A Cell-Free Assay for the Functional Analysis of Variants of the Mismatch Repair Protein MLH1

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Communicated by Rolf Sijmons

Received 16 September 2009; accepted revised manuscript 23 November 2009.

ABSTRACT: The hereditary colon and endometrium cancer predisposition Lynch Syndrome (also called HNPPC) is caused by a germ-line mutation in one of the DNA mismatch repair (MMR) genes. A significant fraction of the gene alterations detected in suspected Lynch Syndrome patients is comprised of amino acid substitutions. The relevance for cancer risk of these variants is difficult to assess, as currently no time- and cost-effective, validated, and widely applicable functional assays for the measurement of MMR activity are available. Here we describe a rapid, cell-free, and easily quantifiable MMR activity assay for the diagnostic assessment of variants of the MLH1 MMR protein. This assay allows the parallel generation and functional analysis of a series of variants of the MLH1 protein in vitro using readily available, or preprepared, reagents. Using this assay we have tested 26 MLH1 variants and of these, 15 had lost activity. These results are in concordance with those obtained from first-generation assays and with in silico and pathology data. After its multifocal technical and clinical validation this assay could have great impact for the diagnosis and counseling of carriers of an MLH1 variant and their relatives. Hum Mutat 31:247–253, 2010. © 2009 Wiley-Liss, Inc.

KEY WORDS: Lynch syndrome; DNA mismatch repair; MMR; variants of uncertain significance; functional assay; MLH1

Introduction

Lynch Syndrome (or Hereditary Nonpolyposis Colorectal Cancer, HNPPC; MIM#s 120435, 609310) is an autosomal dominant disorder that predominantly predisposes to colon and endometrial cancer [Stoffel et al., 2009; Vasen et al., 2007]. Lynch Syndrome is caused by a germ-line mutation in one of the DNA mismatch repair (MMR) genes. In a Lynch syndrome patient stochastic loss of the wild-type allele leads to MMR deficiency, which results in a spontaneous mutator phenotype that drives rapid carcinogenesis [Li, 2008].

A definitive diagnosis of Lynch syndrome can only be made by finding a causative germ-line mutation in an MMR gene [Stoffel et al., 2009; Vasen et al., 2007]. Additionally, the identification of a causative mutation allows the presymptomatic screening of affected relatives [Vasen et al., 2007]. Of all mutations found in MMR genes in suspected Lynch Syndrome patients, ~15% in MSH2 (MIM# 609309), ~30% in MLH1 (MIM# 120436), and ~40% in MSH6 (MIM# 600678) give rise to single amino acid alterations [Lagerstedt Robinson et al., 2007; Peltoniäki and Vasen, 2004] of which the pathological significance is often unclear (so-called Variants of Uncertain Significance, or VUS). Lack of classification of these variants precludes diagnosis for carriers and their relatives. Therefore, when a suspected Lynch syndrome patient carries a VUS, all first-degree relatives currently enroll lifelong periodic screening, irrespective of their mutation status [Castells et al., 2009]. Screening poses a physical and psychological burden on these families, but also a burden on the preventive health care apparatus. To enable correct diagnosis and to avoid unnecessary screening it is of great relevance to develop a widely applicable, validated, and standardized approach for the diagnostic assessment of VUS in MMR genes.

The MMR pathway corrects misincorporations arising during DNA replication, and thereby prevents the accumulation of spontaneous mutations [Hsieh and Yamane, 2008; Li, 2008]. MMR is initiated by the recognition of a misincorporation by the heterodimeric MutSα protein, consisting of the MSH2/MSH6 subunits. This triggers the recruitment of the MLH1/PM2 heterodimeric protein (called MutLβ) and of Exonuclease 1 that removes the misincorporation-containing DNA strand. Removal is directed toward the misincorporation-containing daughter strand by the presence of a strand discontinuity ("nick"), 5’ or 3’ of the mismatch. The excised DNA strand is subsequently resynthesized.

Functional MMR activity of a VUS is considered an important diagnostic criterion [Couch et al., 2008; Goldgar et al., 2008; Plon et al., 2008]. In vitro MMR activity assays have been developed [Li and Modrich, 1995], and in such a complementation assay the inability of a variant to restore MMR activity to a MMR-deficient cell extract almost certainly assigns pathogenicity to the VUS [Couch et al., 2008; Ou et al., 2007]. However, the requirement for molecular cloning, cell culture, and the use of bacteria precludes their independent validation and wide
applicability, complicating diagnosis and screening of suspected patients and their relatives.

Here, we present the analysis of in vitro MMR activity of 26 MLH1 VUS using a completely cell-free MMR complementation assay. The results from this assay are consistent with those from other functional assays and with in vitro predictions of protein function. We infer that this assay is amenable to independent validation and applicable in molecular diagnostic laboratories, and therefore could greatly contribute to the diagnostic classification of MLH1 VUS.

Materials and Methods

Substrate Preparation

The plasmid pUC19CPD has been used for the generation of MMR substrates [Wang and Hays, 2006], and was modified by introducing additional recognition sites for nicking endonucleases. The resulting plasmid was named pJH and, after the introduction of a mismatch (see below), allows to measure MMR activity at each DNA strand, directed by the presence of a nick either at the 5’ or the 3’ side of the mismatch (Fig. 1A and Supp. Fig. S1). The substrate for MMR was prepared from pJH, essentially as described [Wang and Hays, 2006], with modifications. Briefly, a 26 basepair (bp) single-stranded DNA stretch between two Nt. BstNBI sites was removed by heating and annealing to an excess of complementary oligonucleotide. The resulting gapped pH was purified on Sephacryl S-400 HR columns (GE Healthcare, Piscataway, NJ, USA). Phosphorylated oligonucleotide G/T-Fam, containing a mismatching nucleotide and an internal 6-FAM fluorescent label, was annealed into the gapped plasmid and ligated. After purification the resulting mismatched substrate was nicked at the bottom strand, 138 bp 3’ to the mismatch with Nb.BsmI. Finally, the substrate, named pHGT3’InFAM (Fig. 1B, top panel), was purified, aliquoted, and frozen until use. All enzymes were purchased from New England Biolabs (Boston, MA, USA).

Preparation of Nuclear Extracts

HCT116 MutLx-deficient colon cancer cells [Jiricny, 2003] were grown at 37°C in RPMI-1640 (Gibco, Paisley, UK) supplemented with 10% FCS (PAA, Pasching, Austria), penicillin, streptomycin, and Pyruvate (Gibco). Cells were harvested in log-phase and stored for prolonged periods. These extracts can be prepared in large quantities, distributed, and stored for future use. All enzymes were purchased from New England Biolabs (Boston, MA, USA).

In Vitro MMR Complementation Assays

After estimation of quantities of expressed VUS MLH1 proteins by Western blotting, the proteins were diluted to similar concentrations in expression mix. This was followed by dimerization with in vitro produced wild-type PMS2 protein in expression mix (1:1 vol/vol), for 30 min at room temperature.

Assay reactions were performed in a total volume of 25μL containing 75 ng of HCT116 nuclear extract. The extract was complemented with 12 μL of in vitro produced MutLxz and 100 ng of substrate pHGT3’InFAM. Reaction conditions for MMR were as described [Wang and Hays, 2006]. After the MMR reaction and purification using the MinElute kit (Qiagen, Germantown, MD, USA), the substrate was digested with HindIII and BsrBI (both Fermentas, Burlington, Ontario, Canada). One-fifth (2μL) of digested substrate was mixed with 8 μL Hi-Di Formamide containing 0.2 μL GeneScan-500 ROX size standards (Applied Biosystems, Bedford, MA, USA) and fragment analysis was performed on a 3730 DNA analyzer (Applied Biosystems) at the Leiden Genome Technology Center. Data was analyzed using PeakScan Software (Applied Biosystems). Repair levels are calculated by dividing the height of the MMR-specific peak by the total fluorescent signal.

Generation of Cloned Mutants

Most mutations were additionally introduced into MLH1, cloned in pCITE4a, by site-directed mutagenesis (Quick Change Site-Directed Mutagenesis, Stratagene, LaJolla, CA, USA). Primer sets (Biolegio, Nijmegen, The Netherlands) were designed (Supp. Table S2) and PCR was performed using PfX Platinum polymerase (Invitrogen). PCR products were ligated and used to transform Escherichia coli. Resulting plasmids were verified by direct sequencing of the entire mutant MLH1 insert.

In Silico Analysis

All MLH1 VUS tested in this work were subjected to in silico analyses [Tavtigian et al., 2008] using the the Sorting Intolerant From Tolerant (SIFT) [Ng and Henikoff, 2003] and the MAPP-MMR [Chao et al., 2008] algorithms.
Results

Construction of Fluorescent Heteroduplex Substrate for MMR Assays

For the current study we constructed a T/G mismatch-containing substrate, called pJHGT3'InFAM (Fig. 1B, top panel). MMR is directed toward the G-containing (bottom) strand by a nick at that strand, 3' from the mismatch. Repair of the T/G mismatch to T/A by MMR creates a recognition site for HinDIII, within a 174-bp BsrBI restriction fragment. This results in the generation of a 75-bp fluorescent diagnostic fragment (Fig. 1B and C, bottom panels). A 174-bp BsrBI fragment represents the unrepaired substrate.

A Functional Assay Using In Vitro-Produced MutLα

Wild-type MLH1 and PMS2 were cloned into pCITE4A. The MLH1 clone was used as a template to amplify by PCR the open reading frame of the gene, together with sequences required for in
vitro transcription/translation. MLH1 and PMS2 were expressed from the PCR fragment and from the expression vector, respectively, as judged by Western blotting (Fig. 1D, left panel). Importantly, the reticulocyte lysate was devoid of detectable rabbit MLH1 or PMS2 that might give false-positive results. Both proteins were synthesized in approximately equimolar amounts as judged by 35S-Methionine levels in both proteins, using a parallel expression reaction (Fig. 1D, right panel). Next, MLH1 and PMS2 were used to complement an MLH1-deficient HCT116 nuclear extract to test for the repair of substrate pJHGT3'lnFAM (Fig. 1B). Using this assay, nearly half of the substrate was repaired (Fig. 1C, left panel). This result is similar to the repair efficiency in other assays that use nonfluorescent substrates and in vivo produced MutLa [Plotz et al., 2006; Raevaara et al., 2005]. Importantly, mock-expression reactions and reactions containing only MLH1 or PMS2 did not result in significant repair (Fig. 1C, right panel, see also Fig. 3). Moreover, the MMR protein stoichiometry in our MMR assay mimics the in vivo stoichiometry, as evidenced by comparing MLH1:MSH6 ratios between an MMR complementation assay and wild-type cells (Fig. 1E). Taken together, these results indicate that our cell-free assay is suited for testing MLH1 activity.

**Production of MLH1 Mutants in a Two-Step PCR Procedure**

To create MLH1-mutant genes suited for transcription/translation in vitro without the need of prior cloning we developed a two-step PCR-based method to produce 26 MLH1 mutants together with regulatory sequences (Table 1, Fig. 2A–D, Supp. Table S1, and Methods and Materials). This procedure was successful for all mutants, although final DNA yields varied somewhat (Fig. 2E). Sequencing of the MLH1-mutant fragments showed no visible contamination with wild-type input MLH1 (Fig. 2F and Supp. Fig. S2). To investigate the PCR fidelity more extensively, a PCR product of one of the mutants was cloned and 10 independent clones were fully sequenced. This revealed no mutations, additional to the desired one (not shown). We conclude that the PCR-based approach to generate mutant MLH1 genes for expression in vitro is efficient and free of detectable artifacts.

**MMR Activities of In Vitro Produced MLH1 VUS**

The panel of 26 MLH1 mutants included three putative polymorphisms and four presumed pathogenic mutants (for
All mutants are described in database of the International Society for Gastrointestinal Hereditary Tumours [Peltomäki and Väsen, 2004] (http://chromium.liacs.nl/LOVD2/colon_cancer) and most of them also in the MMR Gene Unclassified Variants Database [Ou et al., 2008] (http://www.mmrmissense.net). An overview of pathology data is shown in Table 1. Mutant MLH1

Table 1. In Silico Data, Pathology Data, and References for All MLH1 VUS Tested in This Work

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Pathologyb</th>
<th>In vitro MMR</th>
<th>In silico analysis</th>
<th>References to functional studies e</th>
</tr>
</thead>
<tbody>
<tr>
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<td>DNA</td>
<td>MSI</td>
<td>IHC</td>
<td>SIFTc</td>
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<tr>
<td>A31C</td>
<td>c.91_92delinsTG</td>
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<td>0 3.26</td>
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<td>E37K</td>
<td>c.109G&gt;A</td>
<td>H + - -</td>
<td>0 18.32</td>
<td></td>
</tr>
<tr>
<td>N38H</td>
<td>c.112A&gt;C</td>
<td>H + + -</td>
<td>0 14.29</td>
<td></td>
</tr>
<tr>
<td>N38K</td>
<td>c.114C&gt;G</td>
<td>H + + +</td>
<td>0 25.31</td>
<td></td>
</tr>
<tr>
<td>S44F</td>
<td>c.131C&gt;T</td>
<td>H - NA -</td>
<td>0 16.74</td>
<td>1</td>
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<tr>
<td>G67R</td>
<td>c.199G&gt;A</td>
<td>H - - -</td>
<td>0 36.52</td>
<td>1, 5</td>
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<tr>
<td>S93G</td>
<td>c.277A&gt;G</td>
<td>NA + + +</td>
<td>0.06 2.47</td>
<td>1, 5, 8</td>
</tr>
<tr>
<td>H109P</td>
<td>c.326A&gt;C</td>
<td>H NA NA -</td>
<td>0 9.36</td>
<td></td>
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<tr>
<td>A111P</td>
<td>c.331G&gt;C</td>
<td>H - NA -</td>
<td>0.01 14.1</td>
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<td>T117M</td>
<td>c.350C&gt;T</td>
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<td>0 21.05</td>
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<tr>
<td>I219V</td>
<td>c.655A&gt;G</td>
<td>NA NA NA +</td>
<td>0.36 3.51</td>
<td>1, 5, 11, 12</td>
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<tr>
<td>R265S</td>
<td>c.793C&gt;A</td>
<td>NA NA NA +</td>
<td>0 21.16</td>
<td>1</td>
</tr>
<tr>
<td>R265C</td>
<td>c.793C&gt;T</td>
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<td>0 27.43</td>
<td>1, 10</td>
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<tr>
<td>P403S</td>
<td>c.1207C&gt;T</td>
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<tr>
<td>S406N</td>
<td>c.1217G&gt;A</td>
<td>H NA NA +</td>
<td>1 1.07</td>
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<td>K443Q</td>
<td>c.1327A&gt;C</td>
<td>NA - NA +</td>
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</tr>
<tr>
<td>L550P</td>
<td>c.1649C&gt;T</td>
<td>H NA NA -</td>
<td>0.04 8.88</td>
<td></td>
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<tr>
<td>E578G</td>
<td>c.1731A&gt;G</td>
<td>H + NA +</td>
<td>0.21 4.85</td>
<td>1</td>
</tr>
<tr>
<td>L582F</td>
<td>c.1744C&gt;T</td>
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<td>0.02 2.24</td>
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<tr>
<td>A589D</td>
<td>c.1766C&gt;A</td>
<td>H - NA -</td>
<td>0.01 8.32</td>
<td></td>
</tr>
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<td>K618A</td>
<td>c.1852_1853delinsGC</td>
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<td>0.01 5.06</td>
<td>1, 5</td>
</tr>
<tr>
<td>Y646C</td>
<td>c.1937A&gt;G</td>
<td>L + NA +</td>
<td>0.02 3.69</td>
<td></td>
</tr>
<tr>
<td>N648L</td>
<td>c.1943C&gt;T</td>
<td>H NA NA -</td>
<td>0 6.87</td>
<td></td>
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<tr>
<td>P645L</td>
<td>c.1961C&gt;T</td>
<td>H - NA -</td>
<td>0 19.2</td>
<td>1</td>
</tr>
<tr>
<td>R659P</td>
<td>c.1976G&gt;C</td>
<td>H NA NA -</td>
<td>0.01 11.7</td>
<td>1, 5, 8</td>
</tr>
<tr>
<td>V716M</td>
<td>c.2146G&gt;A</td>
<td>H - NA +</td>
<td>0.16 2.78</td>
<td></td>
</tr>
</tbody>
</table>

aNucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the translation initiation codon in the GenBank reference sequence, amino acid numbering is based on the cDNA with +1 corresponding to the translation initiation codon.

bMLH1: GenBank NG_007109.1. MSI = microsatellite instability; H = high; L = low; NA = not available; IHC = immunohistochemistry.

cSIFT scores as calculated on the SIFT webpage (http://sift.jcvi.org/). Scores <0.06 are considered pathogenic/not tolerated.

dMAPP-MMR [Chao et al., 2008] scores were calculated from http://mappmmr.blueankh.com/. Values >4.55 are considered pathogenic.

References: 1 [Takahashi et al., 2007], 2 [Shimodaira et al., 1998], 3 [Kondo et al., 2003], 4 [Guerrette et al., 1999], 5 [Raevaara et al., 2003], 6 [Blassi et al., 2006], 7 [Avdievich et al., 2008], 8 [Nystrom-Lahti et al., 2002], 9 [Wanat et al., 2007], 10 [Plotz et al., 2006], 11 [Spina et al., 2008], 12 [Trojan et al., 2002], 13 [Perera and Bapat, 2008]. For references employing more than in vitro MMR assays alone, only the result for the MMR assay has been included in this table.

fThe K618A mutant does not fit either category in reference 9 [Wanat et al., 2007].
Protein Levels

MMR VUS Classification Does Not Critically Depend on Efficiencies of PCR-Based Mutants

Identifying pathogenic black bars and Supp. Fig. S3). This difference allows to selectively identify pathogenic MLH1 mutants. In the other 19 MLH1 mutants we found a large variation in MMR activity (Fig. 3). As a tentative cutoff for MMR deficiency we assumed a repair level that was not significantly higher (Student’s two-tailed t-test, p < 0.05) than the average repair efficiency of the presumed pathogenic mutants. In support, all repair deficient VUS were predicted to be deleterious using SIFT analysis and most also by MAPP-MMR, an algorithm focused at MMR gene variants (Table 1).


table

Replication Efficiencies of Cloned Mutants Mirror Repair Efficiencies of PCR-Based Mutants

To further investigate the robustness of the PCR product-based expression system, we investigated the activity of MLH1 VUS, expressed after their cloning. Although the activities of cloned VUS reflected those of PCR-generated VUS (Fig. 3), repair levels for the MMR-proficient cloned VUS were somewhat higher than those of the PCR-based VUS, due to the presence of MMR-inhibitory contaminants in the PCR-based expressions (not shown). Nevertheless, the generation of MLH1 VUS in a two-step PCR followed by their direct expression provides a rapid and reliable alternative for cloning.

MMR VUS Classification Does Not Critically Depend on Protein Levels

As the expression levels between individual variant proteins was subject to some random variation (see above) we wanted to investigate whether the MMR assay is tolerant to variations in MLH1 levels. To this end, wild-type MLH1, MMR-proficient VUS E578G and MMR-deficient VUS G67R were expressed from PCR products (Fig. 2G). Then, the reactions were diluted up to twofold such that each contained a similar concentration of MLH1. During further serial dilution, both the wild-type protein and E578G retained activity that discriminates them from G67R (Fig. 4). Remarkably, at increasing dilutions the activity of E578G was more affected than of wild-type MLH1, indicating that the mutation may confer partial loss of function. This result shows that the assay is tolerant to stochastic variations in in vitro expression levels of the MLH1 variants.

Discussion

Proper genetic counseling of suspected Lynch Syndrome patients requires an accurate diagnosis based on the nature of the germ line mutation found. Cancer risk resulting from nontruncating, and especially missense, variants is difficult to predict. This results in anxiety, and frequently, unnecessary periodic screening in carriers of such a variant, but also in both VUS-carrying and noncarrying relatives. In this work we present a cell-free assay for the functional analysis of MLH1 VUS that enables the rapid identification of pathogenic variants.

Repair deficiency for a VUS in our assay was, with few exceptions, consistent with the in silico prediction of pathogenicity and with pathology data (Table 1). Some of the same VUS were analyzed before by others and most MLH1 VUS that were inactive in our assay were also inactive in other assays. One notable exception is VUS R265C that, although classified repair-deficient in three studies [Plotz et al., 2006; Takahashi et al., 2007; Wanat et al., 2007], was classified proficient in another [Trojan et al., 2002]. Additionally, L550P, A589D, and P654L were active in another assay [Raevaara et al., 2005] but inactive in yeast-based assays [Takahashi et al., 2007; Wanat et al., 2007]. The latter VUS are located at the protein–protein interaction domain (Fig. 2G). In vitro binding studies (Andersen et al., personal communication) support a defect for these variants and another study has shown binding defects for other VUS in this region of the protein [Guerrette et al., 1999]. Furthermore, the observed lack of nuclear import of PMS2 by these VUS [Raevaara et al., 2005] supports a dimerization defect, since heterodimerization of MLH1 and PMS2 stimulates nuclear import of MutLα [Knudsen et al., 2009]. Additionally, all four VUS that show discrepant results (R265C, L550P, A589D, and P654L) are predicted to be defective by in silico analyses and were associated with microsatellite instability, a hallmark of MMR deficiency (Table 1). For these reasons, we conclude that a lack of activity in our assay is in agreement with most other functional data, with pathology data and with in silico analysis.

Because a loss of MMR is strongly predictive for Lynch Syndrome, the positive predictive value of our assay is bound to be high. Further validation of our assay seems warranted, using a much larger group of accepted polymorphisms and pathogenic mutations from well-studied patients. This could aid in determining the relation between MMR activity and disease penetrance, enabling to assess pathogenicity of VUS that display a low to intermediate level of repair. However, as our assay does not address specific in vivo MMR defects, such as a splicing, stability, or intracellular localization, nor subtle in vitro defects, the negative predictive value will probably be lower. As an example, E578G displayed reduction in MMR activity only after its dilution (Fig. 4) that may reflect a subtle dimerization defect. We infer that, in addition to a cell-free complementation assay as described here, additional functional data might be used for final diagnosis of VUS that display residual activity. This may include nuclear localization assays [Raevaara et al., 2005], splicing assays [Tournier et al., 2008], protein–protein interaction studies [Guerrette et al., 1999], or stability studies [Perera and Bapat, 2008]. A combination of different analytical approaches, pathological, and family data should result in a
comprehensive, validated, diagnostic procedure of VUS in MLH1 and other MMR genes [Plon et al., 2008].

In conclusion, with this assay, we have developed a simple, rapid, cost-effective, and reliable method that may fill a niche in diagnostic labs [Heinen, 2009]. Cell extracts, substrate, and other ingredients can be prepared, stored, and distributed in large quantities. The full assay (recreation of the mutants by PCR, in vitro expression and in vitro MMR assay) can be performed in 3 to 4 days, and many mutants can be tested in parallel. The procedure can be used to screen the large amount of mutants already detected, a number that, due to the advent of personalized genomics, is likely to rise sharply. Alternatively, the assay may be used for high-resolution a priori VUS analyses as was done for p53 [Kato et al., 2003]. Currently we are developing and testing very similar cell-free assays to test functional activity of VUS in the MSH2 and MSH6 MMR genes.

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This work was supported by grants from the Nuts/Ohra foundation (SNO-T-03-06), the Dutch Digestive Foundation (MWO 05-16), and the European Union (FP6-018754). We acknowledge Prof. John B. Hays and Prof. Josef Jiricny for providing plasmds and Dr. Giancarlo Marra for help. Drs. Anne Lutzen, Sofie Dabros Andersen and Prof. Lene Juel Rasmussen are thanked for MLH1-mutant plasmds, sharing unpublished data, and helpful discussions. We thank Drs. Anastasia Tsaiabi-Shyltyk, Cristina Ferras, Jaap Jansen, and Prof. Robert Hofstra for critical reading.

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