Protocol for genome-wide analysis of palindrome formation

Scott Diede
Fred Hutchinson Cancer Research Center
Stephen Tapscott
Fred Hutchinson Cancer Research Center

Method Article

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Abstract

Earlier work from our laboratory focused on identifying regions of the genome susceptible to DNA palindrome formation, a rate-limiting step in gene amplification. We described a method to obtain a genome-wide analysis of palindrome formation \(GAPF) based on the efficient intrastrand base pairing in large palindromic sequences[1]. Palindromic sequences can rapidly anneal intramolecularly to form 'snap-back' DNA under conditions that do not favor intermolecular annealing. This snap-back property is used to enrich for palindromic sequences in total genomic DNA by denaturing the DNA at 100˚C, rapidly renaturing it by cooling, and then digesting the mixture with the single-strand specific nuclease S1. Snap-back DNA formed from palindromes is double-stranded and resistant to S1, whereas the remainder of genomic DNA is single-stranded and thus is sensitive to S1 digestion. Using GAPF, we have shown that de novo palindromes can form in cancers, and direct molecular analysis validated that a subset of these GAPF-positive signals represent cancer-specific palindromes located at the boundary of gene amplicons[1,2]. We recently discovered that the original GAPF protocol also enriches for differentially methylated DNA[3]. We now describe a modification of GAPF to increase the stringency of denaturation using 50% formamide to make the assay specific for DNA palindromes.

Reagents

KpnI SbfI Formamide 3M NaCl \(Invitrogen; included in S1 nuclease kit) S1 nuclease \(Invitrogen) ChargeSwitch PCR Clean-Up Kit \(Invitrogen) MseI MspI T4 DNA Ligase \(NEB) Amicon Ultra-0.5, Ultracel-50 Membrane, 50 kDa \(Millipore) Amicon Ultra-0.5, Ultracel-30 Membrane, 30 kDa \(Millipore) 1/10 TE \(1 mM Tris, 0.1 mM EDTA, pH 8.0) FastStart Taq DNA polymerase \(Roche) 2 mM dNTPs for PCR DNase I \(NEB) GeneChip WT Double-stranded Target Labeling Kit \(Affymetrix) Tiling Array \(Affymetrix) **Primers** JW-102g GCGGTGACCCGGGAGATCTGAATTG JW103pcTA \[Phos]TACAATTCAGATCTCCCG JW103pc-2 \[Phos]CGCAATTCAGATCTCCCG JW-102gMse GCGGTGACCCGGGAGATCTGAATTGTAA JW-102gMsp GCGGTGACCCGGGAGATCTGAATTGCGG **Linker annealing** MseI linker: JW-102g is annealed to JW103pcTA. MspI linker: JW-102g is annealed to JW103pc-2 Mix 100 µl of 100 pmol/µl of each appropriate primer Add 6.9 µl 3M NaCl and mix Place in boiling water bath for 7 minutes Turn off heat, let sit on hot plate >2 hours to cool Add 20 µl 3M NaOAc, pH 5.2 Add 400 µl 100% cold EtOH Mix and place at -20˚C for >2 hours Spin at 4˚C for 20 minutes - discard supernatant Wash with 0.7 ml 70% cold EtOH Spin at 4˚C for 20 minutes - discard supernatant - SpeedVac without heat to dryness Dissolve DNA in 500 µl water

Equipment

Microcentrifuge PCR thermocycler MagnaRack magnetic rack \(Invitrogen) Heat plate Nanodrop \(or other UV spec) _Optional_: Bioanalyzer \(Agilent)

Procedure

Restriction digest 1. A total of 2 micrograms of genomic DNA is used as input into the GAPF assay. 2. Split sample evenly into two 0.2 ml thin-walled PCR tubes. 3. Using manufacturer's supplied buffers, digest 1 µg of genomic DNA with KpnI \(10 U) in final volume of 20 µl. In the other tube, digest 1 µg with SbfI \(10 U) in a final volume of 20 µl. Incubate at 37˚C for 6 hours. 4. Heat inactivate restriction enzymes by incubating samples at 65˚C for 20 minutes. _May freeze sample \(-20˚C) at this point_ **Snap-back** 1. From above step, combine restriction digestions into one tube \(final volume = 40 µl). 2. Add 3 µl of 3M NaCl, 45 µl formamide, and 2 µl water to tube. _May make master mix if performing multiple reactions._ 3. Place in thermocycler preheated to 100˚C. Incubate for 7 minutes to denature DNA. 4. Remove tube from thermocycler and immediately place in ice-water bath. Incubate for 7 minutes. **S1 digest** 1. To sample from previous step, add 8 µl 3M NaCl, 12 µl 10x S1 nuclease buffer, 8 µl water and 1 µl S1 nuclease \(200 U/µl). _May make master mix if performing multiple reactions._ **Note:** Dilute stock of S1 nuclease \(~1000 U/µl) in Dilution buffer to obtain 200 U/µl. 2. Incubate at 37˚C for 60 minutes. **DNA clean-up** 1. For most consistent results, use ChargeSwitch PCR Clean-Up Kit \(Invitrogen) to purify DNA. _Alternatively, one may purify DNA using phenol:chloroform extraction followed by ethanol precipitation \(use glycogen as carrier). Resuspend DNA pellet in 84 µl 1/10 TE._ 2. Transfer S1 digested DNA to 1.7 ml microfuge tube. 3. Add 120 µl Purification Buffer \(N5) and mix. 4. Add 10 µl Magnetic Beads and mix by pipetting up and down gently. 5. Incubate 1 minute. 6. Place sample on MagnaRack for 1 minute to pellet beads. 7. Without removing tube from MagnaRack, remove and discard supernatant. 8. Remove tube from MagnaRack. 9. Add 150 µl of Wash Buffer. 10. Pipet up and down gently to mix. 11. Place sample on MagnaRack for 1 minute to pellet beads. 12. Without removing tube from MagnaRack, remove and discard supernatant. 13. Remove tube from MagnaRack. 14. Add 150 µl of Wash Buffer. 15. Pipet up and down gently to mix. 16. Place sample on MagnaRack for 1 minute to pellet beads. 17. Without removing tube from MagnaRack, remove and discard supernatant. 18. Remove tube from MagnaRack. 19. Add 84 µl of Elution Buffer \(E5). 20. Pipet up and down gently to mix. 21. Incubate 1 minute. 22. Place sample on MagnaRack for 1 minute to pellet beads. 23. Without removing tube from MagnaRack, remove and save sample. Discard beads. A total of 80 µl will be used for next step in protocol. The leftover 4 µl can be used for PCR-based enrichment assays for quality control or troubleshooting. _May freeze sample \(-20˚C) at this point_ **Ligation-mediated PCR** _Restriction digestion_ 1. Using manufacturer's supplied buffers, digest 40 µl of purified DNA from previous step with MseI \(10 U) in final volume of 50 µl, and digest the other 40 µl of purified DNA with MspI \(40 U) in final volume of 50 µl. 2. Incubate for 37˚C for 2 hours. 3. Heat inactivate MseI digest by incubating at 65˚C for 20 minutes; MspI digest requires incubation at 80˚C for 20 minutes for inactivation. _May freeze sample \(-20˚C) at this point_ _Linker ligation_ 1. Add the following to the 50 µl restriction digest from previous step: 5 µl of appropriate linker \(MseI or MspI linker), 7 µl 10x T4 DNA Ligase buffer, 7 µl water, and 1 µl T4 DNA ligase \(400 U/µl). 2. Incubate at 16˚C for 3 hours. 3. Heat inactivate ligase by incubating at 70˚C for 20 minutes. _Remove unligated linkers_ 1. To Amicon Ultracel-50 \(50 kDa cutoff) Membrane 0.5 mL column, add 160 µl 1/10 TE and entire ligation mix \(70 µl of either MseI or MspI ligation) from previous step. 2. Spin 14,000 x g for 5-10 minutes until volume concentrated to ~20 µl. 3. Recover DNA by placing inverted column into new tube and spinning at 1,000 x g for 2 minutes. _PCR amplification_ Set up the following PCR reaction: 4 µl template DNA \(from above) 10 µl 10x PCR Buffer 10 µl 2 mM dNTPs 12 µl

Linker specific primer \(10 pmol/µl; use either JW-102gMse or JW-102gMsp) 0.8 µl FastStart Taq \(Roche) 20 µl 5x GC-rich solution 43.2 µl water _Cycling conditions_ 96˚C 6 minutes Then 30 cycles of: 96˚C 30 seconds 55˚C 30 seconds 72˚C 30 seconds Then 72˚C 7 minutes 4˚C Hold Run 1 µl of 100 µl PCR sample on agarose gel for quality control \(Average size range of Mse PCR $_{300\text{-}800\ bp;\ Msp\ PCR}$ 200-600 bp). _PCR clean-up_ 1. To Amicon Ultracel-30 \(30 kDa cutoff) Membrane 0.5 mL column, add 300 µl 1/10 TE and combined MseI and MspI PCR samples \(~198 µl) from previous step. 2. Spin 14,000 x g for 20-30 minutes until volume concentrated to ~20 µl. 3. Recover DNA by placing inverted column into new tube and spinning at 1,000 x g for 2 minutes. 4. Quantitate DNA by Nanodrop. Should get a little greater than 7.5 µg in total. _May freeze sample \(-20˚C) at this point_ _**The following protocol is for labeling DNA for the Affymetrix Tiling Array platform. If a different platform is desired, please modify protocol from this point forward as needed.**_ **DNA fragmentation** Perform in 0.2 mL PCR tube in a thermocycler: 42.2 µl DNA \(7.5 µg from above in water to 42.2 µl final volume) 4.8 µl 10x DNase I buffer 1 µl Diluted DNase I \(0.0167 U; NEB) _\(Dilute 2 U/µl stock => 1 µl stock + 107 µl water + 12 µl 10x DNase I buffer)_ 37°C for 35 minutes 95°C for 15 minutes 4˚C hold _3 µl of DNA may be used on Bioanalyzer \(Agilent) to measure DNA fragmentation profile for quality control purposes. Results of the Bioanalyzer analysis of fragmented DNA should show that the majority of fragmented DNA is between 25 to 200 bases, with the peak of the distribution between 25 to 100 bases._ **DNA labeling** Perform in 0.2 mL PCR tube in a thermocycler; Affy GeneChip WT Double-stranded Target Labeling Kit) 45 µl Fragmented DNA \(from above) 12 µl 5x TdT buffer 2 µl TdT 1 µl DNA labeling reagent 37°C for 1 hour 70°C for 10 minutes 4˚C hold Sample now ready for hybridization to Tiling array and processing per Affymetrix protocol

Timing

2-3 days.

References

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