Enhanced RiboAmp™ RNA Amplification Kit (round one)

Box 1: enzymes and mixes (-20°C)
Box 2: columns and buffers (RT)
Co RNA = T-RNA

1st Strand Synthesis

Prepare RNA in 10µl H_2O in a 200µl tube (e.g. 50ng mRNA or not less than 200ng total RNA)

Add 1µl primer **A** (-20°C) ***(primer A must be mixed and spun down)***

Heat at 65°C for 5 min

Chill the sample at 4°C for at least 1 min then spin the samples until ready to proceed

(mix and spind down 1st strand reagents)

1st strand mix	per sample
1st strand master mix (-20°C)	7µl
1st strand enzyme mix (-20°C)	2µl
total	9µl

Add 9µl 1st strand mix per sample mix well and spin down

Incubate at 42°C for 45 min (20ul)

Chill the sample to 4°C for at least 1 min

Remove a 2µl aliquot at this point for further quantitative RT-PCR analysis (put in a 0.5ml tube and add 8ul nuclease free water).

Add 2µl of 1st strand nuclease mix (=RNase) (-20°C) ***(mix must be mixed and spun down)***

Incubate at 37°C for 20 min (20ul)

Incubate at 95° C for 5 minutes

Put the sample on ice

OK *Sample may be stored at –20°C overnight*

2nd Strand Synthesis

Add 1µl of **primer B** (-20°C) ***(primer B must be mixed and spun down)***

random hexamers

Incubate at 95°C for 2 min then put the sample on ice for at least 2 minutes

(mix and spin down reagents)

2nd strand mix	per sample
2nd strand reaction master mix (-20°C)	29µl
2nd strand reaction enzyme mix (-20°C)	1µl
total	30µl

Add 30µl 2nd strand mix to the sample mix and spin down

Incubate in the thermo-cycler as follows (program: "sec. strand")

- 25°C 5 min
- 37°C 10 min
- 70°C 5 min
- 4°C hold (up to 30 min)

cDNA purification

Add 250µl of DNA Binding Buffer (**DB**) to a purification column (without sample)

(DNA binding buffer must be mixed)

Hold for 5 min at RT

Spin at 16000 g for 1 min

Discard flow-through

Add 200µl of **DB** to the 2nd strand sample, mix well and pipette the sample into the purification column (gelbe Spitze)

Centrifuge at **100 g** for 2 min, followed by 10.000 g for 30 sec

Discard flow-through

Add 250µl of DNA Wash Buffer (**DW)**

Spin at 16000 g for 2 min

Discard flow-through

Place column into a fresh 1.5ml collection-tube provided in the kit

Add 24µl (*neuer Kit 16ul !!)* of DNA Elution Buffer (**DE**)

Incubate for 3 min at RT

Spin at 1000 g for 1 min

Spin at 16000 g for 1 min

OK *You may store the sample at –20°C overnight*

Transfer the sample to a new PCR tube (0.2ml)

***in vitro* Transcription (IVT)**

IVT reagents at RT, components should be added in order of the list

(mix and spin down reagents)

IVT reaction mix	per sample
IVT buffer	8µl
IVT master mix	12µl
IVT enzyme mix	4µl
Total	24µl

Add 24µl of IVT reaction mix to each sample

Incubate at 42°C for 2.5 hours (program: "IVT")

Chill the samples

OK *You may hold the sample(s) at 4°C in the thermal cycler overnight*

Add 2µl DNase mix ***(mix and spin down)***

Incubate at 37°C for 15 min

Chill the samples

aRNA Purification

Add 250µl of RNA Binding Buffer (**RB**) to a new DNA/RNA purification column (***RB must be mixed)***

Hold for 5 min at RT

Spin at 16000 g for 1 min

Discard flow-through

Add 200µl of RB to the IVT reaction sample, mix well and pipette the entire sample into the purification column

Centrifuge at 100 g for 2 min, followed by 10.000 g for 30 sec

Discard flow-through

Add 200µl of RNA Wash Buffer (**RW**)

Centrifuge at 10.000 g for 1 min

Discard flow-through

Add 200 µl of **RW**

Spin at 16000 g for 2 min

Discard flow-through

Place the purification column into a new tube (provided in the kit)

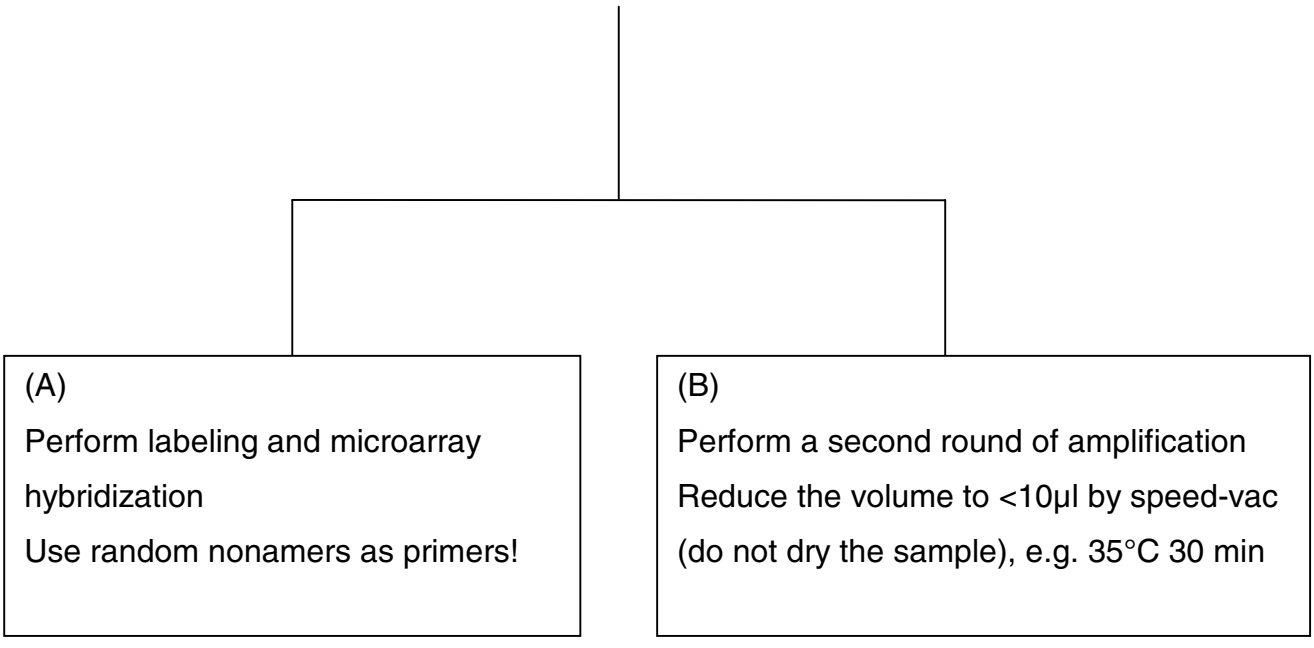
Add 30µl RNA Elution Buffer (**RE**) elution volume=30ul; if a second round is performed use 11ul

Incubate at RT for 3 min

Spin the pufication column at 1000g for 1min,followed by 16000 g for 1 min

Measure the $OD_{260/280}$ (dilute 1µl with 49µl TE-Buffer ph=7.6)

Store aRNA at -70ºC



Enhanced RiboAmp™ RNA Amplification Kit (round two)

Dilute Co aRNA 1:1000

1st Strand Synthesis

Prepare RNA in 10µl H_2O in a new PCR tube

Add **1µl primer B** (-20°C) ***(Primer B must be mixed and spun down)***

Heat at 65°C for 5 min

Chill the sample at 4°C for at least 1 min

mix components

1st strand mix	per sample
1st strand master mix (-20°C)	7µl
1st strand enzyme mix (-20°C)	2µl
total	9µl

Add 9µl 1st strand mix per sample mix well and spin down

Incubate at **25°C for 10 min, then at 37°C for 45 min** (20ul, cycler 1st strand round 2)

Chill the sample to 4°C for at least 1 min (less than 30 min)

OK *Sample may be stored at –20°C overnight*

2nd Strand Synthesis

Add **1µl of primer A** (-20°C) (***Primer a must be mixed and spun down)***

Incubate at **95°C for 2** min then put the sample immediately on ice for 2 minutes (21ul)

mix components

2nd strand mix	per sample
2nd strand reaction master mix (-20°C)	29µl
2nd strand reaction enzyme mix (-20°C)	1µl
total	30µl

Add **30**µl 2nd strand mix to the sample mix well

Incubate in the thermo-cycler as follows (50ul, 2nd strand round 2)

- **37°C 15 min**
- **70°C 5 min**
- **4°C hold (up to 30 min)**

cDNA purification

Add 250µl of DNA Binding Buffer (**DB**) to a purification column (without sample)

Hold for 5 min at RT

Spin at 16000 g for 1 min

Discard flow-through

Add 200 µl of **DB** to the 2nd strand sample, mix well and pipette the sample into the purification column

Centrifuge at 100 g for 2 min and then at 10000 g for 30 sec

Discard flow-through

Add 250µl of DNA Wash Buffer (**DW**)

Spin at 16000 g for 2 min

Discard flow-through

Place column into a fresh 1.5ml collection-tube provided in the kit

Add **24** ul *(neuer Kit 16 ul!!)* of DNA Elution Buffer (**DE**)

Incubate for 3 min at RT

Spin at 16000 g for 1 min

OK *You may store the sample at –20°C overnight*

Transfer the sample to a new PCR tube (0.2 ml)

***in vitro* Transcription (IVT)**

IVT reagents at RT, components should be added in order of the list

mix all reagents

IVT reaction mix	per sample
IVT buffer	**8 µl**
IVT master mix	**12 µl**
IVT enzyme mix	**4 µl**
Total	**24 µl**

Add **24** µl of IVT reaction mix to each sample

Incubate at 42°C for **4 hours**

Chill the samples

OK *You may hold the sample(s) at 4°C in the thermal cycler overnight*

Add 2 µl DNase mix *(must be mixed and spun down)*

Incubate at 37°C for 15 min

Chill the samples

aRNA Purification

Add 250µl of RNA Binding Buffer (**RB**) to a new DNA/RNA purification column

Hold for 5 min at RT

Spin at 16000 g for 1 min

Discard flow-through

Add 200 µl of **RB** to the IVT reaction sample, mix well and pipette the entire sample into the purification column

Centrifuge at 100 g for 2 min and then at 10.000 g for 30 sec

Discard flow-through

Add 200µl of RNA Wash Buffer (**RW**)

Centrifuge at 10.000 g for 1min

Discard flow-through

Add 200 µl of **RW**

Spin at 16000 g for 2 min

Discard flow-through

Place the purification column into a new tube (provided in the kit)

Add **30 µl** RNA Elution Buffer (**RE**)

Incubate at RT for 3 min

Spin the pufication column at 16000 g for 1 min

Measure the $OD_{260/280}$ (dilute 1µl with 49µl TE-Buffer)