Haplotype-Fusion PCR

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Method Article

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Abstract

Introduction

Inversions frequently result from non allelic homologous recombination between long \(>10kb) inverted repeats, making them difficult to detect with long PCR or RFLP analysis. We have developed PCR-based single molecule haplotyping methods that enable both surveys for novel inversion variants, and population-scale genotyping of known inversions \(Turner _et al_ 2006). The inversion genotype of an individual can be deduced from their genomic DNA if single molecule haplotyping is performed on the DNA fragments that span the inversion breakpoints. Fusion PCR \(Yon and Fried 1989) allows sequences at either side of an inversion breakpoint to be joined together, and performing the reaction in an oil : water emulsion \(Ghadessy _et al_ 2001) ensures that the DNA templates are single molecules in the overwhelming majority of cases. In practice, the haplotyping method used depends on the length of the inverted repeat relative to the size of DNA fragments in the genomic DNA preparation. If the repeat is shorter than the template fragment lengths \(around 100kb), single copy sequences on either side of an inverted repeat can be joined and the inversion genotyped by generating fused products of different sizes for the two orientations. For longer repeats, paralog-specific nucleotides are analysed in a magnetic bead-based version of the assay.

Reagents

Reagents: Genomic DNA samples 10X Phusion HF buffer \(Finnzymes) 25mM $MgCl_2$ 2.5mM dNTPs 10µM primers \(outer forward and reverse) 1µM fusion primer Phusion DNA polymerase \(Finnzymes) Span 80 \(Sigma) Tween 80 \(Sigma) Triton X-100 \(Sigma) Light mineral oil \(Sigma) Hexane \(Aldrich) agarose

Equipment

2ml Cryo-Vial \(Corning) Magnetic stirring bar \(8 x 3mm with a pivot ring; VWR International) Magnetic stirrer MJ Research thermocycler Agarose gel electrophoresis and gel visualisation equipment

Procedure

1. Prepare PCR reactions \("aqueous phase") on ice in a total volume of 100µl using 1X Phusion HF buffer, an additional 1mM $MgCl_2$, 250 µM dNTPs, 1µM outside primers and 10nM fusion PCR primer, 200 ng genomic DNA and 16 units Phusion polymerase. 2. Put 200 µl oil phase \(4.5% v/v Span 80, 0.4% v/v Tween 80 and 0.05% Triton X-100 dissolved in light mineral oil) in a 2 ml Cryo-Vial and stirring at 1000 rpm with a magnetic bar \(8 x 3mm with a pivot ring). 3. Add 1 drop of aqueous phase to oil phase every 5 seconds and continue stirring for 5 minutes after addition of the first drop. 4. Transfer 125 µl of emulsion to a clean 200 µl, thin wall PCR tube and overlay with 1 drop of mineral oil. Run standard Phusion polymerase PCR cycle. 5. Transfer completed PCR reactions to a 1.5 ml tube and add 400 µl

hexane. Vortex thoroughly and centrifuge at full speed for 3 minutes. Remove and discard hexane layer \(upper layer). Repeat with 400 µl fresh hexane. PAUSE POINT: reactions can be stored at -20 ^{o}C for several days. 6. Dilute PCR products 100x with water and reamplify in solution omitting the fusion primer. 7. Separate PCR products using gel electrophoresis.

Timing

6-7 hours \(including reamplification and gel)

Critical Steps

All steps are critical to success of the procedure unless otherwise stated in the 'Troubleshooting' section. The efficiency of the reaction is maximised by fusing short amplicons \(<= 150 bp each).

Troubleshooting

The given protocol is used when the inverted repeats that give rise to the inversion are short \(<100 kb). For longer repeats it is necessary to quantify the fusion PCR products and to use bead-based PCR in emulsion followed by single base extension and microscopy. This increases the length of time for the procedure considerably. 1. The use of a polymerase that does not leave an A-tail is unnecessary for the technique to work but not doing so can affect subsequent steps. 2i. Preparing larger volumes of oil phase \(e.g. 20 ml) can help to avoid pipetting inconsistencies caused by the high viscosity of the components. 2ii. Stirring speed is just estimated from looking at the dial. 3. Addition of occasional drops sooner or later than after 5 seconds does not seem to affect the stability of the emulsion significantly. 4. It is probably not necessary to overlay reactions with mineral oil. No special cycling conditions are required. 5. Following cycling some separation of the emulsion usually occurs. The degree of separation is affected by precise stirring speed and the rate of aqueous phase addition. It is important to perform the hexane cleanup on the thick, opaque emulsion and to leave any clear liquid at the bottom of the tube untouched. The mineral oil on top of the emulsion can be included in the cleanup. It is probably fine to perform a single hexane step. Ethoxy ethane can be used instead of hexane, and is more efficient at disrupting emulsions but presents more of a potential hazard. 6. Because the efficiency of performing a single step fusion PCR in an emulsion is low, PCR products will probably not be visible in a gel even after ethanol precipitation and running of the entire reaction, so reamplification with just the outer primers is necessary to increase the quantity of fused product. Dilution of fused PCR products gives cleaner products after reamplification. Reamplification can be performed using a standard polymerase.

Anticipated Results

An assay designed to distinguish between wild-type and pathogenic orientations of the haemophilia-causing X-chromosome Factor VIII gene inversion can produce two differently sized fusion products, indicating the different orientations. In female carriers of the inversion both products are obtained

whereas a single product is obtained from female wild-type and male individuals. When the PCR is performed in solution rather than emulsion, both fusion products are obtained from all individuals. Using this assay we genotyped blind a mixed population of ten males with and without the int22h inversion with 100% concordance with the known inversion genotypes.

References

Ghadessy, F. J., Ong, J. L. & Holliger, P. Directed evolution of polymerase function by compartmentalized self-replication. _Proc Natl Acad Sci U S A_ **98**, 4552-7 \(2001). Turner, D. J., Shendure, J., Porreca, G., Church, G., Green, P., Tyler-Smith, C. & Hurles, M. E. Assaying chrommosomal inversions by single molecule haplotyping. _Nat. Methods_ **3**, 439-445 \(2006). Wetmur, J. G. et al. Molecular haplotyping by linking emulsion PCR: analysis of paraoxonase 1 haplotypes and phenotypes. _Nucleic Acids Res_ **33**, 2615-9 \(2005). Yon, J. & Fried, M. Precise gene fusion by PCR. _Nucleic Acids Res_ **17**, 4895 \(1989).

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Figures

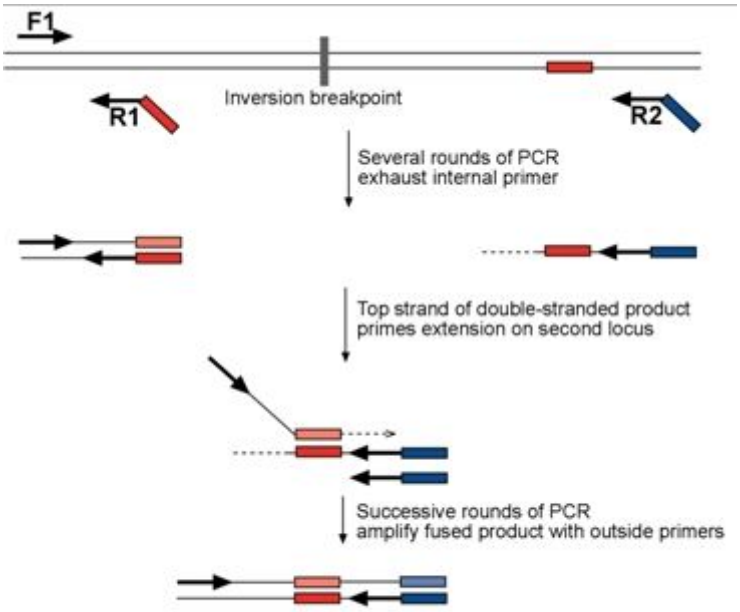

Figure 1

Fusion PCR. When Primer F1 extends over the tail of Primer R1 it generates a 3' end that can itself act as a forward primer on the second locus. In this way sequences at either side of the breakpoint can be

joined.

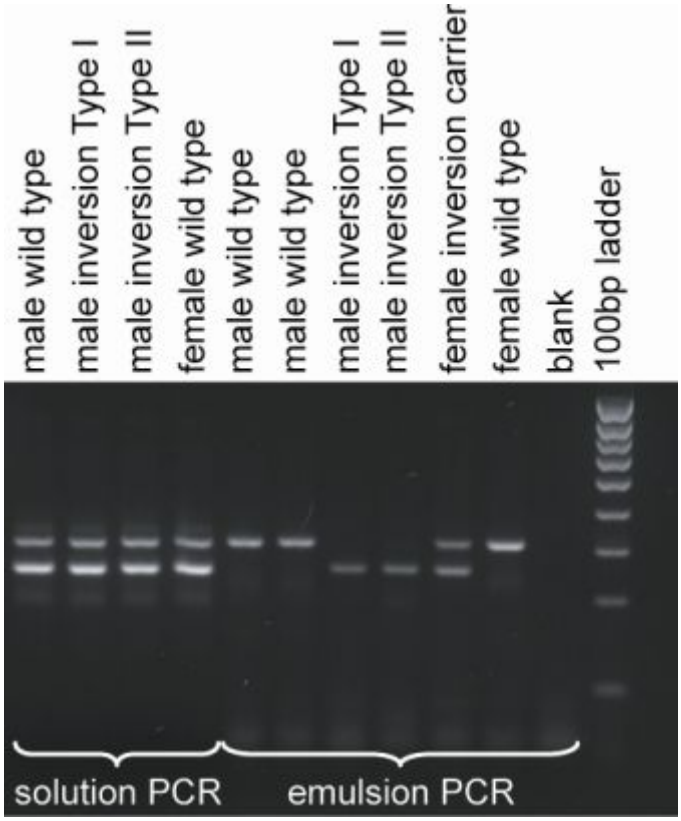

Figure 2

Genotyping the int22h haemophilia inversion. When the fusion PCR reactions are performed in solution under standard PCR conditions both fusion products are generated in all individuals. However, when the fusion PCR is performed in emulsions, products from different templates can no longer fuse and only the fusion products expected for that specific inversion genotype are generated. Thus the assay distinguishes between males with and without the inversion, female carriers of the inversion, and females homozygous for the wildtype orientation.