Technical Info

Well advised with Roth.

Phenolic DNA Purification – Background and Protocol

(acc. to Sambrook and Russell, Molecular Cloning, Third Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 2001)

Background

In molecular cloning, one of the most basic procedures is the purification of nuclear acids. During this process, it is often necessary to inactivate and/or remove enzymes or other proteins that derive from cell extracts or that were needed during a step of cloning procedure. This removal of proteins can often be carried out by simply extracting the aqueous solution of nucleic acids with phenol, phenol/chloroform or phenol/chloroform/ isoamyl alcohol.
The most standardised version of this process is to first extract with phenol/chloroform (1:1), optionally containing hydroxyquinoiine at 0.1 %, followed by one or two purification steps with chloroform. This additional chloroform extraction removes any residual phenol from the solution containing the nucleic acids. For a detailed protocol, see below.

> **Note:** Deproteinisation is more efficient when two different organic solvents are used instead of one during the process.

In 1956, Kirby published the first use of phenol to purify nucleic acids [1]. His idea based on the observation of Grassmann and Deffner (1953), who had previously reported the potency of phenol to extract proteins from aqueous solutions [2]. In his paper, Kirby showed that extraction of homogenates of mammalian tissue with a two-phase phenol-H_2O mixture at room temperature led to partitioning of RNA into the aqueous layer. DNA remained associated with the proteins in the interphase. Kirby then showed that after replacement of pure water by solutions of anionic salts both molecules, RNA as well as DNA, could be found in the aqueous phase. Subsequently, anionic salts in their function as liberators of nucleic acids from proteins were replaced by strong anionic detergents like SDS. The basic approach published in this primary paper, however, was never profoundly altered and still forms the basis of many purification methods presently in use.

> **Note**: The function of phenol during this process is most probably the same as that of a protein solvent – extraction of proteins that have been dissociated from nucleic acid by detergents.

Phenol isolation of nucleic acids is highly efficient: Pure preparations of nucleic acid are obtained after just two or three extractions with phenol. Denatured proteins collect at the interface between the two phases. Lipids partition efficiently into the organic layer. DNA or, depending on the phenol used, whole nucleic acids highly enrich in the aqueous phase and can easily be removed from the organic phase and interphase.
For extraction of DNA, Tris- or TE-saturated phenol of a pH of approx. 7.8 is used. Phenol of this pH value suppresses partitioning of DNA into the organic phase and, therefore, both kinds of nucleic acids are isolated equally. Hence, for RNA isolation, pure water is used as solvent for phenol which then has a pH of about 4.8. At this pH, DNA is caused to enrich in the organic phase and in the protein-interphase, reducing the DNA contamination of RNA isolates significantly.

Purified, solubilized phenol has a specific gravity of 1.07 and, therefore, forms the lower phase when mixed with water or aqueous solutions. Normally, the aqueous phase is on top. However, those two phases are sometimes difficult to separate or may even invert.

> **Note**: Phenolic and aqueous phases may invert when phenol is used to extract nucleic acids from aqueous solutions containing high concentrations of solutes (e.g. >0.5 M salt or >10 % sucrose).

This problem can be avoided by using a 1:1 mixture of phenol and chloroform, because the higher density of chloroform (1.47) ensures a clear differentiation and separation of the two phases. Removal of the upper phase can often easily be carried out. In order to avoid foaming of the organic phase that may also hinder pipetting, a small amount of isoamyl alcohol ma be added. Additionally, this mixture also completely inhibits RNAses and prohibits enrichment of poly(A)-RNA in the phenolic phase.

Note: Although pure phenol denatures proteins efficiently, it does not completely inhibit RNAse activity and serves as a solvent of DNA with long tracts of poly(A) [3], which, subsequently, may be lost during purification.

Protocol

Note: Phenolic solutions should be completely clear and non-coloured and can be stored for approx. 1 year in a refrigerator (dark and under steady temperature). In case a more or less pronounced reddish or pink stain is visible, the phenol has started oxidising and is likely to destroy the nucleic acids during the purification process. Discard and replace by a new batch!

1. Transfer the sample to a reaction tube. Add one volume of phenol/chloroform or phenol/chloroform/ isoamyl alcohol

   Note: Phenol should have a pH of 7.5-8.0 for DNA isolation and of 4.5-5.0 for RNA isolation.

2. Mix the contents of the tube vigorously until an emulsion forms.
   Note: Mix as follows: for isolation of a) small DNA molecules (<100 kb) by vortexing, b) middle sized DNA molecules (10-30 kb) by gently shaking or by rapid inverting, c) large DNA molecules (>30 kb) by rotation on a wheel.

3. Centrifuge the tube at 80 % of the max. speed that the tube can bear (normally 14.000 g) for 1 min. at room temperature. Repeat centrifugation (for up to 10 mins), if the two phases are not yet well separated. At the interface, a more or less pronounced and light coloured protein layer is visible.
   Note: Normally, the aqueous phase is on top. However, special composition of the aqueous phase may lead to inversion of the phases (see above). The organic phase is easily identifiable because of the yellow colour resulting from the hydroxyquinoline added to the phenol.

4. Pipet the aqueous phase to a fresh tube. Discard the interface and organic phase.
   Note: In order to enhance recovery rates, the organic phase may be “back-extracted” as follows: Add an equal volume of TE (pH 7.8) to the organic phase and interface. Mix well. Separate the phases by centrifugation as in step 3. Combine this second aqueous phase with the first and proceed to step 5.

5. Repeat steps 1-4 with the aqueous phase until no protein is visible at the interface of the phases.

6. To the aqueous phase add one volume of chloroform and repeat steps 2-4.
   Note: In case pure phenol is used for the extraction steps, purify twice with pure chloroform.

7. Recover the nucleic acid by standard precipitation with 2 volumes ethanol.

References

[1] Kirby (1956) *Biochem. J.* 64:405

[2] Grassmann and Deffner (1953) *Hoppe-Seylor’s Z. Physiol. Chem.* 293: 89-98

[3] Brawerman *et al.* (1972) *Biochemistry* 11:637-41

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Short Protocol for the Bench

Phenol Chloroform Extraction

- Add 1 vol. phenol-chloroform (1:1) or pure phenol (pH 7.5-8.0) to the sample
- Vortex (ca. 3 sec.)
- Centrifuge for 2 min. at 14.000 rpm (table-top centrifuge)
- Transfer upper (hydrohilic) phase including the DNA completely (!) into a new reaction tube. Interphase must not be irritated. Discard lower phase*.

Reextraction of Hydrophilic Phase with Chloroform (2x)

- Add 1 vol. chloroform to the sample
- Vortex (ca. 3 sec.)
- Centrifuge for 2 min. at 14.000 rpm (table-top centrifuge)
- Transfer upper (hydrohilic) phase including the DNA completely (!) into a new reaction tube. Interphase must not be irritated. Discard lower phase*.
- Add 1 vol. chloroform to the sample
- Vortex (ca. 3 sec.)
- Centrifuge for 2 min. at 14.000 rpm (table-top centrifuge)
- Transfer upper (hydrohilic) phase including the DNA completely (!) into a new reaction tube. Interphase must not be irritated. Discard lower phase*.

Precipitation of DNA

- *Optionally*: + 1µl Roti®-PinkDNA (Art. No. HP54) for making the pellet visible. (Very helpful, particularly if low DNA amounts are to be precipitated.)
- + 1/10 vol. 3 M sodium acetate
  + 2 vol. 100 % ethanol (p.a.)
- Incubate for >1 h at -20 °C (optionally: >20 min. at -80 °C)
- Centrifuge for 15 min. at 14.000 rpm (table-top centrifuge)
- Remove and discard supernatant*
- Rinse pellet with 70% Ethanol
- Centrifuge for 3 min. at 14.000 rpm (table-top centrifuge)
- Remove and discard supernatant*
- Centrifuge for 10 sec. at 14.000 rpm (table-top centrifuge)
- Completely remove residual ethanol*
- Leave reaction tube open on table top for air drying of the pellet (ca. 5 min.)
- Resolubilise pellet in chosen amount of liquid (5 min. at room temperature, 10 mM Tris buffer (pH ca. 8), diluted Roti®-Stock 100x TE buffer, water for molecular biology (Art. No. T143))
- Freeze DNA for storage at -20 °C (optionally in aliquots)

* If desired, the material may be stored for a few hours and may finally be discarded after the isolation has been proven to be successful.

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