ExpressArt® N-Carrier

For Isolation of total RNA in the Nanogram & Picogram Range

Catalogue No. 8997-A100 (100 samples)

Reagents

Contents (store at −20°C)

N-Carrier 120 µl

Note for EtOH precipitation
Occasionally we observed difficulties in the recovery of EtOH pellets, because they did not stick to the wall of tubes from some manufacturers. We recommend 1.5 ml reaction tubes from Sarstedt (Micro tubes 072.690).

Storage

Immediately upon arrival: store at −20°C. Avoid repeated freeze thawing.

Reagents are stable for 6 months after receipt.
Additionally required materials

- RNase-free pipette-tips (filter-tips recommended)**
- RNase-free reaction tubes (0.5 / 1.5 ml)**

**Note for EtOH precipitation**
Occasionally we observed difficulties in the recovery of EtOH pellets, because they did not stick to the wall of tubes from some manufacturers. We recommend 1.5 ml reaction tubes from Sarstedt (Micro tubes #72.690).

- 100% Ethanol and 75% Ethanol (both chilled at -20°C)
- Microcentrifuge
- Mixer (Vortex, Thermomixer)

**See section "Before you start" (page 5)**

**Option 1:**
- Water-saturated phenol
- Chloroform
- Isopropanol

**Option 2:**
- Trizol (or RNA-Bee)
- Chloroform
- Isopropanol

**Option 3:**
RNA isolation kit of your choice: RNAready Kit

**Quality control**
All reagents are tested to be compatible with ExpressArt® mRNA amplification kits.
General

The ExpressArt® N-Carrier is a recommended supplement for small scale RNA isolation, with expected yields of total RNA below 20 ng of total RNA and especially in the range below 1 ng. It should be noted that about 100 pg input total RNA can be amplified with the ExpressArt® mRNA amplification Pico kit, to obtain amplified RNA in the high μg range. RNA quality and yield can be improved with the provided reagent N-Carrier.

The N-Carrier is a short RNA (<100 nt) with a hairpin structure. 100 ng of this RNA is added per sample and this protects against RNA degradation in the lysis step and against loss due to unspecific surface adsorption, especially on the matrix of spin columns. Due to its small size (<100 nt), more than 99% of the N-Carrier are washed off from the column matrix, and there is no interference with subsequent RNA quality control with the Agilent Bioanalyzer.

This reagent does not interfere with ExpressArt® mRNA amplification: no inhibition and no amplification artefacts (Baugh 2003; personal communication).

It can be combined with the RNA isolation method of your choice, and used for fresh, frozen, as well as laser microdissected samples. One of two options can be chosen (see section "Detailed Protocol"). The first option should be used only for samples with intact cells (e.g. sorted cells) or whole organisms (e.g. embryos). In the second option, standard RNA isolation kits are supplemented with ExpressArt reagents.

References (use of ExpressArt Pico RNA Care or N-Carrier)
Before you start

how to store and handle reaction tubes
- do not autoclave
- do not remove from bag by inserting your hand (not even with gloves!)
- instead, pour onto fresh tissue on bench
- never touch inside of cap when opening or closing

how to store and handle pipette tips
- do not autoclave
- always replace pipette box cover after finishing work

how to store and handle stock solutions
- do not insert pipette
- instead, pour small aliquot in tube
- always, replace cap after finishing work

how to thaw liquids in small tubes
- note, no homogeneous solution, freezing generates concentration gradient
- always mix thoroughly
e.g., by thawing on a Thermomixer (1000 rpm)
or by inverting and flicking tube

how to mix small volumes in reaction tubes
- note, small enzyme volumes “precipitate” at the bottom of the tube
- always, mix by flicking tube or by pipet mixing the complete reaction volume

how to perform EtOH precipitation
- always, in this order: RNA solution + salt + carrier, mix thoroughly, then, add EtOH
- do not overdry pellet in Speed-Vac
- instead, air dry pellet

how to use spin columns
- do not touch surface of matrix
- do not use collection tube and cap from last spin
- instead, transfer eluate into fresh tube

how to perform phenol (Trizol, RNA-Be) extractions
- do not spin at room temperature (samples warm up during spin, aqueous phase becomes turbid upon cooling)
- carefully avoid transfer of any interphase material
  (preferably leave behind some aqueous phase)
RNA Quality Control

Successful application of any RNA technology is dependent on the use of high quality RNA. Therefore, stringent RNA quality control is crucial. In addition to conventional gel electrophoresis, the Agilent 2100 bioanalyzer in combination with RNA 6000 Nano and Pico LabChip kits is widely used for high-resolution analysis of small and very small RNA samples. The expected electropherograms vary, depending on species, tissue type and method of RNA isolation. The following table provides known rRNA lengths and expected rRNA ratios.

<table>
<thead>
<tr>
<th>Species</th>
<th>rRNAs</th>
<th>lengths[kb]</th>
<th>ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>18S / 28S</td>
<td>1.9 / 5.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Mouse</td>
<td>18S / 28S</td>
<td>1.9 / 4.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Teleost fish (Zebrafish, Danio rerio)</td>
<td>18S / 28S</td>
<td>1.8 / 4.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>18S / 28S</td>
<td>2.0 / 4.1**</td>
<td>n/a</td>
</tr>
<tr>
<td>Plant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cytoplasmic chloroplasts*†(a)</td>
<td>18S / 25S</td>
<td>1.9 / 3.7</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>16S / 23S</td>
<td>1.5 / 2.7†</td>
<td>1.8†</td>
</tr>
<tr>
<td>Caenorhabditis elegans</td>
<td>18S / 26S</td>
<td>1.75 / 3.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Yeast (Saccharomyces cerevisiae)</td>
<td>18S / 26S</td>
<td>2.0 / 3.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Bacteria</td>
<td>16S / 23S</td>
<td>1.5 / 2.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* theoretical rRNA ratio
** Drosophila 28S rRNA is split in 2 fragments, co-migrating with 18S rRNA
† Only in green tissues with high chloroplast content.
(a) Note: three bands are observed with green tissues from Arabidopsis thaliana.

Stringent RNA quality control must assure that fragmented rRNAs and other RNA aggregates are resolved and do not erroneously migrate as one band. This can be achieved by denaturing electrophoresis conditions, or simply by heating the RNA sample for 2 min at 70°C, immediately before performing native electrophoresis.
with a gel or with the Agilent 2100 bioanalyzer. The following electropherograms with an RNA sample of lower quality illustrate the importance of the heat treatment. The first electropherogram (at the top) was obtained without, and the second electropherogram after heat treatment. Note the decreased rRNA ratio (from 1.9 to 1.4) and the increased signal intensities of RNA fragments migrating between both rRNA bands and faster than 18S rRNA in the second electropherogram.
DETAILED PROTOCOLS

One of two options can be chosen. The first option option should be used only for samples with intact cells (e.g. sorted cells) or whole organisms (e.g. embryos). In the second option, standard RNA isolation kits are supplemented with the ExpressArt reagents.

A: First Option

Notes: Observe general guidelines in section "Before you start".

This option should be used only for samples with intact cells (e.g. sorted cells) or whole organisms (e.g. embryos). It is not advised for samples with partially ruptured cells, like biopsies or microdissected samples.

Per 1 mm$^2$ (or per 1 mg) of sample, a maximum of 15 μl isotonic buffer should be used. If possible, proceed immediately with subsequent isolation and amplification steps.

If necessary, samples can be flash frozen in liquid nitrogen and stored at -80°C.

- If working with more than one reaction at a time, prepare Master Mixes.

Prepare Lysis Reagent Mix 01, and use an appropriate Master mix volume for processing multiple samples.

When calculating the required volume of Mix 01, bear in mind that 100 μl of Mix 01 are required per 10 μl of each sample.

All volumes given below are based on using 100 μl Mix 01 per sample.

<table>
<thead>
<tr>
<th>Lysis Reagent Mix 01</th>
<th>Trizol (Invitrogen) or RNA-Bee (amsbio)</th>
<th>not supplied</th>
<th>100 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Carrier</td>
<td></td>
<td></td>
<td>1.0 μl</td>
</tr>
</tbody>
</table>

Per 10 μl of sample, add 101 μl of Mix 01 and mix vigorously for 10 sec.

Per 10 μl of initial sample volume, add 20 μl chloroform and mix vigorously for 30 sec.
After centrifugation at 4°C for 20 min at 14,000g, carefully remove the upper aqueous phase (approx 60 µl per 100 µl of Mix 01), avoiding the transfer of any material collected at the interface.

Transfer the upper phase to a fresh tube. We strongly advise to use the suggested tubes for EtOH precipitation. Occasionally we observed difficulties in the recovery of EtOH pellets, because they did not stick to the wall of tubes from some manufacturers. [We recommend 1.5 ml reaction tubes from Sarstedt (Micro tubes #72.690)].

Add 60 µl isopropanol per 60 µl aqueous phase, mix well and incubate at room temperature for 10 min.

Spin at 4°C for 20 min at 14,000g, carefully remove the supernatant with a pipette (the pellet is transparent).

Wash pellet with 500 µl of 75% ethanol (pellet is pink).

Re-insert tubes in the rotor, rotated at 180° relative to the previous spin. This results in transition of the pelleted material through the liquid which ensures the complete removal of phenol.

Centrifuge at 4°C for 10 min at 14,000g.

Carefully remove the supernatant with a pipette.

Allow pellet to air dry for 2 min and dissolve pellet in 4 µl of DEPC water. Mixing with a pipette ensures formation of a homogeneously pink solution.

Immediately amplify with the appropriate ExpressArt® mRNA amplification kit version.

**B: Second Option**

**Notes**: Observe general guidelines in section "Before you start".

Use the standard RNA isolation kit of your choice, e.g. RNAready, RNeasy or PicoPure (Arcturus/MDS Pharmaservice).

**Notes**: The kit lysis buffer is supplemented with 1 µl N-Carrier (Tube 02). No other changes are made in the instructions of the manufacturer.

Isolated RNA samples are ready for amplification with the appropriate ExpressArt® mRNA amplification kit version. These kits do not require an rRNA depletion step.
T: Troubleshooting

T1. RNA Isolation

RNA should be free of contaminating DNA. In general we have very good results with the LCM RNAready Kit (amsbio Catalogue code# 9001-A100) which includes the RNase-Free DNase Set. Using this kit protocol, traces of DNA are directly removed on the spin column, followed by an additional wash step and final RNA elution.

When using very low amounts of RNA, as expected with microdissected samples (a few hundred cells), elution efficiency may vary significantly.

Another supplier, Qiagen recommends the addition of a nucleic acid carrier (poly-A) to avoid unacceptable sample loss of RNA, due to unspecific binding to the matrix of spin columns. This is problematic, because this nucleic acid carrier masks RNA profiles in subsequent RNA quality control with the Agilent Bioanalyzer and it interferes with ExpressArt™ mRNA amplification and other downstream applications.

Therefore, the use of the ExpressArt™ N-Carrier is strongly recommended. It contains RNA carrier for total RNA preparations in the picogram range. This carrier is proven to be compatible with subsequent ExpressArt™ mRNA amplification no inhibition, no amplification artefacts (Baugh, 2003; personal communication).

T2. RNA quality with larger samples

The importance of RNA quality can not be overemphasised. Success and reproducibility of mRNA amplification and consequent array hybridisation are highly dependent on RNA quality. Whenever possible, the integrity of purified RNA should be controlled by gel electrophoresis. Well-defined bands of the 28S and 18S rRNA species should be visible as indicators of reasonable integrity of the RNA samples. About 200-500 ng of total RNA will be sufficient for agarose gel electrophoresis followed by ethidium bromide staining. For less RNA you may use more sensitive nucleic acid staining dyes or different technologies like the Agilent 2100 Bioanalyzer (with the recently available RNA 6000 Pico LabChip).
For the isolation of intact RNA, it is important to eliminate internal and external RNase activities. As soon as cells are damaged, intracellular RNase activities will start RNA degradation. After collecting tissue samples, or cells from cell culture, it is important to **immediately** (1) shock-freeze the samples with liquid nitrogen, followed by further storage at –80°C or by direct lysis. Never place your samples directly in a freezer after collection.

RNA degradation can be minimised by complete and rapid sample lysis in strong denaturing agents like phenol, Trizol, RNA-Beet or guanidine thiocyanate (GTC). During microdissection, collected specimens should be transferred immediately into a lysis reagent which has been supplemented with 1 µl per sample of the N-Carrier of the ExpressArt® RNA Care reagents (8999-A100) and 1% of NucleoGuard (8998-M50).

External RNases are accidental contaminations. It is important to know that human finger-tips are an extremely rich source of external RNases. Thus, never touch any equipment for RNA preparations without wearing gloves.

For guidelines to eliminate external RNases see section "Before You Start" (above).

**T3. Control of RNA quality and quantity with very small samples, including microdissected cells**

The isolation of RNA from microdissected cells is certainly more demanding than standard RNA preparations, due to the various steps of sample preparation, storage, staining and microdissection. Unfortunately, control the RNA quantity or quality is not always possible if only small cell numbers were collected (see section 3.2).

Furthermore, our experience has shown that it is difficult to predict RNA yields when working with microdissected cells. Yields can vary between 5% and close to 100% of the theoretical yield of about 10 pg of total RNA per cell [see also Quality assurance of RNA derived from laser microdissected samples obtained by the PALM MicroBeam system using the RNA 6000 Pico LabChip kit Agilent Application Note No. 5988-9128EN (2003)].
Fortunately, the ExpressArt® PICO RNA Care reagents, in combination with the NucleoGuard additive, ensure optimal RNA yields and quality. Furthermore, with ExpressArt® mRNA Amplification kits, there is no need for accurate quantitation of input total RNA.

For RNA quality control with tiny samples, we recommend to perform two amplification rounds with the ExpressArt® mRNA Amplification Kit of your choice. Subsequently, RNA quality control can be performed as described in the Core kit protocols. If there is no amplified RNA of satisfying quality, the yield or quality of your sample RNA preparation might not have been as high as expected. If possible, repeat RNA preparation with higher cell numbers.