

Rapid DNA Extraction, Amplification, and Detection System Effective for Mouse Genotyping

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Abstract

Most mouse genotyping protocols are tedious and time consuming, starting with an overnight digestion and DNA purification, and ending with gel detection. Demonstrated here is a simple, coupled system for rapid extraction, amplification and quantification of genomic DNA from mouse tissue. This can be used in genotyping studies that do not require DNA purification or gel analysis. This system, Extract-N-Amp™, uses a novel extraction treatment that releases sufficient quantities of DNA from tissue (for example, a mouse tail snip) in 10 minutes at room temperature for quantitative PCR analysis. After a 3-minute incubation at 95 °C and the addition of a solution that neutralizes substances inhibitory to PCR, extracted DNA is directly added to a PCR ReadyMix™ that is specially formulated to accommodate the extract for SYBR® Green or fluorescent probe-based real-time qPCR assays. Results show that single copy gene dosage resolution is possible. This single-copy resolution will be demonstrated in two different systems. In the first system, the Extract-N-Amp procedure is used to distinguish between one and two copies of an allele, and in the second, to differentiate extracts from mice that are wildtype, heterozygous, and homozygous for an IL2 gene disruption. Either SYBR Green or dual-labeled probes may be used for detection during real-time PCR. The results with these two systems demonstrate that extensive tissue digestion and DNA purification are not necessary, and that high-resolution genotyping is possible using a simple, rapid tissue extraction and real-time qPCR with Extract-N-Amp.

Materials

Unless otherwise indicated, all reagents and materials used in this work were obtained from Sigma-Aldrich (St. Louis, MO). The PCR primers and dual-labeled probes were obtained from Sigma-Genosys (Woodlands, TX). Extract-N-Amp™ Tissue PCR Kit (Cat. No. XNATG or XNATR) was used for genomic DNA isolation from mouse tissues and PCR setup. GenElute™ Mammalian Genomic DNA Miniprep Kit (Cat. No. G1N10) was used for traditional genomic DNA isolation from mouse tails for positive controls and efficiency studies. Reference Dye (Cat. No. R4526) was used for quantitative PCR.

Whole mice and mouse tails were obtained cryogenically preserved from either Pel-Freez (Rogers, AR) or Jackson Laboratories (Bar Harbor, ME).

Primer Sequence

SYBR Green	Forward (400 nM)	Reverse (400 nM)	TaqMan	Forward (500 nM)	Reverse (500 nM)	Probes (250 nM)
Phex	5'-TGATTTCCCTTGAATCC	5'-CCAAGTGGCTTTAAACCAT	Diap2	5'-GCTGCGTGGCTTTGTC	5'-CCCAGACTCCCAAGAATGTTTC	5'-HEX - CCCACCCCATGTGCTCAC - BH1
Cox1	5'-ATTACAGCCGCTACTGCTCTAT	5'-CCCAAGAATCAGAAACAGATGC	Phactr1	5'-CCCCAAGACTAGGGTCACTCA	5'-GCCAAGTGGCAGAGTGAATCT	5'-FAM - CCTCTTCAGAGCACTGCCCTCC - BH1
IL2 (SYBR)	5'-CTAGGCCACAGAATTGAAAGATCT	5'-GTAGGTGGAAATCTAGCATCATCC	IL2 (TaqMan)	5'-CTGAGCATCTCCAATTGAAAGC	5'-AGGCCACAGAATTGAAAGATCTTC	5'-HEX - CAAATCCAGAACATGCCGAGAGTCC - BH1
Neomycin (SYBR)	5'-TCGAATTCGCCAATGACAGAGCGCT	5'-GTAGGTGGAAATCTAGCATCATCC	Neomycin (TaqMan)	5'-GGCTAGTACTGGGCAACA	5'-CGCTGACAGCGGGAACAC	5'-HEX - CAATCGGCTGCTCTGATGCCG - BH1

Method

Genomic DNA Extraction

The rapid extraction in our system was completed using Extract-N-Amp™ Tissue (see scheme to right). Half-centimeter tail pieces released sufficient quantities of DNA for quantitative PCR analysis. Tissue was placed in a mixture of 100 µl of Extraction Solution and 25 µl Tissue Preparation Solution for 10 minutes at room temperature. After a 3-minute incubation at 95 °C and the addition of a solution that neutralizes substances inhibitory to PCR, extracted DNA is ready to be directly added to a 2x PCR ReadyMix™ that is specially formulated to accommodate the extract for SYBR Green or fluorescent probe-based real-time qPCR assays. Traditional genomic DNA extractions were performed following the supplier's provided protocol.

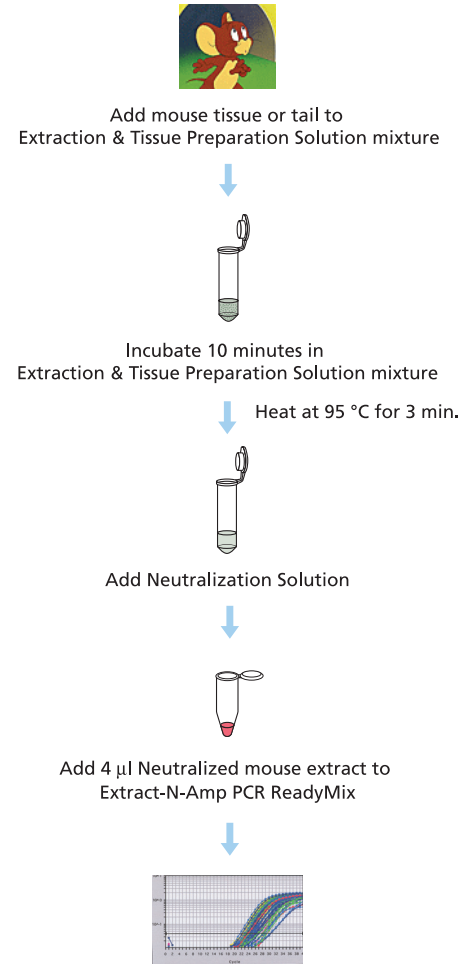
Quantitative PCR Amplification

All real-time product amplification and detection were performed either with dual-labeled probes or SYBR Green on a Stratagene Mx3000p. Quantitative SYBR Green reactions included 4 µl of mouse DNA extracts generated using Extract-N-Amp system, 2x SYBR® Green Extract-N-Amp PCR ReadyMix, primers, and Reference Dye for a total reaction volume of 20 µl. Multiplex dual-labeled reactions included 4 µl of mouse DNA extracts generated using Extract-N-Amp system, 2x Extract-N-Amp PCR ReadyMix, 2 primers/probe sets (specific and normalizer) and Reference Dye for a total reaction volume of 20 µl. Primer/probe sequences and the concentrations used can be found above.

Similar PCR efficiencies were verified for all reactions being compared using purified genomic DNA from a traditional DNA isolation method.

For negative controls, 2 µl Extraction Solution and 2 µl Neutralization Solution were used in place of 4 µl of DNA extract.

Extract-N-Amp DNA Extraction Scheme



Extract-N-Amp Time Savings

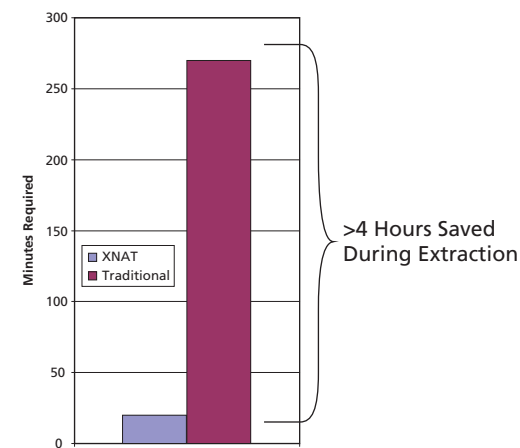


Figure 1: Significant time-savings is achieved using the Extract-N-Amp method for genomic DNA extraction over traditional silica-based methods.

Detection of Gene Copy Number

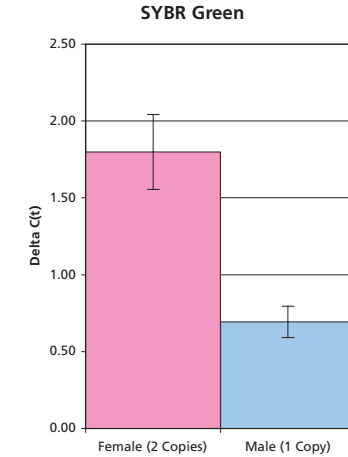


Figure 2: Genomic DNA extracts generated using the Extract-N-Amp system can be directly added to SYBR Green quantitative PCR to distinguish between 1 and 2 copies of the X-linked phosphate-regulating gene, Phex. Female mouse tails provided the source for 2-copy extracts and male for single genomic samples. Four extracts were created from each mouse type. Copy number of the X-linked gene as determined using the delta C(t) method of normalization. In this method, the C(t)s from SYBR Green PCR reactions using primers specific for the Phex gene were normalized by subtracting the C(t)s of the constant copy number, autosomal gene, Cox1. Numbers are reported as an average of duplicate PCR reactions. N=4

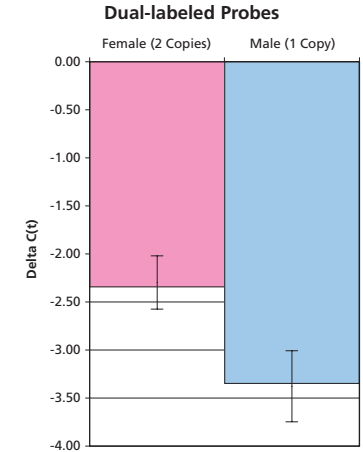


Figure 3: Dual-labeled detection can distinguish between 1 and 2 copies of an X-linked gene (Diap2) in neat Extract-N-Amp extracts. Female mouse tails provided the source for 2-copy extracts and male for single genomic samples. Four extracts were created from each mouse type. Copy number of the X-linked gene as determined using the delta C(t) method of normalization. In this method, the C(t)s from a dual-labeled primer/probe combination specific for the Diap2 gene were normalized by subtracting the C(t)s of the primer/probe set specific for the constant copy number, autosomal gene, Phactr1, in the same multiplex reaction. Numbers are reported as the average of duplicate PCR reactions. N=10

Detection of Gene Disruption

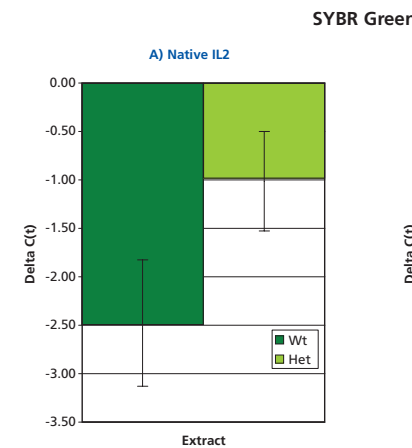


Figure 4: SYBR Green detection can be used to distinguish between hetero and homozygosity of both an IL2 or neomycin gene. The IL2 gene was disrupted by the insertion of neomycin. Three extracts were created from each mouse type. A) Copy number of the intact IL2 gene as determined using the delta C(t) method of normalization. In this method, the C(t)s from SYBR Green PCR using primers specific for the IL2 gene were normalized by subtracting the C(t)s of the constant copy number gene, Phex. Numbers are reported as the average of duplicate PCR. B) Copy numbers of the inserted neomycin gene were determined using the delta C(t) method of normalization. In this method, the C(t)s from SYBR Green PCR reactions using primers specific for the neomycin gene were normalized by subtracting the C(t)s of the constant copy number gene, Phex. Numbers are reported as the average of duplicate PCR reactions.

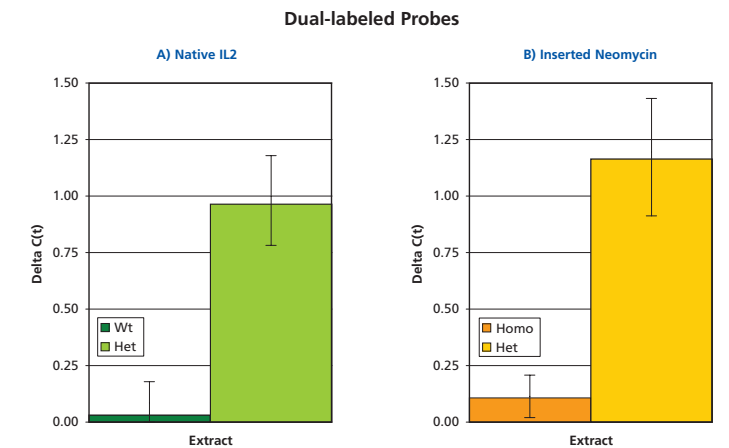


Figure 5: Dual-labeled detection can distinguish between 1 and 2 copies of an IL2 or neomycin gene in neat Extract-N-Amp extracts. The IL2 gene was disrupted by the insertion of neomycin. Four extracts were created from each mouse type. A) Copy number of the intact IL2 gene as determined using the delta C(t) method of normalization. In this method, the C(t)s from PCR using dual-labeled probes and primers specific for the IL2 gene were normalized by subtracting the C(t)s of the constant copy number gene, Phactr1. Numbers are reported as the average of duplicate PCR reactions. B) Copy numbers of the inserted neomycin gene were determined using the delta C(t) method of normalization. In this method, the C(t)s from PCR reactions using dual-labeled probes and primers specific for the neomycin gene were normalized by subtracting the C(t)s of the constant copy number gene, Phactr1. Numbers are reported as the average of duplicate PCR reactions.

Conclusion

Demonstrated above is a streamlined genotyping system capable of distinguishing single copy gene dosage differences. Extraction is completed in less than 20 minutes. Simple amplification and quantitation reactions are possible with a 2x ReadyMix formulated with SYBR Green or ready for dual-labeled probes. The use of real-time detection eliminates the need for agarose gel analysis. The results from these two assays demonstrate that extensive tissue digestion and DNA purification are not necessary and that high-resolution genotyping is possible using a simple, rapid tissue extraction and real-time qPCR with Extract-N-Amp.