Reference reagents for genetic testing: development of plasmid based mutation detection reagents by the UK National Genetics Reference Laboratory (Wessex)

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Background

- National Health Service genetic diagnostic laboratories in the UK perform thousands of mutation detection assays every month using diverse technologies
- Laboratories generally use locally sourced controls as standards to confirm that an assay is working correctly. Hence there is variation in the number and type of controls used in different laboratories which could potentially compromise quality assurance
- To address this problem the NGRL (Wessex) has generated 193 plasmid constructs that harbour defined sequence changes for every exon of hMLH1, MSH2, BRCA1 and BRCA2. These have been field trialled for performance evaluation using a wide range of mutation assays
- To facilitate the evaluation of high throughput mutation detection strategies we have also developed a generic set of 52 reference reagents which can be used to assess the sensitivity and specificity of new and existing mutation detection techniques. These have been field trialled for performance evaluation using a range of mutation assays
- To comply with the European IVD directive (98/79/EC), we are currently exploring the options available for distributing our plasmid based reference reagents as certified reference material (CRM) or CE marked products in conjunction with NGRL (Manchester), the National Institute for Biological Standards and Controls and EuroGentest.

Methodology

Blood collected from 8 individuals (consent obtained)

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DNA Extracted and Pooled

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Normal DNA sequences amplified by PCR

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PCR products cloned into plasmid vector

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Normal sequence verified against reference sequence

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Mutations introduced using site directed mutagenesis

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Mutant sequence verified

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Plasmid mixes produced (1 tube / gene)

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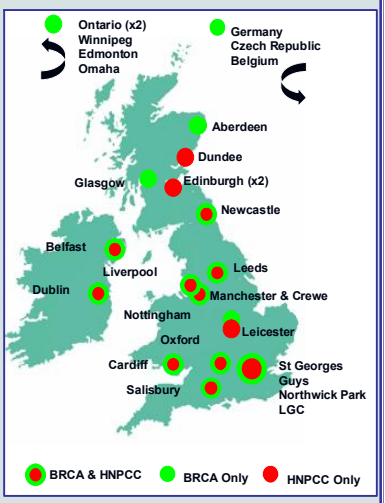
Constructs evaluated using a variety of mutation detection techniques

Plasmid Based Reagents for Mutation Screening of BRCA1, BRCA2, hMLH1 and hMSH2

- NGRL (Wessex) has produced sets of plasmid constructs that harbour defined sequence changes in all exons of BRCA1, BRCA2, hMLH1 and MSH2. These can be used as controls for a wide range of mutation scanning assays.
- Plasmid controls were distributed to 34 individuals in 26 labs (May 2003 – October 2005) for field trial.
- Reagents were analysed using six mutation scanning techniques: dHPLC, sequencing, SSCP/HD, CSCE, PTT and MALDI-TOF
- Data from the 20 field trial participants who returned questionnaires showed that:
  - 80% have used the controls in routine testing
  - 65% have used the reagents to develop new assays or validate existing screens
  - 30% have altered diagnostic protocols as a results of using the controls
  - 95% found plasmid DNA to be an acceptable alternative to genomic DNA
  - 100% thought that the reagents were a useful resource
  - 85% agreed that the reagents should be produced as reference material



Field Trial Participants Comments

" We didn't have control DNA for all exons of hMLH1 so especially useful for these to check that we could detect shift by dHPLC. Trainee is setting up CSCE for MLH1 and is including the plasmid controls in her workup" Dundee

"Plasmids work well when freshly diluted but working stocks do not last. Don't amplify as well as patient DNA regardless of amount used. On the whole a useful product and we would like to continue using them" Liverpool

"Found the controls a very useful resource during our BRCA and HNPCC test development. I find the DNA amplfies consistently in our lab and gives good clean sequence. I feel very confident about using these control plasmids as a reliable source of control DNA for the further development of our service along side family mutation controls" Belfast

Plasmid Based Generic Mutation Detection Reference Reagents

- To facilitate the evaluation of high throughput mutation detection strategies that are currently being introduced into UK Genetic Testing Network labs NGRL (Wessex) has developed a generic set of reference reagents which can be used to assess both new and existing mutation detection techniques.
- Plasmid controls have been produced which can be used to determine the sensitivity and specificity of these techniques by analysing factors that are of general importance for all technologies including: the type of base substitution, the GC content of the amplicon and the location of the mutation in the fragment.
- The controls can be used to amplify fragments ranging from 400-450bp with an average GC sequence content of 20%, 40%, 60% and 80%. The wild type sequence has been mutated to produce every possible heteroduplex (8 in total) at three positions within the amplicon (Figure 1)

Mutation created	Sequence generated	Heteroduplex produced
A > C	nnnCGnnn	C:T & G:A
A > T	nnnTGnnn	T:T & A:A
G > A	nnnAAnnn	A:C & G:T
G > C	nnnACnnn	C:C & G:G

Figure 1: Four wild type plasmids have been constructed which contain inserts with a 20%, 40%, 60% and 80% GC content. Each of these plasmids has been mutated at three positions within the amplicon (as shown above) to introduce the base changes listed in the table. When the mutated plasmids are mixed with the corresponding wild type plasmid the resulting 48 controls can be used to validate mutation detection techniques by analysing how effectively each of the possible heteroduplex configurations are detected at three different positions within amplicons of varying GC content.

- Amplicons from 52 plasmid reagents were produced and sent to 16 laboratories for performance evaluation.
- 5 mutation detection techniques were performed; sequencing, dHPLC, TGCE, CSCE and MALDI-TOF.
- In general, the reagents performed well in most laboratories and comments from participants suggest that the reagents are a useful resource for evaluating the sensitivity and specificity of laboratory mutation detection systems.
- Although this field trial was not designed to compare different mutation detection techniques or laboratory performance, it is notable that the sensitivity and specificity of mutation detection showed marked variation ranging from 75% - 100% and 68 – 100% respectively.
- Further work regarding the stability and final format of the reagents is ongoing as a prelude to establishing these reagents as reference materials.

For further information and copies of our field trial reports please visit our website:

www.ngrl.org.uk/Wessex