Emission Characteristics of Fluorescent Labels with Respect to Temperature Changes and Subsequent Effects on DNA Microchip Studies

Wen-Tso Liu,* Jer-Horng Wu, Emily Sze-Ying Li, and Ezrein Shah Selamat; APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2005, p. 6453–6457

What did they do?

- Evaluate the effect of hybr. paremeters on common fluorescent labels to improve the results of melting curve analysis in DNA microarray studies.

Why did they do this?

- The technique has show promising results in bacterial detection with minimal false-positive signals.
- But needs further optimisation:
  - dyes exhibit different stabilities and intensities under changing external parameters, affecting the results.

Detection by melting curve analysis

- Increased sensitivity required to reliably detect closely related bacteria.
- Detection by 16S rRNA often needs 1MM discrimination.
- Utilises Td determined in non-equilibrium dissociation analysis (melting curve analysis).

Tm and Td difference

- Td – temp. 50% starting duplex intact.
  - Tm – duplex is separated solely by temperature.
  - Td – temperature at which a duplex bound to a substrate dissociates due to temperature and washing effects.
  - Td is lower than Tm (7,6 C^o, Rychlic and Rhoads (1989)).

Detection by melting curve analysis

- Fragmentation of rRNA targets
- Labeling
- Hybridization (~15-25 probes)
- Thermal heating (10 to 80C^{o}, step 2,5C^{o} / 3 min.)
- Capture images (800ms, ca 25 s total)
- Determine Td
- Compare Td-s with (+) controls

Goal of this study

- Measure the effects of hybridisation parameters on emission of 8 common dyes and find a way to normalise any bias created.
  - Measure emission characteristics
  - Temperature on melting curve analysis
  - Normalise the results

Gel-pad arrays

- 300x300x10 um pads
- test probes
  - PM, 2x1MM, 1x2MM
  - 5’ linker + 15 T + 20 nt 3’
- control probes
  - 5’ linker + 15 T + 4 G + label 3’
- 4 replicates

Determine emission characteristics

- Control probes with 8 standard labels

TABLE 1. Fluorophore excitation/emission characteristics and filters used in this study

Fluorophore	Fluorophore characteristic		Wavelengths of microscope filter used	
	Excitation (nm)	Emission (nm)	Excitation/ bandwidth (nm)	Emission/ bandwidth (nm)
Oregon Green	490	514	470/40	525/50
Cy2	489	506	470/40	525/50
FAM	492	517	470/40	525/50
FITC	494	518	470/40	525/50
Cy3	550	565	555/50	610/75
TAMRA	542	568	555/50	610/75
Rhodamine red	570	590	555/50	610/75
Texas Red	596	615	555/50	610/75

Determine emission characteristics

- Variable parameters
  - NaCl – 10, 100, 500 mM
  - Formamide – 0, 10, 20, 30%
  - Temperature – 7.5 – 80 C^o (2.5 / 3 min)
- 800 ms exposure (total < 25 s)
- place grids at first capture
- quantify, subtract bg -> Excel
- average replicates

Temperature and salt effects on fluorophores

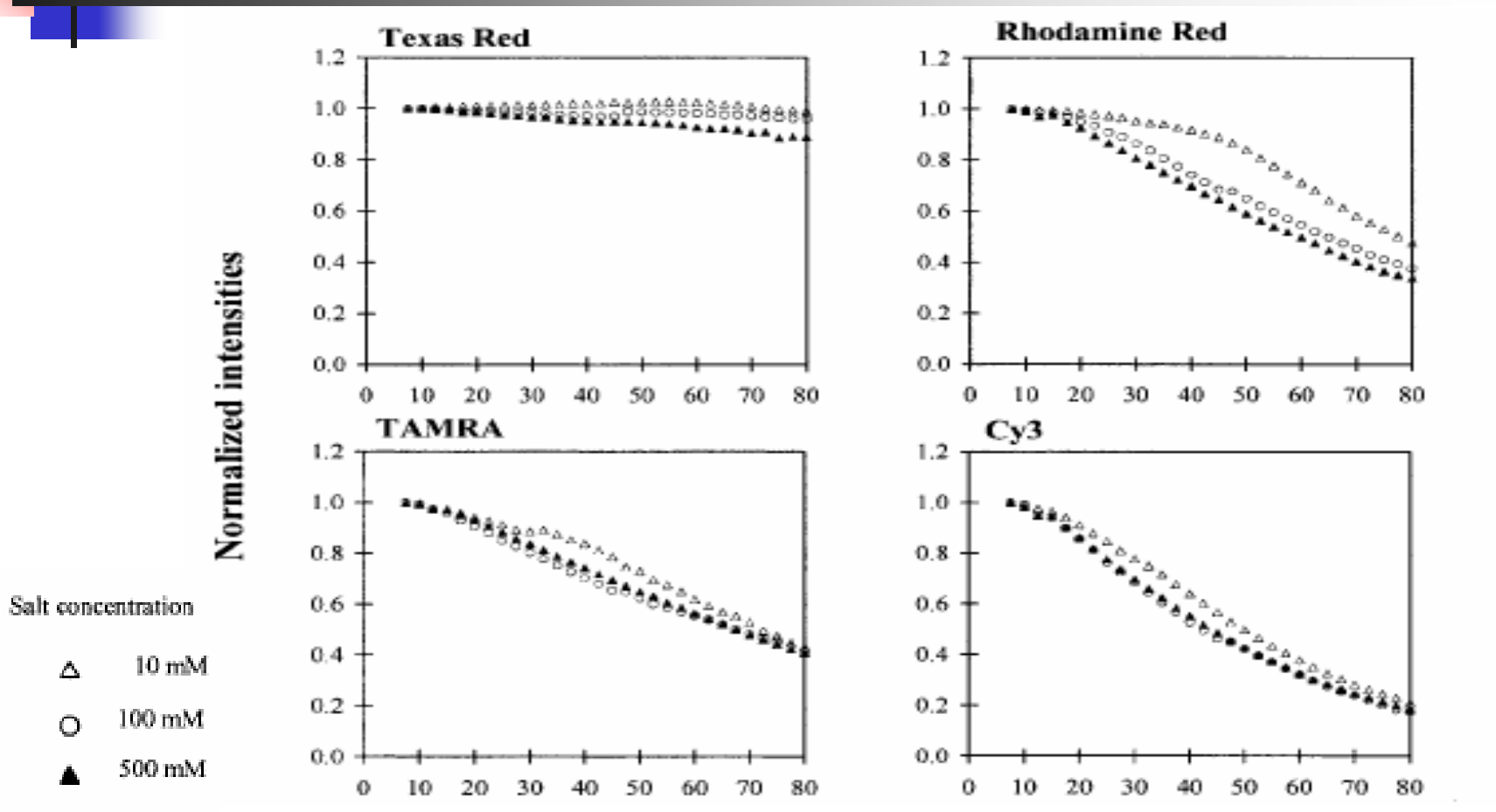
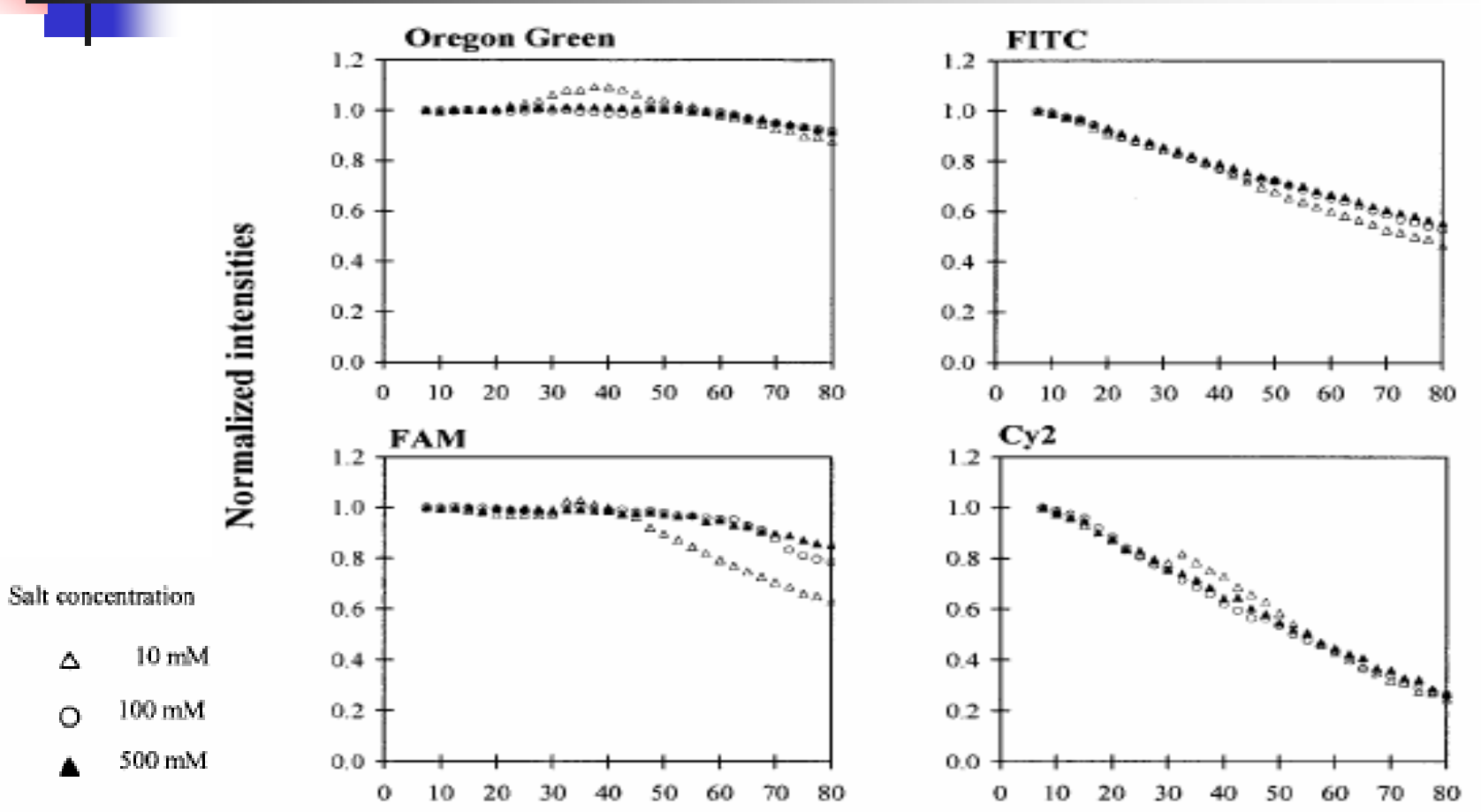


FIG. 1. Effects of salt concentration on fluorophore stabilities with respect to temperature on gel-based microchips. The formamide concentration in the buffer was fixed at 0%. (A) Cy3 filter (excitation, 555/50 nm; 610/75 nm); (B) GFP filter (excitation, 470/40 nm; emission, 525/50 nm).

Temperature and salt effects on fluorophores



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Effect of temperature on probe-target duplexes

- 4 labels tested (TAMARA, Tex. Red, Cy3, Rhod. red)
- Target – 0,5 nM E.Coli 16S rDNA 1492-1511
- Melting curve analysis
  - 15 – 80 C^o, 2.5 / 3 min
  - averaged raw intensities (each duplex)
  - intensity at start -> 1
  - 50% intensity -> Td

Melting curves before and after normalisation

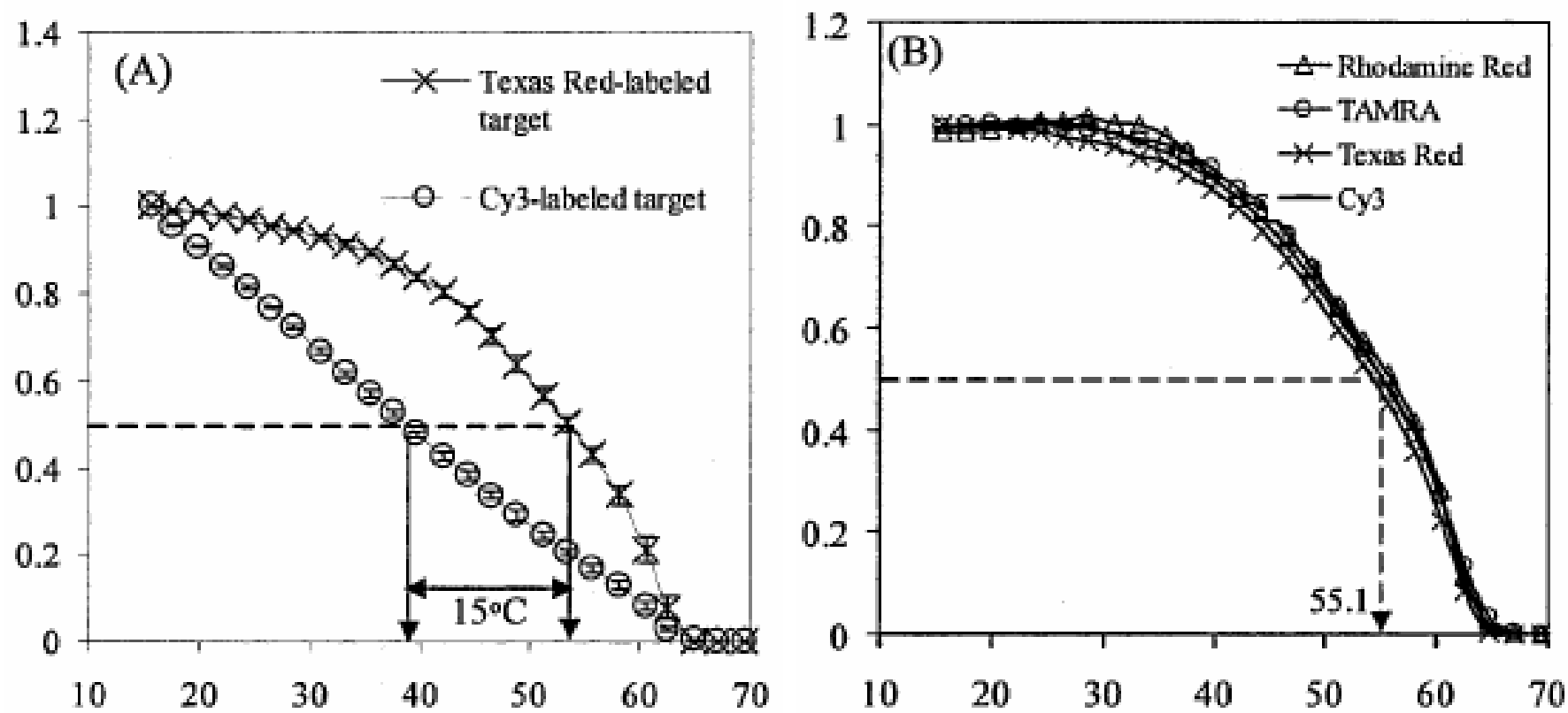
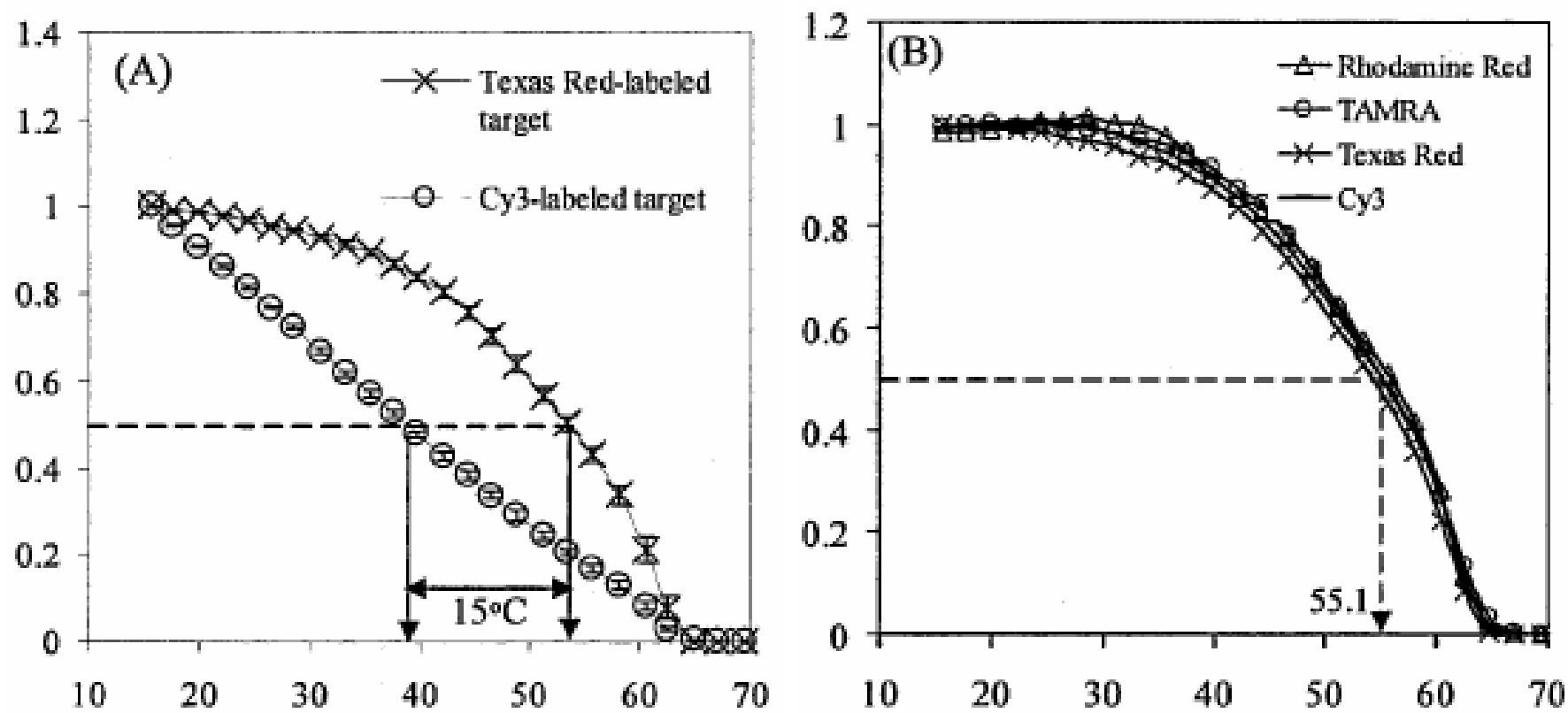


FIG. 2. Melting curves of PM BC probe-target duplexes. (A) Texas Red- and Cy3-labeled targets without normalization (signal intensity at the initial temperature was set to 1); (B) Texas Red-, Cy3-, rhodamine red-, and TAMRA-labeled targets normalized against control probes labeled with the same fluorophores. The formamide and salt concentrations in the wash buffer were fixed at 0% and 50 mM, respectively. The key in panel B indicates the fluorophore-labeled target or the type of fluorophore-labeled control probe used for normalization.

Melting curve results (PM duplex)

- Melting curves different!
- Texas Red – S-shaped curve, Td = 54C^{o}
- Cy3 – linear, Td = 39C^{o}
- Need to normalise (dif. 15 C^{o})

Melting curves before and after normalisation



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2-step normalisation

- 1. Corrects raw intensities against respective control probe at each temp.
- 2. Set max intensity to 1 and determine Td

2-step normalisation

- 1. $I_{sc,t} = I_{s,t} / I_{c,t}$
  - Isc,t – test spot intensity relative to control spot at given temperature
  - Is,t – test spot intensity at a given temperature
  - Ic,t – control spot intensity at at given temperature
- 2. $I_N = I_{sc,t} / I_{sc,max}$
  - Isc,max – maximum relative test spot intensity through all temperatures
  - N – normalised intensity

MM discrimination

Fluorophore	PM duplex	
	T_d	SD
Texas Red	54.20	0.50
Cy3	55.08	0.73
TAMARA	55.83	0.65
Rhodamine red	55.62	0.66

MM9AG duplex		MM9GG duplex		MM9GG10AA duplex	
ΔT_d	SD	ΔT_d	SD	ΔT_d	SD
−3.98	0.37	−4.24	0.53	−10.34	1.03
−4.09	0.45	−3.89	0.43	−10.77	0.84
−4.38	0.43	−4.37	0.54	−10.40	0.69
−5.02	0.61	−4.82	0.51	−12.27	0.24

TABLE 2. T_ds of PM duplexes and ΔT_ds between MM duplexes and the PM duplex under different fluorophore labeling conditions[a]

Summary

- It was shown that commonly used fluorescent dyes exhibit different emission characteristics with respect to changes in important hybridisation parameters.
- Formamide harmless: SD < +- 5%
- The cause is not clear ...

Summary

- It has been previously suggested that different melting curves from different dyes (FITC/TAMRI vs Texas Red) resulted from the change in RNA/DNA duplex stability had different melting curves.
- Current study shows that it is more likely due to the temperature-dependant decrease in dye emission.*

Suggestions

- When using different dyes in one experiment, use dyes with similar emission characteristics to reduce bias in the comparison of results.
- If neccessary, bias resulting from different emission characteristics can be removed using proper calibration curves.

Suggestions

- Fluorophores with linear temperature dependancy can be used as temperature sensors in microfluidic devices where direct physical methods are difficult to use.

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