The ULYSIS® kits provide an optimized, one-step direct DNA labeling method with a new family of fluorescent dyes that span the visible spectrum

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Abstract

The Universal Linkage System (ULS®) is a novel DNA labeling method utilizing platinum-based chemistry to directly label nucleic acids with fluorescent dyes or haptens. We synthesized ten new ULS reagents, including conjugates of eight Alexa Fluor® dyes and Pacific Blue™ dye, for use as fluorescent labels, and Oregon Green 488® dye, for use as a hapten in combination with anti-fluorescein antibodies. We optimized the labeling reaction employing these reagents, and developed a protocol that is compatible with a variety of applications, including multicolor fluorescence *in situ* hybridization. We identified and optimized eight parameters that are critical to the labeling reaction, including temperature, pH, buffer, time, ULS reagent and DNA concentration, DNA size and DNA denaturation. In addition, we optimized the purification procedure. We found that the concentration of ULS reagent in the reaction determined the degree of labeling achieved, and that the degree of labeling was highly reproducible from experiment to experiment, regardless of the source of the DNA. We performed hybridization studies to determine the optimal degree of labeling needed. Using calf thymus DNA to probe dot blots, we found that, depending upon the dye, probes having an average of one dye per 8–50 bases gave the best signals. Furthermore, this same level of labeling was found to be optimal for performing *in situ* hybridization with alpha satellite probes to human chromosome 17. Importantly, while it was possible to achieve still higher degrees of labeling using this method, we found that probe over-labeling decreased hybridization efficiencies, resulting in lower signals. The optimized ULS protocol yielded sensitive probes compatible with a wide variety of instrumentation and filters. This labeling technique combined with the high intensity and photostability of the Alexa Fluor dyes provided a simple, rapid, and versatile DNA labeling method, with excellent reproducibility.

Introduction

Traditional DNA labeling methods (e.g., random priming or nick translation) are enzyme-mediated and inherently inconsistent. The efficiency of these reactions is dictated by the enzyme's ability to incorporate a labeled nucleotide into a growing nucleic acid chain. Labeled nucleotides are relatively poor substrates for DNA and RNA polymerases, because the dye itself, as well as the dye-nucleotide linker, can dramatically affect the incorporation efficiency of the modified nucleotide. Consequently, the efficiency of these reactions is relatively low and is highly variable. Furthermore, these inconsistencies in generating probes make comparative experiments difficult and interpretation of hybridization assays problematic. Low incorporation rates can lead to low signals requiring more probes per target, and decreased sensitivity to low copy number targets. In addition, these types of methods require large numbers of reagents and expensive enzymes, and are time-intensive. Reaction inconsistencies can require a great deal of optimization on the researcher's part to overcome experimental irreproducibility.

Using KREATECH Diagnostics' ULS chemistry and Molecular Probe's fluorophores that span the visible spectrum, we have optimized a protocol for reliably chemically labeling DNA with Alexa Fluor dye ULS labeling reagents. Direct and consistent labeling is complete in only 15 minutes. Labeled probes are indefinitely stable and are compatible with a wide range of molecular biology and molecular cytogenetic applications, including chromosome FISH experiments, comparative genome hybridization (CGH) studies, mutation detection on arrays, and other hybridization techniques.

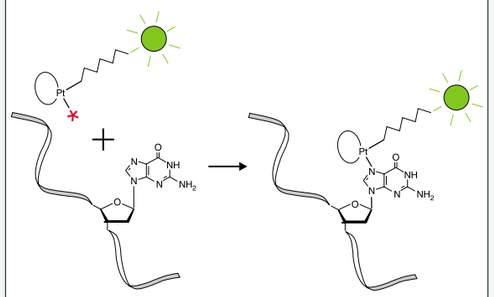


FIGURE 1. The ULS reagent in the ULYSIS Nucleic Acid Labeling Kits reacts with the N_7 position of guanine residues to provide a stable coordination complex between the nucleic acid and the fluorophore label.

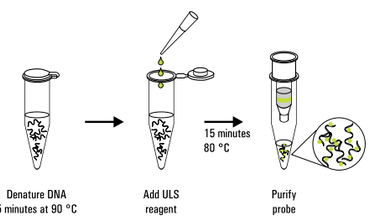


FIGURE 2. The Procedure for using the ULYSIS nucleic acid labeling kits.

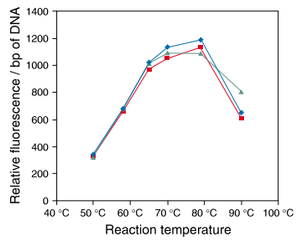


FIGURE 3. ***Optimization of reaction temperature***
Triplicate reactions of Alexa Fluor 532 ULS labeling reagent with calf thymus DNA were carried out at each temperature for 15 minutes. Labeled DNA was purified using Micro Bio-Spin® P-30 Tris spin columns (Bio-Rad Laboratories). Relative fluorescence values were determined using a SpectraMax Gemini microplate reader (Molecular Devices, Inc.). Increased fluorescence per base pair of DNA indicated an increase in labeling efficiency as the temperature increased. The fluorescence decline above 80YC denoted a noticeable loss in labeling efficiency. All ULS labeling reagents tested showed similar temperature responses, indicating that 80YC was the optimal temperature for the reaction.

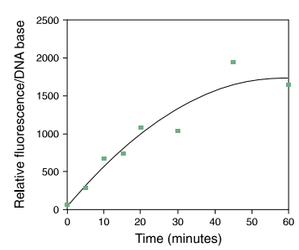


FIGURE 4. ***Effects of reaction duration on labeling efficiency***
Identical reactions containing calf thymus DNA and Oregon Green 488 ULS labeling reagent were incubated at 80YC for varying amounts of time and then purified, and relative fluorescence values measured using a fluorescence microplate reader. Although the data indicate that higher degrees of labeling can be achieved by allowing the reaction to proceed for longer periods of time, we elected to standardize all reactions by limiting the reaction time to 15 minutes. This has proven to provide a highly controlled and reproducible level of labeling.

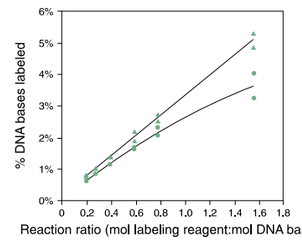


FIGURE 5. ***Effects of DNA heat denaturation on labeling efficiency***
Calf thymus DNA was stored on ice (●) or alternatively, was heat denatured at 95YC for 5′ and then snap-cooled on ice (▲), prior to labeling with increasing amounts of Oregon Green 488 ULS labeling reagent for 15 minutes at 80YC. Absorbance measurements were performed on the purified labeled DNAs with a HTS 7000 microplate reader (Perkin Elmer, Inc.) to determine the percentage of labeling. We found heat denaturation of the sample DNA prior to labeling with the ULS labeling reagent increased labeling efficiency by an average of 20%, suggesting that an increase in DNA accessibility to the labeling reagent by removing secondary structures increases labeling efficiency.

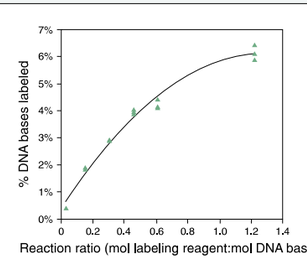


FIGURE 6. ***Labeling efficiency of Alexa Fluor 488 ULS labeling reagent on calf thymus DNA***
Triplicate reactions of Alexa Fluor 488 ULS labeling reagent with purified calf thymus DNA were performed with varying molar ratios of labeling reagent to DNA bases at 80YC for 15 minutes in 5mM Tris, 1mM EDTA pH 8.0, and purified by spin column. Absorbance measurements of the samples were performed in a microplate reader to determine the percentage of labeling. We found that increasing the amount of ULS labeling reagent in the reaction corresponded with an increase in labeling efficiency, peaking near 6% labeling efficiency (averaging 1 dye for every 17 bases).

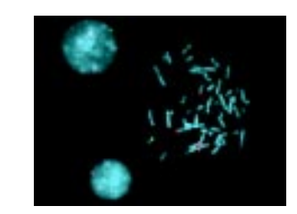


FIGURE 7. ***Probes for multi-color fluorescence in situ hybridization (mFISH) prepared using ULYSIS Nucleic Acid Labeling Kits.***
Centromere probes to chromosome 15, chromosome 17 and chromosome 1 were labeled with ULYSIS Alexa Fluor 532, Alexa Fluor 594 and Oregon Green 488 Nucleic Acid Labeling Kits, respectively, and hybridized to human metaphase chromosomes. The chromosomes were counterstained with DAPI. The multiple-exposure image was acquired using filter sets appropriate for fluorescein, Alexa Fluor 532, and Alexa Fluor 594.

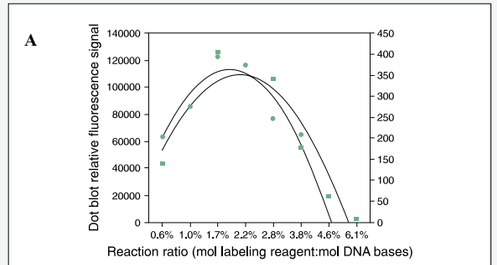
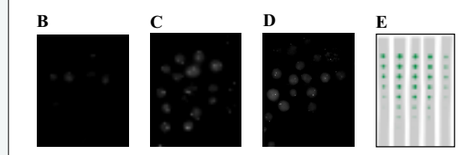


FIGURE 8. ***Determination of optimal labeling ratio for Alexa Fluor 488 ULS labeling using dot blot and FISH hybridization techniques***
We produced calf thymus DNA probes labeled with increasing amounts of Alexa Fluor 488 ULS labeling reagent. We hybridized these probes to calf thymus DNA dot blots using standard hybridization protocols and stringent wash conditions. Blots were imaged on the Wallac ARTHUR™ multi-wavelength fluoroimager (Perkin Elmer, Inc.) and relative fluorescent values were determined by integration of fluorescent signals across the area of the dot (■) using ImageGauge software (FUJIFilm, Inc.). Similarly labeled alpha satellite probes to human chromosome 17 were hybridized to human chromosome spreads, and imaged with a fluorescence microscope equipped with a CCD camera and appropriate filter sets. Fluorescence intensity was integrated using MetaMorph software (Universal Imaging, Co.) across the area of the signal (●). We observed a high degree of correlation between the dot blot and FISH hybridization studies, with both showing optimal signals at 2% degree of labeling (1 dye for every 50 DNA bases) for this particular dye (panel A). Higher and lower degrees of labeling gave a markedly poorer signal. We hypothesize that higher than optimal degrees of labeling inhibit probe-target hybridization stability and results in a lower number of total probes per target region.



Alpha satellite probes to human chromosome 17 were produced using Alexa Fluor 488 ULS labeling reagent and used for FISH experiments on human chromosome spreads with 1 dye per 156 DNA bases (panel B), 1 dye in 60 bases (panel C), and 1 dye in 27 bases (panel D). Probe signal intensity differences are easily seen across the three panels. These data correlates with the dot blots (panel E) probed with DNA's labeled with (from left to right) 1 dye per 156 bases, 1 dye per 60 bases, 1 dye per 36 bases, 1 dye per 27 bases, and 1 dye per 22 bases. Hybridized probe fluorescent signal intensity increases as the number of fluorophores per probe increase until the optimal labeling level is reached (1 dye per 60 bases), after which point additional Alexa Fluor 488 dyes inhibit hybridization and decrease total signal intensity.

Fluorophore	Ex/Em *	ε_{Max} (cm^{-1} M^{-1}) †	CF_{260} ††	Labeling Ratio for Optimal Hybridization Signal ‡
Pacific Blue	410/455	36,000	0.15	1 dye per 12 bases
Oregon Green 488	496/520	80,000	0.31	1 dye per 40 bases
Alexa Fluor 488	492/520	62,000	0.30	1 dye per 50 bases
Alexa Fluor 532	525/550	82,300	0.24	1 dye per 8 bases
Alexa Fluor 546	555/570	104,000	0.21	1 dye per 50 bases
Alexa Fluor 568	576/600	93,000	0.45	1 dye per 50 bases
Alexa Fluor 594	588/615	80,400	0.43	1 dye per 50 bases
Alexa Fluor 647	647/670	239,000	0.00	1 dye per 20 bases
Alexa Fluor 660	660/690	107,000	0.00	1 dye per 30 bases
Alexa Fluor 680	680/700	164,000	0.00	1 dye per 18 bases

* Approximate excitation (ex) and emission (em) maxima in nm determined in buffer
† Extinction coefficient for the dye
†† Correction Factor = A_{260} for the free labeling reagent / A_{max} for the labeling reagent
‡ As determined by dot-blot hybridization signal studies

FIGURE 9. ***Key characteristics of ULS labeling reagents in ULYSIS kits***
Labeling reagents provided in the ULYSIS kits retain the spectral properties of their dye counterpart. Optimal labeling ratios for peak hybridization signal intensities were individually determined for each labeling reagent by dot-blot hybridization experiments.

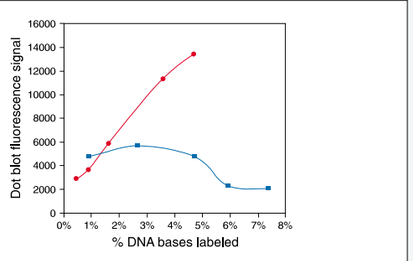


FIGURE 10. ***Comparative hybridized fluorescence of Alexa Fluor 647 dye–labeled probe versus Cy5 dye–labeled probe***
To compare the new Alexa Fluor 647 dye to the spectrally similar Cy5 dye, we labeled calf thymus DNA to varying degrees with Alexa Fluor 647 ULS labeling reagent and Cy5 ULS labeling reagent. Probes were then used for dot blot hybridization comparisons using standard procedures and stringent wash conditions. With increased degree of labeling, the Alexa Fluor 647 dye–labeled probes (●) showed a steady increase in fluorescence signal. In sharp contrast, the Cy5 dye–labeled probes (■) showed no appreciable increase in fluorescence as labeling level increased, and at the highest levels of labeling showed a dramatic decrease in fluorescence. Solution-based experiments suggest this is not a hybridization inhibition event, but rather a dye quenching phenomena (data not shown).

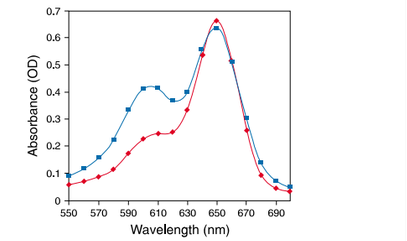


FIGURE 11. ***Spectral absorbance shift of Cy5 dye–labeled probe versus free Cy5 dye***
Previously published work by Hermann Gruber at the Johannes Kepler University in Austria (Gruber HJ, et al, Bioconjug Chem 2000 Sep-Oct; 11(5): 696-704) has shown that high degrees of Cy5 dye labeling of antibodies shows a marked quenching of fluorescent signal characterized by an associated increase of the 600nm component of the Cy5 absorbance spectra. Our data indicates a similar correlation between increased quenching of Cy5 dye–labeled DNA probes and changes in the absorbance spectra of the labeled dye (■) compared to free Cy5 dye (◆).

Summary

- ULYSIS nucleic acid labeling kits provide a rapid and reliable alternative to current enzymatic labeling procedures.
- Time, temperature, and quantity of ULS labeling reagent have been optimized to yield the highest signal possible for hybridization experiments.
- Available ULS labeling reagents spanning the visible spectrum are compatible with many multicolor molecular biology and cytogenetic application including mFISH experiments.
- Oregon Green ULS labeling reagent may be used as a hapten for amplification strategies with antifluorescein antibodies.
- Alexa Fluor 647 ULS labeling reagent is a brighter alternative as a deep red dye than Cy5 ULS labeling reagent.

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