Letters to the Editor

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5. Adamczyk M, Brashear RJ, Mattingly PG. Noninvasive prenatal diagnosis of genetic diseases has yet to attain a routine application in clinical diagnostics. An innovative strategy that has been reported is based on COLD-PCR (coamplification at a lower denaturation temperature PCR),1 which exploits melting temperature (Tm) differences between variant or mismatched sequences and wild-type sequences. This approach uses a critical denaturation temperature (Tc) lower than the Tm to selectively amplify minority mutated alleles (2).

We have developed assays for the identification of fetal paternally inherited mutations in maternal plasma. These assays use full COLD-PCR for the detection of IVSL.I110 (G>A) and Cd39 (C>T) (3).

Full COLD-PCR is based on the generation of heteroduplexes between mutant and wild-type sequences. These heteroduplexes melt at lower temperatures than the wild-type homoduplexes. They are selectively denatured at the Tc and then subsequently amplified. Given that both the fetal and maternal DNA content in maternal plasma has been demonstrated to vary from pregnancy to pregnancy (3), we used a range of Tc values and several replicates of the same sample to develop a protocol that was specific for the identification of each mutation. To simplify the Tc evaluation, we amplified plasma DNA from wild-type controls with several replicates instead of a single Tc, ensuring that at least 1 replicate exhibited differential denaturation of mutant and wild-type alleles, thereby allowing robust PCR amplification.

The study was approved by the local ethics review boards. After obtaining written informed consent from couples in which the parents carried different mutations, we collected 6 mL of maternal blood before chorionic villus sampling. DNA was extracted from 500 µL of plasma and eluted in a 60-µL volume. COLD-PCR was performed in a 25-µL final volume containing 5 µL of eluted DNA, 200 µmol/L of each deoxynucleoside triphosphate, 10 mmol/L Tris–HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 1.5 U of FastStart Taq DNA polymerase (Roche Applied Science), and 10 pmol of each primer. Cycling conditions were as follows: 95 °C for 4 min; 25 cycles of 95 °C for 15 s, 54 °C for 30 s, and 72 °C for 1 min; 72 °C for 10 min; 95 °C for 4 min; 35 cycles of 95 °C for 15 s, 70 °C for 8 min (to generate heteroduplexes), specific Tc temperature for 3 s, and 72 °C for 1 min; 72 °C for 7 min. For IVSL.I110, the Tc interval was 81.9 °C–80.9 °C; for Cd39, the Tc interval was 83.8 °C–82.8 °C. The amplification primers for IVSL.I110 were 5'-TAAGGAGAC CAATAGAAACT-3' (forward) and 5'-GTAGACACCCGCAGC-3' (reverse); the amplicon size was 119 bp. The primers for Cd39 were 5'-GTCTATTTTCCACCCTT-3' (forward) and 5'-AGCACCTTTCTTG

* Address correspondence to this author at: Universitätsinstitut für Medizinisch-Chemische Labordiagnostik Gemeinnützige Salzburger Landeskliniken Betriebsges.m.b.H Müllner Hauptstraße 48 5020 Salzburg, Austria Fax 0662-4482-885 E-mail j.cadamuro@salk.at

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Full COLD-PCR Protocol for Noninvasive Prenatal Diagnosis of Genetic Diseases

To the Editor:

After the discovery of fetal DNA in maternal plasma, investigators reported different strategies for the noninvasive prenatal diagnosis of genetic diseases (1). Despite the advances in improving the analytical sensitivity of methods, distinguishing between fetal and ma-
CCATGA-3’ (reverse); the amplicon size was 134 bp. After COLD-PCR, samples were sequenced directly with the 3730 DNA Analyzer (Applied Biosystems). We performed 35 diagnoses—for 21 cases in which the father carried the Cd39 mutation (the fetus inherited the paternal mutation in 10 of these cases) and for 14 cases in which the father carried the IVSL.110 mutation (the fetus inherited the paternal mutation in 12 of these cases). In all cases, the fetal paternal mutated allele was not detectable by conventional PCR, whereas the mutation was evident after COLD-PCR. Fig. 1 shows examples of samples lacking the paternal inherited mutated allele, as well as the lowest and highest enrichments of fetal paternally inherited mutated alleles for both mutations. The results obtained with COLD-PCR were in complete concordance with those obtained for fetal DNA extracted from chorionic villi (Fig. 1).

Du et al. described the use of fetal circulating DNA and fast COLD-PCR to detect congenital paternal abnormalities in a single individual (5). Fast COLD-PCR cannot detect all mutations, however. To our knowledge, the present report is the first to indicate the feasibility of using full COLD-PCR for prenatal diagnosis in a comprehensive study.

We have provided evidence that COLD-PCR enables straightforward and reliable identification of inherited mutated alleles without the need for sophisticated and costly equipment. The method might be extended to noninvasive prenatal diagnosis of genetic diseases, and it has the potential to be easily transferable to clinical diagnostic laboratories.

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References

The Effects of Sampling Procedures and Storage Conditions on Short-term Stability of Blood-Based Biochemical Markers of Bone Metabolism

To the Editor:

The prevalence and incidence of metabolic bone disease are rising as the population ages (1). In conjunction with imaging and clinical history, biochemical bone markers have become increasingly important in diagnosis and for monitoring bone disease (2, 3).

Little evidence is available regarding preanalytical influences on short-term stability of bone markers. We have evaluated the effects of sample type, processing, and storage procedures on the stability of biochemical markers of bone turnover [C-terminal telopeptide of type 1 collagen (β-CTX), N-terminal propeptide of procollagen type 1 (P1NP), osteocalcin (OC), bone-specific alkaline phosphatase (bone ALP), and parathyroid hormone (PTH)].

After obtaining informed consent, we collected venous blood samples from 18 patients with metabolic bone disease into lithium heparin–containing, EDTA-containing, and serum Sarstedt blood-collection systems.

We investigated the use of 3 sample-processing protocols (6 patients each): (a) delayed separation from cells (samples stored at room temperature for 1, 2, 4, 8, 24, or 48 h after venesection before separation); (b) immediate separation and storage at room temperature (plasma and serum aliquots left at room temperature for 2 h, 4 h, 8 h, 24 h, 48 h, or 7 days); and (c) immediate separation and storage at 2 °C–8 °C (plasma and serum aliquots stored at 2 °C–8 °C for 24 h, 48 h, 7 days, 14 days, or 28 days).

All samples were then stored at −70 °C until analysis of each batch. Baseline samples for each sample type (produced by immediate separation and storage of plasma or serum aliquots at −70 °C) were analyzed for all patients. We measured β-CTX, P1NP, OC, and PTH on the Modular Analytics E 170/Elecsys® platform (Roche). In addition, we used the Metra Biosystems enzymatic immunoassay to measure bone ALP (in duplicate, serum samples only) in 3 patients from each sample-processing protocol. The assays were assessed for intraassay imprecision by duplicate analysis of each sample for each container type, and interassay imprecision was assessed with QC material. For all assays, mean CVs were <10%.

After analysis, we used 1-way ANOVA with Bonferroni-corrected post hoc t-tests to compare analyte concentrations at each time point with the baseline concentration (Table 1). Mean values were considered significantly different at P values <0.05.

The stability of each analyte was evaluated according to the criteria of the 2002 WHO guidelines (4, 5) and was defined as the amount of time the change in concentration remained less than half of the total error of the sum of the biological and analytical variation. The calculated value was <5.4%

Silvia Galbiati2
Angela Brisci2
Faustina Lalatta3
Manuela Seia4
G. Mike Makrigiorgos5
Maurizio Ferrari2,6,7†
Laura Cremonesi2‡

2 San Raffaele Scientific Institute
Genomic Unit for the Diagnosis of Human Pathologies
Center for Genomics and Bioinformatics
Milan, Italy
3 UO Dipartimentale di Genetica Medica and
Laboratorio di Genetica Medica
Fondazione IRCCS Ca’ Granda
Ospedale Maggiore Policlinico
Milan, Italy
4 Radiation Therapy
Dana-Farber/Brigham and
Women’s Cancer Center
Brigham and Women’s Hospital
Boston, MA
6 Università Vita-Salute San Raffaele
Milan, Italy
7 Diagnostica e Ricerca San Raffaele SpA
Milan, Italy

† These authors contributed equally to the work.
‡ Address correspondence to this author at:
Genomic Unit for the Diagnosis of Human Pathologies
San Raffaele Scientific Institute
Via Olgettina 60
20132 Milan, Italy
Fax +39-02-26434351
E-mail cremonesi.laura@hsr.it
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1 Nonstandard abbreviations: β-CTX, C-terminal telopeptide of type 1 collagen; P1NP, N-terminal propeptide of procollagen type 1; OC, osteocalcin; bone ALP, bone-specific alkaline phosphatase; PTH, parathyroid hormone.