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ARTEMIS Pharmaceuticals GmbH an EXELIXIS Co.

# Automated sorting of genetically engineered embryonic stem cells for generation of mouse models

Although genetically modified mice have huge potential for the analysis of gene function, the creation of such mice via homologous recombination in embryonic stem cells (ESCs) is a tedious process. A combination of the CytoClone™ system from Evotec Technologies for cell selection with the ArteMice™ platform from Artemis Pharmaceuticals for rapid generation of genetically modified mouse models from engineered ESCs offers a fast and gentle way to isolate such cells.

## Challenges in mouse model generation

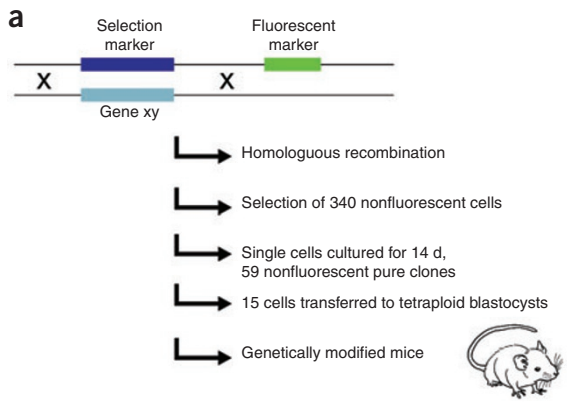
A key property of mouse ESCs is that they can be maintained indefinitely *in vitro*, retain the capacity to participate normally in embryogenesis and contribute to all tissues of the mouse embryo when introduced into host blastocysts, including the germ line. Retention of germline transmission competence, however, depends absolutely on adherence to a rigorous tissue-culture regime, with avoidance of any untoward selective pressures. ESCs can easily experience alterations during cell manipulation that cause a loss of totipotency. Furthermore, ESCs are only modestly able to amplify to form clonal populations if plated as single cells.

The ArteMice technology platform is a comprehensive collection of techniques for the rapid and technically superior production of genetically modified mouse models. It includes the use of a proprietary vector system carrying a fluorescence marker. Cells carrying a random integration show fluorescence. During correct homologous recombination, however, this fluorescence tag is lost. Selecting nonfluorescent ESCs thus leads to an enrichment of cells with the desired genetic modification. The generation of knockout mice that are solely derived from these genetically engineered ESCs is based on the use of Artemis' Tetraploid Complementation Technology (**Fig. 1**).

Laborious manual clone picking has been the method of choice for isolating genetically modified nonfluorescent ESC clones. Standard flow-cytometry cell sorting could not be applied because of the loss of totipotency of the targeted ESCs. It is in this selection step that Evotec Technologies' CytoClone offers substantial new value.

## Clonal cell isolation using the CytoClone

The CytoClone sorts cells using the proprietary CellProcessor™ technology[1]. During the run, all cells are analyzed by high-resolution




c

| | Targeted ES clone OMP 3 cyto 1-B6 |
|---|---|
| Number of infected blatocysts | 60 |
| Number of ES mice born | 3 |

**Figure 1** | Description of embryonic stem mouse generation using CellProcessor technology. (**a**) The F1 Hybrid ESC Art4.12 was chosen for a gene-targeting experiment with a targeting vector comprising a neomycin-resistance cassette as a positive-selection marker and a ZsGreen cassette for counterselection. Nonfluorescent single cells were isolated using the CellProcessor technology of the CytoClone. Cells were plated in 96–well plates for clonal expansion. Correctly targeted ESCs were identified, and genetically modified mice were produced via the ESC-Tetraploid Blastocyst Complementation Technology. (**b**) ES mouse recovered from blastocyst injection for ESC isolation. (**c**) Results from blastocyst injections of clones isolated with CellProcessor technology.

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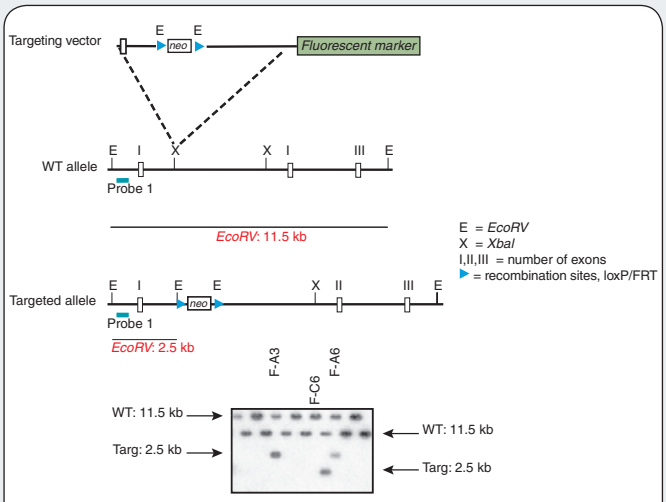


**Figure 2 |** Disruption of the *Gt(ROSA)26Sor* locus. A targeting vector harboring a fluorescence marker gene was constructed for insertion into the *Gt(ROSA)26Sor* locus by homologous recombination. The DNA of ESCs was digested with *EcoRV* and hybridized with probe 1 specific for the *Gt(ROSA)26Sor* locus. Probes were applied at two different heights so that individual bands could be easily distinguished. The sizes of the wild-type and the targeted allele are 11.5 kb and 2.5 kb, respectively. The picture shows the analysis of identified nonfluorescent

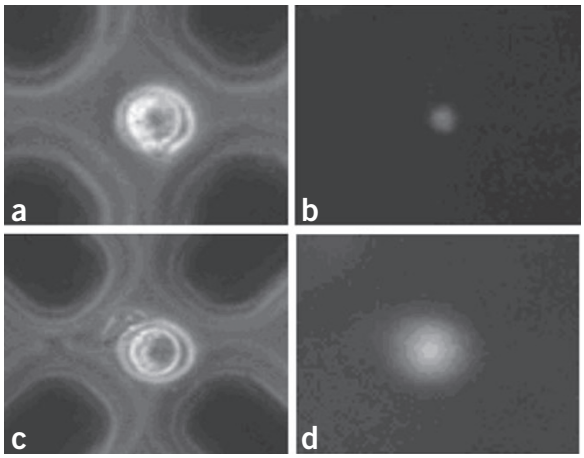


**Figure 3** | CytoClone images of sorted cells. (**a**,**b**) Cells with correct homologous integration of the target appear viable in phase-contrast images (**a**), but do not show fluorescence of the fluorescent marker protein when imaged after 488-nm excitation (**b**). (**c**,**d**) Cells with random integration do survive the selection process and are viable (**c**), but show autofluorescent protein expression (**d**).

phase-contrast and fluorescence imaging for size, viability, single-cell status and amount of fluorescence—all of which can be used as sorting criteria. The imaging capability and automated image analysis routines make it very easy to identify and characterize target cells as well as distinguish them from aggregates or debris. Viable, single-target cells are selected automatically and placed into a microtiter plate for growing pure, high-quality clones.

Experiments under asceptic conditions are facilitated by a removable fluidic panel that contains all fluidic components and the microfluidic sorting chip. It can be assembled and primed off-instrument in a laminar flow box. Additionally, the CytoClone requires only a minimal number of cells. A few hundred are enough for each run.

## Automated generation of totipotent mouse ESC clones

Single, nonfluorescent mouse ESCs were isolated to grow clones in which the *Gt(ROSA)26Sor* (also known as *ROSA26*) gene was targeted (**Fig. 2**). For this purpose we used the phase-contrast and fluorescence imaging capability of the CytoClone to identify single, viable cells that lack GFP expression. After selection with G418, the ESC population was sorted, and phase-contrast and fluorescence images were acquired from each target cell identified by high phase-contrast signal and low green fluorescence value (**Fig. 3**). Nonfluorescent live ESCs were plated as single cells, achieving a plating efficiency of 34% on feeder layers. Clonal growth properties were comparable to those of cells isolated by standard manual procedures. Upon identification and expansion of correctly targeted ESC clones live mice were produced via the ESC-Tetraploid Blastocyst Complementation Technology[2].

## Conclusion

Low plating efficiency, differentiation accompanied by loss of germline competence and subsequent failure to produce genetically modified mice are still key obstacles for the use of automated sorting methodology for ESCs in gene-targeting studies. The results presented here clearly demonstrate the suitability of the CytoClone system for automated clonal selection even of sensitive cell samples such as naive stem cells. The CellProcessor technology did not affect the competence of genetically engineered ESCs. The contact-free procedure and gentle cell handling maintained targeted ESCs germline-competent and allowed the production of fully ESC-derived mice by tetraploid complementation. Taking advantage of the image data available with the CytoClone system makes the process very reliable, and this will open up new possibilities for gene targeting in the future.

This method allows for the first time automated gene targeting, from transfection of the gene-targeting vector to establishment of totipotent targeted ESC clones. Higher-frequency gene targeting and isolation of single cells with defined characteristics in an automated fashion are both big advantages to improve effectiveness and precision of mouse-model generation.

Additional information is available online at the company websites (http://www.artemispharma.de, http://www.evotec-technologies.com).

1. Müller, T. *et al.* The potential of dielectrophoresis for single cell experiments. *IEEE Eng. Med. Biol. Mag.* **22**, 51–61 (2003).
2. Eggan, K. *et al.* Male and female mice derived from the same embryonic stem cell clone by tetraploid embryo complementation. *Nat. Biotechnol.* **20**, 455–459 (2002).

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