

Detection of the Pro12Ala PPAR-g2 Allele by PCR-RFLP Analysis

Genomic DNA will serve as template for polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis (PCR-RFLP), as designed and previously described by our group. Briefly, a 270 bp fragment of the hPPAR-g2 gene encompassing the site of the polymorphism will be generated using a mutagenic downstream primer that introduces a BstU-I restriction site only when the variant Ala12 allele (a C>G substitution at nucleotide 34) is present. The digested PCR products will be visualized by agarose gel electrophoreses with ethidium bromide staining. Easily distinguished bands are expected at sizes of 270 bp for normal Pro12 homozygotes (P/P), 227 bp and 43 bp for Ala12 homozygotes (A/A), and 270 bp, 227 bp and 43 bp for heterozygotes (P/A).

Pro12Ala PPARγ2 PCR-RFLP with BstU-1 Digest

“**gamma 2 - up**” = upstream PCR primer for RFLP –5’- GCCAATTCAAGCCCAGTC –3’

“**X05c**” = mutagenic downstream PCR-RFLP primer

5’-GATATGTTTGCAGACAGTGTATCAGTGAAGGAATCGCTTTCCG-3’,

The second “RFLP” downstream primer “**X05c**” introduces a BstU-I restriction site (...CG||CG...) only if the C>G substitution is present (wild = GAC CCA GAA >> P12A variant = GAC GCA GAA >> double mutant GAC G||CG GAA) (the especially long primer aides in genotyping by agarose gel)

PCR-RFLP Assay to detect Pro12Ala PPAR-γ2 variant

For 20 uL PCR reaction:

For 20 uL PCR reaction	for 1 rxn	10 rxn	100 rxn	30 rxn
PCR Buffer (@ 10X)	2 uL	20	200	60
dNTP (@ 2.5 mM each X 4)	0.6 uL	6	60	18
primer X05c (@ 5uM = 5pmol/ul)	1uL	10	100	30
primer γ2 up (@ 5uM = 5pmol/ul)	1 uL	10	100	30
autoclaved ddH2O	14.3 uL	143	1,430	429

mix the above - may freeze

add taq on ice immediately before use:	0.1 uL	1	100	30
per each well use above mix + taq:	19uL	19	19	19
per each well use genomic DNA	1uL	1	1	1
per each well add “clean” mineral oil	1 drop	1	1	1

*****NEVER bring any PCR products or plasmids into clean room - if using as + control - add it under the oil in the dirty room just before going into machine**

***** keep gDNA always in clean room**

***** Remember that taq is a very fragile (and expensive) enzyme - keep frozen or on ice at all times - keep mixes containing it on ice until put into machine**

***** Each plate should have 1 well with (-) control with no DNA at all, just master mix plus taq**

***** Each plate should have 1 well with (+) control = plasmid or PCR product with known “22”**

Run on thermalcycler @:

94d x 5m

(55d x 30s - 72d x 30s - 94d x 30s) x 35 cycle

55d x 2m

72d x 7 m

4d x hours or days

- We use 1.5mM Mg (though we didn't play with it much – it may work with other concentrations)

-We use cycling temps of 55-72-94, with 30 sec for anneal and extension (here we have played around to accommodate other reactions in the same machine – it is a pretty generous reaction and tolerates just about any temps and times we have used)

BstU1 digest: PCR-RFLP Assay to detect Pro12Ala PPAR- γ 2 variant

For 25 uL digest (20 uL PCR reaction)	for 1 rxn	10 rxn	100 rxn	30 rxn
NEB2 buffer (@ 10X)	2.5 uL	25	250	75
BstU-1 enzyme	0.3 uL	3	30	9
autoclaved ddH ₂ O	2.2 uL	22	220	66

**add 5 uL of above mix to each well
digest at 60 degrees overnight
add running dye
run on 2.5% agarose gel with EtBr**

No need to remove oil or extract PCR product - when we do large scale genotyping it is all done in the same plate (1st load gDNA, then PCR mix with taq, then oil, then positive control in “dirty” room - PCR - add RE buffer and RE under oil - digest - add dye - load gel)

-The expected products after digestion with BstU-I are:
271 bp for the indigestible homozygous wild-type;
271/ 230/ 41 for heterozygous;
230bp/ 41bp for homozygous variant.
*** note that the 41 bp fragment is generally NOT seen

Reference:

Yen C-J, Beamer B, Negri C, Silver K, Brown K, Yarnell D, Burns D, Roth J, Shuldiner A, Molecular Scanning of the Human Peroxisome Proliferator Activated Receptor γ (hPPAR γ) Gene in Diabetic Caucasians: Identification of a Pro12Ala PPAR γ 2 Missense Mutation, Biochemical and Biophysical Research Communications **241**, 270–274 (1997)