

Fractionation of nucleic acids into single-stranded and double-stranded forms

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We describe a rapid and efficient procedure for the fractionation of mixtures of nucleic acids (NA) into double-stranded (ds) and single-stranded (ss) forms regardless of the nature of the nucleic acid (DNA or RNA). The procedure is based on the differential binding of ds- and ss-NA forms to silica particles in different lysis/binding buffers which have in common that they contain a high concentration of the chaotropic agent guanidinium thiocyanate (GuSCN).

Previously we reported on a procedure (protocol Y) for the routine purification of total NA from clinical specimens (1). The method is based on the lysing and nuclease-inactivating properties of the chaotropic agent GuSCN together with the NA-binding properties of silica particles (or diatoms) in the presence of this agent. Comparison of different GuSCN-containing lysis/binding buffers with respect to the binding of different NA-types to silica particles revealed that only ds-forms were bound when using lysis/binding buffer L11 (see below) whereas both ds- and ss-forms were bound in lysis/binding buffer L6 (1). This observation formed the basis for the development of a procedure (protocol R) for the fractionation of mixtures of ss- and ds-NA. The procedure is summarised in Figure 1. A 50 µl specimen (containing a mixture of NA-types in TE buffer) was added to a mixture of 900 µl lysis/binding buffer L11 and 40 µl size-fractionated silica particles (SC) in an Eppendorf tube and subsequently homogenized by vortexing. After a 10 min binding step at room temperature, the tube was centrifuged (2 min at ~12 000 g) which resulted in a silica/ds-NA pellet ('initial silica pellet') and a supernatant containing ss-NA. To recover ss-NA forms (protocol R-sup) 900 µl of the supernatant were added to a mixture of 400 µl binding buffer L10 (see below) and 40 µl SC. Thereafter the ss-NA was bound during a 10 min binding step at room temperature. The tube was subsequently centrifuged (15 s at ~12 000 g), and the supernatant discarded (by suction). The resulting pellet was subsequently washed twice with 1 ml of washing buffer L2 (1), twice with 1 ml ethanol 70% (vol/vol) and once with 1 ml acetone. The silica pellet was dried (10 min at 56°C with open lid in an Eppendorf heating block) and eluted (10 min at 56°C; closed lid) in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After centrifugation (2 min at ~12 000 g) the supernatant contained the ss-NA fraction. To recover ds-NA forms (protocol R-pellet) from the initial silica-pellet, the remaining supernatant was discarded, and the silica pellet was washed twice with 1 ml lysis/binding buffer L11 to remove unbound ss-NA. The resulting silica pellet

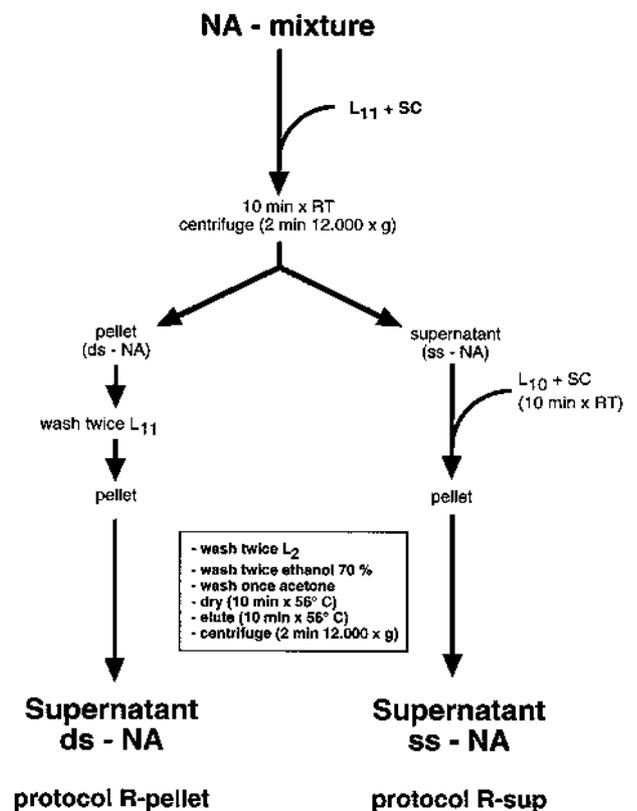


Figure 1. Outline of protocol R. Recovery of ds-NA takes place from the initial pellet (R-pellet), recovery of ss-NA takes place from the initial supernatant (R-sup). L11, L10, L6 and L2 are GuSCN containing buffers; SC is silica particle suspension. For details see text.

was subsequently washed twice with washing buffer L2, twice with ethanol 70%, once with acetone, dried and eluted as described for protocol R-sup. After centrifugation (2 min at ~12 000 g) the supernatant contained the ds-NA fraction.

Lysis/binding buffer L11 was made by dissolving 120 g of GuSCN (Fluka) in 100 ml 0.2 M EDTA (Merck), pH 8.0. Dissolution of the GuSCN was facilitated by heating in a 60°C water bath under continuous shaking. Binding buffer L10 was

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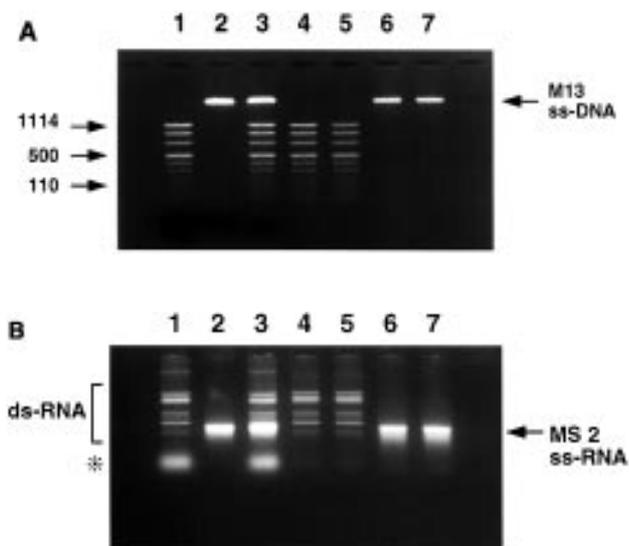


Figure 2. Fractionation of NA by protocol R. (A) Fractionation of ds-DNA and ss-DNA. NA was purified (in duplicate) by protocol R from a mixture of 1 μ g ds-DNA (molecular weight marker VIII, Boehringer) and 1 μ g ss-DNA (phage M13 DNA, Boehringer). Half (25 μ l) of the eluate was electrophoresed (3) through a 1.8% agarose gel (containing ethidium bromide) which was subsequently photographed (Polaroid) under UV-illumination. Lane 1, 100% recovery marker for ds-DNA fragments (500 ng); lane 2, 100% recovery marker ss-DNA (500 ng); lane 3, 100% recovery marker mixture ds-DNA/ss-DNA. Lanes 4 and 5, output protocol R-pellet; lanes 6 and 7, output protocol R-sup. Molecular weight marker VIII ds-DNA fragments 1114, 500 and 110 (bp) and phage M 13 ss-DNA are indicated. (B) Fractionation of ds-RNA and ss-RNA. NA was purified (in duplicate) by protocol R from a mixture of ds-RNA (Rotavirus ds-RNA, previously purified from faeces of an infected individual) and 800 ng ss-RNA (phage MS2 RNA, Boehringer). Half (25 μ l) of the eluate was electrophoresed through a 1% agarose gel (containing ethidium bromide) which was subsequently photographed under UV-illumination. Lane 1, 100% recovery marker for ds-RNA segments; lane 2, 100% recovery marker ss-RNA (400 ng); lane 3, 100% recovery marker ds RNA/ss-RNA mixture. Lanes 4 and 5, output protocol R-pellet; lanes 6 and 7, output protocol R-sup. The ds-RNA segments (segments 2 and 3, and segments 7, 8 and 9 comigrate) and MS2 ss-RNA are indicated. Asterisk indicates low molecular weight NA, presumably breakdown products of DNA and RNA present in the faeces specimen used for isolation of Rotavirus ds-RNA.

prepared by dissolving 120 g GuSCN in 100 ml 0.35 M Tris-HCl (Boehringer), pH 6.4. Dissolution of GuSCN was facilitated by heating in a 60°C water bath under continuous shaking. Subsequently 22 ml 0.2 M EDTA (pH 8.0) and 9.1 g Triton X-100 (Packard) were added and the solution was homogenized. Finally 11 g of solid MgCl₂·6H₂O (Merck) was added and the resulting solution was homogenized. Buffer L6 and washing buffer L2 (both containing GuSCN) and size-fractionated silica particles (SC) were prepared as described (1).

To illustrate the fractionation of ds-DNA and ss-DNA, a mixture of ds-DNA and ss-DNA was subjected to fractionation (in duplicate) by protocol R (Fig. 2A). This experiment showed that the fractionation of a mixture containing ds-DNA and ss-DNA into ds-DNA (R-pellet) and ss-DNA (R-sup) was complete at the level of detection by UV-illumination.

For fractionation of a mixture of ds-RNA and ss-RNA into ds-forms and ss-forms, we used a mixture containing rotavirus

ds-RNA [the human Rotavirus genome consists of 11 ds-RNA segments; for a review see (2)] previously purified from faeces of an infected individual by protocol Y (1) and phage MS2 ss-RNA as input material for protocol R. The data (Fig. 2B) show complete fractionation of the ds-RNA/ss-RNA mixture into ds-forms (R-pellet) and ss-forms (R-sup) at the level of detection by UV-illumination.

Double-stranded DNA could also be efficiently separated from ss-RNA (phage MS2 ss-RNA) by protocol R; similar results were obtained when *Escherichia coli* rRNA (23S and 16S) was used as ss-RNA input (data not shown). The detection limit of the system used (UV-illumination of ethidium bromide stained gels) was ~5 ng for ds-DNA fragments. In order to estimate the amount of ds-DNA still present in the ss-NA fraction, we performed Southern blotting with a homologous ³²P-labelled probe, for detection. This experiment showed that the ss-RNA fraction contained <0.1% of the ds-DNA present in the starting mixture (data not shown). When the separation of high molecular-weight genomic ds-DNA and ss-RNA was examined by direct fractionation using *E.coli* bacteria as input for protocol R, it appeared that the ds-DNA fraction was contaminated with rRNA and ss-RNA recovery was low (data not shown). This was presumably due to trapping of ss-NA by high molecular weight genomic DNA during binding of ds-NA to the silica particles. This phenomenon could be circumvented by first isolating total NA by protocol Y/D (1) which uses diatoms rather than silica particles for NA binding. Genomic DNA thus purified is sheared to an estimated mean size of ~40 kb (1) and trapping of ss-RNA into the ds-DNA fraction was significantly reduced when total NA purified from *E.coli* bacteria was used as input material for fractionation by protocol R (data not shown).

We have shown that mixtures of ds- and ss-NA forms can be efficiently separated into ss- and ds-fractions, regardless of the nature of the nucleic acid (DNA or RNA), in the described 1 h procedure (protocol R). It was also seen that DNA/RNA hybrids behaved like ds-NA (data not shown). The mechanism of differential binding is not understood. In our experiments recoveries of ss- and ds-NA exceeded 50%, and the ss-NA fraction contained <0.1% of ds-DNA present in the starting mixture. NA purified by protocol R has appeared to be a good substrate in RT-PCR experiments (data not shown). The procedure may have wide applications in the separation of ss-NA probes from ds-NA hybrids or in the separation of *in vitro* synthesized ss-RNA transcripts from ds-DNA templates.

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