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MultiCode®-RTx for Quantifying Mixed Populations of Drug-Resistant HIV

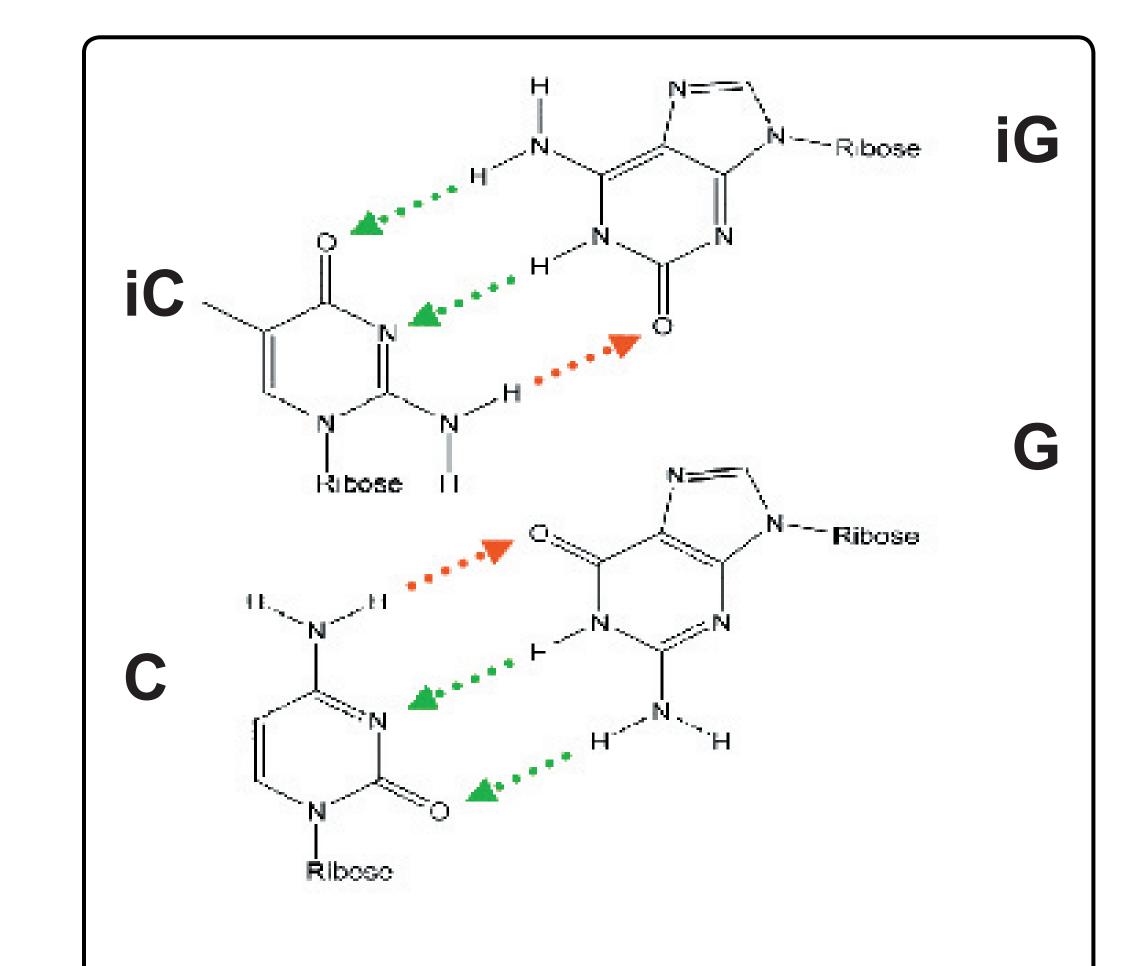
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ombined modalities. Some of these emerging mutations are more common than others and have become markers for antiretroviral resistance.

Methods: For the early detection of these markers, a novel MultiCode RTx one-step PCR testing system to rapidly and simultaneously characterize mixtures of HIV-1 targets was designed.

as 1 to 10,000. Mixed population standard curves using 1-100% of mutant or wild-type generated over four logs of total viral particle input produced consistent results.

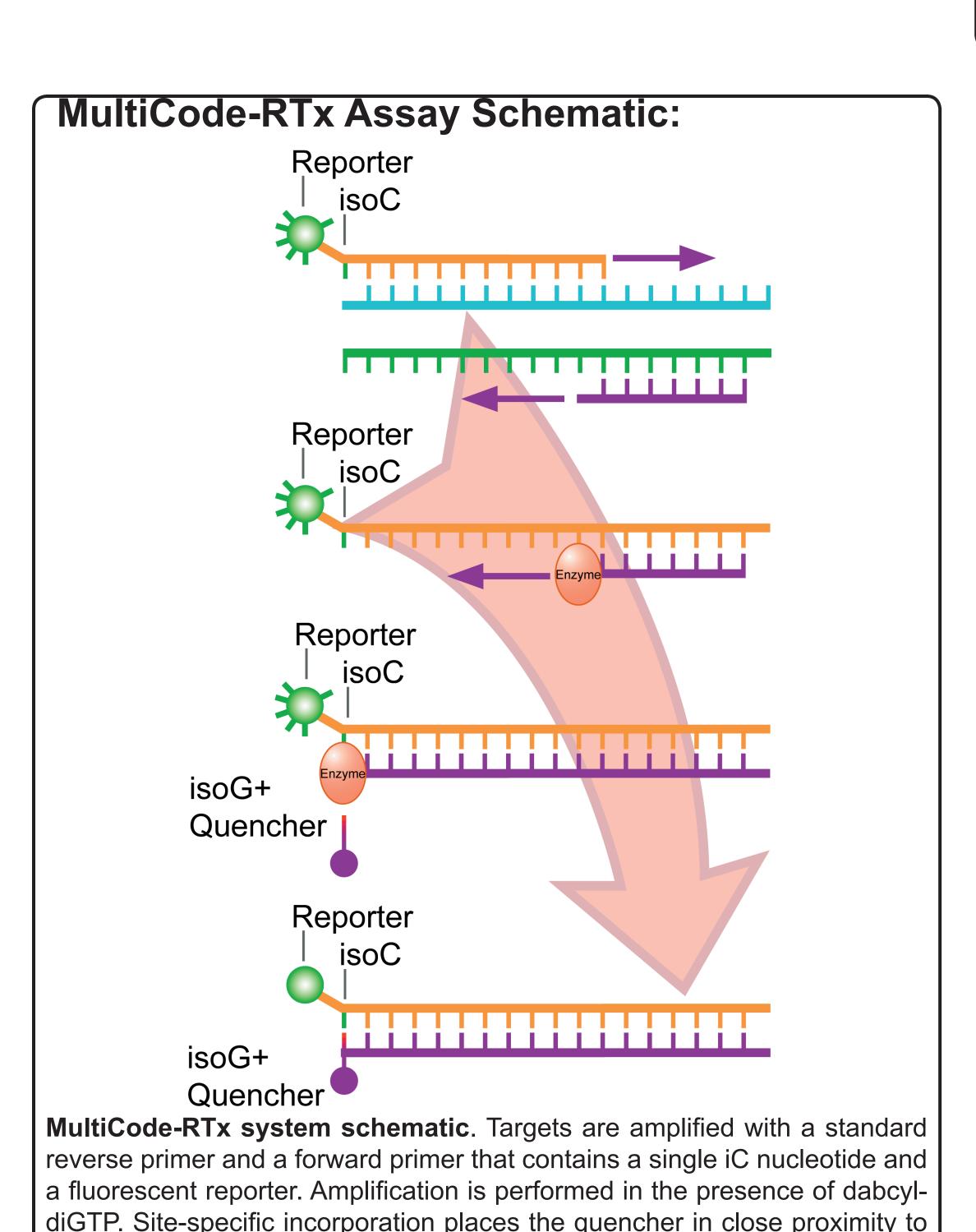
Conclusions: MultiCode RTx could be applied to other drug selected mutations in the viral genome or for other applications where single base changes in DNA or RNA occur at frequencies reaching 0.01% to 1% respectively. Supported by NIH grant AI-058888



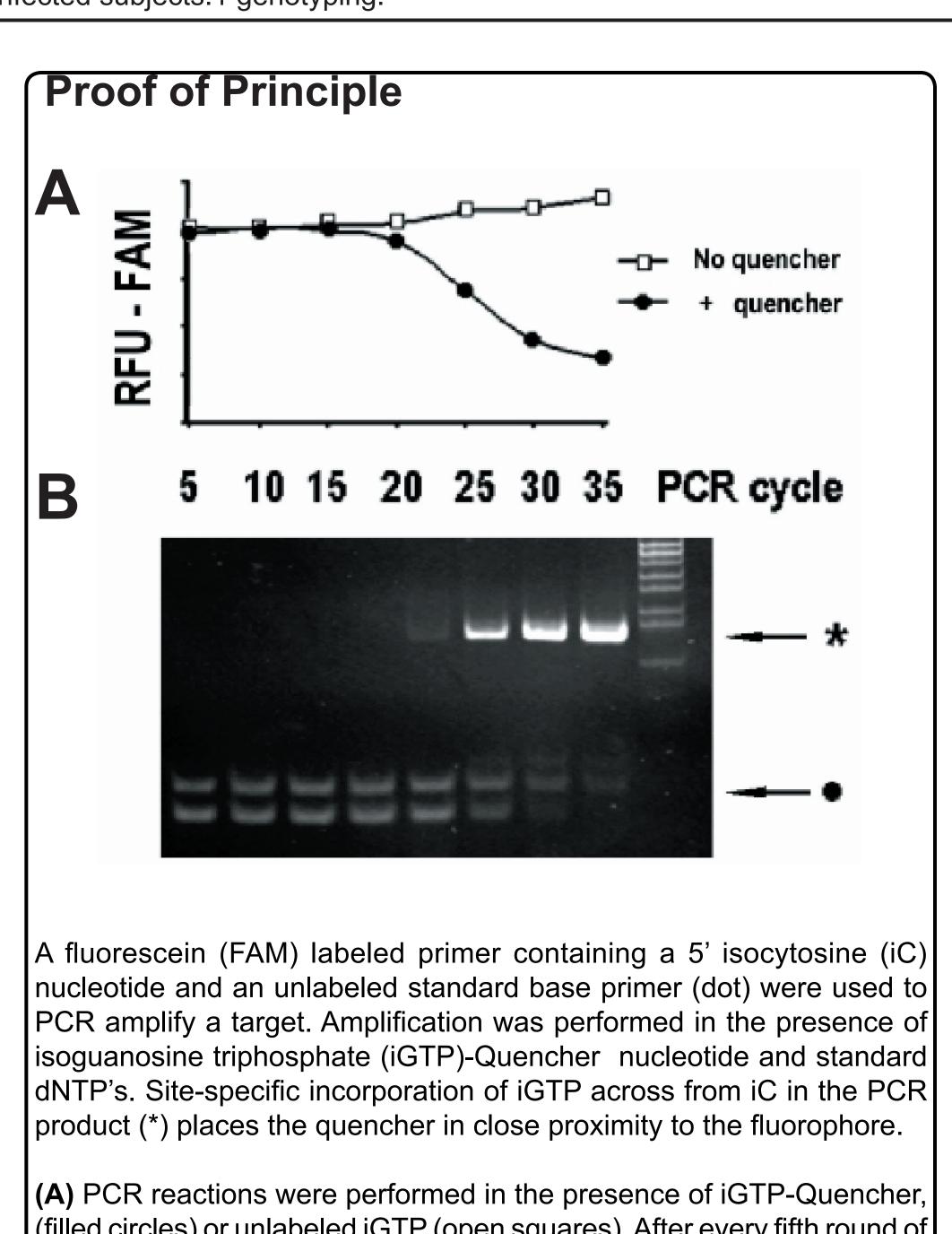
MultiCode bases are structurally quite similar to standard nucleobases but have altered base-pairing specificity. The pattern of hydrogen bond acceptors and donors is rearranged for the MultiCode base pair between 5-methyl-isocytosine (iC) and isoguanosine (iG) (top) as compared to a standard cytosine (C) guanosine (G) base pair (bottom).

Cono	Mutation	
<u>Gene</u>	Mutation M41L	Drug(s) AZT
pol		
pol	T215Y, T215F	AZT
pol	K65R	DDI, TDF, DAPD
pol	L74V	DDI, DAPD
pol	T69D	DDC
pol	E44D	3TC
pol	V118I	3TC
pol	M184V, M184I	3TC, FTC
pol	L100I	Multi-NNRTI
pol	K103N	NNRTI
pol	Y181C, Y181I	NNRTI
pol	Y188L	NNRTI
•		
protease	M46I	Multi-drug resistance
protease	L90M	Multi-drug resistance
protease	G48V	Saquinavir
protease	D30N	Nelfinavir
protease	I50V	Aprenavir
-		- -

List of mutations selected for MultiCode-RTx quantitative genotyping. HIV Reverse transcriptase (RT) inhibitors such as the nucleoside analogs (-)-(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclo pentene-1-methanol (ABC; abacavir), 9-[2-(phosphonomethoxy)propyl]adenine (TDF; tenofovir), 2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC, emtricitabine) 39-azido-39-deoxythymidine (AZT; zidovudine), 29,39-dideoxyinosine (ddl; didanosine), 29,39-dideoxycytidine (ddC;zalcitabine), (2)-b-L-29,39-dideoxy-39-thiacytidine (3TC; lamivudine), and 29.39-didehvdro-39-deoxvthvmidine (d4T: stavudine) are currently approved for the treatment of advanced human immunodeficiency virus (HIV) type 1 (HIV-1) infections. All these nucleosides act in a similar way, namely, as chain terminators of the RT reaction after phosphorylation by intracellular kinases. Treatment of HIV infection with these nucleoside inhibitors invariably leads to selection and accumulation drug-resistant mutations that arise due to the intrinsically low fidelity of the polymerase. Perhaps the most well known of a very large number of mutations observed in HIV RT is HIV M184V. It is believed that these new drug resistant mutants may start from a single virus and slowly increase in population. Yet there is little data to prove this since current technologies have not been capable of analyzing the mutants when they are present in an overwhelming population of the drug sensitive viruses. New technologies that would allow for early quantitative and specific detection of drug resistant infectious disease strains in a mixed population of wild-type particles would be valuable in drug discovery, clinical trial assessment and in monitoring HIV infected subjects.1 genotyping.

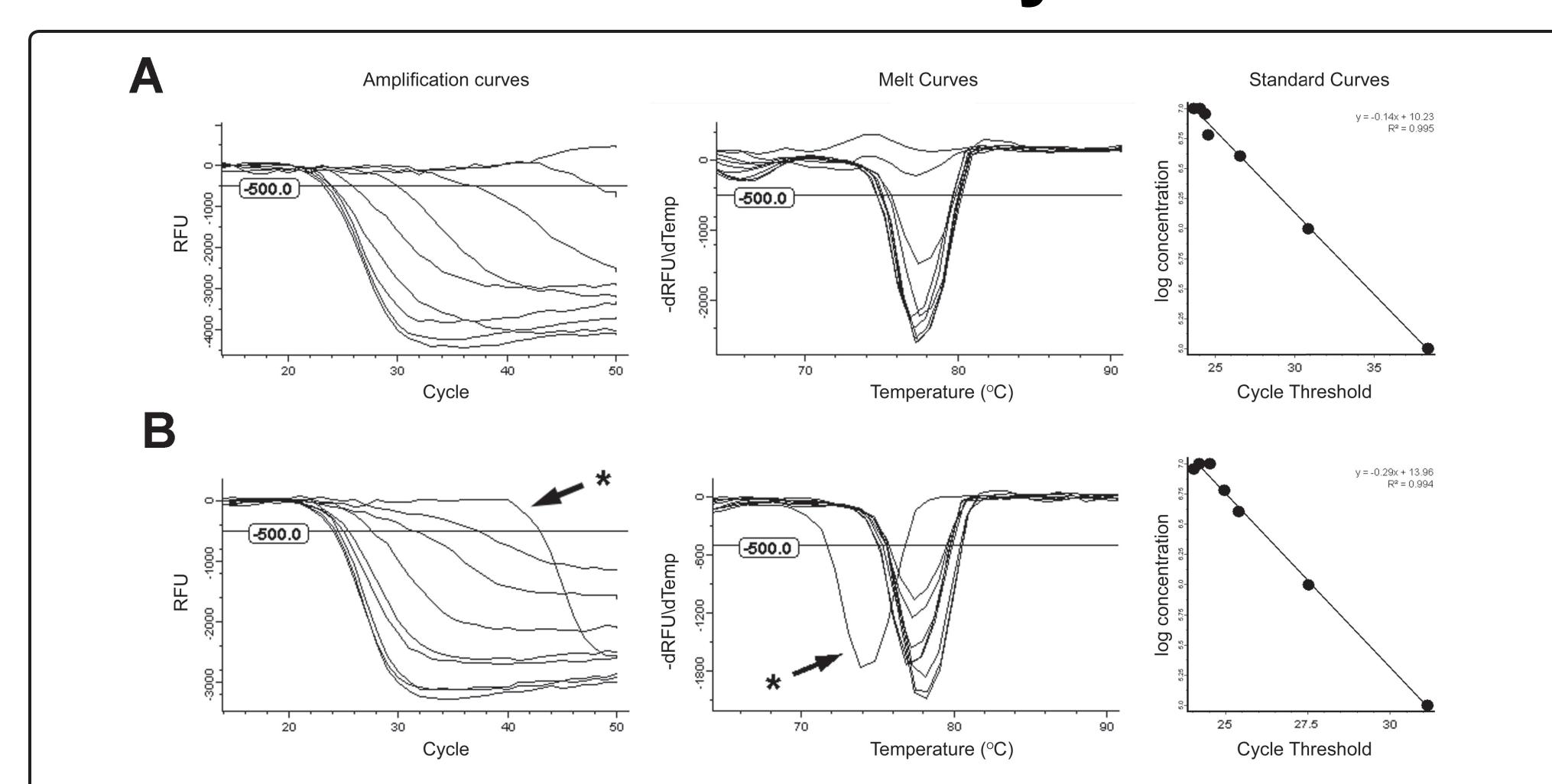


the reporter that leads to a decrease in fluorescence.



(filled circles) or unlabeled iGTP (open squares). After every fifth round of cycling a 5 µl aliquot was removed and analyzed using a Cytofluor 4000 fluorescent plate reader. (B) The 5 μ l aliquots were then used for PAGE analysis. The gel was stained with EtBr and scanned for fluorescence.

EraGen MultiCode-RTx System for Advanced Closed-Tube Real-Time RT-PCR



Real-time, melt and standard curve data of RNA assay in mixed populations of viral RNA. A. Data from wild-type channel. B. Data from M184V channel. (*) Note: Late non-specific amplification in no target control reaction of M184V channel is distinguished by melt Tm.

Cycling conditions ^a	K65K Slope ^b , R ²	K65R Slope ^b , R ²	M184M Slope ^b , R ²	M184V Slope ^b , R ²
1.) 5 sec anneal, Slow ramp to extension all rounds of PCR, 50 rounds PCR	-0.14, 0.995	-0.15, 0.984	-0.19, 0.998	-0.18, 0.997
2.) 5 sec anneal, Fast ramp to extension first round of PCR, 50 rounds of PCR	-0.11, 0.992	-0.14, 0.981	-0.18, 0.997	-0.17, 0.990
3.) 1 sec anneal, Fast ramp to extension all rounds of PCR, 100 rounds PCR	-0.07, 0.949	-0.06, 0.977	-0.09, 0.991	-0.13, 0.990
4.) ABI 7900, 5 sec anneal, slow ramp all 45 rounds of PCR	-0.14, 0.995	-0.13, 0.994	-0.20, 0.994	-0.19, 0.997

Standard curve slope and linear regression R² values from runs with varying cycling conditions and instruments. To test for assay robustness, the M184V and K65R specific assays were performed using four cycling conditions on two instruments as described in methods. Mixtures of cloned DNA targets were tested to obtain Ct curves. We used 10-fold increases and decreases of both mutant and wild-type DNA targets from 103 to 107 copies per reaction with a total of 107 copies per reaction. This set-up provided mixtures that varied in wild-type and mutant targets from 0.01 to 100 percent. We then determined linear regression analyses for individual channels of log[copy number] vs. Ct. Ct values for each of the two specific channels of both the K65R and the M184M systems were plotted vs. log[target copy number]. Linear regressions were performed and linear best fit slopes and R² values are presented.

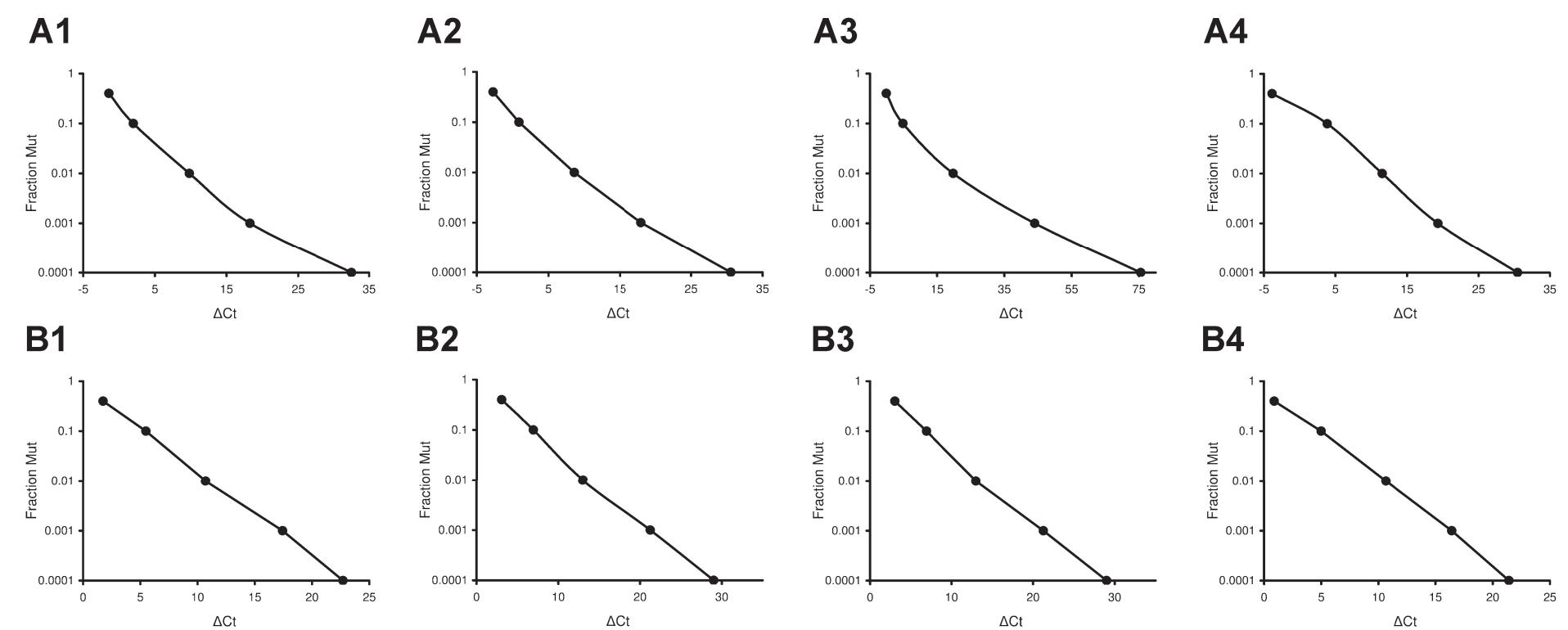
^a For each assay, PCR primers were used at the following concentrations: forward specific primers at 200 nM reverse primers at 400 nM. PCR conditions were as follows: 25 µL reactions in 1x ISOlution buffer (EraGen, Madison, WI) and Titanium Tag DNA polymerase (Clontech, CA) at manufacturers recommended concentration. For one step MultiCode RTx reverse transcriptase PCR assays the conditions included the following: 0.5 Units/µL Maloney Murine Reverse Transcriptase (M-MLV RT) and 5 mM dithiothreitol and an additional 5 minutes at 50°C added prior to the denaturation step. PCR cycling parameters with ramp rates of 20°C/sec unless otherwise specified on the Roche LightCycler (Roche, Indianapolis, IN) were:

1.) 2m @ 95°C, 1 cycle of 1sec @ 95°C, 1 sec @ 45°C, 1°C/sec ramp to 20 sec @ 72° C, 50 cycles 5 sec @ 95°C, 5 sec @ 55°C, 1°C/sec ramp to 20 sec @ 72° C (SINGLE read), Melt 60-95°C 0.4°C/sec ramp; (STEP read); 2.) 2 min @ 95°C, 1 cycle of 1 sec @ 95°C, 1 sec @ 45°C, 20 sec @ 72°C, 50 cycles of 5 sec @ 95°C, 5 sec @ 55°C, 1°C/sec ramp to 20 sec

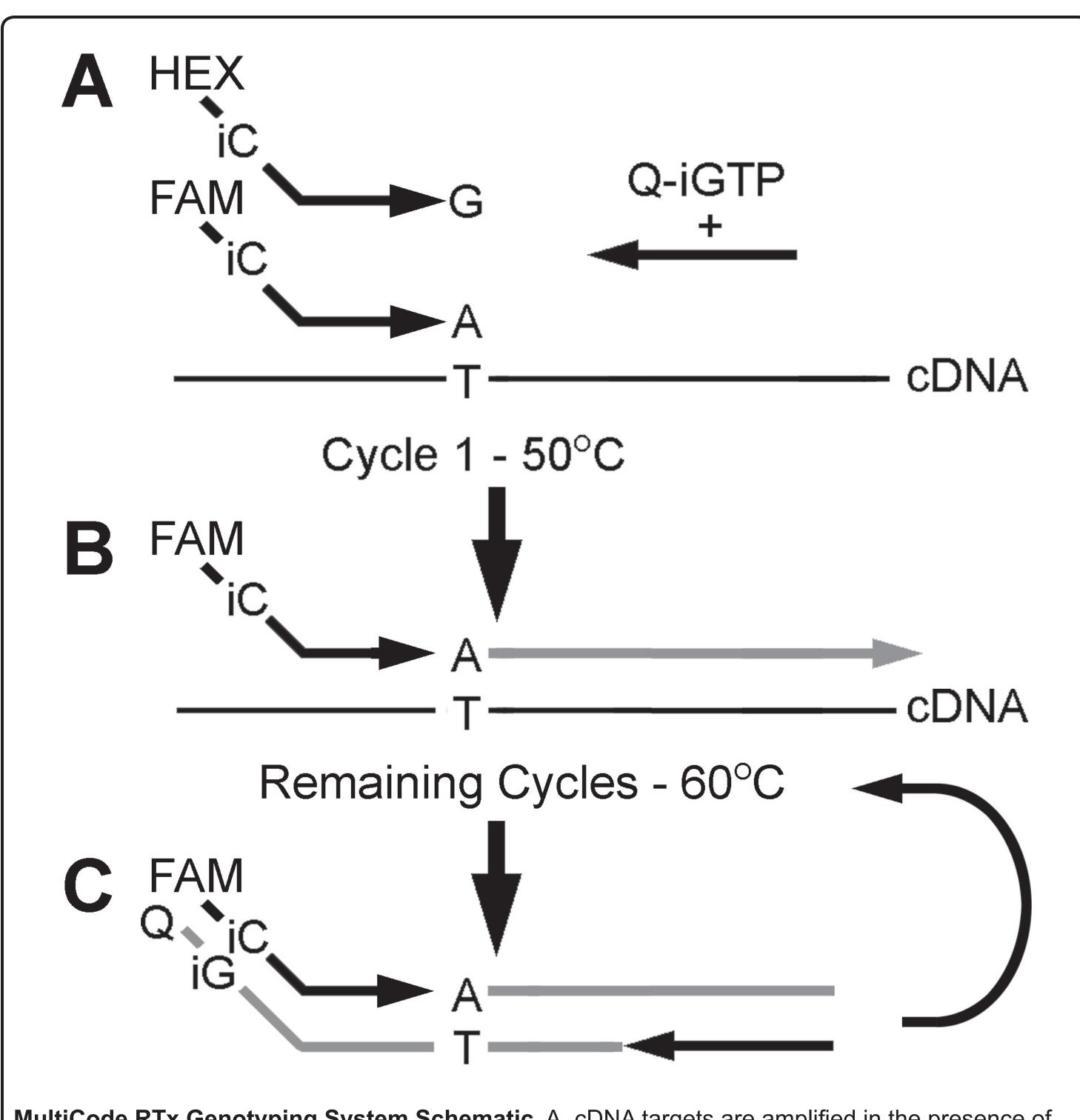
@ 72°C (SINGLE read), Melt 60-95°C 0.4°C/sec ramp (STEP read); 3.) 2m @ 95°C, 1 cycle of 1 sec @ 95°C, 1 sec @ 45°C, 20s @ 72°C, 100 cycles of 1 sec @ 95°C, 1 sec @ 55°C, to 20 sec @ 72°C (SINGLE

read). Melt 60-95°C 0.4°C/sec ramp (STEP read). 4.) PCR cycling parameters on the ABI Prism 7900 (ABI, Foster City, CA) real-time thermal cycler were 2 minutes denature at 95°C, 1 cycle of 5 sec @ 95°C, 5 sec @ 45°C, 20 sec @72°C, followed by 45 cycles of 5 sec @ 95°C, 5 sec @ 60°C, 20 sec @72°C with optical read. A thermal melt at 7% ramp rate with optical read from 60 to 95°C was performed directly following the last 72°C step of thermal cycling.

b Slope expressed as log[copy number]/Ct, slope of -0.31 corresponds to 100 % PCR efficiency, greater values indicate reduced PCR



ΔCt vs. fraction mutant plots for real-time PCR runs with varying cycling conditions and instruments. Fraction Mut is fraction of mutant DNA template in a mixture with wild-type DNA at a total concentration of 10⁷ copies. ΔCt is the Ct of the mutant channel fluorescence minus the Ct of the wild-type channel fluorescence on the LightCycler or the ABI 7900. A1-A4 K65R and B1-B4 M184V. A1-A3, B1-B3 were performed on the Roche LightCycler and A4, B4 were performed on the ABI 7900. See Table legend for details on reaction and cycling conditions.



MultiCode RTx Genotyping System Schematic. A. cDNA targets are amplified in the presence of iGTP-dabcyl (Q-iGTP), one standard reverse and two RTx forward primers. The two forward primers are bipartite. The 5'-parts contain single iCs, separable fluorescent reporters and target independent sequence tails that add 10°C to the annealing temperature of each primer. The 3'-parts are targetspecific, contain a 3' mutation-specific base (A or G) and have an annealing temperature of 50°C to the cDNA targets. B. A single round of competitive priming at 50°C results in specific extension (gray arrow) creating the A:T target. C. Remaining cycles at 60°C inhibit annealing of the G-primer and place quenchers in close proximity to the reporter.

		WT		MUT	
prote	ein site	Sensitivity	Slope	Sensitivity	Best Mixture
prote	ase 30N	10	-0.23	100	0.1%
prote	ease 46I	10000	-0.25	not detected	MUT Fails
prote	ease 48V	100000	-0.25	1000	0.1%
prote	ase 50V	100	-0.24	100	0.1%
prote	ase 90M	10000	-0.27	1000	0.1%
RT	41L-CTG	100	-0.22	100	0.01%
RT	44D	10	-0.23	100	0.01%
RT	65R (1)	100	-0.16	100	0.01%
RT	65R (2)	10	-0.16	100	0.01%
RT	69D	1000	-0.19	not detected	MUT Fails
RT	74V	10000	-0.22	100	1%
RT	103N	10000	-0.25	100	1%
RT	118I	10000	-0.32	1000	MUT Fails
RT	181C	10	-0.23	not detected	MUT Fails
RT	181I	10	-0.28	100	0.01%
RT	184I	100	-0.24	100	0.01%
RT	184V (1)	10	-0.20	100	0.01%
RT	184V (2)	100	-0.20	100	0.01%
RT	188L-CTT	10	-0.23	100	0.1%
RT	188L-TTA	10	-0.23	1000	0.1%
RT	215F	10	-0.22	100	1%
RT	215Y	10	-0.26	100	1%

13 functional systems 20 total mutations tested

65% percentage functional systems

Results of MultiCode RTx HIV-1 Mixed Population Genotyping testing on RNA targets.

HIV-1 drug resistance mutant RNA targets were mixed at 100 or 1000 copies with wild-type RNA target in a 10-fold dilution series from 0 to 106 copies and subjected to MultiCode RTx HIV-1 Mixed Population Genotyping testing on the ABI 7900. Sensitivity to both wild type and mutant targets in copy numbers is presented, "not detected" indicates sensitivity greater than 1000 mutant RNA targets. Bes mixture is the lowest percentage mutant target in a mixture with wild-type target that could be detected. Slope is the slope wild-type standard curve in log copy number vs. cycle threshold. A slope of -0.32 indicates 100% PCR efficiency with greater values indicating reduced PCR efficiency. Functional systems are defined as those that can detect 100 copies of both wild-type and mutant RNA in a mixture of 1% or less. RT indicates HIV-1 reverse transcriptase.

		ABI 7900 WT		I MUT		LightCycler WT		I MUT	
Protein	Site	Sensitivity	Slope	Sensitivity	Best Mixture		Slope	Sensitivity	Best Mixture
protease	30N	10	-0.29	1000	0.1%	1000	-0.14	1000	0.1%
protease		10	-0.24	100	0.1%	10	-0.12	100	0.01%
protease	48V	100	-0.23	100	0.01%	100	-0.09	100	0.01%
protease		1000	-0.24	1000	50%	100	-0.16	1000	90%
protease		100000	-0.17	not detected	MUT Fails	1000	-0.08	100	0.1%
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RT	41L-TTG	100	-0.22	100	0.01%	1000	-0.12	100	0.01%
RT	41L-CTG	100	-0.22	100	0.01%	10000	-0.09	100	0.01%
RT	44D	1000	-0.21	1000	90%	100	-0.09	100	0.1%
RT	65R (1)	1000	-0.28	1000	0.1%	100	-0.22	1000	0.1%
RT	65R (2)	1000	-0.23	1000	0.1%	1000	-0.08	1000	0.01%
RT	69D `	100	-0.24	1000	0.01%	100	-0.08	100	0.01%
RT	74V	100	-0.27	100	0.1%	1000	-0.18	100	0.01%
RT	100L	10000	-0.24	1000	0.1%	1000	-0.15	100	0.01%
RT	103N	100	-0.24	100	0.1%	100	-0.16	100	0.1%
RT	1181	100	-0.26	100	50%	10	-0.12	1000	0.1%
RT	181C	10	-0.23	not detected	MUT Fails	1000	-0.12	not detected	MUT Fails
RT	181I	100	-0.26	not detected	MUT Fails	100	-0.20	100	90%
RT	184 I	100	-0.24	100	0.01%	100	-0.09	100	0.01%
RT	184V (1)	100	-0.25	100	0.01%	100	-0.23	100	0.1%
RT	184V (2)	100	-0.24	100	0.01%	100	-0.15	100	0.1%
RT	188L-CTT	10	-0.24	1000	10%	1000	-0.16	100	0.01%
RT	188L-TTA	100	-0.22	100	0.1%	100	-0.14	100	0.01%
RT	215F	10	-0.27	100	0.01%	100	-0.12	100	0.01%
RT	215Y	10	-0.26	100	1%	10	-0.14	100	0.01%

14 functional systems 20 total mutations tested 70% percentage functional systems

Results of MultiCode RTx HIV-1 Mixed Population Genotyping testing on DNA targets.

HIV-1 drug resistance mutant plasmid DNA clone targets were mixed at 100 or 1000 copies with wild-type DNA target in a 10 fold dilution series from 0 to 10⁶ copies and subjected to MultiCode-RTx HIV-1 Mixed Population Genotyping testing on the ABI 7900 and the Roche LightCycler. Sensitivity to both wild-type and mutant targets in copy numbers is presented, "not detected indicates sensitivity greater than 1000 mutant RNA targets. Best mixture is the lowest percentage mutant target in a mixture with wild-type target that was detected. Slope is the slope wild-type standard curve in log copy number vs. cycle threshold. A slope of -0.32 indicates 100% PCR efficiency with greater values indicating reduced PCR efficiency. Functional systems are defined as those that can detect 100 copies of both wild-type and mutant RNA in a mixture of 1% or less on either the ABI 7900 or the Roche LightCycler. Two independent systems for the 65R and 184V loci were evaluated. RT indicates HIV-1 reverse transcriptase.

	Calls			Mismatches		
Mutation	Correct	Not Called	Miscalled	Correct	Not Called	Miscalled
M41L	9	4	0	37	30	0
K65R	8	5	0	4	14	0
T69D	4	8	1	4	32	1
L74V	8	5	0	12	22	0
V118I	10	3	0	18	20	0
M184V	6	7	0	17	38	0
T215F,Y	7	6	0	25	37	0
Total	52	38	1	117	193	0
Percentage						
and Mean	57%	42%	1%	2.3	5.1	1.0

Results from MultiCode-RTx HIV-1 Mixed Population Genotyping of HIV-1 patient sample clones. Plasmid DNA of 12 clones from the NIH Multidrug Resistant HIV-1 Reverse Transcriptase (RT) Panel and a wild-type clone were tested on the ABI 7900 using The MultiCode RTx HIV-1 Mixed Population Genotyping systems listed. The results of 91 potential genotype calls were compared to the known genotypes of each sample as determined by sequencing and classified into three categories: correct calls. not called, or miscalled (n = 1, sample was T69S). Sequences of the discriminatory primers for each system were aligned with the sequences of each of the RT Panel clones to determine the number of mismatched bases between the primer and template sequences. The total number of mismatches in each category was divided by the number of calls made to determine the mean number of primer-target mismatches for each group.

Conclusions:

Other methods for biallelic polymorphism testing from pooled samples exist. The first was discussed by Kwok. Since then a number of other techniques have been proposed to further simplify genome wide association studies, some more complicated then others. But easy to use and ultra-specific methodologies do not seem to be available for mixed population testing. One method called "Needle-in-a-haystack" exceeds the specificity described here with sensitivities down to 1 in 10⁶ yet requires complicated multiple steps. Other real-time PCR methods such as SYBR green have been used for mixed population analysis. However, SYBR green methodology does not allow for the simultaneous real-time detection of multiple targets which limits the specificity. For example, SNP allele frequency was measured be dividing the pools between two PCR reaction each containing a primer pair specific to one or the other variant. Mismatch amplification under these conditions is typically delayed by 10 cycles Mixtures of 19:1 could be identified using the multi-well approach. This level in mixture detection range may be due to well to well variation, lack of competition between primers for the target or reaction conditions. There are other techniques that enable differential fluorescent labeling of the primers. These should also allow for mixed population genotyping, yet to our knowledge I there are no published reports to demonstrate specificity near what is described for the RTx method. Other techniques employ-Ing amplicon specific hybridization probes are well entrenched within the science communit. Ironically this is due to the perception that probes are needed to gain specificity. Yet no literature reports showing single base specificity in mixed populations below the standard 50:50 SNP studies could be found. This is not to say that these probe based systems could not be used in such studies, but the limits placed on hybridization methods may make this difficult.