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Simple and fast genotyping workflow

Introduction

PCR is a common genotyping technique used to detect the presence or absence of genes of interest. However, it can be time-consuming and can delay the start of downstream applications and analysis. In this application note, we present a fast high-throughput protocol for genotyping plants and mice using the Thermo Scientific™ E-Gel™ Power Snap Plus Electrophoresis System in combination with the Invitrogen™ Platinum™ Direct PCR Universal Master Mix. The master mix enables DNA amplification directly from various tissues without purification of genomic DNA. Direct PCR in combination with the E-Gel Power Snap Plus Electrophoresis System allows for high-throughput amplification analysis and visualization of up to 96 samples at once in less than 2 hours.

Materials and methods

- Platinum Direct PCR Universal Master Mix (Cat. No. A44647500)
- Mouse, plant, and food samples (Table 1)
- Forward and reverse primers (Table 2)
- E-Gel Power Snap Plus Electrophoresis System (Cat. No. G9301)
- Invitrogen™ E-Gel™ 48- and 96-well precast agarose gels with SYBR™ Safe DNA Gel Stain (Cat. No. G820802 and G720802)
- Thermo Scientific™ GeneJET™ Genomic DNA Purification Kit (Cat. No. K0721)
- Thermo Scientific™ GeneJET™ Plant Genomic DNA Purification Kit (Cat. No. K0791)

Key components of an efficient genotyping process

- Direct PCR enables DNA amplification directly from a sample; no DNA purification is required
- Universal primer annealing feature allows running reactions on one block and requires less optimization
- Fast PCR run time (<1 hr) due to fast DNA synthesis (20 sec/kb)
- Platinum Direct PCR Universal Master Mix includes gel loading dye—no time spent preparing samples for gel electrophoresis
- Electrophoresis can be performed as fast as in 12 min at high-throughput scale using precast E-Gel agarose gels
- Integrated imaging system in the E-Gel Power Snap Plus device saves operation time and allows real-time visualization of electrophoresis

Table 1. Samples and protocols used.

	Sample	Protocol
Mouse	4 male ears and tissues	Lysis and direct
	4 female ears and tissues	
Plant and food	Soy seed	Lysis
	GM soy feedstock	Lysis
	Maize seed	Lysis
	Soy drink	Direct
	Soy cream	Direct
	GMO-free soy drink	Direct
	Livestock feedstock	Lysis
	Fishing bait	Lysis
	Pet food	Lysis
	Fish food	Lysis
	Soy sauce	Direct

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Table 2. Primer sequences.

Sample type	Gene or sequence	Description	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplicon size
Mouse	SRY	Sex-determining gene on the Y chromosome	AACAACTGGGCTTTGCACATTG	GTTTATCAGGGTTTCTCTCTAGC	144 and 166 bp duplex
	Rosa26	Rosa26 locus in the mouse genome	AAAGTCGCTCTGAGTTGTTAT	GGAGCGGGAGAAATGGATATG	650 bp
Plant	P2	Photosystem II gene	AGCAATCCAAGGACGCATAC	TTACCCAATCTGGGAAGCTG	150 bp
	Soy lectin	Soy species–specific gene	AACCGGTAGCGTTGCCAG	AGCCCATCTGCAAGCCTTT	81 bp
	Cauliflower mosaic virus 35S	Cauliflower mosaic virus (CaMV) promoter 35S	GCTCCTACAAATGCCATCA	GATAGTGGGATTGTGCGTCA	195 bp
	GTS 40-3-2	5′ integration border region (IBR) between the insert of soybean event glyphosate-tolerant soybean (GTS) 40-3-2 and the soybean host genome	TTCATTCAAAATAAGATCATACATACAGGTT	GGCATTTGTAGGAGCCACCTT	84 bp
	A2704-12	Junction region containing a 3′ *bla* sequence and an inverted 5′ *bla* sequence	GCAAAAAAGCGGTTAGCTCCT	ATTCAGGCTGCGCAACTGTT	64 bp

Platinum Direct PCR Universal Master Mix provides two protocols to amplify target DNA from crude samples (Figure 1). The direct protocol offers a shorter workflow with minimal hands-on time, whereas the lysis protocol allows for a more flexible workflow with the ability to perform multiple PCRs from one sample, and a sample storage option.

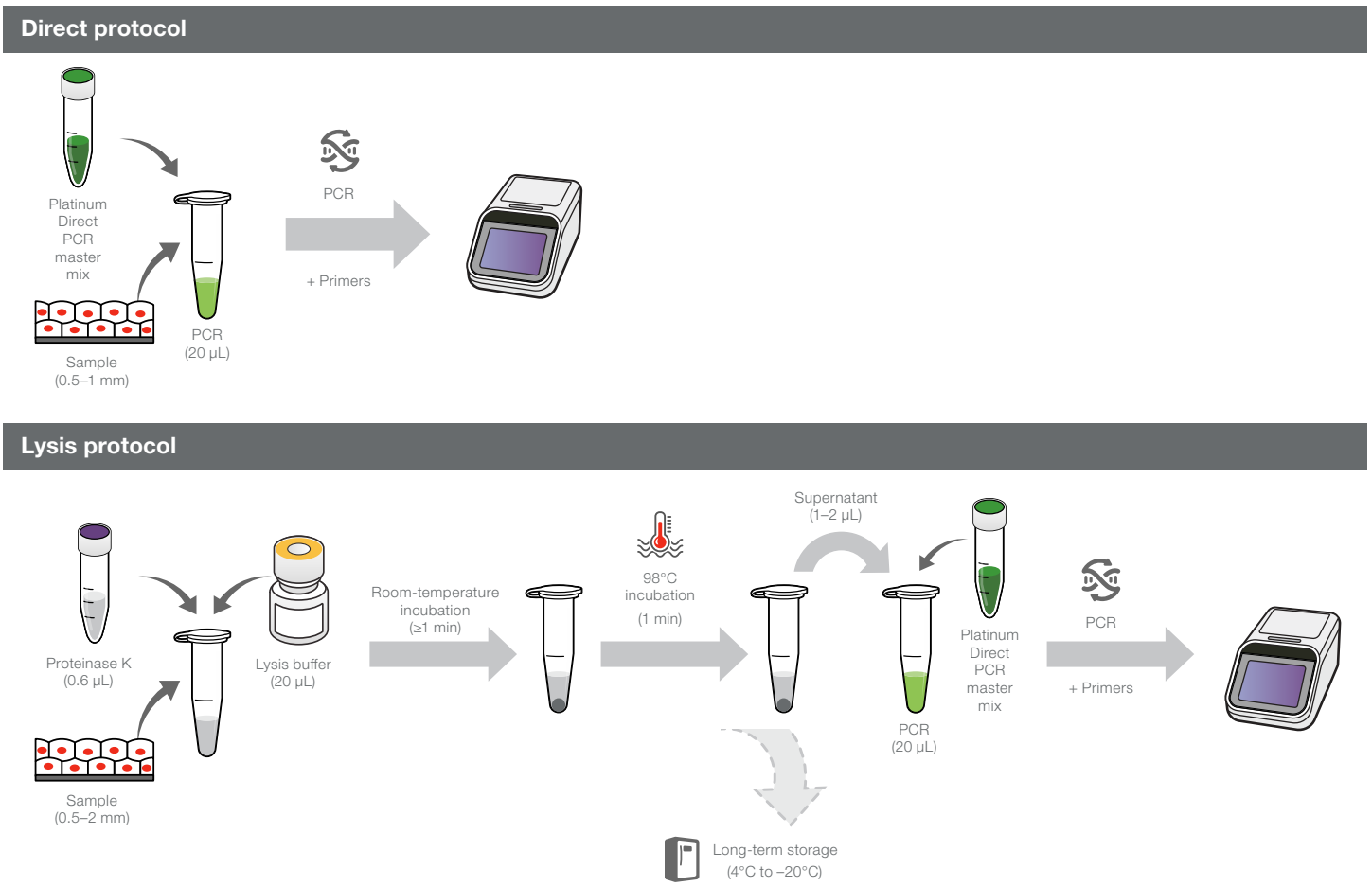

Figure 1. Workflows for direct and lysis protocols.

Direct protocol

Liquid food or a tissue sample of 0.5 mm diameter was added directly to the PCR master mix (Table 3). After PCR (cycling conditions in Table 4), Proteinase K (included in the kit) was added to the PCR reaction mix before loading the sample on the E-Gel Power Snap Plus system. The Platinum Direct PCR Universal Master Mix contains gel loading dye and density reagent; therefore, PCR samples were diluted 1:2 (plant samples) or 1:4 (mouse samples) with only nuclease-free water before loading 15 µL (for 48-well gel) or 20 µL (for 96-well gel) of diluted sample in the E-Gel agarose gel wells.

Lysis protocol

1 mm tissue, a few ground grains from seed, or feedstock was lysed using lysis buffer and Proteinase K (included in the kit). Then, the supernatant containing the crude DNA extract was frozen. A fraction of thawed supernatant was used for PCR. Reaction setup and cycling conditions are shown in Tables 3 and 4. After PCR, samples were diluted 1:2 with nuclease-free water before loading 15 µL (for 48-well gel) or 20 µL (for 96-well gel) in the E-Gel agarose gel wells.

Genomic DNA was used in PCR reactions as a positive control for comparison. Mouse genomic DNA was extracted from the ear and tail of 1 male and 1 female mouse using the GeneJET Genomic DNA Purification Kit (Cat. No. K0721). Soy genomic DNA was extracted from seed and genetically modified soy-based feedstock using the GeneJET Plant Genomic DNA Purification Kit (Cat. No. K0791).

Table 3. Reaction compositions for PCR using the Platinum Direct PCR Universal Master Mix.

Component	20 µL reaction	50 µL reaction	Final concentration
Platinum Direct PCR Universal Master Mix (2X)	10 µL	25 µL	1X
2 µM forward primer	1 µL	5 µL	0.2 µM* each
2 µM reverse primer	1 µL	5 µL	0.2 µM* each
Sample	1 µL lysate	1–3 µL of liquid food** or 0.5 mm tissue sample	1–10%
Platinum GC Enhancer†	5 µL	10 µL	1X
Water, nuclease-free	To 20 µL	To 50 µL	–

* Less primer (0.04 µM) was used for multiplex reactions with the internal control (P2) primer.
** 3 µL of sample was used for soy drink samples, since they have low soy content. 1 µL each of soy cream and soy sauce was used, since samples were viscous or had high salt concentrations.
† Recommended for target sequences with ≥65% GC content.

Table 4. Cycling protocol.

Step	Number of cycles	Temperature	Duration
Activation	1	94°C	2 min
Denaturation	35–40	94°C	15 sec
Annealing		60°C	15 sec
Extension		68°C	20 sec/kb
Hold	1	4°C	Hold

Results

Mouse sex genotyping using ear and tail tissues

To demonstrate direct DNA amplification from crude animal tissues with the Platinum Direct PCR Universal Master Mix, we used ear and tail samples from 4 male and 4 female BALB/c mice. The master mix was used to amplify a pair of fragments, 144 bp and 166 bp long, from a sex-determining gene on the Y chromosome. A non–sex related fragment (650 bp) of the Rosa26 locus in the mouse genome was used as a positive control for multiplex PCR.

PCR samples were diluted 1:4 with nuclease-free water and loaded straight into the precast 96-well 2% E-Gel agarose gel. Electrophoresis was run for 12 minutes, as suggested by the standard protocol pre-programmed in the E-Gel Power Snap Plus system. The results were imaged using the instrument's built-in camera (Figure 2). The images were then exported to the Thermo Fisher™ Connect Platform and deconvoluted to a preferred format using the automated script in Invitrogen™ iBright™ Image Analysis Software.

The Platinum Direct PCR Universal Master Mix amplified the positive control (650 bp fragment) from both female and male ear and tail samples, while the sex-determining target (144 and 166 bp duplex) was specifically amplified only from the male samples, consistent with the known sex of the mouse (Figure 2). Results are comparable when using genomic DNA control or lysis (lanes B–D) and direct (lanes E–H) protocols from mouse ear and tail tissue samples. This provides flexibility to choose a protocol depending on whether minimizing time for genotyping (direct protocol) or saving DNA samples for additional testing (lysis protocol) is required. Some additional low molecular weight bands can be seen in multiplex samples for only the male samples, indicating there is no interference for sex determination of female tissue samples.

Genotyping experiments were completed in less than 2 hours from acquiring samples. Consistent results from direct PCR indicate that the DNA purification step can be omitted from a standard genotyping approach without the loss of detection sensitivity. Omitting this step alone can save several hours that are typically required to sufficiently lyse tissue samples for purification of genomic DNA. The E-Gel Power Snap Plus system saved additional time by eliminating the need to make the agarose gel. Compatibility of the E-Gel 96-well precast gels with multichannel pipettes is beneficial in high-throughput screening applications.

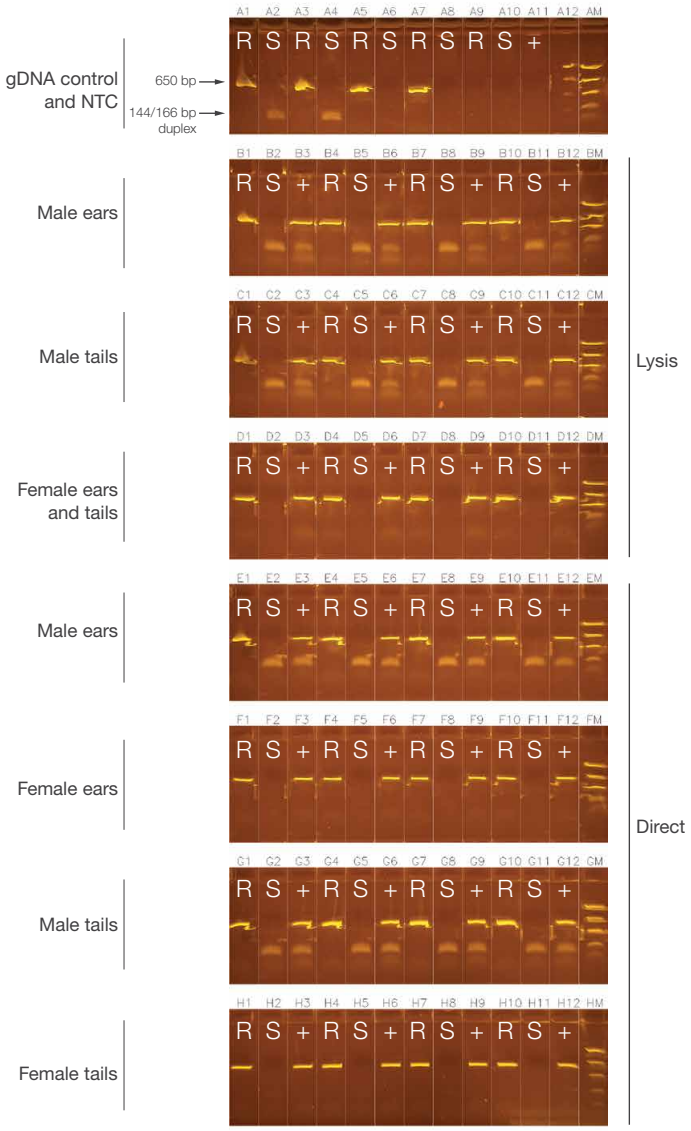

Figure 2. Sex genotyping of mouse tissues. Amplification of the sex-determining target (S) and universal target (R) fragment, or multiplex PCR of both targets (+) was performed using the Platinum Direct PCR Universal Master Mix. Lanes B–H contain samples from 4 male or female mice. The Invitrogen™ E-gel™ Low Rage Quantitative Ladder was used as a size standard. Lanes for gDNA controls: A1–A2: male ear; A3–A4: male tail; A5–A6: female ear; A7–A8: female tail. Lanes for no-template controls (NTC): A9–A11.

GMO detection in plant and plant-based food samples

Plant tissue or plant-based food samples were used to detect the presence of soy and genetically modified (GM) events to demonstrate that the Platinum Direct PCR Universal Master Mix can directly amplify target DNA sequences in plants. Plants have a high concentration of compounds that inhibit PCR reactions and are difficult to extract genomic DNA from, making direct PCR an appealing option.

The aim of this experiment was to see if direct PCR can be used to successfully identify soy-related GM events. Soy-based feedstock (F1) was acquired from the State Food and Veterinary Service (Lithuania) with confirmed GM events GTS 40-3-2 and A2704-12. Therefore, in this experiment we searched for these two mutations in other food products with unconfirmed GM status. Since the cauliflower mosaic virus promoter 35S is present in a majority of approved transgenic constructs, primers targeting it were used in this experiment as a control. The 35S promoter is often used as primary indicator of a GM event, but further testing for specific GM events is required. Store-bought soy seeds (S1) were a non–genetically modified organism (GMO) and served as a negative control for GMO detection.

Store-bought food with unknown GMO status used for this experiment included soy drink, soy cream, soy sauce, pet food, fish food, and fish bait. A universally found plant sequence (P2) was used as an internal control for multiplex PCR, to ensure a negative result was not acquired from a failed or inhibited PCR. The concentrations of P2 gene primers were reduced 5 times from the recommended protocol to balance the resulting amplicon amount. Additionally, all store-bought food was initially screened for soy using primers for the soy lectin gene. Then, only the samples that tested positive for soy were tested for GMO status. GM events can be rare within non-homogeneous sample types such as feedstock; therefore, all dry food samples were tested using the lysis protocol, which allows for a larger sample amount to be screened. Liquid samples—soy drink, soy cream, and soy sauce—were tested using the direct protocol only.

After PCR, samples were diluted 1:2 with nuclease-free water and loaded straight into the E-Gel 48-well 2% precast agarose gel using a multichannel pipette. The gel was electrophoresed for 12 minutes, as suggested by the E-Gel Power Snap Plus instrument protocol. The results were documented using the built-in camera.

Genotyping results were achieved in less than 2 hours from acquiring the samples. The Platinum Direct PCR Universal Master Mix amplified the internal control (150 bp fragment) in all samples (Figure 3). The soy lectin gene was amplified in all samples except maize seed (S2), livestock feedstock (F2), and fish food (F5) that were declared to be predominantly maize based. No GMO-related genes were found in GMO-free seed (S1) or soy drink (M2). All GMO-related genes were identified in the feedstock (F1). Other samples were found to have one or both GM events specific to soy; however, not all samples that were tested had the same 35S promoter sequence. It is worth noting that the 35S promoter sequence can vary between different GMOs and can result in visible nonspecific amplicons. As we have observed, the 35S sequence is generally used as a primary indicator for a possible GMO but not as a conclusive result. Comparable PCR results were achieved from amplification of purified genomic DNA and direct amplification with lysis or direct protocols. The lysis protocol is a flexible and useful protocol to overcome the heterogeneous nature of solid food or feedstock (data not shown).

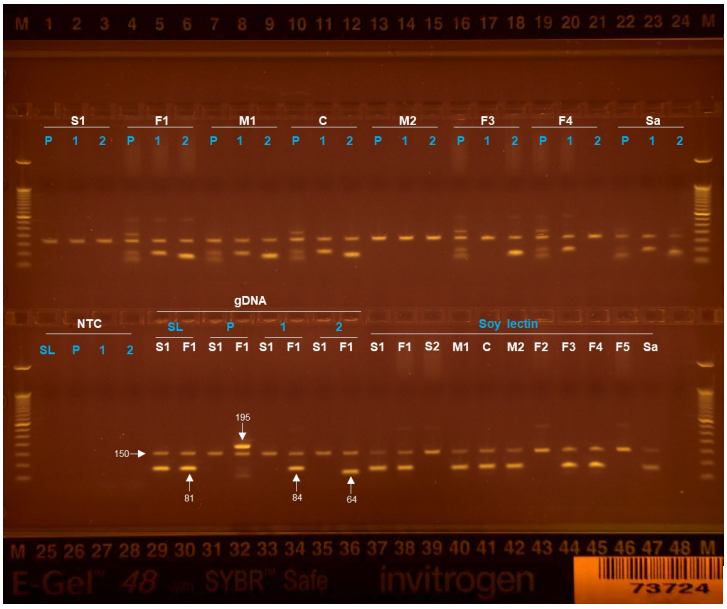

Figure 3. Direct amplification to identify the presence of soy or GM events in soy and soy-based food samples. All reactions were run in multiplex with primers for the P2 universal plant marker. Blue labels are for the following primer sets: SL: soy lectin gene; P: 35S promoter region; 1: GTS 40-3-2 GM event in soy; 2: A2704-12 GM event in soy. White labels are for the following samples: S1: soy seed from seed shop; F1: GMO soy feedstock with mutation confirmed by controlling agency; S2: maize seed from seed shop; M1: soy drink with non-declared GMO status; C: soy cream with non-declared GMO status; M2: soy drink with GMO-free status; F2: livestock feedstock; F3: fishing bait; F4: pet food; F5: fish food; Sa: soy sauce. The E-Gel 50 bp DNA ladder was used as a size standard. gDNA controls are in lanes 29–36. No-template controls (NTC) are in lanes 25–28.

Summary

Here we demonstrate a streamlined workflow for PCR-based genotyping from different mouse tissues and plant and food samples. The Platinum Direct PCR Universal Master Mix in tandem with the E-Gel Power Snap Plus Electrophoresis System enables a fast and high-throughput method to genotype in less than 2 hours. Applying the direct PCR method can save several hours and can help obtain genotyping results on the same day as sample acquisition. This is in contrast with a day or more when genomic DNA extraction is performed. We also show that this protocol can be used for multiplex PCR. The Precast E-Gel agarose gels save additional hands-on time by removing the need to prepare gels and providing shorter run times. The SYBR Safe DNA stain used in these gels makes them easier to dispose of when compared to products containing ethidium bromide. The E-Gel agarose gels and integrated electrophoresis and imaging functionality of the E-Gel Power Snap Plus system streamline genotyping analysis.

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