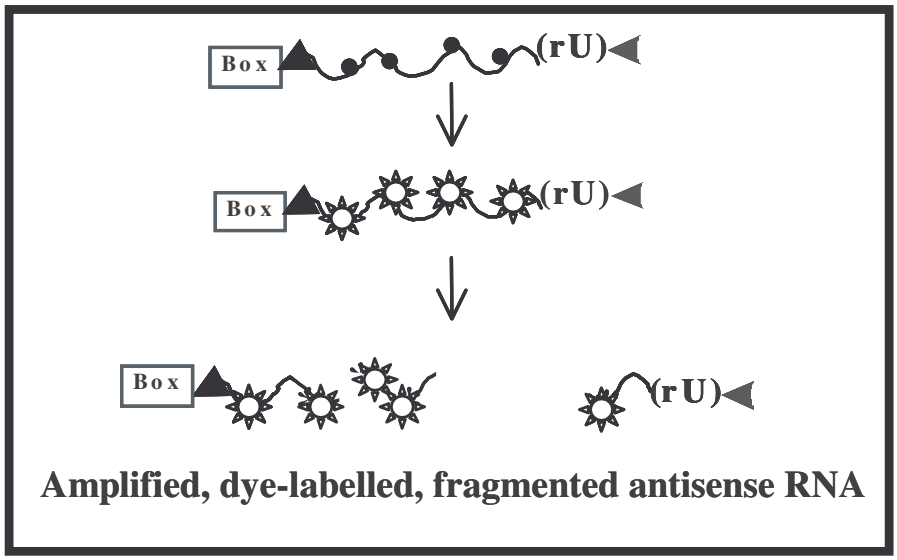
ExpressArt®
AminoAllyl Add-on Module AA15

Cat.-No: 2000-AA15

Supplementary Manual for

In vitro transcription to generate Aminoallyl-labelled RNA for Dye coupling via monoreactive NHS-ester



As detailed in the protocol of your C&A ExpressArt® mRNA Amplification kit, prepare the DNA template for *in vitro* transcription.

As appropriate,

use the DNA template for the **first (step A3), second (step B3),** or **third amplification round (step C4)**

Contents **page**

Reagents

Materials are provided for 12x aminoallyl-labelling reactions via *in vitro* transcription, reagents for subsequent dye coupling (but not NHS-activated dyes), and for RNA fragmentation.

Content Kit box AA-I

Tube A1:	**ATP, CTP, GTP Mix (25 mM each)**	**90 µl**
Tube A2:	**UTP (90 mM)**	**20 µl**
Tube A3:	**AA-UTP (50 mM)**	**15 µl**
Tube A4:	**Sodium Acetate (3M, pH 5)**	**300 µl**
Tube A5:	**Precipitation Carrier (Pellet Paint®)**	**55 µl**
Tube A6:	**Coupling Buffer**	**150 µl**
Tube A7:	**DMSO**	**150 µl**
Tube A8:	**4M Hydroxylamine**	**150 µl**
Tube A9:	**5x Fragmentation Buffer**	**150 µl**
Tube A10:	**10x Stop solution**	**50 µl**
Tube A11:	**DEPC-H_2O**	**1000 µl**
Tube A12:	**P-Carrier**	**15 µl**

Storage

Immediately upon arrival: store all reagents of **Kit box AA-I at -20°C**. Avoid repeated freeze thawing.
Reagents are stable for 6 months (Expiry date on kit box).

Additionally required materials

- RNeasy Mini Kit from (Qiagen Catalogue No. 74104)
- NHS-activated Labelling Reagent.
  We routinely use the CyDye Post-Labelling Reactive Dye Pack (Amersham Cat.-No. RPN 5661, contains 12 vials each of NHS-Cy3 and NHS-Cy5)
- Eppendorf or Gilson pipette 0.5 – 2µl (strongly recommended)
- Thermocycler with adjustable lid temperature (set at 45°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 reaction tubes (0.5 / 1.5 ml)
- 100% ethanol and 70% ethanol
- Microcentrifuge

Quality control

All components of the kit are tested in amplification and labelling reactions, using the Positive Control RNA (Core kit, **Tube 23)**, provided with the core kit. All reagents are tested for the absence of nuclease activity.

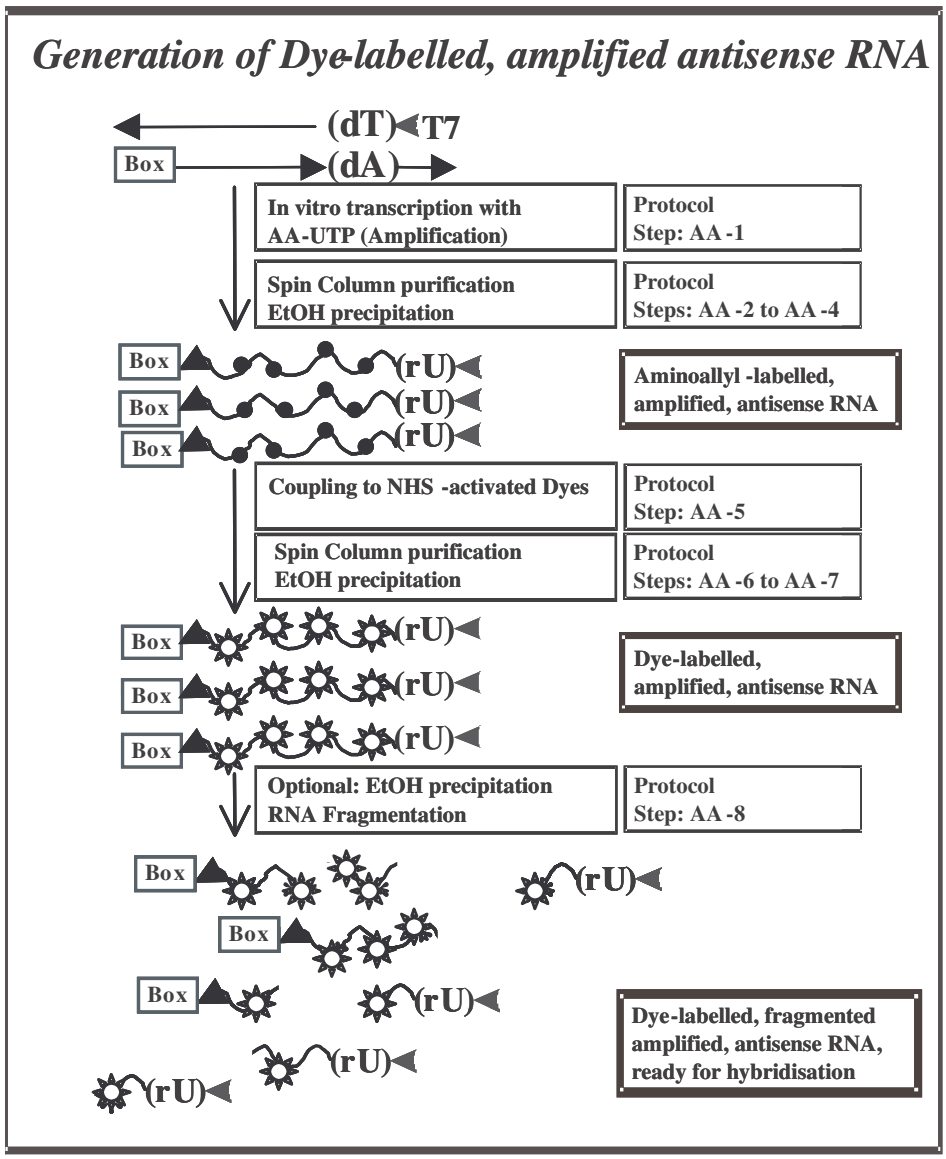
Introduction

This supplementary kit provides reagents to incorporate the modified nucleoside 5-(3-aminoallyl)-U (AA-U) into the amplified RNA during *in vitro* transcription. The aminoallyl-group at the C5-position of uracil contains a reactive primary amino group. Via this group, the AA-labelled RNA can be coupled with activated dyes (or other moieties), using the monoreactive NHS-ester derivatives of the dyes. This reaction is simple, efficient and it yields a stable covalent bond between the RNA and the dye molecules.

Typically, long RNA molecules can form secondary structures with extensive internal base pairing. However, for efficient hybridisation (especially with oligonucleotide microarrays), the sequences of labelled probes should be fully accessible and not masked by intramolecular base pairing. This can be achieved by fragmenting the labelled RNA. In this fragmentation step (metal catalysed cleavage at elevated pH and temperature), the RNA molecules are "nicked". This means, no RNA segments are lost and the resulting short RNA fragments (100-200 nucleotides long) retain essentially all sequence information.

In general, very similar or identical hybridisation conditions can be used for labelled cDNA (antisense orientation relative to mRNA) and fragmented, labelled, amplified RNA (also antisense orientation).

Flow sheet


Generation of Dye-labelled, amplified antisense RNA
(dT)T7
Box
(dA)
In vitro transcription with AA-UTP (Amplification)
Protocol Step: AA -1
Spin Column purification EtOH precipitation
Protocol Steps: AA -2 to AA -4
Box
(rU)
Box
(rU)
Box
(rU)
Aminoallyl -labelled, amplified, antisense RNA
Coupling to NHS -activated Dyes
Protocol Step: AA -5
Spin Column purification EtOH precipitation
Protocol Steps: AA -6 to AA -7
Box
(rU)
Box
(rU)
Box
(rU)
Dye-labelled, amplified, antisense RNA
Optional: EtOH precipitation RNA Fragmentation
Protocol Step: AA -8
Box
(rU)
Box
Box
(rU)
(rU)
(rU)
Dye-labelled, fragmented amplified, antisense RNA, ready for hybridisation

DETAILED PROTOCOL

AA-1. Aminoallyl labelling and amplification by *in vitro* transcription

Required materials: Your DNA template; ATP,CTP,GTP Mix (**Tube A1**), UTP (**Tube A2**), AA-UTP (**Tube A3**); 10x Buffer (**Core kit: Tube 19**); RNA Polymerase (**Core kit: Tube 20**).

Please note: The ExpressArt® Core kits contain the required reagents for *in vitro* transcription (10x buffer, RNA polymerase). The supplementary Amino Allyl kit contains unmodified NTP's and AA-UTP to generate aminoallyl-labelled transcripts.

AA-NTP-Mix: Recommended composition (per 20 µl *in vitro* transcription reaction; please use mastermix for multiple reactions): 6 µl ATP,CTP,GTP Mix (**Tube A1**), 1.25 µl UTP (**Tube A2**), 0.75 µl AA-UTP (**Tube A3**).

In vitro-Transcription Mix AA-1		
AA-NTP-Mix	**see above**	6.6 µl
10x Buffer	Core kit: Tube 19	1.7 µl
RNA Polymerase	Core kit: Tube 20	1.7 µl

- Prepare the in vitro-Transcription Mix by adding the components **at room temperature**, never on ice, because spermidine in the buffer can cause precipitation of DNA template.
- Add 10 µl in vitro-Transcription **Mix AA-1** to your template DNA.
- Consult the table of expected yields (manual of your core kit) for choosing your reaction time:
  Incubate the transcription reaction for **4 h or overnight** at 37°C in a thermocycler with heating lid adjusted to 42°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!

AA-2. RNA-Purification using RNeasy Mini Kit

Required materials: Your AA-labelled RNA; RNeasy Mini Kit (from Qiagen; not provided with the kit); Ethanol

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

aRNA Purification Mix AA-2	
RNase-free water	82 µl
RLT (Lysis Buffer)	350 µl

- Insert the purification columns in the collection tubes.
- Add 432 µl of **Mix AA-2** to each **in vitro-Transcription Reaction**. Mix thoroughly and add 250 µl 100% EtOH. 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 as above. Discard the flow-through, re-insert the columns in the same collection tubes and wash with 500 µl **RPE Buffer**. Centrifuge for 2 min. Discard the flow-through, re-insert the 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 RNase-free reaction tubes and add 50 µl of RNase-free water to the columns (make sure to pipette the water exactly in the middle of the column, without disturbing the matrix with the pipette tip). Incubate the column for 1 min and centrifuge for 1 min at 10,000 rpm.
- Elute the columns a second time with 50 µl RNase-free water in the same collection tube, incubate 1 min, and centrifuge again for 1 min at 10,000 rpm.

Transfer eluate to fresh RNase-free reaction tube for further processing.

AA-3. Control of aminoallyl-RNA product quantity and quality

Expected yields should be > 20 µg (see your ExpressArt® Core kit), accordingly, 1 µl will contain > 200 ng aminoallyl-RNA.

Photometric quantification

Dilute 1 µl of the eluted aminoallyl-RNA in up to 50 µl low salt buffer or water. This is suitable for photometric quantification (expected $A_{260} > 0.1$), measuring against a blank using the same buffer.

Quality Control with Agilent 2100 bioanalyzer

Note: all ionic compounds interfere with capillary electrophoresis. The signal may be significantly compressed by residual salt in the ethanol precipitate.

If a broad size distribution is expected, the minimum recommended RNA concentration is 50 – 100 ng/µl (lower concentrations are possible for total RNA with its prominent rRNA peaks). The RNA size distribution can be monitored with the bioanalyzer profiles, but quantification may indicate too low RNA amounts.

Example electropherograms of first, second and third round amplified human RNAs are shown in section **"RNA Quality Control"** (see manual of your Core kit).

AA-4. Ethanol precipitation of the purified aRNA

Required materials: Sodium acetate (**Tube A4**); Precipitation Carrier (**Tube A5**); DEPC-H_2O (**Tube A11**); 100% ethanol

Always store the Precipitation Carrier (**Tube A5**) in the dark. For long-term storage, keep at –20°C. Smaller aliquots can be kept at 4°C for about 1 month.

Precipitation Mix AA-3		
Sodium Acetate	Tube A4	10 µl
Precipitation Carrier	Tube A5	2 µl

- Add 12 µl of **Mix AA-3** to each eluate (100 µl from A7) and mix well.
- Add 220 µl of 100% ethanol, mix again, and incubate for 2 min at room temperature.
- Centrifuge the cDNA at maximum speed for 10 min at room temperature.
- Discard the supernatant and wash the pink-coloured pellet with 200 µl of 70% ethanol (room temperature). Centrifuge for 5 min at maximum speed and completely remove the supernatant with a pipette.
- To ensure that all liquid is removed, spin briefly to collect liquid, and remove all remaining liquid with a pipette tip.
- Air dry the pellets by leaving the tubes open, but covered with fresh tissue paper, for about 5 min at room temperature. **Do not dry in a speed vac!**
- Dissolve the pellet in an appropriate volume of DEPC-H_2O (**Tube A11**) to obtain an RNA-concentration of 3 µg/µl. Keep on ice.

AA-5. Coupling of fluorescent dye

Required materials: Your aminoallyl-labelled RNA; Coupling Buffer (**Tube A6**); DMSO (**Tube A7**); 4M hydroxylamine (**Tube A8**)
Not provided with the kit: CyDye Post-Labelling Reactive Dye Pack (Amersham # RPN 5661, contains 12 vials each of NHS-Cy3 and NHS-Cy5)

Transfer 7µl of dissolved aminoallyl-RNA (~20µg) into a fresh reaction tube

- Add 9µl of Coupling Buffer (**Tube A6**) and mix well
- To avoid absorption of air moisture, allow the dye packs to reach room temperature before opening. Resuspend one ready-to-use aliquot of CyDye in 4µl DMSO (**Tube A7**) and add the dissolved fluorescent dye to the aminoallyl-RNA
- Mix well and place the tube in the dark. Incubate for 30 min at room temperature
- Add 4.5µl of 4M hydroxylamine (**Tube A8**) to each coupling reaction
- Mix well and incubate the tube in the dark for 15 min at room temperature.

AA-6. Purification of CyDye-labelled cRNA with RNeasy Mini kit

Required materials: Your CyDye-labelled RNA; RNeasy Mini Kit (from Qiagen; not provided with the kit); Ethanol

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

aRNA Purification Mix AA-2	
RNase-free water	80 µl
RLT (Lysis Buffer)	350 µl

- Insert the purification columns in the collection tubes.

- Add 430 µl of **Mix AA-2** to each **RNA Labelling Reaction.** Mix thoroughly and add 250 µl 100% EtOH. 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 as above. Discard the flow-through, re-insert the columns in the same collection tubes and wash with 500 µl **RPE Buffer**. Centrifuge for 2 min. Discard the flow-through, re-insert the 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 RNase-free reaction tubes and add 50 µl of RNase-free water to the columns (make sure to pipette the water exactly in the middle of the column, without disturbing the matrix with the pipette tip). Incubate the column for 1 min and centrifuge for 1 min at 10,000 rpm.
- Elute the columns a second time with the flow-through (**eluate**). Reinsert in the same collection tube, incubate 1 min, and centrifuge again for 1 min at 10,000 rpm.

- Transfer eluate to fresh RNase-free reaction tube for further processing.

AA-7. Photometric Analysis of Dye incorporation

Use an aliquot of the eluate to determine the concentration of the dye-labelled cRNA and to control the CyDye incorporation by spectrophotometry.
For Cy3-labelled samples, obtain the absorbance reading at 550 nm and for Cy5-labelled samples at 650nm.
Calculate N, the number of dye molecules incorporated per 1000 nucleotides of labelled RNA.

$$N \text{ (for Cy5)} = 36 \times A_{650}/A_{260}$$

$$N \text{ (for Cy3)} = 60 \times A_{550}/A_{260}$$

For successful reactions, N should be in the range of 8-15.

AA-8. Fragmentation of CyDye-labelled cRNA

Required materials: Your CyDye-labelled cRNA; 5x Fragmentation buffer (**Tube A9**); 10x Stop solution (**Tube A10**)

Preparing your CyDye-labelled RNAs for co-hybridization experiments: combine 10 µg each of differentially labelled cRNA [10 µg Cy3-labelled cRNA + 10 µg Cy5-labelled cRNA] and adjust the volume to 32 µl. As appropriate, use Vacuum centrifuge (but do not dry completely), and add water to a final volume of 32 µl.

Fragmentation Reaction

Combine the following reagents on ice in 0.2 ml PCR reaction tubes:
32 µl of labelled cRNAs (Mix of Cy3 and Cy5)
8 µl of 5x Fragmentation-Buffer (**Tube A9**)

- Mix carefully and spin down briefly
- Incubate at 94°C for 15 min in a thermocycler
- Immediately, place the sample on ice
- Add 2 µl of 10x Stop solution (**Tube A10**), and mix well

The CyDye-labelled RNA is ready for hybridisation.

If desired, a purification step with Microcon YM-10 can be used to remove buffer and salts. Add 60 µl RNase-free water, mix and transfer onto a Microcon concentrator. Centrifuge at 8600x g for approximately 15 min until only a small layer of liquid remains (do not run dry!). Place the concentrator upside down into a new 1.5 ml collection tube to elute the RNA. Spin at 1200x g for 3 min.

Combine with your appropriate hybridisation solution.

Troubleshooting

Inefficient Dye Coupling

Although the coupling reaction with NHS-activated dyes is a quite robust chemical reaction, storage and handling problems can occur.
The NHS-dyes can be inactivated by hydrolysis: avoid opening of frozen or cold vials, this can lead to trapped air moisture. Once, the dye aliquots are dissolved in DMSO, they should be used immediately. If purchasing vials with larger quantities, immediately dispense aliquots for single use in tightly sealed and dark vials (wrap in aluminium foil) and store frozen at –20°C.
Dyes are sensitive to bleaching, especially at elevated temperatures. Improper storage and shipment conditions can result in reduced performance.
Please refer to your NHS-dye supplier for further information.

AmpTec
Amplification Technologies

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 >