C&E Version
ExpressArt TR*inucleotide* mRNA amplification Pico 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. 6399-A15

(45 amplification reactions, 3 rounds for 15 samples)

The **C&E version (more convenience and higher efficiency)** of the ExpressArt® TR*inucleotide* mRNA amplification Pico kit is suitable for extremely low amounts of less than 1 ng of input total RNA. According to the amount of input total RNA and the required yields of aRNA, it can be used for 2-rounds (input ≥1 ng total RNA) or 3-rounds (input <1 ng total RNA), with aRNA yields in the range of >10 µg.
AmpTec's proprietary TR*inucleotide* priming technology results in preferential amplification of mRNAs (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* Pico kit)

Tube 1:	**Primer TR**	**24 µl**
Tube 2:	**dNTP-Mix**	**160.0 µl**
Tube 3:	**DEPC-H_2O**	**1500 µl**
Tube 4:	**5x RT Buffer**	**350.0 µl**
Tube 5:	**RNase Inhibitor**	**36.0 µl**
Tube 6:	**RT Enzyme**	**36.0 µl**
Tube 7:	**RNase**	**30.0 µl**
Tube 8:	**Primer B**	**38.0 µl**
Tube 9:	**5x Extender Buffer**	**380.0 µl**
Tube 10:	**Polymerase buffer**	**360.0 µl**
Tube 11:	**Primer Erase**	**15.0 µl**
Tube 12:	**Primer C**	**370.0 µl**
Tube 13:	**Extender Enzyme B**	**30.0 µl**
Tube 14:	**Carrier DNA**	**90.0 µl**
Tube 15:	**Primer D**	**75 µl**
Tube 16:	**Reaction Additive**	**75 µl**
Tube 17:	**Positive Control RNA**	**15.0 µl**
Tube 18:	**NTP-Mix**	**297.0 µl**
Tube 19:	**10x Transcription Buffer**	**77.0 µl**
Tube 20:	**RNA Polymerase**	**77.0 µl**
Tube 21:	**DNase I**	**30.0 µl**
Tube 22:	**Polymerase A**	**48.0 µl**
Tube 23:	**Polymerase B**	**12.0 µl**
Tube 24:	**Polymerase C**	**12.0 µl**

Content Kit box II

cDNA Purification Spin Columns	**45 pcs**
Collection Tubes	**45 pcs**
Binding Buffer	**11 ml**
Washing Buffer (salt concentrate)	**4 ml**
Elution Buffer	**10 ml**

Storage

Immediately upon arrival: store all reagents of **Kit box I at -20 °C**. Avoid repeated freeze thawing.
Content of **Kit box II is stored at room temperature**.
Reagents are stable for 6 months (see **Expiry date** on the kit box).

Additionally required materials

- RNeasy MiniKit (Qiagen) or equivalent !
- 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® TR*inucleotide* 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. The TR*inucleotide* 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.
- Program a thermocycler with the temperatures and times, given in this protocol. See “Thermocycler profiles”.
- Range of input total RNA for the **TR*inucleotide* Pico kit** with 3 amplification rounds: appr. 0.1 to 1 ng.
- Also available: **TR*inucleotide* Micro kit** (Cat.-No. 6199-A30) with 1 amplification round for higher amounts in the range of 300 ng to 3 µg.
- Also available: **TR*inucleotide* Nano kit** (Cat.-No. 6299-A15) with 2 amplification rounds for amounts in the range of 1 ng to 700 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
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°C in a thermocycler **(with heating lid! use standard setting, e.g. 110°C)**

Cool samples to 37°C.

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.
Incubate the samples in a thermocycler:

37°C / 45 min
45°C / 15 min
50°C / 5 min
70°C / 10 min
4°C / 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**.**

Continue incubations: 16°C / 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).
- Alternatively, store the samples at –20°C for later use.

A4. Amplification via *in vitro* Transcription

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!**
- Add 1 µl DNase **(Tube 21)** to each reaction, mix thoroughly and incubate further at 37°C for 15 min.
- Continue with purification of amplified RNA (step A5).

A5. aRNA-Purification using RNeasy Mini Kit

(Qiagen, not provided with the ExpressArt® Kit)

Note: Add 4 volumes of 100% ethanol to RPE buffer, as indicated on the bottle.

aRNA Purification Mix 6	
RNase-free water	82 µl
RLT (Lysis Buffer)	350 µl

- Insert the purification columns in the collection tubes.
- Add 432 µl of **Mix 6** to each **in vitro Transcription Reaction**. Mix thoroughly and add 250 µl 100% EtOH (room temperature). Pipette this mixture onto the column. Centrifuge for 15 sec at 10,000 rpm in a table top centrifuge.
- Discard the flow-through and re-insert the column in the same collection tube. Add 500 µl **RPE Buffer (with EtOH added!)** to the column and centrifuge for 2 min.
- Discard the flow-through, and re-insert the column in the same collection tube. Add 500 µl **80% EtOH** to the column and centrifuge for 2 min.
- Discard the flow-through, re-insert the column in the same collection tubes and centrifuge for 1 min to get rid of residual RPE Buffer.
- Insert the columns in new 1.5 ml reaction tubes and add **30 µl of RNase-free water** to the columns.
- Incubate the column for 2 min and centrifuge for 1 min at 10,000 rpm.
- Use amplified RNA for a second amplification round (section B) or store at -80°C.

A6. Control of aRNA product quantity and quality

For input amounts of <1 ng total RNA, quality control measurements are not recommended after the first amplification round.

B: Second Amplification Round

Amplified RNA is again reverse transcribed into cDNA to produce higher yields of aRNA via a 2nd round of amplification.

B1. First strand cDNA synthesis

Use all of the amplified RNA obtained after the first amplification round.

Use Third thermocycler program for step B1-B2.

First Strand cDNA Synthesis Mix 2-1		
dNTP-Mix	Tube 2	2.5 µl
Primer B	Tube 8	2.5 µl

Add 5 µl **Mix 2-1** to 25 µl of each RNA (for smaller volumes, adjust to with water to a reaction volume of 30 µl).
Incubate 4 minutes at 65°C in a thermocycler **(with heating lid! use standard setting, e.g. 110°C)**

Cool samples to 37°C.

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

First Strand cDNA Synthesis Mix 2-2		
DEPC-H_2O	Tube 3	8.4 µl
5x RT Buffer	Tube 4	10.0 µl
RNase Inhibitor	Tube 5	0.8 µl
RT Enzyme	Tube 6	0.8 µl

Add the First Strand cDNA Synthesis **Mix 2-2** (20 µl) to each sample and mix well by gently flicking the tube.
Incubate the samples in a thermocycler:

37°C / 45 min
45°C / 15 min
50°C / 5 min
37°C / HOLD

Primer Erase Mix 2-3		
DEPC-H_2O	Tube 3	3 µl
5x Extender Buffer	Tube 9	1 µl
Primer Erase	Tube 11	1 µl

Then add 5 µl Primer Erase **Mix 2-3**, and continue incubations:
37°C / 5 min
80°C / 15 min
37°C / HOLD

RNase Mix 2-4		
DEPC-H_2O	Tube 3	3 µl
5x Extender Buffer	Tube 9	1 µl
RNase	Tube 7	1 µl

Add 5 µl of RNase **Mix 2-4** to First Strand cDNA Reaction.

Incubate 20 minutes at 37°C.

B2. Template DNA synthesis

Second Strand cDNA Synthesis Mix 2-5		
DEPC-H_2O	Tube 3	25,0 µl
5x Extender Buffer	Tube 9	10,0 µl
Primer C	Tube 12	12.5 µl
dNTP-Mix	Tube 2	2.5 µl

Add 50 µl of **Mix 2-5** to each First Strand cDNA Synthesis Reaction and incubate as follows in a thermocycler:

96°C / 1 min
37°C / 1 min

Extender Enzyme B Mix 2-6		
DEPC-H_2O	Tube 3	3 µl
5x Extender Buffer	Tube 9	1 µl
Extender Enzyme B	Tube 13	1 µl

Add 5 µl of Extender Enzyme B **Mix 2-6** to each sample and mix well by gently flicking the tube. Continue the incubation:

37°C / 30 min
65°C / 15 min
4°C / HOLD

After incubation, place tubes on ice. **Proceed to step B3.**

B3. Purification of Template DNA with Spin Columns

Purification Mix 2-7		
Binding Buffer	(box II)	234 µl
Carrier DNA	Tube 14	2 µl

- Add 236 µl of **Mix 2-7** to each **Template DNA Reaction** (115 µl from step B2). 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 (see step B4).
- **Optional:** Use 10% of the template DNA for an in vitro transcription "test reaction" for determining expected yields and size distribution (see step B6).
- Alternatively, store the samples at –20°C for later use.

B4. Four Options for Microarray Applications

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

Option 1) If you have used the **AT-Micro Add-On Module (Cat.-No. 2010-A15)** for the generation of **Archival Templates,** use the template DNA obtained in **step B3** (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. If the RNA yields are not sufficient, a third amplification round (see section C) can be performed with the unmodified RNA. If sufficient, 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. Purification of amplified RNAs can be performed with RNeasy Mini Kit (Qiagen), or equivalent.

Option 2) **For Affymetrix microarrays,** use the template DNA (from **step B3**) 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 3) **For Affymetrix Whole Transcript microarrays (Exon Arrays & Gene Arrays)** use the template DNA (from **step B3**) for standard in vitro transcription (steps B5, B6) 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 4) Dye-labelled amplified RNAs: Use the template DNA (**step B3**) with the **Amino-Allyl Module (Cat.-No. 2000-A15** for 15 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).

B5. Amplification via *in vitro* Transcription

In vitro-Transcription Mix 5		
NTP-Mix	Tube 18	6.6 µl
10x Buffer	Tube 19	1.7 µl
T7 RNA Polymerase	Tube 20	1.7 µl

- Prepare the in vitro-Transcription Mix by adding the components in the given order. **Work at room temperature**, never on ice, because spermidine in the buffer can cause precipitation of DNA template.
- Add 10 µl *in vitro*-Transcription **Mix 5** to template DNA (from B3).
- 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 amplified RNA (step B6).

B6. RNA-Purification using RNeasy Mini Kit

(Qiagen, not provided with the ExpressArt® Kit)

Note: Add 4 volumes of 100% ethanol to RPE buffer, as indicated on the bottle.

aRNA Purification Mix 6	
RNase-free water	82 µl
RLT (Lysis Buffer)	350 µl

- Add 432 µl of **Mix 6** to each **in vitro-Transcription Reaction**. Mix thoroughly and add 250 µl 100% EtOH (room temperature). Pipette this mixture onto the column. Centrifuge for 15 sec at 10,000 rpm in a table top centrifuge.

- Discard the flow-through and re-insert the column in the same collection tube. Add 500 µl **RPE Buffer (with EtOH added!)** to the column and centrifuge for 2 min.

- Discard the flow-through, and re-insert the column in the same collection tube. Add 500 µl **80% EtOH** to the column and centrifuge for 2 min.

- Discard the flow-through, re-insert the column in the same collection tubes and centrifuge for 1 min at maximum speed to get rid of residual **RPE Buffer**.

- Insert the columns in new 1.5 ml reaction tubes and add 30 µl of RNase-free water to the columns.

- Incubate the column for 2 min and centrifuge for 1 min at 10,000 rpm.

- Use amplified RNA for quality control (see step B6) or store at -80°C.

B7. 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.

If an **additional third amplification round** (see section C) is required, a maximum 0.8 µg of amplified RNA can be used – RNA yields should be confirmed by electrophoresis.

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).

C: Third Amplification Round

Amplified RNA is again reverse transcribed into cDNA to produce higher yields of aRNA via a 3rd amplification round.

For generation of **labelled antisense RNA**, use the template DNA (see below, steps C3, C4) for *in vitro* transcription with an RNA labelling kit (see options in step C5).

C1. First strand cDNA synthesis

RNA yields should be determined by photometric **and** electrophoretic measurements (see step B6).

We recommend to use approximately 200 ng amplified RNA from the second amplification round (a maximum volume of 15 µl of amplified RNA from step B5). Please do not use more than 800 ng of amplified RNA.

First Strand Mix 3-1		
dNTP-Mix	Tube 2	2.5 µl
Primer D	Tube 15	5.0 µl
Reaction Additive	Tube 16	5.0 µl

Add 12.5 µl of **Mix 3-1** to 17.5 µl RNA (for smaller volumes, adjust with water to a reaction volume of 30 µl).
Incubate 4 min at 65°C in a thermocycler (with heating lid! use standard temperature setting, e.g. 110°C), then immediately cool the samples to 45°C.

In the meantime, prepare the First Strand cDNA Synthesis **Mix 3-2**, at room temperature.

cDNA Synthesis Mix 3-2		
DEPC-H_2O	Tube 3	8.4 µl
5x RT Buffer	Tube 4	10.0 µl
RNase Inhibitor	Tube 5	0.8 µl
RT Enzyme	Tube 6	0.8 µl

Add 20 µl of **Mix 3-2** to each sample. **Remove one sample at a time** with continued incubation of the other samples in the 45ºC hot thermocycler.

Continue incubation in a thermocycler:

45°C / 30 min
70°C / 15 min

C2. RNA removal

RNase Mix 3-3		
DEPC-H_2O	Tube 3	3 µl
5x Extender Buffer	Tube 9	1 µl
RNase	Tube 7	1 µl

Add 4 µl of RNase **Mix 3-3** to 50 µl of First Strand cDNA Reaction (from C1).
Incubate 20 minutes at 37°C.

C3. Second strand cDNA synthesis

Second Strand cDNA Synthesis Mix 3-4		
DEPC-H_2O	Tube 3	25.5 µl
Primer C	Tube 12	12.0 µl
5x Extender Buffer	Tube 9	10.0 µl
dNTP-Mix	Tube 2	2.5 µl

Add 50 µl of **Mix 3-4** to each sample (from C2), then incubate:

96°C / 1 min
37°C / 1 min

Extender Enzyme B Mix 3-5		
DEPC-H_2O	Tube 3	3 µl
5x Extender Buffer	Tube 9	1 µl
Extender Enzyme B	Tube 13	1 µl

Add 5 µl of Extender Enzyme B **Mix 3-5** to each sample and mix well by gently flicking the tube. Continue the incubation:

37°C / 30 min
65°C / 15 min

Place the samples on ice. Spin briefly to collect liquid. Continue immediately with purification of template DNA (step C4).

C4. Purification of Template DNA with Spin Columns

DNA Purification Mix 3-6		
Binding Buffer	(box II)	224 µl
Carrier DNA	Tube 14	2 µl

- Add 226 µl of **Mix 3-6** to each **Template DNA Reaction** (110 µl from step C3). 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 (see step B4).
- **Optional:** Use 10% of the template DNA for an in vitro transcription "test reaction" for determining expected yields and size distribution (see step A6).
- Or store the samples at -20°C for later use.

C5. Four Options for in vitro Transcriptions & Labelled RNAs

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

See **step B4 for the fully outlined options**.

C6. Amplification via *in vitro* Transcription

In vitro-Transcription Mix 5		
NTP-Mix	Tube 18	6.6 µl
10x Buffer	Tube 19	1.7 µl
T7 RNA Polymerase	Tube 20	1.7 µl

- Prepare the in vitro-Transcription Mix by adding the components in the given order. **Work at room temperature**, never on ice, because spermidine in the buffer can cause precipitation of DNA template.
- Add 10 µl *in vitro*-Transcription **Mix 5** to template DNA (from C4).
- 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 amplified RNA (step C7).

C7. RNA-Purification using RNeasy Mini Kit

(Qiagen, not provided with the ExpressArt® Kit)

Note: Add 4 volumes of 100% ethanol to RPE buffer, as indicated on the bottle.

aRNA Purification Mix 6	
RNase-free water	82 µl
RLT (Lysis Buffer)	350 µl

- Add 432 µl of **Mix 6** to each **in vitro-Transcription Reaction**. Mix thoroughly and add 250 µl 100% EtOH (room temperature). Pipette this mixture onto the column. Centrifuge for 15 sec at 10,000 rpm in a table top centrifuge.

- Discard the flow-through and re-insert the column in the same collection tube. Add 500 µl **RPE Buffer (with EtOH added!)** to the column and centrifuge for 2 min.

- Discard the flow-through, and re-insert the column in the same collection tube. Add 500 µl **80% EtOH** to the column and centrifuge for 2 min.

- Discard the flow-through, re-insert the column in the same collection tubes and centrifuge for 1 min at maximum speed to get rid of residual **RPE Buffer**.

- Insert the columns in new 1.5 ml reaction tubes and add 30 µl of RNase-free water to the columns.

- Incubate the column for 2 min and centrifuge for 1 min at 10,000 rpm.

Use amplified RNA for quality control (see step B6) or store at -80°C.

Expected yields of amplified RNA

Input total RNA	**RNA-a1 1st round**	**RNA-a2 2nd round**	**RNA-a3 3rd round**
200 ng	10 ± 2 µg	with 200 ng* RNA-a1: **50 ± 20 µg**	n/a
100 ng	5 ± 1 µg	with 200 ng* RNA-a1: **50 ± 20 µg**	n/a
50 ng	3 ± 0.5 µg	with 200 ng* RNA-a1: **50 ± 20 µg**	n/a
10 ng	1 ± 0.5 µg*	with 200 ng* RNA-a1: **50 ± 20 µg**	n/a
≤ 1 ng	0.2 ± 0.1 µg*	using all of RNA-a1: **20 ± 10 µg**	with 200 ng* RNA-a2: **50 ± 20 µg**
~ 100 pg	n/a	using all of RNA-a1: **2 - 10 µg**	with 200 ng* RNA-a2: **50 ± 20 µg**

* **Note:** Please see step B6 for combined photometric and electrophoretic measuremens.

Thermocycler profiles

Before starting the ExpressArt® TR*inucleotide* mRNA amplification kit protocol, program a thermocycler with the following temperatures and times. HOLD steps are included to provide time for thermal ramping or for adding reagents.

First Amplification round

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

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

Required time: appr. 3.5 h

Second Amplification round

Third thermocycler program for steps B1-B2 *For 2nd Amplification Round*			
Step	**Temperature**	**Time**	**Action**
1	**65℃**	HOLD	Start of first cDNA synthesis Add **25µl RNA-a1** to **5µl Mix 2-1**
2	**65℃**	**4 min**	
3	**37℃**	**1 min**	
4	**37℃**	HOLD	add **20µl Mix 2-2**
5	**37℃**	**45 min**	
6	**45℃**	**15 min**	
7	**50℃**	**5 min**	
8	**37℃**	**1 min**	
9	**37℃**	HOLD	add **5µl Mix 2-3**
10	**37℃**	**5 min**	
11	**80℃**	**15 min**	
12	**37℃**	**1 min**	
13	**37℃**	HOLD	add **5µl Mix 2-4**
14	**37℃**	**20 min**	
15	**37℃**	HOLD	add **50µl Mix 2-5**
16	**96℃**	**1 min**	
17	**37℃**	**1 min**	
18	**37℃**	HOLD	add **5µl Mix 2-6**
19	**37℃**	**30 min**	
20	**65℃**	**15 min**	
21	**4℃**	HOLD	
End of Template DNA synthesis **Spin** to collect liquid **Continue** with DNA purification			

Required time: appr. 2.8 h

Third Amplification round

Fourth thermocycler program for steps C1-C3 *For 3rd Amplification Round*			
Step	**Temperature**	**Time**	**Action**
1	**65°C**	HOLD	Start of first cDNA synthesis Add **17.5µl RNA-a1** to **12.5µl Mix 3-1**
2	**65°C**	**4 min**	
3	**45°C**	**1 min**	
4	**45°C**	HOLD	add **20µl Mix 3-2**
5	**45°C**	**30 min**	
6	**70°C**	**15 min**	
7	**37°C**	**1 min**	
8	**37°C**	HOLD	add **5µl Mix 3-3**
9	**37°C**	**20 min**	
10	**37°C**	HOLD	add **50µl Mix 3-4**
11	**96°C**	**1 min**	
12	**37°C**	**1 min**	
13	**37°C**	HOLD	add **5µl Mix 3-5**
14	**37°C**	**30 min**	
15	**65°C**	**15 min**	
16	**4°C**	HOLD	
End of Template DNA synthesis **Spin** to collect liquid **Continue** with DNA purification			

Required time: appr. 2.2 h

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