

# ExpressArt<sup>®</sup> Technology

## for whole transcript amplification

e.g. for use with Affymetrix Exon or Gene ST arrays

This short brochure provides general information.

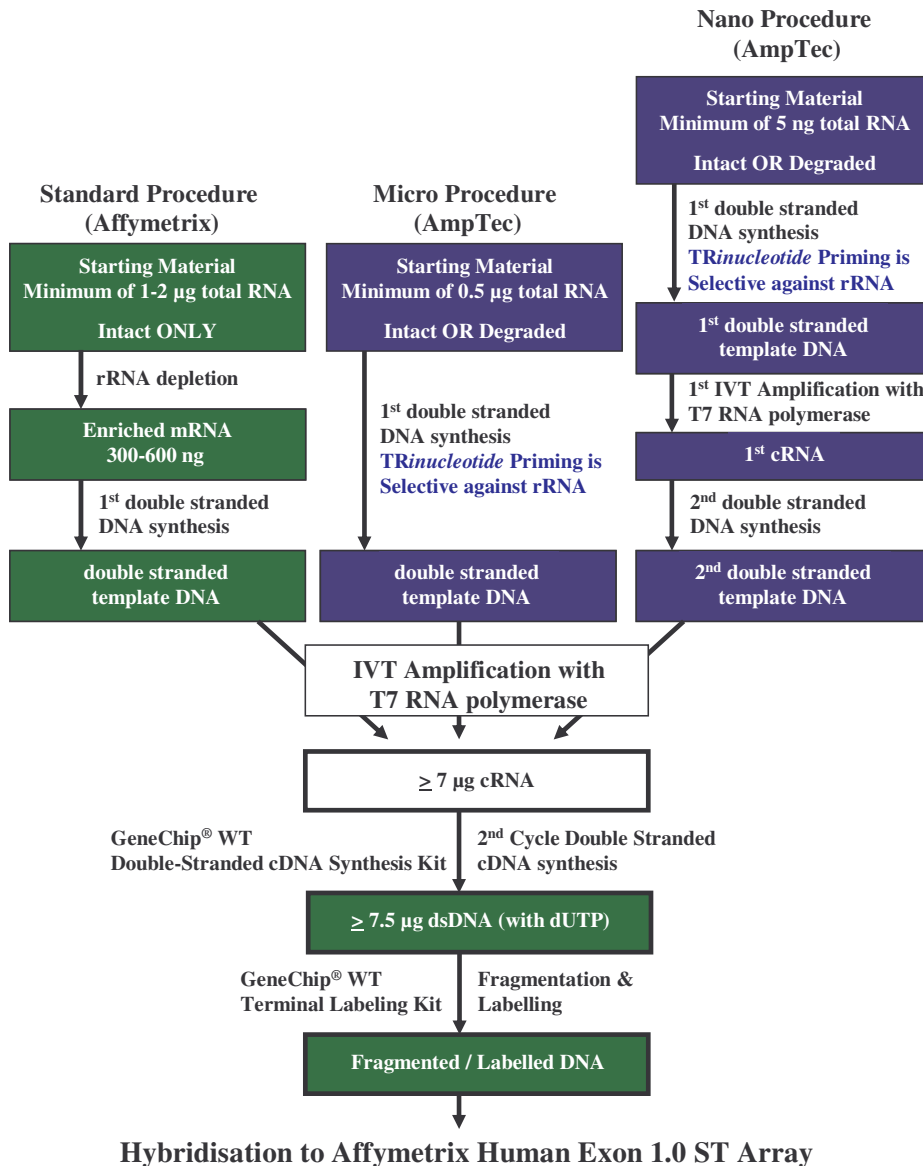
Detailed laboratory protocols are available for the kit which fits your needs:

**Micro** (#6199-A30 for RNA samples of  $\geq 300$  ng), **Nano** (#6299-A15 for  $\geq 1$  ng) or **Pico kits** (#6399-A15 for  $\geq 100$  pg).

### Comparison of Flow Diagrams for Whole Transcript (WT) Amplified Double-Stranded Target Assays

AmpTec's *TRinucleotide* priming technology results in full-length reverse transcription of mRNA sequences, independent of a 3'-polyA sequence. This permits the use of degraded RNA samples.

In a second step, a unique 3'-sequence is introduced in all amplified RNAs. This permits the use of two amplification rounds.

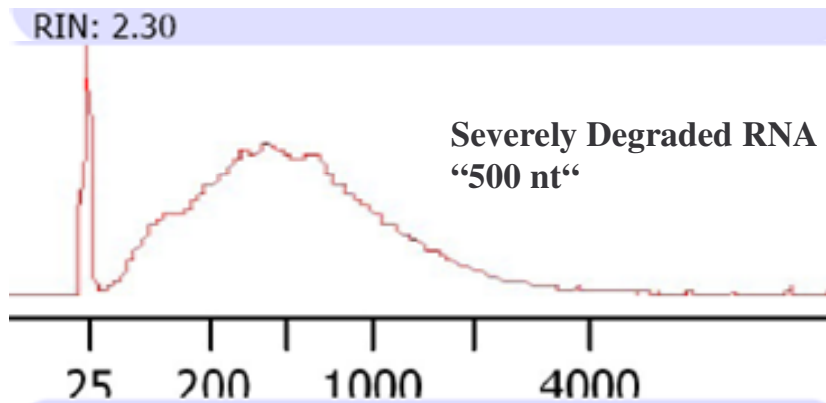


**Intact and degraded RNA samples** can be used, based on these minimal criteria for Bioanalyzer Profiles of suitable RNA samples:

The rRNA peaks can be completely absent.

RNA Integrity Number RIN can be as low as ~ 2.

Median RNA size should peak at  $\geq 500$  nucleotides, extending to  $> 1,000$  nt.



This RNA quality may be used also for RNA amplification technologies, that are based on random priming in the first reverse transcription step. However, the unique unique *TRinucleotide* primer technology of ExpressArt® *TRinucleotide* mRNA amplification kits offer two special advantages:

- **Selectivity against rRNAs and rRNA fragments** eliminates need of rRNA removal steps – with more convenience and even improved signal/background ratios & no compromise, no lower sensitivities in processing of small RNA samples.
- **Suppression of rRNA amplification:** less than 2% rRNAs in amplified RNAs
- **Minimum input RNA amounts of ~ 1 ng total RNA.** *TRinucleotide* technology permits two amplification rounds.

## **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<sup>®</sup> kits for amplifying your mRNA!**

## Introduction

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

**Initially**, the ExpressArt<sup>®</sup> **TRinucleotide 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.

Instead of oligo(dT), the first cDNA synthesis is performed with an especially designed *TRinucleotide* primer (5'-Box-Random-Trinucleotide-3' primer) that results in **preferential priming near the 3'-end** of any nucleic acid [see Hu et al. (2008) *Clinical Chemistry* **54**: 824-832].

**Subsequent observations** have shown that *TRinucleotide* primers respond to high local polymerase concentrations: these occur at the *TRinucleotide*-generated fork-like structures **near free 3'-ends**, due to transient polymerase binding, but also at **internal positions** where the polymerase has stalled and remains associated with its template. In consequence and as required for **whole transcript analysis** methods, mRNA sequences in intact RNAs are fully recovered, extending from the 3'-ends [independent of the 3'-poly(A)], as well as from internal stalling sites

- **Very low priming with rRNA**  
this means, no loss in signal intensity, no loss in presence calls
- **no need to remove rRNAs: < 2% rRNAs in amplified RNAs**
- **intact mRNAs and mRNA fragments are amplified with or without 3'-poly(A)**
- **exceptionally high signal/background ratios**
- **high number of detected exon sequences**

**Now, this new technology enables whole transcript mRNA amplification with**

- 1) **a simplified procedure**
- 2) **very small samples**
- 3) **severely degraded RNAs**

**See section 4 (pages 18 & 19) for example results.**

## **General properties**

The ExpressArt<sup>®</sup> 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<sup>®</sup> mRNA Amplification Kits are unique and based on proprietary AmpTec technology (patents and patents pending).

## **General Advantages of ExpressArt<sup>®</sup> 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<sup>®</sup>, 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***

## 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<sup>®</sup> mRNA Amplification Kits provide a technology, which solves these problems. With this *TRinucleotide* mRNA amplification kit, intact mRNAs as well as all mRNA fragment are converted to cDNAs with a special "*TRinucleotide* 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 "*TRinucleotide* 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.

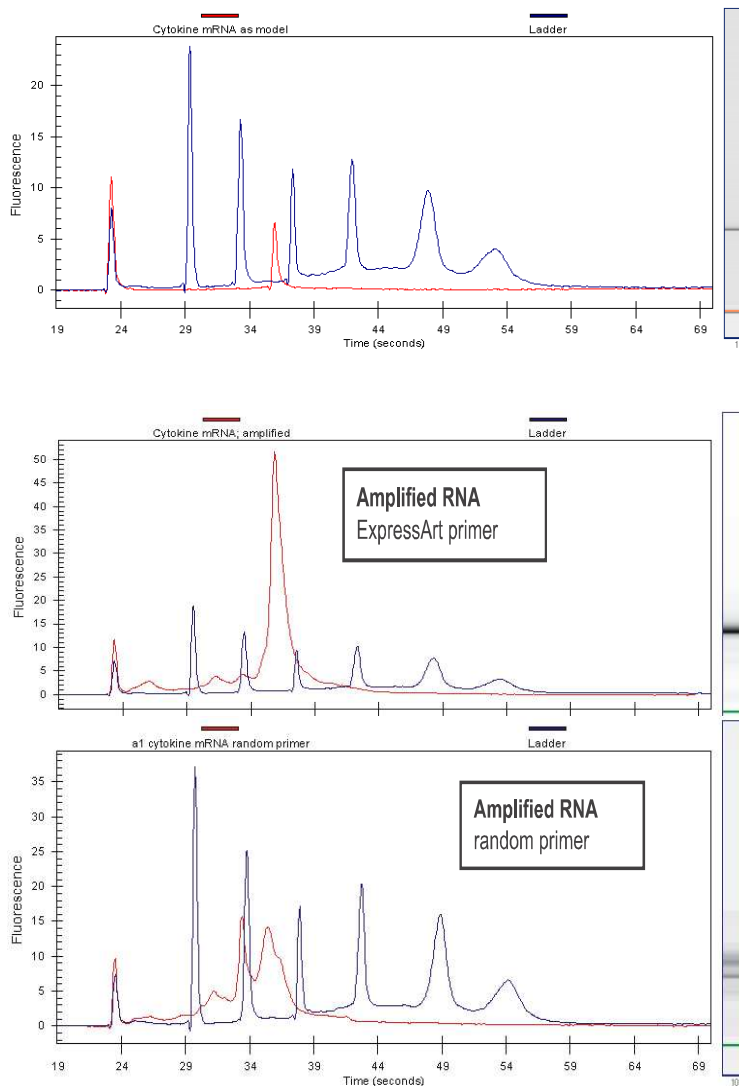
After denaturation, the second cDNA strand will be primed in reverse orientation, using a T7-promoter/Box-1 primer. This leads to double stranded cDNA with a functional T7-promotor at one end and the Box sequence tag at the other end. This dsDNA product is used as template for in vitro transcription, 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.

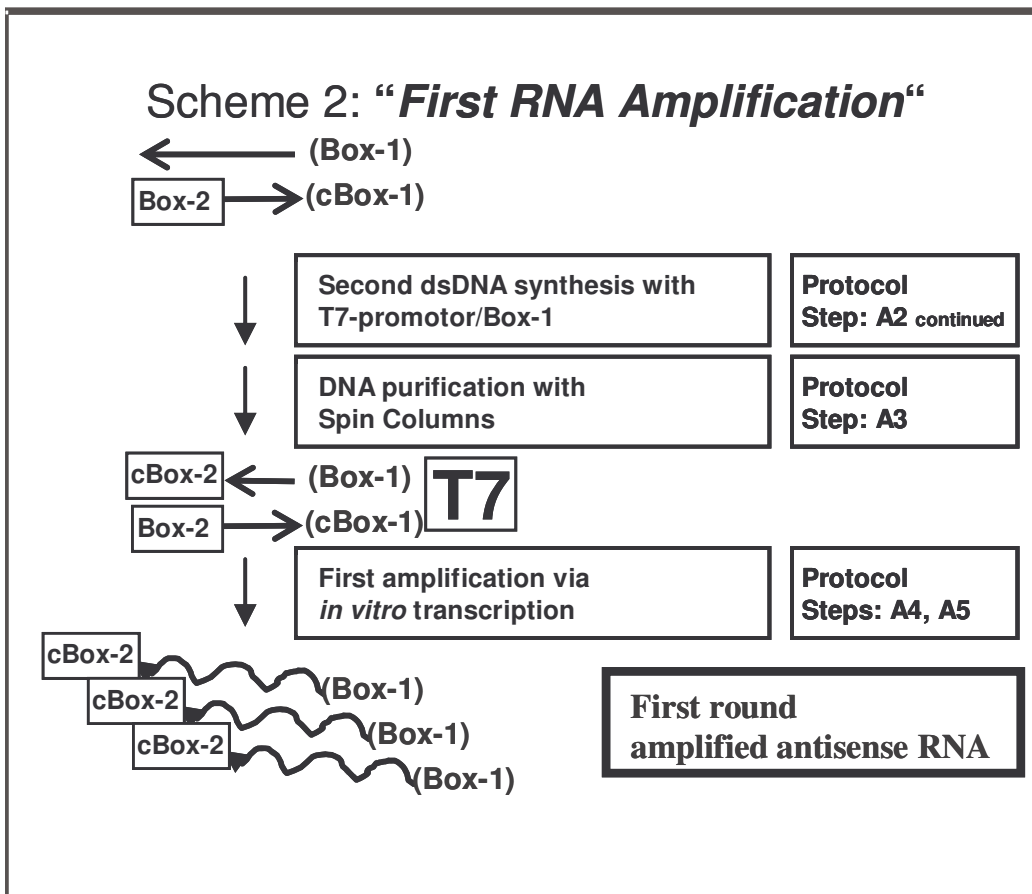
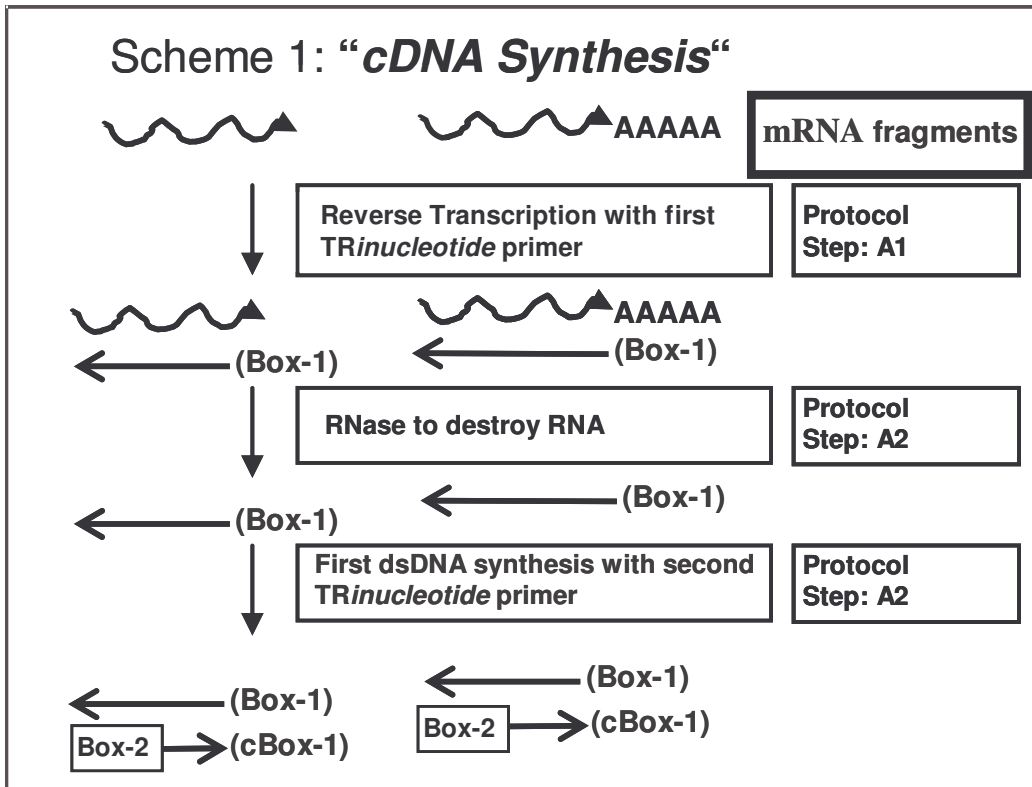
Now, it is not only possible to perform highly reproducible array hybridisations with a few cells, e.g. 4-cell embryos of *C.elegans* (**Baugh (2004)** Genomic analysis of embryogenesis in the nematode *C. elegans*. Ph.D. thesis, Harvard University, Dept. Mol Cell Biol.; **Yanai, Baugh, et al. (2008)** 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<sup>®</sup> TRinucleotide primers

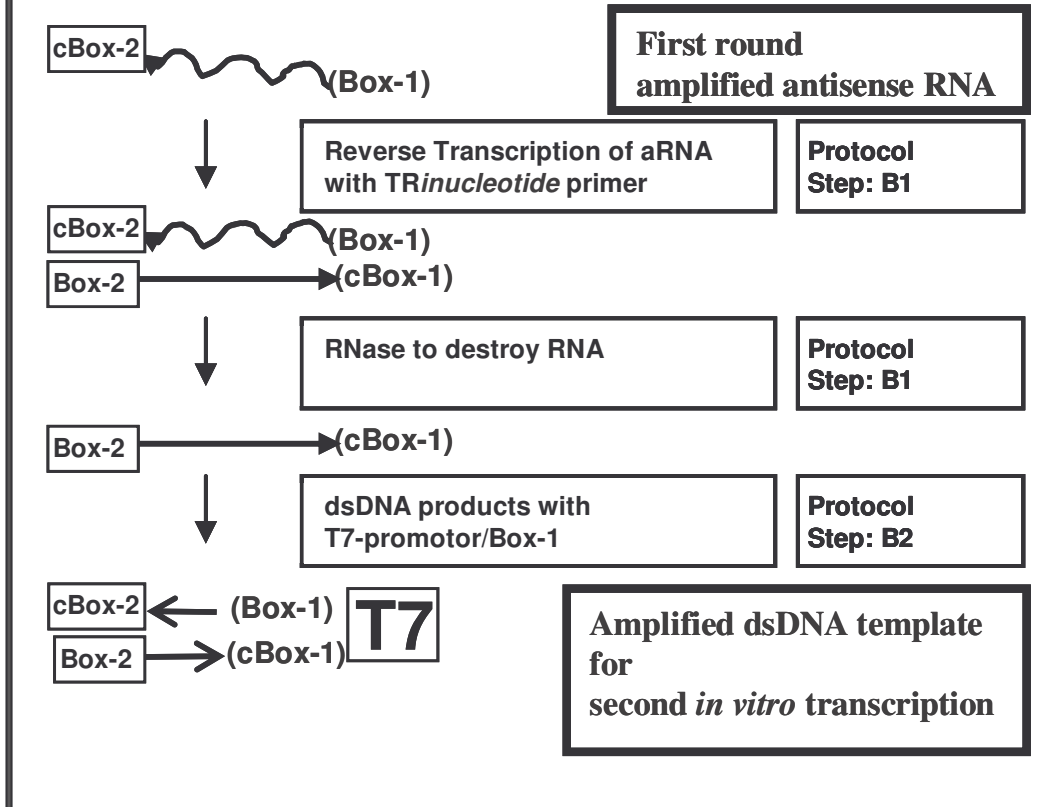
A defined in vitro transcript of 800 nt length was used as input mRNA model (red tracing in top electropherogram). Amplification with ExpressArt<sup>®</sup> technology and the TRinucleotide primer (Box-random-trinucleotide primer) resulted in essentially full-length aRNA (red tracing in middle electropherogram). For comparison, the same reaction steps were used, but the 3'-terminal trinucleotide in the TRinucleotide 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 2<sup>nd</sup> RNA Amplification*”



## RNA Quality Control

### RNAs should meet these minimal criteria:

Peak of RNA at appr. 200 nucleotides with high molecular weight tail; RIN (Agilent) is not really meaningful for RNAs without rRNA peaks.

**Historically:** Successful application of any **standard RNA technology** was dependent on the use of high quality RNA. Therefore, stringent RNA quality control was crucial.

**Now and for the future:** With the introduction of the **ExpressArt® TRinucleotide kits**, this is a problem of the past.

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](mailto:krupp@amp-tec.com)].

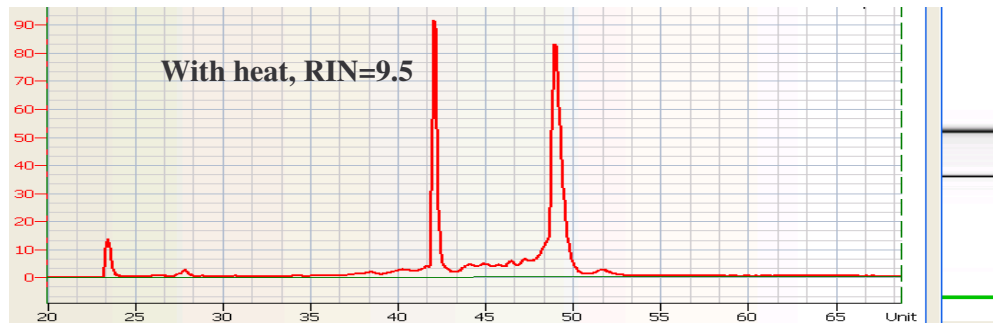
For RNA isolation in the low nanogram and picogram range, use of the **ExpressArt Pico RNA Care** reagents (# 8999-A100) is recommended.

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 introduced [**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.



The **ExpressArt<sup>®</sup> TRinucleotide kits** have overcome another limitation of the past: **Now**, all sequence information retained in your precious samples with severely degraded RNA is amplified.

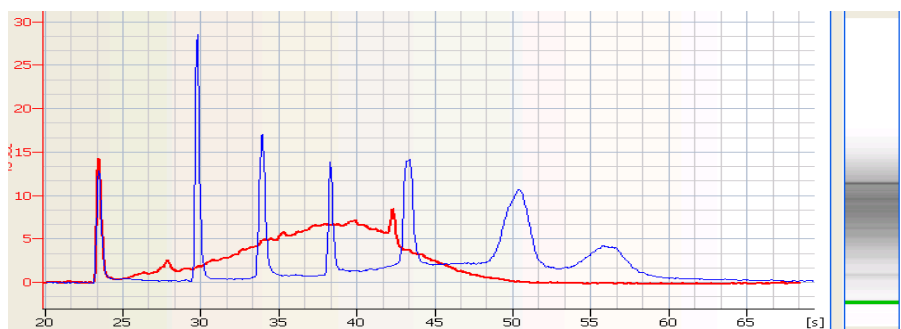
A **model experiment** with artificially degraded RNAs (nicking by metal-catalysed cleavage) demonstrates the potential of the *TRinucleotide* technology: all mRNA sequences were retained in the degraded RNAs and could be fully recovered and amplified.

### 1. Electropherograms of severely degraded input RNAs

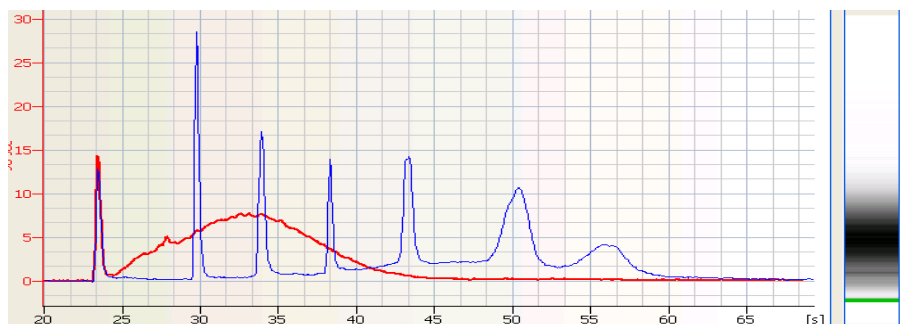
For RNA quality assessment, the profiles were assigned with their **RNA Integrity Numbers, RIN** (see also above).



**Model Example 1:** Extensive degradation of RNA (**RIN = 3.8**)



**Model Example 2:** Extremely poor RNA quality (**RIN = 3.1**)



**Model Example 3:** Extremely poor RNA quality (**RIN = 2.2**)

## **2. TRinucleotide amplified RNAs: electropherograms and Affymetrix hybridisation results.**

Electropherograms are shown for "Model Examples 1–3". Using 100 ng input total RNA, two amplification rounds were performed.

Biotinylated, amplified RNAs were hybridised to Affymetrix HG-U133A GeneChips. Quality of the data was assessed, based on (i) the relative percentage values of presence calls (data of parallel samples with good RNA quality were defined as 100%),

(ii) the 3'/5'-ratios and

(iii) scatter plots comparing hybridisation results with good quality RNA versus the derived, degraded RNA samples.

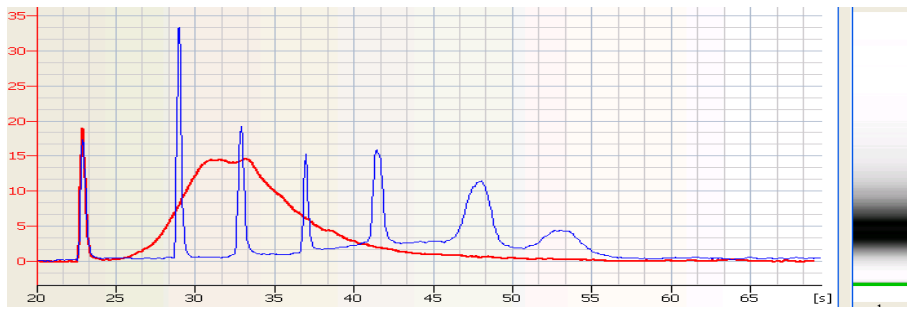
### **2.1. Amplification of mRNA fragments without 3'-poly(A)**

**Standard mRNA amplification technology:** For comparison, the degraded RNA samples were amplified using oligo(dT) as primer in the first cDNA synthesis reaction. As a consequence with degraded RNA samples, amplification is limited to the 3'-terminal mRNA fragments that retained a poly(A) sequence. Severe mRNA degradation and loss of mRNA sequence information were evident after hybridisations with Affymetrix HG-U133A GeneChips. Very high 3'-5'-ratios were observed, especially for "Example 3" with GAP-DH > 15 and  $\beta$ -actin > 50 (effectively, middle and 5' probe sets were "called absent").

**ExpressArt<sup>®</sup> TRinucleotide amplification** results in efficient priming with all mRNA fragments. As a consequence for degraded RNA samples, 3'-5'-ratios remain very low (observed values are included in the following examples).

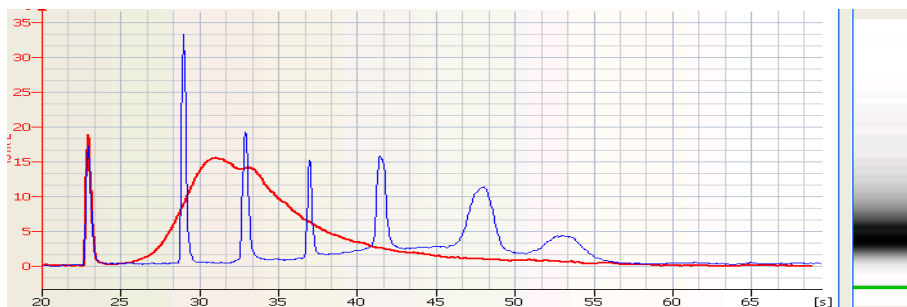
Occasionally, priming with 5'-proximal mRNA fragments can be more efficient, and increased RNA degradation can result in even lower 3'/5'-ratios. As actually observed, values below 1.0 can occur.

**Please note:** TRinucleotide amplification prevents **loss of 5'-proximal sequence** information and fully retains all sequence information present in RNA samples.



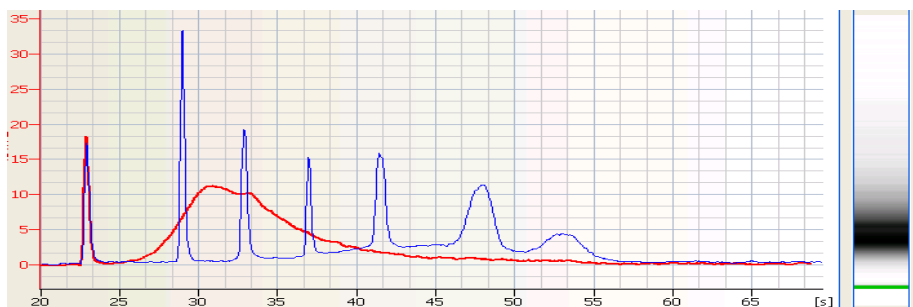
**Amplified RNA from Example 1: "Degraded RNA, RIN 3.8"**

Relative presence calls: >96%, 3'/5'-ratios: GAP-DH = 2.8,  $\beta$ -actin = 1.1



**Amplified RNA from Example 2: " Degraded RNA, RIN 3.1"**

Relative presence calls: >96%, 3'/5'-ratios: GAP-DH = 2.0,  $\beta$ -actin = 0.9



**Amplified RNA from Example 3: " Degraded RNA, RIN 2.2"**

Relative presence calls: >96%, 3'/5'-ratios: GAP-DH = 2.5,  $\beta$ -actin = 0.9

**2.2. Amplification of rRNAs?**

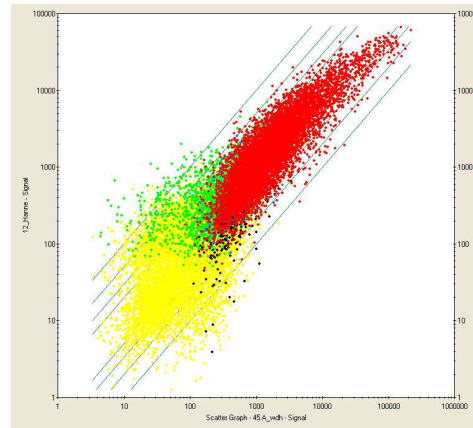
18S and 28S rRNAs and derived rRNA fragments in degraded RNAs are the bulk of total RNA (> 90%). This means, non-selective labelling or amplification requires very stringent hybridisation conditions and can result in low detection sensitivity.

With ExpressArt<sup>®</sup> TRinucleotide amplification, a strong selection against rRNA amplification leads to very low hybridisation signals for rRNAs: **amounts of rRNAs in amplified RNAs are below 2%.**

### 3. Comparative hybridisations: samples with intact and severely degraded RNAs

#### 3.1. Standard mRNA amplification technology

Samples with intact and with artificially degraded RNAs were amplified with standard mRNA amplification technology, using oligo(dT) as primer in the first cDNA synthesis reaction. Loss of mRNA sequence information makes direct comparisons impossible.

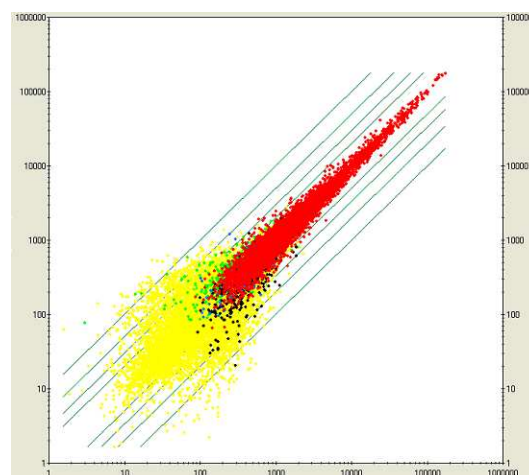


#### Scatter Plot with standard mRNA amplification technology:

Inconsistency of data (**Pearson value,  $r = 0.793$** ) obtained with intact RNA (RIN=9.5; page 12) and severely degraded RNA (RIN=2.2; "Example 3").

#### 3.2. *TRinucleotide* mRNA amplification technology

Scatter plots illustrate high concordance of microarray data obtained with intact RNA (RIN=9.5) and with artificially degraded "Examples 1 to 3" (section 1).



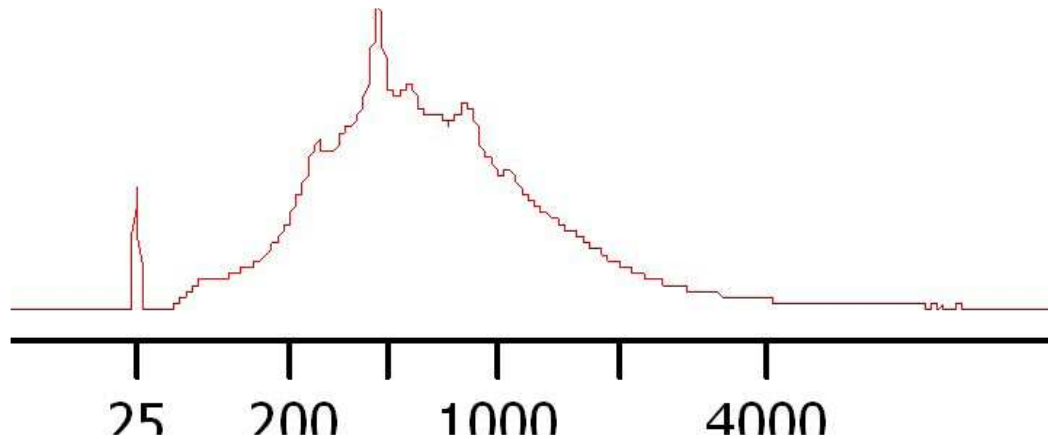
#### Scatter Plot with *TRinucleotide* mRNA amplification technology

Concordance of data (**Pearson value,  $r = 0.993$** ) with intact RNA (RIN=9.5) and severely degraded RNA (RIN=2.2; "Example 3").

## 4. Improved performance with Affymetrix Exon ST arrays

### 4.1. Example electropherogram of 2-rounds amplified RNA

RNA profile illustrates a median size of 500 nt, and a characteristic display of multiple peaks.



### 4.2. Overview of QC data

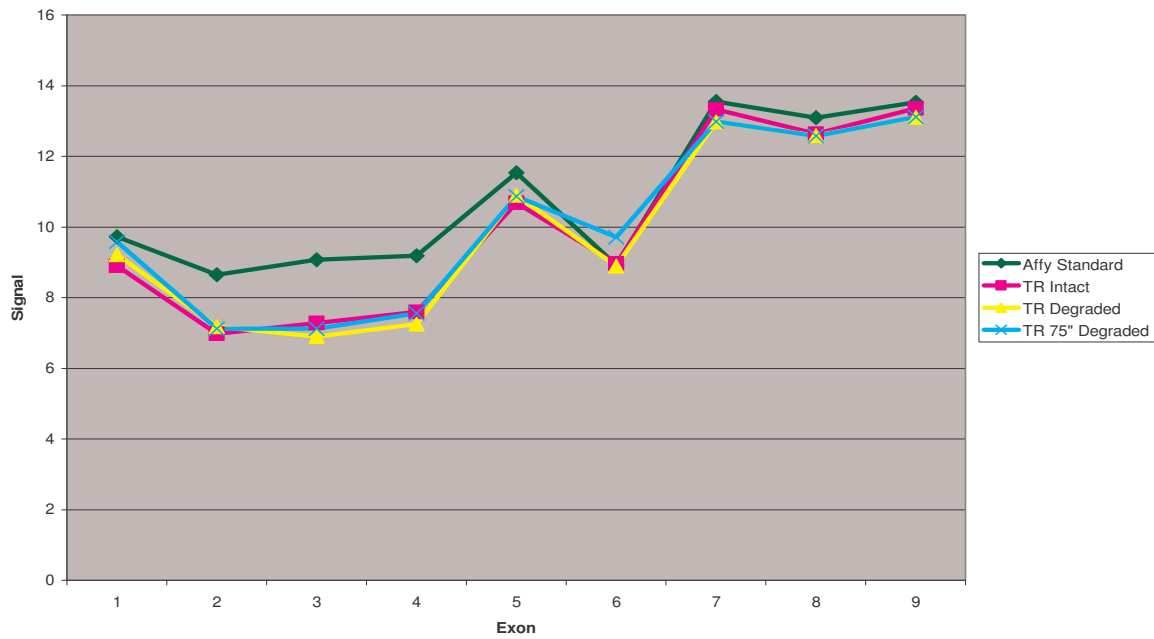
Three independently obtained biological samples (RNAs from human cell cultures) were processed and hybridised to Human Exon 1.0 ST Arrays. In all samples, the low rRNA amounts result in high sensitivity and in acceptable or good signal vs background ratios, as well as good ROC values (a measure of the false positive rate).

Method for generating cRNAs	Starting material RNA amount / RNA quality	cRNA yields [ $\mu$ g]	Sensitivity [% P]	Mean Signal vs Background (Ratio)	Biological replicates [Pearson values]	ROC pos-neg cont
<b>Standard Affymetrix</b>	<b>2.0 <math>\mu</math>g / intact</b>	<b>21 <math>\pm</math> 5</b>	<b>51 <math>\pm</math> 1</b>	<b>280 vs 310 (0.9)</b>	<b>0.98</b>	<b>0.881 <math>\pm</math> 0.003</b>
<b>AmpTec TRinucleotide</b>	<b>50 ng / intact</b>	<b>2 rounds* 62 <math>\pm</math> 10</b>	<b>64 <math>\pm</math> 2</b>	<b>360 vs 210 (1.7)</b>	<b>0.98</b>	<b>0.901 <math>\pm</math> 0.004</b>
<b>AmpTec TRinucleotide</b>	<b>50 ng / degraded ("1000nt")</b>	<b>2 rounds* 58 <math>\pm</math> 10</b>	<b>53 <math>\pm</math> 2</b>	<b>280 vs 200 (1.5)</b>	<b>0.96</b>	<b>0.892 <math>\pm</math> 0.004</b>
<b>AmpTec TRinucleotide</b>	<b>50 ng / severely degraded ("500nt")</b>	<b>2 rounds* 52 <math>\pm</math> 5</b>	<b>47 <math>\pm</math> 3</b>	<b>265 vs 250 (1.1)</b>	<b>0.95</b>	<b>0.875 <math>\pm</math> 0.003</b>

\*Note: Only 50% of the amplified RNAs from the first round were used for the second round.

## 4.2. Complete representation of exon sequences

Signal Intensities for Exon Probes over the Complete Length of GAP-DH mRNA. Some variability (presumably structure related) was observed for all samples. Very high consistency for all AmpTec TRinucleotide amplified RNAs, irrespective of divergent RNA qualities in the starting material.



## T: 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 *TRinucleotide* 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. Isolation of very low RNA amounts

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 isolated from human saliva samples, 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.

**Addition of RNA carriers** 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<sup>®</sup> *TRinucleotide* amplification, RNA profiling with the Agilent Bioanalyzer and other down-stream applications.

Therefore, the use of the ExpressArt<sup>®</sup> **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<sup>®</sup> *TRinucleotide* amplification: no inhibition, no activity as primers or as templates, no amplification artefacts (**Baugh**, personal communication).

### **T3. RNA quality with large samples**

Standardised RNA quality is an important issue, but only for standard RNA technology.

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<sup>®</sup> 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](mailto: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  $-80^{\circ}\text{C}$  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<sup>®</sup> 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).

#### **T4. 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 T2).

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<sup>®</sup> PICO RNA Care reagents, in combination with the NucleoGuard additive, ensure optimal RNA yields and quality. Furthermore, with ExpressArt<sup>®</sup> 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<sup>®</sup> 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.

## **T5.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  $\mu$ l) 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 time).

**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 (see also section 4.1). 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  $\mu$ l 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**.

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