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Phenol-chloroform Extraction

Introduction

A **phenol-chloroform extraction** is a liquid-liquid extraction. A liquid-liquid extraction is a method that separates mixtures of molecules based on the differential solubilities of the individual molecules in two different immiscible liquids (28). Liquid-liquid extractions are widely used to isolate RNA, DNA, or proteins[1].

Brief History

Volkin & Carter reported the first use of guanidinium chloride in the isolation of RNA in 1951 (30). In 1953, Grassmann & Defner described the efficacy of phenol at extracting proteins from aqueous solution (16). Utilizing this find, Kirby demonstrated the use of phenol to separate nucleic acids from proteins in 1956 (18). Cox and others renewed interest in the use of guanidinium chloride in the isolation of RNA from ribonucleoproteins in the 1960s (11,12,13). From then on, guanidinium extractions were the method of choice for RNA purification, replacing phenol extraction. The use of guanidinium thiocyanate instead of guanidinium chloride was first briefly mentioned by Ullrich et al. in 1977 (29), and later successfully employed by Chirgwin et al. in 1979 (8). Chirgwin et al. used guanidinium thiocyanate to isolate undegraded RNA from ribonuclease-rich tissues like pancreas. A combination of guanidinium thiocyanate and hot phenol for RNA isolation was reported by Feramisco et al. in 1981 (14). In 1987, Chomczynski & Sacchi combined guanidinium thiocyanate with phenol-chloroform extraction under acidic conditions (9). Since its inception, the Chomczynski & Sacchi method has been the method of choice to isolate RNA from cultured cells and most animal tissues (10).

Extraction of Nucleic Acids

The extraction of nucleic acids involves adding an equal volume of phenol-chloroform to an aqueous solution of lysed cells or homogenized tissue, mixing the two phases, and allowing the phases to separate by centrifugation (**Figure 1**). Centrifugation of the mixture yields two phases: the lower organic phase and the upper aqueous phase.

Chloroform mixed with phenol is more efficient at denaturing proteins than either reagent is alone. The phenol-chloroform combination reduces the partitioning of poly(A)+ mRNA into

[1] Abbreviations used: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; poly(A)+, polyadenylated; phenol:$CHCl_3$, phenol-chloroform; mRNA, messenger ribonucleic acid; RNase(s), ribonuclease(s).

the organic phase and reduces the formation of insoluble RNA-protein complexes at the interphase (24). Moreover, phenol retains about 10-15% of the aqueous phase, which results in a similar loss of RNA; chloroform prevents this retention of water and thus improves RNA yield (22). Typical mixtures of phenol to chloroform are 1:1 and 5:1 (v/v). At acidic pH, a 5:1 ratio results in the absence of DNA from the upper aqueous phase; whereas a 1:1 ratio, while providing maximal recovery of all RNAs, will maintain some DNA present in the upper aqueous phase (17). Isoamyl alcohol is sometimes added to prevent foaming (typically in a ratio of 24 parts chloroform to 1 part isoamyl alcohol). Guanidinium salts are used to reduce the effect of nucleases.

Purified phenol has a density of 1.07 g/cm^3 and therefore forms the lower phase when mixed with water (1.00 g/cm^3) (21). Chloroform ensures phase separation of the two liquids because chloroform is miscible with phenol and it has a higher density (1.47 g/cm^3) than phenol (21); it forces a sharper separation of the organic and aqueous phases thereby assisting in the removal of the aqueous phase with minimal cross contamination from the organic phase.

In general, a solute dissolves best in a solvent that is most similar in chemical structure to itself. The overall solvation capacity of a solvent depends primarily on its polarity (20). For example, a very polar solute such as urea is very soluble in highly polar water, less soluble in fairly polar methanol, and almost insoluble in non-polar solvents such as chloroform and ether (21). Nucleic acids are polar because of their negatively charged phosphate backbone, and therefore nucleic acids are soluble in the upper aqueous phase instead of the lower organic phase (water is more polar than phenol) (20). Conversely, proteins contain varying proportions of charged and uncharged domains, producing hydrophobic and hydrophilic regions (3). In the presence of phenol, the hydrophobic cores interact with phenol, causing precipitation of proteins and polymers (including carbohydrates) to collect at the interface between the two phases (often as a white flocculent) or for lipids to dissolve in the lower organic phase (3).

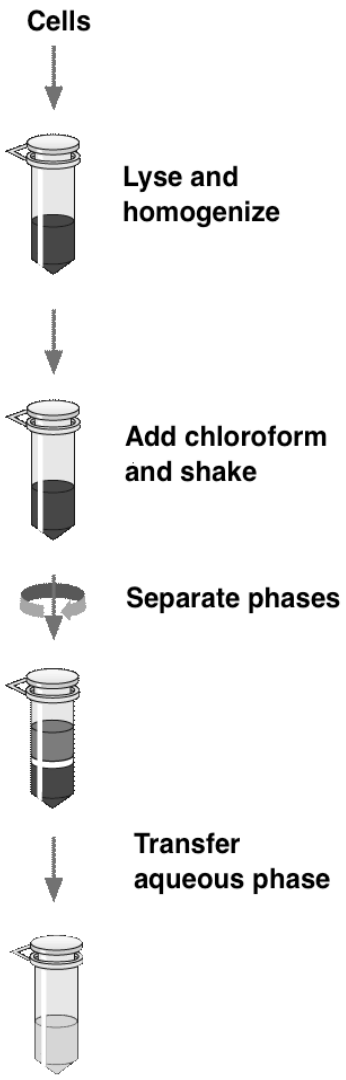

FIGURE 1. The procedure of phenol–$CHCl_3$ extraction. Afterward, the product is cleaned by alcohol precipitation or column purification.

The pH of phenol determines the partitioning of DNA and RNA between the organic phase and the aqueous phase (6,23). At neutral or slightly alkaline pH (pH 7-8), the phosphate diesters in nucleic acids are negatively charged, and thus DNA and RNA both partition into the aqueous phase. DNA is removed from the aqueous layer with increasing efficiency as the pH is lowered with a maximum efficiency at pH 4.8. At this acidic pH, most proteins and small DNA fragments (<10 kb) fractionate into the organic phase and large DNA fragments and some proteins remain at the interphase between the organic and aqueous phases

(6,9,25). Acidic phenol retains RNA in the aqueous phase, but moves DNA into the phenol phase, because the phosphate groups on the DNA are more easily neutralized than those in RNA (i.e., DNA is less acidic/has a greater pKa than RNA) (**Figure 2**) (5,26). An acid pH also minimizes RNase activity (7).

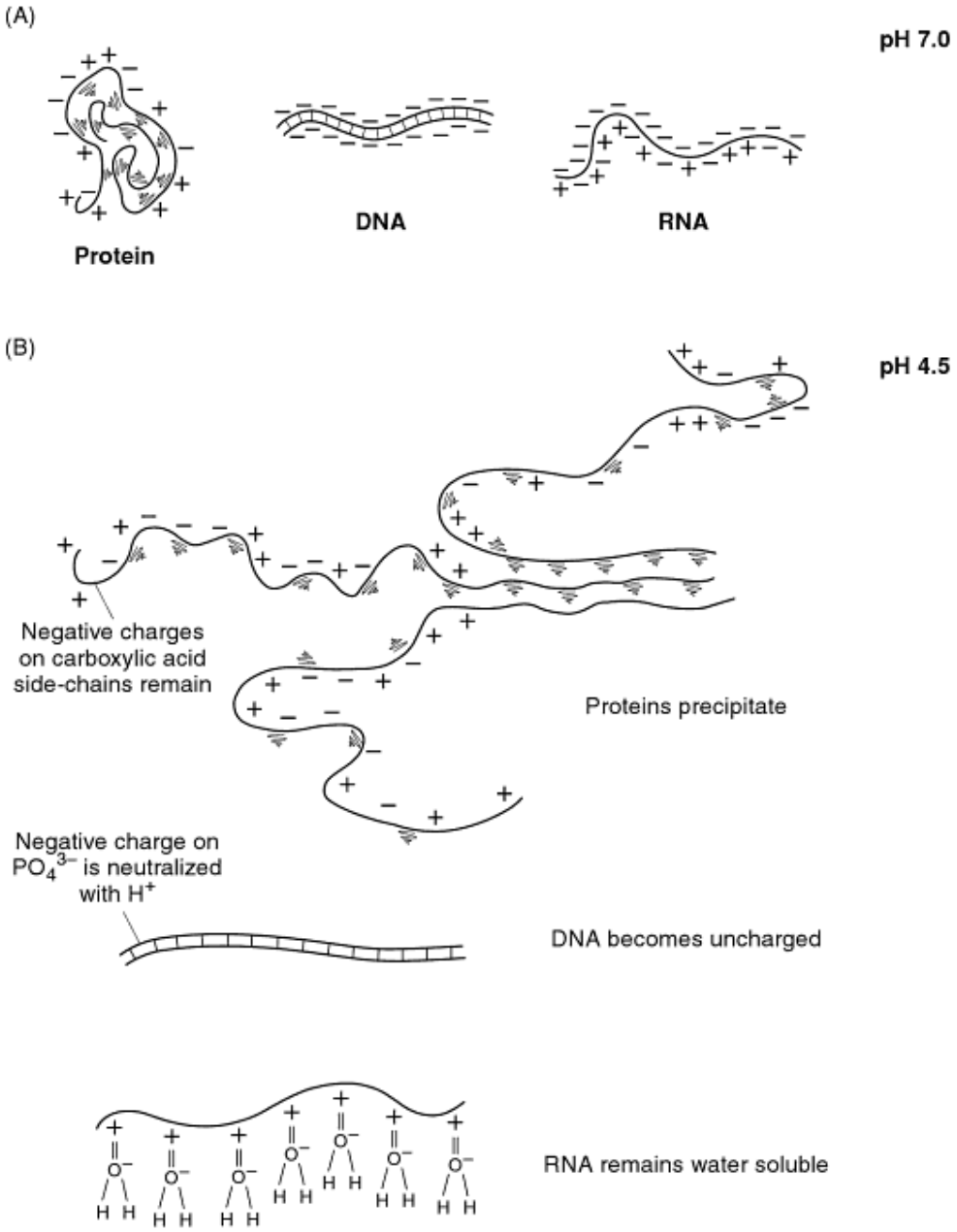

FIGURE 2. Acid phenol specifically leaves RNA in the aqueous phase. As the pH decreases, the concentration of protons increases. DNA carries a negative charge because of the phosphate groups in its sugar-phosphate backbone, which are neutralized in acid by protonation. In this case, DNA dissolves in the organic phase (*like dissolves like*). RNA, on the other hand, is not neutralized in acid because, even though it also has a negative charge, it has exposed nitrogenous bases (it is single-stranded), which can form hydrogen bonds with water, keeping it in the aqueous phase. (3)

Protocol for Isolation of RNA from Animal Cells or Tissues

Materials

Reagents

- TRIzol reagent
- Chloroform

Miscellanea

You will need access to a fume hood, vortexer, micropipettes, chilled microcentrifuge, pellet pestle homogenizer, and all standard equipment of a biochemistry-molecular biology laboratory. ▲ **CRITICAL** Maximum care should be taken to not contaminate samples with RNases. For this reason, use pipettes reserved for RNA work to prevent cross-contamination with RNases from shared equipment. Always wear disposable gloves, as skin cells as well as bacteria and molds can contaminate samples and can be sources of RNases. Use either disposable, sterile plasticware or nondisposable glassware or plasticware that is RNase-free. For this, glassware should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240 °C for at least 4 h. Since autoclaving alone will not fully inactivate many RNases, glassware can be treated with diethylpyrocarbonate (DEPC). Fill glassware with 0.1% DEPC (0.1% in water), incubate for 12 h at 37 °C, and then autoclave or heat to 100 °C for 15 min to eliminate residual DEPC. Plasticware can be soaked for 10 min in 0.5 M NaOH, 1 mM EDTA followed by RNase-free water. Chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases. (15) **! CAUTION** TRIzol reagent and chloroform are both toxic and therefore should be handled under a fume hood (1,2).

Setup

Before beginning, clean the workstation (pipettor shaft, benchtop) with a surface decontamination solution that destroys RNases (such as RNase-zap); and chill the microcentrifuge to 4 °C.

Procedure

Homogenization

1| Use option A for tissue or option B for cultured cells.

▲**CRITICAL** Procedure is done at room temperature (15-25 °C) unless otherwise indicated.

(A) Fresh tissue is preferable for optimal RNA isolation. Alternatively, tissue should be submerged in RNAlater stabilization reagent immediately after dissection and stored at –80 °C. Add 1 ml TRIzol reagent per 100 mg fresh tissue, mince on ice using sterile scalpels, and homogenize with a sterile pellet pestle probe. Frozen tissue should be thawed and removed from RNAlater stabilization reagent prior to the addition of TRIzol.

(B) Cell cultures should be processed immediately after removal from the incubator. Either centrifuge cells grown in suspension at 300 x g for 5 min at RT (15-25 °C) and discard supernatant or remove the culture medium from cells grown in monolayer. In both cases, it is not necessary to wash the cells with saline. Add 1 ml TRIzol reagent per 1×10^7 cells to cell pellets or directly to the culture dish or flask for cells grown in monolayer. Resuspend the lysate with a sterile, disposable 1-ml pipette tip.

Extraction

2| Transfer the tissue or cell lysate to a 2-ml siliconized low-retention tube.

3| Pass sample through sterile, disposable 21 g needle 10 times. In doing so, you will fragment high-molecular weight cellular components (DNA), thus minimizing their presence in the aqueous phase.

4| Let homogenate sit at RT (15-25 °C) for 5 min for complete dissociation of nucleoprotein complexes.

■ **PAUSE POINT** You can stop at this point, store your samples at –20 °C, and complete the procedure later.

5| Add 200 ul chloroform. Vortex vigorously for 15 s.

▲**CRITICAL** Thorough mixing is important for subsequent phase separation.

▲**CRITICAL** When mixing and shaking, make sure that the caps are tightly closed!

6| Let homogenate sit at RT (15-25 °C) for 3 min.

7| Centrifuge at 12,000 x g for 15 min at 4°C. After centrifugation, heat the centrifuge to room temperature (15–25°C).

8| Transfer upper aqueous phase (600 ul) to new 1.5 ml RNase-free tube.

▲**CRITICAL** Volume of lysate may be less than 600 ul due to loss during homogenization and centrifugation.

9| Proceed to either silica-gel membrane purification or alcohol precipitation.

● **TIMING**

Cell or tissue homogenization and RNA extraction: less than 1 h.

? TROUBLESHOOTING

Troubleshooting is discussed in **Table 1**.

TABLE 1 | Troubleshooting table.

PROBLEM	POSSIBLE REASON	SOLUTION
Incomplete phase separation	a) No chloroform added or chloroform not pure	Make sure to add chloroform that does not contain additives.
	b) Homogenate not sufficiently mixed before centrifugation	After addition of chloroform, the homogenate must be vigorously shaken. If the phases are not well separated, shake the tube vigorously for at least 15 s, and repeat the incubation and centrifugation in steps 6 and 7.
	c) Organic solvents in samples used for RNA purification	Ensure starting sample does not contain organic solvents (e.g., ethanol, DMSO), strong buffers, or alkaline reagents. These can interfere with phase separation.

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