ExpressArt® Technology
for mRNA amplification

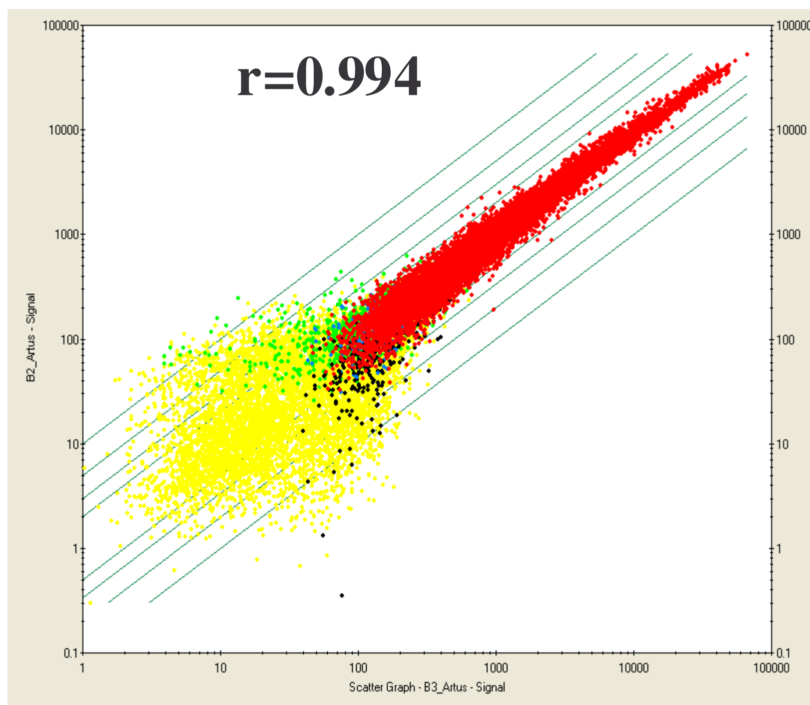
This short brochure provides general information.

Detailed laboratory protocols are available for the kit which fits your needs:

Micro (#7199-A30 for RNA samples of **≥300 ng**), **Nano** (#7299-A15 for **≥1 ng**) or **Pico kits** (#7399-A15 for **≥100 pg**).

Small and very different RNA amounts can be used in sample series, without limitations in direct comparabilities.

ExpressArt®



2 rounds:
100ng versus 10ng

Before you start:
please follow these general instructions

how to store and handle reaction tubes

- do not autoclave (serious contamination risk)
- do not remove from bag by inserting your hand (not even with gloves!)
- instead, pour tubes onto fresh tissue on the bench
- never touch inside of cap when opening or closing

how to store and handle pipette tips

- do not autoclave (serious contamination risk)
- preferably, use filtered pipette tips
- 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 after thawing, 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 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

Bearing these essentials in mind, you will enjoy the advantages of using the ExpressArt® kits for amplifying your mRNA!

1. Introduction

For **good quality eukaryotic total RNA samples,** the standard ExpressArt® mRNA Amplification Kits are available: an oligo(dT) primer anneals with the 3'-poly(A) tail of intact eukaryotic mRNAs.

As an alternative, the ExpressArt® **TR*inucleotide* mRNA amplification kits** were developed for amplification of mRNAs or mRNA fragments without poly(A), for severely degraded eukaryotic RNAs, like FFPE-RNAs, *or* for bacterial mRNAs.

General properties

The ExpressArt® Kits and reagents provide a highly sensitive and reproducible technology for **linear** mRNA amplification, as well as RNA isolation, in combination with microarray hybridisation or for multiple quantitative analyses with RT-qPCR.

The ExpressArt® mRNA Amplification Kits are unique and based on proprietory AmpTec technology (patents and patents pending).

General Advantages of ExpressArt® mRNA Amplification

1. ***No primer derived artefacts***
   cDNA synthesis is uncoupled from insertion of T7-promotor

With **other** systems, the frequently observed large amounts of template-independent high molecular weight amplification artefacts are a severe limitation in the amplification of very low amounts of input RNA. With ExpressArt®, the “no-template-control“ is free of high molecular weight background products, even after two and three amplification rounds. This enables the amplification of sub-nanogram amounts of input total RNA, as demonstrated by the amplification of RNA from 4-cell embryos of *C. elegans* **[see Yanai, Baugh, et al. (2008) Molecular Systems Biology 4:163]**.

2. ***No continuous shortening with loss of mRNA sequences***
   ”TRinucleotide priming” *(Box-random-trinucleotide primer sections)* ***ensures 3’-proximal priming****, not possible with random primers*

Three amplification rounds as faithful as two. Full comparability is obtained.

3. *Absolutely unique flexibility*

No need for careful control of input RNA amounts. Small and large amounts can be directly compared. This includes even mixed sample sets that required two or three amplification rounds.
Flexible transition between laser microdissection, cryosections, biopsies etc.
Rescue of drop-outs in series with two amplification rounds. A third round can be performed, but it is not necessary for all samples, only for those with insufficient yields after the second round.

4. *Improved detection*

Hundreds of additional genes are amplified above expression threshold and more differentially expressed genes are identified.

5. *Archival templates*

Perform multiple solid phase in vitro transcription reactions using the same, immobilised template DNA.

Check yields and qualities of amplified RNAs for the crucial decision: Use unmodified NTP's for the first IVT, and if yields and qualities are satisfying, perform a second IVT with the same template DNA, using labelled NTP's.

With insufficient yields: perform several IVT's with labelled NTP's or if appropriate, use unmodified RNAs for an additional amplification round.

Store template DNA for later recovery of amplified RNA to use for new microarray generations and other applications.

6. *Amplified RNAs contain defined sequences at both ends*

7. *Faithful reproduction of dynamic gene expression levels*

2. Methodology

Now, highly reproducible array hybridisations can be performed with a few cells, e.g. 4-cell embryos of *C.elegans* [**Yanai, Baugh, et al. (2008)** Pairing of competitive and topologically distinct regulatory modules enhances patterned gene expression. Molecular Systems Biology 4:163].

Historically, a linear, isothermal amplification strategy based on in vitro transcription with T7 RNA-polymerase was used [**Van Gelder (1990)** Amplified RNA synthesised from limited quantities of heterogeneous cDNA. Proc. Natl. Acad. Sci. 87: 1663-1667; **Eberwine et al. (1992)** Analysis of gene expression in single life neurones. Proc. Natl. Acad. Sci. 89: 3010-3014**].**

In this procedure, mRNA was converted into double stranded cDNA, using a T7-promoter/oligo(dT) primer for first strand cDNA-synthesis and limited RNase H digestion for self-priming during second strand synthesis. For amplification, these dsDNA-molecules were used as templates for in vitro transcription. Resulting in linear amplification and maintaining the expression patterns of the original mRNAs [**Poirier et al. (1997)** Screening differentially expressed cDNA clones obtained by differential display using amplified RNA. Nucleic Acids Res. 25: 913-914; **Puskas et al. (2002)** RNA amplification results in reproducible microarray data with slight ratio bias. BioTechniques 32: 1330-1340.

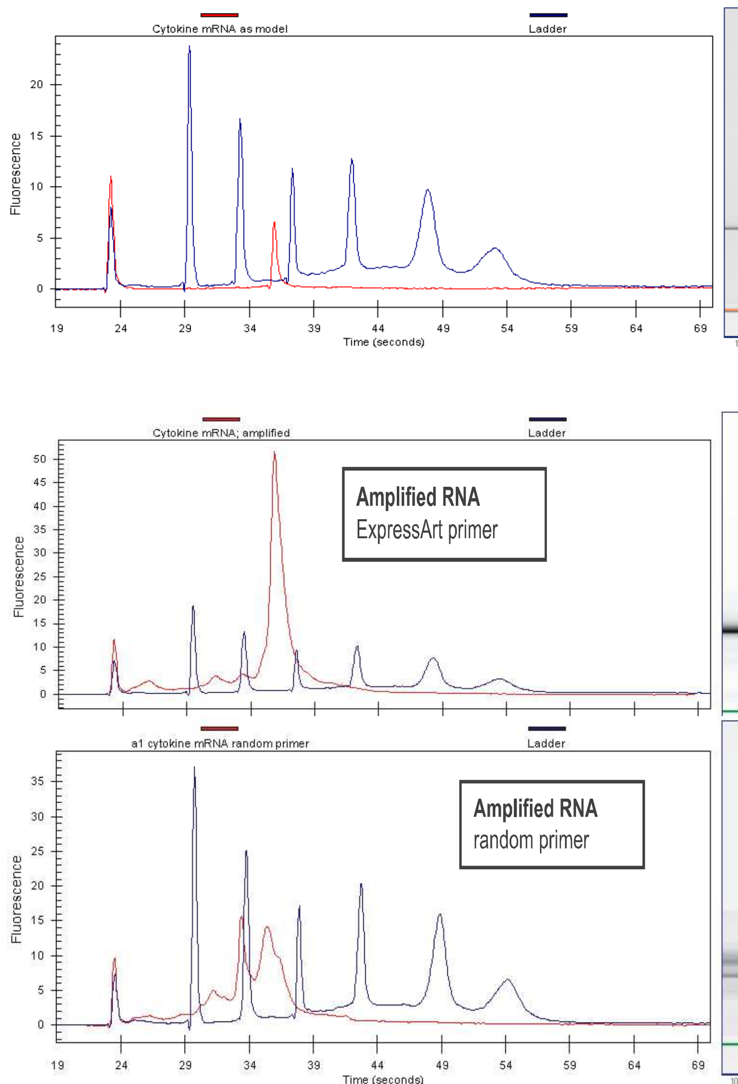
A number of problems were observed with this approach:
(i) amplified RNA (aRNA) is 3´-biased, since transcription and cDNA-synthesis with the T7-promoter/oligo(dT) primer start at the poly(A)-tail of the original mRNA. **(ii)** a second amplification was based on random priming, causing reduction of fragment length, which was even more pronounced when only small amounts of input RNA were available. **(iii)** the use of the T7-promoter/oligo(dT) primer in the first cDNA-synthesis could lead to large amounts of non-template high molecular weight artefacts, which became dominant with low amounts of input RNA [**Baugh et al. (2001)** Quantitative analysis of mRNA by in vitro transcription. Nucleic Acids Res. 29:E29]. **(iv)** only high quality RNA samples with intact RNA could be used.

Now and for the future, the ExpressArt® mRNA Amplification Kits provide a technology, which solves these problems. With this TR*inucleotide* mRNA amplification kit, intact mRNAs as well as all mRNA fragment are converted to cDNAs with a special "TR*inucleotide* primer" (Box-1-random-trinucleotide primer; without T7-promoter). This primer permits preferential priming near the 3'-ends of all nucleic acid molecules. A model experiment illustrates its performance (see below).
To minimise further 3´-bias in the next step, double stranded cDNA is generated with a second "TR*inucleotide* primer" (Box-random-trinucleotide primer), again with preferential priming near the 3'-ends of the cDNAs.
This feature results in the generation of almost full-length double stranded cDNAs.
After denaturation, the second cDNA strand will be primed in reverse orientation, using a T7-promoter/Box-1 primer. This leads to double stranded cDNA with a functional T7-promotor at one end and the Box sequence tag at the other end. This dsDNA product is used as template for in vitro transcription, generating amplified, ***antisense*** oriented RNA with defined sequences at both ends.
This is a major advantage for second and especially for third round amplifications, where size reductions of amplified RNAs are avoided. This is crucial and enables the comparison of samples that contain very divergent amounts of input RNA.
Now, it is not only possible to perform highly reproducible array hybridisations with a few cells, e.g. 4-cell embryos of *C.elegans* (**Baugh (2004)** Genomic analysis of embryogenesis in the nematode C. elegans. Ph.D. thesis, Harvard University, Dept. Mol Cell Biol.**; Yanai, Baugh, et al. (2008)** Pairing of competitive and topologically distinct regulatory modules enhances patterned gene expression. Molecular Systems Biology 4:163]**.** Furthermore, ***even severely degraded RNAs yield excellent results***.

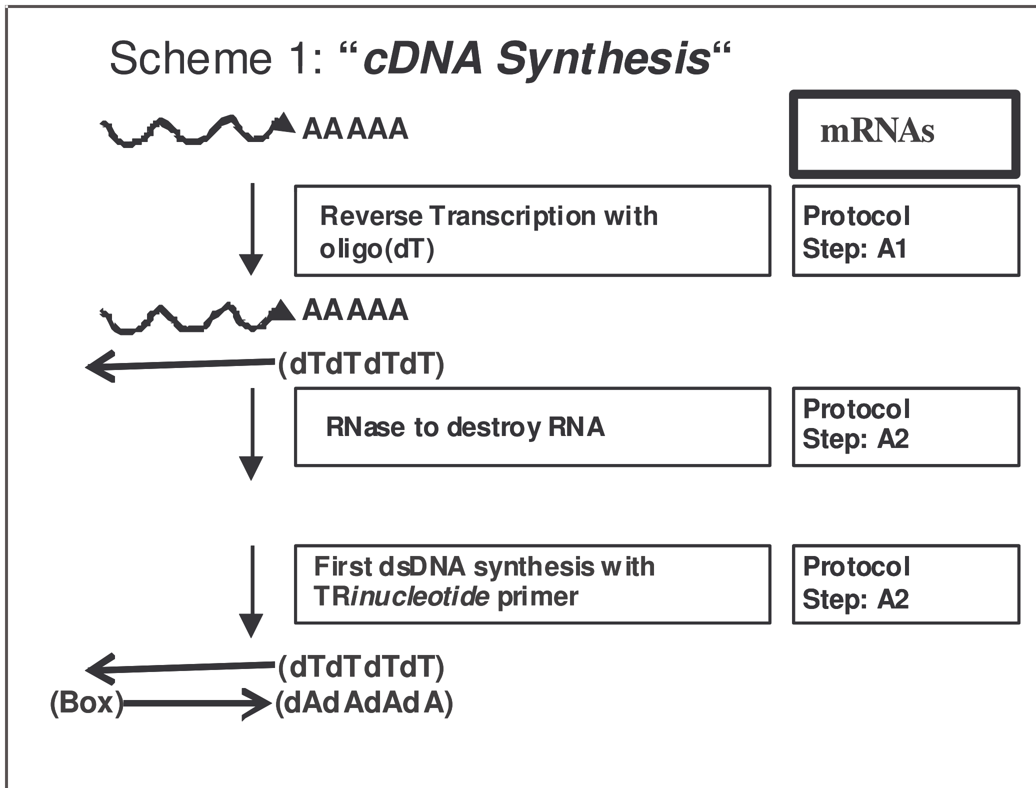
.

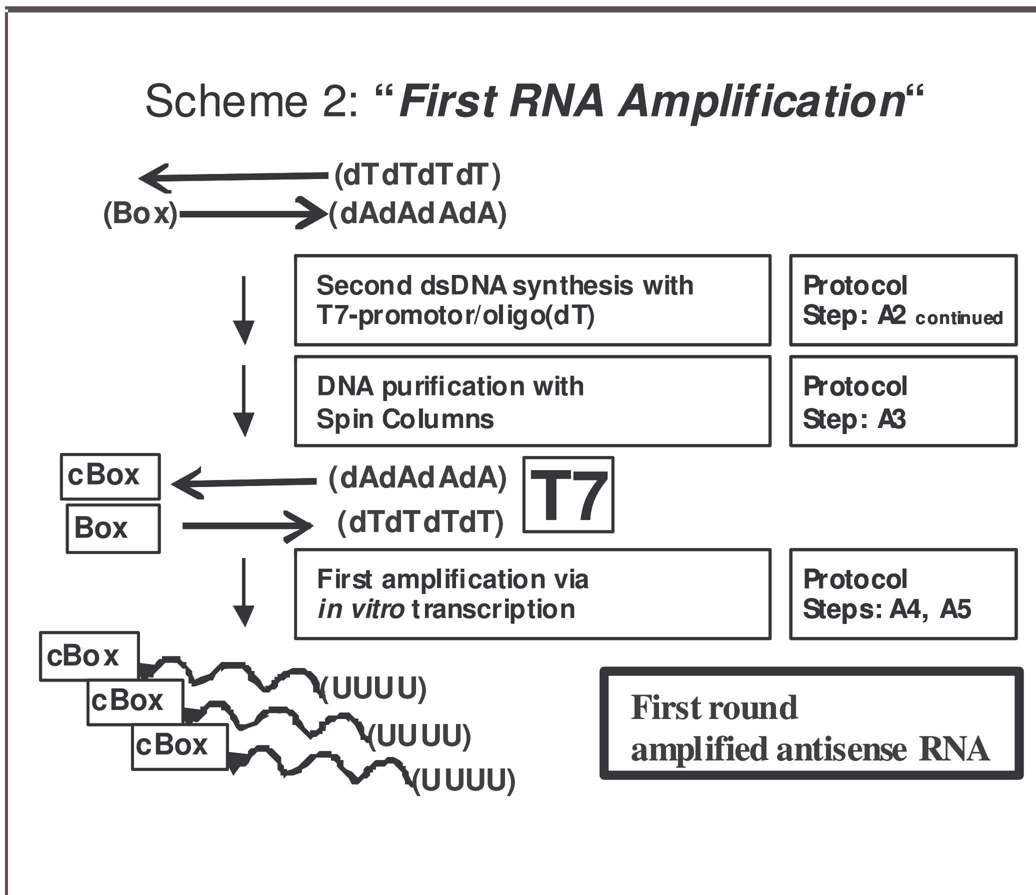
Model experiment to illustrate one of the unique properties of ExpressArt® TR*inucleotide* primers

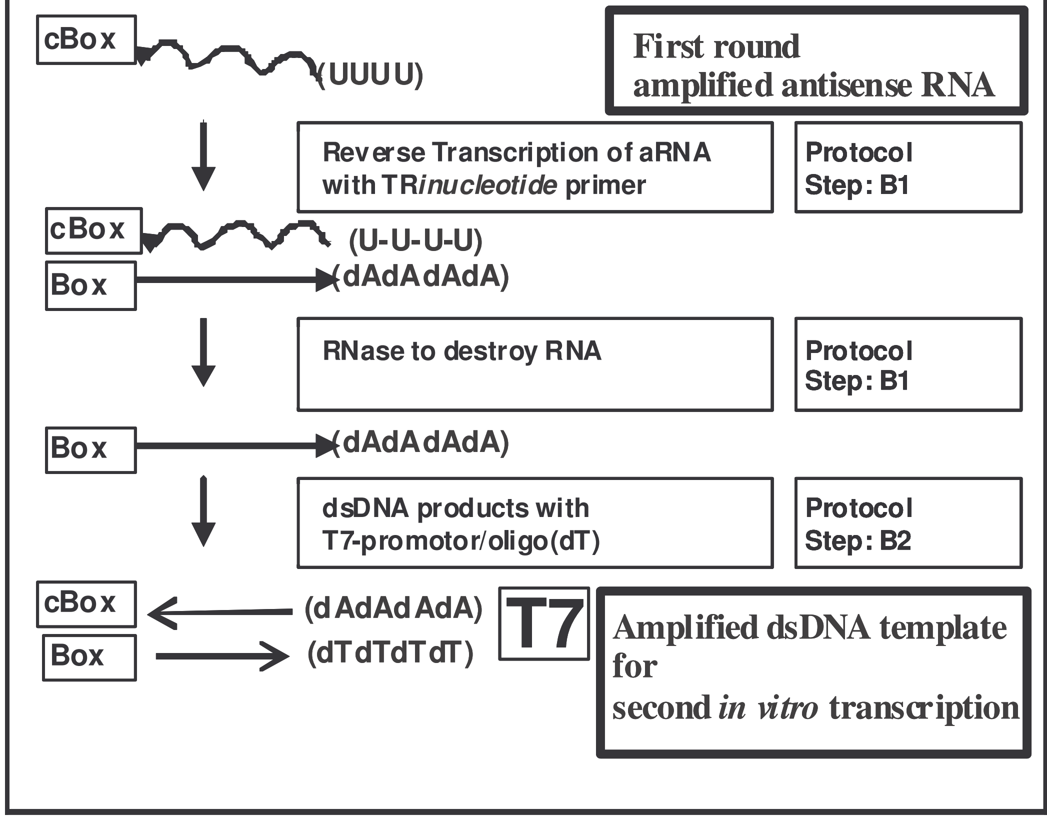
A defined in vitro transcript of 800 nt length was used as input mRNA model (red tracing in top electropherogram). Amplification with ExpressArt® technology and the TR*inucleotide* primer (Box-random-trinucleotide primer) resulted in essentially full-length aRNA (red tracing in middle electropherogram). For comparison, the same reaction steps were used, but the 3'-terminal trinucleotide in the TR*inucleotide* primer was replaced by a fully random trinucleotide sequence. This resulted in a mixture of shorter aRNAs with a minor fraction (if any) of full-length product (red tracing in bottom electropherogram).



Flow Sheets






Scheme 3: "Template for 2nd RNA Amplification"
cBox
(UUUU)
First round
amplified antisense RNA
Reverse Transcription of aRNA
with TRinucleotide primer
Protocol
Step: B1
cBox
(U-U-U-U)
Box
(dAdAdAdA)
RNase to destroy RNA
Protocol
Step: B1
Box
(dAdAdAdA)
dsDNA products with
T7-promotor/oligo(dT)
Protocol
Step: B2
cBox
(dAdAdAdA)
Box
(dTdTdTdT)
T7
Amplified dsDNA template
for
second in vitro transcription

3. RNA Quality Control

3.1. RNAs should meet these minimal criteria

Successful application of any **standard RNA technology**, based on priming with oligo(dT) in the first reverse transcriptase step, is dependent on the use of high quality RNA. Therefore, stringent RNA quality control is crucial.

In addition to gel electrophoresis, the Agilent 2100 bioanalyzer combined with RNA 6000 Nano and Pico LabChips is widely used for high-resolution analysis of small and very small RNA samples. Expected electropherograms vary, depending on species, tissue type and RNA isolation method [see **Krupp (2004)** Stringent RNA Quality Control using the Agilent 2100 Bioanalyzer. Agilent Application Note; available from krupp@amp-tec.com].

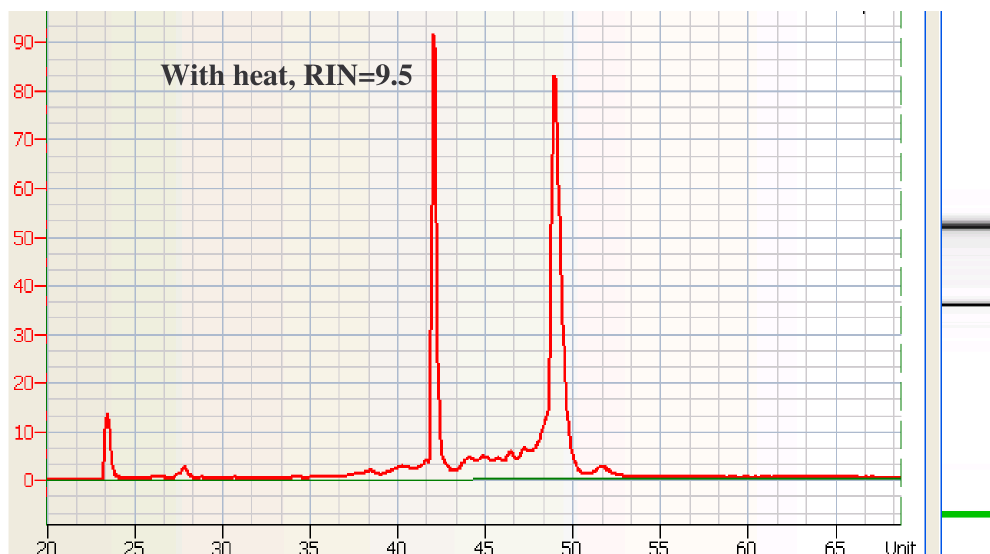
Peaks of 28S and 18S rRNA should be clearly visible, a ratio of 1:1 or higher is desired. A minimal RIN value (Agilent 2100 Bioanalyzer) of 6 is desired.

For RNA isolation in the low nanogram and picogram range, use of the **ExpressArt Pico RNA Care** reagents (# 8999-A100) is recommended.

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.

RNA profiling with the Agilent 2100 Bioanalyzer have become the method of choice for RNA quality control. Please note, this capillary electrophoresis uses native or **non-denaturing conditions**. However, it is sufficient to simply heat the RNA samples for 2 min at 70°C, followed by a brief spin to collect the liquid and cool the samples just prior to performing the electrophoresis. At this temperature, RNA structures are opened up and can refold in their thermodynamically favoured, native structures to result in well defined, sharp bands for intact rRNAs, but this also ensures disruption of aggregated rRNA fragments,

Recently, an improved general RNA quality assessment was introdueced [**Mueller et al. (2004)** RNA Integrity Number (RIN) - Standardization of RNA Quality Control. Agilent Application Note 5989-1165EN]. A RIN value is derived from the RNA profiles in electropherograms**,** with a range of 1 to 10 and with RIN = 10 for the highest RNA quality.
An RNA sample with good quality (RIN=9.5) is shown below.

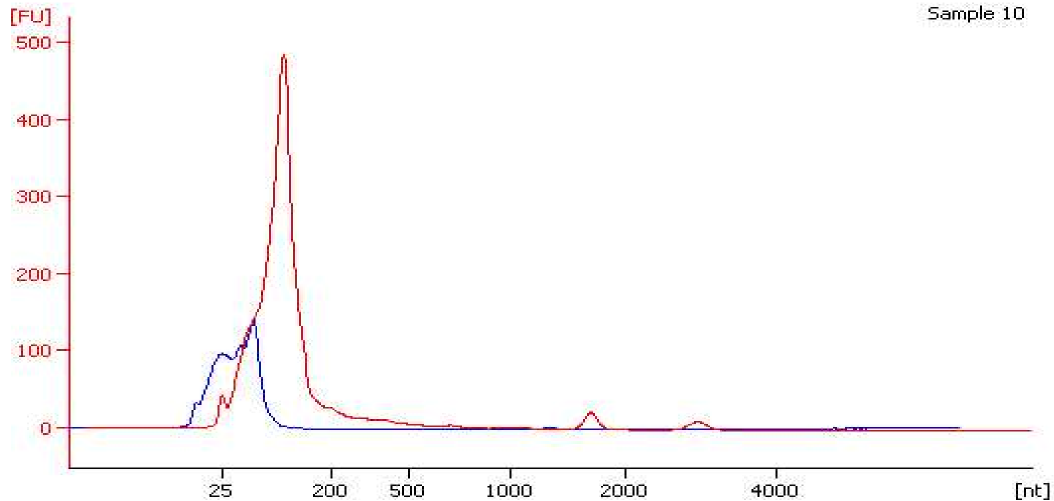


3.2. Isolation of very small RNA samples (Laser Microdissection, FACS cell sorting, micro-biopsies)

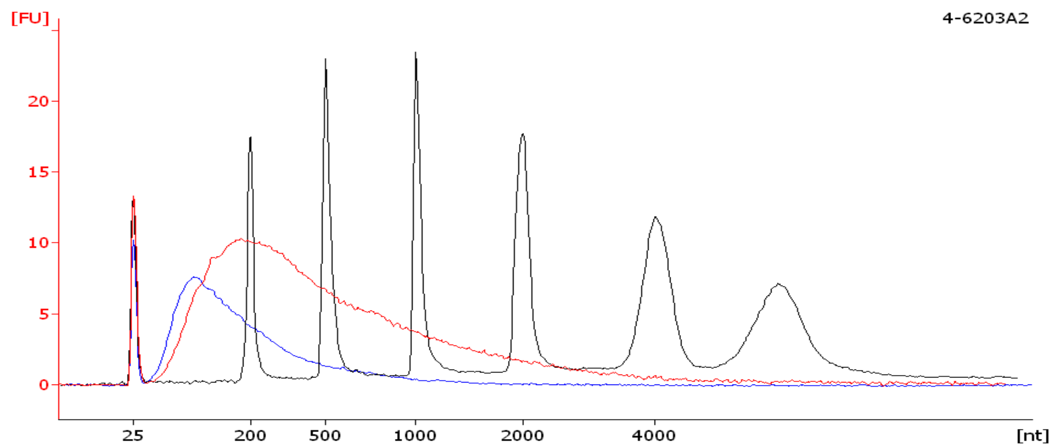
Addition of RNase inhibitors: the universal inhibitor Nucleo-Guard

With very low amounts of RNA, as they are expected with microdissected samples (a few hundred cells) or FACS-sorted cells, elution efficiency may vary significantly.
Although standard lysis buffers contain the strong denaturing agent GTC, its inhibition of enzymes (nucleases) is not 100%, as evident in the use of proteinase K digestion in these buffer conditions. Standard RNase-inhibitors are protein-based and not fully active (if at all) under these conditions. With ExpressArt **NucleoGuard** (#8998-M50) we offer a completely different inhibitor type, based on a low molecular-weight chemical. It is truly universal (competitive inhibition by its action as nucleic acid analogue) and fully active in GTC or all other aqueous buffer formulations. Its effect was recently demonstrated with RNA isolation from **very challenging samples**:

RNA from human saliva: See **Hu et al. (2008)** Clin.Chem. 54:824-832. In Supplementary Figure 2 it is shown that rRNA peaks could be recovered – but only if NucleoGuard had been included in the lysis step. Another example is shown with RNA from paraffin (FFPE) samples.



RNA profiles of representative saliva samples
RNAs (~ 4 ng) were prepared without (**blue**: 0.98 ng/µl) and with the addition of 1% NucleoGuard in RLT lysis buffer (**red:** 3.65 ng/µl). Only in the presence of NucleoGuard it was possible to recover RNA with small rRNA peaks. This figure shows the improvement of RNA yield and recovery of long transcripts by using the universal RNase inhibitor NucleoGuard.



RNA profiles of FFPE samples
RNAs were prepared without (**blue**: 151 ng/µl) and with the addition of 1% NucleoGuard in the lysis step (**red:** 356 ng/µl). In the presence of NucleoGuard it was possible to recover higher amounts of RNA with higher quality.

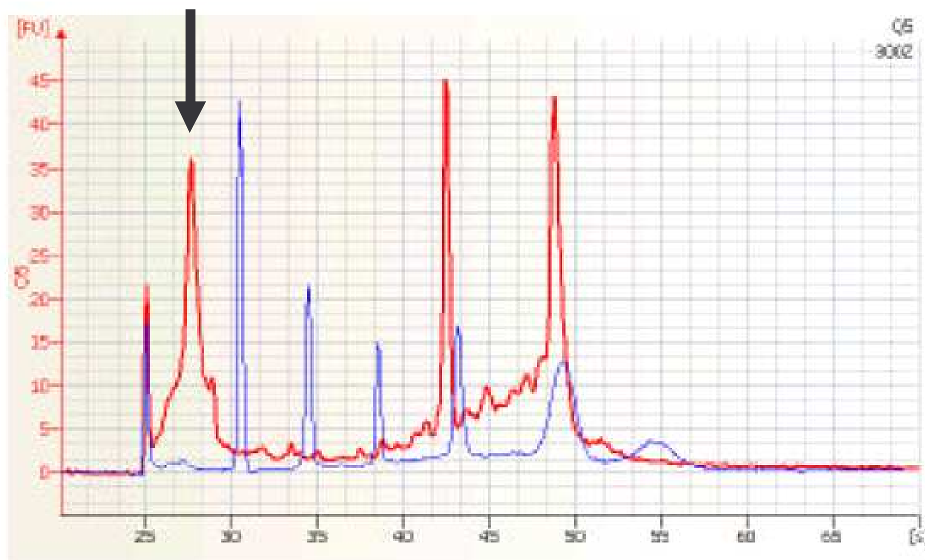
Addition of RNA carriers

This seems a reasonable strategy to keep RNases at bay and to reduce loss of RNA by unspecific adsorption to surfaces, especially when spin columns are used.

Qiagen recommends the addition of the RNA carrier Poly(A). This is problematic, because Poly-A interferes with ExpressArt® TR*inucleotide* amplification, RNA profiling with the Agilent Bioanalyzer and other down-stream applications.

Therefore, the use of the ExpressArt® **RNA Care** reagents (#8999-A100) is strongly recommended. These carrier reagents enable the reliable preparation of total RNAs in the picogram range. These carriers are proven to be compatible with subsequent ExpressArt® TR*inucleotide* amplification: no inhibition, no activity as primers or as templates, no amplification artefacts (**Baugh,** personal communication).

The N-Carrier is a small RNA with hairpin structure. Its structure makes it an inert RNA, its size results in >99% removal by spin column purification (like the RNeasy kit from Qiagen, or the PicoPure kit from Arcturus/MDS-Pharma-Service). The N-Carrier is present when needed: during lysis and during adsorption to the column matrix, but is essentially absent in subsequent QC with the Agilent Bioanalyzer.

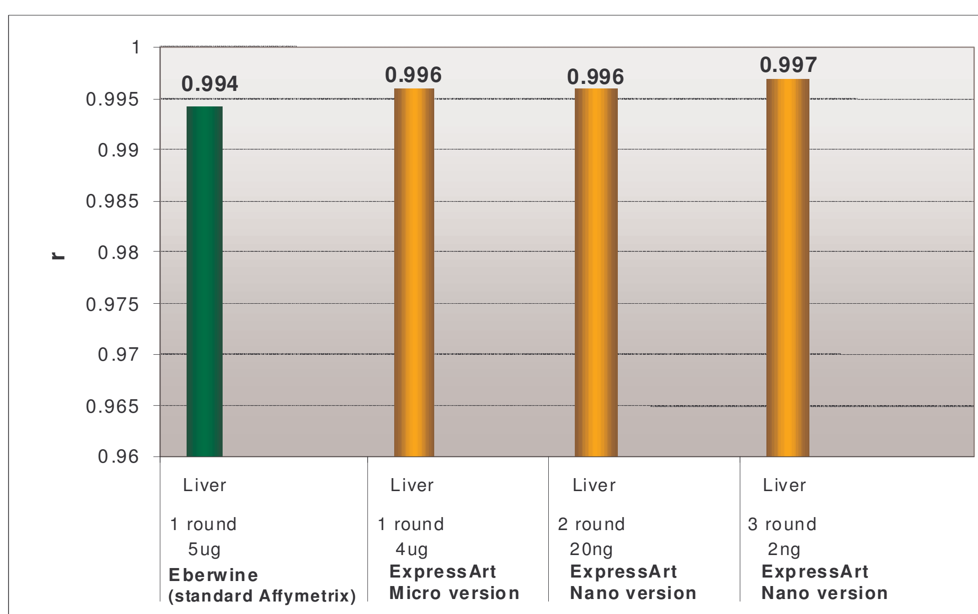


This RNA profile demonstrates the use of N-Carrier in combination with the Bioanalyzer. Approximately 300 mouse liver cells were

collected, by adding 1% NucleoGuard and 1 µl N-Carrier (100 ng) in the lysis buffer. A small aliquot (10%) was analysed on an RNA 6000 PicoChip. The rRNA peaks are clearly visible in this small aliquot (~ 0.3 ng RNA). Although 100 ng N-Carrier were added initially, only a minor amount (<0.1 ng in this aliquot, indicated by the arrow) remains and this is no limitation in RNA profiling of very small samples.

4. Example results

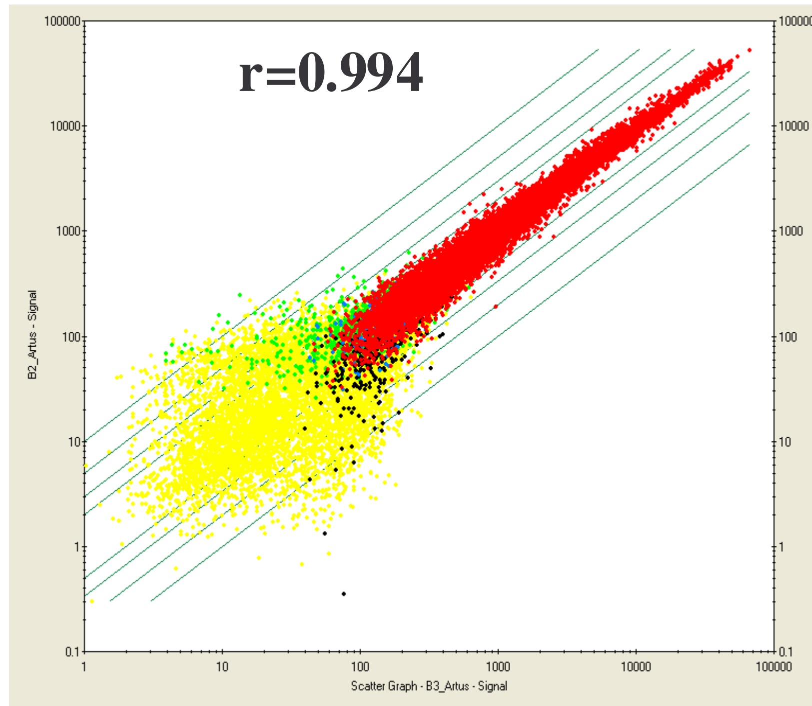
4.1. Excellent reproducibility of technical replicates



These sample series were analyzed with Affymetrix GeneChips HG-U133v2.0 and Pearson values of replicate pairs are indicated. Regardless of the ExpressArt kit version and the use of 1, 2 or 3 amplification rounds, very high reproducibility was observed, quite comparable to data with the standard Eberwine technology.

4.2. Different RNA amounts in sample series

It is a unique advantage that RNA quantisation and calibration are not required prior to ExpressArt amplification.



This scatter plot compares microarray of two samples with very different amounts (10 ng and 100 ng of input total RNA). The samples were amplified by 2 rounds of ExpressArt and hybridised to Affymetrix GeneChips HG-U133v2.0. Very high reproducibility was observed, very similar to standard technical replicates with identical RNA amounts (see above).

Troubleshooting

T1. RNA Isolation

In addition to your choice of commercial RNA isolation kit, we recommend the ExpressArt additive **NucleoGuard (#8998-M50)**.

Ideally, RNA should be free of contaminating DNA. The TR*inucleotide* mRNA amplification kits are extremely sensitive to contaminating DNA fragments. A DNase treatment should be combined with a spin column purification to remove all fragments of digested DNA.
In general we have very good results with a modified protocol for the RNeasy FFPE Kit from Qiagen (Qiagen Catalogue No. 74104) in combination with the RNase-Free DNase Set (Qiagen Catalogue No. 79254) for DNA removal: simply include 1% NucleoGuard reagent in the RLT lysis buffer.

Please note, **Trizol (or RNA-Stat)** are not suitable for removal of DNA in samples with degraded nucleic acids. The degraded DNA fragments will co-purify with RNA.

T2. RNA quality with large samples

Standardised RNA quality is an important issue. RNA isolation procedures should maintain, as far possible, the RNA quality in your samples. Whenever possible, the quality of purified RNA should be controlled by gel electrophoresis or RNA profiling with the Agilent 2100 bioanalyzer.
Standard RNA technology and the standard ExpressArt® mRNA Amplification kits require total RNA with well-defined bands of the 28S and 18S rRNA species, and an intensity ratio of at least 1:1. 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 the Agilent 2100 bioanalyzer. See example results in **Krupp (2004)** Stringent RNA Quality Control using the Agilent 2100 Bioanalyzer. Agilent Application Note, available from krupp@amp-tec.com.

For maintaining RNA quality during the isolation procedures, it is important to eliminate internal and external RNase activities. As soon as the cells are damaged, intracellular RNase activities will start RNA degradation. After collecting tissue samples, or cells from cell

culture, it is important to **immediately (!)** 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, RNAStat 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.

T4. Problems with mRNA Amplification

- **No amplified RNA**

With **50-100 ng input total RNA**, the first amplification round should yield enough material to detect an intense smear of amplified RNA in the gel with an aliquot (1-2 µl) of the transcription reaction (see also bioanalyzer RNA profiles, as shown above). If you do not see any amplified material, we recommend performing the kit reaction again with the Positive Control RNA, provided with your ExpressArt kit. If the control works properly, your sample RNA might have been RNase-contaminated. If the control did not work, make sure that you carefully follow the protocol, especially make sure that you thoroughly mix all thawed solutions and the Master Mixes (for example by inverting the tubes; avoid vortexing of enzymes), as well as all samples after adding new reagents (pipetting up and down and stirring at the same ime).
Starting with less than 50 ng total RNA, only the second round of amplification may yield visible amounts of amplified RNA.

- **Low yield of amplified RNA**

Among different cell lines and tissues or cell types, significant variations in the mRNA content can occur. Estimates range from < 1% to 5% of total RNA, thus leading to different amplification yields, despite of using the same amount of input total RNA. If you obtain only a faint, hardly visible, smear of amplified RNA in the gel, but with the expected length distribution, you may consider an additional amplification round (this option is another advantage of our amplified RNA with defined sequences at each end).

- **Amplified RNA length too small**

With the **TR mRNA Amplification kit**, amplified RNAs should have a centre-of-mass at appr. 0.5 kb. Maybe the quality of your RNA was low and this will result in more lost mRNA sequence

information. Try a different RNA isolation protocol, if not done before, include 1 µl per sample of ExpressArt N-Carrier (#8999-A100) and 1% of the ExpressArt reagent NucleoGuard (#8998-M50) in the lysis step.

- **Comparison of expression patterns**

Note: Avoid direct comparison of array patterns from samples with different pre-treatments, only samples without amplification or samples subjected to the same amplification procedures should be compared directly. Then differential gene expression patterns are largely unaffected.
However, a **unique advantage of ExpressArt® technology** is the possibility to directly compare amplified RNA samples, obtained with one, and especially with **two or three amplification rounds**.

AmpTec GmbH
Koenigstrasse 4a
22767 Hamburg
Germany
Tel: +49 (0)40 – 636 747 22
Fax:+49 (0)40 – 636 747 19
Technical Support:
"Dr. Guido Krupp" <krupp@amp-tec.com>
"Dr. Peter Scheinert" < scheinert@amp-tec.com >
www.amp-tec.com