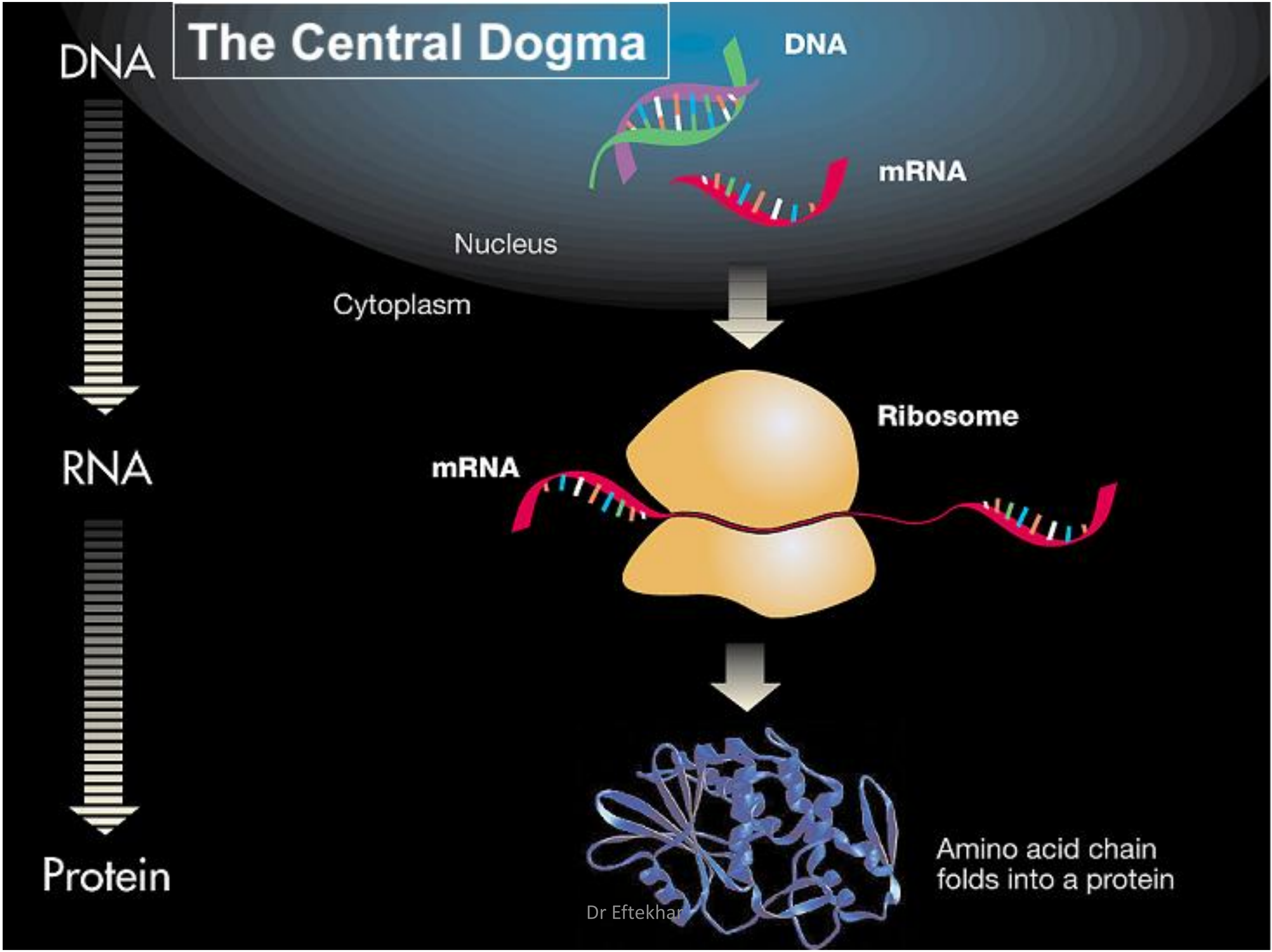

The Central Dogma
DNA
RNA
Protein
DNA
mRNA
Nucleus
Cytoplasm
Ribosome
mRNA
Amino acid chain
folds into a protein

TABLE 36–1 Classes of Eukaryotic RNA

RNA	Types	Abundance	Stability
Ribosomal (rRNA)	28S, 18S, 5.8S, 5S	80% of total	Very stable
Messenger (mRNA)	~10^5 different species	2–5% of total	Unstable to very stable
Transfer (tRNA)	~60 different species	~15% of total	Very stable
Small RNAs			
Small nuclear (snRNA)	~30 different species	≤1% of total	Very stable
Micro (miRNA)	100s–1000	<1% of total	Stable

Total cellular RNA

TABLE 1. RNA species and their relative abundance

RNA type	Relative abundance %	Picogram/ cell
rRNA	80	10–30
snRNA	15	2–5
tRNA		
mRNA	5	0.5–2

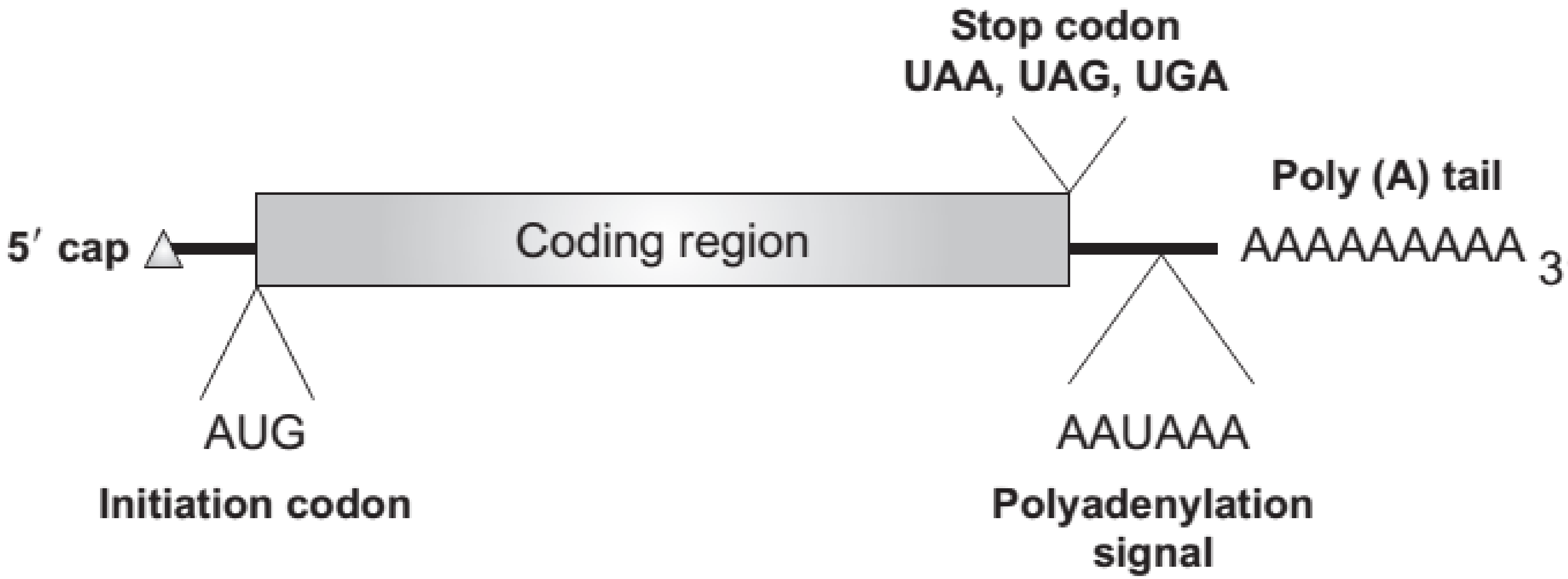


Figure 1.7 Topology of a typical eukaryotic mRNA molecule.

RNA Isolation

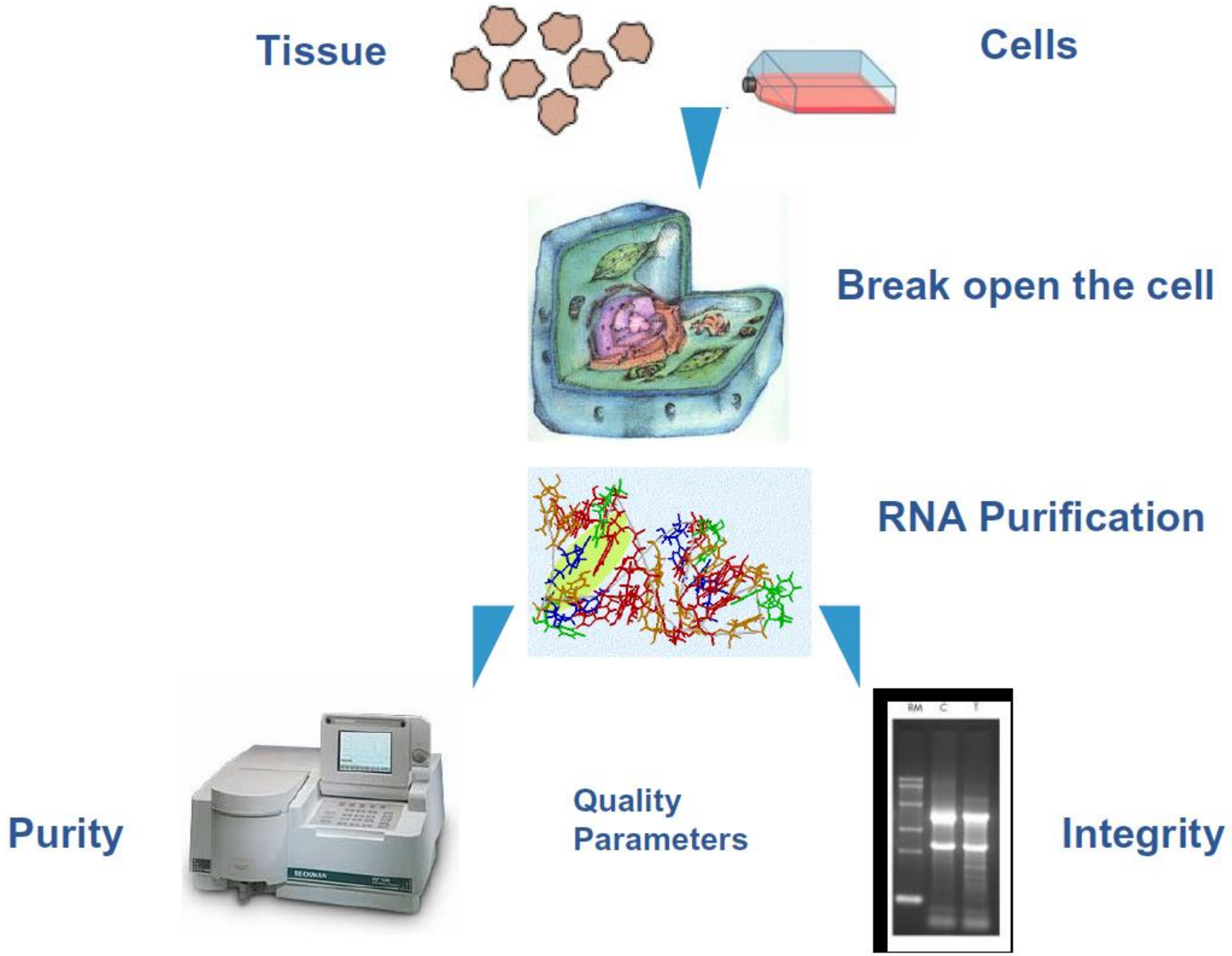
- Isolation of pure, intact RNA is one of the central techniques in today's molecular biology.

- Obtaining pure RNA is an essential step in the analysis of patterns of gene expression and understanding the mechanism of gene expression.

Table 2.1 Subpopulations of Eukaryotic RNA

mRNA Subpopulation	Composition
Total cellular RNA	All RNA from the cell
Total cytoplasmic RNA	Cytoplasmic RNA, excluding mtRNA
Nuclear RNA	Transcripts (mature and immature) isolated directly from intact nuclei.
mRNA	Transcripts that have matured and been exported to the cytoplasm. This subpopulation includes both poly(A)$^+$ and poly(A)$^-$ transcripts.
Cellular Poly(A)$^+$ RNA	Nuclear and cytoplasmic polyadenylated transcripts
Cytoplasmic Poly(A)$^+$ RNA	Cytoplasmic polyadenylated mRNA
Poly(A)$^-$ RNA	Non-polyadenylated RNA (mostly rRNA and tRNA)
mtRNA	Mitochondrial RNA (all types)
cpRNA	Chloroplast RNA (all types; plants only)

RNA isolation



Elimination of Ribonuclease activity

- the control of RNase activity must be considered from two prespective:
- 1- Extrinsic RNase activity must be controlled.

- external sources of potential RNase contamination must be identified and neutralized from the onset of the experiment and all reagents maintained RNase-free at all times.
- external sources include: bottles and containers in which chemicals are packaged, non-nuclease-free water, gel boxes and combs, and microbial and fungal contamination of buffers.

- The most commonly used inhibitors included in extraction buffers for inhibiting endogenous RNAse are as follows:

1-Strong protein Denaturation agents. These include guanidinium hydrochloride and guanidinium isothiocyanate used at a concentration of 4M.

These agents can quickly inactivate endogenous RNAses and contribute to denaturation of nucleoprotein complexes.

- In order to denature RNAse irreversibly by these compounds, a high concentration of 2-mercapthoethanol is also included.


P/N: AM9780, AM9782
L/N: 1204062
MADE IN USA
RNaseZap
Completely removes RNase contamination from glass and plastic surfaces
Directions: Apply directly to work surfaces and apparatus to be cleaned, or dampen a paper towel, wipe thoroughly, rinse and dry. For labware vessels, add enough solution to coat surface. Follow with two rinses of distilled water. See instruction sheet for further information.

5- **RNAlater®**

is a aqueous formulation (Ambion) that is well known in RNA circles for its ability to prevent chemical and RNase-mediated deterioration of RNA in cells, tissues, and organs until such time as it is convenient for the investigator to process the biological material and recover high-quality RNA for any of several uses.

RNA later rapidly permeates tissues, offering several strategic advantages with respect to the collection of samples, especially when working out in the field.

RNA later is compatible with plant and animal cells and tissues, as well as E. coli .

RNA isolation strategies for various tissues

- There is no single fool-proof method for the expedient isolation of RNA from tissue samples.

- Each tissue type has qualities that make the isolation methodology unique to that sample.

- The tissue mass, the cell type(s) involved, the age of the tissue, the status of the tissue (fresh, flash-frozen, formalin-fixed, animal or plant) are all important considerations

Fresh tissue

- Immediately upon harvesting, the tissue should be rinsed in ice-cold phosphate-buffered saline (PBS), weighed, and then rapidly minced into small pieces using a nuclease-free scalpel.
- It is wise to avoid reusable scalpel blades, a fresh disposable blade should be used for each tissue sample. The minced tissue is then dropped into lysis buffer as quickly as possible.
- When transferring minced tissue into lysis buffer, it is best to transfer as little PBS as possible; if the lysis buffer is diluted too much it will lose its ability

Frozen tissue

- If samples must be stored for later processing, manageable-sized cell pellets or tissues should be snap-frozen using a liquid nitrogen and then maintained at -80 ° (When stored as such, RNA can be purified up to a year later).

- When processing frozen material extreme care must be taken to avoid even minimal amounts of thawing, which could detrimentally affect the integrity of an RNA preparation.

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Two homogenization methods

- 1- Polytron disruption

- Is a motorized device used to rapidly homogenize all types of plant and animal tissue, as well as bacterial cultures, in large and small volumes.
- Attached to the motor is a stainless steel probe, properly known as a “ generator ” .

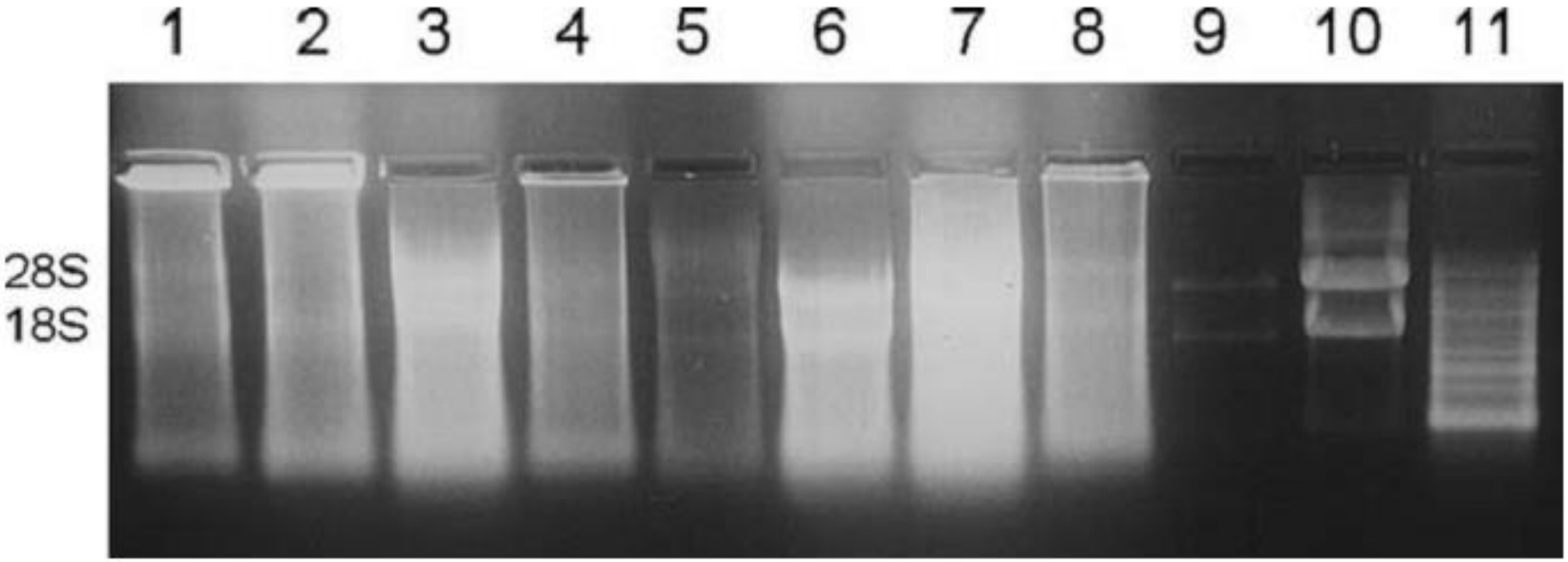
- The end of the generator is characterized by very sharp, knifelike, stainless steel saw-tooth blades that are designed to shred tissue efficiently.

- 3- Dounce homogenization

- is a hand-held glass implement that resembles a test tube. is a much more gentle and economical means of breaking up tissue.
- glass homogenizers are thoroughly scrubbed and rinsed, and then soaked in 3% H_2O_2 for 20 minutes before being rinsed with copious amounts of autoclaved water.

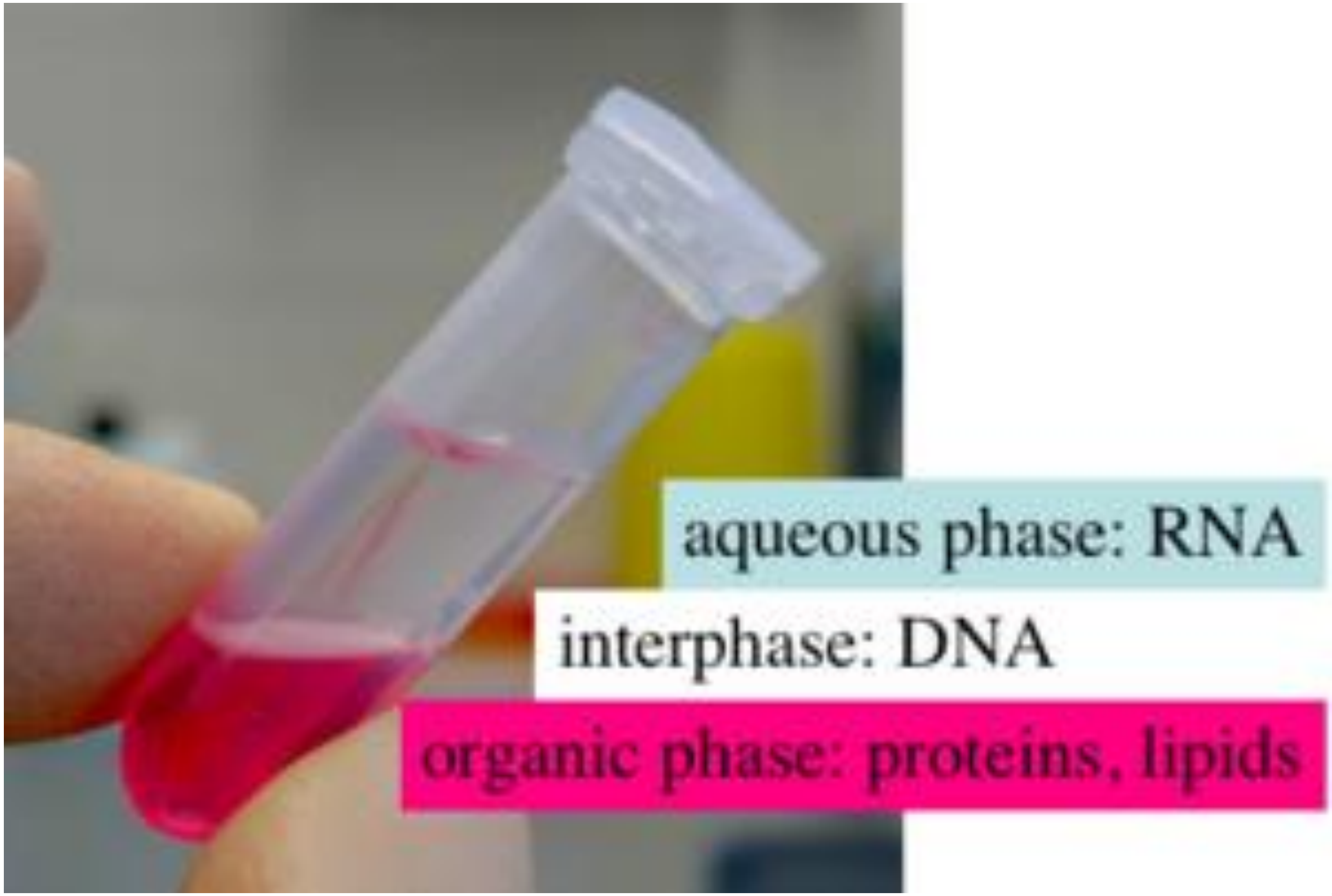
- After air-drying, each homogenizer and its pestle are individually wrapped in foil and autoclaved to ensure that the homogenizer is nuclease-free the next time it is used.

Dounce homogenizer

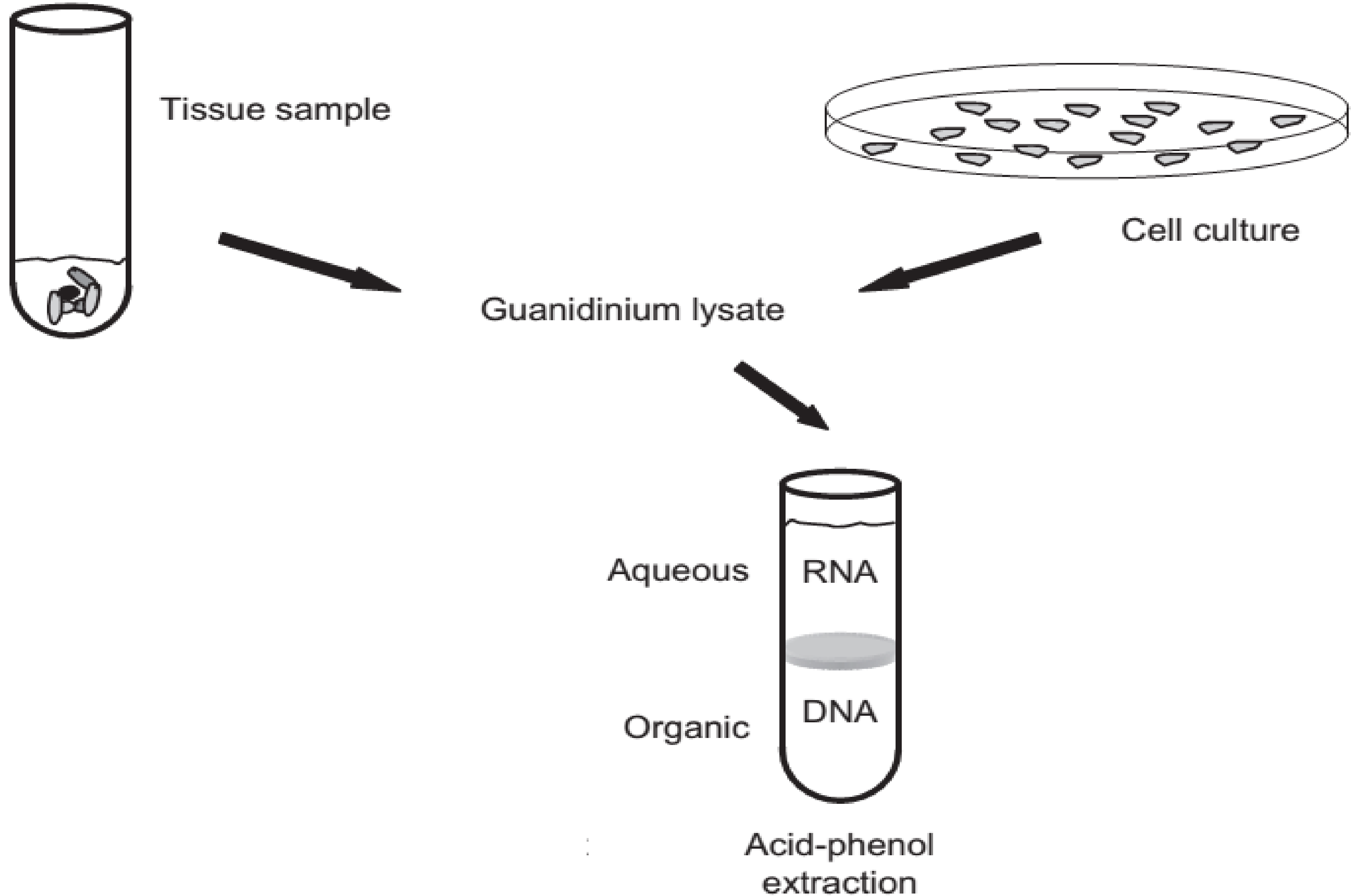


- Appearance of incompletely dissolved RNA. Lanes 1 – 8: RNA was isolated from various human tissues Since the amount of RNA recovered from the tissue was so great, 500 µl was not enough to completely dissolve it, which also compromised the accuracy of spectrophotometric concentration measurements.
- Aliquots of the RNA samples were removed from the master tube and diluted further until the 28S and 18S rRNAs were plainly visible upon electrophoresis. Lane 10: RNA that was isolated from human tissue at an earlier date and dissolved properly.

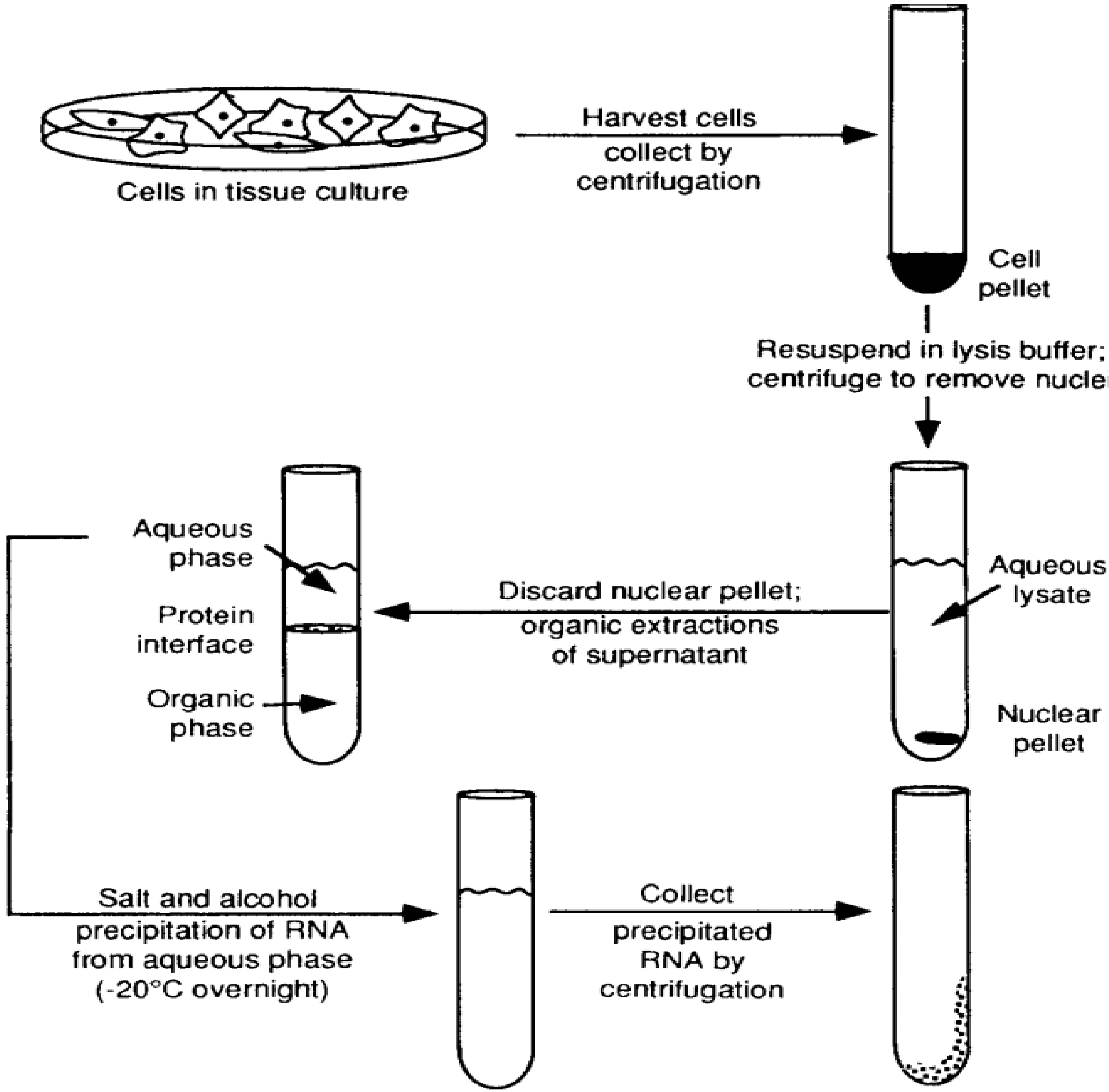
Methods of RNA isolation



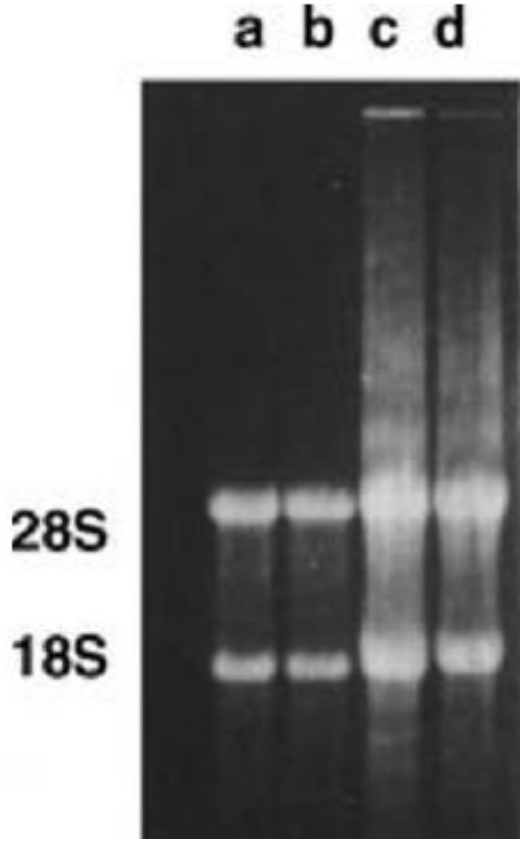
TRIsol: when used, it resembles cough syrup, bright pink



Methods for fractionation of total cellular RNA following cellular disruption with guanidinium buffer and acid-phenol extraction.

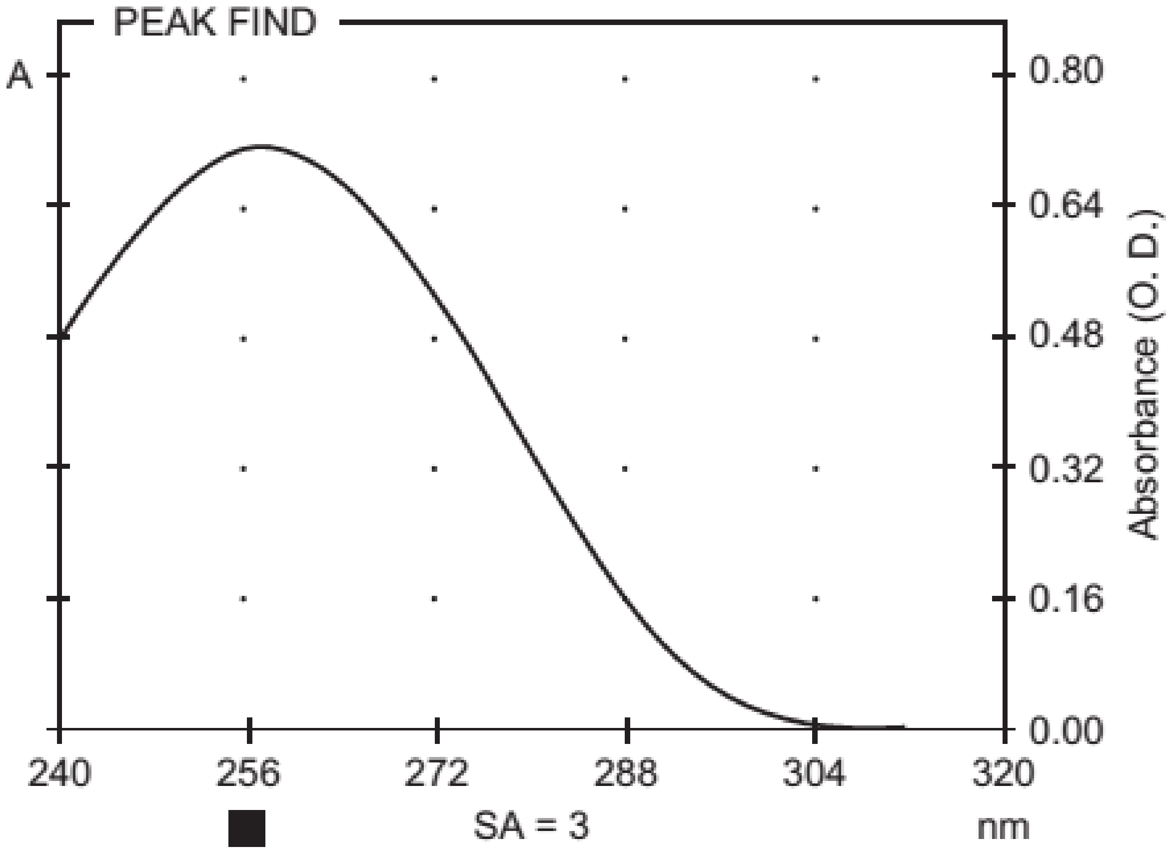


Preparation of total cytoplasmic RNA by gentle hypotonic. This method is best suited for cells grown in culture; soft tissue samples may require a gentle, nonaggressive homogenization step.



Comparison of size distribution of total cellular RNA and total cytoplasmic RNA. Lanes a and b: 20µg total cytoplasmic RNA isolated with NP-40 lysis buffer . Lanes c and d: 25 µg of total cellular RNA isolated by guanidinium-acid-phenol extraction. The higher molecular weight species (hnRNA) are clearly visible above the 28S rRNA in lanes c and d. The obvious fluorescence in the wells of lanes c and d suggests a small amount of contaminating DNA.

Quality Control for RNA Preparations



Typical UV absorbance spectrum of purified RNA. The spectrophotometric profile for purified DNA is similar. Note the positive slope of the curve below 260 nm, and the negative slope above 260 nm

- Quality control technique 1: Determination of nucleic acid concentration and purity by UV spectrophotometry
- The extinction coefficient for RNA is 0.025 $\mu g^{-1} cm^{-1}$ when measured at neutral or slightly basic pH.
- Thus, an absorbance of 1.0 at 260 nm gives a RNA concentration of 40 µg/ml.(1/0.025 =40).

- [RNA] µg/ml= A260x dilutionx40

where

A_{260} = absorbance, in optical densities, at 260 nm (OD_{260})
Dilution = dilution factor
40 = average extinction coefficient of RNA (40 µg/OD_{260})

	Expected A_{260}/A_{280} Ratio
Double-stranded DNA	1.7-1.9
RNA	1.8-2.0
	pH Impact on Ratio
DEPC treated water (pH 5-6)	1.6
Nuclease free water (pH 6-7)	1.85
TE (pH 8)	2.14
	Contaminant Impact on Ratio
Phenol contamination	↓ ratio
High protein contamination	↓ ratio
Guanidine isothiocyanate contamination	↓ ratio

- The pH of the diluent does, in fact influence the absorbance ratio, and is most reliable when measurements are made between pH 7.5 – 8.5.

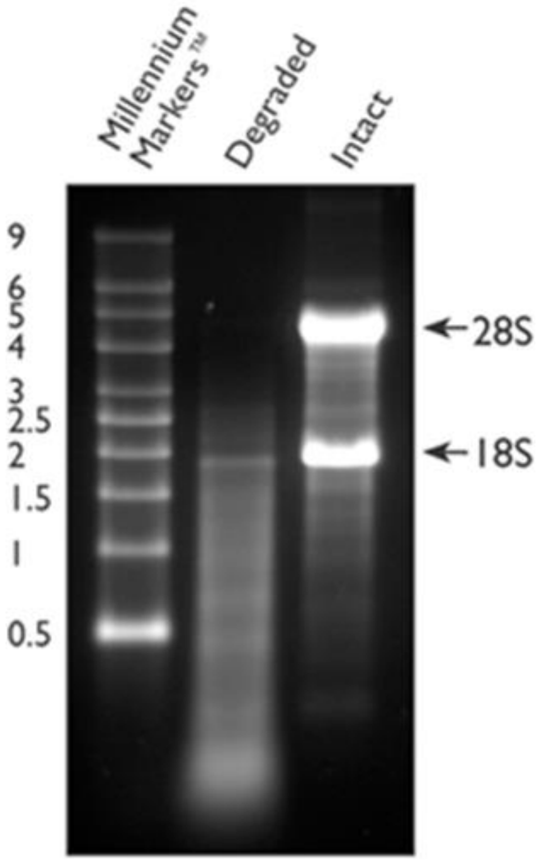
- It is now commonplace to dilute both DNA and RNA samples in TE- or 1-5mM phosphate buffer (Na_2HPO_4),but not water, to establish a pH within this range.

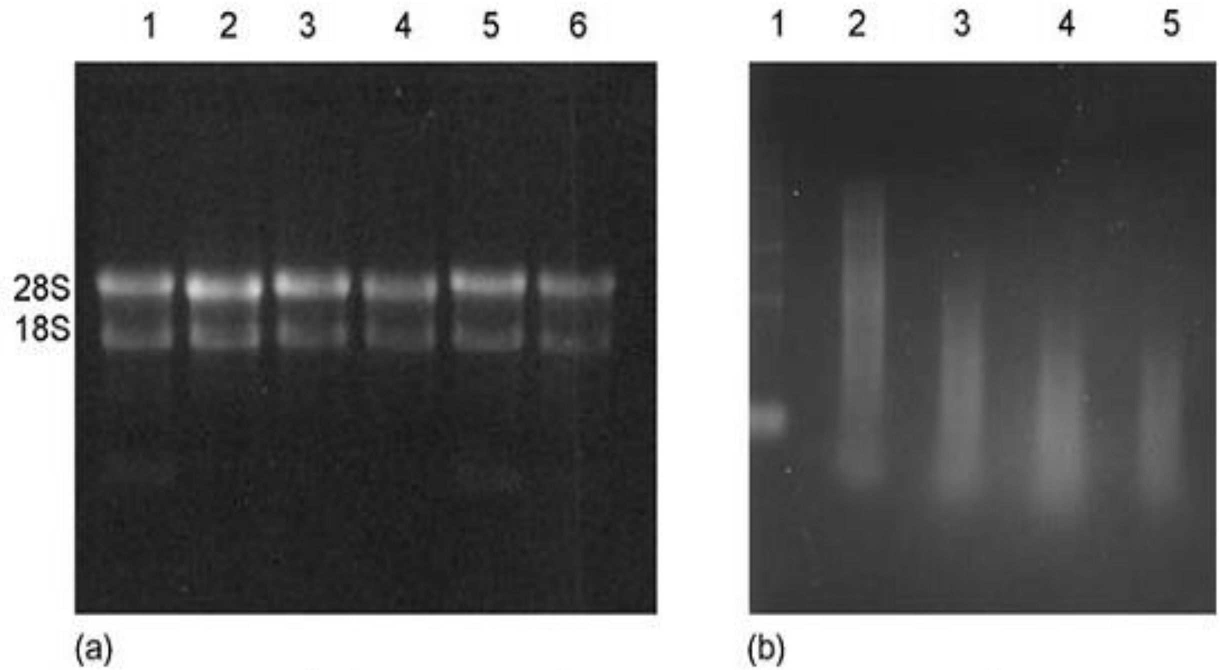
- The take-home lesson is that biochemical quality water, which is usually slightly acidic with pH around 6.0, is not the best choice for accurate measurement of the concentration of nucleic acid in a sample

Quality control technique 2: Electrophoretic profile of the RNA

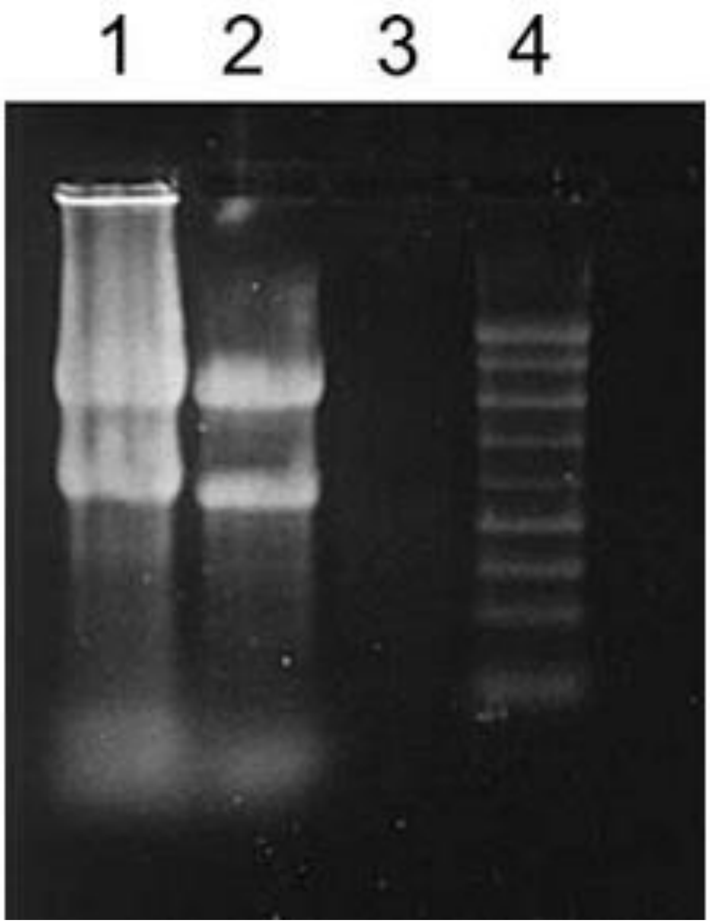
- running out an RNA sample on an agarose gel is the single best diagnostic that the investigator has at his disposal.
- There is no better indicator of the probable utility of an RNA sample than measurement of the fluorescence ratio between the 28S rRNA and the 18S rRNA.

- For mammalian RNA, a minimum fluorescence ratio of 2:1 is desirable and a ratio of 2.5:1 or greater is better. it is interesting to note that this is not the case for all eukaryotes.





Assessment of the integrity of RNA samples. (a). Five micrograms of purified RNA was electrophoresed in a 1.2% agarose-formaldehyde gel . The clear definition of the ribosomal 28S and 18S rRNA species and the greater fluorescence of the 28S rRNA in lanes 1 – 6 demonstrates the integrity of the samples. (b) Samples in lanes 2 – 5 show increasing degrees of degradation



- Electrophoresis of RNA sample.Fluorescence confined to a well (lane 1) into which a sample was loaded strongly suggests genomic DNA contamination

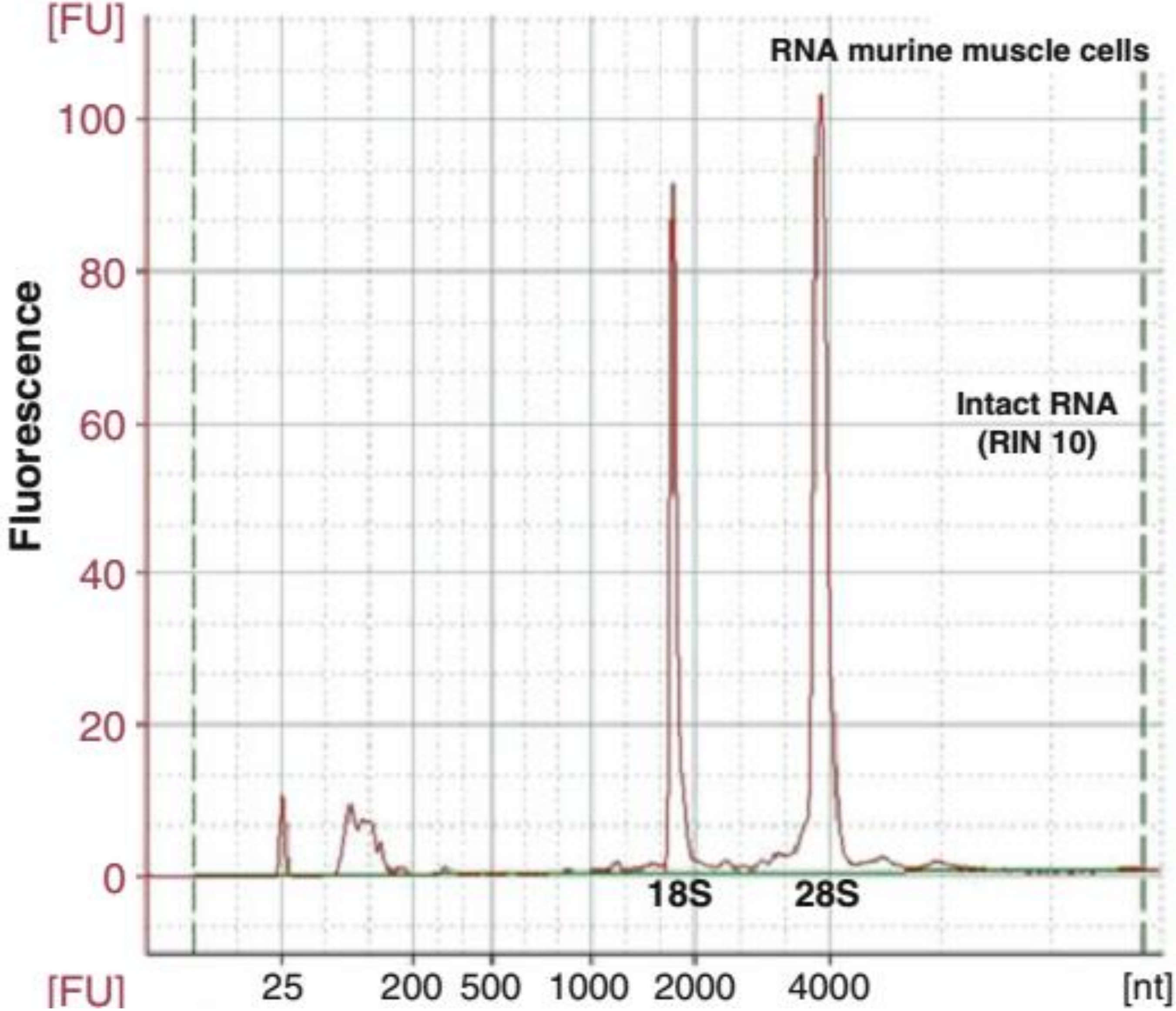
- The Agilent 2100 Bioanalyzer also provides an RNA Integrity Number (RIN) as a useful measure of RNA integrity.

- Ideally, the RIN should be close to 10, but in many cases (particularly with tissue samples), RNA quality is greatly influenced by how well the original sample was preserved

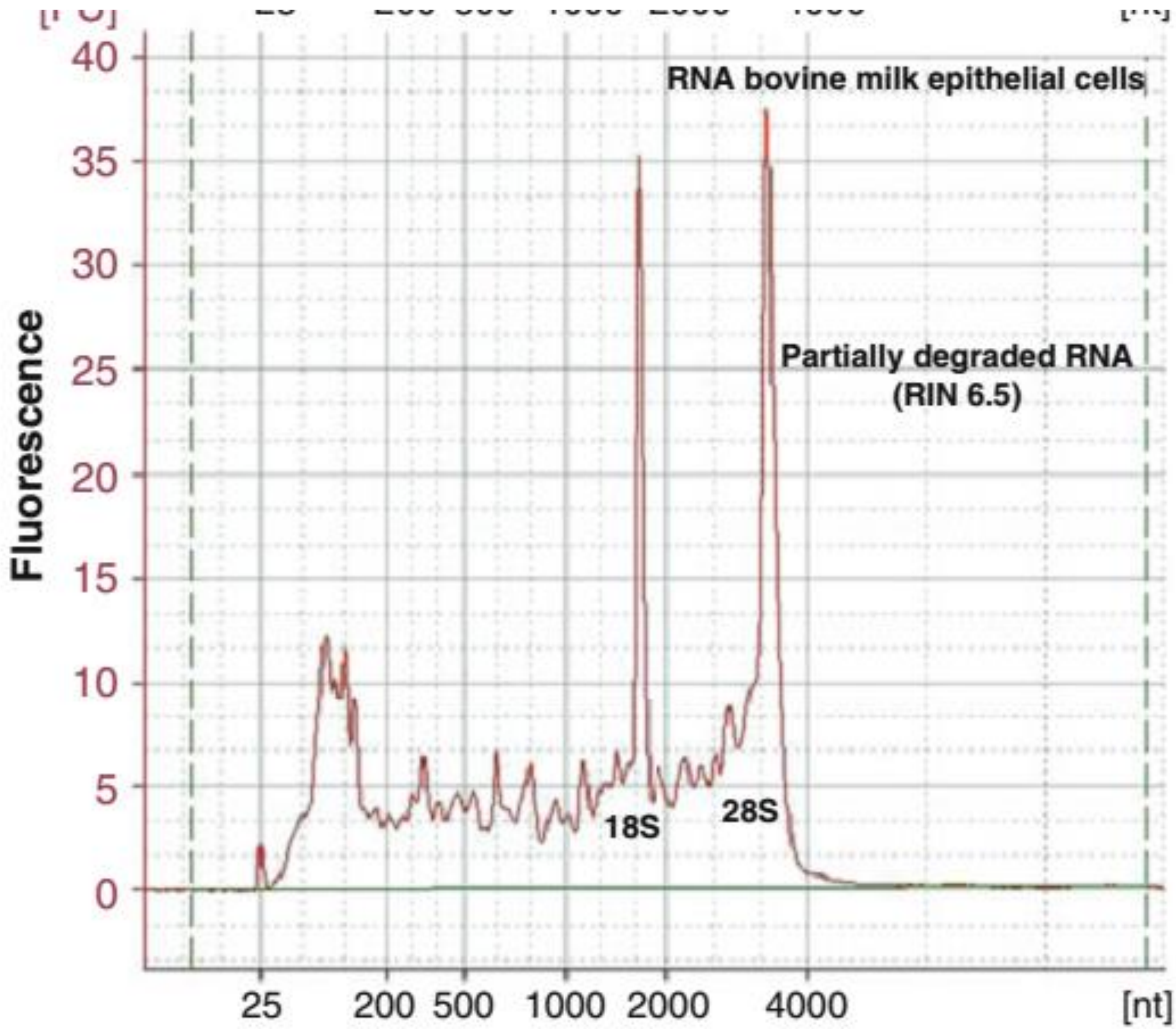
- instruments such as Agilent Technologies' 2100 Bioanalyzer or Bio-Rad Laboratories' Experion has become the golden standard for RNA quality control.

- RNA samples are separated electrophoretically on a microfabricated chip, and fragments are detected via laser-induced fluorescence measurement.

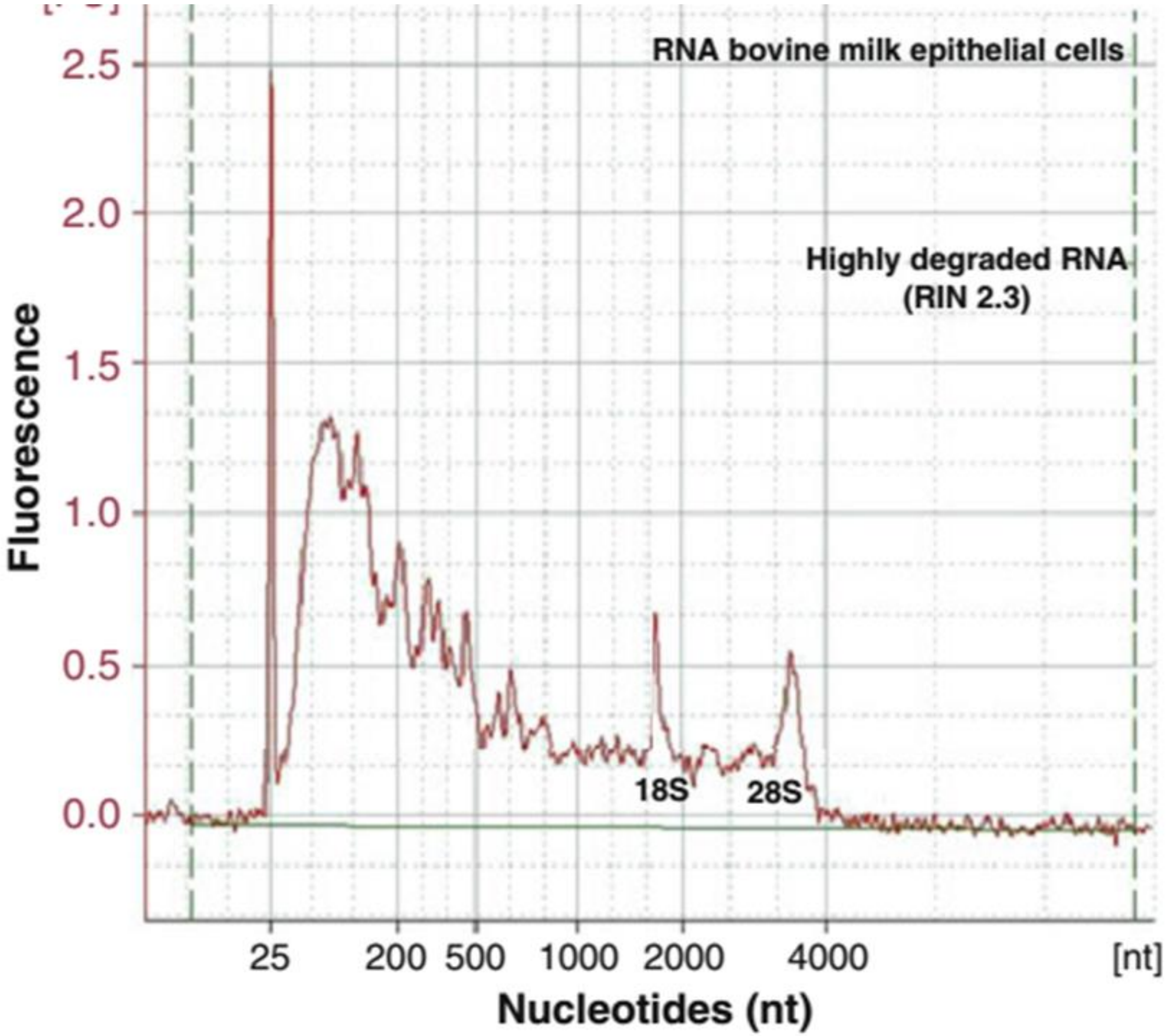
- Estimation of RNA band sizes and total concentration is achieved by using an RNA ladder as a mass and size standard.



[FU]
100
80
60
40
20
0
Fluorescence
RNA murine muscle cells
Intact RNA
(RIN 10)
18S
28S
[FU]
25
200
500
1000
2000
4000
[nt]



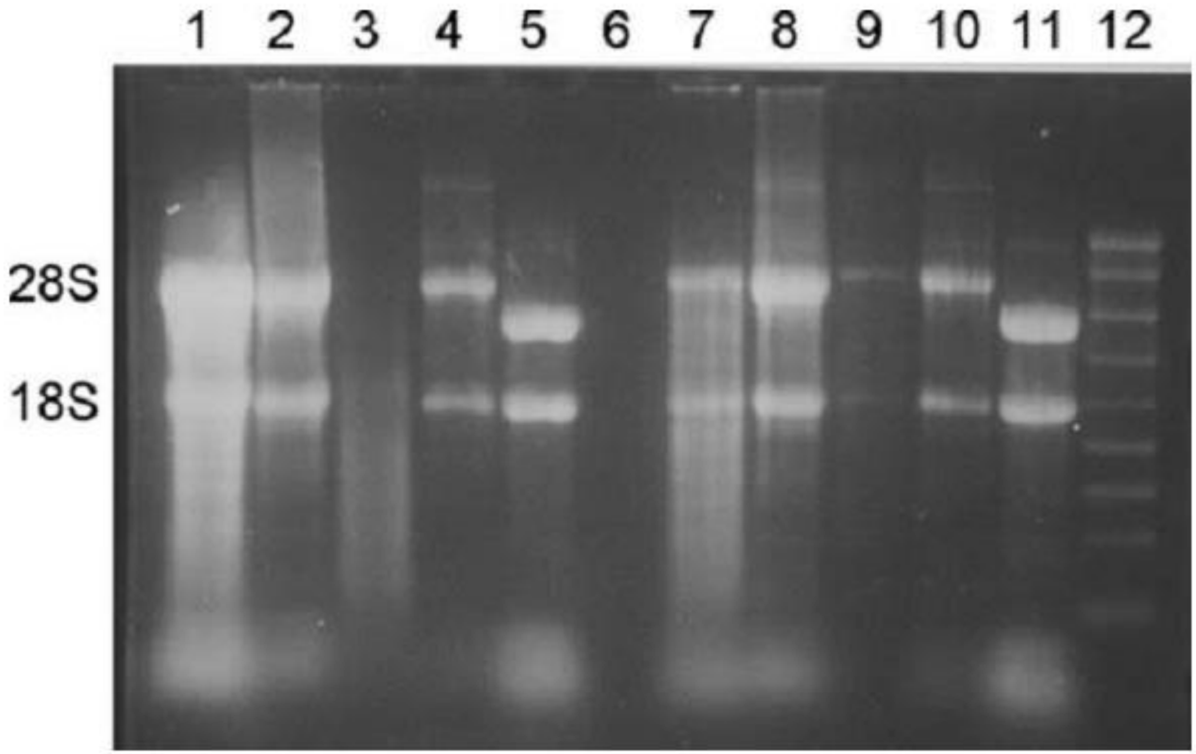
RNA bovine milk epithelial cells
Partially degraded RNA
(RIN 6.5)
Fluorescence
18S
28S
40
35
30
25
20
15
10
5
0
25
200
500
1000
2000
4000
[nt]



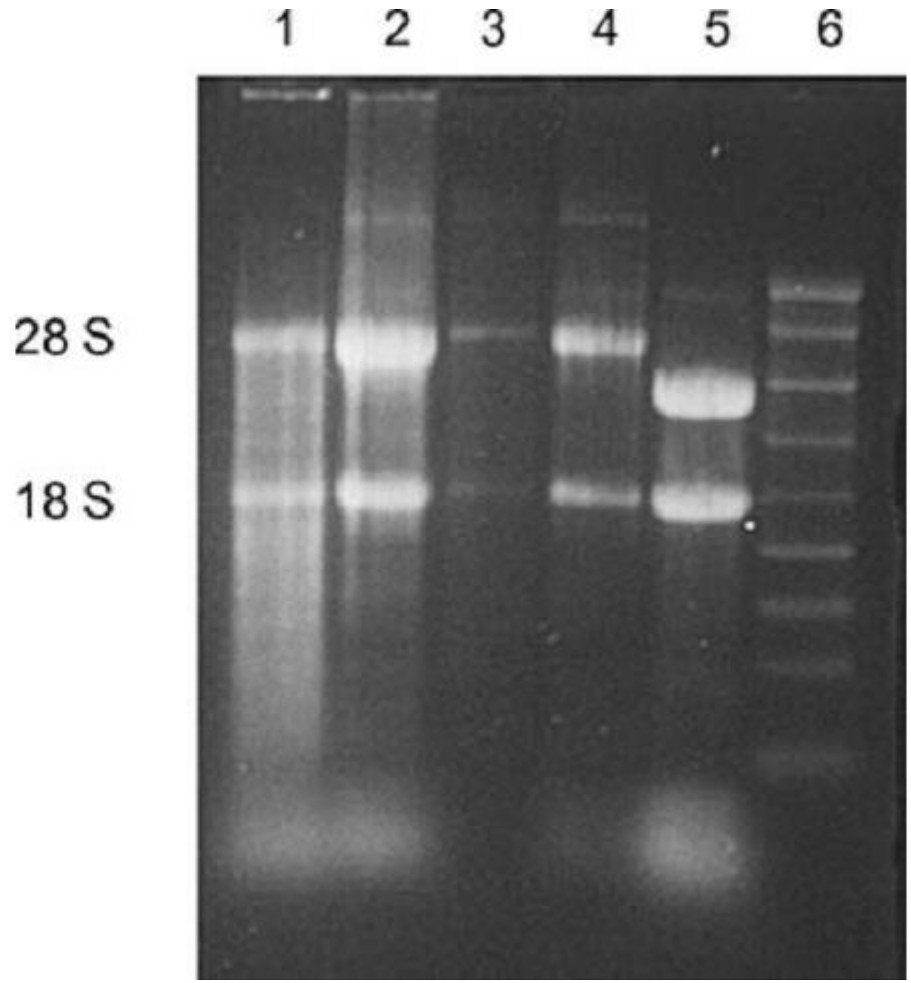
Electropherograms of total RNA with varying degradation levels using the 2100 Bioanalyzer (Agilent Technologies)

Quality control technique 3: Sample capacity to support RT-PCR

- one standard protocol in this laboratory is to use primers that are known to work all the time when RNA is isolated intact and clean, from a particular biological source.
- Testing a sample in this way can be performed using primers, such as those for β -actin or GAPDH, just to show that one can at least amplify something from the RNA in question.
- If a PCR product is evident with control primers, then the reason for failure to generate a product from experimental primers can be investigated more intelligently.



Comparison of eukaryotic RNA profiles from different species. RNAfrom CHO-K1 cells (lanes 1 – 4 and 7 – 10) and yeast (lanes 5 and 11) on a denaturing 1.2% agarose gel. (Lane 1) Total cytoplasmic RNA, – good quality, though the lane is overloaded. (Lane 2) Total cellular RNA – fair quality, with evidence of high molecular weight hnRNA. (Lane 3) Poly(A)$^+$ RNA – sample is degraded. (Lane 4) Poly(A)− RNA – good quality. (Lane 5) Total yeast RNA–good quality. (Lane 7) Total cytoplasmic RNA, – poor quality, showing signs of degradation. (Lane 8) Total cellular RNA purified – good quality, with evidence of high molecular weight hnRNA. (Lane 9) Poly(A)$^+$ RNA .(Lane 11) Total yeast RNA. (Lane 12) Single-stranded RNA molecular weight standard from Promega. Notice the shift (greater mobility) in the 28S rRNA in the yeast sample in lanes 5 and 11, compared to the CHO-K1 samples. Notice also the presence of hnRNA and the precursor 45S rRNA in lanes 2, 4, 8, and 10. Dr Eftekhar



samples of RNA from CHO-K1 cells (lanes 1 – 4) and yeast (lane 5) were electrophoresed on a denaturing 1.2% agarose gel. (Lane 1) Total cytoplasmic RNA, – poor quality. (Lane 2) Total cellular RNA , good quality, with evidence of high molecular weight hnRNA. (Lane 3) Poly(A)$^+$ RNA – good quality, with traces of the major rRNA species. (Lane 4) Poly(A)$^-$RNA – good quality. (Lane 5) Total yeast RNA – good quality. (Lane 6) RNA molecular weight standard from Promega. The 28S rRNA in yeast is smaller than in mammals, and thus has greater mobility, compared to the electrophoretic migration of the 28S transcript in the CHO-K1 samples.

Organism	Type	Length (in bases)	MW (in kDa)
E. coli	tRNA	75	26
	5S rRNA	120	41
	16S rRNA	1,541	523
	23S rRNA	2,904	987
Drosophila	18S rRNA	1,976	672
	28S rRNA	3,898	1.3×10^3
Mouse	18S rRNA	1,869	635
	28S rRNA	4,712	1.6×10^3
Human	18S rRNA	1,868	635
	28S rRNA	5,025	1.7×10^3
Rabbit	18S rRNA	2,366	804
	28S rRNA	6,333	2.15×10^3

Product	Quantity of starting material	Typical Yield
High Pure RNA Tissue Kit	➢ Tissue: 1 – 10 mg	➢ 0.5 – 3.0 μg/mg[a]
High Pure RNA Isolation Kit	➢ Blood: 200 – 500 μl ➢ Cultured cells: 10^6 cells ➢ Yeast: 10^8 cells ➢ Bacteria: 10^9 cells	➢ for 10 RT-PCR Reactions ➢ 20 μg[b] ➢ 20 μg ➢ 35 – 50 μg
High Pure Viral RNA Kit	➢ 200 – 600 μl of serum, plasma, urine, cell culture supernatant	➢ Product detectable by RT-PCR
mRNA Isolation Kit for Blood/Bone Marrow	➢ 1.5 – 5 ml of blood, bone marrow aspirate	➢ 50 – 200 ng/ml blood
mRNA Isolation Kit for White Blood Cells	➢ Blood: 1.5 – 5 ml	➢ 50 – 200 ng/ml blood
mRNA Isolation Kit for Tissue	➢ Tissue: 10 – 100 mg ➢ Cells: 10^6 – 10^7	➢ 5 – 35 μg/100 mg[a,b]

[a] depends on type of tissue
[b] depends on type of cells

Size of ribosomal RNAs from various sources (Qiagene)

Source	rRNA	Size (kb)
E. coli	16S	1.5
	23S	2.9
S. cerevisiae	18S	2.0
	26S	3.8
Mouse	18S	1.9
	28S	4.7
Human	18S	1.9
	28S	5.0