Amplification and Labeling Kit

ULS™

RNA ampULSe: Amplification and Labeling Kit for Agilent arrays

For 20 reactions
(Fluorescent labels)

Product code
GEA-014/GEA-016/GEA-018

For laboratory use only
Research purposes only

KREATECH BIOTECHNOLOGY

Instruction manual

KREATECH's ULS™ RNA ampULSe: Amplification and Labeling Kit for Agilent arrays

This kit is intended for RESEARCH USE ONLY. IT IS NOT INTENDED FOR DIAGNOSTIC APPLICATIONS and/or COMMERCIAL PURPOSES.

Important

Open the kit immediately and store all components as instructed on page 7 and 8

- Read the entire Instruction manual before starting your experiment.
- Do not mix reagents from different kits.
- During the preparation of reagents and throughout the entire procedure please observe the safety regulations issued for laboratories concerning handling of samples.
- Dispose of reagents according to relevant local regulations. Take appropriate safety precautions such as wearing a lab coat, gloves and eye protection.

Limited Product Warranty – Patent Disclaimer

This warranty limits our liability to replacement of this product. No other warranties of any kind expressed or implied, including without limitation, implied warranties or fitness for a particular purpose, are provided by KREATECH. KREATECH shall have no liability for any direct, indirect, consequential, or incidental damages arising out of use, or the inability to use this product.

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Table of Contents

A. Assay Materials

I. Components Provided with the Kit and Storage Conditions

The RNA ampULSe: amplification and labeling kit includes reagents for single-round amplification and labeling of 20 samples or double-round amplification of 10 samples.

Box 1: cDNA Synthesis and In Vitro Transcription

Amount	Component	Storage
60 µL	T7 Oligo(dT) Primer	–20°C
22 µL	ArrayScript™	–20°C
22 µL	RNase Inhibitor	–20°C
42 µL	10X First Strand Buffer	–20°C
170 µL	dNTP Mix	–20°C
210 µL	10X Second Strand Buffer	–20°C
42 µL	DNA Polymerase	–20°C
22 µL	RNase H	–20°C
84 µL	T7 10X Reaction Buffer	–20°C
84 µL	T7 Enzyme Mix	–20°C
336 µL	T7 rNTP mix (75 mM)	–20°C
40 µL	Second Round Primers	–20°C
10 µL	Control RNA (1 mg/mL HeLa total RNA)	–20°C
3 mL	KREA*block*™	–20°C
1.75 mL	Nuclease-free Water	any temp

Box 2: cDNA and aRNA Purification

Amount	Component	Storage
10 mL	Nuclease-free Water	any temp
30 mL	Wash Buffer (Add 24 ml 100% ethanol before use)	4°C or room temp
7 mL	cDNA Binding Buffer	room temp**
9 mL	aRNA Binding Buffer	room temp
20	aRNA Filter Cartridges	room temp
40	aRNA Collection Tubes	room temp
20	cDNA Filter Cartridges + Tubes	room temp
20	cDNA Elution Tubes	room temp

***The cDNA Binding Buffer may form a precipitate if stored below room temp. If a precipitate is visible, redissolve it by warming the solution to 37°C for up to 10 min and vortexing vigorously. Cool to room temp before use.*

Box 3: For product code GEA-014

Component	Amount	Storage
Cy5-ULS	10 reactions	4°C
Cy3-ULS	10 reactions	4°C
10 x Labeling Buffer	100 µL	4°C
KREA*pure*™ columns	20 pcs	4°C

Box 3: For product code GEA-016 or GEA-018

Component	Amount	Storage
Cy5-ULS (GEA-016) or Cy3-ULS (GEA-018)	20 reactions	4°C
10 x Labeling Buffer	100 µL	4°C
KREA*pure*™ columns	20 pcs	4°C

II. Components Not Provided with the Kit

- Fragmentation reagents (Ambion Cat # 8740)
- 100% Ethanol (to prepare the Wash Buffer)
- Thermal cycler with adjustable temperature heated lid, hybridization oven, or heat blocks set at 85°C, 70°C, 42°C, 37°C, and 16°C
- Vacuum centrifuge concentrator

B. General Information

I. Product Description and Background

Gene expression profiling using DNA-microarrays has been growing rapidly over the last decade. When working with material from fine needle biopsy, laser capture micro-dissection material and with patient material in general, the amount of target material is limited. This has made it necessary to carry out target amplification, generally by linear amplification based on a protocol first described by van Gelder and Eberwine[1]. The RNA ampULSe: amplification and labeling kit combines two successful technologies.

The aRNA generation employs Ambion's widely used MessageAmp™ II aRNA amplification reagents followed by fluorescent labeling with KREATECH's proprietary Universal Linkage System (ULS) technology. Using the RNA ampULSe: amplification and labeling kit the need for bulky, modified nucleotides that are used for traditional amplification and labeling is avoided. This results in higher yields and a better representation of the transcriptome. With this kit unmodified aRNA is generated which is subsequently labeled with ULS labels. Another appealing feature of generating unmodified aRNA is the possibility to store unused aRNA and label it at a later date for follow-up or validation studies. In addition since unmodified nucleotides are used in this kit, a second round of amplification is always an option which is useful when unsure if two rounds of amplification are needed. Low amounts of RNA samples (0.1-100 ng) can be put through two rounds of amplification if desired. This strategy makes the production of microarray samples from picogram amounts of total RNA entirely possible[2].

In addition to RNA ampULSe: RNA amplification and labeling kits for Agilent microarrays, KREATECH Biotechnology also provides kits without the amplification module to label unmodified aRNA for Agilent microarrays. There are three available formats for use with Agilent microarrays:

Product Code	Name	Labeling Reactions
	Labeling Kits	
EA-021	ULS labeling kit with Cy3 and Cy5 ULS for Agilent gene expression microarrays	25 dual
EA-022	ULS labeling kit with Cy5 ULS for Agilent gene expression microarrays	25 single
EA-023	ULS labeling kit with Cy3 ULS for Agilent gene expression microarrays	25 single

II. Principle of Universal Linkage System (ULS)

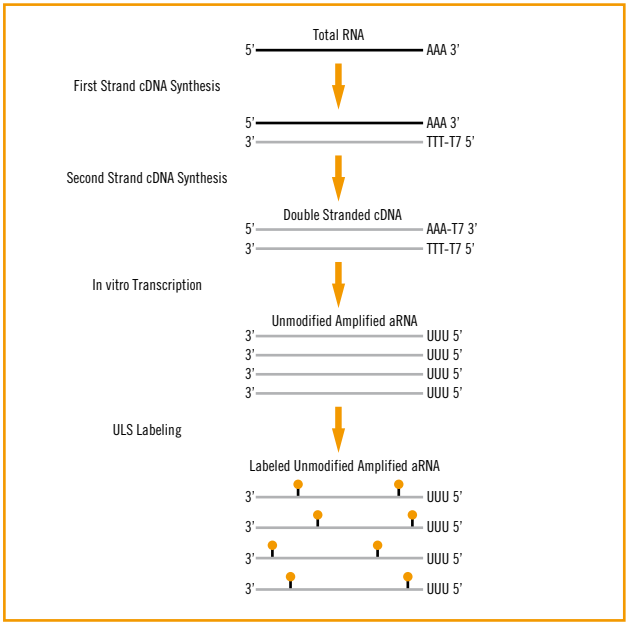
The proprietary ULS technology is based on the stable binding properties of platinum to biomolecules. The ULS molecule consists of a platinum complex, a detectable molecule and a leaving group which is displaced upon reaction with the target. This ULS molecule forms a co-ordinative bond, firmly coupling the ULS to the target. ULS labels DNA and RNA by binding to the N7 position of guanine. In proteins, ULS binds to nitrogen and sulphur containing side chains of methionine, cysteine and histidine (see figure inside back cover).

ULS is available coupled to a variety of labels and haptens, including fluorochromes and biotin. ULS thereby enables one-step non-enzymatic labeling of nucleic acids to be achieved within 15-30 minutes. ULS labeling of RNA and DNA is compatible with all DNA microarray platforms. This kit is specially formatted to be convenient for use with the Agilent gene expression microarrays..

III. Overview of RNA ampULSe: Amplification and Labeling Kit Procedure

1. Starting material is eukaryotic Total RNA or poly (A) selected RNA
2. Reverse transcription to synthesize first strand cDNA using T7 Oligo(dT) primers (2 hr)
3. Second strand synthesis (2 hr)
4. Clean up cDNA and use as template for aRNA synthesis
5. In vitro transcription to synthesize aRNA (4-14 hr)
6. Clean up aRNA
7. ULS labeling of aRNA (15-30 min)
8. aRNA fragmentation (15 min)
9. Hybridization

IV. Schematic Overview of RNA ampULSe: Amplification and Labeling Kit Procedure

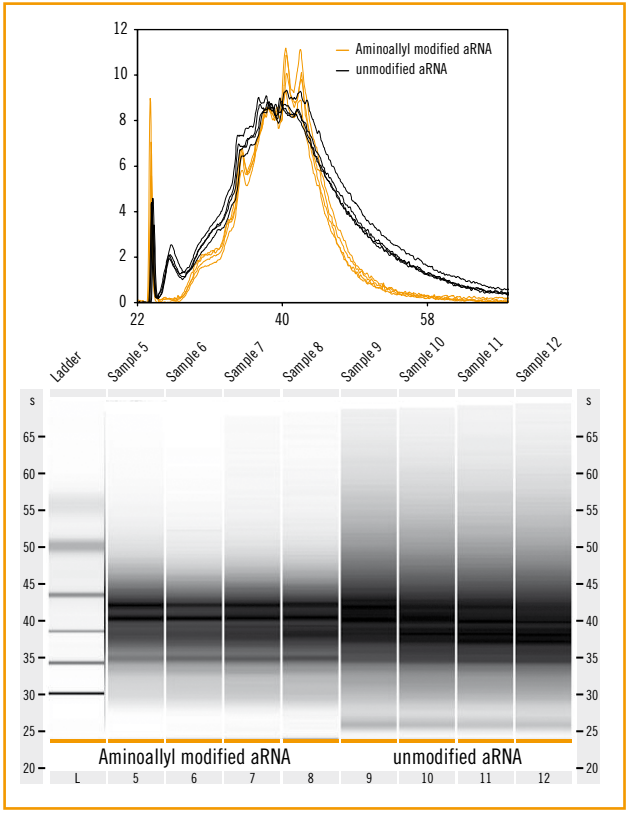


C. The RNA ampULSe: Amplification and Labeling Kit Advantage

The RNA ampULSe: amplification and labeling kit employs the ULS technology which allows labeling of your unmodified aRNA in a one step non enzymatic labeling reaction. ULS labels the aRNA forming a stable coordinative bond, which does not interfere with downstream hybridization. ULS labeling in the RNA ampULSe: amplification and labeling kit has been optimized to give extremely reproducible labeling densities which are optimal for gene expression analysis. Also the unique KREA*pure* columns which have been specially designed to remove unreacted ULS and have no affinity for aRNA result in very clean samples and ensure high recovery of aRNA.

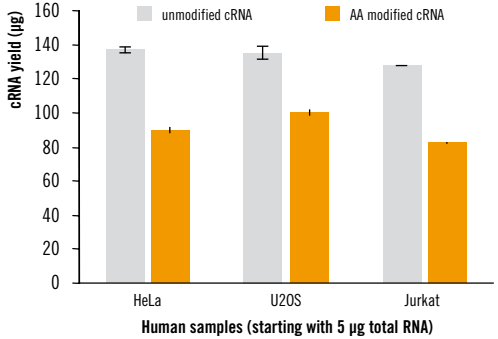
In addition, since the RNA ampULSe: amplification and labeling kit does not require modified nucleotides in order to label aRNA, users can work with unmodified aRNA which in itself has many advantages (see box). These include; higher aRNA yields therefore more experiments per IVT, better representation of the transcriptome by avoiding the strain on the enzyme to incorporate modified nucleotides. Unmodified aRNA is also more stable for storage and therefore after the IVT reaction only the amount of aRNA needed is labeled, the rest of the aRNA can be kept or archived for later use such as RT-PCR, more dedicated chips or other validation experiments. Since unmodified nucleotides are used in this kit a second round of amplification is always an option which is useful when unsure if two rounds of amplification are needed.

Use of unmodified nucleotides increases your aRNA yield and gives a more even representation of the transcriptome



After IVT aRNA synthesis and clean up, the aRNA samples (4 replicates of both modified and unmodified aRNA) were evaluated on an Agilent 2100 bioanalyzer. Top figure;

aminoallyl modified aRNA (light line) and unmodified aRNA (dark line). The curve of the unmodified aRNA is much broader and appears to yield longer fragments of aRNA. The aminoallyl modified aRNA on the other hand seems to give a narrower range of fragment length and it is dominated by two or three particular lengths. Bottom figure (page13); gel view of aminoallyl modified aRNA and unmodified aRNA. The left 4 lanes contain the aminoallyl modified aRNA, compared to the right 4 lanes which are the unmodified aRNA. There is a clear difference in size distribution and relative abundance of some aRNA fragments. Modified aRNA gave a narrower and slightly altered fragment distribution from natural unmodified aRNA.



aRNA yields from three human cell lines generated with natural unmodified nucleotides (light bar) and modified nucleotides (dark bar). These results show that use of modified nucleotides reduces yield by about 40%.

D. Protocol

I. Total RNA Isolation

A wide variety of RNA isolation techniques are available, e.g. using Trizol (Invitrogen) extraction followed by RNeasy column purification (QIAGEN) or other silica based columns. Irrespective of which isolation procedure is used the RNA material should be free from DNA and other contaminants. Assessment of the purity and yield of your RNA should be carried out by;

a. Running your RNA on a 1% agarose gel. The integrity of the total RNA is determined by observing the ribosomal bands
b. Determining the OD_{260}. For all RNAs $OD_{260/280}$ should be >1.9 and $OD_{260/230}$ should be >2.1

II. RNA Amplification

a. Reverse Transcription First Strand cDNA Synthesis

Incubators needed:

- 70°C: thermal cycler recommended
- 42°C: hybridization oven or air incubator recommended

1. Place a maximum volume of 10 µL of total RNA (1000 ng recommended) or poly(A) selected RNA (typically 10-100 ng) into a nonstick, sterile, RNase-free, 0.5 mL tube. RNA must be in high quality water or TE. (See Table on page 21 for minimum and maximum RNA input amounts)
2. Add 1 µL of T7 Oligo(dT) Primer
3. Add Nuclease-free Water to a final volume of 12 µL, vortex briefly to mix, then centrifuge to collect the mixture at the bottom of the tube

4. Incubate for 10 min at 70°C in a thermal cycler
5. Centrifuge samples briefly (~5 sec) to collect them at the bottom of the tube
6. Place the mixture on ice
7. At room temp, prepare Reverse Transcription Master Mix in a nuclease-free tube. Assemble enough to synthesize first strand cDNA from all the RNA samples in the experiment, including ≤ 5% overage to cover pipetting error

Reverse Transcription Master Mix (for a single 20 µL reaction)

Amount	Component
2 µL	10X First Strand Buffer
4 µL	dNTP Mix
1 µL	RNase Inhibitor
1 µL	ArrayScript

8. Mix well by gently vortexing
9. Centrifuge briefly (~5 sec) to collect the Reverse Transcription Master Mix at the bottom of the tube and place on ice
10. Transfer 8 µL of Reverse Transcription Master Mix to each RNA sample
11. Mix thoroughly by pipetting up and down 2-3 times and flicking the tube 3-4 times, then centrifuge briefly to collect the reaction in the bottom of the tube
12. Place the samples in a 42°C incubator
13. Incubate reactions for 2 hr at 42°C (hybridization oven or air incubator is recommended). After the incubation, centrifuge briefly (~5 sec) to collect the reaction at the bottom of the tube
14. Place the tubes on ice and immediately proceed to second strand cDNA synthesis (below)

b. Reverse Transcription; Second Strand cDNA Synthesis

Incubator needed:

- 16°C: thermal cycler recommended

1. On ice, prepare a Second Strand Master Mix in a nuclease-free tube in the order listed below. Prepare enough to synthesize second strand cDNA from all the samples in the experiment, including ≤ 5% overage to cover pipetting error

Second Strand Master Mix (for a single 100 µL reaction)

Amount	Component
63 µL	Nuclease-free Water
10 µL	10X Second Strand Buffer
4 µL	dNTP Mix
2 µL	DNA Polymerase
1 µL	RNase H

2. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the Second Strand Master Mix at the bottom of the tube and place on ice
3. Transfer 80 µL of Second Strand Master Mix to each sample. Mix thoroughly by pipetting up and down 2-3 times and flicking the tube 3-4 times, then centrifuge briefly to collect the reaction in the bottom of the tube
4. Place the tubes in a 16°C thermal cycler. It is important to cool the thermal cycler block to 16°C before adding the reaction tubes because subjecting the reactions to temperatures >16°C will compromise aRNA yield
5. Incubate for 2 hr in a 16°C thermal cycler

Note: If the lid temperature cannot be adjusted to match the 16°C block temperature, cover the reactions with the heated lid turned off, or if the lid cannot be turned off—do not cover the tubes with it. (Do not use a water bath or a heat block in a 4°C refrigerator for this incubation because the temperature will fluctuate too much)

6. After the 2 hr incubation at 16°C, place the reactions on ice and proceed to cDNA Purification (below), or immediately freeze reactions at –20°C. Do not leave the reactions on ice for more than 1 hr
   This is a potential stopping point (at –20°C), but it is better to complete the cDNA purification (next section) before stopping

c. cDNA Purification

Note: All centrifugations in this purification procedure should be done at 10,000 x g (typically ~10,000 rpm) and at room temp. cDNA Filter Cartridges should not be subjected to RCFs over 16,000 x g because the force could cause mechanical damage and/or may deposit glass filter fiber in the eluate.

Before beginning the cDNA purification

- Preheat the 10 mL bottle of Nuclease-free Water to 50-55°C for at least 10 min. Note: Temperatures above 58°C can partially denature the cDNA, compromising final aRNA yield
- Check the cDNA Binding Buffer for precipitation before using it. If a precipitate is visible, redissolve it by warming the solution to 37°C for up to 10 min and vortexing vigorously. Cool to room temp before use
- Make sure that the ethanol has been added to the bottle of Wash Buffer before use

1. Add 250 µL of cDNA Binding Buffer to each sample, and mix thoroughly by pipetting up and down 2-3 times, then flicking the tube 3-4 times. Follow up with a quick spin to collect the reaction in the bottom of the tube. Proceed quickly to the next step
2. Check that the cDNA Filter Cartridge is firmly seated in its wash tube (supplied). Pipette the cDNA sample\cDNA Binding Buffer (from step 1) onto the center of the cDNA Filter Cartridge
3. Centrifuge for ~1 min at 10,000 x g, or until the mixture is through the filter
4. Discard the flow-through and replace the cDNA Filter Cartridge in the wash tube
5. Apply 500 µL Wash Buffer to each cDNA Filter Cartridge
6. Centrifuge for ~1 min at 10,000 x g, or until all the Wash Buffer is through the filter
7. Discard the flow-through and spin the cDNA Filter Cartridge for an additional minute to remove trace amounts of Wash Buffer
8. Transfer cDNA Filter Cartridge to a cDNA Elution Tube
9. Apply 10 µL of Nuclease-free Water (preheated to 50-55°C) to the center of the filter in the cDNA Filter Cartridge. Note: It is important to use Nuclease-free Water that is at 50-55°C for the cDNA elution. Colder water will be less efficient at eluting the cDNA, and using hotter water (≤58°C) may result in reduced aRNA yield
10. Leave at room temperature for 2 min and then centrifuge for ~1.5 min at 10,000 x g, or until all the Nuclease-free Water is through the filter
11. Elute with a second 10 µL of preheated Nuclease-free Water. The double-stranded cDNA will now be in the eluate (~16 µL)
12. Proceed directly to In Vitro Transcription to synthesize aRNA, or place the cDNA at –20°C

Note: The purified cDNA can be stored overnight at –20°C at this point if desired

d. In Vitro Transcription; aRNA Synthesis

Incubator needed:

- 37°C (hybridization oven or air incubator recommended)

1. At room temp, prepare an IVT Master Mix by adding the following reagents to a nuclease-free microfuge tube in the order listed below. Assemble enough for all the samples in the experiment, including ≤ 5% overage to cover pipetting error

IVT Master Mix for a single reaction (40µL)

40 µL rxn	Component
16 µL	Double-stranded cDNA in Nuclease-free Water
16 µL	T7 rNTP mix (75mM)
4 µL	T7 10X Reaction Buffer
4 µL	T7 Enzyme Mix

2. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the IVT Master Mix at the bottom of the tube and place on ice
3. Transfer IVT Master Mix to each sample
4. Mix thoroughly by pipetting up and down 2-3 times and flicking the tube 3-4 times, then centrifuge briefly to collect the reaction in the bottom of the tube
   Note: If the cDNA mixture was vacuum dried, resuspend the template by washing the sides of the tube with the IVT Master Mix as you add it to the tube. It is important to make sure that all of the material is resuspended in the IVT reaction mix

5. Once assembled, place the tubes at 37°C
   Note: It is important to maintain a constant 37°C incubation temperature. We recommend incubating in a hybridization oven because it is extremely important that condensation does not form inside the tubes; this would change the reagent concentrations and reduce yield

The minimum recommended incubation time is 4 hr; the maximum is 14 hr. Use the table below as a guide to determine how long to continue your IVT reaction

aRNA Needed	Input Total RNA	IVT Incubation
10-100 µg	1-5 µg	4 hr
1-10 µg	0.1–1 µg	8 hr
0.1-1 µg	≤100 ng	14 hr

6. Stop the reaction by adding Nuclease-free Water to each aRNA sample to bring the final volume to 100 µL. Mix thoroughly by gentle vortexing. Proceed to the aRNA purification step (below) or store at –20°C. Note: The aRNA can be stored overnight at –20°C at this point if desired

Add Nuclease-free Water to bring each sample to 100 µL

e. aRNA Purification

This purification removes enzymes, salts and unincorporated nucleotides from the aRNA. At the end of the purification, the aRNA is eluted from the filter with Nuclease-free Water
Note: All centrifugations in this purification procedure should be done at 10,000 x g (typically ~10,000 rpm) and at room temp. aRNA Filter Cartridges should not be subjected to RCFs over 16,000 x g because the force could cause mechanical damage and/or may deposit glass filter fiber in the eluate.

Before beginning the aRNA purification

- Preheat the 10 mL bottle Nuclease-free Water to 50-60°C for at least 10 min
- Prepare 80% ethanol solution (not provided)

For each sample, place an aRNA Filter Cartridge into an aRNA Collection Tube, and set aside for use in step 3

1. Check to make sure that each IVT reaction was brought to 100 µL with Nuclease-free Water. Add 350 µL of aRNA Binding Buffer to each aRNA sample. Proceed to the next step immediately
2. Add 250 µL of ACS grade 100% ethanol to each aRNA sample, and mix by pipetting the mixture up and down 3 times. Do NOT vortex to mix and do NOT centrifuge. Proceed immediately to the next step as soon as you have mixed the ethanol into each sample. Any delay in proceeding could result in loss of aRNA because once the ethanol is added; the aRNA will be in a semi precipitated state
3. Pipette each sample mixture onto the center of the filter in the aRNA Filter Cartridge
4. Centrifuge for ~1 min at 10,000 x g. Continue until the mixture has passed through the filter
5. Discard the flow-through and replace the aRNA Filter Cartridge back into the aRNA Collection Tube
6. Apply 650 µL Wash Buffer to each aRNA Filter Cartridge
7. Centrifuge for ~1 min at 10,000 x g, or until all the Wash Buffer is through the filter
8. Discard the flow-through and replace the aRNA Filter Cartridge back into the aRNA Collection Tube
9. Apply 650 µL 80% ethanol to each aRNA Filter Cartridge
10. Centrifuge for ~1 min at 10,000 x g, or until all the ethanol is through the filter

11. Discard the flow-through and spin the aRNA Filter Cartridge for an additional ~1 min to remove trace amounts of ethanol and Wash Buffer
12. Transfer Filter Cartridge(s) to a fresh aRNA Collection Tube
13. To the center of the filter, add 100 µL Nuclease-free Water (preheated to 50-60°C)
14. Leave at room temp for 2 min and then centrifuge for ~1.5 min at 10,000 x g, or until the Nuclease-free Water is through the filter
15. The aRNA will now be in the aRNA Collection Tube in ~100 µL of Nuclease-free Water

Store aRNA at –80°C for up to 1 year, and minimize repeated freeze-thawing. Splitting samples into 5-20 µg aliquots for microarray labeling and hybridizations is a good way to prevent multiple freeze-thaw events.

f. Assessing aRNA Yield and Quality

Assessing aRNA yield

The concentration of an aRNA solution can be determined by measuring its absorbance at 260 nm Find the concentration in µg/mL by multiplying the A_{260} by the dilution factor and the extinction coefficient. (1 A_{260} = 40 µg RNA/mL)
For all RNAs $OD_{260/280}$ should be >1.9 and $OD_{260/230}$ should be >2.1

Expected yield

The aRNA yield will depend on the amount and quality of poly(A) RNA in the input total RNA. Since the proportion of poly(A) RNA in total RNA is affected by influences such as health of the organism, and the organ from which it is isolated, aRNA yield from equal amounts of total RNA may vary considerably.

Assessing aRNA Quality

The size distribution of aRNA can be evaluated using an Agilent 2100 bioanalyzer with Caliper's LabChip® technology, or by conventional denaturing agarose gel analysis. The bioanalyzer can provide a fast and accurate size distribution profile of aRNA samples, but aRNA yield should be determined by UV absorbance or RiboGreen analysis. To analyze aRNA size using a bioanalyzer, follow the manufacturer's instructions for running the assay using purified aRNA. Alternatively run a denaturing agarose gel electrophoresis.

Expected aRNA size Agilent bioanalyzer analysis

The expected aRNA profile is a distribution of sizes from 250-5500 nt with most of the aRNA at 1000-1500 nt.

Denaturing agarose gel analysis

Amplified aRNA should appear as a smear from 250 to 5000 nt. The average size of biotin labeled aRNA should be approximately 1400 nt; the average size of unmodified aRNA should be ~1150 nt.

g. Optional Second Round Amplification

If one cycle of amplification does not yield the amount of aRNA necessary for your experiments, a second round of amplification can be conducted to generate additional aRNA. The procedure is similar to the first round of amplification, and the reaction products are equivalent, but different primers are used for first and second strand synthesis, and the reaction setup is slightly different. Second round amplification products are typically shorter than first round amplification products.

h. Synthesis of First Strand cDNA (second round)

Incubators needed:

- 70°C (thermal cycler recommended)
- 42°C (hybridization oven or air incubator recommended)
- 37°C (hybridization oven or air incubator recommended)

1. Place up to 2 µg of purified aRNA from the first round of amplification into a sterile RNase-free microfuge tube. With very small RNA samples, we have dried the entire first round amplification reaction, and used it as starting material for the second round amplification. Note The volume of the aRNA must be ≤ 10 µL. If necessary, concentrate the aRNA in a vacuum centrifuge. Do not dry the aRNA completely, as this could impede reverse transcription
2. Add 2 µL of Second Round Primers
3. Add Nuclease-free Water to bring the volume to 12 µL, vortex briefly to mix, and centrifuge briefly to collect the reaction in the bottom of the tube
4. Incubate for 10 minutes at 70°C in a thermal cycler
5. Remove the RNA samples from the 70°C incubation and centrifuge briefly (~5 sec) to collect the reaction at the bottom of the tube. Place the reaction on ice briefly before starting continuing

At room temp, prepare Reverse Transcription Master Mix in a nuclease-free tube. Assemble enough master mix for all of the samples in the experiment, including ≤ 5% overage to cover pipetting error.

Reverse Transcription Master Mix (for a single 20 µL reaction)

Amount	Component
2 µL	10 x First Strand Buffer
4 µL	dNTP Mix
1 µL	RNase Inhibitor
1 µL	ArrayScript

6. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the Reverse Transcription Master Mix at the bottom of the tube and place on ice
7. Transfer 8 µL of Reverse Transcription Master Mix to each sample. Mix thoroughly by pipetting up and down 2-3 times and flicking the tube 3-4 times, then centrifuge briefly to collect the reaction in the bottom of the tube
8. Place the tubes in a 42°C incubator
9. Incubate reactions for 2 hr at 42°C (hybridization oven or air incubator recommended). After the incubation, centrifuge briefly (~5 sec) to collect the reaction at the bottom of the tube
10. Add 1 µL RNase H to each reaction. Mix thoroughly by pipetting up and down 2-3 times and flicking the tube 3-4 times, then centrifuge briefly to collect the reaction in the bottom of the tube. Note: RNase H specifically degrades the aRNA leaving only the cDNA as template for second strand synthesis. This helps assure that the second strand synthesis reaction will be primed exclusively by the T7 Oligo(dT)Primer
11. Incubate for 30 min at 37°C in a hybridization oven or air incubator. After the incubation, proceed directly to Second Strand Synthesis

i. Synthesis of Second Strand cDNA (second round)

Incubator needed:

- 70°C (thermal cycler recommended)
- 16°C (thermal cycler recommended)

1. Add 5 µL T7 Oligo(dT) Primer to cDNA sample
2. Mix well by gently vortexing, then centrifuge briefly (~5 sec) to collect the sample at the bottom of the tube
3. Incubate 10 min at 70°C in a thermal cycler
4. Place the reaction on ice briefly before adding the remaining second strand cDNA synthesis reagents

On ice, prepare a Second Strand Master Mix by adding the following reagents in the order listed below. Assemble master mix for all the samples in the experiment, including ≤ 5% overage to cover pipetting error

Second Strand Master Mix (for a single 100 µL reaction)

Amount	Component
58 µL	Nuclease-free Water
10 µL	10 x Second Strand Buffer
4 µL	dNTP Mix
2 µL	DNA Polymerase

5. Mix well by gently vortexing. Centrifuge briefly (~5 sec) to collect the Second Strand Master Mix at the bottom of the tube and place on ice
6. Transfer 74 µL of Second Strand Master Mix to each sample. Mix thoroughly by pipetting up and down 2-3 times, then flicking the tube 3-4 times, and centrifuge briefly to collect the reaction in the bottom of the tube

7. Place the tubes in a pre-cooled 16°C thermal cycler. It is important to cool the thermal cycler block to 16°C before placing the reaction tubes; subjecting the reactions to temperatures >16°C could compromise aRNA yield
8. Incubate the tubes for 2 hr in a 16°C thermal cycler.
   Note: If the lid temperature cannot be adjusted to match the 16°C block temperature, cover the reactions with the heated lid turned off, or if the lid cannot be turned off – do not cover the reactions. (Do not use a water bath or a heat block in a 4°C refrigerator for this incubation because the temperature will fluctuate too much)

After the 2 hr incubation at 16°C, place the reactions on ice and proceed to section cDNA Purification on page 18, or immediately freeze reactions at –20°C. Do not leave the reactions on ice for long periods of time. Complete the rest of the second round amplification.

III. ULS Labeling Procedure

Depending on the Agilent microarray being use the amount needed for hybridization may vary between 0.25µg and 1µg. This kit is set up to allow labeling of up to 2µg per labeling reaction. We recommend that where possible 1-2µg is labeled per reaction. This allows for controls such as labeling density measurements to be carried out. Afterwards the desired amount can be taken for hybridization

Incubator needed:

- 85°C (thermal cycler recommended)

Briefly spin all required reagents to collect contents of tubes

1. Take 1-2 µg of purified aRNA. Ensure final concentration in labeling reaction is above 50 ng/µL
   Note: In general optimal modification degrees of the labeled material are not achieved if final concentration of the aRNA in the labeling mixture is below 50 ng/µL
2. Add 1 µL of Cy5 or Cy3 per 1 µg aRNA (Always keep the ratio of µg of aRNA to µL ULS 1:1 when increasing or decreasing the amount to be labeled)
3. Add 1/10 volume of 10x Labeling solution
4. Adjust with RNase-free water to final volume and mix by pipetting (see example set-up below)
5. Label sample by incubation for 15 min at 85°C
6. Place samples on ice, spin down to collect contents of tube before proceeding with purification using the KREA*pure* columns

Example of ULS labeling of 2 µg aRNA

	Cy3-ULS	Cy5-ULS
aRNA (2 µg) + RNAse free water	16 µL	16 µL
ULS -dye	2 µL	2 µL
10 x labeling solution	2 µL	2 µL
Total volume	2 µL	2 µL

a. Dye removal using KREA*pure* columns

Removal of free ULS label using KREA*pure* columns
(20800 xg is equivalent to 14,000 rpm on eppendorf 5417C)

1. Resuspend column material by vortexing
2. Loosen cap ¼ turn and snap off the bottom closure
3. Place the column in a 2 mL collection tube
4. Pre-spin the column for 1 minute at 20800 xg
5. Discard flow through and re-use collection tube
6. Wash the column with 300 µL RNase free water
7. Spin column for 1 minute at 20800 xg
8. Discard collection tube and flow-through
9. Put column in a new (RNase free) 1.5 mL micro centrifuge tube
10. Add ULS-labeled aRNA on to column bed - careful not to pipette on the sides of the column but directly on the column material
11. Spin column for 1 minute at 20800 xg
12. Flow through is purified labeled aRNA
13. At this point the degree of labeling (DOL) can be measured (see appendix E.II)

IV. aRNA Fragmentation

(Below describes the protocol using the fragmentation reagents from Ambion #8740)

1. If carrying out a dual color hybridization both colors can be pooled for fragmentation
2. Transfer the mixture to a microfuge tube and add 1/10 volume of 10 x fragmentation buffer (Ambion) to decrease the fragment size to 60-200 bases. (e.g. 4 µL in final volume of 40 µL)
3. Incubate at 70°C for 15 minutes
4. Spin the vial briefly and add 1µL of stop solution (Ambion), mix by pipetting (the labeled aRNA can form aggregates which dissolve by pipetting) and place on ice until further use
5. If necessary concentrate samples to volume needed for hybridization. We recommend to concentrate under vacuum e.g. Savant SpeedVac

V. Preparation of Labeled Material for Hybridization

This kit supplies a KREA*block* solution which can help to reduce background on your array. If background is an issue we suggest that KREA*block* is used in your hybridization mixture.

Use of KREA*block*

1. KREA*block* should be added to ¼ final volume of the hybridization mixture (e.g. 25 µL of KREA*block* in a 100 µL hybridization volume)
2. Hybridize and wash slides according to own protocol (we recommend that the KREA*block* solution be used to provide the moisture in the hybridization chamber if applicable)

E. Trouble Shooting

I. RNA Amplification

Problem	Possible Cause	Suggestions
Low yield and small average aRNA size	Impure RNA samples	RNA samples with significant amounts of contaminating DNA, protein, phenol, ethanol, or salts are reverse transcribed poorly and subsequently generates less aRNA than pure RNA samples. Phenol extract and ethanol precipitate your RNA.
	Lower than suspected input RNA concentration	Take another A_{260} reading of your RNA sample or try using more RNA in the aRNA amplification procedure.
	RNA integrity is compromised	RNA that is partially degraded generates cDNA that is relatively short. This will reduce the average size of the aRNA population and subsequently reduce the yield of aRNA. You can assess the integrity of an RNA sample by determining the size of the 18S and 28S rRNA bands and the relative abundance of 28S to 18S rRNA.
	The mRNA content of your total RNA sample is lower than expected	Different RNA samples contain different amounts of mRNA. In healthy cells, mRNA constitutes 1-3% of total cellular RNA. The actual amount of mRNA depends on the cell type and the physiological state of the sample. When calculating the amount of amplification, the starting mass of mRNA in a total RNA prep should always be considered a range from 10-30 ng per µg of total RNA (assuming good RNA quality). Most total RNA samples can be amplified up to 1000 fold producing 10-30 µg of aRNA from 1 µg of total RNA.
aRNA is not efficiently reverse transcribed	The cDNA procedure relies on Oligo(dT) priming	The aRNA has a poly (U) tract near the 5′ end but lacks a poly (A) tract at its 3′ end. Thus any reverse transcription procedures that rely on Oligo(dT) primers will not effectively convert aRNA to cDNA. Try using gene specific or random primers.
	The filter in the aRNA Filter Cartridge was not completely dried after the wash steps	If the aRNA contains ethanol carried over from the Wash Buffer, it can inhibit reverse transcription. Make sure that the filter is completely dry just before eluting the aRNA.
	Absorbance readings are inaccurate	Confirm that your spectrophotometer is accurate by measuring the absorbance of an RNA or DNA sample of known concentration. Alternatively, assess the quantity of aRNA by a different method such as fractionating on an agarose gel adjacent to an RNA sample whose concentration is known and comparing the ethidium bromide staining or using a sensitive RNA dye like RiboGreen.

II. Total RNA and aRNA Preparation and Analysis

Problem	Possible Cause	Suggestions
OD_{260} not within parameters	Impure RNA	Repeat RNA clean-up kit using commercial kit or precipitate RNA and dissolve again.

III. ULS Labeling

Problem	Possible Cause	Suggestions
Degree of labeling too low	There may be salt present which disturbs labeling	Clean up aRNA and ensure final 80% ethanol wash step is used with silica based columns.
	Incorrect ratio of labeling reagent to aRNA	Ensure use of instructed amount of ULS-dye per µg of aRNA.
	Concentration of the labeling reaction was under 50 ng/µL	Ensure concentration of the labeling reaction is above 50 ng/µL.
Degree of labeling too high	Incorrect ratio of labeling reagent to aRNA	Ensure use of instructed amount of ULS-dye per µg of aRNA.

IV. Array Hybridization and Detection

Problem	Possible Cause	Suggestions
Background on the slide	Too much sample added to microarray	Reduce sample amount.
	Insufficient blocking	Add more or alternative blockers to pre-hybridization or hybridization buffer. If not added previously then use KREA*block* in the hybridization mixture.
	Partial drying of hybridization buffer during hybridization due to insufficient amount of moisture in hybridization vessel	Ensure sufficient moisture is added to hybridization chamber and vessel is sealed tightly. KREA*block* can be used to provide moisture in hybridization chamber.

F. Appendix

I. Determination of RNA Quality

Measure A_{260} and A_{280} nm using a spectrophotometer and calculate $OD_{260/280}$. For good quality RNA this value should be between 1.9 and 2.1.

II. Determination of the Degree of Labeling (DOL)

Measure A_{260} and A_{550} for determining the DOL of Cy3-ULS labeled aRNA

Measure A_{260} and A_{650} for determining the DOL of Cy5-ULS labeled aRNA

$$\text{ng / µL} = \frac{A_{260} \text{ *dilution factor* } 40}{\text{cuvet length (in cm)}}$$

$$\text{pmol / µL} = \frac{A_{\text{dye at max}} \text{* dilution factor}}{\text{cuvet length *} \varepsilon_{\text{dye}} \text{*} 10^{-6}}$$

εdye Cy3 Reagent = 150,000
εdye Cy5 Reagent = 250,000

Degree of labeling (amount of dyes per 100 nucleotides)

$$\text{Labeling \%} = \frac{340 * \text{pmol}_{\text{dye}}}{\text{ng}_{\text{ nucleic acid}} \text{*1000}} \text{ x } 100\%$$

G. References

1. Van Gelder RN, von Xastrow ME, Yool A, Dement DC, Barchas JD, Eberwine JH (1990) *Amplified RNA*
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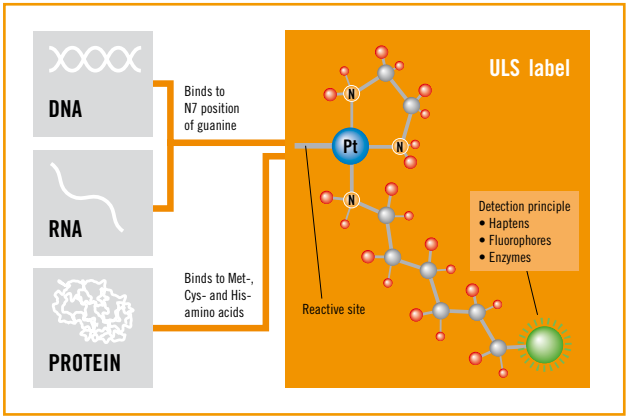

Figure. ULS, the Universal Linkage System that labels your DNA, RNA and proteins.

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