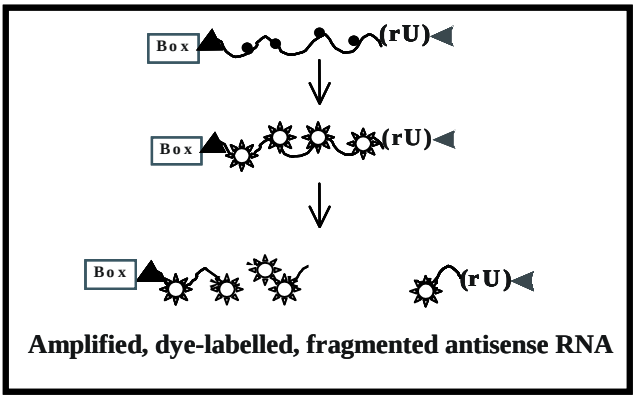
ExpressArt®
AminoAllyl Add-on Module AA30

Cat.-No: 2000-AA30

Supplementary Manual for

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



As detailed in the manual of your Core 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)**

Reagents

Materials are provided for 30 aminoallyl-labelling reactions via *in vitro* transcription, reagents for subsequent dye coupling (but not the NHS-activated dyes), and for RNA fragmentation.

Content Kit box AA-I

Tube A1:	**ATP, CTP, GTP Mix (25 mM each)**	**180 µl**
Tube A2:	**UTP (90 mM)**	**40 µl**
Tube A3:	**AA-UTP (50 mM)**	**25 µl**
Tube A4:	**Sodium Acetate (3M, pH 5)**	**300 µl**
Tube A5:	**Precipitation Carrier (Pellet Paint®)**	**60 µl**
Tube A6:	**Coupling Buffer**	**300 µl**
Tube A7:	**DMSO**	**120 µl**
Tube A8:	**4M Hydroxylamine**	**140 µl**
Tube A9:	**5x Fragmentation Buffer**	**250 µl**
Tube A10:	**10x Stop solution**	**60 µl**
Tube A11:	**DEPC-H_2O**	**1000 µl**

Kit box III *new* (second box, in addition to Core kit)

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 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

- 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.

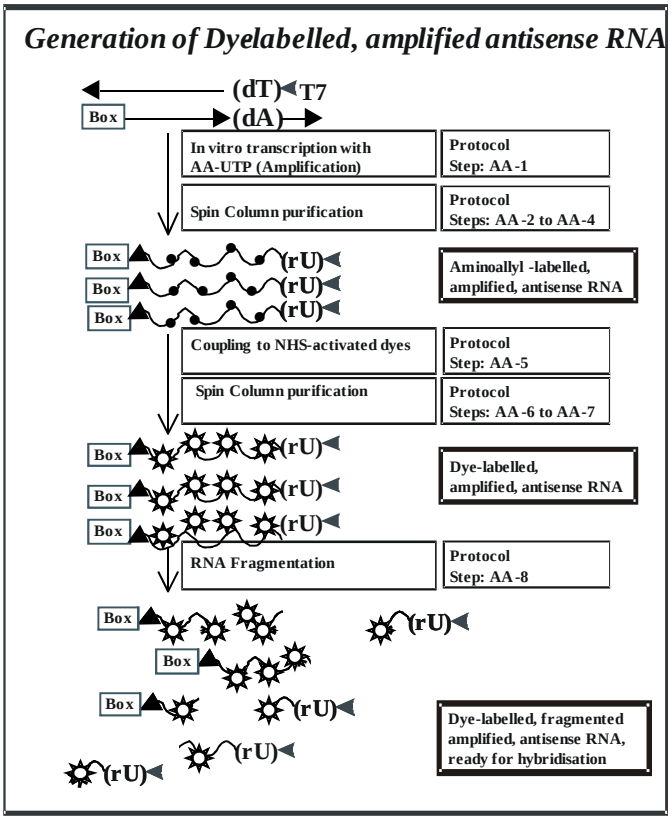
Introduction

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 Dyelabelled, amplified antisense RNA
(dT)T7
Box
(dA)
In vitro transcription with AA-UTP (Amplification)
Protocol Step: AA-1
Spin Column purification
Protocol Steps: AA-2 to AA-4
Box
Box
Box
(rU)
(rU)
(rU)
Aminoallyl -labelled, amplified, antisense RNA
Coupling to NHS-activated dyes
Protocol Step: AA-5
Spin Column purification
Protocol Steps: AA-6 to AA-7
Box
Box
Box
(rU)
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(rU)
Dye-labelled, amplified, antisense RNA
RNA Fragmentation
Protocol Step: AA-8
Box
Box
Box
(rU)
(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 (for one *in vitro* transcription reaction; please use mastermix for multiple reactions): 5 µl ATP,CTP,GTP Mix (**Tube A1**), 1 µl UTP (**Tube A2**), 0.6 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 in the given order **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 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!

AA-2. RNA-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°C.

aRNA Purification Mix AA-2	
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 AA-2** 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°C)
- 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°C.

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

Expected yields should be > 10 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.

AA-4. <u>Optional:</u> Ethanol precipitation of the purified aRNA
but see comment at begin of step AA-5.

<u>Required materials</u>: 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 <u>Mix AA-3</u>		
Sodium Acetate	Tube A4	10 µl
Precipitation Carrier	Tube A5	2 µl

- Add 12 µl of **<u>Mix AA-3</u>** 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)

The aminoallyl-RNA (~20µg) should be in a volume of ~7µl.
For larger volumes, reduce volume in a vacuum centrifuge ("SpeedVac"); be careful and do not allow samples to dry completely!
As alternative, the optional EtOH precipitation can be used (see step AA-4, above).

- 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 labelled RNA with 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°C.

aRNA Purification Mix AA-4	
RNA LB Buffer (box III)	350 µl
RNA DeS Buffer (box III)	90 µl
100% Ethanol (at room temperature; not supplied)	575 µl

- Add 1015 µl of **Mix AA-4** to each **Labelling 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°C)
- 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 labelled RNA immediately or store at -80°C.

- **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: If desired, 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, reduce volume in a vacuum centrifuge ("SpeedVac"); be careful and do not allow samples to dry completely! 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.

A purification step with Microcon YM-10 is recommended 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.

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