

# Modification Form for Permit BIO-UWO-0227

**Permit Holder: Peter Rogan**

**Approved Personnel**

**(Please stroke out any personnel to be removed)**

Heather Tarnowski

Eliseos John Mucaki

**Additional Personnel**

**(Please list additional personnel here)**

**Please stroke out any approved Biological Agent(s) to be removed**

**Write additional Biological Agent(s) for approval below. Give the full name**

**Approved Microorganisms**

E.coli

**Approved Primary and Established Cells**

Human (primary): lymphocyte, fibroblast, hepatocyte. Human (established): lymphoblastoid, HepG2, CaCo2. Rodent (established): rarely. Non-Human primate (established): cos7, cos293, CV1

**Approved Use of Human Source Material**

Human blood (whole). Human organs or tissues (unpreserved): small fibroblast sample. Human organs or tissues (preserved): fixed lymphocytes tissue sections.

**Approved Genetic Modifications (Plasmids/Vectors)**

PCR 2.1 Topo, pFxP2-DEJT, pDEJT26, pHuR195

PCAS-2  
pCDNA-Dup

} plasmids from insert

**Approved Use of Animals**

**Approved Biological Toxin(s)**

\* PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOLOGICAL AGENTS.  
\*\* PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOLOGICAL AGENTS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF..

As the Principal Investigator, I have ensured that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1\_2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at <http://www.shs.uwo.ca/workplace/newposition.htm>

Signature of Permit Holder:  \_\_\_\_\_

Current Classification: 2 Containment Level for Added Biohazards: 1

Date of Last Biohazardous Agents Registry Form: Dec 9, 2008

Date of Last Modification (if applicable): Feb 18, 2011

BioSafety Officer(s): \_\_\_\_\_

Chair, Biohazards Subcommittee: \_\_\_\_\_ Date: \_\_\_\_\_

----- Original Message -----

**Subject:**Re: Biological Agents Registry Form: Rogan

**Date:**Mon, 21 Nov 2011 16:26:23 -0500

**From:**Peter Rogan <progan@uwo.ca>

**To:**Jennifer Stanley <jstanle2@uwo.ca>

New Info

Jennifer,

We are not predicting genes. We are using the constructs to assess predicted mutations.

The present study will predict splicing mutations in breast cancer genes in patients previously screened for protein coding variants using our information theory-based software (for analysis of high throughput sequencing, and which has been validated for BRCA mutations; Mucaki et al. 2011). This software filters out polymorphic variants, insignificant  $\Delta R_i$  values, and distant and weak cryptic sites. Previously derived and re-sequenced patient DNA sequences will be assessed for splicing mutations identified in our BIC. Novel VUS within exons and flanking intron regions of the BRCA (or *CHEK2*, *TP53*, *CDH1*, *ATM*, and *PALB2*) genes will also be assessed. Individual information analysis will be carried out after each set of sequencing runs. Information analysis has predicted and validated leaky, splicing mutations and their presence in *BRCA1/2* is consistent with the idea that a high level expression may be essential for tumour suppression.

*Prediction of mutant splice isoforms.* An innovative approach will be used to predict the structures and abundance of mutant splice isoforms. These predicted “molecular phenotypes” are based on mechanisms of exon definition prescribing recognition of both cognate donor and acceptor splice sites for inclusion in mature transcripts. Bipartite entropy minimization (Bi and Rogan 2004) can represent sequence conservation at exon boundaries as a pair of half sites separated by a gap (of coding sequence). It identifies the majority (80%) of internal exons in human genes, producing weight matrices for donor and acceptor half sites that are indistinguishable from those of individual splice sites. The splice sites of internal exons occur in close proximity, typically 30 to 400 nucleotides in length, to enable crosstalk between the U1 and U2 splicesomes. Activated cryptic splice sites are recognized by the same mechanism as natural sites. We define normal, mutated, and cryptic exons from the total individual information contents of the donor and acceptor sequences,  $R_{i(total)}$ , as  $R_{i(acceptor)} + R_{i(donor)} + g(L)$ , where  $L$  is the length of the exon and  $g(L)$  is a gap surprisal penalty, which adjusts  $R_{i(total)}$  for the distance between the splice acceptor and donor site sequences, and ensures that splice sites from the same exon are paired. The gap surprisal is:  $g(L) = -\log(1/p(L)) + C$ , where  $p(L)$  is the likelihood of an exon of length  $L$  in the genome and  $C$  is a scaling factor that ensures  $R_{i(total)} > 0$  for known exons. The same approach is used to compute  $R_{i(total)}$  for terminal exons, except that exon length distributions and gap surprisals are determined separately. The abundance of normal and mutant isoforms are compared based on

differences in their  $R_{i(total)}$  values [ $\Delta R_{i(total)}$ ]. Expression levels are expected to correspond to the fold differences ( $\geq 2^{\Delta R_{i(total)}}$ ) in exon abundance (Kodolitsch et al 1999). Spurious predicted exons which do not conform to mRNA splicing mechanisms are eliminated, and very low abundance exons are filtered out (<1% of the maximum  $R_{i(total)}$  isoform). Exon definition analysis is available on the ASSA server (Nalla and Rogan 2005). We have validated predicted molecular phenotypes for 15 *BRCA1* mutations and the predicted isoforms generally mirror reported splice isoforms.

Assessing gene expression changes due to suspected splicing or promoter mutations. *In vitro* analysis of the processed transcripts from a patient is the most reliable method of establishing whether a particular mutation affects splicing. *BRCA1/2* studies of leukocyte mRNA are consistent with the expression patterns in breast tissue.

The predicted molecular phenotypes will be assessed by qRT-PCR. Predicted phenotypes can suggest potential changes in natural exon abundance, detect unobvious cryptic isoforms, and avoid testing for unlikely mRNA species. Dr. Peter Ainsworth has stored RNA from patients tested for BRCA mutations. Each predicted isoform will be reverse transcribed, amplified and detected with SYBR Green dye.  $\Delta\Delta C_T$  values of natural, skipped or cryptically spliced products determine the relative abundance of the isoforms. We have validated this approach for 30 pathogenic SNPs and the 4 BRCA splicing mutations, which were concordant with predictions for diminished natural site use, exon skipping, or cryptic splicing.

*In vitro* mutation analysis is not practical to carry out for many mutations, as patients cannot be reascertained or are unavailable for other reasons (eg. they may be deceased).

Our collaborator, Dr. Mario Tosi (Rouen, France), has developed minigene assays for *BRCA1/2* splicing abnormalities and has provided the necessary plasmid constructs which both his laboratory and our own will use to assess the mutations that we predict. The plasmids we have received from Dr. Tosi will be used to perform *ex vivo* minigene assays will be used to assess variants affecting splicing if mutation carriers are unavailable (Gaildrat et al 2009; Tournier et al 2008). Each mutation and surrounding sequences is cloned into a minigene, transfected into a breast cancer cell line and analyzed by endpoint RT-PCR and cDNA sequencing.

The Mucaki et al. 2011, Gaildrat et al 2009, Tournier et al 2008 references were included with the original submission.

Kind regards,  
Peter K. Rogan, Ph.D.,  
Canada Research Chair in Genome Bioinformatics, Tier I  
Professor of Biochemistry & Computer Science  
The University of Western Ontario

The plasmids will be used to assess predicted mutations in breast cancer and other cancer related genes by ex vivo RT-PCR assays.

**Inserm**

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**Material Transfer Agreement between**

**Inserm U614, Faculty of Medicine, University of Rouen (France)**

And (recipient)

.....

Concerning the splicing reporter minigene pCAS-2, described in Gaildrat et al. Methods in Molecular Biology, vol 653, published by Springer October 2010

This DNA construct is transferred under the agreement that:

- 1) it will be used for research by the Recipient and not for commercial purposes
- 2) this material is not transferred to third parties

Date:

For Recipient:

For Inserm U614, Rouen  
Pr Mario Tosi

A handwritten signature in black ink, appearing to read 'Mario Tosi', is written over a light grey circular stamp.

**Inserm**



Institut national  
de la santé et de la recherche médicale



## Material Transfer Agreement

between

**Inserm U614 Laboratory, Rouen Medical School, University of Rouen**  
22 boulevard Gambetta, 76183 Cedex 1, France  
Phone: +33(0)235148313, Fax: +33(0)235148237  
Email : inserm.u614@univ-rouen.fr

and

.....  
(Recipient Scientist, Recipient Institution, address, contact information)

Concerning the plasmid **pcDNA-Dup**, described in Tournier *et al.*, 2008, Human Mutation, 29: 1412-1424.

This DNA construct is transferred under the agreement that:

- 1) it will be used for research only by the Recipient and not for commercial purposes
- 2) it will not be transferred to third parties

Recipient's name (print), signature and date

Provider's name (print), signature and date

Alexandra Martins, PhD  
Inserm Researcher, CR1  
Inserm U614, Rouen  
alexandra.martins@univ-rouen.fr

March 11th, 2011



Peter Rogan &lt;p1k2r3@gmail.com&gt;

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## TRANSFER OF PLASMIDS AND SUGGESTED COLLABORATION

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Mario Tosi &lt;Mario.Tosi@univ-rouen.fr&gt;

Thu, Sep 8, 2011 at 9:24 AM

To: Peter Rogan &lt;progan@uwo.ca&gt;

Cc: Alexandra Martins &lt;alexandra.martins@univ-rouen.fr&gt;, Pascaline Gaildrat &lt;pascaline.gaildrat@univ-rouen.fr&gt;, thierry.frebourg@chu-rouen.fr, mario.tosi@orange.fr

Dear Dr Rogan,

I have received your message while I was studying in detail the Supplementary tables of your recent paper in Human Mutation. It seems that many of those variants have already been tested at the RNA level in French laboratories.

Concerning your practical questions:

- 1) There is no background from endogenous BRCA expression, because RT-PCR primers are in the exons of the minigene, which are derived from SerpinG1; moreover there is no background from the endogenous SerpinG1, because the sequence at the priming sites has been slightly modified
- 2) If you wish to test in parallel much larger flanking intronic sequences it might be better to use the pSpliceExpress from Stefan Stamm, Lexington KT (E-mail: [s\\_stamm1@yahoo.de](mailto:s_stamm1@yahoo.de)). This minigene has sites for cloning by in vitro recombination. In our experience it takes up easily fragments up to 4 kb.

We are interested in discussing possibilities of collaboration because we think that all algorithms for splicing predictions should be tested against large sets of experimental data. We have a commitment to provide the community of medical geneticists with the most reliable bioinformatics tools for prediction of splicing defects. We could help you not only in setting up the minigene assays, but also by providing unpublished results of splicing assays of variants of BRCA genes and Mismatch repair genes for comparison with your splicing predictions.

As you know, we have used your program in the past. More recently, we have been using the algorithms SSF-like, MES, NNS and HSF, mainly because they are very often used by the medical genetics community through the program Alamut, developed here in Rouen by André Blavier (Interactive Biosoftware <http://www.interactive-biosoftware.com/>).

We can send you the plasmids pCAS-2 and pcDNA-Dup as soon as we receive the enclosed MTAs with your signatures. We have developed these constructs and are using them in an academic context

and our research is supported by public funding. Therefore it will be important to discuss if there are potential conflicts of interest concerning your company Cytognomix Inc.

Please write me if you have further questions on these matters.

Best regards

Prof. Mario Tosi

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**De :** [p1k2r3@gmail.com](mailto:p1k2r3@gmail.com) [mailto:[p1k2r3@gmail.com](mailto:p1k2r3@gmail.com)] **De la part de** Peter Rogan

**Envoyé :** lundi 5 septembre 2011 20:05

**À :** [mario.tosi@univ-rouen.fr](mailto:mario.tosi@univ-rouen.fr)

**Cc :** Peter Ainsworth

**Objet :** minigene constructs

Dear Dr. Tosi,

I would like to set up minigene assays for BRCA1 and BRCA2 with the constructs that you have published (pCAS1/2 and pcDNA-Dup) to assess mutations in patients we have ascertained, from which mRNA is not available. Would you be willing to share these plasmids with us?

We have recently carried out information theory based analysis of mutations in BIC ([Mucaki et al.](#)

# Chapter 15

## Use of Splicing Reporter Minigene Assay to Evaluate the Effect on Splicing of Unclassified Genetic Variants

Pascaline Gaildrat, Audrey Killian, Alexandra Martins, Isabelle Tournier, Thierry Frébourg, and Mario Tosi

### Abstract

The interpretation of the numerous sequence variants of unknown biological and clinical significance (UV for “unclassified variant”) found in genetic screenings represents a major challenge in the molecular diagnosis of genetic disease, including cancer susceptibility. A fraction of UVs may be deleterious because they affect mRNA splicing. Here, we describe a functional splicing assay based on a minigene construct that assesses the impact of sequence variants on splicing. A genomic segment encompassing the variant sequence of interest along with flanking intronic sequences is PCR-amplified from patient genomic DNA and is cloned into a minigene vector. After transient transfection into cultured cells, the splicing patterns of the transcripts generated from the wild-type and from the variant constructs are compared by reverse transcription-PCR analysis and sequencing. This method represents a complementary approach to reverse transcription-PCR analyses of patient RNA, for the identification of pathogenic splicing mutations.

**Key words:** Cancer molecular diagnosis, Minigene construct, Splicing mutations, Unclassified genetic variants

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### 1. Introduction

Functional splicing reporter minigene assays represent a powerful tool to assess the impact of sequence variants on splicing (1, 2). These assays are very useful to diagnostic laboratories for determining the biological and the pathological significance of certain sequence variations detected in genetic screenings of disease-predisposing genes. The protocol provided here is used routinely in our laboratory to evaluate whether unclassified variants (UVs) identified in genes associated with predisposition to Lynch syndrome (*MLH1/MSH2*) or to hereditary breast-ovarian cancer (*BRCA1/BRCA2*) lead to splicing defects (3, 4).

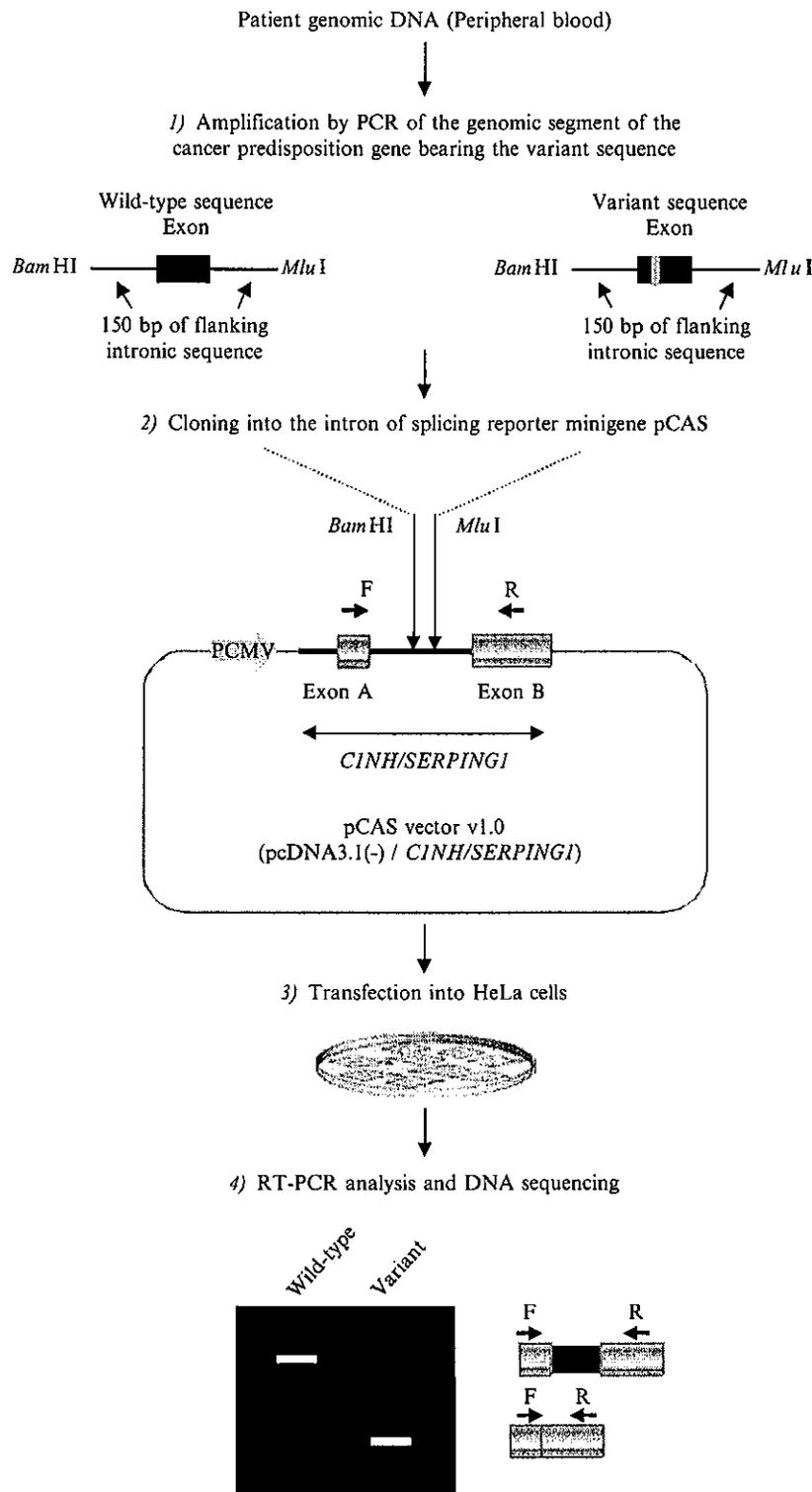


Fig. 1. Schematic representation of the functional splicing reporter minigene assay. (1) The wild-type and the variant exonic sequences of interest are PCR-amplified from patient genomic DNA together with ~150 bp of their 5' and 3' intronic flanking sequences, using specific primers carrying 5' tails with BamHI and MluI restriction sites. (2) The amplicons are cloned into the pCAS1 reporter vector, which is based on the pcDNA3.1 plasmid and contains a minigene composed of two exons (here, named A and B). The minigene contains 114 bp of intron 1, exon 2, the entire intron 2, and exon 3, fused to partial exon 4 of the *SERPING1/C1NH* gene. In a recent version of the minigene construct, named pCAS2,

8. Sequencing. ABI PRISM BigDye Terminator v3.1 cycle Sequencing kit (Applied Biosystems).

### **2.3. Transfection of the pCAS Vector into HeLa Cells**

1. Cell Culture. HeLa cells (ATCC), Dulbecco's Modified Eagle's Medium (D-MEM) with L-Glutamine, 4,500 mg/L D-Glucose, without Sodium Pyruvate (Gibco), Fetal bovine serum (FBS) (Biowest).
2. Transfection. FuGENE 6 Transfection Reagent (Roche Applied Science).
3. Treatment by puromycin (Sigma-Aldrich).

### **2.4. RT-PCR Analysis**

1. RNA extraction. TriPure Isolation Reagent (Roche Applied Science).
2. DNase treatment. Dcoxyribonuclease Amplification Grade RNase-free (Sigma-Aldrich).
3. First-strand cDNA synthesis. SuperScript II Reverse Transcriptase (Invitrogen), Oligo(dT)<sub>18</sub> mRNA primer (New England Biolabs), 100 mM dNTP Set PCR Grade (Invitrogen), RNase OUT (Invitrogen).
4. Amplification by PCR. Thermoprime plus DNA polymerase (ABgene), 100 mM dNTP Set PCR Grade (Invitrogen).
5. Electrophoresis. SeaKem LE Agarose (Tebu-bio), TBE buffer: 89 mM Tris-HCl, 89 mM Boric Acid, 2 mM EDTA (Euromedex), Ethidium bromide (Qbiogene), DNA size marker (New England Biolabs).
6. DNA purification from agarose gel: NucleoSpin Extract II kit (Macherey-Nagel).
7. Sequencing: ABI PRISM BigDye Terminator v3.1 cycle Sequencing kit (Applied Biosystems).

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## **3. Methods**

### **3.1. Cloning of the Variant Sequence into the pCAS Vector**

1. Amplification of the genomic fragments by PCR: The wild-type and the variant exonic sequences of interest are PCR-amplified from patient genomic DNA together with approximately 150 bp of their 5' and 3' intronic flanking sequences (see Notes 1 and 2). Specific forward and reverse primers carrying 5' tails that contain sites for BamHI and MluI restriction enzymes are used (see Note 3). To a 0.2 mL Eppendorf tube, add 5  $\mu$ L of 10 $\times$  reaction buffer, 0.4  $\mu$ M of each forward and reverse primers, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 1.25 U Thermoprime plus DNA Polymerase (ABgene) (see Note 4), and 100 ng genomic DNA, and add

is added to the culture 5.5 h before harvesting. Cells are collected 24 h post-transfection. Puromycin treatment inhibits translation, thus preventing the degradation of transcripts containing a premature stop codon that are targets of the nonsense-mediated mRNA decay (NMD).

### 3.3. RT-PCR Analysis

1. Total RNAs are isolated from transfected cells using the TriPure Isolation Reagent (Roche), according to the manufacturer's instructions. Total RNAs are quantified by spectrophotometry (optical density at 260 nm,  $OD_{260}$ ). In order to eliminate contaminating DNA, RNAs are treated with Amplification Grade RNase-free DNase I (Sigma-Aldrich), as described by the manufacturer.
2. First-strand cDNAs are synthesized from 1 to 2  $\mu$ g of each DNase-treated total RNA sample using oligo(dT)<sub>18</sub> mRNA primer and the SuperScript<sup>TM</sup> II Reverse Transcriptase in a 20  $\mu$ L reaction volume, as described by the manufacturer (see Note 11).
3. PCR amplifications are performed from 6  $\mu$ L of the first-strand cDNA reaction mixture using primers F and R located, respectively, in exon A and exon B of the minigene pCAS1 (see Fig. 1). Thermoprime plus DNA Polymerase (ABgene) is used for the PCR reaction in a 50  $\mu$ L volume, under the same conditions as described in step 1 of Subheading 3.1. PCRs are performed as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 10 s, 57°C for 20 s, and 72°C for 50 s, with a final elongation step at 72°C for 10 min (see Note 12).
4. RT-PCR products are separated, alongside the DNA size marker, by electrophoresis through an agarose gel containing ethidium bromide (0.5  $\mu$ g/mL) in 1 $\times$  TBE buffer and visualized by the exposure to ultraviolet light (see Note 6). Each DNA band is gel-purified using Nucleospin Extract II kit and sequenced using Big Dye Terminator cycle sequencing kit and ABI Prism 3100 automated sequencer, as described by the manufacturers (see Note 13).

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## 4. Notes

1. In most cases studied, the sequence variant to be tested is present in the heterozygous state. Therefore, the wild-type and the variant exonic sequences are coamplified by PCR from patient genomic DNA and subsequently selected after molecular cloning and sequencing.

## RESEARCH ARTICLE

# A Large Fraction of Unclassified Variants of the Mismatch Repair Genes *MLH1* and *MSH2* Is Associated With Splicing Defects

Isabelle Tournier,<sup>1</sup> Myriam Vezain,<sup>1</sup> Alexandra Martins,<sup>1</sup> Françoise Charbonnier,<sup>1</sup> Stéphanie Baert-Desurmont,<sup>1,2</sup> Sylviane Olschwang,<sup>3</sup> Qing Wang,<sup>4</sup> Marie Pierre Buisine,<sup>5</sup> Johann Soret,<sup>6</sup> Jamal Tazi,<sup>6</sup> Thierry Frébourg,<sup>1,2</sup> and Mario Tosi<sup>1\*</sup>

<sup>1</sup>Inserm U614, Federate Institute for Multidisciplinary Research on Peptides, Faculty of Medicine, University of Rouen, Department of Genetics and Institute for Biomedical Research, Rouen University Hospital, Rouen, France; <sup>2</sup>Department of Genetics, Rouen University Hospital, Rouen, France; <sup>3</sup>Inserm UMR 599, Institut Paoli-Calmettes, Marseille, France; <sup>4</sup>Molecular Oncology Unit, Centre Léon Bérard, Lyon, France; <sup>5</sup>Laboratory of Biochemistry and Molecular Biology, Lille University Hospital, Lille, France; <sup>6</sup>Institut de Génétique Moléculaire de Montpellier, UMR 5535, Centre National de la Recherche Scientifique, Montpellier, France

Communicated by Riccardo Fodde

Numerous unclassified variants (UVs) have been found in the mismatch repair genes *MLH1* and *MSH2* involved in hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome). Some of these variants may have an effect on pre-mRNA splicing, either by altering degenerate positions of splice site sequences or by affecting intronic or exonic splicing regulatory sequences such as exonic splicing enhancers (ESEs). In order to determine the consequences of UVs on splicing, we used a functional assay of exon inclusion. For each variant, mutant and wild-type exons to be tested were PCR-amplified from patient genomic DNA together with ~150 bp of flanking sequences and were inserted into a splicing reporter minigene. After transfection into HeLa cells, the effects on splicing were evaluated by RT-PCR analysis and systematic sequencing. A total of 22 UVs out of 85 different variant alleles examined in 82 families affected splicing, including four exonic variants that affected putative splicing regulatory elements. We analyzed short stretches spanning the latter variants by cloning them into the ESE-dependent central exon of a three-exon splicing minigene and we showed in cell transfection experiments that the wild-type sequences indeed contain functional ESEs. We then used this construct to query for ESE elements in the *MLH1* or *MSH2* regions affected by 14 previously reported exonic splicing mutations and showed that they also contain functional ESEs. These splicing assays represent a valuable tool for the interpretation of UVs and should contribute to the optimization of the molecular diagnosis of the Lynch syndrome and of other genetic diseases. *Hum Mutat* 29(12), 1412–1424, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: *MLH1*; *MSH2*; variant of unknown significance; Lynch syndrome; splicing mutation; splicing assay

## INTRODUCTION

Identification of the mismatch repair (MMR) gene mutations responsible for hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome; MIM# 120435) is essential for genetic counseling and for follow-up aimed at cancer prevention in affected family members, but it is complicated by the genetic heterogeneity of the syndrome [Wijnen et al., 1997, 1998; Lynch and de la Chapelle, 2003; Wagner et al., 2003]. While the sensitivity of mutation detection has improved, especially since inclusion of a screen for large rearrangements of the *MLH1* (MIM# 120436) and *MSH2* (MIM# 609309) genes [Di Fiore et al., 2004], a major difficulty in the diagnosis of Lynch syndrome, like of other mendelian diseases, is the interpretation of numerous variants of unknown clinical and biological significance. In most cases the pathogenic role of the unclassified variants (UVs) cannot be established from clinical data and segregation analysis, because of the small number of family members available, the incomplete penetrance of MMR mutations, and the possibility of additional undetected

mutations in the MMR genes. Several assays of protein function have been developed to evaluate the consequences of missense mutations. These assays include the use of in vitro systems [Plotz et al., 2006], yeast cells [Ellison et al., 2001], or mammalian cells [Blasi et al., 2006] to test specific activities or interactions of the *MLH1* or the *MSH2* proteins that are relevant to MMR function [reviewed in Ou et al., 2007]. The protein assays provide

The Supplementary Material referred to in this article can be accessed at <http://www.interscience.wiley.com/jpages/1059-7794/suppmat>.

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\*Correspondence to: Mario Tosi, Inserm U614, Faculty of Medicine, 22 Boulevard Gambetta, 76183 Rouen, France. E-mail: [mario.tosi@univ-rouen.fr](mailto:mario.tosi@univ-rouen.fr)

Grant sponsors: French North West Canceropole; Fondation de France.

DOI 10.1002/humu.20796

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TABLE 1. Continued

Variant description <sup>a</sup>			Ex vivo splicing assay	Patient RNA <sup>b</sup>
Exon 11	c.1680T>C (p.Asn560Asn)	Synonymous	No effect	-
Exon 11	c.1737A>G (p.Lys579Lys)	Synonymous	No effect	Concordant <sup>1</sup>
Exon 11	c.1759G>C (p.Gly587Arg)	Missense (DS)	Exon 11 skipping	-
Intron 11	c.1760-62G>A, c.1760-112.1760-111insAGG	Intronic <sup>c,d</sup> /Intronic	No effect	-
Exon 12	c.1786-1788del (p.Asn596del)	Codon deletion	No effect	Concordant <sup>1</sup>
Exon 12	c.1828C>A (p.His610Asn)	Missense	No effect	-
Exon 12	c.1912A>G (p.Arg638Gly)	Missense	No effect	Concordant (*)
Exon 12	c.1933C>G (p.Gln645Glu)	Missense	No effect	Concordant (*)
Intron 12	c.2006-6T>C	Intronic (AS-Py) <sup>d</sup>	Partial exon 13 skipping	Not concordant (*)
Exon 13	c.2011A>T (p.Asn671Tyr)	Missense	No effect	Concordant <sup>3</sup>
Exon 13	c.2087C>T (p.Pro696Leu)	Missense	No effect	-
Exon 13	c.2089T>C (p.Cys697Arg)	Missense	No effect	Concordant <sup>1</sup>
Exon 13	c.2168C>T (p.Ser723Phe)	Missense	No effect	Concordant (*)
Exon 14	c.2242G>T (p.Asp748Tyr)	Missense	No effect	-
Exon 15	c.2517T>A (p.His839Gln)	Missense	No effect	-
Intron 15	c.2634+5G>T	Intronic (DS)	Exon 15 skipping	Concordant <sup>4</sup>

<sup>a</sup>Nucleotide numbering reflects cDNA numbering, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

<sup>b</sup>RT-PCR analyses of patient RNA performed by us (\*) or published by others during this work (<sup>1</sup>Auclair et al. [2006]; <sup>2</sup>Pagenstecher et al. [2006]; <sup>3</sup>Sharp et al. [2004]; <sup>4</sup>Barnetson et al. [2008]).

<sup>c</sup>Variant used for the genetic counseling.

<sup>d</sup>Variant with a RS number listed in the NCBI SNP database or in the SNPer database.

DS, donor splice site; AS, acceptor splice site (as defined in the text); Py, polypyrimidine tract.

TABLE 2. Mutations Affecting Splicing by Disrupting Putative Enhancer or Silencer Elements

Gene-exon	Mutation	ESE finder <sup>a</sup>	Rescue ESE <sup>b</sup>	PESX <sup>c</sup>	Automated splice site analyses <sup>d</sup>	Ex vivo splicing assay	ESE-dependent splicing assay	
							Wild-type	Mutant
<i>MLH1</i> -intron 9	c.790+10A>G	-	3/3 ESE	0/4 ESE	+Ds-0.3/3.2 (c.790+4)	Weak exon 9 skipping	-	-
<i>MLH1</i> -exon 10	c.793C>T	-	-	-	-	Partial exon 10 skipping	Inclusion	Partial exclusion
<i>MLH1</i> -exon 10	c.794G>A	SRp40 3.85/-, SC35 3.31/-	-	-	SC35 3.8/0.8	Partial exon 10 skipping	Inclusion	No effect
<i>MLH1</i> -exon 11	c.988.990del	-	-	-	-	Weak exon 11 skipping	Inclusion	No effect
<i>MSH2</i> -exon 5	c.815C>T	SRp40 3.38/-, SF2 3.27/-, SRp55 2.76/-, SRp40 -/3.31	-	1/0 ESE	SF2 4/2.6	Partial exon 5 skipping	Inclusion	Partial exclusion

<sup>a</sup>Mutation effects on the scores predicted by the ESE Finder algorithm. Only scores that are above the default threshold and differ between the wild-type and variant were indicated as (wild-type sequence score)/(mutant sequence score).

<sup>b</sup>(Number of ESE motifs predicted from the wild type sequence)/(number of ESE motifs predicted from the mutant sequence).

<sup>c</sup>(Number of ESE or ESS sequences predicted from the normal allele)/(number of ESE or ESS motifs predicted from the mutation). Only sequences scores above the default threshold were indicated.

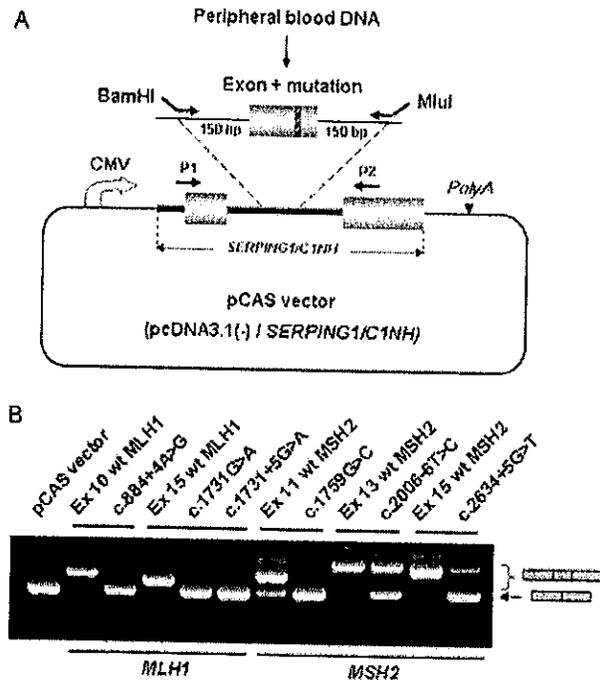
<sup>d</sup>Automated splice site analyses predicts units of bits [Nalla and Rogan, 2005] for ESE motifs as well as for splice sites. Predicted changes are expressed as bits for the wild-type sequence/bits for the variant. +DSbits(position) point to an additional donor site generated by the variant at the position shown in parentheses. Only predictions that differ between the wild-type and variant are shown.

important functional information, which is, however, insufficient to establish if a given variant is pathogenic. Not only are the results sometimes contradictory between assays and intermediate effects are often observed, but there is also the possibility that the pathogenic effect of the mutation may be at the level of mRNA maturation and processing. Considering the primary importance of early diagnosis of Lynch syndrome for cancer prevention, the consequences of each uncharacterized sequence variant of the MMR genes should be explored both at the protein and at the mRNA level.

We hypothesized that a fraction of the UVs detected in *MLH1* and *MSH2* could be pathogenic by affecting splice sites at the exon/intron boundaries or by disrupting exonic or intronic splicing regulatory cis-elements. Exonic and intronic splicing enhancers (ESE and ISE) and exonic and intronic splicing silencers (ESS and ISS) have been described in many genes [Cartegni et al., 2002]. They consist of short (6-8 bp) degenerate sequences that are recognized by splicing factors that either favor or inhibit recognition of suboptimal splice sites. Enhancer elements are

frequently recognized by members of the serine-arginine-rich protein family (SR proteins), while silencers are mostly recognized by heterogeneous nuclear ribonucleoproteins (hnRNP). In recent years, it has become increasingly clear that mutations affecting such elements may cause disease [Faustino and Cooper, 2003]. ESE elements have been proposed to be frequent in the MMR genes *MLH1* and *MSH2* [Gorlov et al., 2003], but they remain to be identified and characterized.

We used an ex vivo functional splicing assay based on a novel splicing reporter minigene to test 87 intronic or exonic variants (20 and 67, respectively) corresponding to 85 alleles found in *MLH1* or *MSH2* by the French HNPCC consortium. All but six variants were of unknown biological significance. Several minigene systems have been described for the analysis of cis-elements affecting splicing [reviewed in Cooper, 2005]. While a minigene approach has recently been used on 20 selected *MLH1* or *MSH2* exonic variants to evaluate bioinformatics predictions of ESE sequences [Lastella et al., 2006], this is, to our knowledge, the first systematic study of the effect of UVs on splicing, on a large cohort



**FIGURE 1.** Ex vivo splicing assay. **A:** Schematic representation of the pCAS reporter vector used in the ex vivo splicing assay. The pCAS reporter construct is a pCDNA3.1-based vector that contains exons 2 and 3 of the *SERPING1/C1NH* gene separated by their natural intron into which we have inserted appropriate cloning sites (BamHI, MluI). Wild-type and mutant *MLH1* or *MSH2* exons, PCR-amplified from patient genomic DNA together with 5' and 3' intronic flanking sequences are cloned into the pCAS reporter and wild-type and mutant clones are isolated by sequencing. **B:** Typical examples of ex vivo splicing assays for different *MLH1* and *MSH2* variants. After transfection into HeLa cells of the pCAS constructs corresponding to the wild-type and mutant alleles, total RNA is extracted and the transcripts are analyzed by RT-PCR, using primers P1 and P2. RT-PCR products are analyzed by ethidium bromide agarose gel separation followed by direct sequencing of the different bands. The position of PCR products corresponding to full-length spliced RNA and RNA lacking the exon examined are indicated on the right.

containing ethidium bromide, and DNA from each band was purified (Qiaquick Gel Extraction Kit; Qiagen) and sequenced directly.

### ESE-Dependent Splicing Assay

*MLH1* and *MSH2* exonic fragments carrying putative splicing regulatory sequences were tested for the presence of ESE elements by using an ESE-dependent splicing assay. Briefly, exonic fragments, as indicated, were obtained by annealing complementary 5'-phosphorylated oligonucleotides designed to encompass approximately 30 nucleotides around the mutation position, within the same exon, and to carry 5'-EcoRI and 3'-BamHI compatible ends. Then, the duplexes were inserted into the EcoRI and BamHI sites of pCDNA-Dup plasmid, which contains a splicing cassette consisting of a  $\beta$ -globin-derived three-exon minigene (Dup) [Labourier et al., 1999] under the control of the cytomegalovirus (CMV) promoter to generate the plasmids pCDNA-Dup-*MLH1* and pCDNA-Dup-*MSH2*. For details on plasmid constructs and for in silico predictions of splicing see Supplementary Material and Table S1, respectively available online at <http://www.interscience.wiley.com/jpages/1059-7794/suppmat>. The constructs pCDNA-Dup-SC35-3x and pCDNA-

Dup-SF2/ASF-3x were generated by insertion of fragments containing triplets of binding sites (underlined) for the SR proteins SC35 (5'-GGGCTCCTGTTGGCCCCTGTTGGCTCCTGTT-3') [Cartegni et al., 2003] and SF2/ASF (5'-AGAAGAACAAGAA GAACAAGAAGAACG-3') [Labourier et al., 1999; Tacke and Manley, 1995], respectively. The sequence of the insert of pCDNA-Dup-BR2In11 (5'-TGTTGTCCAGGTCACATTCAATTATAG-3') was derived from intron 11 of *BRCA2*. An empty pCDNA-Dup plasmid was prepared by filling in the termini of the EcoRI-BamHI digested plasmid, using the Klenow polymerase, and ligating the Klenow-generated blunt ends. The pCDNA-Dup-derived plasmids were sequenced to confirm the presence of the desired inserts and to ensure that no extra mutations were introduced into the middle exon and flanking intronic sequences during cloning. Wild-type and mutant constructs were transiently transfected into HeLa cells, as described above. At least two independent transfection experiments were performed for each construct. Cells were harvested 24 hr after transfection and total RNA was extracted using the TriPure Isolation Reagent (Roche Diagnostics). The RNA preparations were treated with DNase I (AMP-DI; Sigma-Aldrich), according to the manufacturer's instructions and subjected to RT-PCR analysis. The RT step was performed using 5  $\mu$ l of RNA, the Superscript<sup>TM</sup> II Reverse Transcriptase (Invitrogen), and 500 ng of oligonucleotide d(T)18 (New England Biolabs) in a 20- $\mu$ l reaction volume. The cDNA amplification reaction was performed with 25 cycles using 2  $\mu$ l of the RT reaction mixture, the forward primer T7-Pro (5'-TAATACGACTCACTATAGGG-3'), which binds immediately upstream of the Dup minigene, and the reverse primer Dup-2R (5'-GGACTCAAAGAACCCTCTGGG-3') that binds to the last exon of the minigene (Ex2 in Fig. 3). RT-PCR products were separated by electrophoresis on 2.5% agarose gel containing ethidium bromide and visualized by exposure to ultraviolet light. Each DNA band was extracted from the gel by using the Qiaquick Gel Extraction Kit (Qiagen) and sequenced directly with the forward primer T7-Pro.

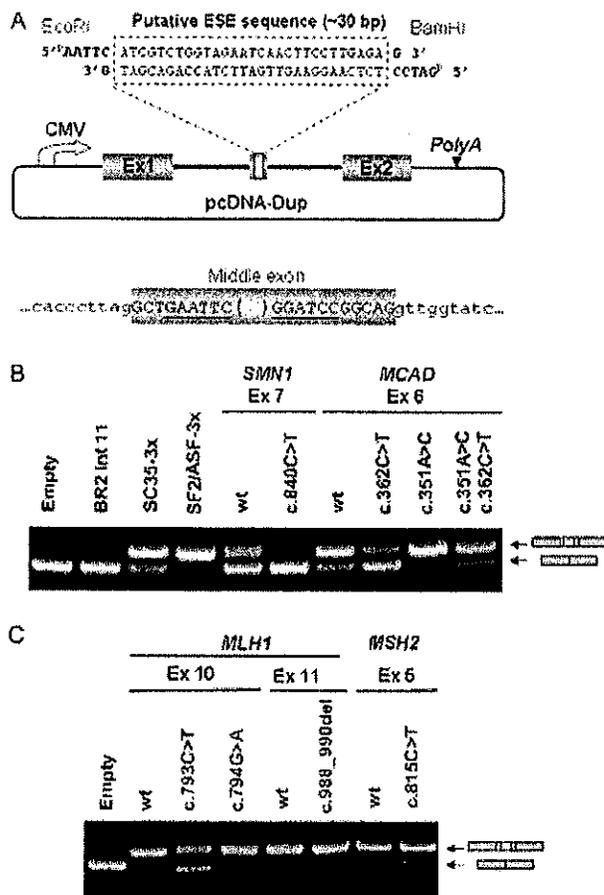
### Mutation Nomenclature

The official cDNA nomenclature is used with reference sequences NM\_000249.2 and NM\_000251.1 for *MLH1* and for *MSH2*, respectively. Nucleotide numbering starts at the A of the ATG initiator codon.

## RESULTS

### Detection, Using an Ex Vivo Splicing Assay, of Sequence Variants Affecting Splicing

As RNA samples from patients are not always available, we have developed an ex vivo splicing assay that relies on the PCR-amplification, from patient genomic DNA, of *MLH1* or *MSH2* wild-type and mutant sequences spanning the exon(s) of interest and approximately 150 bp of intronic flanking sequences. PCR fragments were inserted into the intron of a two-exon expression construct (denoted pCAS; Fig. 1A) that carries exons 2 and 3 of the *SERPING1/C1NH* gene separated by the natural intron, into which we have introduced appropriate cloning sites. Wild-type or mutant constructs were transiently transfected into HeLa cells either in the absence or presence of puromycin to avoid degradation by nonsense-mediated mRNA decay (NMD) of transcripts that may bear a premature stop codon. No difference was found in this study between puromycin-treated and -untreated cells. After 24 hr, cells were harvested and total RNA was extracted and analyzed by RT-PCR and agarose gel electrophoresis.



**FIGURE 3.** ESE-dependent splicing assay. **A:** Schematic representation of the pcDNA-Dup plasmid. The plasmid pcDNA-Dup has a pcDNA3.1(-) backbone and a  $\beta$ -globin-derived three-exon minigene under the control of the CMV promoter as indicated by the arrow. Gray boxes in solid lines represent the exons and the thick lines in between indicate the introns. The first (Ex1) and the last exon (Ex2) are the  $\beta$ -globin exons 1 and 2, respectively. The middle exon is flanked upstream and downstream by the 130 nucleotides of human  $\beta$ -globin intron 1. Sequences of interest were inserted into the EcoRI and BamHI sites of the minigene middle exon as indicated by the broken lines. An example of a fragment cloned into pcDNA-Dup consisting of a duplex of phosphorylated complementary oligonucleotides with EcoRI and BamHI ends is shown. The intron-exon boundaries of the middle exon are shown below the diagram. Intronic and exonic sequences are indicated in lower and upper case, respectively, restriction sites used in cloning are underlined and the position of the sequences tested is indicated between brackets. **B:** Detection of known ESEs. HeLa cells were transfected with either a pcDNA-Dup empty vector or pcDNA-Dup constructs carrying exonic fragments containing known wild-type (wt) or mutated splicing regulatory sequences of *SMN1* and *MCAD* as indicated. The fragments tested were c.835–c.864 from *SMN1* exon 7 (Ex 7) and c.347–c.376 from *MCAD* exon 6 (Ex 6). BR2In11 contained a short sequence from intron 11 of *BRCA2*. The constructs SC35-3x and SF2/ASF-3x contained triplets of binding sites for the SR proteins SC35 and SF2/ASF, respectively. Total RNA was collected 24 hr after transfection, subjected to RT-PCR and analyzed on a 2.5% agarose gel. The position of PCR products corresponding to full-length spliced RNA and RNA lacking the middle exon are indicated on the right. **C:** Discovery of ESEs in *MLH1* and *MSH2* regions affected by mutations identified in HNPCC patients. HeLa cells were transfected with either a pcDNA-Dup empty vector or pcDNA-Dup constructs carrying *MLH1* or *MSH2* exonic fragments affected by splicing mutations detected by using the pCAS assay. Wild-type (wt) and mutant sequences are indicated. The fragments tested were c.791–c.819 from *MLH1* exon 10 (Ex 10), c.975–c.1004 from *MLH1* exon 11 (Ex 11), and c.801–c.829 from *MSH2* exon 5 (Ex 5). Total RNA was collected 24 hr after transfection, subjected to RT-PCR and analyzed as described in (B).

boundaries, respectively, and a contribution of these positions to the definition and/or the strength of acceptor splice sites cannot be excluded [Rogan et al. 2003]. Mutations of the highly conserved GT and AG dinucleotides at the 5' and 3' extremities of introns, respectively, were not included in this study, since nonpathogenic variants at these positions are very rare [Krawczak et al., 2007], although some T>C transitions at the second position of donor splice sites may have minor effects on splicing [Nalla and Rogan, 2005]. Six variants listed in Table 1 (marked by <sup>c</sup> in Table 1), are usually considered pathogenic on the basis of phylogenetic, structural, or functional arguments or of segregation data. For example, Gly67Arg and Thr117Met of *MLH1* have previously been reported to affect protein function (Supplementary Table S2).

We also included in our study some variants that are listed as single nucleotide polymorphisms in the NCBI SNP database or in the SNPper database, because, while it is quite unlikely that they yield a major splicing defect, they could nevertheless produce weak splicing defects and act as modifiers of the effect of other mutations.

All wild-type *MLH1* or *MSH2* exons tested in the pCAS assay were predominantly included in the mature transcript, as illustrated in Fig. 1B. Traces of exon exclusion were observed with the following wild-type exons: 9, 10, and 16 (not shown) of *MLH1* and 11, 13, and 15 of *MSH2*. The wild-type exon 2 of the *MSH2* gene exhibited exon inclusion but also yielded a band corresponding to the activation of a cryptic 3' splice site within the

by NMD. The c.794G>A transition is expected to lead not only to partial exon 10 skipping, causing a frameshift that introduces a premature stop codon at position 263+2, but possibly also to an increase of simultaneous skipping of exons 9 and 10, since an alternatively-spliced form excluding both exons has previously been described [Charbonnier et al., 1995; Genuardi et al., 1998]. We initially carried out RT-PCRs with primers encompassing exons 9 and 10. As expected, the sample from a control individual showed RT-PCR products corresponding to inclusion of exons 9 and 10, skipping of exon 10, and skipping of both exon 9 and exon 10. The sample from the patient carrying the c.794G>A variant showed a slight increase of products corresponding to exon 10 skipping (data not shown). Considering that the latter transcripts are very likely unstable because of activation of the NMD system, we decided to evaluate quantitatively the consequences of the c.794G>A transition by measuring the reduction of transcripts carrying the A allele in exon 10 and used the SNaPshot method, which is based on allele-specific primer extension (see Supplementary Methods). As shown in Fig. 2D, we carried out an RT-PCR with primers in *MLH1* exon 8 and exon 11 and then extended with fluorescent allele-specific dideoxynucleotides a reverse primer positioned immediately downstream of the c.794G>A transition in exon 10. The extension products were then separated by electrophoresis and analyzed quantitatively. As the fluorescence intensities differ between dideoxynucleotides, the data obtained with reverse transcribed RNA were normalized using a similar primer extension on a PCR product from genomic DNA. After normalization, we found that approximately 40% of the transcripts corresponding to the c.794A allele had lost exon 10 sequences, compared to those corresponding to the c.794G allele (Fig. 2D), indicating that the c.794G>A transition detected by using the pCAS ex vivo splicing assay has measurable consequences in vivo. We were also able to analyze by RT-PCR the RNA from a patient carrying the c.790+10A>G transition in *MLH1* that yielded weak exon 9 skipping in the pCAS assay, but failed to detect any significant increase in the exclusion of *MLH1* exon 9 (data not shown). Note that the sensitive SNaPshot method described above could not be applied in this case, for lack of an informative exonic variant to discriminate the two alleles. Blood samples suitable for RNA extraction were not available from patients carrying the exonic *MLH1* variants c.793C>T or c.988\_990del nor for the *MSH2* variant c.815C>T shown in Fig. 2.

### An Ex Vivo Splicing Assay to Detect Exonic Splicing Enhancers

Mutations that cause exon skipping and that are located at positions different from the canonical splice sites are likely to affect splicing regulatory elements. Among the 87 UVs of *MLH1* and *MSH2* tested in the pCAS assay, five fall into this category, namely: *MLH1* variants c.790+10A>G, c.793C>T, c.794G>A, and c.988\_990del, and *MSH2* variant c.815C>T (Table 2). It is possible that these variants affect the maturation of the RNA because they either disrupt an enhancer motif (ESE or ISE) or they create a silencer motif (ESS or ISS) within the transcript sequence.

To determine if the exonic regions affected by *MLH1* and *MSH2* splicing mutations contain exonic splicing enhancer elements (ESEs) we performed an ex vivo ESE-dependent splicing assay. Briefly, a short fragment (~30 bp) of the genomic region of interest was inserted into the middle exon of a three-exon minigene under the control of the CMV promoter (Fig. 3A). Due to a weak 3' splice site, this middle exon is not recognized by the splicing

machinery and therefore is excluded from the processed transcript, unless enhancer elements are inserted within its sequence [Labourier et al., 1999]. The minigenes carrying inserts (either wild-type or mutant sequences) were transfected into HeLa cells and the splicing products of the minigene transcripts were analyzed by RT-PCR using RNA extracted from the transfected cells as template.

Because the middle exon in our assay has been modified for cloning purposes to contain EcoRI and BamHI restriction sites, we decided to verify if its inclusion in the mature transcripts remained ESE-dependent. As can be seen from the RT-PCR pattern shown in Fig. 3B, in the absence of enhancer elements, the modified middle exon is indeed excluded from the spliced transcripts. More precisely, middle exon exclusion was observed when the exon did not contain an inserted sequence, or when it contained a randomly chosen intronic sequence, such as a 27-bp fragment from the intron 11 of the *BRCA2* gene, or an *MLH1* exonic sequence not affected by splicing mutations (data not shown). In contrast, the middle exon was included in the minigene transcripts when it contained sequences carrying triplets of ESE elements known to bind to the splicing regulatory SR proteins SC35 or SF2/ASF (Fig. 3B).

To further validate the ex vivo ESE-dependent splicing assay we analyzed the effect produced by well characterized naturally occurring ESE sequences. We found that gene fragments known for containing an ESE motif recognized by the protein SF2/ASF, such as a segment of the exon 7 of *SMN1* [Cartegni and Krainer, 2002] or a segment of the exon 5 of *MCAD* [Nielsen et al., 2007] were able to induce inclusion of the middle exon of the minigene (Fig. 3B). Moreover, point mutations within these ESE, which are known to cause exon skipping of the corresponding transcripts, such as the substitution C>T at the position 6 of exon 7 of *SMN1* (corresponding to the sequence found in exon 7 of *SMN2*) or the c.362C>T substitution in exon 5 of *MCAD*, could also induce exon skipping in the context of the ex vivo ESE-dependent splicing assay (Fig. 3B). The inclusion/exclusion of an exon may depend on the combined action of enhancer and silencer elements that often coexist within the same sequence fragment. We verified that this type of interaction could be reproduced in the context of the ex vivo ESE-dependent splicing assay by analyzing the effect of the recently characterized *MCAD* polymorphic variant c.351A>C [Nielsen et al., 2007]. Indeed, we observed that the presence of *MCAD* c.351A>C corrected the splicing defect caused by the downstream *MCAD* c.362C>T transition (Fig. 3B), probably by disrupting an ESS as suggested by Nielsen et al. [2007]. This example illustrates how alleged polymorphic variants may actually have an effect on splicing and how the consequences of a mutation located within a splicing regulatory element may depend on the neighboring sequence environment.

### Detection of ESEs in *MLH1* and *MSH2* Regions Affected by Splicing Mutations Identified in the pCAS Assay

After validating the ex vivo ESE-dependent splicing assay, we applied this test to query for ESE elements within the genomic regions affected by the *MLH1* variants c.793C>T, c.794G>A, and c.988\_990del, and the *MSH2* variant c.815C>T, identified as splicing mutations by using the pCAS assay. While this work was in progress, the c.815C>T transition was independently reported as having an effect on splicing [Lastella et al., 2006]. As shown in Fig. 3C, the wild-type fragments corresponding to the regions c.791–c.819 and c.975–c.1004 from *MLH1*, and c.801–c.829 from

for the c.815C>T SNP, the c.2006–6T>C SNP is observed in 10 to 25% of the general population [Goessl et al., 1997; Hall et al., 1994]. Although it has been associated with an increased risk of developing sporadic colorectal cancer [Goessl et al., 1997], endometrial cancer [Beiner et al., 2006], or lung cancer [Jung et al., 2006], we failed to detect any splicing defect associated with this variant in blood lymphocytes (data not shown), thus confirming the results of Goessl et al. [1997], who tested blood lymphocytes of patients, colorectal tumor tissue, and a brain tumor cell line. However, exon 13 skipping results in a stop codon in exon 14 [Xia et al., 1996] and the transcripts bearing this deletion are likely to be targets for NMD and may not be detectable unless NMD is inhibited.

The hypothesis that exonic variants of *MLH1* or *MSH2* could affect splicing by disrupting splicing regulatory elements of the ESE type has been emphasized by the bioinformatics work of Gorlov et al. [2003]. These authors used the ESEfinder software to show that deleterious missense mutations are colocalized with predicted ESEs more frequently than expected by chance and more frequently than nonpathogenic variants. They proposed that 20 to 55% of the missense mutations of *MSH2* and 16 to 58% of those of *MLH1* may be pathogenic by disrupting ESE elements. However, several studies have already shown that computational predictions of exonic splicing regulatory elements and of their sequence changes lack specificity (see e.g., Lastella et al. [2006]). This may be due to inaccuracy of bioinformatics programs in predicting changes in ESEs or ESSs, but also to the redundancy of such regulatory elements, which may compensate for the alteration of a particular element. Conversely, among the four exonic variants, for which we present functional evidence that they are located within or near ESE elements, only c.794G>A and c.815C>T were predicted to have an effect on splicing (see Table 2). Therefore, our work suggests that bioinformatics predictions of exonic splicing regulatory elements may lack not only specificity but also sensitivity. In contrast, predictions of the definition and the strength of splice sites using NNsplice and SpliceSiteFinder and Automated Splice Site Analysis were in good agreement with the data obtained in the pCAS assay, with only few exceptions.

The effects on splicing of 37 out of the 87 variants listed in Table 1 have been tested by us or by others also by analyzing RNA from patients, or from patient-derived cell lines. The *in vivo* results were consistent with those obtained in the pCAS assay, with the exception of the polymorphic c.2006–6T>C variant in intron 12 of *MSH2* (which yielded a mild splicing defect in the pCAS assay and is predicted to affect splicing by several algorithms, as shown in Supplementary Table S1) and of the c.790+10A>G in intron 9 of *MLH1*, which yielded a very weak exon exclusion in the pCAS assay and was predicted by Automated Splice Site Analyses to generate a new donor site four bases downstream of the natural one. Interference between two donor splice sites, as previously suggested [Rogan et al. 2003], may explain the weak skipping of *MLH1* exon 9 in the pCAS assay. Neither *MSH2* c.2006–6T>C nor *MLH1* c.790+10A>G revealed splicing alterations in the blood cells of patients. However, since the transcripts bearing the splicing defects observed in the pCAS assay are likely to be unstable due to NMD, we cannot rule out the possibility that they do exist but are undetectable unless NMD is inhibited. While the level of concordance between *in vivo* and *ex vivo* results is remarkably good, we cannot exclude that some variants may affect *in vivo* the splicing of neighboring exons rather than that of the exon in which they reside. Altogether, these results indicate that *ex vivo* splicing

assays are a valuable tool allowing accurate detection of splicing mutations when patient RNA is not available. Additionally, *ex vivo* splicing assays represent an interesting way to confirm the causative role of variants associated with a splicing defect observed *in vivo* and to rule out major effects of other undetected changes.

We used an ESE-dependent splicing assay to analyze all the exonic regions of *MLH1* and *MSH2* described to date as being affected by splicing mutations lying outside the canonical splice sites (Fig. 3). Currently, 18 such mutations are known: four have been identified in this study and 14 were described by others (references in Table 3). With the exception of three mutations that were engineered by site-directed mutagenesis in exon 3 [McVety et al., 2006] and in exon 12 of *MLH1* [Lastella et al., 2004], all the variants have been identified in HNPCC patients. The results from the ESE-dependent splicing assay indicate the presence of ESE elements in all the wild-type exonic fragments tested. We have therefore defined ESE-containing sequences, of about 30 nucleotides, in eight different exonic regions of *MLH1* and four exonic regions of *MSH2*. Based on these findings we consider that any mutation identified in HNPCC families in these regions should be analyzed for its impact on splicing. Moreover, we observed that middle exon inclusion was inhibited by 11 out of the 15 HNPCC-associated splicing mutations identified presently in the *MLH1* and *MSH2* ESE-containing regions. Some of the variants tested failed to inhibit middle exon inclusion in the ESE-dependent *ex vivo* splicing assay. The effect of these variants seems to be context-dependent. It is possible that surrounding sequences, absent in the ESE-dependent assay, contain regulatory elements that contribute to exon exclusion when the ESE containing regions are disturbed by a mutation.

This work underlines the importance of functional splicing assays for the interpretation of UVs, especially when patient blood samples for RNA extraction are not available, but also as a tool to confirm the effects detected in the RNA from patients or from patient cell lines. In the absence of fully reliable bioinformatics predictions, especially for the detection of alterations affecting exonic splicing regulatory elements, these splicing assays should contribute to the optimization of the molecular diagnosis of Lynch syndrome as well as of other genetic diseases.

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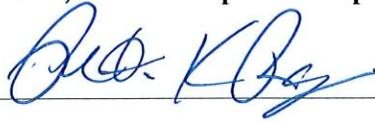
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 marker(s): tetR  
 replicon: pMB1

**Comments:** Restriction digests of the clone give the following sizes (kb): EcoRI--5.5; HindIII--5.5; BamHI--5.5; PstI--4.3, 1.2; Aval--5.5. DNA containing this sequence is underrepresented in most genomic libraries because most restriction enzymes (except HinfI and TaqI) generate fragments with pHuR195 elements larger than 20 kb. [5913]  
 This repeat is a variant of the satellite 2 family with a core sequence of CATCAT followed by 4 divergent GGAAT elements with a spacing of 0 - 49 nt. [5913]

**Media Description:** [ATCC medium 1273](#): LB medium (ATCC medium 1065) with 20 mcg/ml tetracycline

**References:** 5913: Moyzis RK, et al. Human chromosome-specific repetitive DNA sequences: novel markers for genetic analysis. Chromosoma 95: 375-386, 1987. PubMed: [3677921](#)

## Related Links

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[Technical Support](#)

[Media and Antibiotics](#)

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Notices and Disclaimers

ATCC products are intended for laboratory research purposes only, unless noted otherwise. They are not intended for use in humans.

While ATCC uses reasonable efforts to include accurate and up-to-date information on this site, ATCC makes no warranties or representations as to its accuracy. Citations from scientific literature and patents are provided for informational purposes only. ATCC does not warrant that such information has been confirmed to be accurate.

All prices are listed in U.S. dollars and are subject to change without notice. A discount off the current list price will be applied to most cultures for nonprofit institutions in the United States. Cultures that are ordered as test tubes or flasks will carry an additional laboratory fee. Fees for permits, shipping, and handling may apply.

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Modification Form for Permit BIO-UWO-0227

Permit Holder: Peter Rogan

Additional Biohazard – Genetic Modification (Plasmid/Vector)

Plasmid pHuR195, purchased from ATCC (Cedarlane Laboratories). See information sheet from ATCC.

This plasmid contains a 1.2 kilobase insert containing satellite repeat DNA from human chromosome 16q11.2. We will be extracting this plasmid from *Escherichia coli*, and using it in fluorescent *in situ* hybridization experiments to identify chromosome 16 in metaphase cells.

*Escherichia coli* cells containing pHuR195 will be stored as frozen stocks at -80°C. Waste created from cell growth and DNA extraction protocols will be passed onto the hazardous waste disposal team on Thursday mornings.

THE UNIVERSITY OF WESTERN ONTARIO  
BIOHAZARDOUS AGENTS REGISTRY FORM  
Revised Biohazards Subcommittee: April, 2008  
Biosafety Website: [www.uwo.ca/humanresources/biosafety/](http://www.uwo.ca/humanresources/biosafety/)

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biohazardous agents are described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans. This form must also be updated at least every 3 years or when there are changes to the biohazards being used.

Containment Levels will be required in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Health Canada (HC) or Containment Standards for Veterinary Facilities, 1<sup>st</sup> edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety, OHS (Stevenson-Lawson Building, Room 295) for distribution to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135. If there are changes to the information on this form (excluding grant title and funding agencies), modifications must be submitted to Occupational Health and Safety. See website: [www.uwo.ca/humanresources/biosafety/](http://www.uwo.ca/humanresources/biosafety/)

PRINCIPAL INVESTIGATOR Peter K Rogan, PhD  
SIGNATURE [Signature]  
DEPARTMENT Biochemistry  
ADDRESS MSB 389  
PHONE NUMBER x 84255  
EMAIL progan@uwo.ca

Location of experimental work to be carried out: Building(s) MSB Room(s) 381, 365A

\*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to Occupational Health and Safety (See Section 12.0, Approvals). For research being done at Lawson Health Research Institute, London Regional Cancer Program, Child and Parent Research Institute, or Robarts Research Institute, a University Biosafety Committee member can also sign as the Safety Officer for the Institution.

FUNDING AGENCY/AGENCIES: Start-up funds, Ontario Genomic Institute  
GRANT TITLE(S): "Single copy technology for diagnostic & therapeutic applications"

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED.

Names of all personnel working under Principal Investigators supervision in this location:

John Macaki  
graduate student pending

## 1.0 Microorganisms

1.1 Does your work involve the use of microorganisms or biological agents of plant or animal origin (including but not limited to viruses, prions, parasites, bacteria)?  YES  NO  
 If no, please proceed to Section 2.0

1.2 Please complete the table below:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/Supplier	Health Canada or CFIA Containment Level
<i>E. coli</i> (For recomb. DNA)	<input type="radio"/> Yes <input checked="" type="radio"/> No	<input type="radio"/> Yes <input checked="" type="radio"/> No	<input type="radio"/> Yes <input checked="" type="radio"/> No	< 1 L	*TSD	<input checked="" type="radio"/> 1 <input type="radio"/> 2 <input type="radio"/> 3
	<input type="radio"/> Yes <input type="radio"/> No	<input type="radio"/> Yes <input type="radio"/> No	<input type="radio"/> Yes <input type="radio"/> No			<input type="radio"/> 1 <input type="radio"/> 2 <input type="radio"/> 3
	<input type="radio"/> Yes <input type="radio"/> No	<input type="radio"/> Yes <input type="radio"/> No	<input type="radio"/> Yes <input type="radio"/> No			<input type="radio"/> 1 <input type="radio"/> 2 <input type="radio"/> 3
	<input type="radio"/> Yes <input type="radio"/> No	<input type="radio"/> Yes <input type="radio"/> No	<input type="radio"/> Yes <input type="radio"/> No			<input type="radio"/> 1 <input type="radio"/> 2 <input type="radio"/> 3

\*Please attach a Material Safety Data Sheet or equivalent from the supplier.

## 2.0 Cell Culture

2.1 Does your work involve the use of cell cultures?  YES  NO  
 If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture in the table below

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	<input checked="" type="radio"/> Yes <input type="radio"/> No	lymphocyte, fibroblast, hepatocyte	Not applicable
Rodent	<input type="radio"/> Yes <input checked="" type="radio"/> No		
Non-human primate	<input checked="" type="radio"/> Yes <input checked="" type="radio"/> No		
Other (specify)	<input type="radio"/> Yes <input checked="" type="radio"/> No		

2.3 Please indicate the type of established cells that will be grown in culture in the table below.

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Supplier / Source
Human	<input checked="" type="radio"/> Yes <input type="radio"/> No	lymphoblastoid, HepG2, Calo2	ATCC, NIGMS
Rodent	<input checked="" type="radio"/> Yes <input type="radio"/> No		Established by PI at Penn State & Univ. Missouri
Non-human primate	<input checked="" type="radio"/> Yes <input type="radio"/> No	Cost, 293, CV1	" "
Other (specify)	<input type="radio"/> Yes <input checked="" type="radio"/> No		

\*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org) \* see attached examples from ATCC + NIGMS

2.4 For above named cell types(s) indicate HC or CFIA containment level required  1  2  3

### 3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials?  YES  NO  
 If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	Source/Supplier /Company Name	Is Human Source Material Known to Be Infected With An Infectious Agent? YES/NO	Name of Infectious Agent (If applicable)	HC or CFIA Containment Level (Select one)
Human Blood (whole) or other Body Fluid	study participant (520 cc)	<input type="radio"/> Yes <input checked="" type="radio"/> No		<input type="radio"/> 1 <input checked="" type="radio"/> 2 <input type="radio"/> 3
Human Blood (fraction) or other Body Fluid	—	<input type="radio"/> Yes <input type="radio"/> No		<input type="radio"/> 1 <input type="radio"/> 2 <input type="radio"/> 3
Human Organs or Tissues (unpreserved)	small fibroblast sample - study participant	<input type="radio"/> Yes <input checked="" type="radio"/> No		<input type="radio"/> 1 <input checked="" type="radio"/> 2 <input type="radio"/> 3
Human Organs or Tissues (preserved)	fixed lymphocytes tissue sections	<input type="radio"/> Yes <input checked="" type="radio"/> No		<input checked="" type="radio"/> 1 <input type="radio"/> 2 <input type="radio"/> 3

*preserved prior to coming to lab by London Health Sci. Centre, Ontario Tumor Bank, collaborating labs*

### 4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0?  YES  NO  
 If no, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done?  YES, complete table below  NO

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results
competent E. Coli cells	PCR 2.1 Topo pEAR2-pDEST26	cg Invitrogen	Human cDNA + genomic	copy amplification, protein expression

\* Please attach a Material Data Sheet or equivalent if available.

4.3 Will genetic modification(s) involving viral vectors be done?  YES, complete table below  NO

Virus Used for Transduction *	Vector(s) *	Source of Vector	Gene Transfected	Describe the change that results

\* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- ◆ HIV  YES, please specify \_\_\_\_\_  NO
- ◆ HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens  YES, specify \_\_\_\_\_  NO
- ◆ SV 40 Large T antigen  YES  NO
- ◆ E1A oncogene  YES  NO
- ◆ Known oncogenes  YES, please specify *presumed mutation*  NO
- ◆ Other human or animal pathogen and or their toxins  YES, please specify \_\_\_\_\_  NO

4.5 Will virus be replication defective?  YES  NO

4.6 Will virus be infectious to humans or animals?  YES  NO

4.7 Will this be expected to increase the containment level required?  YES  NO

*NOT applicable*  
|

## 5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted using the viral vector in 4.0?  YES  NO  
If no, please proceed to Section 6.0 If YES attach a full description of the make-up of the virus.

5.2 Will virus be able to replicate in the host?  YES  NO

5.3 How will the virus be administered? \_\_\_\_\_

5.4 Please give the Health Care Facility where the clinical trial will be conducted: \_\_\_\_\_

5.5 Has human ethics approval been obtained?  YES, number: \_\_\_\_\_  NO  PENDING

## 6.0 Animal Experiments

6.1 Will live animals be used?  YES  NO If no, please proceed to section 7.0

6.2 Name of animal species to be used \_\_\_\_\_

6.3 AUS protocol # \_\_\_\_\_

6.4 Will any of the agents listed be used in live animals  YES, specify: \_\_\_\_\_  NO

## 7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other body fluids including blood be used?

- ◆ Pound source dogs  YES  NO
- ◆ Pound source cats  YES  NO
- ◆ Cattle, sheep or goats  YES  NO
- ◆ Non- Human Primates  YES, please specify species \_\_\_\_\_  NO
- ◆ Wild caught animals  YES, please specify species & colony # \_\_\_\_\_  NO
- ◆ Birds  YES  NO
- ◆ Others (wild or domestic)  YES, please specify \_\_\_\_\_  NO

## 8.0 Biological Toxins

8.1 Will toxins of biological origin be used?  YES  NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s) \_\_\_\_\_  
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD<sub>50</sub> (specify species) of the toxin \_\_\_\_\_

## 9.0 Import Requirements

9.1 Will the agent be imported?  YES, please give country of origin \_\_\_\_\_  NO  
If no, please proceed to Section 10.0

9.2 Has an Import Permit been obtained from HC for human pathogens?  YES  NO

9.3 Has an import permit been obtained from CFIA for animal pathogens?  YES  NO

9.4 Has the import permit been sent to OHS?  YES, please provide permit # \_\_\_\_\_  NO

Level 2

**10.0 Training Requirements for Personnel Named on Form**

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- ◆ Biosafety
- ◆ Laboratory and Environmental/Waste Management Safety
- ◆ WHMIS
- ◆ Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE [Signature]

*- shared tissue culture with Knoll 365 A lab 9/1*

**11.0 Containment Levels**

11.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required.

*live cells*  
 1  2  3

11.2 Has the facility been certified by OHS for this level of containment?

- YES, permit # if on-campus \_\_\_\_\_
- NO
- NOT REQUIRED

*fixed preserved cells*  
*MSB 365A - cell culture (D. Litchfield Biochem)*  
*MSB 377 - Not yet certified - to be done*

**12.0 Procedures to be Followed**

12.1 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories. I will ensure that workers have an up-to-date Position Hazard Communication Form, found at <http://www.wph.uwo.ca/>

SIGNATURE [Signature] Date: 7-7-08

**13.0 Approvals**

UWO Biohazard Subcommittee: SIGNATURE: [Signature]  
Date: 9 Dec 2008

Safety Officer for Institution where experiments will take place: SIGNATURE: [Signature]  
Date: Dec 4/08

Safety Officer for University of Western Ontario (if different from above): SIGNATURE: \_\_\_\_\_  
Date: \_\_\_\_\_

Approval Number: BIO-UWO-0227 Expiry Date (3 years from Approval): Dec. 09, 2011

Special Conditions of Approval:

My laboratory develops and applies computational methods to interpret single nucleotide polymorphisms and to detect differences in the number of copies of sequences among different individuals. The objectives of my research are to: (a) create computer programs to predict mutations that predispose to disease and then evaluate them by gene and protein expression analysis, (b) to identify and validate genes whose expression responds to drug treatment, and (c) to design and tailor DNA probes to diagnose changes in copy number associated with specific genetic diseases and cancer.

This work utilizes human (and to a lesser extent non-human primate) primary and immortalized cell lines, peripheral blood and fibroblast samples, and suspensions and tissue sections that have been preserved prior to coming to the laboratory. Recombinant human genomic DNA and cDNA are routinely used in the laboratory, some of which are prepared in our own laboratory from nucleic acids extracted from the sources identified above. This entails introduction of plasmids, phage, cosmids and or bacterial artificial chromosomes containing human sequences into laboratory E.coli strains or transfection of common cell lines. Frequently, strains and cell lines containing these cloned constructs are obtained from suppliers such as The Center for Applied Genomics, NIGMS mutant cell repository, American Type Culture Collection or the European Collection of Cell Cultures. If the specific recombinant clone is not available, it will be introduced in to the appropriate vector depending on the length of the target.

The cell lines, peripheral blood and fibroblast samples are used for cell preparation and culture and nucleic acid or protein preparation. Stocks of cell lines and strains are stored in liquid nitrogen cell tanks or ultra low temperature freezers. They will be cultured or handled in a tissue culture facility (MSB 365A, managed by Dr. David Litchfield) prior to fixation and or extraction in the main laboratory (Rm 381, MSB). Lymphoblastoid cell lines were developed previously by the PI at other institutions (Penn State College of Medicine and University of Missouri School of Medicine) and through NIGMS or ATCC human genetic cell repository and occasionally, by collaborating researchers.

Peter K. Rogan Ph.D. 2008-07-07