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Authors: Michio Ogasawara, Takuya Minokawa, Yasunori Sasakura, Hiroki Nishida, and Kazuhiro W. Makabe

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[SHORT COMMUNICATION]

A Large-Scale Whole-Mount *in situ* Hybridization System: Rapid One-Tube Preparation of DIG-Labeled RNA Probes and High Throughput Hybridization using 96-Well Silent Screen Plates

Michio Ogasawara[1※], Takuya Minokawa[2†※], Yasunori Sasakura[1], Hiroki Nishida[2] and Kazuhiro W. Makabe[1]*

[1]*Department of Zoology, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan*
[2]*Department of Biological Science, Tokyo Institute of Technology, Yokohama 226-8501, Japan*

ABSTRACT—Recent progress in multiple and automated-sequencing technology allows large-scale random cDNA sequencing, the so-called EST project, in various fields. In addition to the EST collection, the cDNA project requires analysis of spatiotemporal patterns of gene expression of a large number of clones by whole-mount *in situ* hybridization (WISH). To facilitate the multiple WISH procedures, we developed a protocol for rapid and uniform synthesis of multiple probes and multi-well based WISH processing. A DIG-labeled RNA probe for WISH was synthesized from a PCR-amplified template which contained an RNA promoter. All reactions of PCR and subsequent RNA synthesis were performed in a single tube by sequential addition of the reagents without phenol extraction or ethanol precipitation steps. An RNA probe was purified and condensed by a centrifugal ultrafilter to achieve high and stable purification efficiency. WISH of 96 samples were performed simultaneously in a 96-well plate attached to silent screen filters that were connected with a vacuum exhausting system. These processes eliminated the labor-intensive steps of WISH and provided opportunities to search for novel genes.

INTRODUCTION

As well as progress in EST sequencing analysis, large-scale analysis on expression patterns of genes is necessary in the embryological field. Whole-mount *in situ* hybridization (WISH) is an indispensable tool to directly analyze the spatiotemporal expression patterns of genes. WISH consists of two processes: one is preparation of probes, and the other is a hybridization system. Recently, a non-radioactive DIG-labeled probe has been widely used for WISH because it is safer and more convenient to handle than a radioactive probe. In addition, the sensitivity of detection of a DIG-labeled RNA probe is higher than that of a DIG-labeled DNA. However, synthesis of RNA probes requires several time-consuming steps such as digesting plasmid DNA by an appropriate restriction enzyme to linearize it for making a template DNA; the linearized DNA has to be extracted by phenol to remove enzymes including the restriction enzymes, DNase and RNase; after ethanol precipitation, RNA is transcribed from the template DNA under RNase-free conditions; synthesized RNA has to be precipitated by lithium to remove excess ribonucleotides; purified RNA needs to be subjected to alkaline hydrolysis for a probe of appropriate length; RNA probe is neutralized and precipitated by ethanol, and again dissolved in RNase-free distilled water. These steps make it difficult to prepare a number of different RNA probes of uniform quality. A typical problem is, for example, in the step of linearization of plasmid DNA. Some plasmid DNA cannot be digested by an appropriate restriction enzyme whose site is located in the upstream sequence of the cloning site. Others are cut out inside the cDNA, and most part of the template DNA may be lost. On

* Corresponding author: Tel. +81-75-753-4102;
FAX. +81-75-705-1113.
E-mail: kwmakabe@ascidian.zool.kyoto-u.ac.jp
† Present address: Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan.
※ These authors equally contributed to this work.

the other hand, whole-mount *in situ* hybridization also consists of many steps, most of which are labor-intensive. In WISH for EST analysis, a large number of different genes are analyzed. There is, however, no low-cost method which does not need an expensive automated machine for large-scale analysis on gene expression, except for a sectioned *in situ* hybridization system (Komiya *et al.*, 1997). Thus, we developed a rapid protocol for synthesis of DIG-labeled RNA probes and a multi-processing WISH system.

METHODS, RESULTS AND DISCUSSION

Rapid synthesis of DIG-labeled RNA probes

In general, rather than a large amount of a probe, a large number of probes are needed for WISH in cDNA projects. To synthesize a large number of DIG-labeled RNA probes of a uniform quality from each cDNA for large-scale analysis, we developed a single tube reaction protocol consisting of the following three steps: 1) generation of a template DNA by PCR for subsequent RNA synthesis, 2) synthesis of DIG-labeled RNA probes in the same tube by sequential addition of the reagents, 3) alkaline hydrolysis of the synthesized RNA probes for appropriate length by simple addition of solution. This protocol is not restricted to a microcentrifuge tube but also applicable to a 96-well plate. The RNA probe was then purified using a centrifugal ultrafilter. Since PCR amplification and RNA synthesis are performed in the same tube, the first step of PCR was carried out in a siliconized tube under RNase-free conditions.

The cDNA used was directionally inserted into the *Eco*RI-*Xho*I site of pBluescript SK(–) plasmid vector (STRATAGENE). The antisense RNA probe was synthesized from a T7 promoter. To obtain the linearized template DNA containing the whole cDNA sequence and promoter for T7 RNA polymerase, we used a set of PCR primers; SK (5'-CGCTCTAGAACTAGTGGATC-3') that are located in the upstream multicloning site, and T7 (5'-GCGTAATACGACTCACTATA-3') located on the T7 promoter flanking to poly(A) tail of the cDNA. Ten ng of plasmid DNA of each clone, which can be left over from the sequencing template, was amplified in 10 μl of PCR mixture (Table 1) under the three-step PCR condition (15 cycles at 94°C for 1 min, 50°C for 2 min, 72°C for 3 min). After the PCR amplification, 7.5 μl of PCR reaction was removed and run on a 1% agarose gel to check the amount and the length of the PCR product (Fig. 1A). Subsequently, 7.5 μl of additional DIG mixture (Table 1) containing the DIG RNA labeling mixture (Roche), RNase inhibitor and T7 RNA polymerase (GIBCO BRL) was added and incubated for 4 hr at 37°C. After the RNA synthesis, 1 μl of reaction was run on an agarose gel and the amount of product was checked (Fig. 1B).

Table 1. Protocol for rapid synthesis of DIG-labeled RNA probes and purification

Step 1. generation of linearized template DNA
a. add 9.5 μl of PCR mixture*1 to a siliconized 0.2ml microcentrifuge tube
b. add 0.5 μl of plasmid DNA (5-50 ng, typically 10 ng)
c. PCR (15 cycles at 94°C for 1 min, 50°C for 2 min, 72°C for 3 min)
d. remove 7.5 μl of reaction and run on a 1% agarose gel to check the length of amplified DNA
Step 2. synthesis of DIG labeled RNA probe
a. add 7.5 μl of additional DIG mixture*2 to the reaction
b. incubate at 37°C for 4 hr
c. remove 1 μl of the reaction and run on a 1% agarose gel to check the synthesized RNA
Step 3. alkaline hydrolysis
a. add 41 μl of alkaline mixture*3 to the reaction
b. incubate at 60°C for the appropriate time*4
c. chill on ice
Step 4. purification of DIG labeled RNA probe
a. put the reaction into the filter cup of ultrafilter
b. centrifuge at 4,000 x g for 4 min
c. add 100 μl of RNase-free H_20 to the filter cup and centrifuge at 4,000 x g for 4 min
d. repeat the previous step twice
e. add 6 μl of RNase-free H_20 to the filter cup and recover the probe

*1. PCR mixture (total 9.5 μl): 7.85 μl of RNase-free H_2O, 1.0 μl of 10x PCR buffer, 0.3 μl of 50 mM $MgCl_2$, 0.1 μl of 10 μM SK primer, 0.1 μl of 10 μM T7 primer, 0.1 μl of 2 mM 4dNTPs, 0.05 μl of rTaq DNA polymerase (5 U/μl) (TOYOBO). It is possible and recommended to prepare a large amount of master mix and make aliquots which can be stored at –30°C for at least a month.

*2. additional DIG mixture (total 7.5 μl): 4.0 μl of RNase-free H_2O, 2.0 μl of 5x T7 RNA polymerase buffer, 0.5 μl of 10x DIG RNA labeling mixture (Roche), 0.5 μl of 0.1M DTT, 0.25 μl of RNase inhibitor (40 U/μl)(TOYOBO), 0.25 μl of T7 RNA polymerase (50 U/μl) (GIBCO). Master mix without RNase inhibitor and T7 RNA polymerase can be stored at –30°C.

*3. alkaline mixture (final pH 10.2) (total 41 μl): 1.0 μl of 10 mg/ml yeast tRNA, 0.25 μl of 0.1M DTT, 40 μl of alkaline buffer (50 mM $NaHCO_3$, 75 mM Na_2CO_3, filtered). Prepare before use.

*4. duration time for alkaline hydrolysis: 10 min for 1.0 kb, 13 min for 1.5 kb, 15 min for 2.0 kb, 16 min for 2.5 kb, 16.6 min for 3.0 kb, 17.1 min for 3.5 kb, 17.5 min for 4.0 kb.

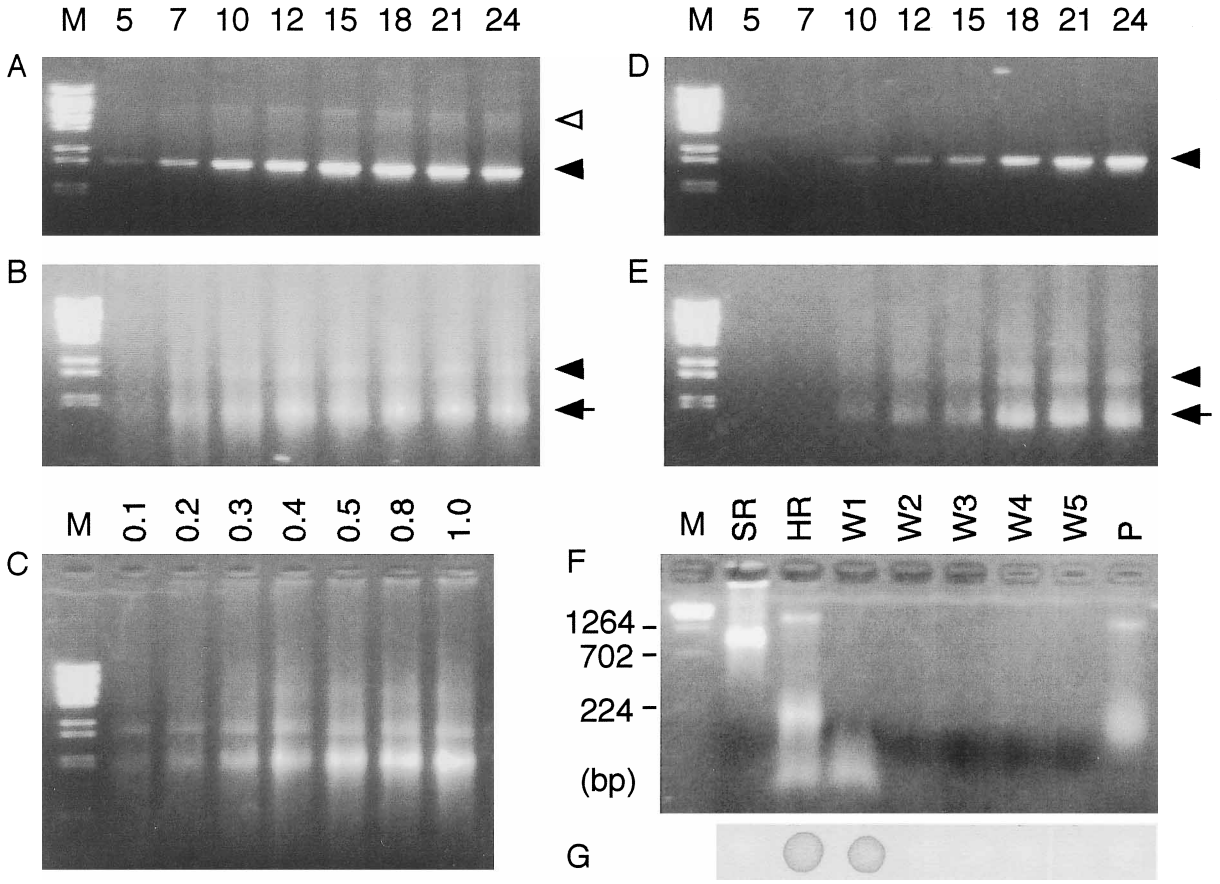

Fig. 1. **A–G**, Setting of conditions for synthesis of DIG-labeled RNA probe. **A–B**, 10 ng of plasmid DNA was amplified in different cycles of PCR (A), and DIG-labeled RNA was synthesized subsequently (B). Number on each lane indicates the cycles of PCR. *White arrowhead*, plasmid DNA; *Arrowhead*, amplified DNA; *Arrow*, synthesized DIG-labeled RNA; M, Lambda-*Bst*PI digested marker. **C**, Dose-dependent efficiency of DIG-labeled RNA synthesis under the different doses of DIG RNA labeling mixtures. Number on each lane indicates the proportion of contained DIG RNA labeling mixtures. Concentration for the original protocol (Roche) is indicated as 1.0. **D–E**, A bacterial colony which contains plasmid DNA was used as a starting material. The template was amplified in different cycles of PCR (D), and DIG-labeled RNA was synthesized subsequently (E). Number on each lane indicates the cycles of PCR. *Arrowhead*, amplified DNA; *Arrow*, synthesized DIG-labeled RNA; *M*, Lambda-*Bst*PI digested marker. **F–G**, Alkaline hydrolysis of synthesized RNA and purification of probe were monitored by agarose electrophoresis (F) and by a BTB pH test paper (dark, alkaline; light, neutral) (G). Samples used in (G) correspond to the above lanes in (F), respectively. *SR*, synthesized RNA; *HR*, hydrolyzed RNA; *W1-5*, wastes of the first-to-fifth wash; *P*, purified probe; *M*, Lambda-*Bst*PI digested marker.

Composition of reagents and condition of reactions were selected from the stand points of time saving, cost reduction and stability of the reaction. The concentrations of primers and 4dNTPs in our PCR mixture (Table 1) were reduced to one-tenth of the conventional concentrations so as to saturate the reaction after 15–20 cycles by shortage of the primers and/or 4dNTPs to obtain a constant amount, typically about 0.5 μg, of amplified product. The appropriate number of cycles of the PCR which was most effective for DIG-labeled RNA synthesis is shown in Fig. 1A and 1B. Fig. 1A shows template DNA amplified in a PCR cycle-dependent manner (arrowhead), and Fig. 1B shows the amount of RNA synthesized from each template DNA obtained from the above lane (arrow). About 12 to 24 PCR cycles were effective for subsequent RNA synthesis. The amount of the amplified template DNA in cycles of 12 to 15 seemed to be the same, and this indicated that the reaction of the PCR was saturated, as we expected. Therefore, we adopted 15 cycles for PCR. Synthetic efficiency, depending on the concentration of DIG RNA labeling mixture, is shown in Fig. C. We used half (0.5 in Fig. C) of the original concentration recommended by the manufacturer (1.0 in Fig. C).

We also performed a similar experiment using a bacterial colony which contained plasmid DNA as a starting material of PCR. As shown in Fig. 1D and 1E, amplified template DNA (arrowhead) and synthesized RNA (arrow) were consistently obtained. These results suggested that non-purified DNA is also suitable as a starting material in this rapid synthesis of DIG-labeled RNA probes. The most appropriate number of PCR cycles for a bacterial colony was 21 in our experiment. Comparison between the amount of PCR product and that of synthesized RNA (18, 21 and 24 cycles in Fig 1D and 1E) indicated that almost the same amount of DIG-labeled RNA was synthesized under these conditions.

Synthesized DIG-labeled RNA was subjected to alkaline hydrolysis at 60°C in the same tube by adding 41 μl of alka-

line mixture. The appropriate time for alkaline treatment depended on the length of the RNA (Table 1). In the conventional methods, synthesized RNA is precipitated once and dissolved in an alkaline mixture for hydrolysis, and then is precipitated and dissolved in water again before use. This time-consuming step of RNA probe purification was avoided by using the centrifugal ultrafilter (MILLIPORE ultrafree-MC centrifugal filter units, UFC3 LTK). Alkaline-hydrolyzed RNA probe was put into the filter cup of the ultrafree-MC centrifugal filter unit, and washed with RNase-free H_20 three times or more by centrifugation for 4 min at 4,000 x g. As shown in Fig. 1F, the size of alkaline-hydrolyzed RNA (HR) was reduced from the original synthesized RNA (SR). Waste 1 (W1), flow-through of the first centrifugation, contained a large number of small molecules causing background signals in WISH. In waste 2 (W2), which was obtained from addition of RNase-free H_2O to the filter cup and centrifugation, the number of small molecules was considerably lower. In wastes 3 to 5 (W3 to W5), we hardly detected any small molecules. After purification by centrifugation, purified probe (P) was sufficiently recovered from the bottom of the filter cup. The DIG-labeled RNA probe is unstable under alkaline conditions. Therefore, we checked the pH values of the wastes and purified probe using BTB pH test paper (TOYO ROSHI CO., LTD). As shown in Fig. 1G, an alkaline-hydrolyzed probe was neutralized after the second round wash (W2). These results demonstrated that purification and neutralization of RNA probe were quickly and simultaneously performed, and enough purified probe was obtained using the centrifugal ultrafilter system. In addition, the probe prepared by this method did not contain RNA that was too short to serve as a probe (< ca. 50 nt) which may affect the background after hybridization.

Using this method, we routinely prepare 48 different probes within 7 hr. Compared with the conventional method, production of the DIG-labeled RNA probe by this method is extremely rapid. Furthermore, the cost of this method was much lower than the conventional method despite the use of the centrifugal ultrafilter system that is relatively expensive.

Table 2. Protocol for 96-well based whole-mount *in situ* hybridization

Step 1. prehybridization (in a 1.5 ml microcentrifuge tube)
a. re-hydrate embryos through 50% and 30% ethanol at RT
b. wash twice with PBST*[1] at RT
c. incubate at 37°C for 25 min in 200 µl of 5 µg/ml proteinase K in PBST
d. wash twice with PBST
e. post-fix at RT for 1 hr in 4% paraformaldehyde in PBST
f. wash three times with PBST
g. rinse in 1:1, PBST:hybridization buffer*[2] at RT for 10 min
h. rinse in hybridization buffer at RT for 10 min
i. prehybridize in hybridizaion buffer at 45°C for 1 hr
Step 2. hybridization
a. transfer the embryos into a 3-cm petri dish containing hybridization buffer
b. pick up 15 embryos by micropipetter under a dissecting microscope
c. transfer embryos to each well of a silent screen plate (ca. 30 µl)
d. prepare 50 µl of hybridization mix containing 1 µg/ml of DIG labeled probe
e. add 50 µl of hybridization mix and seal both surfaces of the plate with sealing tapes
f. place in a plastic chamber and incubate at 45°C overnight (16 hr)
Step 3. post-hybridization wash (take 100 µl each by an 8-channel micropipetter)
a. wash twice with 4xSSC, 50% formamide, 0.1% Tween 20 at 48°C for 15 min
b. wash three times with 2xSSC, 50% formamide, 0.1% Tween 20 at 48°C for 15 min
c. wash twice with 1xSSC, 50% formamide, 0.1% Tween 20 at 48°C for 15 min
d. wash with 3:1, 1xSSC, 50% formamide, 0.1% Tween 20: PBST at RT for 10 min
e. wash with 1:1, 1xSSC, 50% formamide, 0.1% Tween 20: PBST at RT for 10 min
f. wash with 1:3, 1xSSC, 50% formamide, 0.1% Tween 20: PBST at RT for 10 min
g. rinse three times with PBST
Step 4. staining
a. incubate in 100 µl of 0.5% blocking reagent (Roche) in PBST at RT for 30 min
b. incubate in 100 µl of antibody solution*[3] at 4°C overnight
c. wash four times with PBST at RT for 10 min
d. rinse twice with 0.1M Tris-HCl (pH 9.5), 0.1M NaCl, 50 mM $MgCl_2$
e. add 100 µl of freshly prepared coloring solution*[4]
f. incubate in the dark at RT and monitor periodically for color reaction
g. stop reaction by adding 300 µl of 4% formaldehyde in PBST

*1. 8.0 g of NaCl, 0.2 g KCl, 2.9 g Na_2HPO_4-$12H_2O$, 0.2 g KH_2PO_4, 0.1% Tween 20 per liter.
*2. 6xSSC, 50% formamide, 5xDenhardt's, 100 µg/ml yeast tRNA, 0.1% Tween 20.
*3. 1/2000 diluted anti-DIG antibody (Roche) in PBST.
*4. 45 µl of 75 mg/ml NBT in 70% dimethylformamide (DMF), 35 µl of 50 mg/ml BCIP in DMF per 10 ml in solution used in step d.

This was mainly due to reduction of reaction scale of the RNA synthesis, which contains the expensive DIG RNA labeling mixture.

96-well based whole-mount *in situ* hybridization

Ascidian embryos were fixed in 4% paraformaldehyde in 0.1 M MOPS (pH 7.5), 0.5 M NaCl at 4°C overnight as described in Miya *et al.* (1994). Fixation must be optimized depending on species and tissues. A large amount of fixed embryos were dehydrated in a graded series of ethanol and stored in 80% ethanol at –30°C. Before use, the embryos were first manually dechorionated, then rehydrated in an ethanol series and substituted in PBST. During steps before hybridization, the embryos were processed in 1.5 ml microcentrifuge tubes, each of which contained 200 embryos at the same stage. The embryos were treated with 5 μg/ml of proteinase K at 37°C for 25 min, and were post-fixed in 4% paraformaldehyde in PBST at room temperature for 1 hr. After washing with PBST three times, they were rinsed in PBST: hybridization buffer (1:1) at room temperature for 10 min, then in hybridization buffer at 45°C for 10 min, and again in hybridization buffer for 1 hr as a prehybridization step. Conventionally the embryos were subjected to subsequent hybridization in a microcentrifuge tube. However, it is obviously difficult to process a large number of reactions at once. In this study, we adopted a 96-well silent screen plate (Nunc, catalog number 256073), which is attached to a Nylon 66 membrane (pore size, 3.0 μm) at the bottom, so that the simultaneous discharge of solutions out of 96 wells are possible using a vacuum. The embryos after prehybridization were distributed into wells of the 96-well silent screen plate. Each well can hold 12–20 embryos in 30 μl of hybridization buffer (typically 5 embryos each in 3 different developmental stages). Hybridization was carried out in 80 μl of hybridization buffer containing about 1 μg/ml DIG-labeled probe per well (typically prepared by adding 2 μl of probe into 50 μl of hybridization buffer) at 45°C for 16 hr. We found that a probe did not need to be heat-shocked, which saved time. A probe for a positive control was also always added to a few wells so the reaction could be checked. During hybridization, the silent screen plate was attached to a sealing tape (Nunc, catalog number 236366) at both top and bottom surfaces, and was placed in a plastic box to avoid contamination. Washing procedures were carried out as shown in Table 2 using a silent screen-plate vacuum manifold (Nunc, catalog number 256278) for discharge of solutions (Fig. 2). An RNase A-treatment step, which is present in the conventional method, was dispensable. This system, together with use of an 8- or 12-channel micropipetter for addition of solutions, enabled us to change solutions in 96 wells simultaneously and, therefore, much more rapidly compared with conventional ways, such as sucking solutions one by one. Another significant advantage to using this system is that there were no losses of embryos during the procedures, which was an implicit big problem for small embryos in conventional whole-mount *in situ* hybridization methods. After a blocking step, the silent screen plate containing

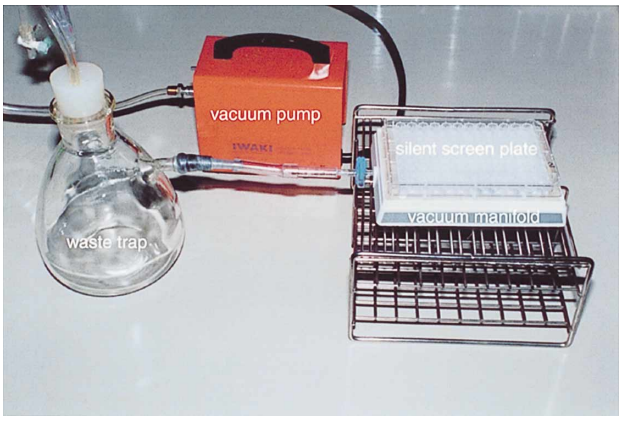

Fig. 2. Photograph shows the 96-well dish with vacuum exhausting system for WISH. A 96-well silent screen plate is attached on a vacuum manifold connected to a vacuum pump via a waste bottle.

anti-DIG antibody was sealed again and placed at 4°C overnight, since the extended duration in the antibody was found to enhance the sensitivity (data not shown). Staining was also carried out in a silent screen plate by adding 100 μl of coloring solution containing BCIP and NBT, and monitoring periodically for coloring reaction under a dissecting microscope up to about 6 hr in the dark at room temperature. During the reaction, the bottom of the plate was sealed to prevent the reagent solution from leaking. One problem with using a multiwell plate for whole-mount staining was the labor needed to stop the reaction by discharge of the coloring reagents and wash the stained wells one by one. We adopted a facile method to reduce labor; wells which were to be stopped also had an excess volume (300 μl) of 4% paraformaldehyde in PBST added. After termination of the reactions in all wells, the solutions were discharged at once and substituted with PBS. The staining of the embryos was stable for at least a few days in PBS. To take photographs, the embryos were transferred into a 3-cm petri dish, and were illuminated by an Olympus ring light (R66) attached to a dissecting microscope (Olympus SZX-12-3131; DF-PLAPO 1.2x; PE 5x). Images were recorded with an Olympus digital camera HC-300Z/OL-2.

We compared the staining images between embryos processed by this method and embryos processed by the conventional method (Miya *et al.*, 1994) using the clone 001A03 as a probe. The 001A03 encodes *HrWnt-5*; the maternal RNA is localized at the posterior pole in the 8-cell embryo (Sasakura *et al.*, 1998). As shown in Fig. 3A and B, there was no essential difference between the results of the two methods in terms of intensity of the signals and the background noise, although sensitivity in the conventional method appears to be slightly higher. The same results were obtained as to the early tailbud embryos using the clone 008F13 as a probe. The 008F13 encodes *HrDoublecortin* which is expressed in a subset of cells in the central nervous system, peripheral neurons and endodermal strand (Yagi and Makabe, unpublished) (Fig. 3C and D). Two other examples processed by this method are shown in Fig. 3E and F, demonstrating that this system can be used for other probes. These results indicated that a mul-

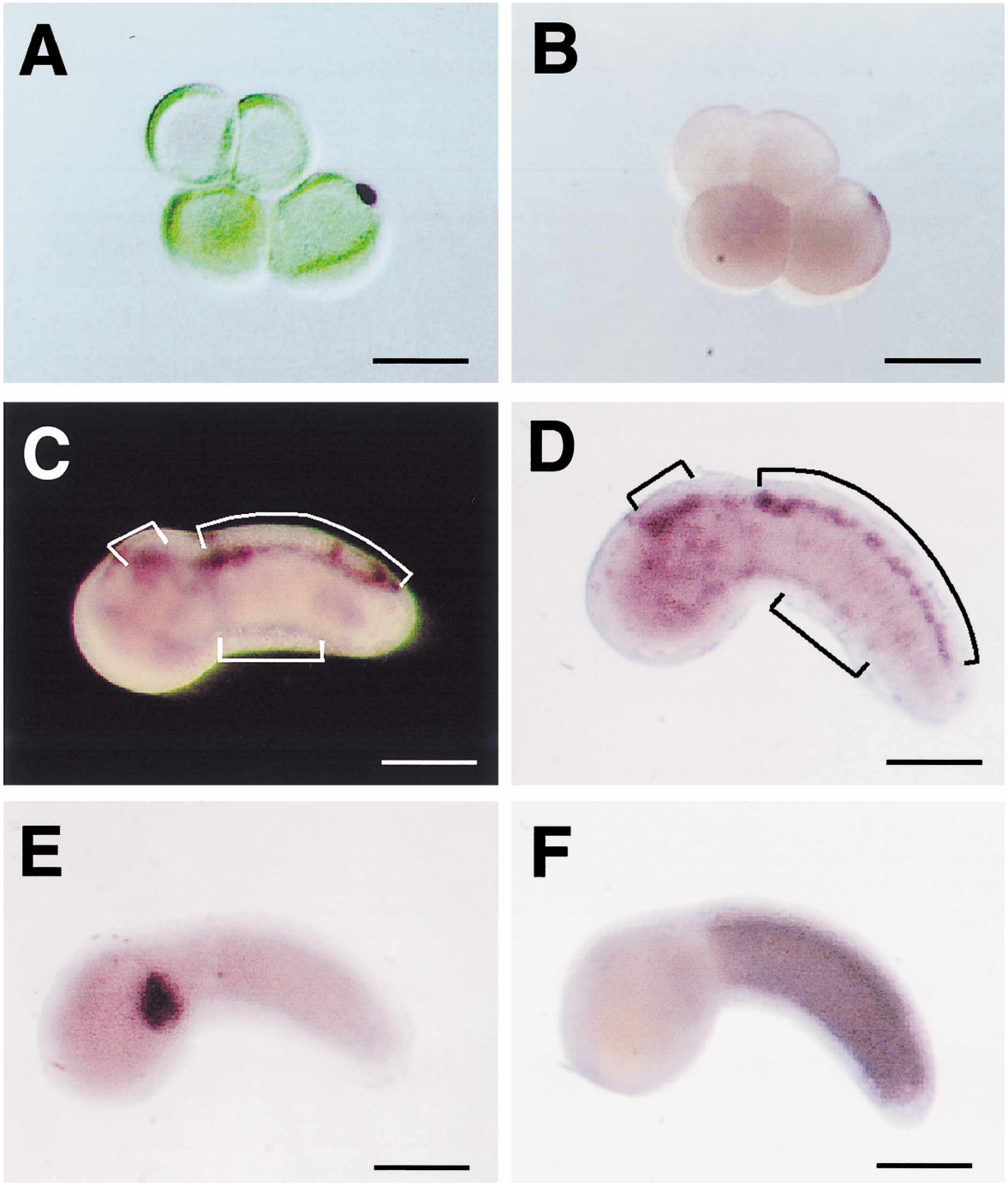

Fig. 3. Hybridization patterns of various probes in developmental stages of ascidian *Halocynthia roretzi*. **AB**, Probe 001A03, 8-cell embryos. **CD**, Probe 008F13, early tailbud embryos. The signals seen in the central neuvous system, peripheral neurons and endodermall strand are indicated by brackets. **AC**, Probe synthesis and in situ hybridization were done by the conventional method (Miya *et al.* 1994). **BD**, Probe synthesis and in situ hybridization were carried out by the method described in this paper. **E**, Probe 008H16, an early tailbud embryo. The signal is clearly seen in mesenchyme. **F**, Probe 006K12, an early tailbud embryo. The signal is seen in muscle. *scale bars* A–F, 100 μm.

tiple whole-mount *in situ* hybridization system, using a 96-well silent screen plate we developed, works as well as the conventional individual method.

The methods we developed are simple, but much time and labor can be saved both in probe preparation and hybridization processes. Using this method, we routinely accomplished a whole-mount *in situ* hybridization experiment of 96 different genes in 7 days, including a 2-day image processing. This is almost ten times as efficient in performance as conventional methods. The results of the large-scale in situ hybridization using the present methods can be seen in our cDNA project of the ascidian *Halocynthia roretzi* maternal mRNA, the MAGEST project (http://www.genome.ad.jp/magest/) (Kawashima *et al.*, 2000). Thus, these techniques

described here help us to carry out a large-scale analysis of gene expression based on an EST project which is widely performed in many animal species.

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