Xpert directXtract PCR Kit

mouse genotyping

Objective: Test performance of the GE60 Xpert directXtract PCR Kit

Biological Material: Mouse Ear punches

primers: (A1/A2 (Ta=62ºC) + B1/B2/B3/B4 (Ta=60ºC) + C1/C2/C3/C4 (Ta=62ºC). Concentration is 10µM (10pmol/µl)

Genotypes:
A: ~200bp and ~1200bp (approx)
B: ~ 600bp and ~ 750bp (approx)
C: ~ 350bp and ~500bp (approx)
[sizes are only indicative]

amplify samples 22,23,24 with A1/A2 e B1/B2/B3/B4 and samples 5790, 5791 with primers C1/C2/C3/C4

a) Extraction (conform protocol)

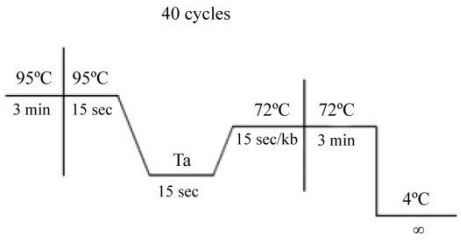
In total 5 samples were supplied (22,23,24, 5790, and 5791).
Hence, prepare pre-mix for 5 samples as follows: 350µl PCR-grade water, 100µl Xpert directXtract buffer A, and 50µl Xpert directXtract buffer B. Cover each sample with 100µl of reaction mixture and incubate at 75ºC for 5 minutes (*lysis*) followed by incubation at 95ºC for 10 minutes (*heat-inactivation*). Centrifuge at max for 1 minute, and transfer the cleared supernatant in a new DNase-free, and store at -20ºC or proceed to PCR immediately. **>total time = 0:15**

b) Optimization of the dilution of DNA extract

The protocol recommends to dilute the extracted DNA (10-fold). *This step not only dilutes DNA but also PCR inhibitors !* Since these particular samples are very, very small, first determine optimal dilution for one of the samples (e.g. nº 22, with primers A1A2), as perhaps 10x is too much. Once optimized for sample 22, presume it will be very similar for the others as size of the ear punches is very similar for all samples.
Prepare a premix for 8 PCR reactions according to the table. (6 dilutions [see Fig 1] plus a negative control) and divide into 7x 24µl PCR tubes.

Component	Volume (25 µl)	Mix (200µl)	Final Conc.
Xpert Fast Hotstart Mastermix (2X) with dye	12.5 µl	100µl	1X
A1 primer (10 pmol/µl)	1 µl	8µl	0.4 µM
A2 primer (10 pmol/µl)	1 µl	8µl	0.4 µM
Diluted DNA extract (1x|2x|5x|10x|20x|100x)	1 µl	8µl	conform sample
PCR–grade water	9.5 µl	76µl	

Add 1µl of diluted target DNA and Set-up initial PCR amplification, on a thermal cycler (*with ~2ºC/s ramp rate*), as follows:



>use Ta=62ºC
>As size could be 1200bp (max), set elongation time above 15 sec (>>25 sec).
>total run time = 1:06

c) Electrophoresis

Analyze 10µl of PCR products by 2% DNA Agarose gel electrophoresis using SGTB as electrophoresis buffer. Samples can be loaded directly onto an agarose gel without the need of adding a loading buffer. Expected amplicon sizes are 200bp and/or 1200bp depending on the genotype. Use Midori Green as DNA stain and GRS Ladder 100bp for size determination. Run 15 minutes at 250V (minigel). >**total time: 0:15**

d) Results

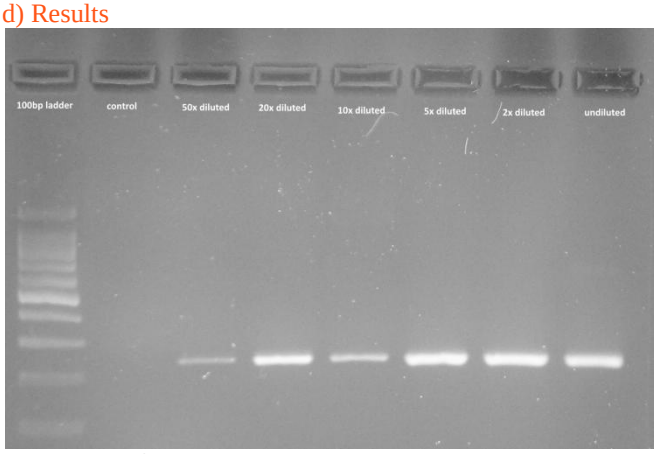


Fig 1. Adapted from IMPG0702

All dilutions, including 50x diluted, amplified perfectly, whereas negative control did not.
Strong bands, no non-specific bands, size as expected (genotype of sample 22 is homozygote: only 200bp band). **All ok !** Afterall, there was no need to optimize, 10x dilution is just fine, just as undiluted and 20x.
Continue genotyping of samples 22,23 and 24 using primers A1 and A2, using a 10x dilution, conform protocol, as indicated in the standard protocol. No further changes.

e) PCR using primers A1 and A2

Amplify samples 22,23, and 24 (use 10-fold dilution) according to above.

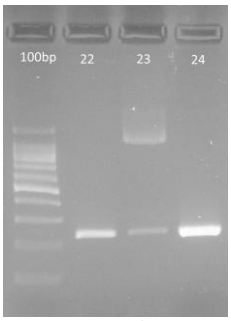


Fig 2. Adapted from IMPG0703

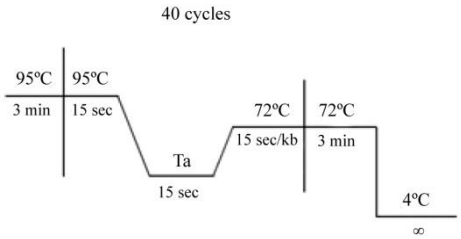
Samples 22 and 24 are homozygotes (200bp band) and samples 23 is heterozygote (1200bp/200bp).
Results are 100% ok.

f) PCR using primers B1, B2, B3 and B4

Since all samples (22,23,24) amplified well with A1 and A2, continue genotyping of the same samples with primers B1, B2, B3 and B4, using the same 10x dilution as before. Include samples 5790 and 5791. Expected amplicon sizes are 600bp and 750bp, so extension time can be lower than 15 sec (>>12 sec). Prepare a premix for 8 PCR reactions (excess) according to the table. (5 samples plus 1 negative control and divide into 7x 24µl PCR tubes.

Component	Volume (25 µl)	Mix (200µl)	Final Conc.
Xpert Fast Hotstart Mastermix (2X) with dye	12.5 µl	100µl	1X
B1 primer (10 pmol/µl)	1 µl	8µl	0.4 µM
B2 primer (10 pmol/µl)	1 µl	8µl	0.4 µM
B3 primer (10 pmol/µl)	1 µl	8µl	0.4 µM
B4 primer (10 pmol/µl)	1 µl	8µl	0.4 µM
Diluted DNA extract (10x)	1 µl	8µl	conform sample
PCR–grade water	7.5 µl	60µl	

Add 1µl of diluted target DNA and Set-up initial PCR amplification as follows:



>use Ta=60ºC
>As size could be 750bp (max), set elongation time below 15 sec (>>12 sec).
>total run time = 1:01

g) Electrophoresis

Analyze 10µl of PCR products by 2% DNA Agarose gel electrophoresis using SGTB as electrophoresis buffer. Samples can be loaded directly onto an agarose gel without the need of adding a loading buffer. Expected amplicon sizes are 750bp and/or 600bp depending on the genotype. Use Midori Green as DNA stain and GRS Ladder 100bp for size determination. Run 15 minutes at 250V (minigel). >**total time: 0:15**

h) Results

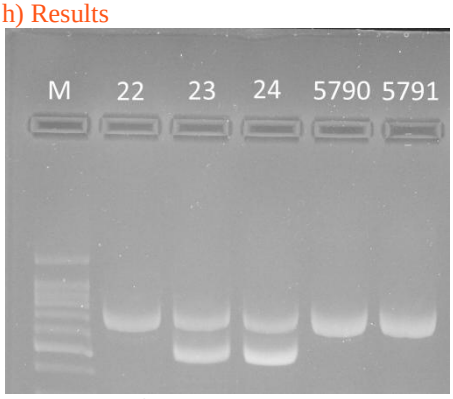


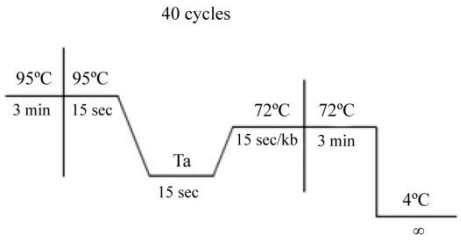
Fig 3. Adapted from IMPG0704
Strong bands ! Samples 22, 5790 and 5791 are homozygotes (750bp band) and samples 23 and 24 heterozygotes (750bp/600bp).

i) PCR using primers C1, C2, C3 and C4

Since all samples (22,23,24, 5790, and 5791) amplified well with B1, B2, B3 and B4, continue genotyping of the same samples with primers C1, C2, C3 and C4, using the same 10x dilution as before. Expected amplicon sizes are 500bp and 350bp, so extension time can be lower than 15 sec (>>10 sec). Prepare a premix for 8 PCR reactions according to the table. (5 samples plus 1 negative control and divide into 7x 24µl PCR tubes.

Component	Volume (25 µl)	Mix (200µl)	Final Conc.
Xpert Fast Hotstart Mastermix (2X) with dye	12.5 µl	100µl	1X
C1 primer (10 pmol/µl)	1 µl	8µl	0.4 µM
C2 primer (10 pmol/µl)	1 µl	8µl	0.4 µM
C3 primer (10 pmol/µl)	1 µl	8µl	0.4 µM
C4 primer (10 pmol/µl)	1 µl	8µl	0.4 µM
Diluted DNA extract (10x)	1 µl	8µl	conform sample
PCR–grade water	7.5 µl	60µl	

Add 1µl of diluted target DNA and Set-up initial PCR amplification as follows:



>use Ta=62ºC
>As size could be 500bp (max), set elongation time below 15 sec (>>10 sec).
>total run time = 1:01

j) Electrophoresis

Analyze 10µl of PCR products by 2% DNA Agarose gel electrophoresis using SGTB as electrophoresis buffer. Samples can be loaded directly onto an agarose gel without the need of adding a loading buffer. Expected amplicon sizes are 500bp and/or 350bp depending on the genotype. Use Midori Green as DNA stain and **GRS Ladder 50bp** for size determination. Run 15 minutes at 250V (minigel).
>total time: 0:15

k) Results

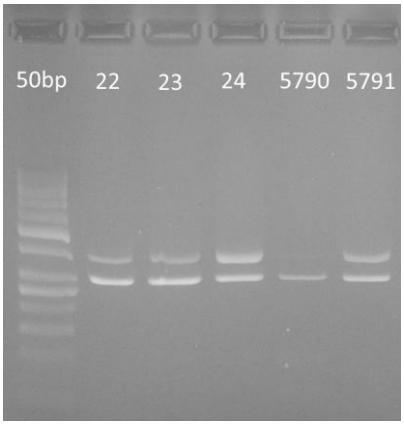


Fig . Adapted from IMPG0704
Strong bands ! Samples 22, 23, 24, and 5791 are heterozygotes (350bp band) and sample 5791 is homozygote (500).

l) Conclusion(s)

In all cases, as confirmed with supplier of biological material, results were as expected. Depending on the sizes of the expected amplicons, genotyping could be carried out in approximately 1h30:
15 min for extraction, 1 hour for PCR, and 15 minutes for electrophoresis.