Simple Genotyping Method Using Ampdirect® Plus and FTA® Technologies: Application to the Identification of Transgenic Animals and Their Routine Genetic Monitoring

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A novel genotyping method, the Amp-FTA method, has been developed by combining the advantages of the Ampdirect® Plus and FTA® technologies. This method allowed the direct PCR amplification of DNA from unpurified blood immobilized on the FTA® card. PCR templates were prepared only by punching out the discs from the FTA® card, which could lead to a foolproof way of genotyping. The PCR products obtained could be subjected to downstream analyses such as restriction enzyme treatment and direct sequencing. The Amp-FTA method was applicable to buccal swabs collected by a simple technique minimizing distress to animals. Therefore, the Amp-FTA method is ideal for the genotyping and genetic monitoring of laboratory animals.

Key words: Genotyping, transgenic, monitoring, Ampdirect®, FTA® technology

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Simple and accurate genotyping for the identification of a transgenic or routine genetic monitoring is required to efficiently maintain a colony of laboratory mice and rats. In addition, animal welfare should be considered in the sampling of tissues as sources of DNA. Ampdirect® Plus is a reagent cocktail that effectively neutralizes inhibitory substances in the blood (Nishimura et al., 2000, 2002). It makes the isolation of DNA for PCR unnecessary, but the storage of samples is space-consuming and has to be done in a freezer. The FTA® card is designed to simplify the collection, shipment, archiving, and purification of nucleic acids from a wide variety of biological sources. Once blood is dropped onto the card, nucleic acids released from the cells are physically entrapped, immobilized, and stabilized for storage at room temperature. Infectious agents in blood samples are also denatured, and thus samples are no longer considered a biohazard. Preparing PCR templates is very easy; just punch out the disc from the FTA® card. But, laborious sample processing is needed to wash away the impurities that inhibit PCR. We believe that the Ampdirect® Plus and FTA® card technologies complement one another.

To evaluate the effect of Ampdirect® Plus on the amplification of DNA from unpurified blood on the FTA® card, we tried to amplify the endogenous Ocif gene and transgenic Neo gene of Ocif heterozygous mice. For blood sampling, the tip (0.5~1 mm) of the tail was cut under anesthesia and the blood leaking (5<µL each) from the tail was dropped onto a FTA® card (Whatman Japan K.K., Tokyo, Japan). After the sampling, the bleeding was stopped by applying pressure to the wound with sterile absorbent cotton. The filters were allowed to dry completely at room temperature for at least 1 hour. Discs were manually removed from the bloody-stained region using a Harris Micro-Punch (Whatman Japan K.K., Tokyo, Japan) (1.2 mm diameter) or standard ear punch (1.5-mm diameter). Un-treated sample discs were placed directly in a 15 µL PCR mixture containing 1×Ampdirect® Plus (Shimadzu Corporation, Kyoto, Japan), 0.2 µM of each primer, and 0.4 units of NovaTM Taq Hot start DNA polymerase (Merck KGaA, Damstadt, Germany). PCR primer sequences used in this study are as follows. PCR primers for detection of the wild-type allele of Ocif (Mizuno et al., 1998); 5'-CCCTGGCACCTGACCATGAC-3' and 5'-CTGACCATCACCACCTCAG-3'. PCR primers for Neo; 5'-GCTGCCATCCGACCCCTTCTGA-3' and 5'-CTGAGCGGCGCTAC-3' and 5'-CTGAGCGGCGCTAC-3'.
Laboratory Animals

Figure 1. Direct PCR with Ampdirect® Plus buffer from the untreated blood sample on the FTA® card. Blood from the Ocf-heterozygous mouse (+/-), carrying both wild-type and knockout alleles, was dropped onto the FTA® card, and then un-treated discs were used as templates with standard PCR buffer or Ampdirect® Plus buffer. The Ocf gene (left) as well as Neo gene (right) was amplified only when using Ampdirect® Plus buffer. Lanes 1 and 4; 100 ng of spleen DNA as a positive control. Lanes 2 and 3; Genomic DNA from the tail purified by phenol/chloroform extraction (positive control). Lanes 3 and 6; water (negative control). m; size marker φX174-Hae III digest. The Ocf knockout mouse (Ocf/Jcl) (Mizuno et al., 1998) was purchased from CLEA Japan, Inc. (Tokyo, Japan).

GCC-3’. PCR primers for the fa allele; 5’-TATGGAAGTCAGAGA TAGATGG-3’ and 5’-CTACGATGAGAATCCTCTAA-3’. PCR primers for the BAC vector sequence; 5’-CAACTCAATGACACACTCAGGGG-3’ and 5’-GGTTTGGCCGTAATGT-3’. The standard PCR buffer was made up to give the following concentrations: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.05% W-1, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 μM of each primer, and 0.4 units of Nova™ Taq Hot start DNA polymerase. PCR was carried out in a thermal cycler (PCR-808, ASTEC, Fukuoka, Japan) using the following program: 30 seconds of 94°C, 30 seconds of 94°C, 30 seconds of 60°C, 60 seconds of 72°C, 35 cycles. After PCR, 1 μL of the reaction mixture was applied to a 4% agarose gel in 0.5xTBE buffer and electrophoresed. The bands were visualized with ethidium bromide staining.

Using 100 ng of spleen DNA as a positive control, a prominent fragment of 203 bp of Ocfα was amplified in both the standard and Ampdirect® Plus buffers. With the unpurified blood DNA on FTA® paper, enough product to be visualized on agarose gel was obtained from the PCR with Ampdirect® Plus but not the standard buffer (Figure 1). Similar results were obtained when amplifying the Neo gene (Figure 1). Amplification of DNA from nontransgenic littermates was consistently negative in both standard and Ampdirect® Plus buffers (data not shown). These findings indicated that Ampdirect® Plus could neutralize PCR inhibitors that existed in the blood and were denatured by chemicals on the FTA® card. The restriction enzyme could work in the Ampdirect® Plus buffer, so, following the PCR, products could be directly digested in the reaction with the appropriate restriction enzymes (figure 2). Furthermore, the PCR products amplified using the Ampdirect® Plus buffer could be used for direct sequencing (data not shown). We would like to call this novel genotyping method the Amp-FTA method.

To minimize distress to animals in the sampling of blood from the tail, several alternative DNA sources have been proposed including samples from saliva (Irwin et al., 1996), stools (Broome et al., 1999), hair (Schmitteckert et al., 1999), and epithelial cells from the rectum (Lahn et al., 1998) or the buccal cavity (Meldgaard et al., 2004). Among them, we adopted sampling from the buccal cavity, because it is simpler than collecting saliva and has a standardized utility for human buccal swabs. Mice or rats were manually restrained in a vertical position by grasping the loose skin around the neck and back with the thumb, forefinger, and long finger of the nondominant hand. Buccal swabs were collected by scraping the inside of the cheek with thin cotton sticks with only 2 mm diameter buds (MEB115P 5P1501, J.C.B. Industry Limited, Tokyo, Japan). Following the scraping of both inner cheeks, a flat cotton tip was pressed within the circle sample area, and squeezed using an axis rotation without lifting the tip from the FTA® card.

As shown in Figure 3, the Amp-FTA method allowed us to amplify an endogenous DNA fragment as well as the transgene from buccal swabs. PCR bands from buccal swabs were weaker than those from blood with 35 PCR cycles (data not shown); so an additional 5 cycles (total 40 cycles) is recommended to obtain clear bands from the buccal swabs. For animal welfare and because of technical advantages, the buccal swab may replace blood collected from the clipped tail.

Figure 2. Genotyping of the rat fatty (fa) mutation by the Amp-FTA method. Blood from KZ-Lepr+/Tky rats (lanes 1-5), which were maintained by mating fa+ heterozygous males and females, was dropped onto the FTA® card, and then un-treated discs were used as templates with Ampdirect® Plus buffer. Following PCR, the restriction enzyme MspI, which recognizes the fa mutation but not the wild-type allele, was directly added to the PCR mixtures. Then the PCR mixtures were incubated at 37°C for 3 hours. DNA from lane 1 was determined as the fa Homozygote, DNAs from lanes 2 and 3 were determined as the wild-type homozygotes, and DNAs from lanes 4 and 5 were determined as the fa+ heterozygotes, respectively. Lane 6; Genomic DNA from the spleen as positive controls, purified by phenol/chloroform extraction (lane 6; fa-, lane 7, fa+, lane 8; +/+), m; size marker φX174-Hae III digest. The KZ-Lepr+/Tky fatty rat was supplied by the National BioResource Project for the Rat (NBRP-Rat) in Japan.
The Amp-FTA method would be ideal for genotyping laboratory animals, because it has the following advantages (Figure 4). First, to our knowledge, the Amp-FTA method is the simplest and most rapid genotyping method available. Preparing DNA templates is very straightforward: just punch out the discs from the FTA® card on which the blood was dropped. There is no need for special chemicals or equipment for the preparation of DNA, thus there is no waste of time. Second, the method is safe for laboratory workers and eco-friendly, because there is no use of harmful chemicals for DNA purification and no discharge of polluting waste. Third, the Amp-FTA method reduces the risk of spread of microbiological contamination to breeding colonies of laboratory animals, because pathogens, existing in the blood or urine if the hosts were infected, can be inactivated by the cell-lytic chemicals on the FTA® card. Lastly, the DNA samples on the FTA® card can be stably stored at room temperature for at least 14 years (unpublished observations in the Whatman data sheet). This allows the re-interrogation of the DNA at any time, in addition to space-efficient storage of samples at room temperature.

These advantages of the Amp-FTA method might be highlighted when the number of samples to be processed increases. Therefore, the method can be recommended for genotyping in facilities maintaining huge numbers of animals such as bio-resource centers. Actually, the National Bio Resource Center for the Rat in Japan has employed the Amp-FTA method for routine genetic monitoring as well as the determination of the transgenes of rat strains. Using the Amp-FTA method, more than 100 strains have been routinely genotyped for identification of their transgene or mutant gene and for scheduled genetic monitoring.

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Japan (http://www.anim.med.kyoto-u.ac.jp/NBR/) for providing KZ-depr+/Tky rat (NBRP Rat No: 0032).

References


