C&E Version
ExpressArt TR*inucleotide* mRNA amplification Micro kit

for degraded RNAs and for FFPE samples
suitable for standard gene expression microarrays
and for whole transcript arrays
"Exon Arrays" & "Gene Arrays"

Catalogue No. 6199-A30

(30 amplification reactions, 1 round for 30 samples)

The **C&E version (more convenience and higher efficiency)** of the ExpressArt® mRNA amplification Micro kit is suitable for a wide range, from approximately ≥300 ng up to 3 µg total RNA, with aRNA yields of >10 µg.
AmpTec's proprietary TR*inucleotide* priming technology results in preferential amplification of mRNAs and mRNA fragments (independent of the universal eukaryotic 3'-poly(A)-sequence), combined with selection against rRNAs.

This protocol provides the required laboratory procedures

Extended manuals
with additional information about ExpressArt technology are available at www.amp-tec.com

Content Kit box I (C&E TR*inucleotide* Micro kit)

Tube 1:	**10x Primer TR**	**24 µl**
Tube 2:	**dNTP-Mix**	**120.0 µl**
Tube 3:	**DEPC-H_2O**	**1500 µl**
Tube 4:	**5x RT Buffer**	**200.0 µl**
Tube 5:	**RNase Inhibitor**	**24.0 µl**
Tube 6:	**RT Enzyme**	**24.0 µl**
Tube 7:	**EMPTY: not required**	**---**
Tube 8:	**EMPTY: not required**	**---**
Tube 9:	**EMPTY: not required**	**---**
Tube 10:	**Polymerase buffer**	**720.0 µl**
Tube 11:	**EMPTY: not required**	**---**
Tube 12:	**EMPTY: not required**	**---**
Tube 13:	**EMPTY: not required**	**---**
Tube 14:	**Carrier DNA**	**90.0 µl**
Tube 15:	**EMPTY: not required**	**---**
Tube 16:	**EMPTY: not required**	**---**
Tube 17:	**Positive Control RNA**	**15.0 µl**
Tube 18:	**NTP-Mix**	**198.0 µl**
Tube 19:	**10x Transcription Buffer**	**51.0 µl**
Tube 20:	**RNA Polymerase**	**51.0 µl**
Tube 21:	**EMPTY: not required**	**---**
Tube 22:	**Polymerase A**	**96.0 µl**
Tube 23:	**Polymerase B**	**24.0 µl**
Tube 24:	**Polymerase C**	**24.0 µl**

Content Kit box II

DNA Purification Spin Columns	**30 pcs**
Collection Tubes	**30 pcs**
Binding Buffer	**7.5 ml**
Washing Buffer (salt concentrate)	**4 ml**
Elution Buffer	**10 ml**

Content Kit box III *new*

RNA Purification Spin Columns	**30 pcs**
Collection Tubes	**30 pcs**
RNA LB Buffer	**15 ml**
RNA DeS Buffer	**5 ml**
RNA Washing Buffer WB1 (salt concentrate)	**12 ml**
RNA Washing Buffer WB2 (salt concentrate)	**4 ml**
RNase-free water	**5 ml**

Storage

Immediately upon arrival:
Store **Kit box I at -20°C**. Avoid repeated freeze thawing.
Kit box II and box III are stored at room temperature.
Reagents are stable for a minimum of 6 months.

Additionally required materials

- Thermocycler. All reactions, apart from the overnight *in vitro* transcription (see below) can be performed in a standard thermocycler (with the lid temperature adjusted to 110°C).
- **Optional:** Hybridisation oven. We strongly recommend using an air incubator for performing overnight in vitro transcription reactions at 37°C. Alternatively, a thermocycler with adjustable heating lid can be used (lid temperature adjusted to 45°C).
- RNase-free pipette-tips (filter-tips recommended)
- RNase-free PCR and reaction tubes (0.2 / 0.5 / 1.5 ml)
- 100% Ethanol
- Microcentrifuge

Positive control

The ExpressArt® mRNA amplification Kit contains total RNA as positive control (100 ng/µl) (**Tube 17**).

Chemical hazards

The Binding Buffer (**box II**) contains guanidine thiocyanate, which is harmful in contact with skin, when inhaled or swallowed. Guanidine thiocyanate also liberates toxic gas, when mixed with strong acids. Always store and use the Binding Buffer away from food. Always wear gloves, and follow standard safety precautions during handling and make sure to comply with the safety rules of your laboratory.

Quality control

All components of the kit are tested in an amplification using the Positive Control RNA (**Tube 17**, provided with the kit). All reagents are tested for the absence of nuclease activity.

DETAILED PROTOCOL

A: First Amplification Round

Notes:

- RNA should be purified with a spin column kit (e.g. RNeasy from Qiagen, or equivalent) to remove small RNAs (tRNAs and 5S rRNAs) that interfere by acting as primers and possibly as templates.
- RNA must be free of any genomic DNA. A DNase treatment should be combined with a spin column purification to remove all fragments of digested DNA.
- Program a thermocycler with the temperatures and times, given in this protocol. See “Thermocycler profiles”.
- Range of input total RNA for the **Micro kit:** 300 ng to 3 µg.
- Also available: Nano kit (Cat.-No. 6299-A15) with two amplification rounds for lower amounts of 1 ng to 700 ng.
- Also available: Pico kit (Cat.-No. 6399-A15) with three amplification rounds for very low amounts below 1 ng.
- If running more than one reaction at a time, prepare **Master Mixes**.
- **Positive Control.** To check the amplification performance, a reaction tube containing Positive Control RNA (provided at 100 ng/µl, **Tube 17**) should be processed in parallel.

A1. First strand cDNA synthesis

Use the first thermocycler program for step A1.

Prepare First Strand cDNA Synthesis **Mix 1**. Use an appropriate Master mix volume for processing multiple samples.

First Strand cDNA Synthesis Mix 1		
H_2O	Tube 3	2.4 µl
dNTP-Mix	Tube 2	0.8 µl
10x Primer TR	Tube 1	0.8 µl

Add 4 µl **Mix 1** to 4 µl of each RNA (and to the optional negative control).

Incubate 4 minutes at 65℃ in a thermocycler **(with heating lid! use standard setting, e.g. 110℃)**

Cool samples to 37℃.

In the meantime, prepare the First Strand cDNA Synthesis **Mix 2** at room temperature.

First Strand cDNA Synthesis Mix 2		
DEPC-H_2O	Tube 3	3.2 µl
5x RT Buffer	Tube 4	3.2 µl
RNase Inhibitor	Tube 5	0.8 µl
RT Enzyme	Tube 6	0.8 µl

Add the First Strand cDNA Synthesis **Mix 2** (8 µl) to each sample and mix well by pipetting and stirring.
Incubate the samples in a thermocycler:

37℃ / 45 min
45℃ / 15 min
50℃ / 5 min
70℃ / 10 min
4℃ / HOLD

Remove samples from the thermocycler, centrifuge the tubes briefly and put the tubes on ice.
Proceed immediately to step A2.

A2. Template DNA synthesis

Use the second thermocycler program for step A2.

On ice prepare the second strand DNA synthesis mix (**Mix 3**) in the given order in a 1.5 ml reaction tube.

Second Strand DNA Synthesis Mix 3		
H_2O	Tube 3	73.0 µl
Polymerase-Buffer	Tube 10	24.0 µl
dNTP-Mix	Tube 2	2.4 µl
Polymerase A	Tube 22	3.2 µl
Polymerase B	Tube 23	0.8 µl
Polymerase C	Tube 24	0.8 µl

On ice, add 104 µl of **Mix 3** to the first strand reaction. Mix gently by pipetting and stirring**.**

Continue incubations: 16℃ / 2 h

NOTE: with heating lid switched off! If your thermocycler does not have this option, do not close the heating lid.

Remove samples from thermocycler, put on ice.

Spin to collect liquid and immediately continue with purification of the Template DNA (step A3).

A3. Purification of Template DNA with Spin Columns

Before starting, add 16 ml of 100% ethanol to the 4 ml Washing Buffer concentrate (Kit box II) and mix well.

Purification Mix 4		
Binding Buffer	(box II)	244 µl
Carrier DNA	Tube 14	2 µl

- Add 246 µl of **Mix 4** to each **Template DNA Reaction** (120 µl from step A2). Mix gently by pipetting.
- Insert DNA Purification Spin Columns in Collection Tubes.
- Pipette the **entire sample** onto each column and centrifuge for 1 min at 10,000 rpm in a table top centrifuge. (**Note:** guanidine thiocyanate in the **Binding Buffer** is an irritant. Always wear gloves and follow standard safety precautions to minimise contact when handling).
- Discard the flow-through and re-insert the columns in the same Collection Tubes. Add 200 µl **Washing Buffer** (with Ethanol added) to the columns and centrifuge for 1 min at 10,000 rpm.
- Discard the flow-through, re-insert the columns in the same Collection Tubes and wash again with 200 µl **Washing Buffer**. Centrifuge for 1 min at 10,000 rpm. Discard the flow-through and the Collection Tubes.
- Insert the columns in fresh 1.5 ml reaction tubes and add 10 µl of **Elution Buffer** to the columns (make sure to pipette the Elution Buffer exactly in the middle of the column, directly on top of the matrix, without disturbing the matrix with the pipette tip). Incubate the column for at least 2 min, then centrifuge for 1 min at 10,000 rpm.
- The purified template DNA (approximately 8 µl) is now ready for in vitro transcription (step A4).
- **Optional:** Use 10% of the template DNA for an in vitro transcription "test reaction" for determining expected yields and size distribution (see steps A5-A7).
- Alternatively, store the samples at –20°C for later use.

A4. Four Options for in vitro Transcriptions & Labelled RNAs

At this stage, there are **4 options** for proceeding with in vitro transcription reactions.

Option 1) If you have used the **AT-Micro Add-On Module (Cat.-No. 2010-A30)** for the generation of **Archival Templates,** use the template DNA obtained in step A3 (see **step AT-1** in the AT Module for necessary changes in the Core kit protocol) to generate immobilised template DNA (**step AT-2**) for solid phase in vitro transcription (**step AT-3**). This allows you to determine yields and qualities of amplified RNAs and the recovered template can be used for a second in vitro transcription that includes the appropriate NTP-mixes for labelled RNA (see options in step AT-3).

Additional suggestion: If you did not use the **AT-Module**, you may use 10% of the template DNA for an in vitro transcription "test reaction" for determining expected yields and size distribution (see steps A5-A7).

Additional use of unlabelled RNA: it can be converted to cDNAs for performing multiple qPCR assays (see protocol at www.amp-tec.com).

Note: Reagents for in vitro transcriptions with unmodified NTPs are included in the kit.

Option 2) **For Affymetrix Whole Transcript microarrays (Exon Arrays & Gene Arrays)** use the template DNA (from **step A3**) for standard in vitro transcription (steps A5, A6) to prepare and purify unmodified, amplified RNA: required yields for WT arrays are at least 7 µg.
Then, use Affymetrix kits to generate labelled WT Double-Stranded DNA: Follow the Affymetrix GeneChip® Whole Transcript (WT) Double-Stranded Target Assay Manual, Chapter 3 "Target Preparation for Model Organisms, Single Tiling Arrays (No Amplification)".
Use the GeneChip® WT Double-Stranded cDNA Synthesis Kit (Affymetrix # 900813) and the GeneChip® WT Double-

Stranded DNA Terminal Labeling Kit (Affymetrix # 900812) and Sample Cleanup Module (Affymetrix # 900371).

Option 3) For Affymetrix microarrays, use the template DNA (from **step A3**) as template for in vitro transcription with the ENZO Bioarray High Yield RNA Transcript Labelling Kit (or equivalent), according to the instructions of the manufacturer.

Option 4) Dye-labelled amplified RNAs: Usethe template DNA (**step A3**) for in vitro transcription with the **Amino-Allyl Labelling Module (Cat.-No. 2000-A30** for 30 samples**).** It contains all required supplementary reagents for obtaining amino-allyl-labelled amplified RNAs and their conversion to purified, dye-coupled and fragmented RNAs, ready for microarray hybridisations (this Module does **not include** the required NHS-activated Dye-derivatives).

A5. Amplification via *in vitro* Transcription

RNA Labelling for microarray hybridisations: **see options in section A4**.

Unlabelled RNA can be converted to cDNAs for performing multiple qPCR assays (see protocol at www.amp-tec.com).

In vitro-Transcription Mix 5		
NTP-Mix	Tube 18	6.6 µl
10x Buffer	Tube 19	1.7 µl
RNA-Polymerase	Tube 20	1.7 µl

- Prepare the *in vitro*-Transcription **Mix 5** by adding the components in the given order. **Work at room temperature**, never on ice, because spermidine in the buffer can cause precipitation of the template DNA.
- Add 10 µl in vitro-Transcription **Mix 5** to template DNA (from A3).
- Incubate the transcription overnight at 37°C in a thermocycler with heating lid adjusted to 45°C; or preferentially in a hybridisation oven. **Do not use a thermocycler WITHOUT adjustable heating lid, because high lid temperature (usually >100°C) will negatively affect the efficiency of the transcription reaction!**
- Continue with purification of the amplified RNA (step A6)

A6. aRNA-Purification using RNA spin columns

Note: Add **6 ml of 100% ethanol to WB1 (12 ml)**, and **16 ml of 100% ethanol to WB2 (4 ml)**, as indicated on the bottles. **At the start:** Put **RNase-free water** in thermoblock at 95℃.

aRNA Purification Mix 6	
RNA LB Buffer (box III)	340 µl
RNA DeS Buffer (box III)	87 µl
100% Ethanol (at room temperature; not supplied)	560 µl

- Add 987 µl of **Mix 6** to each **in vitro Transcription Reaction**. Mix thoroughly.
- Transfer an aliquot of **700 µl of this mixture** on the RNA spin column, centrifuge for 30 sec@10'000 rpm & discard flow-through
- Transfer the **remaining mixture** on column, centrifuge for 30 sec@10'000 rpm & discard flow-through
- Add 500 µl **Wash Buffer 1 (WB 1)**, centrifuge for 30 sec@10'000 rpm & discard flow-through
- Add 500 µl **Wash Buffer 2 (WB 2)**, centrifuge for 30 sec@10'000 rpm & discard flow-through
- Re-insert the column in the same collection tube. Add 500 µl **80% EtOH,** centrifuge for 30 sec@10'000 rpm & discard flow-through
- Re-insert the column in the same collection tube, and centrifuge for 1 min@maximum speed to get rid of residual salt on the spin column matrix.
- **Elution**: Transfer column in fresh 1.5 ml reaction tube and add 30 µl **RNase-free water** (preheated at 95℃)
- Incubate: 2 min and centrifuge for 1 min@maximum speed
- Reapply the eluate on the column
- Incubate: 2 min and centrifuge for 1 min@maximum speed

  RNA is in a total volume of ~ 30 µl. Use amplified RNA immediately or store at -80℃.

A7. Control of aRNA product quantity and quality

Photometric quantification:
Note: The in vitro transcription reactions are performed with very high NTP concentrations (30 mM total) and these NTP's are not removed 100% by RNA clean-up with spin columns.
In consequence: measurements with negative control reactions may indicate – erroneous - values of up to approximately 1.5 µg. The presence of RNA strongly competes and prevents this "background binding" of NTP's, and this means, calculated yields of ≥2 µg are a reliable indication of RNA amounts. A correlation with electrophoretic results (bioanalyzer or agarose gel) is recommended.

Quality Control with Agilent 2100 bioanalyzer:
Note: All ionic compounds interfere with capillary electrophoresis. The RNA size distribution can be monitored with the bioanalyzer, but quantitation may indicate too low RNA amounts, because the signal may be significantly compressed by residual and variable buffer/salt concentrations in the eluted RNAs. For maximum sensitivity and more consistent quantifications, removal of buffer/salt by ethanol precipitation is recommended.
For amplified RNA, a broad size distribution is expected and an RNA concentration >50 ng/µl is recommended for the Agilent RNA 6000 Nano kit (lower concentrations are possible for total RNA, due to the prominent rRNA peaks).

Expected yields* of amplified RNA

Input total RNA	RNA-a1 "1st round"
200 ng	10 ± 2 µg
300 ng	15 ± 2 µg
1 µg	50 ± 10 µg

*** Note:** Please see step A7 for combined photometric and electrophoretic measurements.

Thermocycler profiles

Before starting the kit protocol, program a thermocycler with the following steps. HOLD steps are included to provide time for thermal ramping or for adding reagents.

First thermocycler program for step A1			
Step	**Temperature**	**Time**	**Action**
1	**65℃**	HOLD	Start of first cDNA synthesis Add **4µl RNA** to **4µl Mix 1**
2	**65℃**	**4 min**	
3	**37℃**	**1 min**	
4	**37℃**	HOLD	add **8µl Mix 2**
5	**37℃**	**45 min**	
6	**45℃**	**15 min**	
7	**50℃**	**5 min**	
8	**70℃**	**10 min**	
9	**4℃**	HOLD	Put samples on ice and continue with second thermocycler program for step A2.

Second thermocycler program for step A2			
NOTE: heating lid is switched off			
Step	**Temperature**	**Time**	**Action**
1	**16℃**	HOLD	Add **104µl Mix 3**
2	**16℃**	**2 h**	
3	**4℃**	HOLD	
End of Template DNA synthesis **Spin** to collect liquid **Continue** with DNA purification			

Required time: appr. 3.5 h

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