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Technical Report

Generation of Two Spotted Plasmid cDNA-Libraries of the Head Region of Stage 20 and Whole Stage 17 Medaka Embryos

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Abstract The comparative analysis of vertebrate development relies on the study of homologous genes in different species. To get hand on medaka homologues a rapid isolation of the corresponding medaka cDNA is highly desirable. Here we describe the generation of two spotted plasmid cDNA-libraries of early neurula stage (stage 17, (Iwamatsu, 1994) and the head region of 4 somite stage (stage 20) embryos respectively. The spotting of individual clones onto filters allows the direct identification and isolation of single cDNAs within two days.

Material and Methods

Total RNA was isolated (Chomczinsky and Sacchi, 1987) from the head region of about 450 stage 20 embryos yielding 33 μg total RNA; from 800 stage 17 embryos yielding 113 μg total RNA (Fig. 1). PolyA$^+$ RNA was isolated from total RNA using the Oligotex-RNA-Kit (Qiagen). The Superscript Plasmid System (Life Technologies) was used to construct the library. 300 ng (st. 20 library) and 1 μg (st. 17 library) polyA$^+$ RNA were used for transcription. After second strand synthesis and adapter ligation (5': SalI adapter, 3': NotI primer-adapter $(T)_{15}$) the longest fractions of the cDNA were selected for directional ligation to the plasmid vector pSport1 (NotI-SalI precut). Subsequently the plasmids were electroporated into highly competent ELECTROMAX DH10B E. Coli cells (Life Technologies). The library was not amplified to preserve the original distribution of mRNAs and prevent faster growing clones to outgrow more slowly growing ones. The primary library was plated onto 22 cm × 22 cm agarose plates at a density of 2500 colonies per plate. The colonies were picked by an automated device. Pictures of the colonies were taken by a CCD-camera. Well separated colonies were identified by an image analysis software and picked into 384 well microtiter plates (each well filled with 55 μl 2*x*YT/10%Glycerol/80 μg/ml Ampicilin). These master plates were replicated. Clones from the replica plates were spotted onto 22 cm × 22 cm hybond N$^+$ (Amersham) nylon filters, again by a robot. Each library is represented by one filter containing 27,648 clones. Twelve clones are spotted in a block of 5 × 5 spots (Fig. 2) and each of these twelve clones is spotted twice within this block to increase the security of the system. In the middle of each block there is an ink guide dot. The orientation of two signals to each other in one block indicates the number of the microtiter plate. The position of the block on the filters determines the coordinates of the clone in the microtiter plates.

The spotted colonies were allowed to grow over night on these filters. The colonies were lysed in situ and the DNA was bound to the membrane following standard procedures (Sambrook *et al.*, 1989). In detail the membrane was placed on a blotting paper prewetted with denaturant (0,5 M NaOH; 1,5 M NaCl) for 4 min at room temperature and then incubated for additional 4 min in a 94°C water steam. The membrane was then transferred onto a fresh blotting paper prewetted with neutralization buffer (1 M Tris/HCl pH 7.4) and finally incubated in Pronase buffer (100 μg/ml Pronase in 100 mM NaCl; 50 mM Tris/HCl; 1% Sarkosyl) for 20–40 min. After drying the DNA was crosslinked to the membrane by UV light (Stratagene Stratalinker).

The filters were prehybridized in "Church Buffer" (7% SDS; 0.5 M $NaPO_4$; (Church and Gilbert, 1984) for 1 h in glass tubes at 65°C and hybridized over night in 10 ml "Church Buffer" with 100 μl denatured probe generated by random

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priming (Feinberg and Vogelstein, 1983) (*Pax6*: $28 \cdot 10^6$ dpm, *XlTubulin*: $37 \cdot 10^6$ dpm). The filters were washed twice (30 min., 60°C, 0.5 M SSC, 0.1% SDS; 30 min., 65°C, 0.1 M SSC, 0.1% SDS) and exposed for 90 min. at –80°C to a Kodak Biomax film.

Results

The initial complexity of both libraries was about 400,000 independent clones with an average insert size of 1 kb. The library was tested by hybridizing the filters with a heterologous, abundant probe: neural class *II Xenopus laevis tubulin* (Good *et al.*, 1989) and with a homologous, eye specific probe: *Oryzias latipes Pax6* (Loosli and Wittbrodt, unpublished). For the specific probe (*Pax6*) we obtained two signals on the filter that represents the genes expressed in the head region of stage 17 embryos (Fig. 3.A). The DNA isolated from the corresponding clones was sequenced and encodes *Pax6*.

With the abundant probe (*Xltubulin*) we detected about 30 signals in both libraries (Fig. 3.B). We sequenced 5 of the clones and all of them turned out to be paralogues of *tubulin*. Therefore the higher frequency of *tubulin* signals can be explained by the high expression rate of *tubulin* as well as by the existence of different paralogues of *tubulin*.

Our results show that moderately abundant genes can be identified and rapidly isolated using homologous as well as heterologous probes in a single hybridization step.

The libraries are now freely available. The filters as well as the protocols can be ordered at the Resource Center/Primary Database (RZPD). Identified clones can be obtained through the RZPD (RZPD, Resourcen-Zentrum-Primär-Datenbank, Heubnerweg 6, 14059, Berlin-Charlottenburg, http://www.rzpd.de/, libraryno. 554 and 555).

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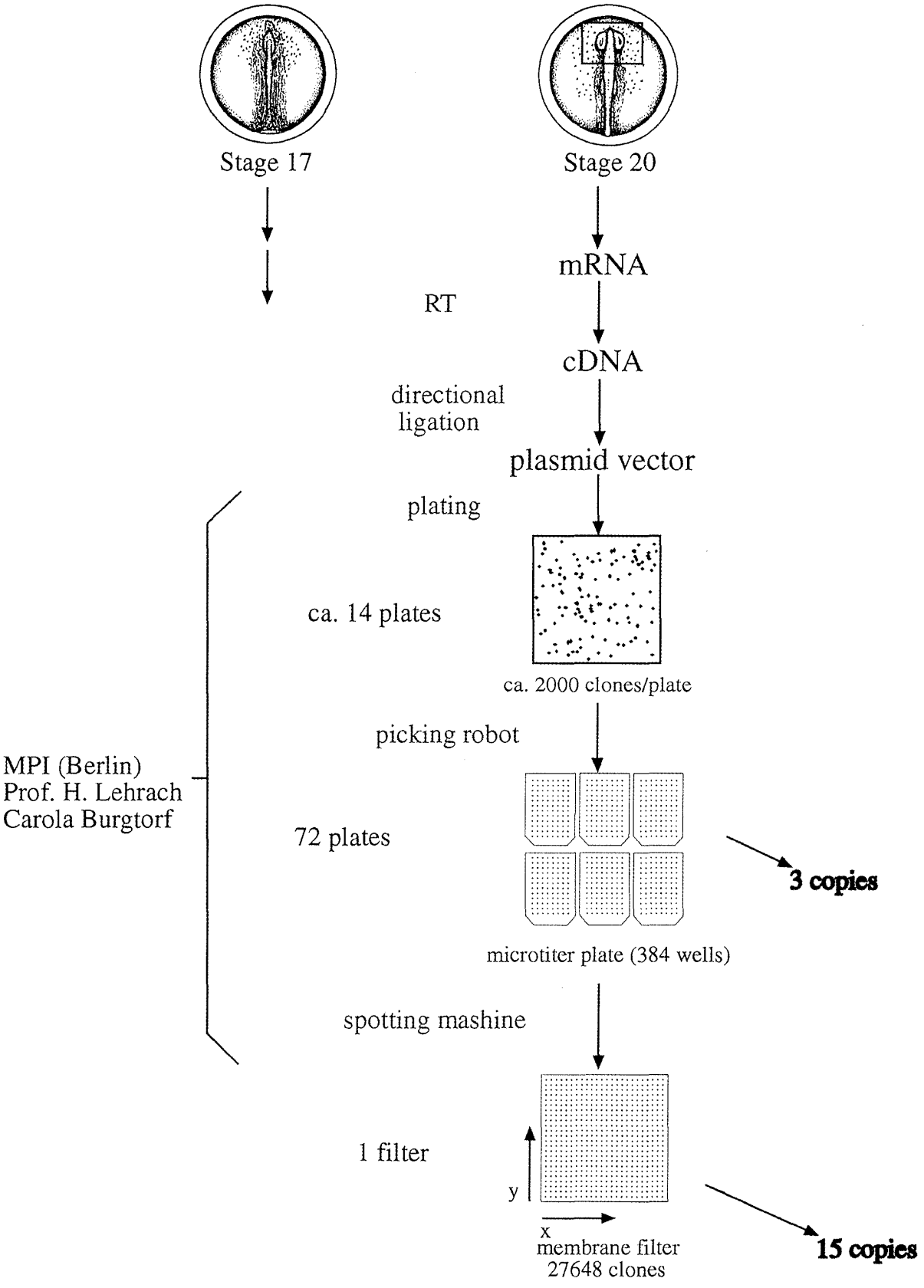

Fig. 1. Schematic drawing outlining the generation of the two spotted cDNA libraries.

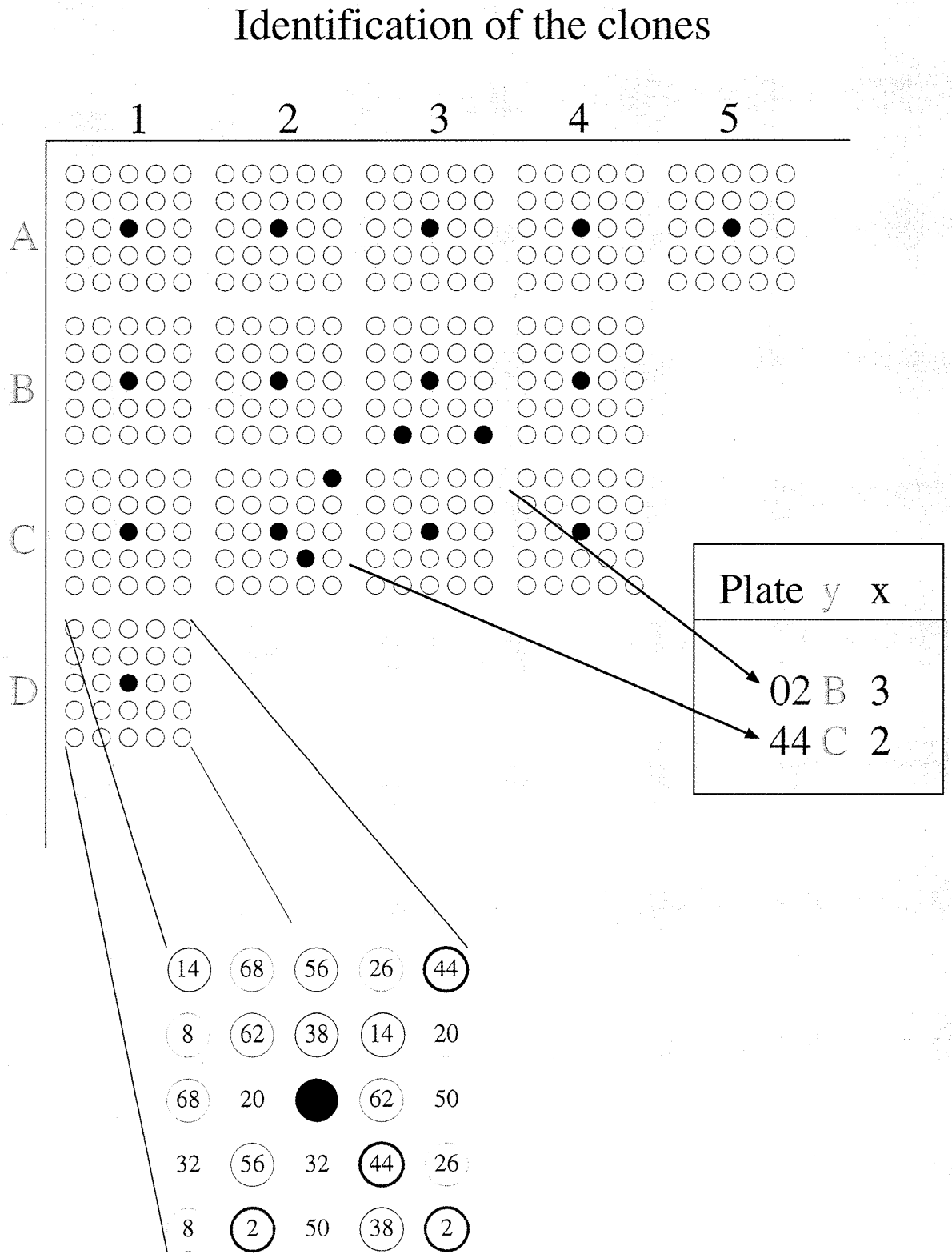

Fig. 2. Identification of individual clones: The relative position of two corresponding signals within a block determines the number of the microtiter plate. The absolute position of the block on the filter indicates the coordinates of the clone in this microtiter plate.

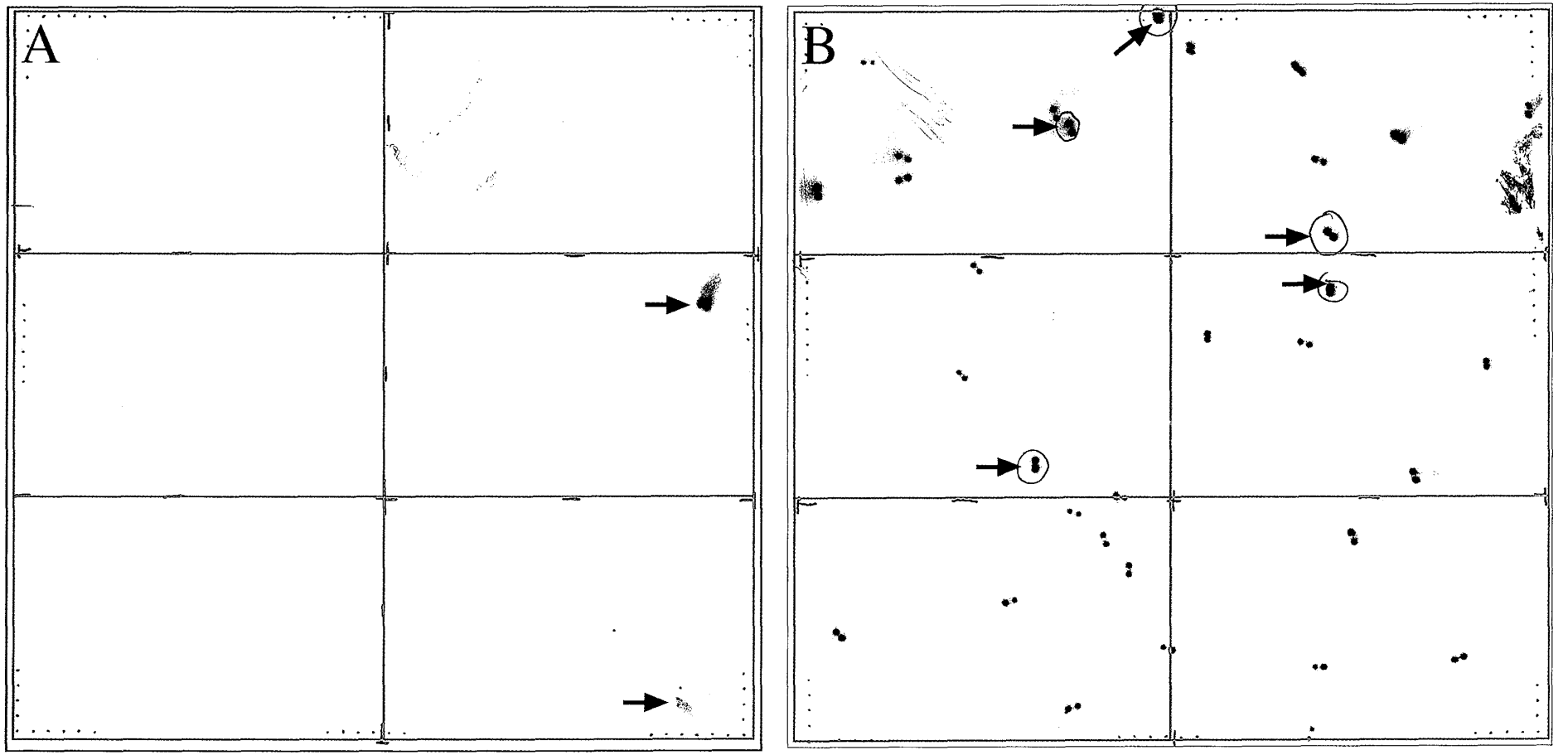

Fig. 3. Examples for hybridized filters. A. *Pax6* probe, stage 20 head specific library (#554) B. *Xltubulin* probe, stage 17 library (#555)
Both were hybridized and washed under high stringency conditions. Arrows indicate clones whose identity was confirmed by sequencing.