MICROWELL ARRAY PCR CHIP FOR STUDY OF GENETICALLY ENGINEERED MOUSE STEM CELLS

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ABSTRACT

We have developed a real-time polymerase chain reaction (rt-PCR) microwell device to screen for homologous recombination in transfected murine embryonic stem (ES) cell colonies. The device facilitates the screening of the approximately 500 ES colonies that survive the selection media after transfection. An rt-PCR loss of allele (LOA) assay has been developed to confirm correct vector integration[1]. The device coupled to a microcup array for cell selection decreases the time required for cell growth and reduces the amount of labor required for the entire transfection process[2].

KEYWORDS: Real-time PCR on Chip, Genetically Engineered Mouse, Stem Cells, Loss of Allele

INTRODUCTION

Transfection of a vector in a murine ES cell can be used for various applications, including gene function studies, human disease models, and cell cycle regulation. Due to the inefficiency of homologous recombination (only ~1 in 10^5 cells are properly transfected), a high number of starting cells increases the chances of obtaining successful results. In addition to the low efficiency of homologous transfection, the time required for cell proliferation, colony picking and long-range PCR analysis, leads to a slow, tedious, and expensive process for production of genetically altered stem cells. Therefore, typically only ~200 out of 500 colonies surviving negative selection strategies are analyzed due to labor and time constraints leaving potentially transfected cells unanalyzed. By mating an rt-PCR microwell device with an automated cell selection process (Figure 1), significant reduction in time, cost and effort required to engineer ES cell lines should be achieved.

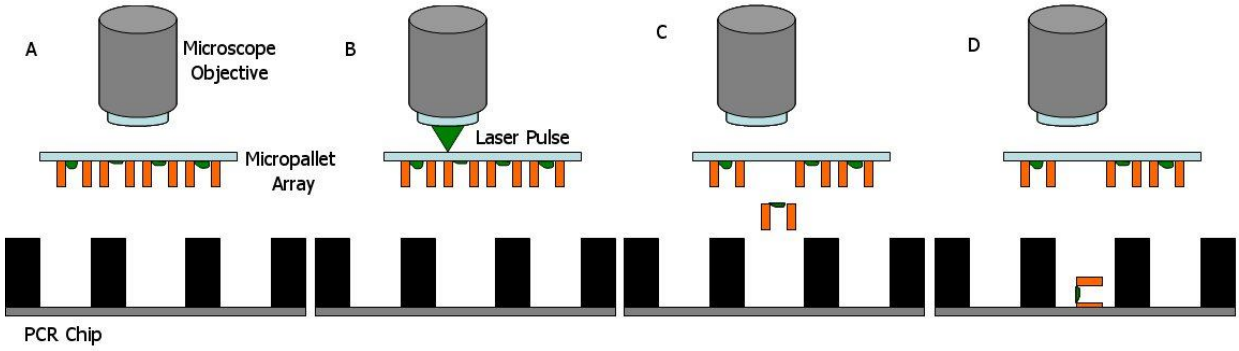

Figure 1: A) rt-PCR microwell array device is secured below a microcup array that contains cell colonies. B) A microcup and microwell are aligned and a laser pulse activated. C) The selected microcup is released and D) drops into the PCR microwell. Cells from different colonies are deposited in individual microwells for later rt-PCR analysis.

Using the microcup array, a portion of cells can be removed from colonies containing fewer than 100 cells, reducing the time for proliferation before genetic testing. The selected colony subsets are deposited in individual microwells such that the LOA assay can be performed simultaneously on all suspected transfected cells. Moreover, the cell selection process and delivery to a microwell array PCR chip can be automated to alleviate tedious manual colony picking.

Upon completion of the microcup transfers, the rt-PCR device is separated from the microcup array and LOA assays performed. For our LOA assay, one set of primers and probe targets the insertion region at the Rosa26 locus. This probe is designed to cross the insertion site with the forward and reverse primers flanking the insertion site. In the case of a successful transfection, the number of amplicons generated should be half as many as the wild-type cell since one copy of the original gene has been knocked-out. A second set of primers and probes targets an unrelated and known homozygous gene. As both genes are present, the number of amplicons generated will be twice that of a successfully transfected cell. Using a multiplexed assay with both sets of primers and probes present, the difference in the cycle threshold (ΔCt) between the Rosa26 locus gene and the homozygous gene should be non-zero for a successfully transfected cell. For our purposes, as the amplification efficiency for each primer set may not be identical, the ΔCt of potentially transfected cells will be compared to the ΔCt of wild-type cells. A non-zero difference in ΔCt ($\Delta\Delta$Ct) between cell lines indicates a successfully transfected cell.

EXPERIMENTAL

The rt-PCR device (Figure 2) consists of a black-dyed polycarbonate chip with CNC machined 1.4 mm diameter wells bound to an aluminum oxide membrane (AOM), which will be used to capture the DNA during analysis[3]. The bottom of the chip is sealed with Kapton tape prior to thermal cycling. The prototype device consists of 37 PCR reaction wells. Experiments have been conducted using purified genomic DNA (gDNA). PCR reagents were Platinum Quantitative PCR SuperMix-UDG (Invitrogen), Platinum Taq Polymerase (Invitrogen), primer and probe sets were from Biosearch Technologies. For gDNA analysis, gDNA was spiked into the PCR reagent mixture prior to loading the reagents on the

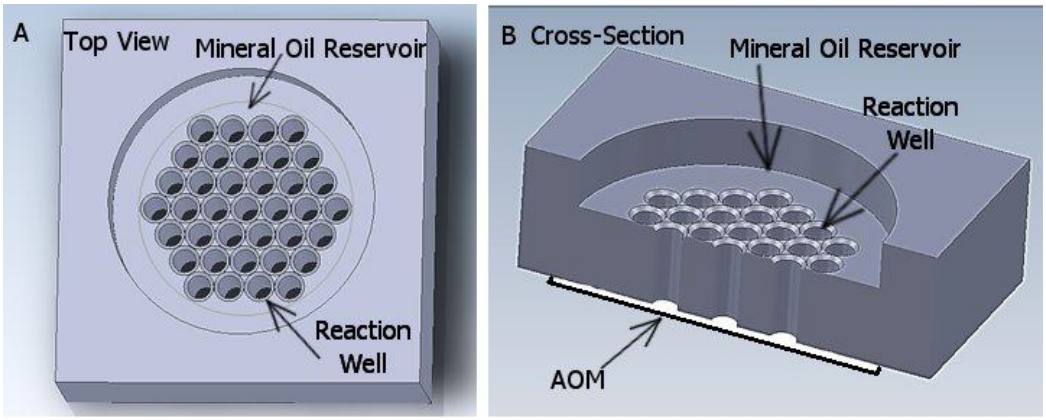

Figure 2: *Chip diagram of the unsealed rt-PCR microwell device a) top view and b) cross-section. Wells are approximately 5 µL in volume, holding a PCR reaction volume of 2.5 µL. The remainder of the reaction well is filled with mineral oil.*

sealed chip using a pipette. After the addition of PCR reagents, chips were centrifuged to remove bubbles produced during reagent loading. Mineral oil was added to the reservoir and the chip was centrifuged to force the mineral oil into the reaction wells. A LabView-controlled Peltier stage is used to control the thermal cycling of the chip for 50-60 cycles (Table 1).

Table 1. Thermal cycling parameters used for the rt-PCR chip.

Stage	Temp (°C)	Time (m:ss)
Initial	50	2:00
Denaturation	95	2:00
Cycle	95 66	0:45 1:00
Final	66	2:00

The rt-PCR is monitored using a CCD equipped Nikon AZ100 fluorescence microscope with a home-built automated filter wheel for multiplexed PCR with multiple fluorescent probes. Fluorescence images are taken at the end of each extension step. The assay uses Taq-man style primer and probe sets from Biosearch Technologies.

RESULTS AND DISCUSSION

The LOA assay targets both the Rosa26 insertion site and the homozygous Mus ß Actin gene. The primer sets were tested with 10 ng (~1000 copies) gDNA from correctly targeted, randomly integrated and wild-type cell lines. Amplification was seen for both the Mus ß Actin and Rosa26 primer sets for wild type and correctly targeted cell lines (Figure 3) as well as the randomly integrated cell line. For the correctly targeted cell line, there are half as many amplicons from Rosa26 as there are from the wild-type or randomly integrated cell line, while the number of amplicons from Mus ß Actin should be the same. As a result, the ΔCt for the correctly targeted cell line should be higher than the wild type cell line. From the amplification plots (Figure 3), the ΔCt for the Rosa26 genes and the homozygous gene (3a) are close to zero with the amplification curves overlapping beyond the Cts indicating a similar number of amplicons. For the correctly targeted cells, a larger ΔCt between the homozygous and Rosa26 gene is present with the amplification curves diverging beyond their Ct values. Figure 4 shows the ΔCt for wild type, successfully targeted and randomly integrated cells. As expected, the ΔCt of the successfully targeted cells are greater than the wild type and randomly integrated cells giving a non-zero ΔΔCt. The wild type and randomly integrated cells show similar ΔCt valves. Therefore, using a wild type cell line as a control and comparing ΔCt valves with the vector exposed cells, successfully transfected cells can be distinguished.

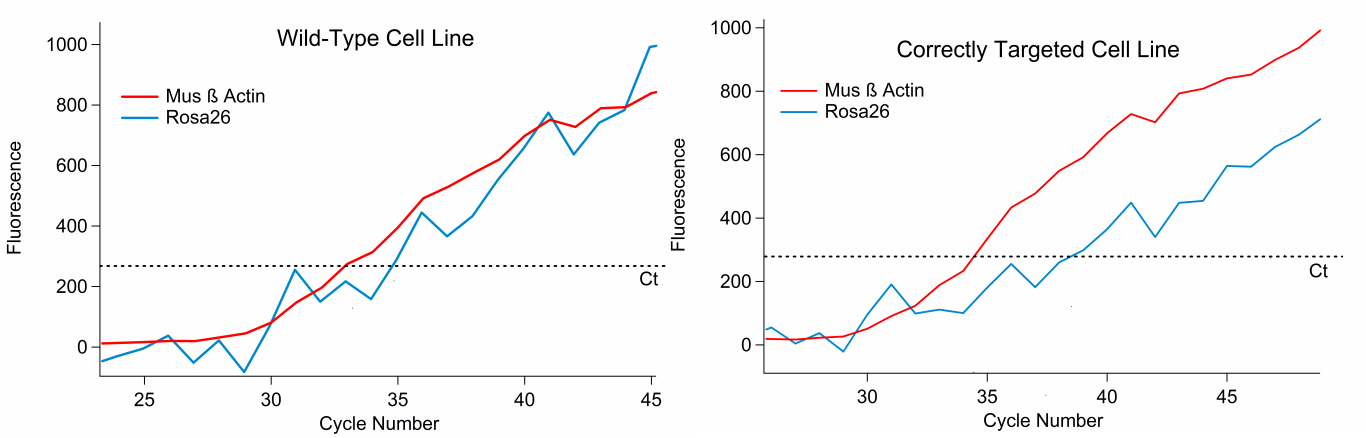

Figure 3: Multiplexed rt-PCR using the device showing detection of the amplicons from the Rosa26 and Mus ß Actin loss of allele assay. The ΔCt *is the difference in the cycle number where each trace crosses the cycle threshold line (Ct). As expected, the (a) wild-type cell line shows a smaller ΔCt than the (b) correctly targeted cell line.*

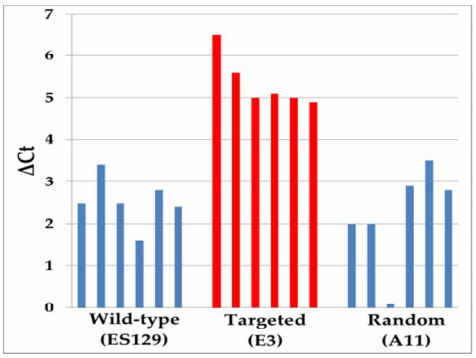

Figure 4: LOA assay for Rosa26 performed on the rt-PCR device using 10 ng gDNA (~1000 cells) showing six replicates for each of the cell lines tested. $\Delta Ct = Ct_{,Rosa} - Ct_{\beta\text{-}actin}$ *The targeted cell line (red) contains only one Rosa26 allele, resulting in a higher ΔCt than the randomly integrated or wild-type cell lines (blue) which contain two Rosa26 alleles.*

CONCLUSION

Based on the data obtained, a LOA assay comparing the ΔCt values of potentially transfected cells to wild-type cells can help identify correctly targeted ES cells. When coupled with the microcup array cell selection, the overall time and cost of creating a transfected cell can be greatly reduced.

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