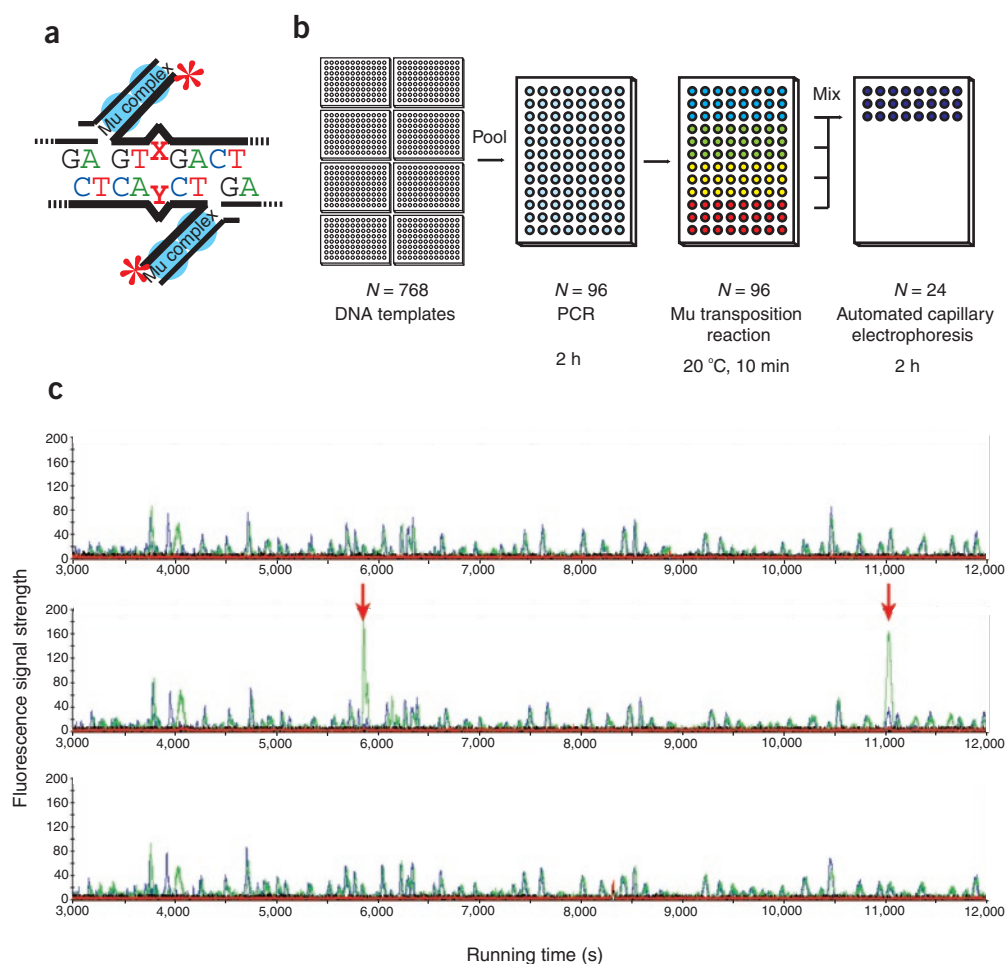


# An ENU-induced mutant archive for gene targeting in rats

## To the editor:

Although the laboratory rat is increasingly being used as a mammalian model in biomedical research, no technology exists thus far for the production of *in vivo* genetically engineered mutations equivalent to knockout or knock-in mice because of the lack of development of functional embryonic stem cells in this species. Rat spermatogonial stem cells may possibly have greater potential use for genetic engineering to produce gene-targeted rats<sup>1,2</sup>. The use of somatic cell nuclear transfer to develop cloned rats as an alternative to using embryonic stem cells has also been reported<sup>3</sup>, but it seems to be a very difficult technique to perform. At present, *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis followed by a screening method to detect single-nucleotide substitutions within the targeted gene seems to be the most promising technology in rats, as previously reported by several groups<sup>4,5,6</sup>. The screening protocol, however, either a yeast-based screening assay<sup>4,5</sup> or a high-throughput resequencing-based screening technique<sup>7</sup>, is expensive. In addition, gene-driven ENU mutagenesis approaches in rats are not a very efficient use of resources because most of the rats generated are usually discarded within a few weeks or months after the targeted genes have been screened owing to a lack of reliable sperm conservation and rederivation technologies.

We have developed a new, efficient approach that combines two methods: a high-throughput, low-



**Figure 1** Screening assay for ENU-mutagenized rats. (a) Detection of single-nucleotide mismatches by Mu transposition. The Mu transpososome is a complex of Mu-end DNA and MuA transposase (blue circles). The Mu-end DNA fragments are labeled at the 5' ends (red asterisks). The mismatched DNAs (X and Y) are targeted by the Mu transpososome to generate two reaction products (thick lines). (b) Schematic of the MuT-POWER high-throughput DNA screening method for ENU-mutagenized G1 rats. Genomic DNA of G1 rats is extracted and pooled by a factor of eight for PCR. Targeted genomic regions are amplified by PCR for 2–3 h. PCR products are then mixed with Mu transposase labeled with four different fluorophores (blue, green, yellow, red), and incubated at 20 °C for 5 min. Thereafter, Mu reaction products are mixed to form one sample. The resulting reaction mixtures are separated by capillary electrophoresis on an automated sequencer. (c) Transposition reaction products are easily visualized as two positive peaks by Gene-Mapper software. The mutations are then confirmed by sequencing each DNA from the eight G1 rats in the pool.

cost screening assay that uses the phage Mu transposition reaction and intracytoplasmic sperm injection (ICSI) for the recovery of the rare heterozygous genotypes from our newly generated frozen sperm repository, the Kyoto University Rat Mutant Archive (KURMA) (Supplementary Fig. 1 online). Phage Mu transposition shows a strong target site preference for all single-nucleotide mismatches<sup>8</sup>. The process involves *in vitro* cleavage of the mismatch target site by the Mu transpososome, which is a complex of Mu-end DNA and MuA transposase, followed by rejoining of the 3' ends of the Mu-end DNA to the target site (Fig. 1a). Despite the unknown mechanism responsible for Mu transposition, this method has several advantages when used for detecting single-nucleotide substitutions. Using the pre-formed transpososome, the reaction starts with only a few components and requires only 5 min. The length of the DNA target varies from a dozen bases to a few kilobases, and labeled Mu-end DNA eliminates the need to label individual target DNA. Finally, this Mu transposition reaction can be combined with DNA pooling and therefore facilitates an efficient screening approach for mutagenized animals, termed MuT-POWER (Mu Transposition Pooling method With sequencER) (Fig. 1b).

In the analysis, the pooling of DNA samples for thousands of first generation (G1) offspring of ENU-mutagenized rats allows a reduction in the number of PCR samples to one-eighth of the original number. Mu transposition reactions are carried out at 20 °C for 5 min in a 96-well plate. The use of multicolor labels on the Mu-end DNA allows a further reduction, by a factor of four, in the number of electrophoresis samples to be analyzed on an automated capillary sequencer. Mismatch DNA is clearly detected as two peaks of cleaved targeted DNA captured by the labeled Mu-end DNA (Fig. 1c). MuT-POWER screening of the 1,735 G1 KURMA samples permitted us to identify ten mutations thus far, including missense, silent and intronic mutations (Supplementary Table 1 online). The MuT-POWER technology,

therefore, is a powerful tool for detecting ENU-induced mutations, owing to its cost effectiveness, time efficiency and ease of application compared to other protocols<sup>4,5,7</sup>.

ICSI technology<sup>9</sup> is very useful for producing an archive of cryopreserved ENU-mutagenized sperm that allows the screening of any targeted gene at any time in the future. Our sperm freezing protocol is very simple to perform, even for a large number of G1 samples. The mutant sperm archive is stored in a -80 °C freezer or a liquid nitrogen tank. Possible fertility problems of ENU-mutagenized rats are alleviated by ICSI. A preliminary injection experiment of such frozen sperm into 234 oocytes from F344 females resulted in the successful recovery of 56 live rats (23.9%) (Supplementary Table 2 and Supplementary Video 1 online), demonstrating the ability of ICSI technology under these circumstances; thus far, we have been able to rederive all detected mutants by ICSI.

We conclude that our systematic approach of gene-driven ENU mutagenesis and the rapid screening for DNA mismatches, complemented by ICSI of archived frozen rat sperm for the recovery of living heterozygous rats (Supplementary Fig. 1) is cost and resource efficient. The KURMA sperm archive has been deposited at the National Bio Resource Project for the Rat in Japan (<http://www.anim.med.kyoto-u.ac.jp/nbr>), and it is open to any interested researcher worldwide. The mutation frequency in KURMA was 1 mutation per 3.7 Mb (unpublished data), which is lower than that usually obtained in the mouse (1 mutation per 1–1.5 Mb)<sup>10</sup>, probably because of the lower dose of injected ENU (two injections of 40 mg per kilogram body weight; see Supplementary Methods online). The number of G1 DNA and sperm samples has already expanded to 5,000, which increases the possibility of finding mutations in a wide variety of genes. Future expansion of the archives, together with potential improvement of the screening protocols, will provide an efficient platform for the generation of any kind of allelic variation or missense or null mutation (knockout). This would allow

the production of rat models of human diseases, including cancer, hypertension, diabetes and various diseases for which the mouse has proven to be less useful.

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