4. Geller RJ, Spyker DA, Herold DA, Bruns DE. Serum osmolal gap and ethanol concentration: a simple and accurate formula. Clin Toxicol 1986;24:77–84.
5. McQuillen KK, Anderson AC. Osmol gaps in the pediatric population. Acad Emerg Med 1999;6:27–30.
6. Glaser DS. Utility of the serum osmol gap in the diagnosis of methanol or ethylene glycol ingestion. Ann Emerg Med 1996;27:343–6.
7. Hoffman RS, Smilkstein MJ, Howland MA, Goldfrank LR. Osmol gaps revisited: normal values and limitations. Clin Toxicol 1993;31:81–93.
8. Osterloh JD, Kelly TJ, Khayam-Bashi H, Romeo R. Discrepancies in osmol gap and calculated alcohol concentrations. Arch Pathol Lab Med 1996;120: 637–41.
9. Purssell RA, Pudek M, Brubacher J, Abu-Laban RB. Derivation and validation of a formula to calculate the contribution of ethanol to the osmol gap. Ann Emerg Med 2001;38:653–9.
10. Purssell RA, Lynd LD, Koga Y. The use of the osmole gap as a screening test for the presence of exogenous substances. Toxicol Rev 2004;23:189–202.
11. Koga Y, Purssell RA, Koga Y, Lynd LD. The irrationality of the present use of the osmole gap. Toxicol Rev 2004;23:203–11.
12. Khajuria A, Krahn J. Osmolality revisited: deriving and validating the best formula for calculated osmolality. Clin Biochem 2005;38:514–9.

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Multiplex Tetra-Primer Amplification Refractory Mutation System PCR to Detect 6 Common Germline Mutations of the *MUTYH* Gene Associated with Polyposis and Colorectal Cancer,

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Background: **We describe a simple tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) for detecting *MUTYH* mutations, which are associated with colorectal adenomas and colorectal cancer.**

Methods: **We designed specific T-ARMS-PCR assays for 6 mutations (Y165C, G382D, 1395_7delGGA, Y90X, 1103delC, and R231H) selected on the basis of the frequency of their occurrence. We also designed a set of 3 multiplex T-ARMS PCR assays, each for detection of 2 mutations. We tested DNA samples from patients with attenuated or classic adenomatous polyposis coli and no detectable *APC* germline mutations.**

Results: **All mutations were easily detected with both the specific and multiplex T-ARMS-PCR assays. Results were confirmed by DNA HPLC analysis in all 54 patients, and each mutation was confirmed by direct DNA sequencing.**

Conclusions: **T-ARMS-PCR does not require any special equipment, and it provides rapid, reproducible, and cost-effective detection of common *MUTYH* mutations. Multiplex T-ARMS-PCR allows the detection of 6 common *MUTYH* mutations with use of as few as 3 single tube PCR reactions. It could be useful to carry out large population-based epidemiologic studies.**

MUTYH-associated polyposis (MAP) is an autosomal recessive syndrome associated with biallelic germline mutations in the base excision repair gene *MUTYH* (OMIM #608456) *(1)*. *MUTYH* biallelic germline mutations have been found in 4%–33% *(2, 3)* of patients with multiple colorectal adenomas and in 7.5%–29% of patients who have attenuated or classic adenomatous polyposis coli and no detectable *APC* germline mutations *(2, 4–6)*. Population-based studies suggest that biallelic *MUTYH* germline mutations might be also responsible for ~0.5% of unselected colorectal cancers *(7, 8)*.

At least 23 different putative pathogenic mutations have been identified as widespread in the *MUTYH* gene *(9)*. Two of these mutations (Y165C in exon 7 and G382D in exon 13) account for at least 70% of the mutant *MUTYH* alleles *(2, 6)*, and at least 1 of them is found in more than 80% of Caucasian MAP patients *(2, 4–6, 10, 11)*. In addition, these 2 mutations have been found in the general Caucasian population with a frequency of ~0.5% *(1, 2, 8, 12, 13)*. Other mutations may be frequent in patients from some populations; e.g., the homozygous E466X (exon 14) mutation has been found in 3 patients from unrelated Indian families *(3)*. Recently, we found that in Italian patients a 3-bp deletion in exon 14 (1395_7delGGA) is relatively frequent *(5)* and that each of the mutations Y90X (exon 3), 1103delC (exon 12), and R231H (exon 9) represents more than 6% of mutant *MUTYH* alleles *(14)*.

The identification of germline mutations in both *MUTYH* alleles in patients with multiple colorectal adenomas or colorectal cancer has clinical relevance because their siblings may also have a very high risk of cancer. Thus, genetic testing for *MUTYH* mutations should be offered, after appropriate counseling, to individuals with multiple colorectal adenomas and to members of their families; it may also be offered to individuals with early-onset colorectal cancer *(9, 15)*. In addition, because some of the pathogenic *MUTYH* mutations have relatively high frequencies in the general population and heterozygotes may also have an increased risk of colorectal cancer *(8, 12)*, more widespread genetic testing for *MUTYH* mutations, perhaps in any individual with colorectal cancer, may be advisable.

Many methods, such as single-strand conformation polymorphism analysis and DNA HPLC (dHPLC), are suitable for *MUTYH* mutation detection. These methods, however, require specialized equipment and, most impor-

tantly, are not designed for the screening of known pathogenic mutations in a large number of samples.

Here we describe a simple tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) *(16, 17)* for screening the more frequent *MUTYH* mutations that does not require specialized equipment. Conventional ARMS-PCR amplifies the 2 alleles in 2 different PCR reactions *(18, 19)*. In contrast, T-ARMS-PCR amplifies both wild-type and mutant alleles, together with a control fragment, in a single tube PCR reaction. The region flanking the mutation is amplified by 2 common (outer) primers, producing a non–allele-specific control amplicon (Fig. 1A). Two allele-specific (inner) primers are designed in opposite orientation (Fig. 1A) and, in combination with the common primers, can simultaneously amplify both the wild-type and the mutant amplicons. The 2 allele-specific amplicons have different lengths and can be easily separated by standard gel electrophoresis (Fig. 1A) because the mutation is asymmetrically located with respect to the common (outer) primers (Fig. 1A). Because the control amplicon and at least 1 of the 2 allele-specific amplicons are always present, T-ARMS-PCR provides an internal control with respect to false negatives as well as amplification failure. In addition, the presence of wild-type and mutant allelic amplicons allows easy interpretation of PCR results.

We designed a T-ARMS-PCR for each of the following 6 mutations selected on the basis of their frequency in the literature *(1, 2, 4, 10, 20)* and in our patient series *(5, 14)*: Y165C, G382D, 1395_7delGGA, Y90X, 1103delC, and R231H (Table 1; also see Table 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol52/issue4/). According to reports on systematic mutation analysis of

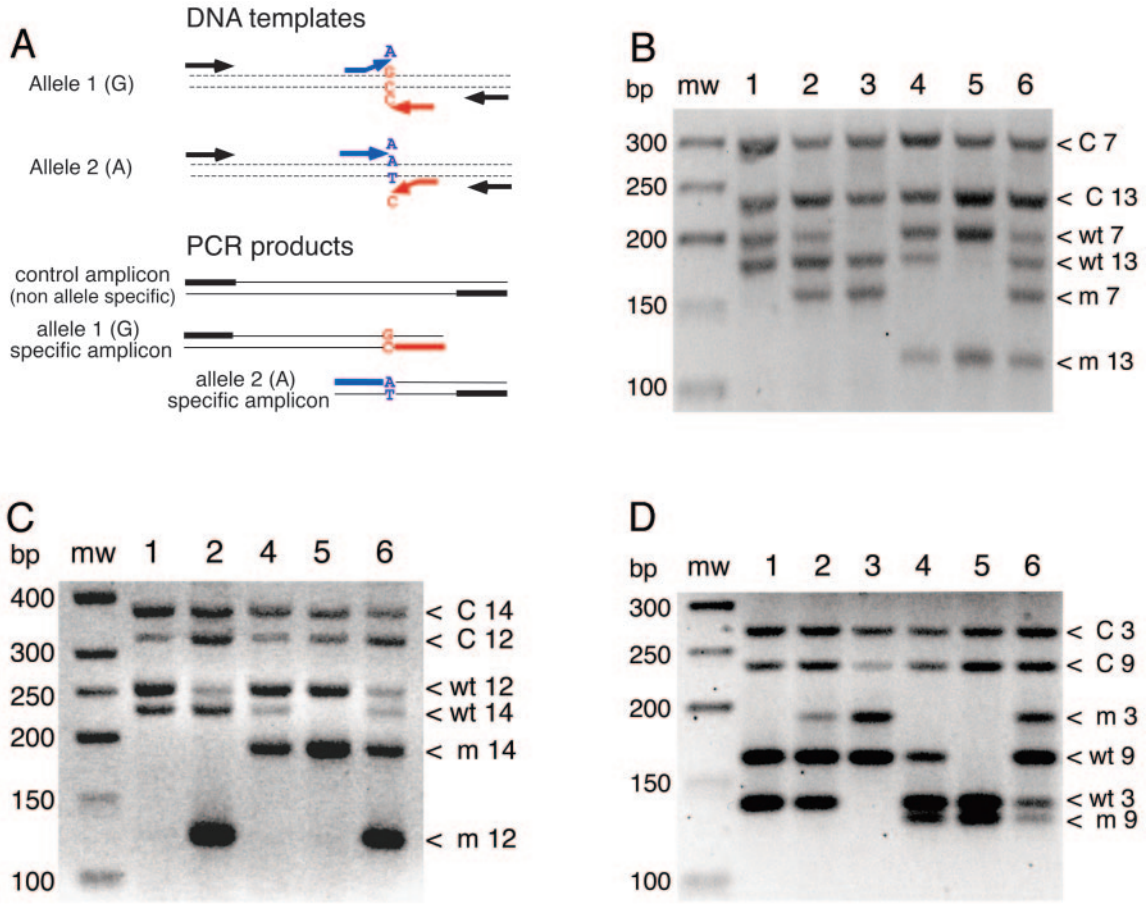

Fig. 1. T-ARMS PCR assay.

(*A*), schematic representation of T-ARMS-PCR. Two different allele-specific amplicons and a larger (non–allele-specific) control amplicon are generated by a pair of 2 common (outer) primers (*black*) and by 2 allele-specific (inner) primers that have opposite orientation [allele 1–specific primer, antisense (*red*), and allele 2–specific primer, sense (*blue*)]. Because common primers are designed in a way that the mutation is located nearer one of them, the 2 allele-specific amplicons will have different lengths and will be easily separated by gel electrophoresis. (*B*), multiplex T-ARMS-PCR for the Y165C (exon 7) and G382D (exon 13) mutations. Representative results for 6 individuals: *lane 1*, wild-type control; *lane 2*, Y165C heterozygote; *lane 3*, Y165C homozygote; *lane 4*, G382D heterozygote; *lane 5*, G382D homozygote; *lane 6*, Y165C/G382D compound heterozygote. (*C*), multiplex T-ARMS-PCR for the 1103delC (exon 12) and 1395_97delGGA (exon 14) mutations. Representative results for 5 individuals: *lane 1*, wild-type control; *lane 2*, 1103delC heterozygote; *lane 4*, 1395_97delGGA heterozygote; *lane 5*, 1395_97delGGA homozygote; *lane 6*, 1103delC /1395_97delGGA compound heterozygote. A person homozygous for 1103delC was not available. (*D*), multiplex T-ARMS-PCR for the Y90X (exon 3) and R231H (exon 9) mutations. Representative results for 6 individuals: *lane 1*, wild-type control; *lane 2*, Y90X heterozygote; *lane 3*, Y90X homozygote; *lane 4*, R231H heterozygote; *lane 5*, R231H homozygote; *lane 6*, Y90X/R231H compound heterozygote. *Lane mw* in *panels B–D*, molecular markers. *C*, non–allele-specific control band; *wt*, wild-type–specific band; *m*, mutation-specific band. The *number* next to each band indicates the mutation (identified by exon).

the entire *MUTYH* gene *(2, 3, 6, 10, 11, 21, 22)*, screening for these 6 mutations would identify at least 85% of patients with biallelic *MUTYH* mutations.

To carry out even more rapid genotyping, we designed a set of 3 multiplex T-ARMS-PCRs for the detection of these 6 frequent *MUTYH* mutations: (*a*) the relatively frequent Y165C and G382D mutations; (*b*) the 1103delC and 1395_7delGGA mutations; and (*c*) the Y90X and R231H mutations (Table 1). Multiplexes with different combinations of mutations can be designed for the screening of populations in which mutations have different frequencies. For example, we have set up a multiplex PCR for the Y90X and Y165C mutations (Table 1).

We obtained DNA samples from patients with attenuated or classic adenomatous polyposis coli and no detectable *APC* germline mutations. Written informed consent was obtained from each patient according to institutional procedures. Genomic DNA was extracted from blood

Table 1. Primers and conditions for the multiplex tetra-primer ARMS-PCR for *MUTYH* mutations.

Mutations (exon)[a]	Primer names[b]	Primer sequences,[c] 5′→3′	Conc,[d] μM	Amplicon size,[e] bp	Annealing conditions[f]
Y90X (3) and R231H (9)	3Fo	CAGGCAGCCGGAAGAGGTGGTATTGCAG	0.2	Y90X	T_a = 66 °C (35 cycles for 30 s)
	3Ro	GCATATGCCCGCCTGTCCAGGTCCATCTC	0.2	*C 268*	
	3Fi (A)	CTTCCGAGGGAGCCTGCTAAGCTGG***C***AA	1.2	**Mut 190**	
	3Ri (C)	CTCCATGGTAGGTCCCGTTTCTCTTGG***C***CG	1.2	WT 135	
	9Fo	GTGCCCAGCCTCCTTCCTCCCAGCCCAG	0.8	R231H	
	9Ro	CTAAAAGAAGGGAACACTGCTGTGAAGCAG	0.8	*C 233*	
	9Fi (G)	ATGGCAACGTAGCACGGGTGCTGTG***G***CG	1.2	WT 162	
	9Ri (A)	CTGGGATCAGCACCAATGGCTCGGA***G***AT	1.2	**Mut 126**	
Y165C (7) and G382d (13)	7Fo[g]	CTGCCTGCCTGTGGCTATAGAAGTGGCCTA	1.2	Y165C	T_a = 67 °C (5 cycles for 40 s);
	7Ro	CCTCTACCACCTGATTGGAGTGCAAGACTC	1.2	*C 302*	62 °C (32 cycles for 40 s)
	7Fi (G)	GGTGAATCAACTCTGGGCTGGCCTGGG***A***TG	2.0	WT 202	
	7Ri (A)	CTGCAGCCGCCGGCCACGAGAAT***C***GT	2.0	**Mut 155**	
	13Fo	GGGCAGTGGCATGAGTAACAAGAGAGAATG	0.2	G382D	
	13Ro[g]	TCCGCTGCTCACTTACCTCCCCAAGGT	1.0	*C 235*	
	13Fi (G)[g]	CCCTGCCT***T***GCTG***T***CCTCCCTCTC***C***GG	2.0	WT 179	
	13Ri (A)[g]	CGGGAACTCCCACAGTCCTGCCAGCA***T***AT 3′	2.0	**Mut 111**	
1103delC (12) and 1395_7delGGA (14)	12Fo[g]	ACCCGGCCAAAGCCCACTCTCTAG	1.0	1103delC	T_a = 65 °C (35 cycles for 30 s)
	12Ro	CCCTCCATTCTCTCTTGTTACTCATGCCAC	0.5	*C 325*	
	12Fi (del)	CCACCTGTGTTCTGGAACAGCCTGGGG***G***CT	1.0	WT 255	
	12Ri (C)	CTGCACCAGCAGAATTTGGGCCCCAAG***T***GC	1.0	**Mut 126**	
	14Fo	GGAGGTAAGTGAGCAGCGGAATAGCCAAG	0.4	1395_7delGGA	
	14Ro	CTCCAGCCTGGGCAACAGAGCGATTCTC	0.4	*C 371*	
	14Fi (del)	AGGTGCTCGCTGGCTGACGCAG***T***AAT	1.0	WT 231	
	14Ri (GGA)	CGGTGGAAACAGCTGCGGTGTGAAATTCC***G***CC	1.0	**Mut 193**	
Y90X (3) and Y165C (7)	3Fo	CAGGCAGCCGGAAGAGGTGGTATTGCAG	0.2	Y90X	T_a = 65 °C (10 cycles for 45 s);
	3Ro[g]	ATGAGGAGTTAGGGTGGAGGGGGCTG	0.6	*C 249*	58 °C (25 cycles 30 s)
	3Fi (A)	CTTCCGAGGGAGCCTGCTAAGCTGG***C***AA	1.0	**Mut 171**	
	3Ri (C)	CTCCATGGTAGGTCCCGTTTCTCTTGG***C***CG	1.0	WT 135	
	7Fo	GGGACTGACGGGTGATCTCTTTGACCTCTG	0.1	Y165C	
	7Ro	CCTCTACCACCTGATTGGAGTGCAAGACTC	1.0	*C 200*	
	7Fi (G)	GGTGAATCAACTCTGGGCTGGCCTGGG***A***TG	2.2	**Mut 155**	
	7Ri (A)	CTGCAGCCGCCGGCCACGAGAAT***C***GT	2.0	WT 100	

[a] GenBank sequence no. U63329.

[b] The number indicates the exon that contains the mutation. F, forward; R, reverse; o, outer (common); i, inner (allele specific). The nucleotide specificity is indicated in parentheses. Bold font indicates the primer specific for the mutant allele.

[c] Deliberate mismatches are in bold italics.

[d] The relative and absolute concentrations (Conc) of each individual primer have been determined experimentally to reduce nonspecific amplification and to produce similar amplification of all amplicons.

[e] C, size of the control fragment (in italics); WT, size of the wild-type fragment (regular text); Mut, size of the mutant fragment (in bold font).

[f] Cycling conditions are 10 min at 95 °C, followed by 35 cycles of 94 °C for 30 s, annealing temperatures (T_a) as given above, 72 °C at 30 s (40 s for Y165C and G382D), and a final cycle 72 °C for 7 min. To reduce nonspecific amplification, in some cases the annealing temperature has been increased for the initial 5–10 cycles.

[g] Primer different from that used in the single-mutation T-ARMS-PCR (see Table 1 in the online Data Supplement).

samples by use of the QIAamp DNA Blood Midi Kit (Qiagen S.p.A) and from paraffin tissue inclusions by phenol–chloroform extraction.

We designed the primers on the basis of the published *MUTYH* genomic sequence (GenBank accession no. U63329). The specificity of allele-specific primers is conferred by the match of the terminal 3′ nucleotide with either the wild-type or the mutant allele, and it is enhanced by the introduction of a deliberate mismatch near the primer 3′ end, usually at the third position *(18, 19)*. The PCR reaction (total volume, 20 μL) contained 200 μM deoxynucleoside triphosphates, 1 U of hot-start DNA polymerase AmpliTaq Gold with its buffer (Applera Europe B.V.), 100 ng of genomic DNA, and the appropriate concentration of each primer (see Table 1; also see the online Data Supplement). PCR amplification was carried out in both an I-cycler® (Bio-Rad) and a Px2® (Hybaid) thermal cycler. After activation of the AmpliTaq Gold DNA polymerase for 10 min at 95 °C, the cycling conditions were as indicated in Table 1 and in the online Data Supplement. PCR products were separated by standard electrophoresis on 2.5% agarose gels containing ethidium bromide.

The method was first tested on samples previously analyzed by direct sequencing: at least 1 homozygous and 2 heterozygous samples were tested for each mutation (for 1103delC, no homozygous samples were available). All mutations were easily detected by both the T-ARMS-PCR specific for one mutation (see Fig. 1 in the online Data Supplement) and the multiplex T-ARMS-PCR (Fig. 1, B–D; also see Fig. 2 in the online Data Supplement).

The T-ARMS-PCR procedures for single-mutation detection were validated by analysis of samples from a new series of 54 patients with polyposis and no detectable *APC* germline mutations. In this series, 7.5%–29% of patients were expected to carry *MUTYH* mutations *(2, 4–6)*, and we identified 3 Y165C homozygotes, 4 compound heterozygotes (2 Y165C/G382D, 1 Y165C/1395_7delGGA, and 1 R231H/G382D), 1 Y90X heterozygote, and 1 G382D heterozygote. These results were confirmed by dHPLC analysis in all 54 patients, and each mutation was confirmed by direct DNA sequencing.

The multiplex T-ARMS-PCR procedures were validated by the analysis of DNA samples from a previously characterized group of 22 patients without and 31 patients with *MUTYH* mutations. We identified 7 Y90X alleles, 14 Y165C alleles, 7 R231H alleles, 4 1103delC alleles, 10 G382D alleles, and 8 1395_7delGGA alleles. Our results were identical to those obtained with direct DNA sequencing.

Overall, these data indicate the reliability of the T-ARMS-PCR method. Repeat analyses demonstrated the reproducibility of the procedure. In addition, an independent laboratory (MDA) that uses different thermal cycler models (PTC-100® from MJ Research and PCR express® from Hybaid) obtained the same results using established PCR conditions. These results confirm the robustness and the reproducibility of the T-ARMS-PCR procedure and show for the first time that T-ARMS-PCR can be been used to detect not only nucleotide substitutions but also small deletions (1395_7delGGA and 1103delC).

This method provides rapid, reproducible, and cost-effective detection of common *MUTYH* mutations without the use of any special equipment. In addition, it is the first time that T-ARMS-PCR has been designed to detect 2 different mutations simultaneously. It is unlikely that this method could test more than 2 mutations in the same reaction; nevertheless, the described multiplex T-ARMS-PCR allows investigation of 6 common *MUTYH* mutations with as few as 3 single-tube PCR reactions. T-ARMS-PCR can be easily adapted for local mutation frequencies. Most importantly, given the short lengths of the amplicons, the method may be used to genotype archival material from paraffin-embedded tissues.

This simple, inexpensive, and accurate method could be used to genotype relatives of patients with known *MUTYH* mutations, to optimize the strategy for identification of *MUTYH* mutations in a diagnostic setting, and to carry out the large population-based epidemiologic studies needed to investigate the possible role of mono-allelic *MUTYH* mutations in predisposing to colorectal cancer.

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References

1. Al-Tassan N, Chmiel NH, Maynard J, Fleming N, Livingston AL, Williams GT, et al. Inherited variants of MYH associated with somatic G:C→T:A mutations in colorectal tumors. Nat Genet 2002;30:227–32.
2. Sieber OM, Lipton L, Crabtree M, Heinimann K, Fidalgo P, Phillips RK, et al. Multiple colorectal adenomas, classic adenomatous polyposis, and germline mutations in MYH. N Engl J Med 2003;348:791–9.
3. Jones S, Emmerson P, Maynard J, Best JM, Jordan S, Williams GT, et al. Biallelic germline mutations in MYH predispose to multiple colorectal adenoma and somatic G:C→T:A mutations. Hum Mol Genet 2002;11:2961–7.
4. Sampson JR, Dolwani S, Jones S, Eccles D, Ellis A, Evans DG, et al. Autosomal recessive colorectal adenomatous polyposis due to inherited mutations of MYH. Lancet 2003;362:39–41.
5. Gismondi V, Meta M, Bonelli L, Radice P, Sala P, Bertario L, et al. Prevalence of the Y165C, G382D, and 1395delGGA germline mutations of the MYH gene in Italian patients with adenomatous polyposis coli and colorectal adenomas. Int J Cancer 2004;109:680–4.
6. Nielsen M, Franken PF, Reinards TH, Weiss MM, Wagner A, van der Klift H, et al. Multiplicity in polyp count and extracolonic manifestations in 40 Dutch patients with MYH-associated polyposis coli (MAP). J Med Genet 2005;42:e54.
7. Fleischmann C, Peto J, Cheadle J, Shah B, Sampson J, Houlston RS. Comprehensive analysis of the contribution of germline MYH variation to early-onset colorectal cancer. Int J Cancer 2004;109:554–8.
8. Farrington SM, Tenesa A, Barnetson R, Wiltshire A, Prendergast J, Porteous M, et al. Germline susceptibility to colorectal cancer due to base-excision repair gene defects. Am J Hum Genet 2005;77:112–9.
9. Chow E, Thirlwell C, Macrae F, Lipton L. Colorectal cancer and inherited mutations in base-excision repair. Lancet Oncol 2004;5:600–6.
10. Isidro G, Laranjeira F, Pires A, Leite J, Regateiro F, Castro e Sousa F, et al. Germline MUTYH (MYH) mutations in Portuguese individuals with multiple colorectal adenomas. Hum Mutat 2004;24:353–4.
11. Eliason K, Hendrickson BC, Judkins T, Norton M, Leclair B, Lyon E, et al. The potential for increased clinical sensitivity in genetic testing for polyposis colorectal cancer through the analysis of MYH mutations in North American patients. J Med Genet 2005;42:95–6.

12. Croitoru ME, Cleary SP, Di Nicola N, Manno M, Selander T, Aronson M, et al. Association between biallelic and monoallelic germline MYH gene mutations and colorectal cancer risk. J Natl Cancer Inst 2004;96:1631–4.
13. Peterlongo P, Mitra N, Chuai S, Kirchhoff T, Palmer C, Huang H, et al. Colorectal cancer risk in individuals with biallelic or monoallelic mutations of MYH. Int J Cancer 2005;114:505–7.
14. Varesco L, Gismond V, Meta M, Bonelli L, Pugliese V, Gatteschi B, et al. Spectrum of *MYH* mutation in the Italian population. Fam Cancer 2005; 4(Suppl 1):136.
15. Wang L, Baudhuin LM, Boardman LA, Steenblock KJ, Petersen GM, Halling KC, et al. MYH mutations in patients with attenuated and classic polyposis and with young-onset colorectal cancer without polyps. Gastroenterology 2004;127:9–16.
16. Ye S, Dhillon S, Ke X, Collins AR, Day IN. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res 2001;29: E88–8.
17. Ji L, Pan S, Marti-Jaun J, Hanseler E, Rentsch K, Hersberger M. Single-step assays to analyze *CYP2D6* gene polymorphisms in Asians: allele frequencies and a novel *14B allele in mainland Chinese. Clin Chem 2002;48: 983–8.
18. Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, et al. Analysis of any point mutation in DNA: the amplification refractory mutation system (ARMS). Nucleic Acids Res 1989;17:2503–16.
19. Old JM, Varawalla NY, Weatherall DJ. Rapid detection and prenatal diagnosis of β-thalassaemia: studies in Indian and Cypriot populations in the UK. Lancet 1990;336:834–7.
20. Enholm S, Hienonen T, Suomalainen A, Lipton L, Tomlinson I, Karja V, et al. Proportion and phenotype of MYH-associated colorectal neoplasia in a population-based series of Finnish colorectal cancer patients. Am J Pathol 2003;163:827–32.
21. Venesio T, Molatore S, Cattaneo F, Arrigoni A, Risio M, Ranzani GN. High frequency of MYH gene mutations in a subset of patients with familial adenomatous polyposis. Gastroenterology 2004;126:1681–5.
22. Aceto G, Cristina Curia M, Veschi S, De Lellis L, Mammarella S, Catalano T, et al. Mutations of APC and MYH in unrelated Italian patients with adenomatous polyposis coli. Hum Mutat 2005;26:394.

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Differentiation of Amphetamine/Methamphetamine and Other Cross-Immunoreactive Sympathomimetic Amines in Urine Samples by Serial Dilution Testing,

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Background: **Immunoassay-based screening for amphetamines has a variable positive predictive value (PPV) for detecting amphetamine abuse. The lack of immunoassay specificity necessitates confirmatory testing by gas chromatography–mass spectrometry (GC/MS), but the technical complexity and expense of GC/MS limit its availability. Physicians may make decisions regarding patient disposition based on unverified results. In this study we assessed the utility of using dose–response properties to distinguish urine samples containing amphetamines from samples containing cross-immunoreactive species.**

Methods: **Urine was supplemented with known concentrations of amphetamine, methamphetamine, methylenedioxymethamphetamine (MDMA), or pseudoephedrine. Using a series of dilutions, we determined the maximum change in rate over the fractional change in concentration for each compound in the Emit® II amphetamine/methamphetamine immunoassay. Patient urine samples that screened positive for amphetamines were diluted 1:1, 1:10, and 1:20, and maximum slope estimates within the dynamic assay range were determined. An optimal slope cutoff that differentiated samples containing (meth)amphetamine from those containing cross-reacting species was determined by ROC analysis.**

Results: **The slope of the dose response was largest for amphetamine and methamphetamine, followed by MDMA and pseudoephedrine. The optimum slope cutoff for identifying patient specimens containing (meth)amphetamine was 320 (sensitivity, 96%; specificity, 90%; PPV, 92%). High concentrations of less reactive compounds may mask low concentrations of amphetamines.**

Conclusions: **Use of the slope of the dose–response relationship in patient urine specimens can enhance the PPV of presumptive positive immunoassay results but does not exclude the presence of low amphetamine concentrations in samples containing high concentrations of cross-reactive species.**

Amphetamines and their analogs are a family of noncatecholamine stimulants that are among the most commonly abused drugs in the United States *(1)*. Urine drug screening for amphetamines is performed in both clinical and workplace settings. The positive predictive value (PPV) of amphetamine immunoassays to detect amphetamine abuse varies from 0% *(2)* to 90% *(3)* because these assays recognize a wide variety of sympathomimetic amines *(4–7)* (see Table 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol52/issue4). Positive immunoassay results should, therefore, be verified by alternative methods such as gas chromatography–mass spectrometry (GC/MS). The technical complexity and expense of GC/MS analysis limit its availability in many clinical laboratories, however. Physicians must often make decisions regarding patient disposition based on unverified screening immunoassay results.

Amphetamine immunoassays display precise dose–response properties from concentrations below the Substance Abuse and Mental Health Services Administration (SAMHSA)-stipulated cutoff to concentrations well above cutoff *(8)*. Within this dynamic range, amphetamine and methamphetamine may exhibit dose–response properties that are distinguishable from other cross-immunoreactive compounds. The AACC has encouraged the US Food and Drug Administration to require reagent manufac-