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Preparation of Microcells For Use in Production of Transchromosomic Animals*

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Abstract Microcell mediated chromosome transfer (MMCT) is a challenging technique for introducing exogenous chromosomes into interested mammalian cells. Combined with the somatic cell nuclear transfer technique, MMCT has been employed for producing transchromosomic animals of medical and agricultural value. Producing high quality of microcells is a key step in the success of MMCT. Eamamined by fluorescin staining and Giemsa staining, 0.2 mg/L colcemid was considered suitable for inducing high percentage of micronuclei in A9 (neo12) cells, without causing death of a mass of cells. Microcells were produced by centrifugation of micronucleated A9 (neo12) cells in Percoll density gradient containing 20 mg/L Cytochalasin B at 39 000 *g*. The resulting mixture of microcells, whole cells, karyoplasts and cytoplast fragments was filtered through 8 μm and 5 μm size membrane pores sequentially to obtain pure preparation of microcells. Microcells were then characterized by Giemsa staining and microcell PCR was first applied for examination of the quality of microcell preparation. The result showed that microcells containing our interest chromosomes-human chromosome 12 were equally distributed in the preparation, the preparation was suitable for use in generation of transchromosomic animals.

Key words A9 (neo12) cell line, human chromosome, microcell, microcell mediated chromosome transfer (MMCT)

Microcell mediated chromosome transfer (MMCT) is a technique for introducing intact chromosomes from one mammalian cell to another mammalian cell[1]. MMCT mainly includes following steps: micronucleation of donor cells by prolonged colcemid treatment; enucleation of micronucleated donor cells on density gradient at high speed centrifugation; isolation of microcells; fusion of microcells to recipient cells by phytohemagglutin-P (PHA-P) and polyethylene glycol (PEG); screening for the desired microcell hybrid clones under certain selective conditions (Figure 1).

Compared with non-mammalian chromosomes, such as YACs, BACs and PACs, mammalian chromosome vectors can overcome the upper size limit, and can transfer megabase-sized chromosomes or mammalian artificial chromosomes (MACs) between different mammalian cells. The transfered chromosomes generally do not integrate into host genome, and tend to remain stable and segregate freely[2]. Because of the size and capability of MACs, it is highly probable the exogenous genes on the MACs will represent a whole pathway.

The MMCT technique has been used in a variety of scientific fields, including the discovery of specific genes related to disease, chromatin structure study, epigenetics study, genome organization, mammalian chromosome engineering, gene therapy and the production of transchromosomic animals[3~8]. MMCT is therefore an invaluable tool in gene function studies in the post-genomic era.

Combined with the somatic cell nuclear transfer technique, MMCT can be employed to generate transchromosomic animals[9~13]. Here we introduce an efficient method of preparing microcells containing human chromosome 12 harboring the human alpha-lactalbumin gene from A9 (neo12) cells, for use in generation of transchromosomic cattle expressing human alpha-lactalbumin.

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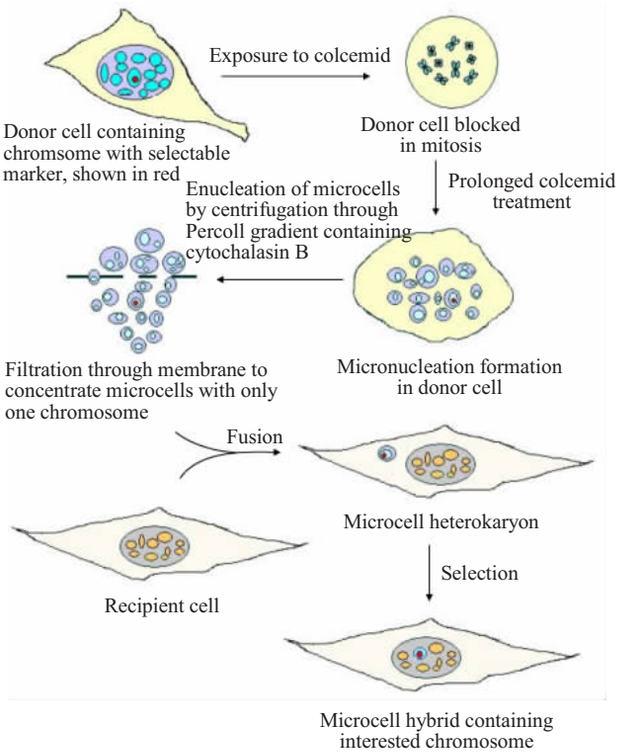

Fig. 1 Diagram of microcell-mediated chromosome transfer protocol

1 Materials and methods

1.1 Cell lines

The A9 (neo12) cell line was purchased from the Japanese Collection of Research Bioresource (JCRB) Cell Bank. A9 (neo12) is a mouse fibroblast cell line contains human chromosome 12 inserted with a neo resistant gene.

1.2 Fluorescent *in situ* hybridization analysis

For fluorescent *in situ* hybridization (FISH) analysis, the chromosome spreads were prepared with standard cytogenetic protocol modified from previous study [14]. The BAC346L Ⅱ (Invitrogen, USA) containing the human alpha-lactalbumin gene was used as probes, which were labeled with biotin-14-dATP (Invitrogen, USA). The denatured probes were dissolved in hybridization solution to a final concentration of 50 mg/L and prehybridized for 40 min at 37℃. Hybridization was performed for 17 h at 37℃ in a humid chamber. Probes were detected with FITC-conjugated avidin (Vector, USA) and signals were amplified by biotinlated antiavidin (Vector). Chromosomes were counterstained with 0.5 mg/L propidium iodide.

1.3 Microcell and micronucleated cell staining

Giemsa stained microcells and micronucleated A9 (neo12) cells were prepared as procedures described in Chromosome spread preparation [14], except for colcemid treatment. Dried microcell slide was stained by Giemsa stain for 8 min, then washed by pure water gently, and air dried.

1.4 Production of microcells

Logarithmic phase grown A9 (neo12) cells in fifteen 150-mm petri dishes were exposed to 0.2 mg/L colcemid for 48 h. Then cells were trypsinized, sedimented pellets were resuspended with 20 ml DMEM (GIBICO). Resuspension was then loaded to two 50-ml steriled centrifuge tubes, each contains 10 ml Percoll added with 1 ml 3 mol/L NaCl and 1 ml HEPES (GIBICO). Cytochalasin B was added to make a final concentration of 20 mg/L. The gradient solution was subjected to 39 000 *g* centrifugation force at 32℃ for 75 min. After centrifugation, all the particles contained in bands were collected using a 10 ml syringe. The collection then was centrifugated at 2 000 *g* for 10 min. Pellets were resuspended in DMEM, and filtered through 8 μm and 5 μm pore size polycarbonate isopore membrane (Millipore) sequentially to obtain the pure microcell preparation.

1.5 Microcell PCR

For microcell detection, 2 μl microcell solution was selected randomly from the preparation, then moved into PCR tube containing following chemicals: 1 μl 10×PCR buffer, 1 μl 1% Tween 20, 1 μl 1% Triton X100, 6 μl double distilled H_2O, and 0.2 μl 10 g/L protease K. Then the solution was covered with 10 μl mineral oil. The mixture was incubated at 45℃ for 15 min, denatured at 96℃ for 20 min. PCR then was performed by adding following compounds into the mixture: 5 μl 10×PCR buffer, 4 μl dNTP, 1 μl primer F1 (5′ GAGTGATGCTTCCATTTCAG 3′), 1 μl primer F2 (5′ CAGAGATGTACAGGATCTGC 3′), 0.25 μl Taq polymerase, 27.55 μl double distilled H_2O. The PCR reactions were run for 35 cycles, with denaturation at 95℃ for 30 s, annealing at 60℃ for 30 s, and extension at 72℃ for 30 s. After the last cycle, a final extension at 72℃ for 10 min was included.

1.6 Statistical analysis

Duncan multiply range test and *T*-test was performed by SAS statistical soft.

2 Results

2.1 Identification of human chromosome 12 in A9 (neo12) cells

We confirmed the presence of human chromosome 12 in A9 (neo12) cells by fluorescent *in situ* hybridization. Figure 2 shows that the human chromosome was stably retained in A9 (neo12) cells.

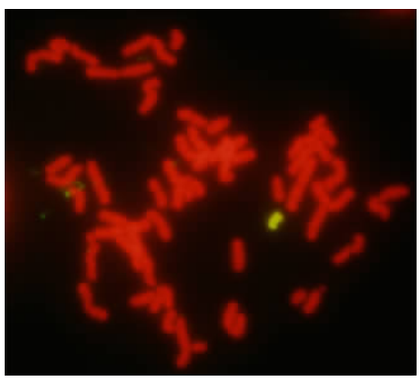

Fig. 2 Human chromosome 12 in A9 (neo12) cells analyzed by fluorescent *in situ* hybridization to metaphase cells

DNA probes were labeled from BAC346LⅡ(Invitrogen) containing the human alpha-lactalbumin gene. Human chromosome 12 was detected by FITC (green) and counterstained by PI (red).

2.2 Concentration and length of colcemid treatment

A range of concentration of colcemid from 0.05 mg/L to 1.5 mg/L were applied to A9 (neo12) cells for 48 h. Acridine orange (AO) staining results showed that 0.05 mg/L colcemid induced the formation of micronucleation less effectively, with the increase of colcemid, more micronuclei were observed within A9 (neo12) cells, but when the colcemid was over 0.2 mg/L, it presented cytotoxic to A9 (neo12) cells (Figure 3).

We found after subjecting micronucleated cells to hypotonic treatment, then stained by Giemsa, micronuclei can be clearly discriminated and counted (Figure 4). On average, about 5～10 micronuclei appeared in one A9 (neo12) cell after exposure to colcemid from 0.05 mg/L to 0.2 mg/L for 48 h (Table 1). From the statistical analysis, 0.2 mg/L colcemid exposure for 48 h was determined to be the optimal concentration for inducing high percentage of micronuclei with minimal cell death in A9 (neo12) cells.

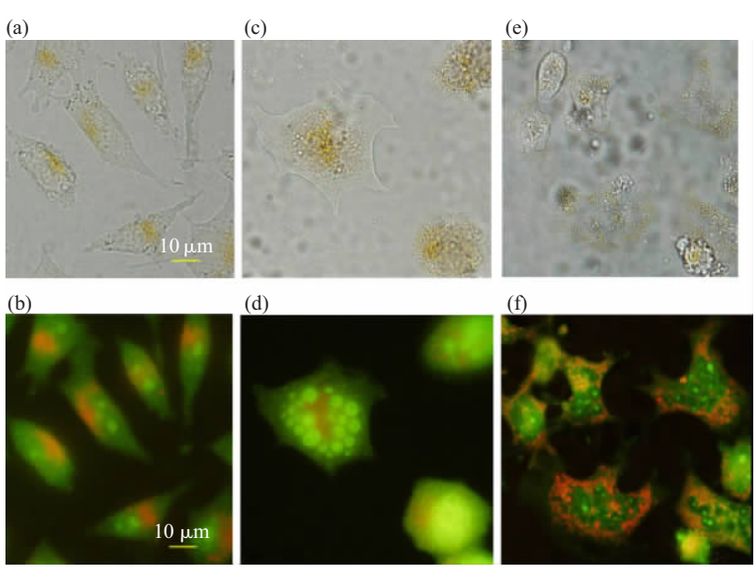

Fig. 3 The formation of micronuclei within A9 (neo12) cells by prolonged exposure to colcemid

Phase-contrast photomicrographs of A9 (neo12) cells without colcemid treatment, no micronucleus was observed (a, Bright field; b, Acridine orange stained cells in fluorescent field). By exposure to 0.2 mg/L colcemid for 48 h, the volume enlargement of cells and micronuclei (green spots) within cells was observed (c, Bright field; d, Fluorescent field). By exposure to 0.5 mg/L colcemid for 48 h, the abnormal morphology of cells was observed (e, Bright field; f, Fluorescent field). Scale bar=10.0 μm.

Table 1 Micronuclei formation

Cell line	Colcemid dose/(mg·L^{-1})	Average No.per cell	Bon grouping[1)]	Cells forming micronuclei/%
A9(neo12)	0	0 ± 0	A	
	0.05	5.48 ± 2.15	B	
	0. 08	6.06 ± 2.64	B C	69%
	0.10	6.09 ± 2.26	B C	
	0.15	7.91 ± 4.75	D C	
	0.20	9.68 ± 3.92	D	89%[2)]

[1)] Duncan multiply range test, means with the same letter are not significantly different. [2)]P<0.01. Only 0.05 mg/L colcemid and 0.2 mg/L colcemid were further investigated for their micronucleation induction efficiency. A9 (neo12) cells stained by AO, in the fluorescent field, the number of micronucleated cells and total cells were counted in each vision field, the percentage of micronucleated cells was figured out. This was performed in more than 20 vision fields, the average percentage of micronucleated cells was determined as the micronucleation induction efficiency.

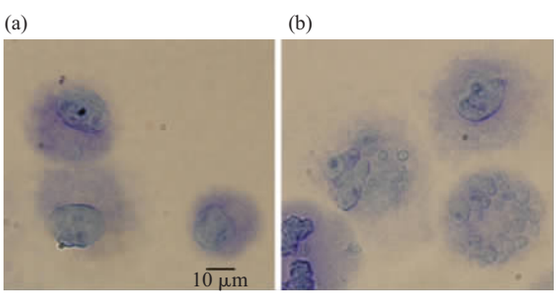

Fig. 4 Giemsa stained preparation of cells and micronucleated cells

(a) Normal growing A9 (neo12) cells; (b) Micronucleated A9 (neo12) cells, micronuclei showed a nuclear like shape, micronuclei number can be clearly conted.

2.3 Enucleation and production of microcells

At the conclusion of centrifugation, three bands of cellular material were distributed on different layers in the tube and the silica pellet was present at the bottom of the tube (Figure 5).

Purified microcells were examined under phase-contrast microscopy, and counted on a hemacytometer (Figure 6a). Giemsa stained microcells showed they were around 3 ～5 μm in diameter, condensed interphase chromatins were presented in the microcells, some remained in dispersed chromatins (Figure 6b,c).

2.4 Microcell PCR

Microcell PCR was first applied to detect whether the preparation of purified microcells contains the interested chromosomes. According to human α-lactalbumin genomic sequence (GenBank X05153), a pair of primers: forward primer F1, 5′GAGTGATG-CTTCCATTTCAG 3′, and reverse primer F2, 5′ CAGAGATGTACAGGATCTGC 3′, was designed to amplify a 790 bp fragment from human chromosome 12. Figure 7 showed that in five randomly picked up 2 μl microcell solution, four contain human chromosome 12. We therefore concluded microcells containing chromosome of interest were universally present in the preparation. We may use the preparation for the following chromosome transfer experiments.

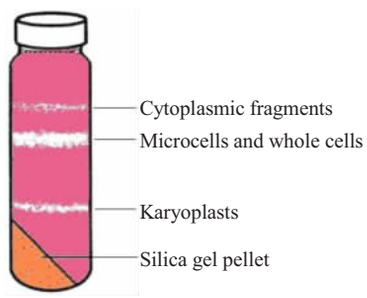

Fig. 5 Diagram of the result appearance of gradient centrifugation

Most microcells were found in the middle band (the widest one), tangled with enucleated cells and whole cells which failed to be enucleated. The upper band with the least width was mainly composed of debris of cytoplasts, membrane fragments and some microcells with larger cytoplasmic component. The lower band with the medium width was composed mainly of enucleated nuclei designated karyoplasts and some whole cells with more nuclear contents.

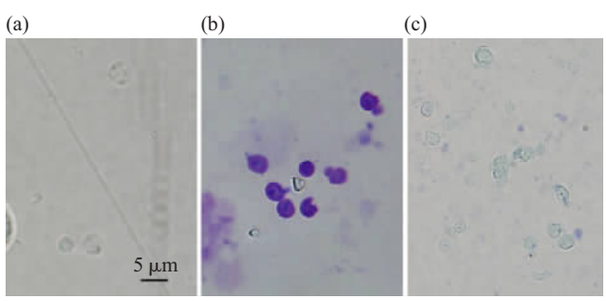

Fig. 6 photomicrograph of microcells

Phase-contrast photomicrograph of microcells on hemacytometer (a); Giemsa stained microcells (b); Less Giemsa stained microcells, the work concentration of Giemsa is 1/10 of standard concentration, the condensed interphase chromatins was clearly discriminated (c).

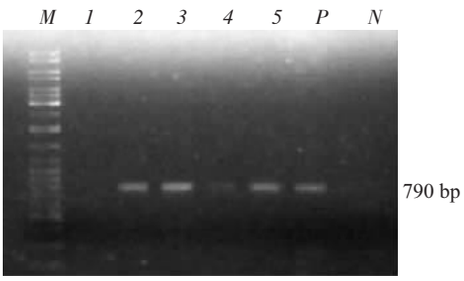

Fig. 7 Microcell PCR

Microcell PCR was applied for amplification of a 790 bp fragment of human α-lactalbumin gene. *M*: 1 000 bp ladder marker; 1～5: Randomly picked up microcell preparation; *P*: BAC346LⅡ; *N*: H_2O.

2.5 Microcell fusion

For microcell fusion, bovine fibroblast cell line was established from a cow fetus.

Two methods of microcell fusion were adopted: PHA-P/PEG monolayer fusion and suspension microcell fusion. In the former, microcells were suspended in PHA-P solution, then the solution was loaded onto the monolayer culture of bovine fibroblast cells, PEG was recruited for the fusion of microcells to bovine fibroblast cells. PHA-P is cytoxic to a spectrum of cell lines, in our experiments, bovine fibroblast cell line show less sensitive to PHA-P, so the usually used concentration of PHA-P 100 mg/L was doubled to 200 mg/L for enhancing the agglutination of microcells to bovine fibroblast cells. The pH of PEG was important for the success of fusion, pH between 7.5～8.0 proved better for fusion efficiency, but when the pH was over 8.0, the alkaline PEG was deleterious to bovine fibroblast cells. In our observation, the over pH 8.0 PEG can broken down plasmic membranes of bovine fibroblast cells, casuse the immediate cell death. Bovine fibroblast cells can endure the pH 7.5～8.0 PEG at least five minutes, so the PEG exposure time was prolonged from one minute to five minutes for a more thorough fusion.

In the suspension microcell fusion, bovine fibroblast cells were trypsinized, the resuspension was pooled to microcell solution, after centrifugation, PEG was added to the pellet for 1min. Immediately after the fusion, the cytoplasmic fusion between microcells and bovine fibroblast cells can be observed under contrast-phase microscopy (Figure 8), the fusion between bovine fibroblast cells also can be observed.

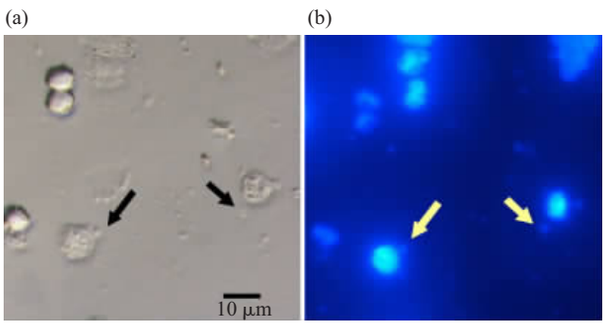

Fig. 8 Phase contrast microphotograph of microcell fusion

Cell particles were stained with Hoechst 33258. The arrows show the position of microcells (a, Bright field; b, Fluorescent field).

3 Discussion

The methodology of creating microcells was developed by Ege and Ringertz [15]. Fournier and Ruddle[1] first successfully used microcells to transfer single intact chromosome from one tumor cell line to another cell line, establishing the technique of microcell mediated chromosome transfer (MMCT). Recently with the success of the somatic nuclear transfer technique and development of stem cell research, MMCT was employed to produce transchromosomic animals, such as chimeric mice contain intact single human chromosome, fragments of human chromosome or artificial human chromosomes[9,11,12]. Transchromosomic large domestic animals has already exist, for example four calves expressing human polycolonal antibodies were born and lived healthily in 2002[13].

The production of cows expressing humanized milk is a challenge. Transchromosomic cows are expected to improve nutritional value for infants. The full length of all human milk protein genes together with their remote regulatory elements can span several mega bases , which is beyond the capacity of YAC, so MMCT is the candidate method for introducing the whole components of the human milk protein into bovine.

Human α-lactalbumin is a very important component in milk. It is a 14 ku whey protein, promotes the synthesis of lactose as a regulatory subunit[16]. Because it is rich of essential amino acids, such as tryptophan and cysteine, human α-lactalbumin plays a important nutritional role for the rapid growth and development of infants[17]. In order to overcome the position effect as to ensure a high level expression of

human α-lactalbumin for the improvement of infant formula, we intend to transfer human chromosome 12 into bovine.

To ensure the success of MMCT, it is essential to produce as many microcells as possible. The first step of microcell preparation is the induction of micronucleation of donor cells by prolonged colcemid treatment. The concentration and exposure length of colcemid are the key factors for effective micronucleation, human cell lines call for higher concentration than murine cell lines[18～20]. the exposure time generally is around 48 h, some lymphoblast cell lines difficult for micronucleation need to prolong the exposure time to around 72 h[18]. In our study, 0.2 mg/L colcemid was found more effective for A9 (neo12) micronucleation than literature value 0.05 mg/L.

The success introducing exogenic chromosomes into finite cell lines was seldom reported. One major problem for finite cell lines such as bovine fibroblast cell line, is the early coming of cell senescence and death under the positive microcell hybrids selective pressure. Bovine fibroblast cells usually grow no more than three weeks after MMCT. This should cause the loss of positive cell colony formation from heterochromosome already transfered cells, due to the less active proliferation ability of finite cells after MMCT. Another problem concerns the success of introducing heterochromosomes into finite cell lines maybe the mismatch cycle phase of microcells and recipient cells. Microcells generated by enucleation of interphase multinucleated cells present in all cycle phase, most in G1 phase[21, 22]. The fast growing bovine fibroblast cells have a shorter G1 phase, most cells are in S and G2 phase. The G1 phase microcells fused into bovine fibroblast cells will enter S phase, the micronuclei will undergo premature chromosome condensation when hybrid cells proceed to mitosis. This is a serious problem for the success incorporation of exogenic chromosomes into bovine fibroblast cells. We plan to alleviate the mismatch of microcells and bovine fibroblast cells, approaches will be developed to synchronize both microcells and bovine fibroblast cells at G1 or early S phase before fusion. Further more, because of the short lifespan of the after MMCT fibroblast cells, modified strategy suitable for positive hybrid selection should be developed in the following experiments.

Our most important finding was that for examination of micronucleation efficiency, Giemsa stained micronucleated cells were found more efficient for exact quantitative analysis than traditional fluorescin stained cells.

Microcell PCR developed from single cell PCR by us was first used for examination of the quality of microcell solution[23]. By using this method, without nesting strategy, PCR product less than 1 kb is suitable for rapid detection of microcells preparation contains the interested chromosomes.

Our ultimate goal is to develop artificial human chromosomes for producing transchromosomic animals expressing valuable complex proteins for the benefits of humankind. The efficient preparation of microcells for chromosome transfer is the basis of achieving our prospective goal.

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一种适用于生产转染色体动物的微细胞制备方法 *

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摘要 微细胞介导的染色体转移技术 (MMCT) 是一项将外源染色体转入哺乳动物细胞的技术，具有广阔的应用前景. 与体细胞核移植技术结合，MMCT 可用于生产具有重要医学药用价值和优良农业生产性状的转染色体动物. 制备高质量的微细胞是关系 MMCT 技术成功的关键步骤之一. 通过荧光染色和吉姆萨染色分析，结果表明，A9 (neo12) 细胞经 0.2 mg/L 秋水仙素酰胺处理 48 h 后，89%的细胞产生微核化，每个细胞平均形成 10 个微核. 微核化的细胞在含有 20 mg/L 细胞松弛 B 的 Percoll 密度梯度介质中，经 39 000 *g* 高速离心后，包含微细胞、完整细胞、细胞核和细胞碎片的混合液，依次通过 8 μm 和 5 μm 孔径的滤膜过滤后可获得纯化的微细胞溶液. 通过光学显微镜和吉姆萨染色观察，可见微细胞为一群直径约为 3～5μm 的类细胞核的球形物质. 微细胞 PCR 技术首次用于检测微细胞溶液的质量，检测结果显示，所制备的溶液中均匀分布着带有目的染色体的微细胞，适用于进一步作转染色体动物实验.

关键词 A9(neo12)细胞系，人类染色体，微细胞，微细胞介导的染色体转移技术 (MMCT)

学科分类号 Q81，Q813

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