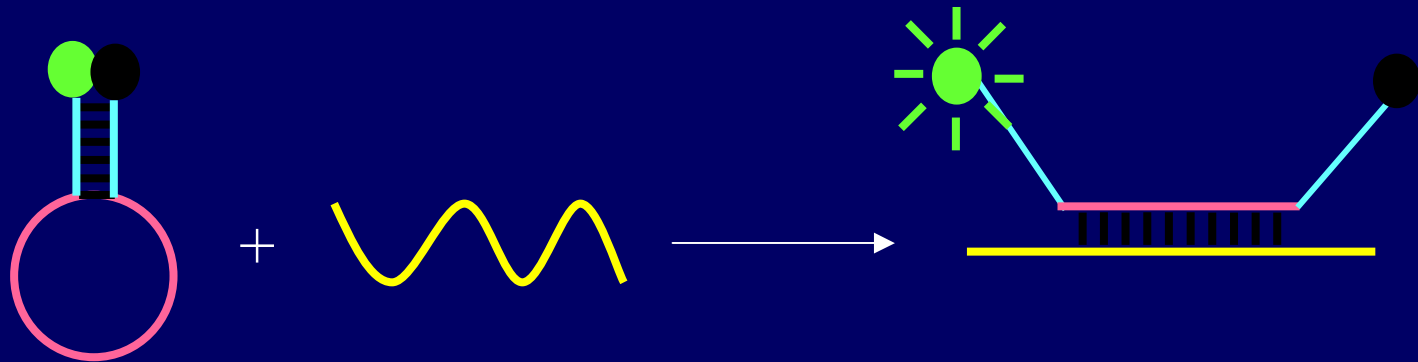


Homogeneous genotyping based on displacement hybridization

Qingge Li*, Jinping Cheng, Yongyou Zhang

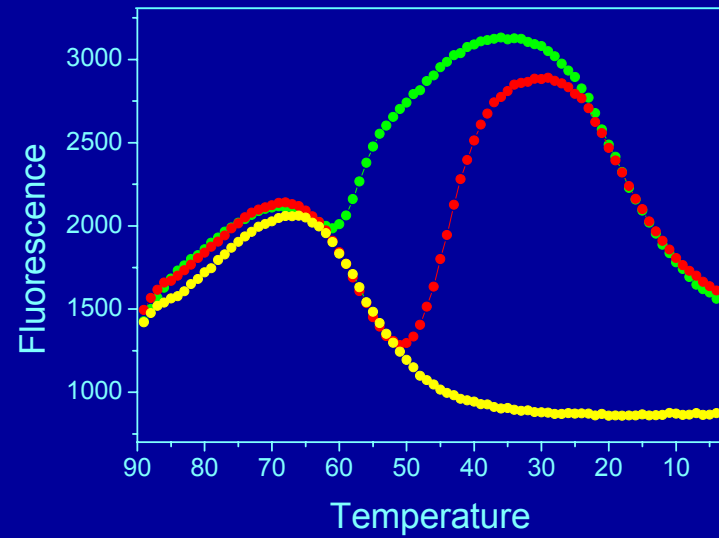
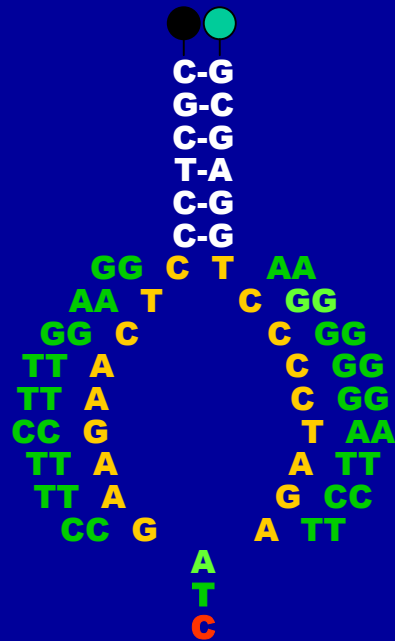
*School of Life Sciences, Xiamen University
Xiamen, Fujian 361005, PRC*

Molecular beacon, invented by Snajay Tyagi and Fred Kramer, PHRI, USA



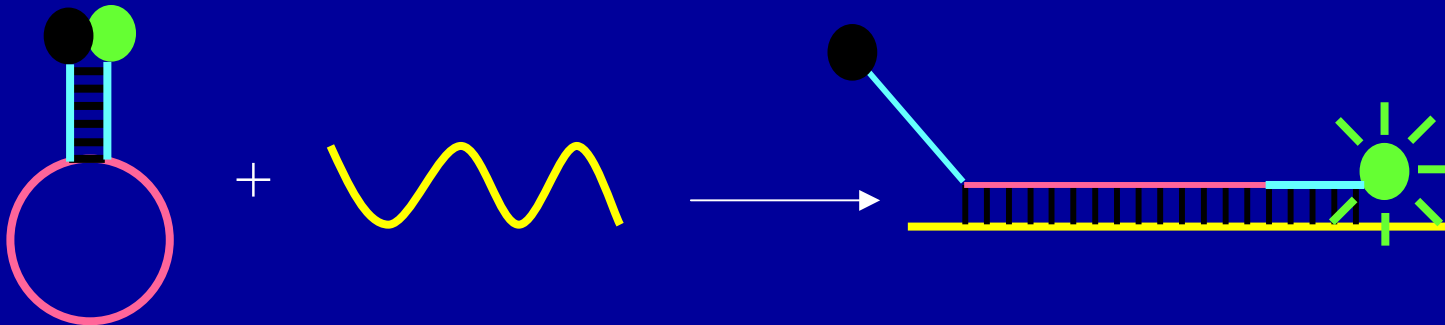
and its problems when detected at RT...

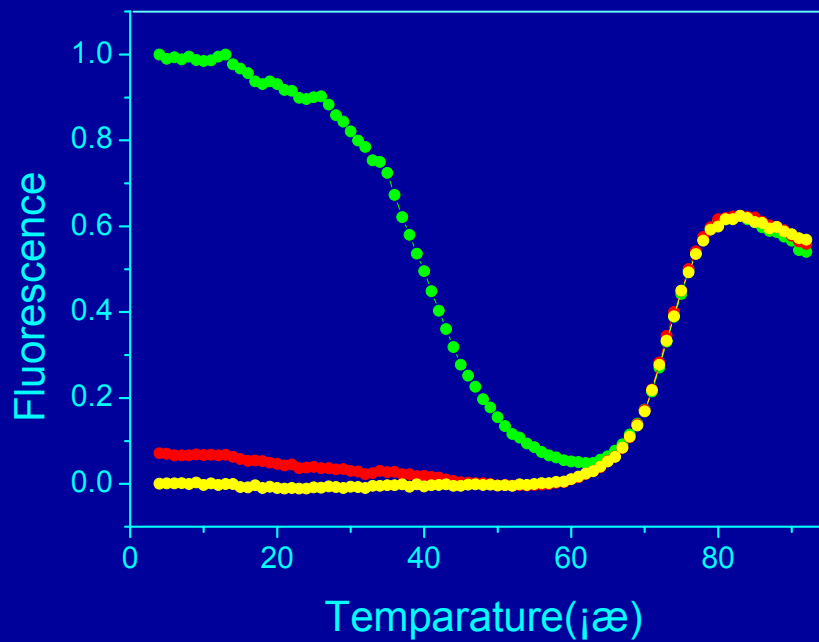
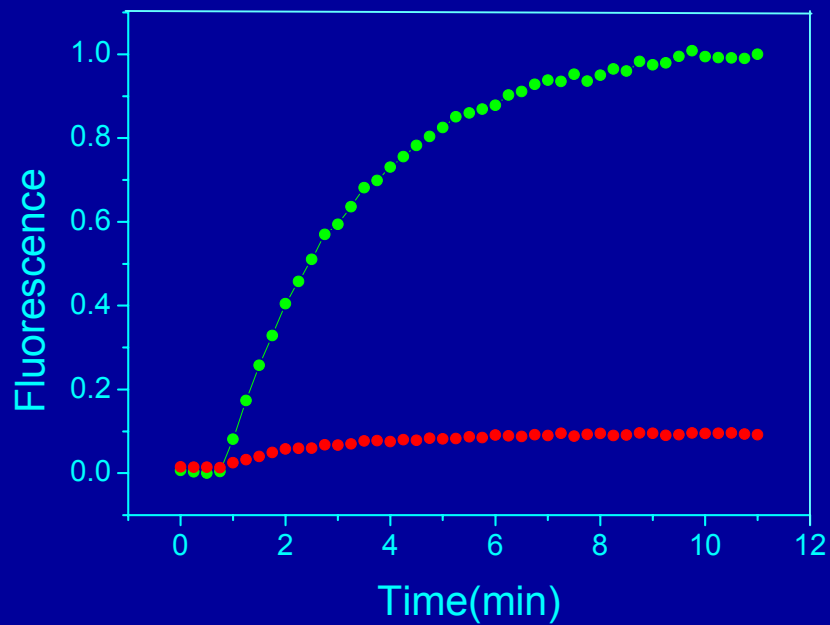
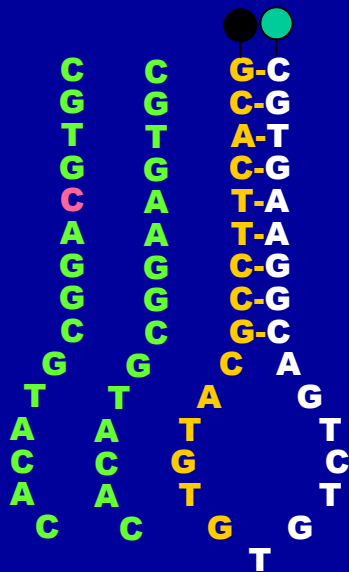
Molecular beacons close at low temperature



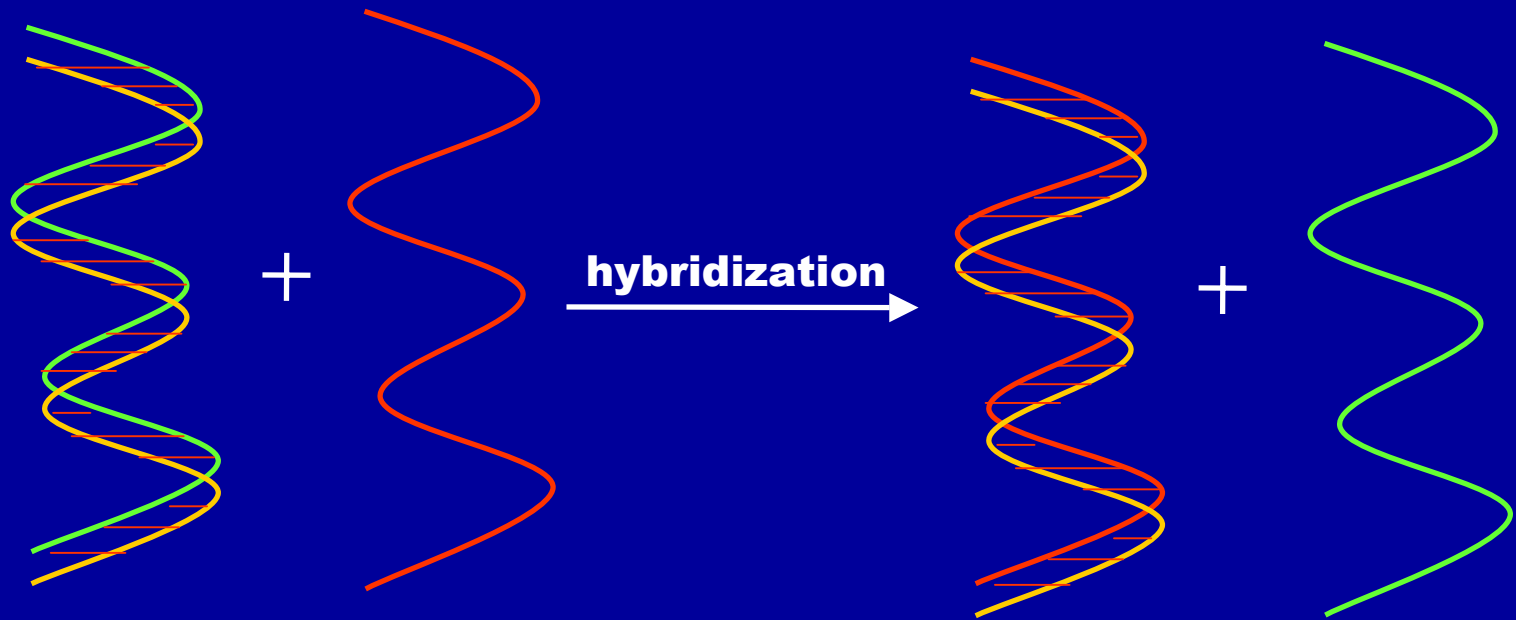
Stem-shared molecular beacons

---Li Q, et al, Anal Sci, 2000





Displacement hybridization

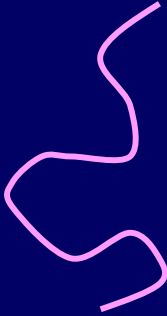


Fluorophore Quencher



Probe

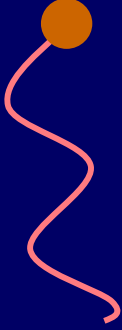
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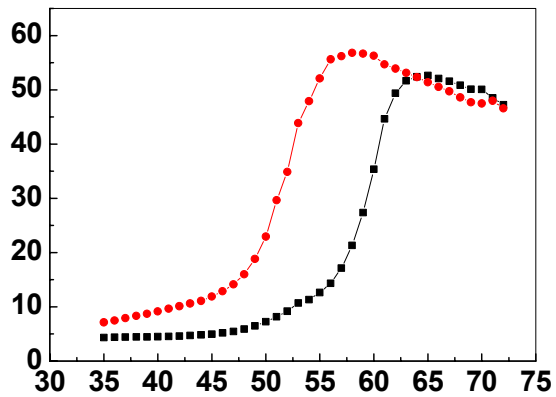
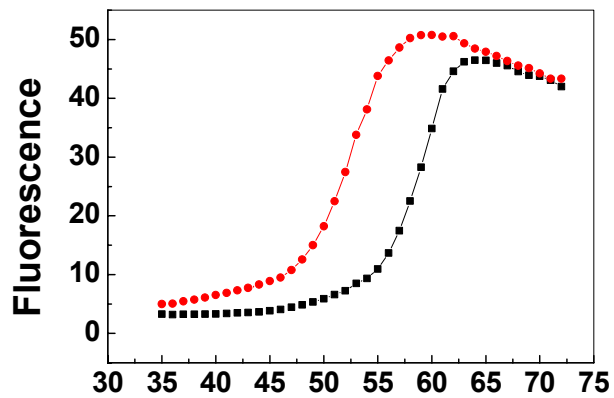
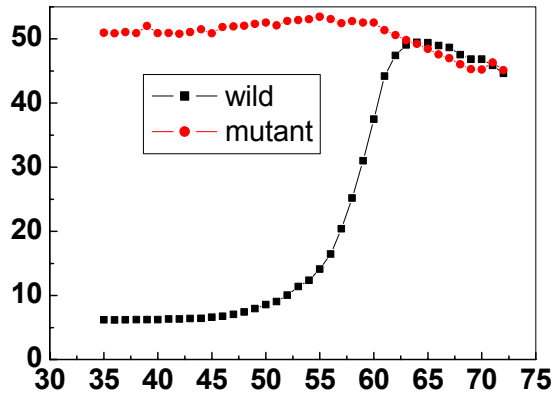
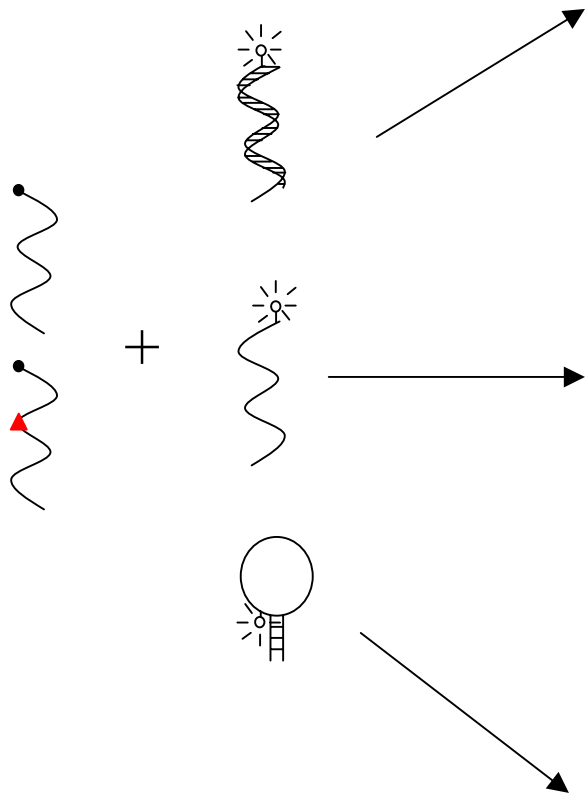


Target



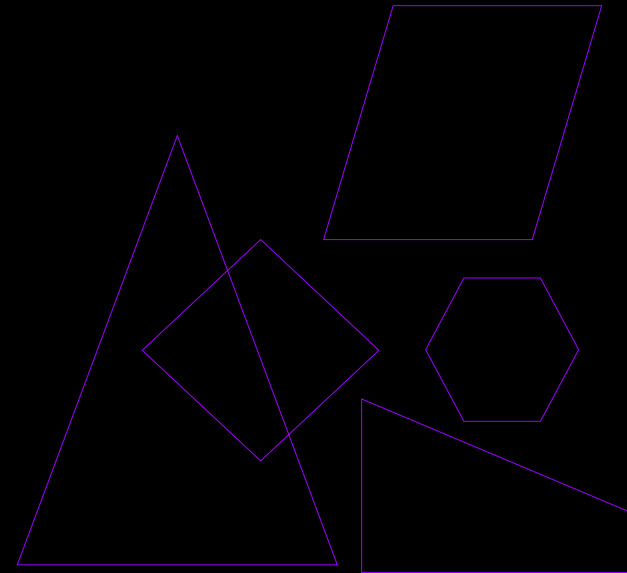
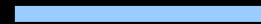
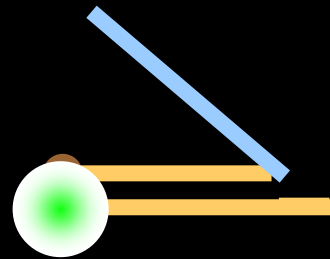
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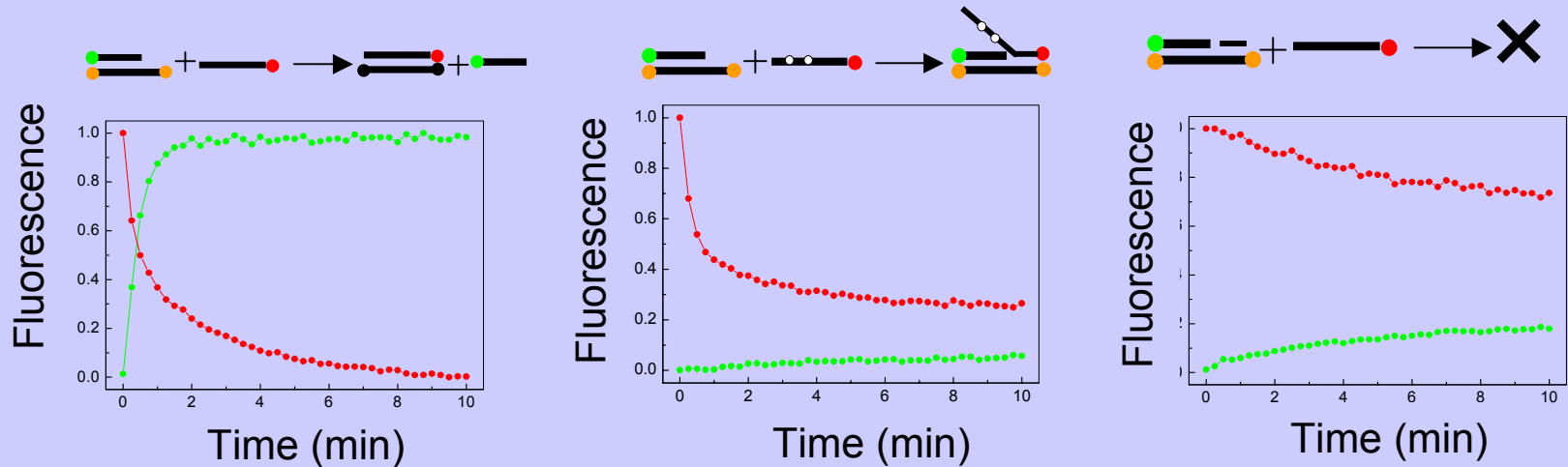


Temperature

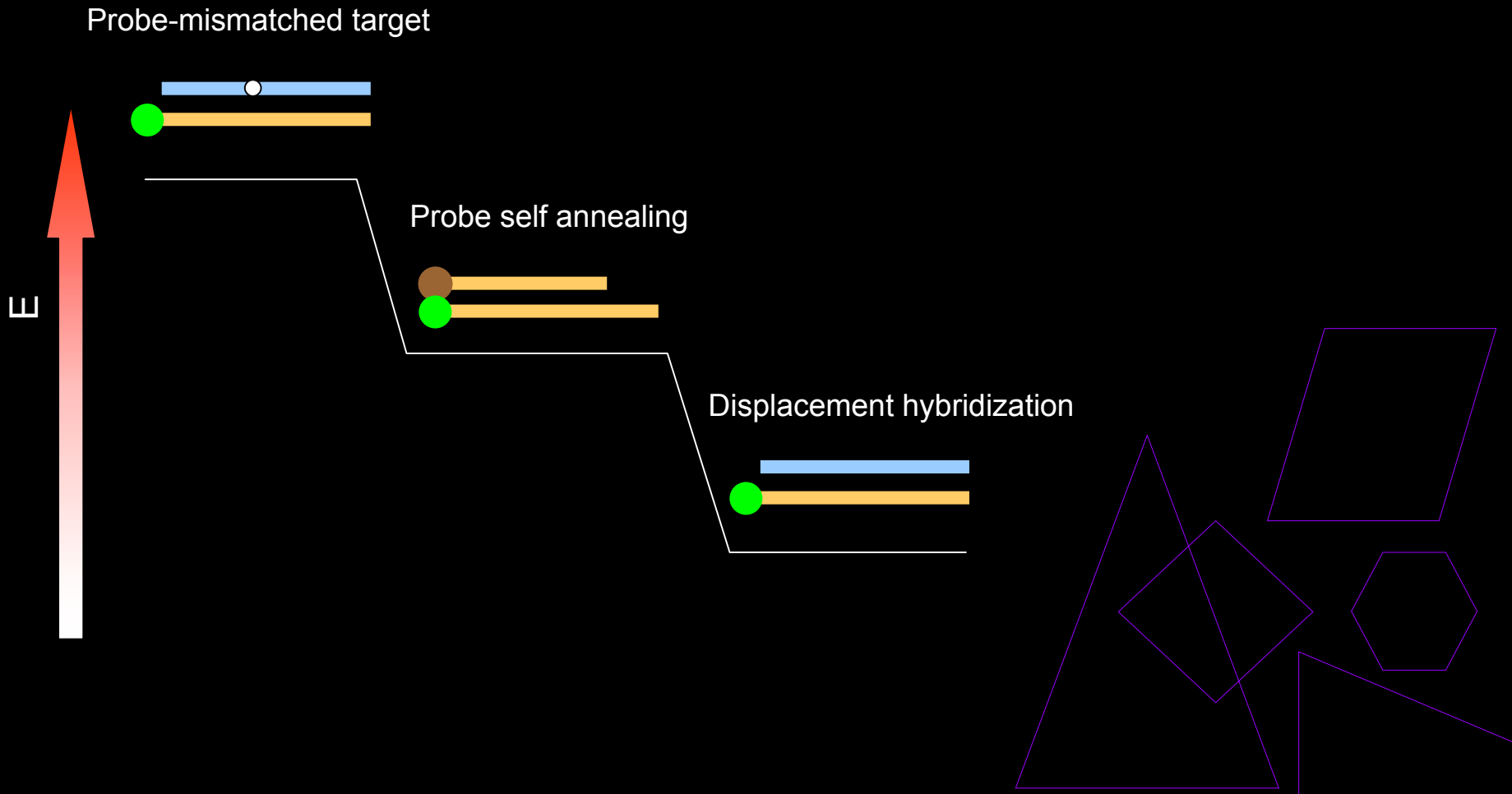
How displacement hybridization happens



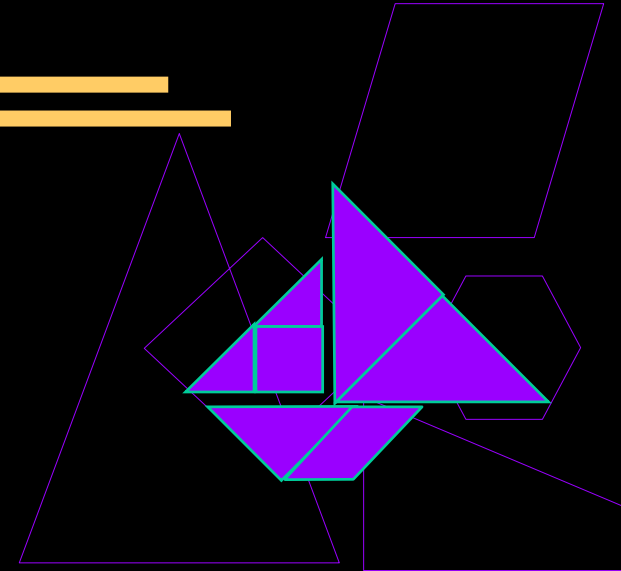
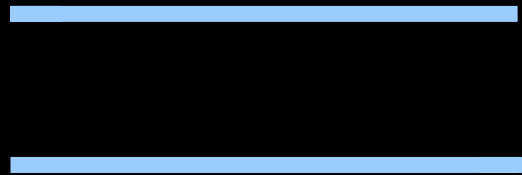
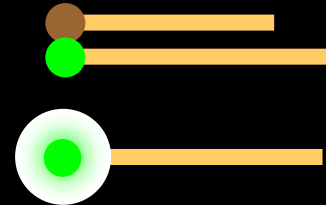
The existence of the transient state



Three states of double-stranded probes

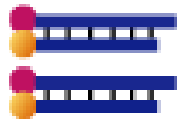
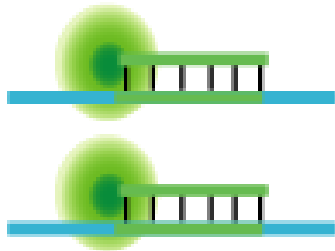


Displacement hybridization in real-time PCR

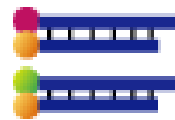
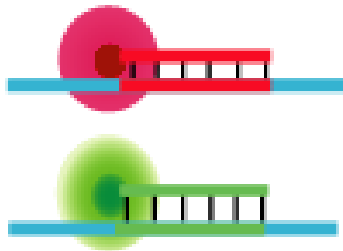


Spectral genotyping with double-stranded Probes

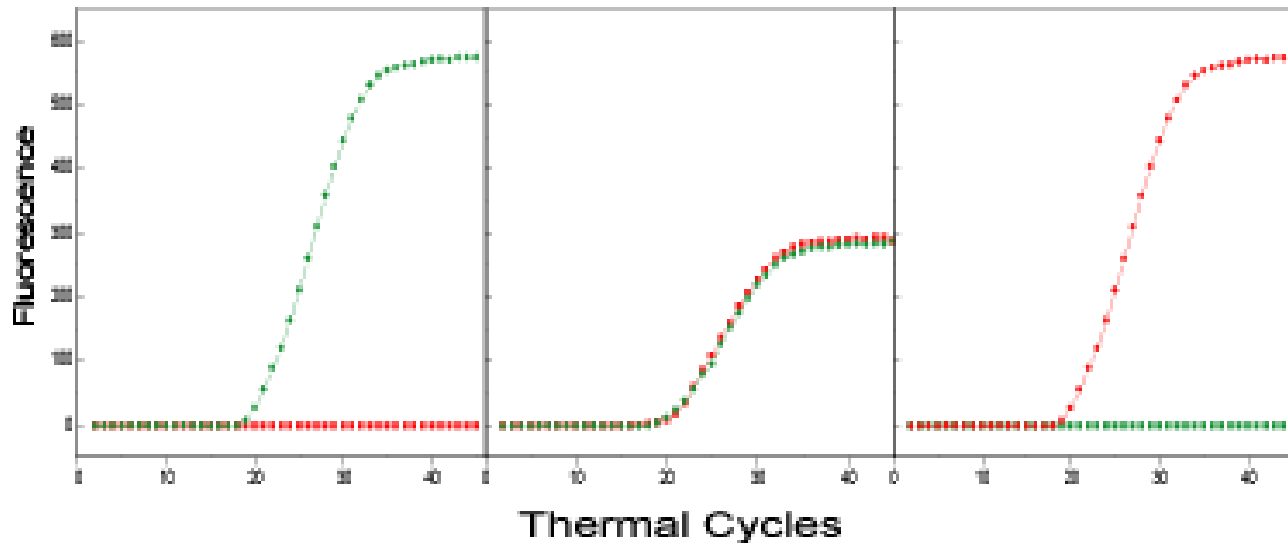
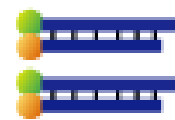
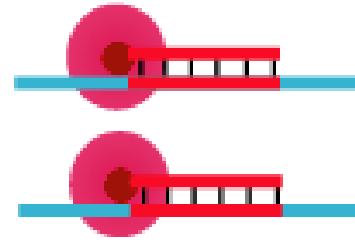
Homozygous wild-type



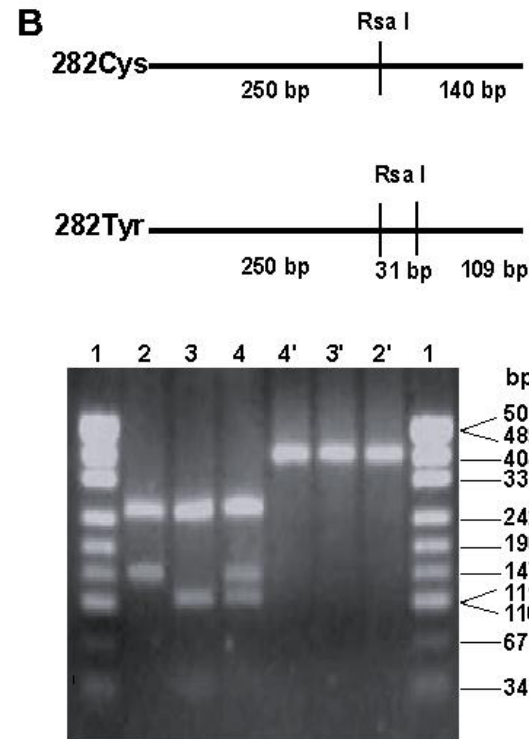
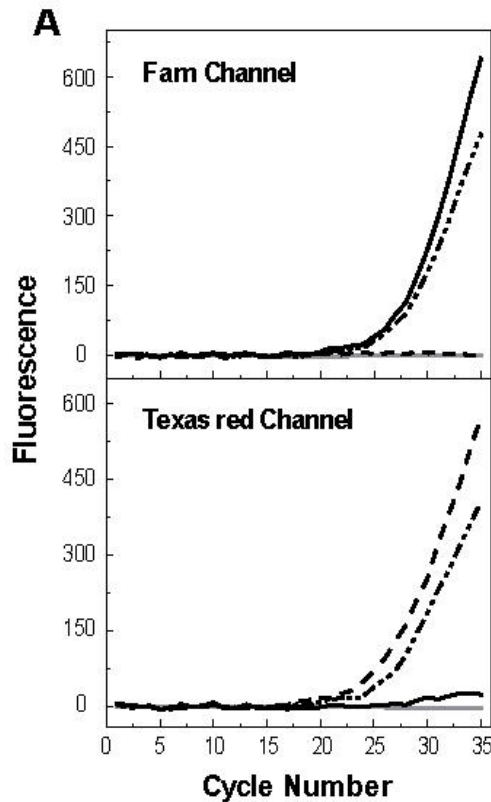
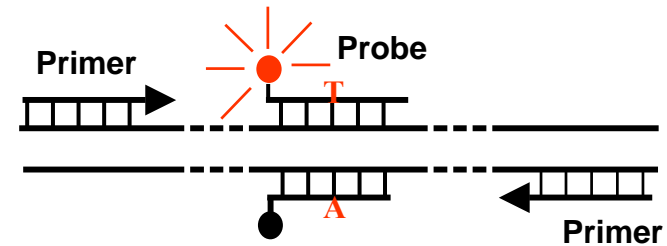
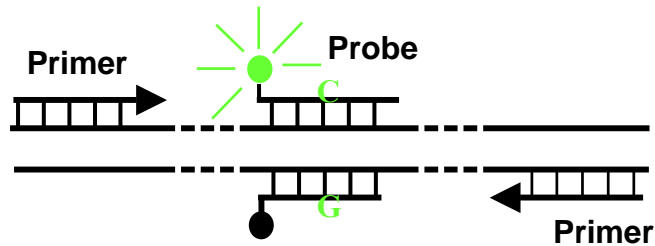
Heterozygote



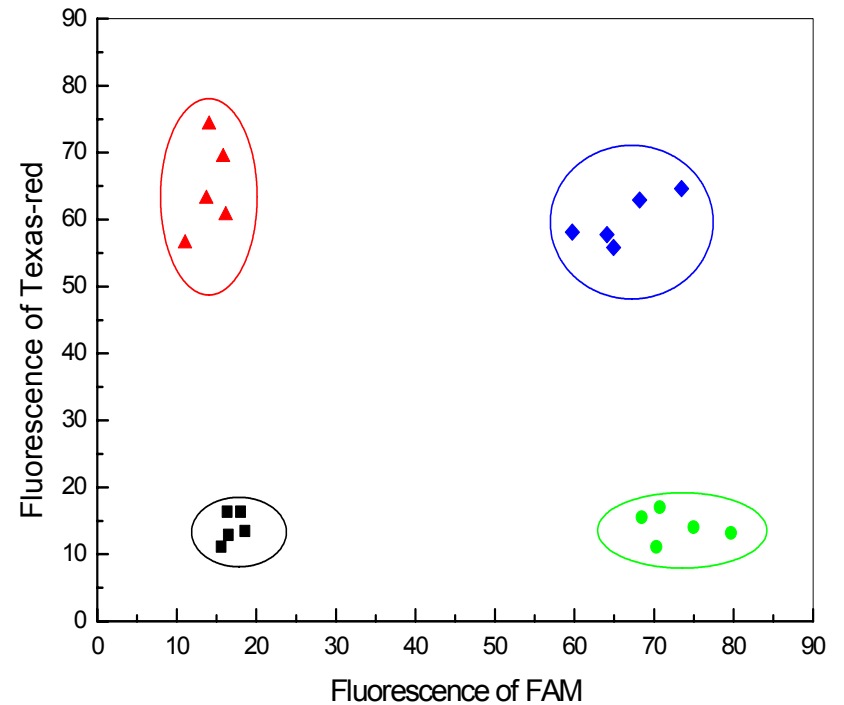
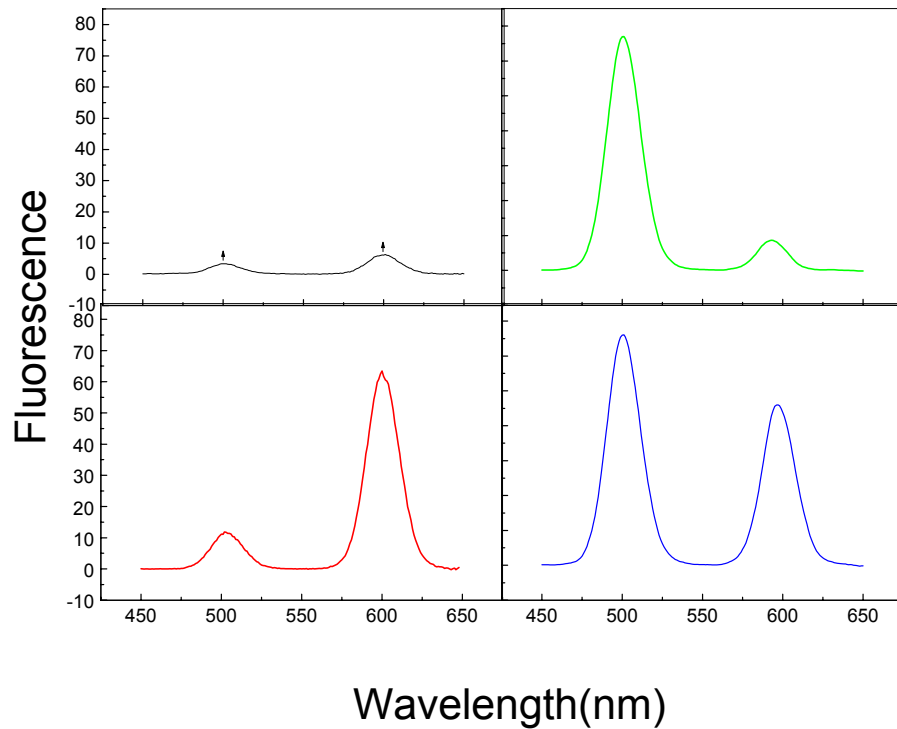
Homozygous mutant



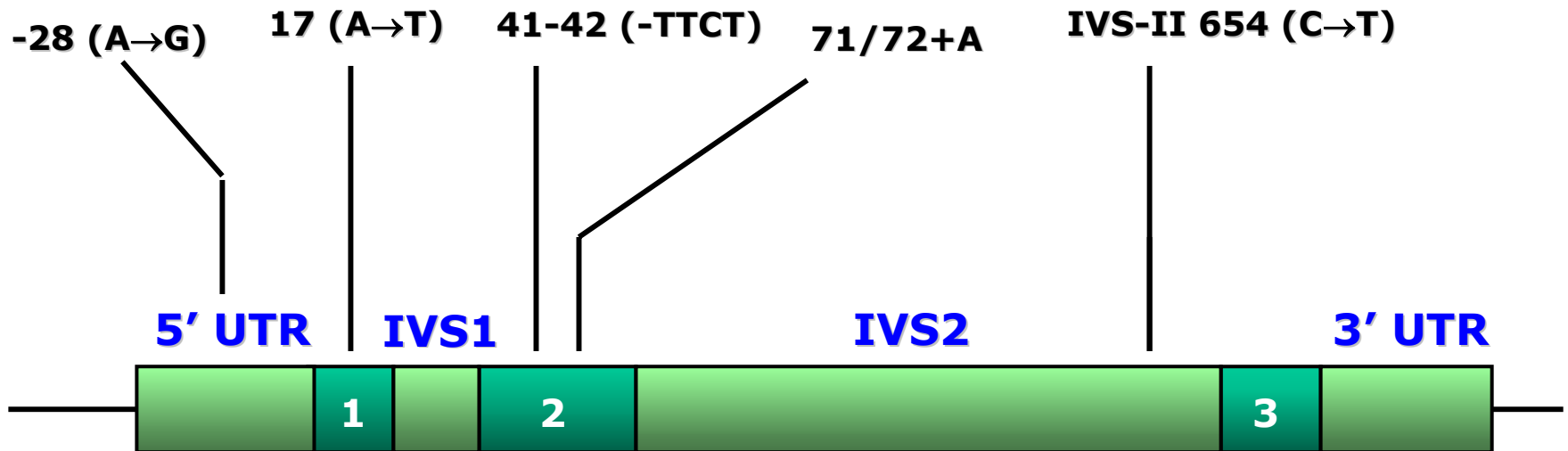
Genotyping of hemochromatosis alleles with real time PCR



Genotyping of hemochromatosis alleles with synchronous fluorometry ---end-point analysis at room temperature



Five most common mutations in β -globin alleles of Chinese population



Human β -globin gene sequence and the mutations locations

HBB gene (GenBank version: U01317.1 GI:455025)

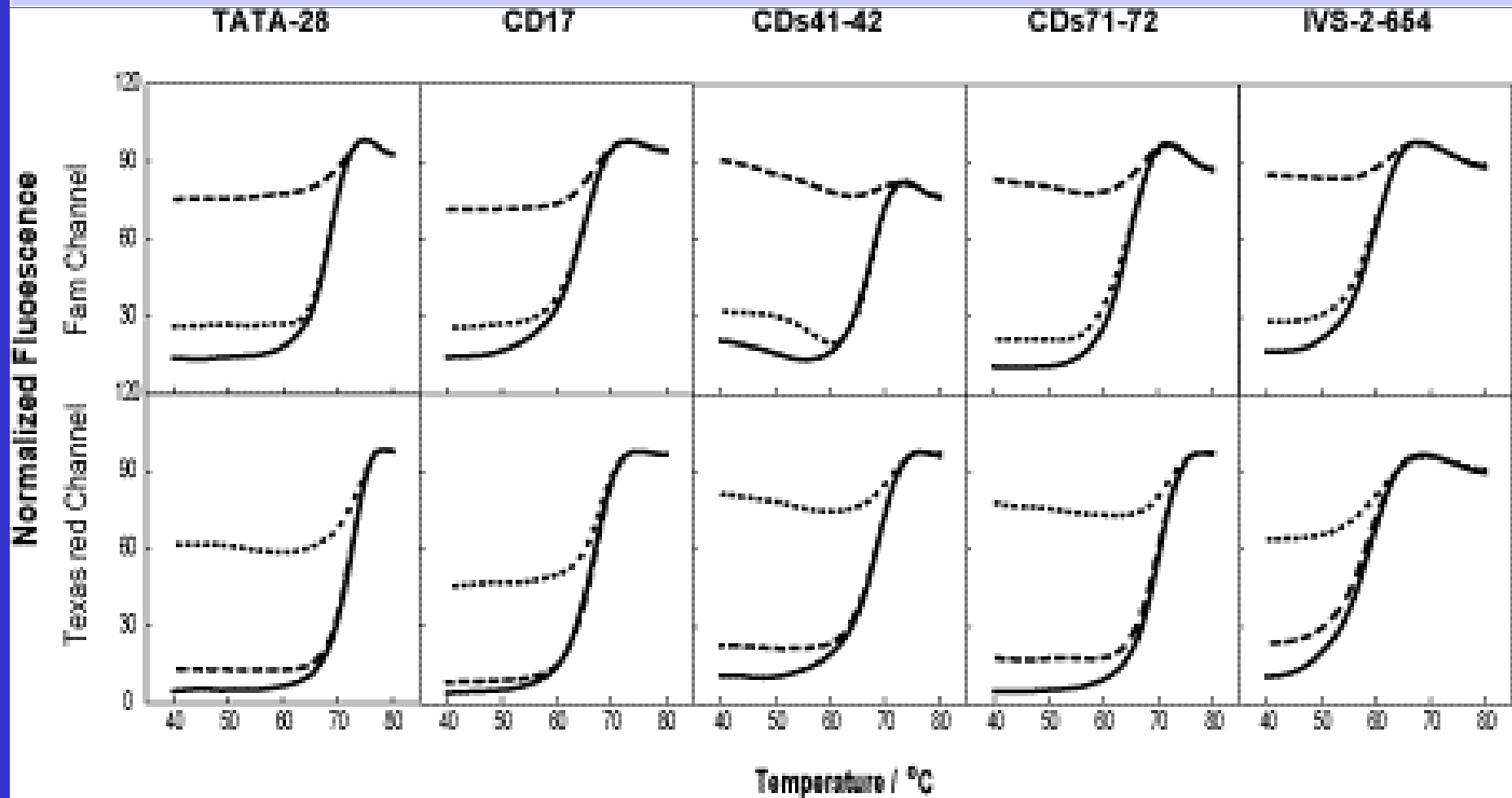
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62161	ttCACTAGCA	ACCTCAAACA	GACACcatgg	tgcacctgac	tcctgaggag	aagctgccg
62221	ttactgccct	gtggggcAag	gtgaacgtgg	atgaaGTTGG	TGGTGAGGCC	CTGGgcaggt
62281	tggatcaag	gttacaagac	aggtttaagg	agaccaatag	aaactgggca	tgtggagaca
62341	gagaagactc	ttgggtttct	gataggcact	gactctcTCT	GCCTATTGGT	CTATTTTCCC
62401	acccttaggc	tgctgggtgt	ctacccttgg	accagaggt	TCTTtgagtc	ctttgggat
62461	cTGTCCACTC	CTGATGCTGT	TATGggcaac	cctaagGTGA	AGGCTCATGG	CAAGAAAGTg
62521	ctcgggtgct	ttAgtgatgg	ccctggctcac	ctgGACAACC	TCAAGGGCAC	CTTtgcaca
62581	ctgagtgagc	tgcactgiga	caagctgcac	gtggatcctg	agaacttcag	ggtgagtcia
62641	tgggaccctt	gatgttttct	ttcccccttct	tttctatggt	taagttcatg	tcataggaag
62701	gggagaagta	acaggttaca	gtttagaatg	ggaaacagac	gaatgattgc	atcagtggtg
62761	aagcttcagg	atcgttttag	tttcttttat	ttgctgttca	taacaattgt	tttctttigt
62821	ttaattcttg	ctttcttttt	ttttcttctc	cgcaattttt	actattatac	ttaatgcctt
62881	aacattgigt	ataacaaaag	gaaataatctc	tgagatacat	taagliaactt	aaaaaaaaaac
62941	tttacacagt	ctgcctagta	cattactatt	tggaatatat	gtgtgcittat	tfgcatattc
63001	ataatctccc	tactttatft	tcttttattt	ttaattgata	cataatcatt	atacatattt
63061	atgggttaaa	gigtlaatgtt	ttaatatgtg	tacacatatt	gaccaaatca	gggiaatttt
63121	gcatttgtaa	ttttaaaaaa	tgccttcttc	ttttaatata	cttttttgtt	tatcttattt
63181	ctaatacttt	ccctaacttc	tttctttcag	ggcaataatg	atacaatgta	tcatgcctct
63241	ttGCACCATT	CTAAAGAATA	ACAGTGATAA	Ttttctgggtt	aaggCaatag	caatatttct
63301	gcatataaat	atttctgcat	atttctgcat	ACTGATGTAA	GAGGTTTCAT	ATTgctaata

Table 1. Primer and probe sequences used in the HBB genotyping assay

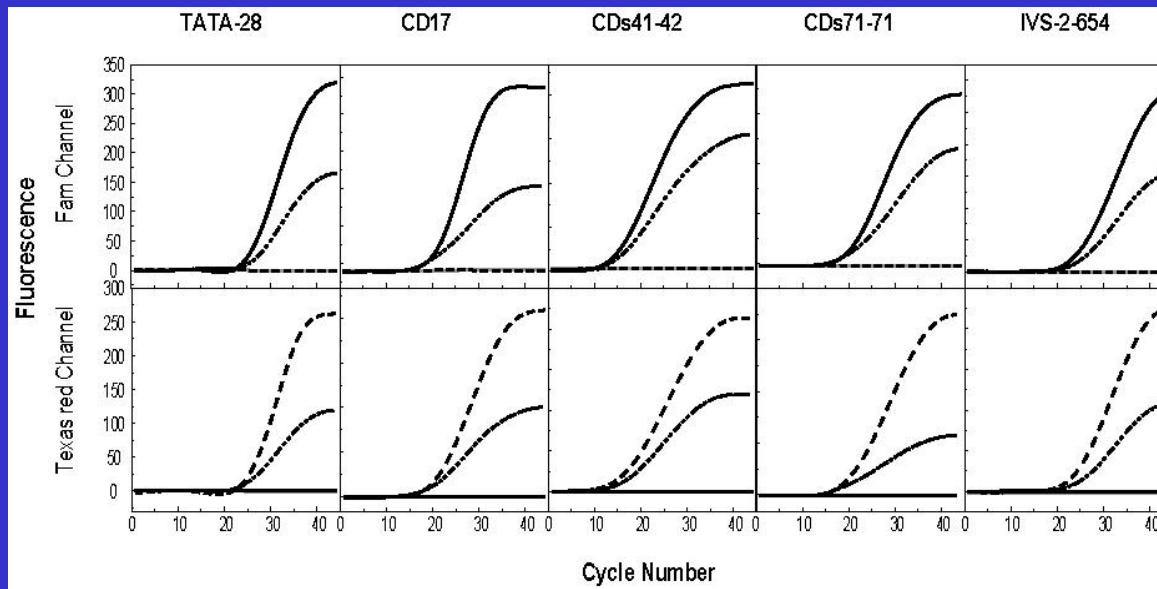
Mutations	Primers	Probes*
TATA-28 (A → G)	5' -GCCAATCTACTCCCAGGAGC-3'	5'-Fam-AGGGCTGGGCATAAAGTCAGGGC-PO ₄ -3' 3'-Dabcyl-TCCCGACCCGTATTTTCAGTCCC-5'
	5' -TGTCAGAAGCAAATGTAAGCAATA-3'	5'-Texas red-AGGGCTGGGCATAGAAAGTCAGGGC-PO ₄ -3' 3' -Dabcyl -TCCCGACCCGTATCTTCAGTCCC-5'
CD17 (A → T)	5' -CACTAGCAACCTCAAACAGACAC-3'	5'-Fam-CCTGTGGGGCAAGGTGAACGT-PO ₄ -3' 3'-Dabcyl-GGACACCCCGTTCCACTTGC-5'
	5' -CCAGGGCCTCACCACCAAC-3'	5'-Texas red-CCTGTGGGGCTAGGTGAACGT-PO ₄ -3' 3' -Dabcyl -GGACACCCCGATCCACTTGC-5'
CDs41/42 (-TCTT)	5' -TCTGCCTATTTGGTCTATTTTCCC-3'	5'-Fam-GACCCAGAGGTTCTTTGAGTCCTT-PO ₄ -3' 3'-Dabcyl-CTGGGTCTCCAAGAACTCAGGA-5'
	5' -CATAACAGCATCAGGAGTGGACA-3'	5'-Texas red-GACCCAGAGGTTGAGTCCTTTGGG-PO ₄ -3' 3' -Dabcyl -CTGGGTCTCCAACTCAGGAAACC-5'
CDs71/72 (+A)	5' -GTGAAGGCTCATGGCAAGAAAGT-3'	5'-Fam-CGGTGCCTTTGTAGTGATGGCC-PO ₄ -3' 3'-Dabcyl-GCCACGGAAATCACTACCG-5'
	5' -AAGGTGCCCTTGAGGTTGTC-3'	5'-Texas red-CGGTGCCTTTAAAGTGATGGCCT-PO ₄ -3' 3' -Dabcyl -GCCACGGAAATTCAC'TACCGG-5'
IVS-2-654 (C → T)	5' -GCACCATTCTAAAGAATAACAGTGATA-3'	5'-Fam-CTGGGTTAAGGCAATAGCAATAT-PO ₄ -3' 3'-Dabcyl-GACCCAATTCCGTTATCGTTAT-5'
	5' -AATATGAAACCTCTTACATCAGTTACAA-3'	5'-Texas red-CTGGGTTAAGGTAATAGCAATAT-PO ₄ -3' 3' -Dabcyl -GACCCAATTCCATATCGTTAT-5'

*Blocked bases denote mutation sites.

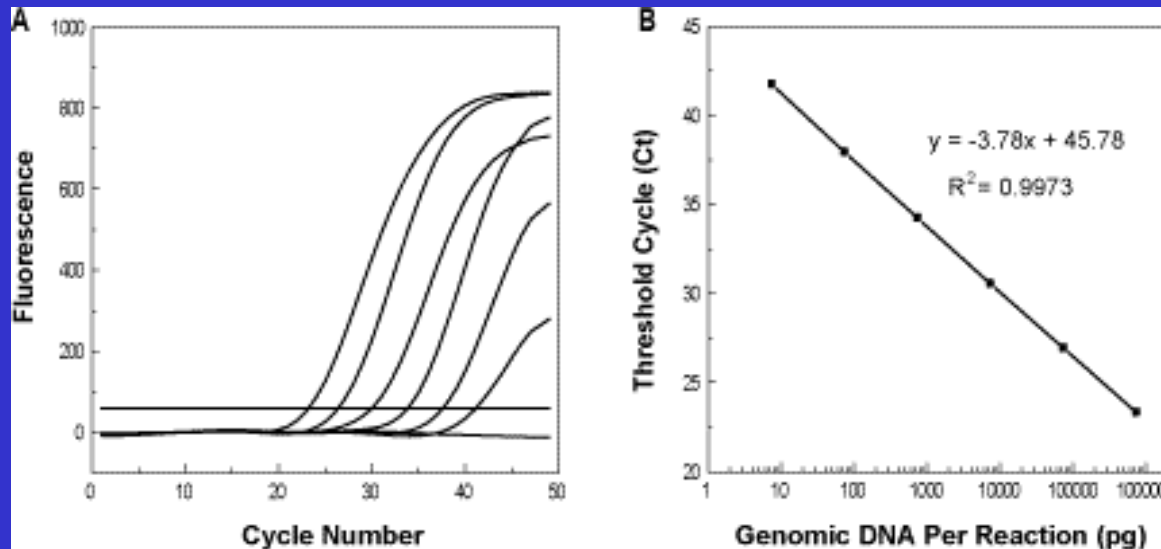
Thermal denaturation analysis of displacement probes



Unequivocal genotyping of the five mutations

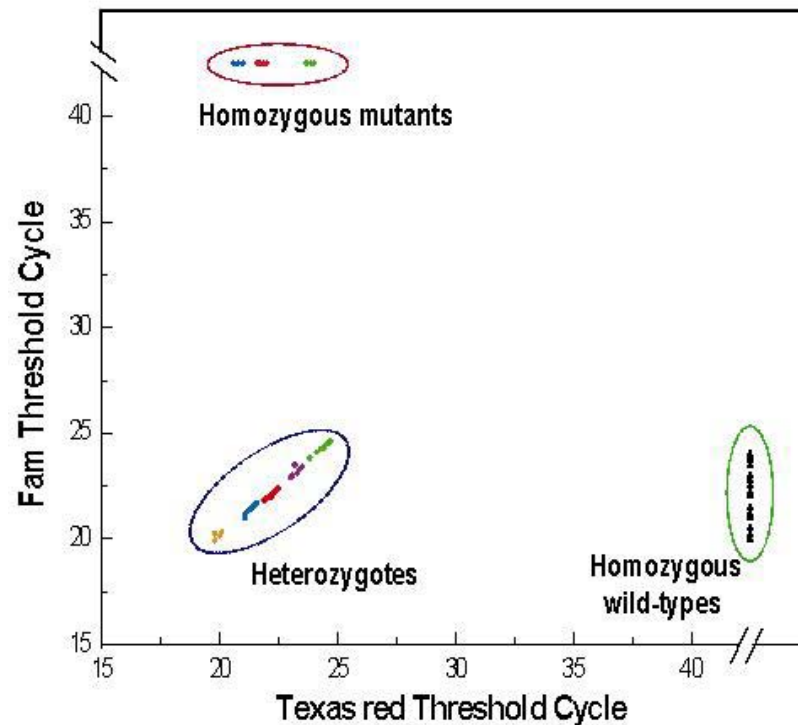


Wide range of linear quantification of displacing probes



Real-Time Genotyping of confirmed samples

List of 62 DNA samples of known β -globin genotype used in a blinded validation analysis of the double-stranded probes in the real-time genotyping assay



Samples	Genotypes	Number
Heterozygous samples	TATA-28 / N	6
	CD17 / N	7
	CDs41-42 / N	7
	CDs71-72 / N	4
	IVS-2-654 / N	7
Homozygous samples	CD17 / CD17	3
	CDs41-42 / CDs41-42	5
	IVS-2-654 / IVS-2-654	3
Normal samples	N / N	20
Total		62

Double-Stranded Design Guidelines Probes for SNP Genotyping

√ Position the polymorphic site approximately in the middle third of the probe sequence

Keep G-C content in the 30–80% range

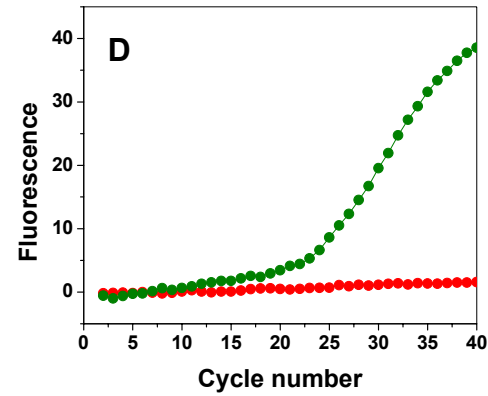
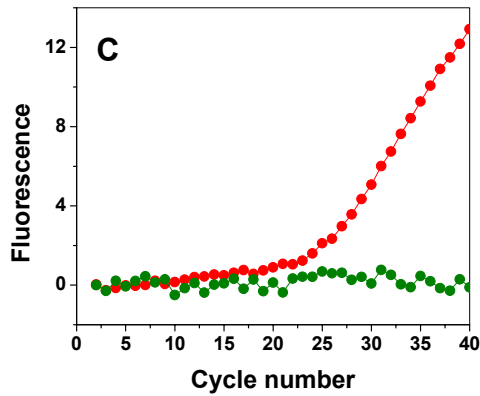
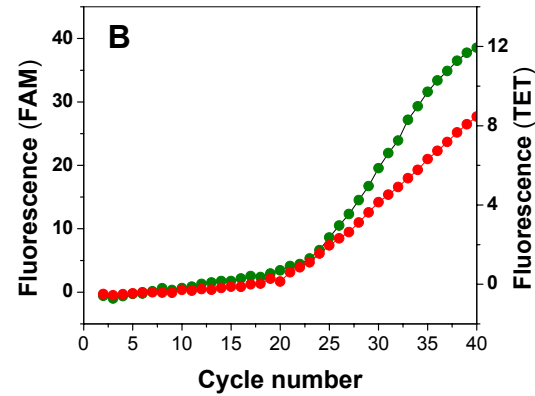
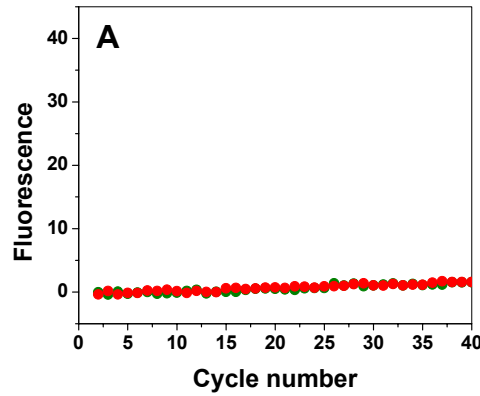
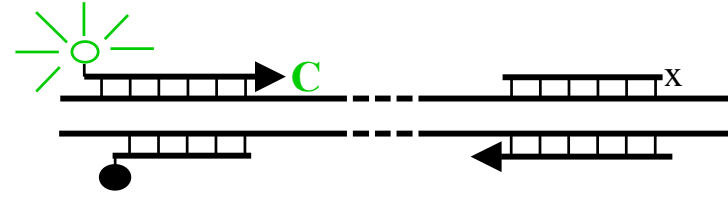
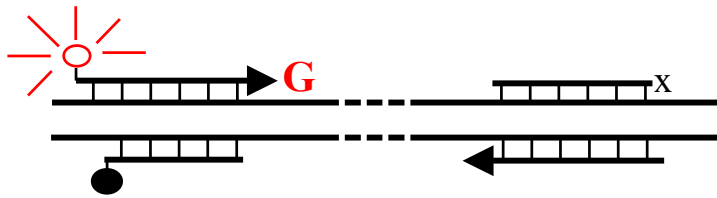
Avoid runs of an identical nucleotide

Do not put Gs on the 5' end as a G residue adjacent to the reporter dye will quench the reporter fluorescence somewhat

Select the strand as positive strand that gives the probe more Cs than Gs

√ The T_m of the probe should be 55-70 °C

√ The length difference between positive strand and negative strand is in the range of 1-3bp



Real-time PCR genotyping with dsPrimer

Conclusions:

- Displacement hybridization is more specific than direct hybridization;
- Novel dsProbe and dsPrimer could be developed based on displacement hybridization;
- Both dsProbe and dsPrimer could be used for real-time PCR genotyping;
- Also, dsProbe and dsPrimer could be used in an end-point detection format genotyping when combined with synchronous fluorometry.
- Multiplex genotyping could be accomplished in a single tube with multicolor dsProbe or dsPrimer.

Current probes for real time amplification assay

Probes	Chemistry	SNP Detection	Multiplex	Cost	Design
Molecular beacons	Proximity quenching	High	Yes	Upper medium	Difficult
TaqMan	Cleave FRET	Ordinary	Minimal	Upper medium	Less difficult
LightCycler	Adjacent FRET	Ordinary	Minimal	Upper medium	Less difficult
Intercalating dyes	Dye binding	None	No	Minimal	Easy
Light up	Dye binding	Ordinary	No	High	Difficult
Cyclons	Proximity quenching	Unknown	Yes	High	Difficult
Scorpions	Proximity quenching	High	Yes	High	Difficult
Amplifluor	Proximity quenching	Ordinary	Yes	High	Difficult
Eclipse Probes	MGB-FRET	High	Ordinary	High	Difficult
?	?	High	Yes	Low	Easy