# Tagging DNA mismatches by selective 2′-amine acylation

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**Background:** Widespread characterization of genetic variation and disease at the gene-sequence level has inaugurated a new era in human biology. Techniques for the molecular analysis of these variations and their linkage with measurable phenotypes will profoundly affect diverse fields of biological chemistry and biology.

**Results:** A chemical tagging method has been developed to detect point mutations and other defects in nucleic acid sequences. The method employs oligodeoxynucleotide probes in which one 2′-ribose position (–H) is substituted with an amine ($–NH_2$) group. 2′-Amine-substituted nucleotides are specifically acylated by succinimidyl esters to form a 2′-amide product. The mutation detection method exploits our observation that 2′-amine groups at the site of a mismatch are acylated more rapidly than amine substitutions at base-paired nucleotides. 2′-Amine acylation is governed primarily by local, rather than global, differences in nucleotide dynamics, such that site-specific tagging of DNA mismatches does not require discriminatory hybridization conditions to be determined.

**Conclusions:** 2′-Amine mismatch tagging offers an approach for chemically interrogating the base-paired state of individual nucleotides in a hybridized duplex and for quantifying nucleicacid hybridization with single-base specificity.

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## Introduction

Much effort has focused on methods for detecting and scoring the thousands of small genetic differences, including single nucleotide polymorphisms (SNPs), that ultimately distinguish one individual from another [1,2]. Important methods for scoring single nucleotide changes in DNA sequences derive a signal from hybridization of a probe oligonucleotide with a target sequence. The presence of a mutation at a given position produces a DNA mismatch or bulge upon probe hybridization. Imperfect duplexes can be subsequently detected through their reduced thermal stability [3–5], by selective amplification [6], as substrates for mismatch repair and endonuclease enzymes [7–9], using differential chemical cleavage [10–12], and with DNA chips [13,14]. Mutation readout can be achieved using chromatographic [1,10,11], fluorescent [4,5,13] or electrochemical [15–17] methods.

One challenge in mutation detection lies in defining discriminatory hybridization conditions. For example, many perfect helices containing a majority A–T base pairs have a lower stability than G–C rich duplexes with one or more mismatches [18–20]. This problem of thermodynamic stringency is a significant consideration in the application of hybridization approaches to SNP scoring.

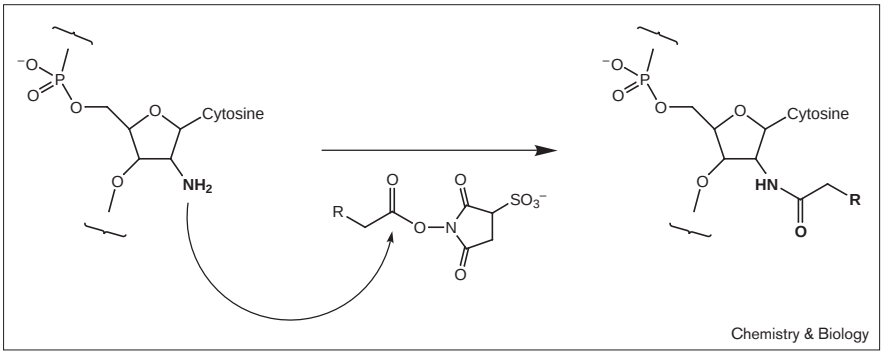
Alternatively, chemical cleavage of mismatches [12] and enzymatic cleavage approaches [7–9], are sensitive to local disruptions in duplex structure. These methods can, in principle, detect any mismatch in the hybridized substrate, which is an advantage when screening for SNPs, but a disadvantage when trying to interrogate a single site in a sequence.

Desired are methods that selectively tag, in a site-specific way, single-base mismatches in DNA without the requirement to identify discriminatory hybridization conditions. Ideally, such a method would also be generically compatible with the most appropriate detection technology.

Acylation of 2′-amine-substituted nucleotides offers an approach for site-specifically querying and tagging a mismatched nucleotide in a hybridized duplex. 2′-Amine-substituted nucleotides in RNA or DNA react with a variety of electrophilic reagents including S-ethyl trifluoroacetate [21], isothiocyanates [22] and succinimidyl esters [23]. Reaction with a succinimidyl ester yields the 2′-amide product (Figure 1).

In the case of $tRNA^{Asp}$, acylation of 2′-amine groups using a succinimidyl ester is gated by the underlying flexibility in the RNA secondary and tertiary structure [24]. Thus, nucleotides involved in base-paired secondary structure or those that form stable tertiary interactions react with the ester more slowly than unconstrained nucleotides. Relative rates of 2′-amine acylation at individual nucleotides are strongly correlated, not with static solvent accessibility, but with local nucleotide flexibility, as measured, for

**Figure 1**




2′-Amine chemistry for tagging DNA mismatches. Reaction of a 2′-amine substituted nucleotide with an activated ester yields the 2′-amide product. The R group could be any detection moiety; in these experiments it is a biotinamido butyl group.

example, by crystallographic temperature factors [24]. Because mismatched DNA nucleotides are more dynamic locally [25,26] than residues forming canonical base pairs, 2′-amine acylation might therefore be used to tag selectively mismatches in hybridized oligonucleotides.

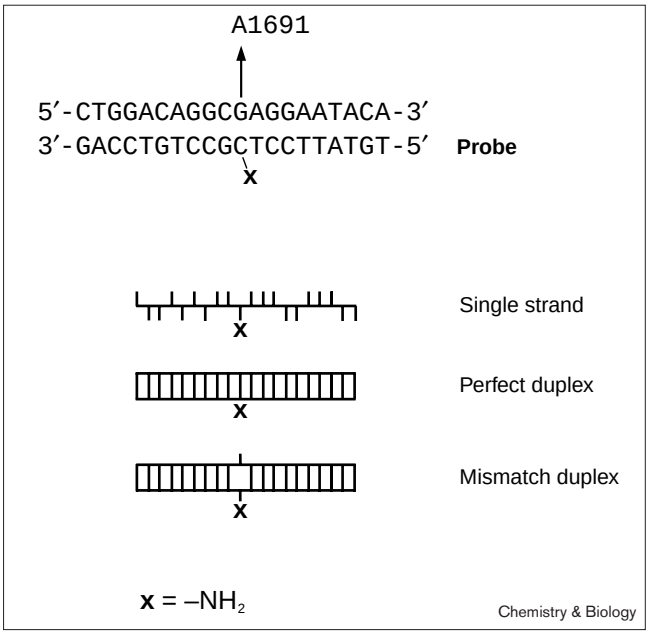
## Results and discussion

### Oligonucleotide model for detecting a biologically important mutation in DNA

For DNA mutation detection, we employed oligodeoxynucleotide probes in which one 2′-ribose position was substituted with an amine group. The reaction is selective for the unique 2′-amino ribonucleotide in the probe (see Figure 1) because of the greater nucleophilicity of the aliphatic amine compared with the base arylamines naturally found in DNA.

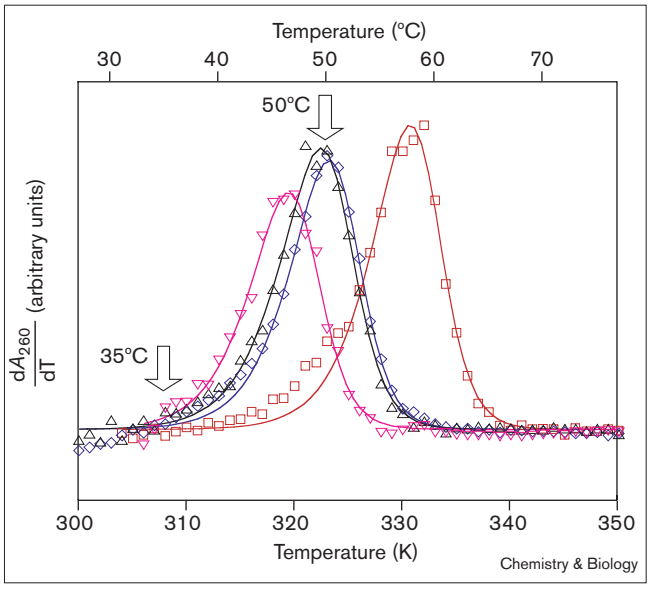
As a representative example, we focused on the DNA sequence corresponding to the human Factor V clotting factor. A single point mutation, a guanine to adenine substitution at position 1691 in the Factor V gene (G1691→A), termed Factor V Leiden (FVL), occurs at a frequency of ~5% in individuals of European descent [27]. This point mutation leads to replacement of

**Figure 2**




Oligonucleotide model system for detection of the Factor V Leiden (G1691→A) mutation. Hybridization of the 2′-amine-containing probe with complementary oligonucleotides containing either the wild-type or the Factor V Leiden allele yields a perfect duplex or a mismatch-containing duplex, respectively. The Factor V sense strand is written in the 5′→3′ direction.

**Figure 3**




Differential thermal melting curves for the 20-base-pair perfect duplex (squares) and for C–A (diamonds), C–T (triangles) and C–C (inverted triangles) mismatch-containing duplexes. Denaturation was monitored in 100 mM Na–Hepes (pH 8) and 10% DMSO, and thermodynamic parameters were obtained by fitting the differential denaturation curves to equations 1–3 (see the Materials and methods section).

**Table 1**

**Thermodynamic parameters and acylation rates for 2′-amine-containing single-stranded probe and duplexes.**

| | | | 35°C | | 50°C | |
|---|---|---|---|---|---|---|
| | $T_m$ (°C) | $\Delta H_{vH}$ (kcal/mol) | $k_{acyl}$ ($min^{-1}$) | $k_{rel}$ | $k_{acyl}$ ($min^{-1}$) | $k_{rel}$ |
| Single-stranded | | | 0.24 | 60 | 0.26 | 37 |
| C–G perfect duplex | 57.1 | −152.2 | 0.004 | 1 | 0.007 | 1 |
| C–A mismatch | 49.4 | −133.5 | 0.066 | 17 | 0.19 | 27 |
| C–T mismatch | 49.6 | −134.8 | 0.037 | 9 | 0.18 | 26 |
| C–C mismatch | 45.5 | −126.2 | 0.088 | 22 | 0.23 | 33 |

Uncertainties in $T_m$ and $\Delta H_{vH}$ (van't Hoff enthalpy) are ±0.5°C and ±6%, respectively. $T_m$ values reflect a total strand concentration of $2 \times 10^{-6}$ M. 2′-Amine acylation rates were determined using 75 or 50 mM succinimidyl ester at 35°C and 50°C, respectively. $k_{rel}$ is defined as $k_{acyl}/k_{acyl}^{duplex}$; larger values indicate faster reactions.

arginine at residue 506 by glutamine and renders the activated form of Factor V resistant to regulated proteolysis by activated protein C. We explored FVL mutation detection in the oligonucleotide model system shown in Figure 2.

Detection employs a 20-residue probe oligonucleotide that contains a unique 2′-amino-cytidine nucleotide. The 2′-amine-containing probe is annealed either to a complementary strand to form a perfect 20-base-pair duplex (wild-type Factor V) or to a strand that forms a duplex containing a C–A mismatch at the position of the 2′-amino cytidine (corresponding to the FVL mutation; Figure 2). To evaluate the generality of 2′-amine acylation for DNA mismatch detection, we also tested the two remaining possibilities such that C–T and C–C mismatches are formed.

### Hybridization specificity

We evaluated the stability of the perfect duplex and of the C–A, C–C and C–T mismatch-containing duplexes in thermal-melting experiments. Melting temperatures ($T_m$) and van't Hoff enthalpies ($\Delta H_{vH}$) were obtained by direct fitting of the differential melting curves (Figure 3). The completely paired C–G duplex has a $T_m$ of 57°C under our reaction conditions. The mismatch-containing duplexes melt 8–11°C lower than the perfect duplex (Table 1).

We therefore explored the selectivity of 2′-amine acylation chemistry at two instructive temperatures, 35°C and 50°C. At 35°C, none of the duplexes show appreciable melting or fraying, whereas 50°C is at or above the $T_m$ for the mismatch-containing duplexes, but below the $T_m$ for the perfect duplex (compare squares with other symbols in Figure 3; see Table 1). Experiments performed at 50°C therefore monitor the global stability of the perfect versus the mismatch-containing duplexes. In contrast, 35°C represents a nonstringent temperature at which hybridization alone using the 2′-amine-containing probe cannot discriminate thermodynamically between the perfect duplex and mismatch-forming target sequences. Thus, 2′-amine acylation will detect the mismatched base pairs at 35°C only if acylation is sensitive to local nucleotide flexibility.

### Mismatch tagging by 2′-amine acylation

Modification at the unique 2′-amine position can be used to incorporate a bulky R group (see the legend to Figure 1). Upon acylation, the oligonucleotide probe is retarded in a denaturing acrylamide gel (Figure 4). Acylation selectivity was first tested at the nonstringent temperature of 35°C. The reaction was initiated by addition of excess succinimidyl ester and quenched at the indicated time points with dithiothreitol (DTT). An oligonucleotide containing all deoxyribose groups shows negligible reactivity, demonstrating that acylation is specific for the nucleophilic 2′-amine group (see all 2′-H lane in Figure 4).

**Figure 4**

Mismatch-dependent 2′-amine acylation. Representative acylation experiments with the single stranded probe, C–G duplex and C–A mismatch at 35°C (in 100 mM Na–Hepes, (pH 8); 10% DMSO; 75 mM succinimidyl ester) are shown. Acylated product is retarded in a denaturing gel compared with free probe. The 'All 2′-H' lane indicates an oligonucleotide in which the unique 2′-amine group has been replaced by a hydrogen atom.

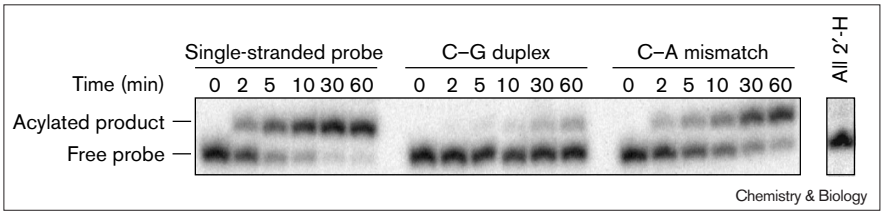
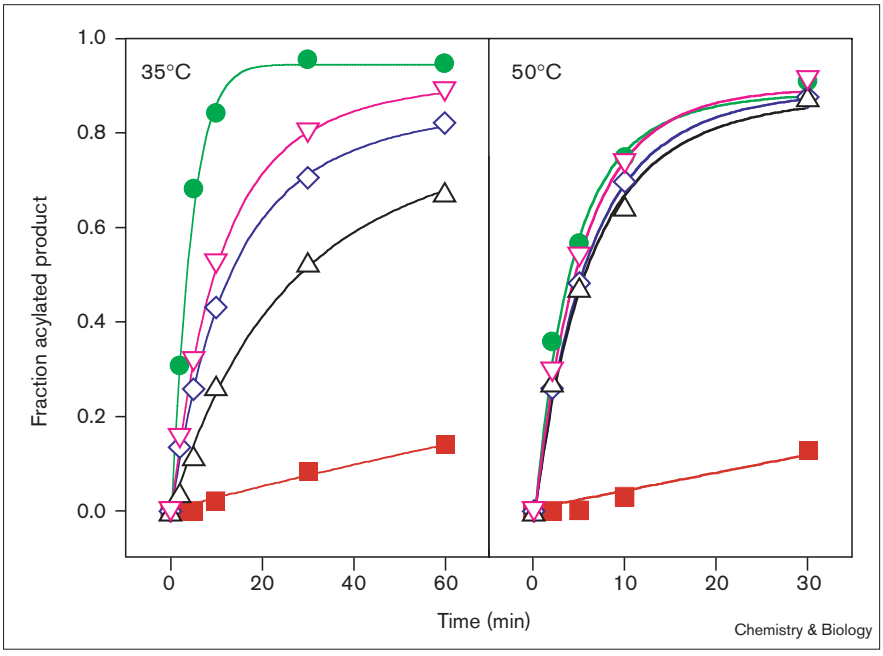


**Figure 5**



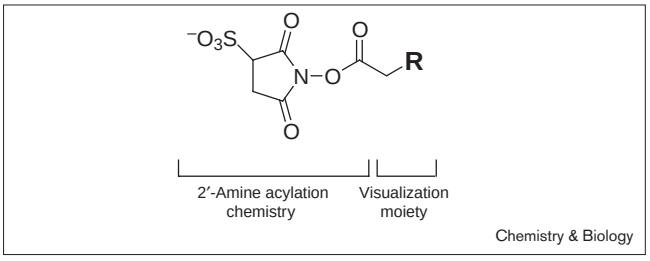

Kinetic analysis of mutation-selective acylation reactions. Mismatched 2′-amino-nucleotides (open symbols: C–A diamonds; C–T, triangles; C–C inverted triangles) react more rapidly than a base-paired 2′-amino-nucleotide (filled squares). Reaction of the single-stranded probe is shown as filled circles. Lines represent a best-fit to an equation that takes into account reaction of the succinimidyl ester by 2′-amine acylation and hydrolysis.

The flexible 2′-amine-containing single-stranded probe oligonucleotide is acylated rapidly ($t_{1/2}$ = 3 min). The C–A mismatch duplex also reacts rapidly; whereas, the 2′-amino cytidine nucleotide constrained in a C–G base pair reacts slowly ($t_{1/2}$ = 170 min; compare single strand, C–G duplex and C–A mismatch panels in Figure 4).

The fraction of 2′-amine-containing probe oligonucleotide that is acylated was calculated for each time point, and best fit curves are shown in Figure 5. The curve fit takes into account parallel reaction of the activated succinimidyl ester by 2′-amine acylation and hydrolysis [24].

Reaction of the C–A mismatch (corresponding to the FVL mutation) is 17-fold and 27-fold faster than reaction of the perfect duplex (wild type) at 35°C and 50°C, respectively (Table 1). The observed kinetic selectivity provides a strong signal for identifying the mutation in potential polymorphism screening applications (Figure 5). All acylation rates are faster at the higher temperature, as expected. Importantly, there is less than a twofold increase in the relative rate of 2′-amine acylation under conditions where neither duplex shows appreciable melting (35°C) versus conditions where the mismatch-containing duplex is half melted (50°C) and the perfect duplex is essentially paired. These data emphasize that 2′-amine acylation is governed primarily by local differences in nucleotide stability. Relative reaction rates for the C–T and C–C mismatches are ninefold and 22-fold faster, respectively, than reaction of the perfect duplex at the nonstringent temperature (35°C). At 50°C, all three mismatched duplexes react ~30-fold more rapidly than the perfect duplex (Figure 5; Table 1).

**Figure 6**




Bifunctional chemical sensor for mutation detection by selective 2′-amine acylation. R, visualization group (biotin, fluorescent reporter group or enzyme conjugate, for example).

### Potential adaptability

The use of gel electrophoresis and radiolabel detection as a scoring method, as used in this work, is not optimal for a practical scoring assay. The succinimidyl ester, however, is a bifunctional molecule (Figure 6) in which the mutation-sensitive acylation chemistry is independent of, and separated from, the visualization moiety by a simple methylene linker. The visualization (R) group could be biotin, a fluorescent reporter group or an enzyme conjugate. Multiplexed scoring of SNPs in a single reaction might be possible by varying the reporter group or probe length for oligonucleotides directed against different target sequences.

## Significance

**Selective acylation of 2′-amine-substituted nucleotides is a promising approach for site-specific recognition and tagging of DNA mismatches. Structural studies emphasize that mismatched base pairs are accommodated within the DNA helix without significant backbone distortion [18,28,29] but differ in local dynamic behavior [25,26]. Because the method is sensitive to local nucleotide flexibility, 2′-amine acylation circumvents difficulties of allele-specific hybridization methods that require careful optimization of stability relative to other competing sequences. Moreover, as acylation is highly selective for the 2′-amine substitution in the probe, mismatch interrogation is specific for the region at the 2′-amino nucleotide. 2′-amine mismatch tagging is compatible with existing oligonucleotide hybridization-based assays, including DNA chip technology, and will be useful as a method for chemical proofreading of hybridization specificity at temperatures below the global melting temperature.**

## Materials and methods

*Reagents and reaction conditions*

Oligodeoxyribonucleotides were synthesized using standard phosphoramidite chemistry by the North Carolina State University (NCSU) Nucleic Acids Facility and purified by denaturing gel electrophoresis in 20% gels (7 M urea, 90 mM Tris-borate). The 2′-amino cytidyl phosphoramidite was purchased from Glen Research (Sterling, VA) and sulfosuccinimidyl-6-(biotinamido) hexanoate was obtained from Pierce Biochemical (Rockford, IL). All thermal denaturation experiments and 2′-amine acylation reactions were carried out under the same conditions (100 mM Na–Hepes, pH 8, 10% dimethylsulfoxide [DMSO]).

*Thermodynamic parameters*

Melting experiments were performed at 1 μM in each strand using an AVIV 17DS spectrophotometer. Melting curves were obtained at a heating rate of 1°C/min using 1 cm path length cells, monitored at 260 nm. Data were collected every 1°C, algebraically differentiated and manually corrected for nonzero baselines. Melting temperature ($T_m$) and van't Hoff enthalpy ($\Delta H_{vH}$) were obtained by direct fitting of the differential melting curve [30] assuming a bimolecular reaction:

$$\frac{dA_{260}}{dT} = a\frac{f(1-f)}{1+f} \qquad (1)$$

where $f$, the fraction duplex, is a function of $T_m$ and $\Delta H_{vH}$ and $a$ is a scaling factor ($A_{260}$ is absorbance at 260 nm). $T_m$ and $\Delta H_{vH}$ are obtained by substituting the equilibrium expression:

$$f = 1 + (KC_T)^{-1} - \left[\left((1 + KC_T)^{-1}\right)^2 - 1\right]^{1/2} \qquad (2)$$

and integrated form of the van't Hoff equation:

$$K = K_{T_m} e^{\frac{\Delta H_{vH}}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right)} \qquad (3)$$

into Equation 1, where $K$ is the equilibrium constant, $C_T$ is the total strand concentration, T is the absolute temperature, R is the gas constant and $K_{Tm} = 2 \times 10^6\ M^{-1}$. Real duplexes undergo pretransition fraying; therefore, points more than 6–8 K below $T_m$ were excluded during fitting.

Although the thermal melting and 2′-amine acylation (see below) experiments are performed at different DNA strand concentrations, calculated $T_m$ values under acylation conditions are the same, within error, as obtained in the thermal melting experiments (see Equation 3 in [19]). Thus, the thermal denaturation and chemical acylation experiments performed in this work may be compared directly.

*Selective acylation*

2′-Amine acylation experiments were performed with excess (0.7 μM) complementary strand and 1–10 nM $^{32}$P-5′-end-labeled probe oligonucleotide. Reactions (16 μl) were initiated by addition of a 10× solution of the succinimidyl ester in DMSO and aliquots (2 μl) were quenched by addition of 8 μl stop solution (125 mM DTT in 85% formamide, 1/2 × TBE, 50 mM EDTA). 2′-Amine acylation at 35°C and 50°C was performed with a final concentration of 75 mM or 50 mM ester, respectively. Reactions were resolved on 20% denaturing gels and quantified using a phosphorimager. The succinimidyl ester reacts by 2′-amine acylation and also by solvent hydrolysis [24]. Acylation rates were obtained by fitting the fraction acylated product to an equation that accounts for these parallel reactions:

$$\text{Fraction product} = 1 - \exp\left\{\frac{k_{acyl}}{k_{hydrolysis}}\left(e^{-k_{hydrolysis}t} - 1\right)\right\} \qquad (4)$$

where $k_{acyl}$ and $k_{hydrolysis}$ are the pseudo-first-order rate constants for 2′-amine acylation and reagent hydrolysis, respectively. $k_{hydrolysis}$ was found to be equal to 0.025 $min^{-1}$.

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