ARES™ DNA Labeling Kits

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage	Stability
5-(3-aminoallyl)-dUTP (Component A)	50 µL	2 mM in TE buffer, pH 7.6	≤–20°C	When stored as directed, product is stable for at least 6 months.
Reactive dye (Component B)	10 vials*	NA	• ≤–20°C • Avoid freeze-thaw cycles • Desiccate • Protect from light	
Dimethylsulfoxide (DMSO) or dimethylformamide (DMF) (Component C)	200 µL		• ≤25°C • Desiccate	
Sodium bicarbonate (MW = 84.0) (Component D)	84 mg		≤25°C	
Nuclease-free water (Component E)	10 mL			
*Each vial of dried reactive dye is packaged separately in a foil pouch with desiccant.				
Number of labelings: 10 labelings total, each containing 1–5 µg of DNA. Each vial of reactive dye contains sufficient reagent for one labeling.				
Approximate fluorescence excitation and emission maxima: See Table 2.				

Introduction

The ARES™ DNA Labeling Kits provide a versatile method for labeling DNA with Molecular Probes' superior fluorescent dyes. This method achieves a high degree of labeling and a uniformity and consistency that is difficult to obtain with conventional enzymatic incorporation of dye-labeled nucleotides.

The ARES™ kits employ a two-step method for labeling DNA (Figure 1). In the first step, an amine-modified nucleotide, 5-(3-aminoallyl)-dUTP, is incorporated into DNA using standard enzymatic labeling methods. Because there is no bulky dye molecule on the nucleotide to restrict the enzymatic labeling rate, this reaction results in a reliably high level of amine-modification for every sample.

In the second step, the amine-modified DNA is chemically labeled using an amine-reactive, fluorescent dye. This chemical reaction varies little in its efficiency from dye to dye, and results in consistent labeling levels, regardless of the dye chosen.[1] The protocols provided here yield a labeling efficiency of about 4–8 dyes per 100 bases, which we have found optimal for fluorescence *in situ* hybridization (FISH) and microarray hybridization.

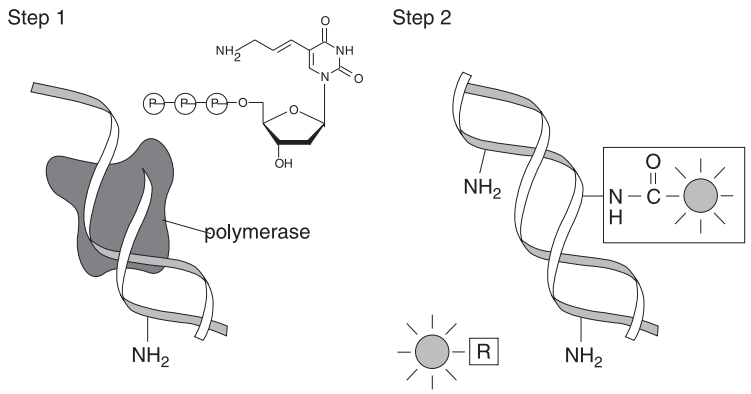

Figure 1. The ARES™ DNA Labeling Kits use a two-step method to label DNA. **Step 1)** The aminoallyl dUTP is enzymatically incorporated. **Step 2)** A reactive fluorophore is used to label the incorporated aminoallyl group.

The kits are supplied with Molecular Probes' state-of-the-art fluorescent dyes, conveniently packaged in single-use vials. The Alexa Fluor® dyes have properties superior to conventional dyes, including exceptionally high quantum yields and large extinction coefficients, excellent photostability, reduced pH sensitivity, and improved water solubility.

These versatile kits work well with reverse transcription, nick translation, and random priming and should also be suitable for other enzymatic labeling techniques. The ARES™ Kits are especially useful for labeling cDNA for microarray applications, where the consistency of labeling between samples is critical for accurate interpretation of results.[2,3] The wide selection of dyes and uniform labeling results also make the kits ideal for other multicolor applications, such as multicolor FISH[4] and CGH (comparative genome hybridization).

Before You Begin

Materials Required but Not Provided

- Reagents required for the enzymatic incorporation reaction, including unlabeled nucleotides (see protocols below)
- PureLink™ PCR Purification Kit (Cat. no. K3100-01)
- 3 M sodium acetate, pH 5.2

Using DMSO

The solvent (Component C), DMSO or DMF, is hygroscopic, absorbing water from the atmosphere quite readily. DMSO is a solid at refrigeration temperatures. To minimize moisture contamination during thawing, warm the vial completely to room temperature before opening and recap it immediately after use.

Spectral Characteristics

For the best results in experiments, it is important to match the light source, excitation filters, and emission filters to the spectral characteristics of the dye. Refer to Table 2 for this information.

Table 2. Spectral characteristics of the fluorescent dyes available in the ARES™ DNA Labeling Kits.

Cat. no	Fluorescent Dye	λ_{max} (nm) *	Em (nm) †	ε_{dye} ($cm^{-1}M^{-1}$) ‡	CF_{260} §	Spectrally Similar Dyes
A21665	Alexa Fluor® 488	492	520	62,000	0.30	Fluorescein (FITC, FAM)
A21667	Alexa Fluor® 546	554	570	112,000	0.21	Cy™3, tetramethylrhodamine (TRITC, TAMRA)
A21677	Alexa Fluor® 555	555	565	150,000	0.04	Cy™3, tetramethylrhodamine (TRITC, TAMRA)
A21669	Alexa Fluor® 594	588	615	80,400	0.43	Texas Red®
A21676	Alexa Fluor® 647	650	670	239,000	0.00	Cy™5

* Excitation (absorption) maximum for the fluorophore. † Emission maximum for the fluorophore. ‡ Extinction coefficient for the dye.
§ Correction factor = A_{260} for the free dye / A_{max} for the free dye.

Enzymatic Labeling Protocols

Reverse Transcription

We have optimized a reverse transcription labeling protocol using 20–30 µg of total human RNA, 5 µg of anchored oligo (dT) primer ($(dT)_{20}VN$), and SuperScript™ II reverse transcriptase (Cat. no. 18064). We have empirically determined that the ratio of dTTP to aminoallyl-dUTP used in this protocol, with subsequent labeling by an amine-reactive dye, results in optimally-labeled samples for hybridization to microarrays.

1.1 Synthesize amine-modified cDNA. Perform cDNA synthesis according to the reverse transcriptase manufacturer's protocol but use the following **final** concentrations of nucleotides: 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.15 mM dTTP, and 0.30 mM aminoallyl-dUTP. A total reaction volume of 20 µL is usually sufficient.

1.2 Remove the RNA. Hydrolyze the RNA using the following protocol:

- Place the reverse transcription reaction at 95°C for 5 minutes to inactivate the reverse transcriptase and denature the RNA:cDNA hybrids. Snap cool by placing the reaction immediately into an ice bath.
- Add 0.43 volumes of 1 M NaOH for a concentration of 0.3 M, mix and incubate at 65°C for 15 minutes.
- Neutralize the solution by adding a volume of 1 M HCl equal to the volume of 1 M NaOH added in the previous step.
- Add 0.11 volumes (relative to the neutralized solution) of 1 M Tris-HCl, pH 7.0, for a final concentration of 0.1 M.

For example, if your cDNA synthesis reaction has a final volume of 10 µL, add 4.3 µL of 1 M NaOH to hydrolyze the RNA, neutralize the solution by adding 4.3 µL of 1 M HCl, and then add ~2 µL of 1 M Tris-HCl, pH 7.0 (0.11 × 18.6 µL).

1.3 Prepare amine-modified DNA for purification. Bring the mixture to a final volume of 100 µL with nuclease-free water (Component E) and proceed to *Purifying Amine-Modified DNA* below.

Nick Translation

We have optimized a nick translation labeling protocol using 1 µg of an 8 kb DNA template and the enzymes listed below. We have empirically determined that the ratio of dTTP to aminoallyl-dUTP used in this protocol, with subsequent labeling by an amine-reactive dye, results in optimal probes for fluorescence *in situ* hybridization (FISH) to metaphase chromosome spreads.

2.1 Prepare 10X nick-translation buffer. This buffer is 0.5 M Tris-HCl, 50 mM $MgCl_2$, 0.5 mg/mL nuclease-free BSA, pH 7.8.

2.2 Prepare the aminoallyl-dUTP. Dilute a portion of the aminoallyl-dUTP (Component A) 4-fold in nuclease-free water (Component E) to a final concentration of 0.5 mM.

2.3 Prepare DNase I stock solution.

a. On ice, dissolve 1 mg of DNase I in 1 mL of cold 20 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol (DTT), 100 µg/mL nuclease-free BSA, 50% glycerol, pH 7.6. This stock solution should have an activity of approximately 2000 Kunitz units/mg.

b. Mix gently. Do not vortex. Store aliquots at ≤–20°C.

2.4 Prepare fresh DNase I working solution. Dilute 1 µL of the 1 mg/mL DNase I stock solution (from step 2.3) into 1 mL of cold 1X nick-translation buffer. Leave on ice.

2.5 Prepare the reaction mixture. Add the following to a microfuge tube in the order indicated, adjusting the volume of water if necessary to achieve a final volume of 50 µL:

- 21.5 µL nuclease-free water (Component E)
- 5 µL 10X nick-translation buffer (from step 2.1)
- 5 µL 0.1 M DTT
- 4 µL d(GAC)TP mix (0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP)
- 1 µL 0.5 mM dTTP
- 6 µL 0.5 mM aminoallyl-dUTP (from step 2.2)
- 1 µL DNA template, 1 µg/µL
- 5 µL DNase I (from step 2.4)
- 1.5 µL DNA polymerase I, 10 U/µL

2.6 Incubate at 15°C for 2 hours.

2.7 Bring to a final volume of 100 µL with 50 µL nuclease-free water (Component E) and proceed to *Purifying Amine-Modified DNA* **below.**

Random Priming

The ARES™ random prime labeling protocol was optimized with 1 µg of a 200 Kb BAC DNA clone using Invitrogen's Random Primers DNA Labeling System (Cat. no. 18187-013), DNA polymerase I, large (Klenow) fragment (Cat. no. 18012-021), and the aminoallyl dUTP-containing nucleotide mix described below.

The ratio of dTTP to aminoallyl dUTP used in this protocol was empirically determined and with subsequent labeling by amine-reactive dye, results in optimal probes for fluorescence in situ hybridization (FISH) to metaphase chromosome spreads.

3.1 Prepare 10X nucleotide mix. Stock nucleotide concentrations are given in parentheses.

- 2 µL dATP (10 mM)
- 2 µL dGTP (10 mM)
- 2 µL dCTP (10 mM)
- 2.8 µL dTTP (1 mM)
- 8.6 µL aminoallyl-dUTP (2 mM)
- 2.6 µL nuclease-free water (to 20 µL total volume)

3.2 Denature DNA template.

a. Combine 1 µg of DNA template and nuclease-free water into a total volume of 8 µL.

b. Heat denature at 95°C for 5 minutes.

c. Immediately cool by incubating the tubes in an ice water slurry for 5 minutes.

3.3 Prepare reaction mixture on ice. Add the following reagents to the denatured DNA on ice in the order indicated:

- 15 µL Random Primers Buffer Mixture
- 5 µL 10X nucleotide mix from step 3.1
- 21 µL nuclease-free water.
- 1 µL Klenow enzyme (3 units/µL).

Mix reaction gently by pipetting reaction up and down several times.

3.4 Incubate reaction at 37°C for 4 hours to overnight. Longer incubation times will not affect the yield or degree of labeling.

3.5 Bring to a final volume of 100 µL with 30 µL of nuclease-free water (Component E) and proceed to *Purifying Amine-Modified DNA* **below.**

Purifying Amine-Modified DNA

4.1 Purify the amine-modified DNA using a PureLink™ PCR Purification Kit (Cat. no. K3100-01 or K3100-02), following the instructions in the kit.

4.2 Perform an ethanol precipitation. This step is very important because it will remove the Tris buffer from the reaction mixture. Tris contains primary amines, which will interfere with the labeling reaction with the reactive fluorescent dye.

a. Precipitate the amine-modified DNA by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol.

b. Incubate at –70°C for 30 minutes and then centrifuge for 15 minutes at 12K rpm.

c. Wash the pellet with 70% ethanol and allow it to air dry.

Note: Do **not** use ammonium acetate for precipitation as the residual ammonium ions will interfere with the reactive dye labeling step. Adding ~20 µg of glycogen helps to precipitate the DNA, and generally results in greater DNA recovery.

Labeling with a Reactive Fluorescent Dye

5.1 Prepare labeling buffer. Add 1 mL of nuclease-free water (Component E) to the vial of sodium bicarbonate (Component D), and vortex the solution until the solid is completely dissolved. Store the labeling buffer at ≤–20°C. When properly stored, labeling buffer are stable for at least 6 months.

5.2 Thaw out the kit components. Warm the DMSO or DMF (Component C), labeling buffer (prepared in step 5.1), and nuclease-free water (Component E) to room temperature. Once thawed, vortex the labeling buffer thoroughly.

5.3 Prepare the amine-modified DNA. Thoroughly dissolve 1–5 µg of the amine-modified DNA (made in the previous section or by another labeling technique) in 5 µL of nuclease-free water (Component E), warming in a 42°C waterbath for 5 minutes if necessary.

Note: Although not essential, prior to labeling, you can denature nick-translated or random-primed amine-modified DNA for 5 minutes at 95°C, and then snap cooled on ice. Denaturing the amine-modified DNA improves the subsequent labeling of the amine-modified DNA with amine-reactive dyes by 10–20%.

5.4 Add labeling buffer. Add 3 µL of labeling buffer (prepared in 5.1) to the amine-modified DNA.

5.5 Prepare the reactive dye.

a. Just before use, dissolve one vial of the reactive dye (Component B) in 2 µL of solvent (Component C, either DMF or DMSO, depending on the kit).

b. Vortex for about 10 seconds to ensure that the dye is completely dissolved. It may be useful to remove the label of the vial in order to see the dye more clearly. Sufficient dye is supplied for labeling 1–5 µg DNA.

c. Once the reactive dye has been dissolved, perform the reaction immediately. You cannot save the dissolved dye for later use.

5.6 Add amine-modified DNA to the reactive dye. Add the 8 µL of the amine-modified DNA (from step 5.4) to the dissolved dye (from step 5.5). Vortex for 15 seconds to ensure that the reaction is well-mixed.

5.7 Incubate the reaction **in the dark** at room temperature for 1 hour.

5.8 Proceed to *Purifying Dye-Labeled DNA* **below.**

Purifying Dye-Labeled DNA

6.1 Purify the dye-labeled DNA. Add 80 µL of nuclease-free water (Component E) and 10 µL of 3 M sodium acetate (pH 5.2) to the reaction mixture, and purify the labeled DNA using a PureLink™ PCR Purification Kit (Cat. no. K3100-01 or K3100-02), following the instructions in the kit.

6.2 Perform an ethanol precipitation. Precipitate the labeled DNA as before (step 4.2). Resuspend the DNA pellet in 20 µL of TE buffer.

Calculating the Labeling Efficiency and Concentration of Nucleic Acid

You can evaluate the relative efficiency of a labeling reaction by calculating the approximate ratio of dye to nucleic acid bases. You can determine this ratio, as described below, by measuring the absorbance of the nucleic acid at 260 nm and the absorbance of the dye at its excitation maximum (λ_{max}). The calculations are based on the Beer-Lambert law:

$$A = \varepsilon \times \text{path length (cm)} \times \text{concentration (M)},$$

where ε is the molar extinction coefficient in $cm^{-1}M^{-1}$.

You can also use the absorbance measurements to determine the concentration of nucleic acid in the sample. You can find the values needed for these calculations in Tables 2 and 3. Alternatively, you can determine the ratio by using the Dye:Base Ratio Calculator on our website (www.invitrogen.com). Optimal labeling for FISH to metaphase chromosomes or hybridization to microarrays is 4–8 dyes per 100 bases. Higher levels of labeling may interfere with hybridization.

Table 3. Values for a base in different nucleic acids.

Nucleic Acid	ε_{base} (cm^{-1} M^{-1}) *	MW_{nt} †
dsDNA	6600	330
ssDNA	8919	330

*Average extinction coefficient for a base. † Average molecular weight of a nucleotide residue (g/mol).

Measuring the Base:Dye Ratio

7.1 Measure the absorbance of the nucleic acid–dye conjugate at 260 nm (A_{260}) and the λ_{max} for the dye (A_{dye}). Measure the background absorbance at 260 nm and λ_{max}, using buffer alone, and subtract these numbers from the raw absorbance values for the sample. You can find the λ_{max} values for the fluorophores used in the ARES™ kits in Table 2.

- To perform these measurements, the nucleic acid–dye conjugate should be at a concentration of at least 5 µg/mL. Depending on the dye used and the degree of labeling, a higher concentration may be required.
- For most applications, it will be necessary to measure the absorbance of the entire sample using a conventional spectrophotometer with a 100 µL or 200 µL cuvette or an absorbance microplate reader with a microplate.
- Use a cuvette or microplate that does not block UV light and that is clean and nuclease-free. Note that most plastic disposable cuvettes and microplates have significant absorption in the UV.

7.2 Correct for the contribution of the dye to the A_{260} reading. Most fluorescent dyes absorb light at 260 nm as well as at their λ_{max}. To obtain an accurate absorbance measurement for the nucleic acid, you must account for the dye absorbance using a correction factor (CF_{260}). Use the CF_{260} values given in Table 2 in the following equation:

$$A_{base} = A_{260} - (A_{dye} \times CF_{260})$$

7.3 Calculate the ratio of dye to nucleic acid bases. Use the following equation:

$$\text{dye:base} = \frac{100}{(A_{base} \times \varepsilon_{dye}) / (A_{dye} \times \varepsilon_{base})}$$

where ε_{dye} is the extinction coefficient for the fluorescent dye (found in Table 2) and ε_{base} is the average extinction coefficient for a base in double stranded DNA (dsDNA) or single-stranded DNA (ssDNA) (found in Table 3). Note that since the calculation is a ratio, the path length has canceled out of the equation. The value determined indicates the number of dyes per 100 bases.

Measuring the Concentration of Nucleic Acid

You may also use the absorbance values A_{260} and A_{dye} to measure the concentration of nucleic acid in the sample ([N.A.]). To obtain an accurate measurement for a dye-labeled nucleic acid, you must use a dye-corrected absorbance value (A_{base}) , as explained in step 7.2. In addition, for concentration measurements, the path length (in cm) is required. If the path length of the cuvette or of the solution in a microplate well is unknown, consult the manufacturer. Follow steps 7.1 and 7.2 above, and then use the following equation:

$$[\text{N.A.}]\ (\text{mg/mL}) = (A_{base} \times MW_{base}) / (\varepsilon_{base} \times \text{path length})$$

References

1. Biotechniques 36, 114 (2004); **2.** J Bacteriology 183, 7027 (2001); **3.** Anal Biochem 331, 243 (2004); **4.** Science 305, 846 (2004).

Patent and Trademark Information

The use of labeled nucleic acid hybridization probes may be covered by patents belonging to third parties. Purchase of these kits does not provide a license to practice inventions covered by those patents.

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat. no.	Product Name	Unit Size
A21665	ARES™ Alexa Fluor® 488 DNA Labeling Kit *10 labelings*	1 kit
A21667	ARES™ Alexa Fluor® 546 DNA Labeling Kit *10 labelings*	1 kit
A21669	ARES™ Alexa Fluor® 594 DNA Labeling Kit *10 labelings*	1 kit
A21676	ARES™ Alexa Fluor® 647 DNA Labeling Kit *10 labelings*	1 kit
A21677	ARES™ Alexa Fluor® 555 DNA Labeling Kit *10 labelings*	1 kit
Related Products		
18012-021	DNA Polymerase I, Large (Klenow) Fragment	100 units
18064-022	SuperScript® II Reverse Transcriptase	2,000 units
18187-013	Random Primers DNA Labeling System *30 reactions*	1 kit
K3100-01	PureLink™ PCR Purification Kit *50 reactions*	1 kit

Contact Information

Molecular Probes, Inc.
29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:
6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
probesorder@invitrogen.com

Toll-Free Ordering for USA:
Order Phone: (800) 438-2209
Order Fax: (800) 438-0228

Technical Service:
8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probestech@invitrogen.com

Invitrogen European Headquarters
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

For country-specific contact information, visit www.invitrogen.com.

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