

ExpressArt[®] Technology for selective amplification of bacterial mRNAs

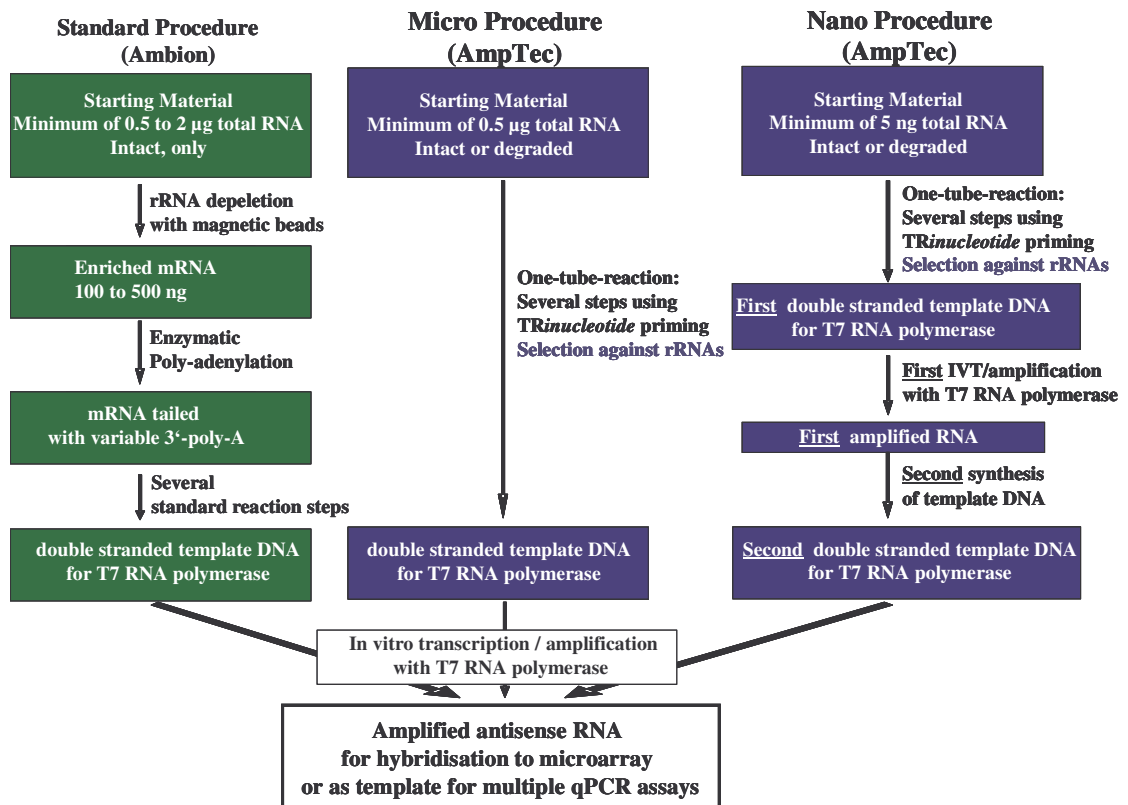
This short brochure provides general information.

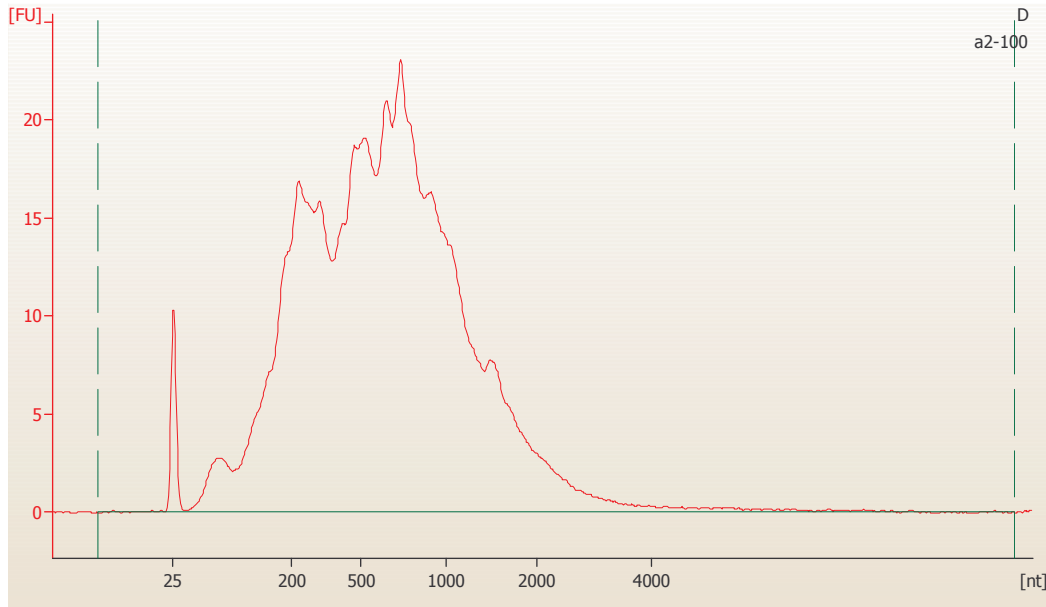
Detailed laboratory protocols are available for the kit which fits your needs:

Micro (#5199-A30 for RNA samples of ≥ 300 ng) or Nano kits (#5299-A15 for ≥ 1 ng).

Comparison of Flow Diagrams for Amplification of Bacterial mRNAs

AmpTec's *TRinucleotide* priming technology results in amplification of all mRNAs, independent of a universal 3'-sequence, and in effective exclusion of rRNA sequences. In a second step, a universal 3'-sequence is introduced in all amplified mRNAs. This permits two amplification rounds.





Electropherogram of 2-rounds amplified RNA from *E. coli*

Hybridisation results with Affymetrix GeneChip *E_coli_2*

42.50% (4336) Presence Calls

Suppression of rRNA amplification: less than 2% rRNAs in amplified RNAs

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.

For severely degraded eukaryotic RNAs, like FFPE-RNAs, *or* for **bacterial mRNAs**, the ExpressArt[®] **TRinucleotide mRNA amplification kits** were developed.

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**].

- **Very low priming with rRNA**
this means very good signal/background ratios, high levels of presence calls
- **no need to remove rRNAs: < 2% rRNAs in amplified RNAs**

Now, this new technology enables specific mRNA amplification with

1) **samples of bacterial total RNA**

- **intact mRNAs and mRNA fragments are amplified**,
no need for a universal 3'-sequence, like 3'-Poly(A);
- **high level of sequence representation**
high number of presence calls
high signal intensity.

2) **specific amplification of bacterial mRNAs.** Although they have no universal 3'-sequence, bacterial mRNAs are selected over rRNAs.

For example results, see section xx (pages

2. 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 proprietary 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*

3. 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 *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.

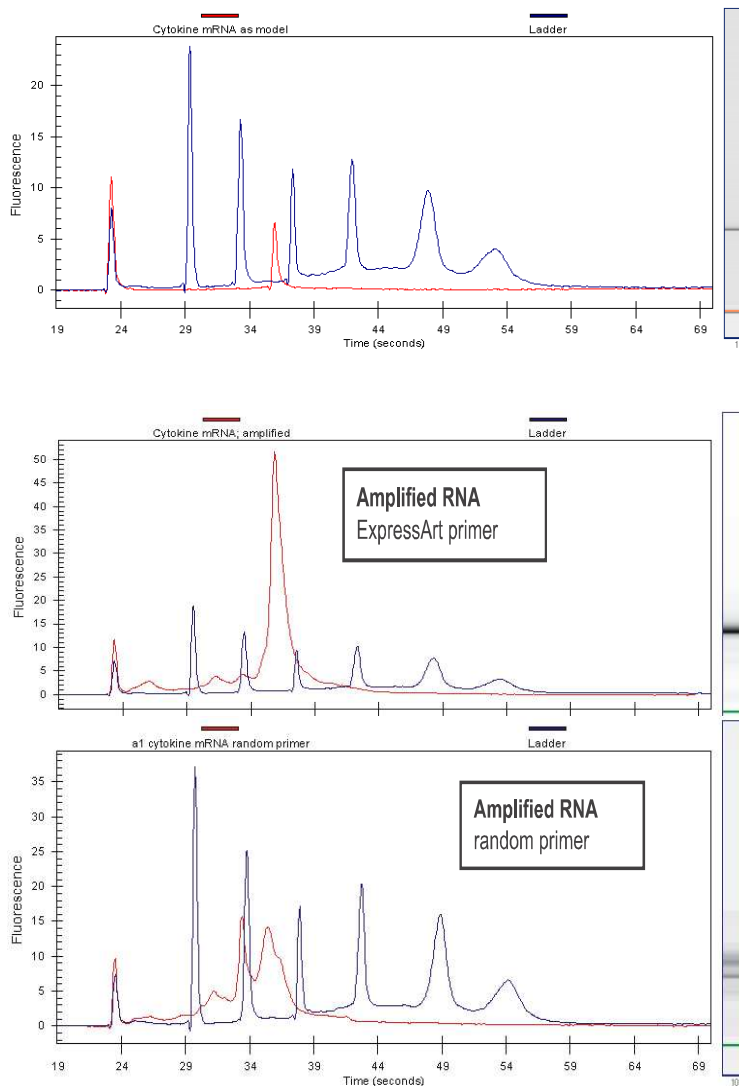
After denaturation, the second cDNA strand will be primed in reverse orientation, using a T7-promoter/Box-1 primer. This leads to double stranded cDNA with a functional T7-promotor at one end and the Box sequence tag at the other end. This dsDNA product is used as template for in vitro transcription,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

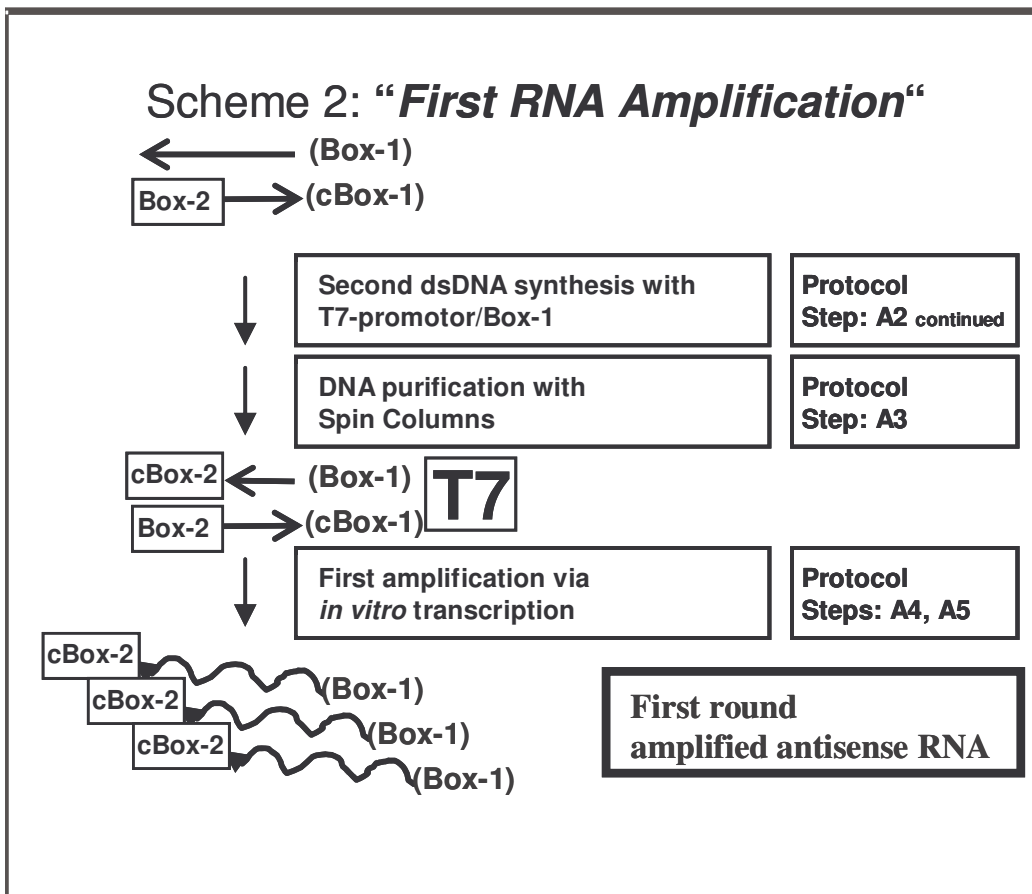
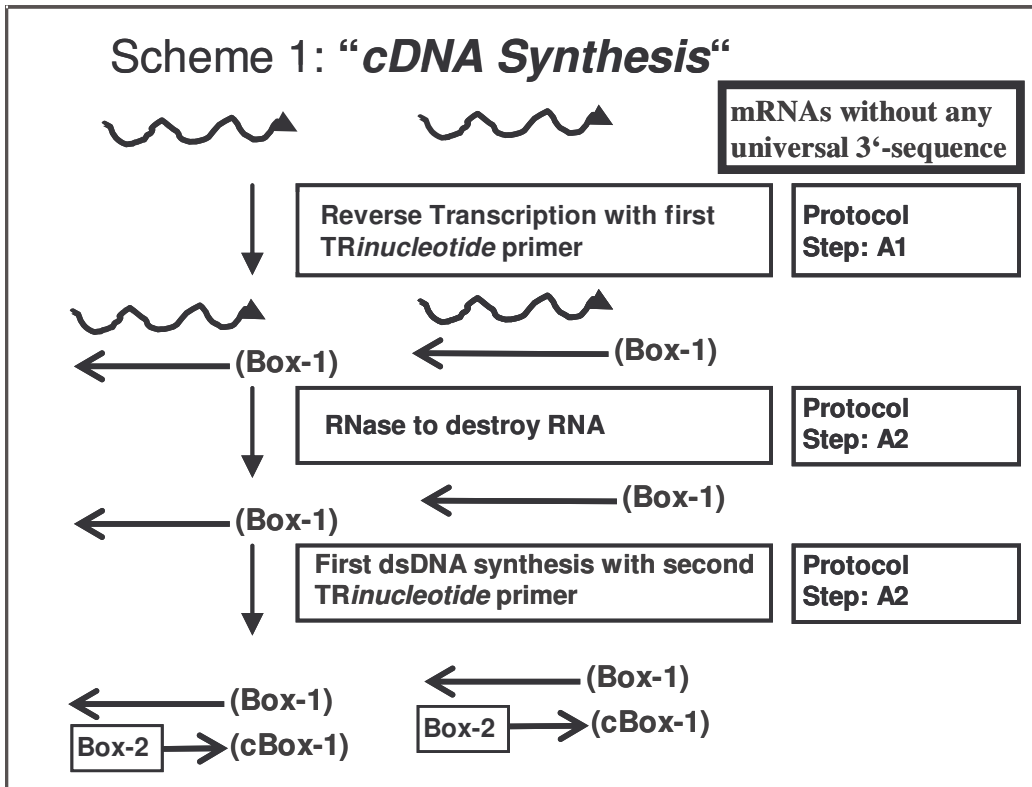
Now, it is not only possible to perform highly reproducible array hybridisations with a few cells, e.g. 4-cell embryos of *C.elegans* (**Baugh (2004)** Genomic analysis of embryogenesis in the nematode *C. elegans*. Ph.D. thesis, Harvard University, Dept. Mol Cell Biol.; **Yanai, Baugh, et al. (2008)**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

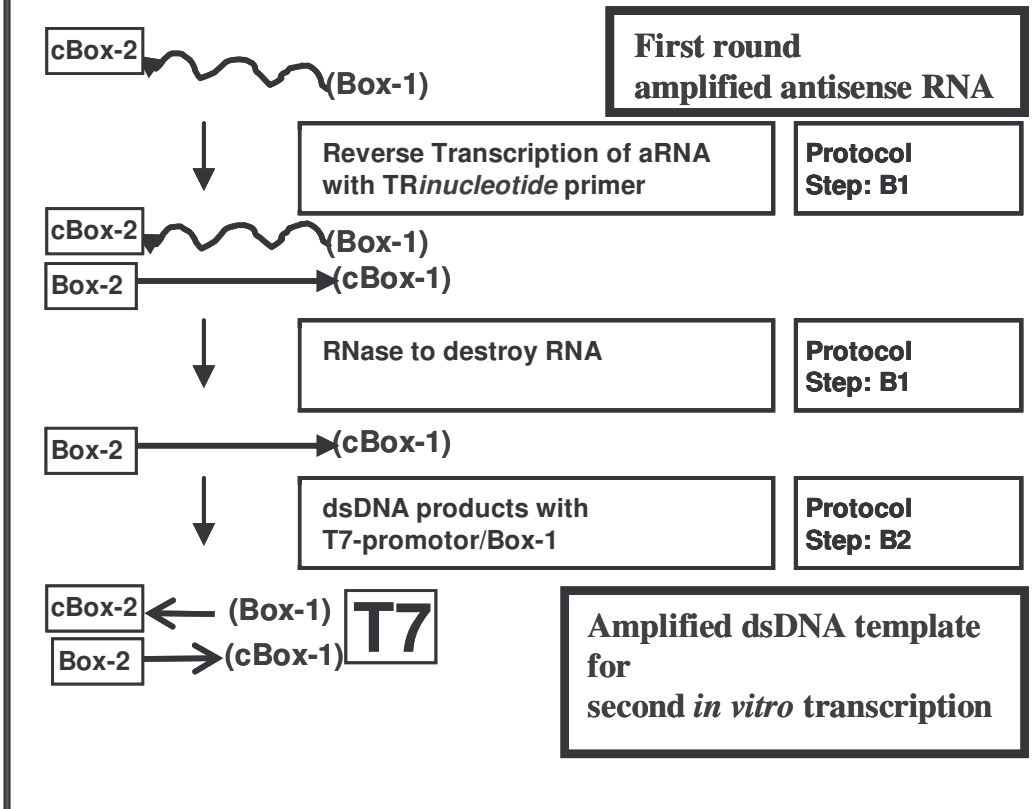
A defined in vitro transcript of 800 nt length was used as input mRNA model (red tracing in top electropherogram). Amplification with ExpressArt[®] technology and the TRinucleotide primer (Box-random-trinucleotide primer) resulted in essentially full-length aRNA (red tracing in middle electropherogram). 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 2nd RNA Amplification*”



RNA Quality

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 and Bacterial kits**, this a problem of the past.

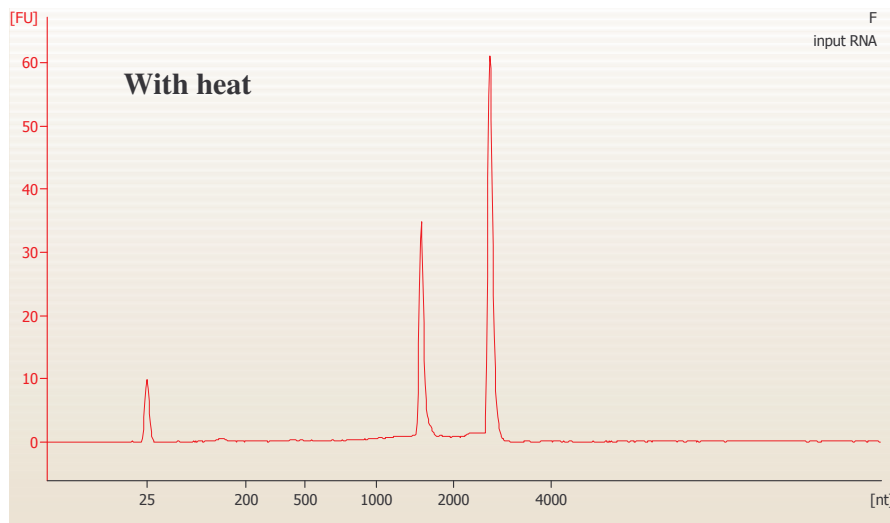
In addition to gel electrophoresis, the Agilent 2100 bioanalyzer combined with RNA 6000 Nano and Pico LabChips is widely used for high-resolution analysis of small and very small RNA samples. Expected electropherograms vary, depending on species, tissue type and RNA isolation method [see **Krupp (2004)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

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,

An RNA sample from *E. coli* with good quality is shown below.



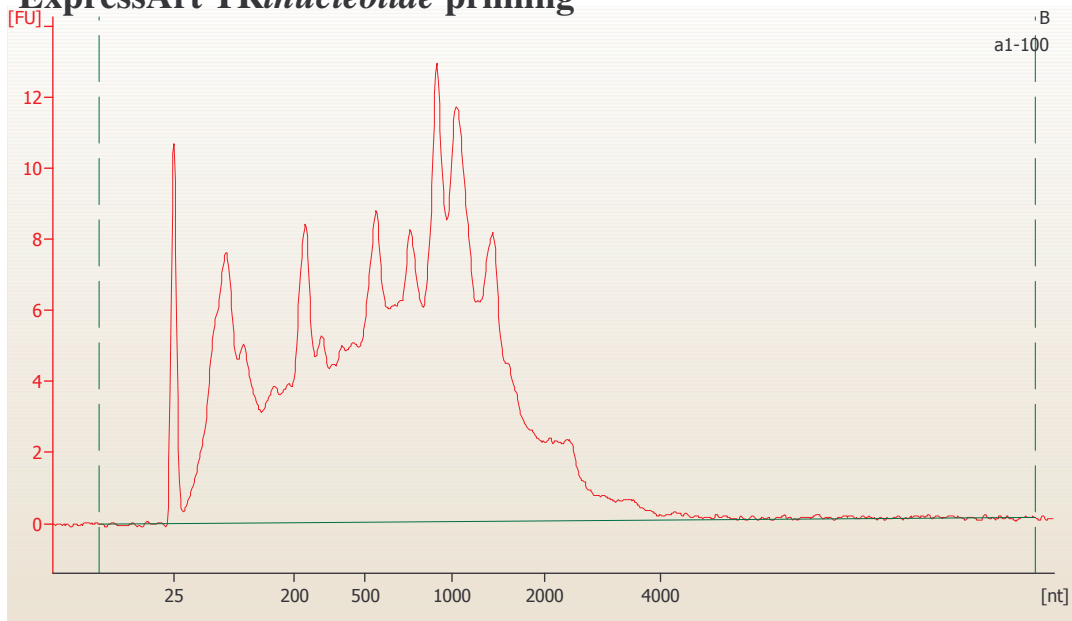
The **ExpressArt[®] TRinucleotide kits** have overcome another limitation of the past: **Now**, all sequence information retained in the mRNA sequences of your precious samples is amplified, you can use total bacterial RNA and get specific amplification of all mRNAs, you can even use degraded RNA samples and fully recover all retained mRNA sequences from your samples, maintaining selection against amplification of rRNA fragments.

4. Example results with amplified *E. coli* total RNAs

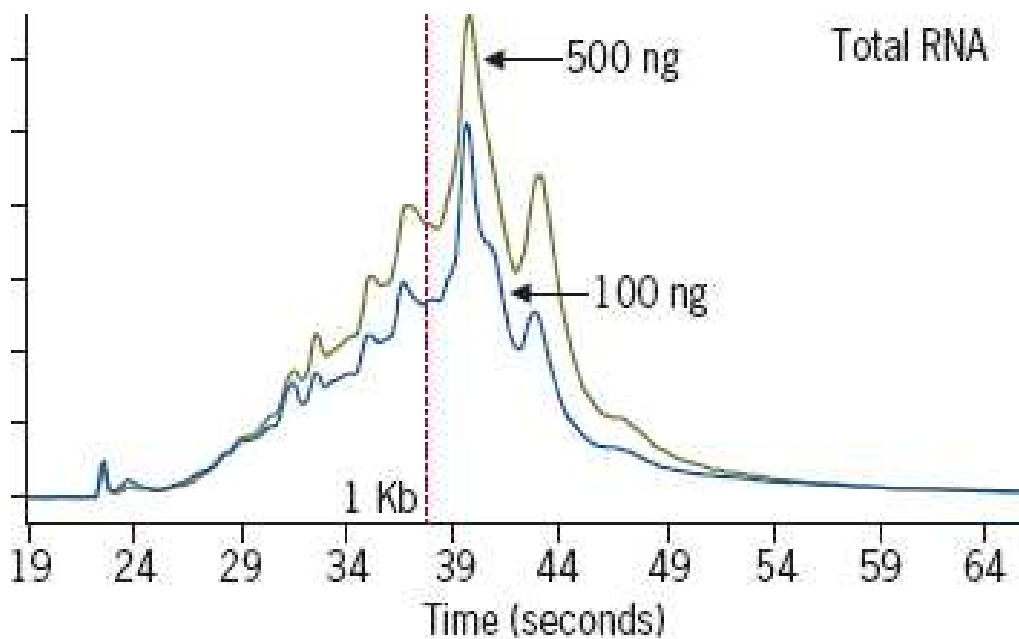
Methods After performing a typical heat shock experiment with 7 min incubations at either 37°C or at 50°C [Richmond et al. (1999) Genome-wide expression profiling in *Escherichia coli* K-12. Nucleic Acids Res. 27: 3821-3835], total RNAs from *E.coli* K12 were extracted with Trizol, followed by spin column purification (RNeasy) and genomic DNA was removed by DNase I treatment (example RNA profile, see above, p. 13). **High amounts of total RNA in combination with conventional labelling of cDNAs:** 50 µg total bacterial RNA were used to generate dye-labelled cDNAs by reverse transcription, using random primers and Cy-3- or Cy-5-labelled dCTP's. **Nanogram amounts of total RNA in combination with ExpressArt TRinucleotide amplification:** 50 ng total bacterial RNA samples were amplified (2-rounds) with the AmpTec ExpressArt Bacterial mRNA Amplification Kit to generate amplified antisense RNAs. **Fluorescence labelling:** Using the ExpressArt AminoAllyl Module (#2000-A30) aminoallyl-modified UTP's were incorporated during in vitro transcription, followed by coupling with Cy-dyes. Fragmented cRNAs were hybridised with Ocimum *E.coli* K12 Arrays. **Biotin labelling:** Biotin-modified UTP's and CTP's were directly incorporated during in vitro transcription. Fragmented cRNAs were hybridised with Affymetrix *E.coli* Genome 2.0 GeneChips.

4.1. Electropherogram of 1-round amplified *E. coli* RNA

ExpressArt TRInucleotide priming

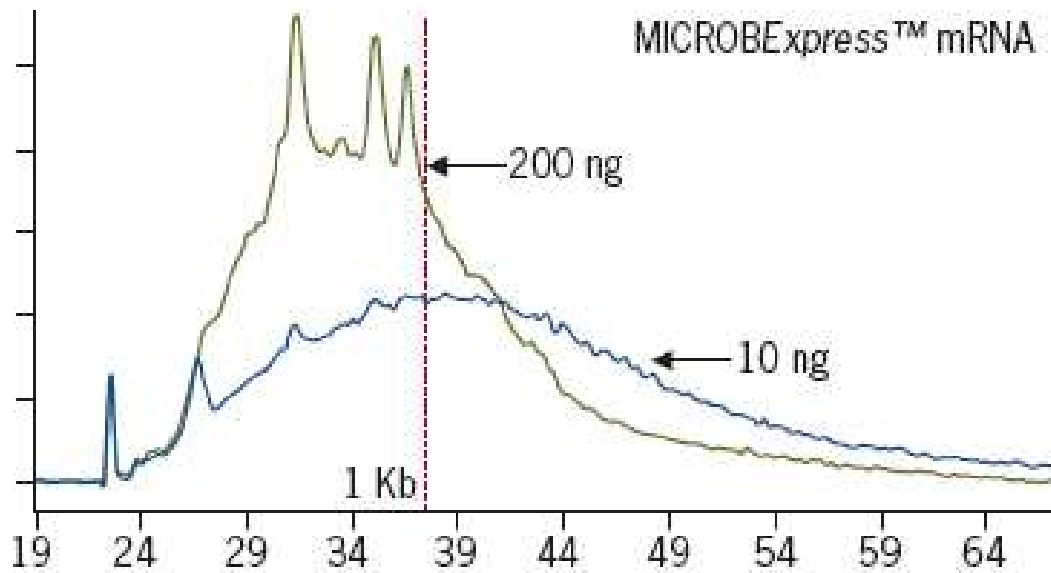


Competitor A Random priming with total RNA

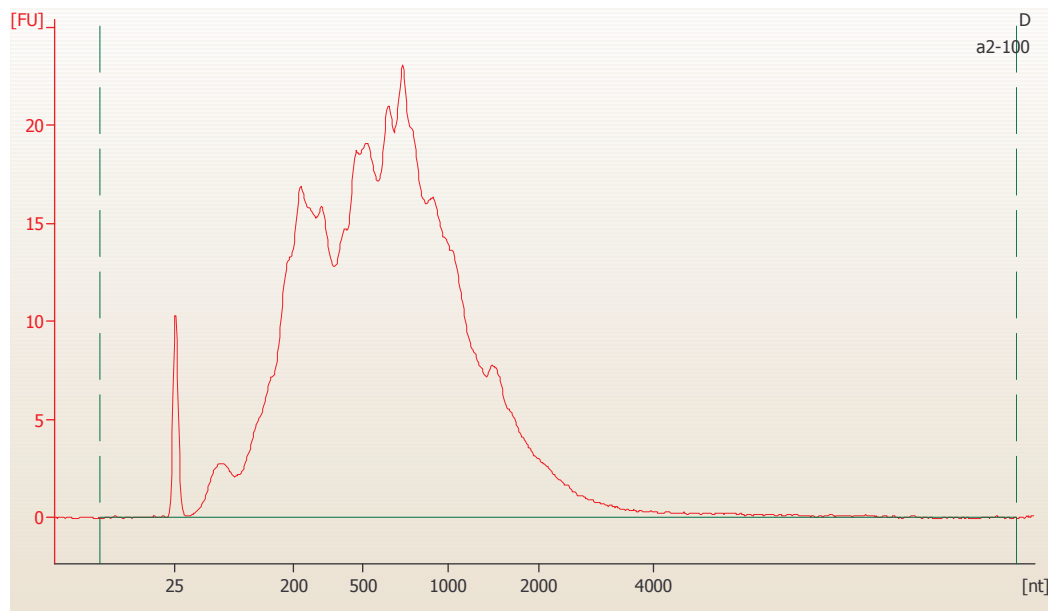


Both RNA profiles illustrate wide size distributions and the display of characteristic peaks. **Top panel:** As indication of low rRNA presence, please note the absence or low level of amplified RNAs in the size range of 16S and 23S rRNAs (>1.6 kb). No rRNA removal step is needed. **Bottom panel:** More amplified RNAs in the rRNA size range indicate the requirement for an rRNA removal step for optimal results.

Competitor A Random priming with enriched mRNA (rRNA removal step with MICROBExpress prior to amplification)



4.2. Electropherogram of 2-rounds amplified *E. coli* RNA ExpressArt TRInucleotide priming



After 2 amplification rounds, the RNA profile illustrates slightly narrower size distribution that still extends up to 2000 nt; the characteristic peaks are less pronounced.

Note: Protocols (kits) based on random priming are not suitable for two amplification rounds.

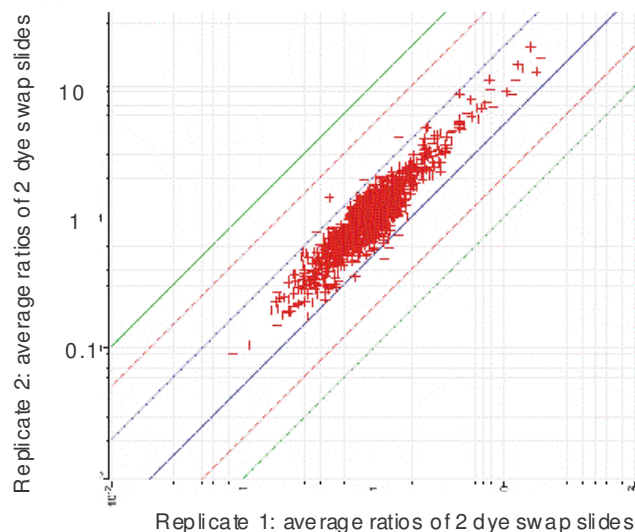
4.3 General quality of Affymetrix microarray data with ExpressArt amplified RNA *E. coli* total RNA

Data with high amounts of total RNA (0.3 µg) and one amplification round. Approximately 8 µg of biotinylated amplified RNA were hybridised with Affymetrix *E.coli* Genome 2.0 GeneChips. With *E.coli* grown at 37°C, the number of presence calls was 4804 (47.1%), signal background ratio was 98, average signals of 3378 with a Scaling Factor of 8.5. The sum of all rRNA intensities was approximately 1.2% of all detected signals. – Please note: these signals derive from hybridisation with sets of local probes, whereas with qPCR assays and different amplicon locations, up to approximately 10% rRNA sequences were estimated.

Data with low amounts of total RNA (5 ng) and two amplification rounds. Approximately 10 µg of biotinylated amplified RNA were hybridised with Affymetrix *E.coli* Genome 2.0 GeneChips. With *E.coli* grown at 37°C, the number of presence calls was 4536 (44.5%), signal background ratio was 82, average signals of 3898 with a Scaling Factor of 7.2. The sum of all rRNA intensities was approximately 1.2% of all detected signals. – Please note: these signals derive from hybridisation with sets of local probes; here we found good agreement with the estimated values derived from qPCR assays and different amplicon locations

4.4. Reproducibility of mRNA amplification reactions

Results are shown with fluorescence-labelled RNAs and Ocimum *E.coli* K12 Arrays. Four slides were hybridised with amplified cRNAs in these dye-swap experiments. The expression ratios of two slides with reverse labelling were averaged and compared to the replication of the experiment. High reproducibility was observed with a correlation coefficient (R) of 0.922.



4.5. Identification of differentially expressed genes

Examples for genes induced during heat-shock in *E.coli*. Comparison of published data with results obtained with conventional cDNA labelling and with ExpressArt TRInucleotide amplification.

Gene	Gene description	published: Richmond et al., 1999	labeled cDNA & Ocimum Array	Amplified RNA & Ocimum Array	Amplified RNA & Affymetrix GeneChip
dnaj	chaperone with dnaK, heat shock protein	high	medium	medium	medium
dnak	chaperone hsp70, dna biosynthesis, heat shock protein	medium	medium	medium	low
grpe	phage lambda replication, host dna synthesis, heat shock protein	medium	low	medium	medium
hslj	heat shock protein	medium	low	low	medium
hslu	heat shock protein hslvu, atpase subunit	low	low	low	low
hslv	heat shock protein hslvu, proteasome-related peptidase subunit	medium	low	medium	medium
htpx	integral membrane protein, heat shock protein	medium	low	low	medium
ibpa	heat shock protein	high	high	high	high
ibpb	heat shock protein	high	high	high	high
lon	dna-binding, atp-dependent protease la, heat shock k-protein	medium	low	medium	low

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[®] 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).

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.

Good quality total RNAs should display well-defined bands of the 23S and 16S 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.

RNA degradation can be minimised by complete and rapid sample lysis in strong denaturing agents like phenol, Trizol, RNASat or guanidine thiocyanate (GTC).

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

Fortunately, 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[®] Bacterial mRNA Amplification Kit. Subsequently, RNA quality control can be performed as described in the Core kit protocol.

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 and consider the inclusion of the ExpressArt N-Carrier (#8999-A100) and the universal RNase inhibitor NucleoGuard (#8998-M50).

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 μ 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**

Significant variations in the mRNA content can occur. Estimates range from <1% to 10% of total RNA, this can lead to different amplification yields, despite of using the same amounts of input total RNA. If you obtain only a faint, hardly visible, smear of amplified RNA, 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**

Amplified RNAs should have a centre-of-mass at appr. 0.9 kb (see also sections 4.1 and 4.2). Maybe the quality of your RNA was low. This will result in more lost mRNA sequence information. Try a different RNA isolation protocol, if not done before, include 1% of the ExpressArt reagents NucleoGuard (#8998-M50) in the cell 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 and correctly identified.

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