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Fluorescence In Situ Hybridization (FISH) Screening of Frozen Cell Lines in Large Numbers

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With the advance in molecular technology and the availability of commercial kits, fluorescence in situ hybridization (FISH) is increasingly being used in research and diagnostic laboratories. The introduction of interphase FISH further reduces the overall turnaround time (4). Most laboratories nowadays possess an archival collection of cellular specimens for record-keeping or research purposes. These specimens can either be in the form of established cell lines kept in liquid nitrogen for long-term storage or in fixed forms. If FISH is used on the former type of specimens for screening purposes, retrieval and resurrection of these cell lines will usually be required. The cells are thawed and cultured. Several passages are needed before the slides can be prepared (5). These steps are time-consuming and demand a waiting period of at least 1–2 weeks before the actual FISH screening can be carried out. Usually, there is an additional step of growing sufficient cells for refreezing to replenish the stock. The workload imposed on the laboratory staff for handling the cell lines is both costly and labor-intensive, especially if a considerable sample size is intended. Subsequently, for FISH analysis, one specimen is conventionally dropped onto a whole slide. This provides a vast excess of metaphase and interphase cells than is generally required for screening and again becomes very inefficient and wasteful. The procedure we describe here greatly simplifies these processes by completely eliminating all cell culture steps and allowing up to 20 samples to be analyzed on a single slide using a very small amount of sample materials.

Immediately after thawing a cell ampule, a 50–100- μ L aliquot is removed, and the ampule is refrozen promptly, according to standard protocol (5). Test studies on transformed lymphoblast cell lines, and a Chinese hamster ovary (CHO) cell line indicat-

ed that the viability of the cells decreases progressively with each successive freeze/thaw cycle. Despite that, an acceptable viability of about 40% was obtained that allowed successful resurrection of the cultures after 4 freeze/thaw cycles. This alleviates the need for stock replenishment every time a specimen is retrieved. The sample aliquot of cells is washed twice in phosphate-buffered saline (PBS) and then incubated in 0.56% KCl (aq) at 37°C for 15 min. This hypotonic treatment step is necessary to provide a more consistent nuclear morphology and better FISH signals, especially in variant in situ techniques such as primed in situ (PRINS) labeling (3). The cells are then fixed with 200 μ L of freshly prepared ice-cold methanol:acetic acid (vol/vol 3:1) fixative, after two washes with the fixative. The washes are conveniently performed in a 1.5-mL microcentrifuge tube at 1500 rpm for 10 min in a Sorvall® MC12V (Amrad® Pharmacia Biotech, Kew, VIC, Australia).

To streamline the screening of large number of samples, 20 specimens are dropped onto one single slide. A concentration of approximately 10^5 cells/mL will be optimal for a good metaphase spread. This is less critical if only interphase FISH is intended. The cell suspension is thoroughly mixed by gentle pipetting with a 20- μ L micropipet. A small volume of the cell suspension (ca. 2 μ L) is taken up into the micropipet by passive capillary action. Then, the specimen is applied onto the slide at the center of each numbered square according to the template grids shown in Figure 1. This template is designed to be aligned under a standard 25- $\times$ 75-mm slide. The grids divide the area under an ordinary 22- $\times$ 50-mm coverslip into 3 $\times$ 7 squares. Only 20 specimens are applied to simplify the numbering and labeling system. A quick touch of the micropipet tip on the glass surface will be sufficient to deliver enough cells onto the slide. The aliquot delivered is highly dependent on the contact time between the pipet tip and the slide. Excess fixative will be seen radiating outward from the point of application. Some spreading is needed to avoid the overlapping of individual nuclei and chromosomes in each specimen. The

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grids on the template delineate sufficient area on the slide for this spreading. The volume taken up by capillary action into the micropipet tip is sufficient for applying cells, on the same numbered grid position, onto 20–40 different slides to generate multiple duplicate slides. After the application of the specimens, the slides are dried overnight at room temperature and can be stored at -20°C with a dessicator for months. FISH can be performed on these slides according to established methods (2,6). Figure 2A represents a typical example of FISH on specimens prepared in this manner, showing well-spaced lymphoblast nuclei and good interphase hybridization signals. The number of metaphases in this type of preparation depends on the initial culturing conditions of the cells before freezing. The chromosomes in these metaphase cells are generally highly clustered (Figure 2A). Some alleviation of such clustering can be achieved by adding KARYOMAX-COLCEMID® Solution (Life Technologies, Melbourne, VIC, Australia) at a final concentration of $0.1\text{ }\mu\text{g/mL}$ to the thawed culture, followed immediately by PBS washing. No incubation is required because the purpose is to dissociate the chromosomes from the mitotic spindles.

In addition to the use of specimens stored under liquid nitrogen, we have also tested for the application of FISH in archival clinical specimens prepared for routine cytogenetic examinations and stored in methanol:acetic acid fixative at -20°C for as long as 8 years. These specimens are often no longer suitable for the routine banding tech-

niques due to the progressive disintegration of the chromosomes, but in general, FISH is still feasible. The chromosomes seem more “sticky”, and a good spread can only be obtained with sufficient dilution of the sample. However, exercise caution to avoid overdilution because this makes it difficult to find the sample spots on our numbered squares. A concentration of approximately 10^3 cells/mL should be appropriate for most situations. Figure 2, B and C, represent examples of FISH in these types of specimens, showing good signals for a unique sequence probe and a chromosome-specific paint probe, in addition to an α -satellite probe.

The above procedure could be used to determine the genomic location of a particular DNA fragment using somatic cell hybrid mapping panels, consisting of spotted hybrid specimens that contain different known human chromosomes or deletion fragments on one slide. Precise genomic location of the DNA fragment of interest could then be deduced by analyzing the concordance of interphase FISH signals in different

somatic cell hybrids on these mapping panel slides. This would provide an alternative approach to the other more conventional ways of cytogenetic and molecular mapping strategies.

The protocol described provides a quick and convenient method using FISH as a screening strategy on a large number of specimens. This protocol has been used successfully in our laboratory on a number of large-scale screening programs involving frozen lymphoblast and fibroblast cell lines. The omission of the culturing steps resolves a major bottleneck in the workflow of any screening procedure involving FISH. The application of 20 specimens on a slide, and the simultaneous hybridization with one probe significantly reduces the time, the labor and the cost of the screening. The reasonably good integrity of cells stored in fixative for up to 8 years offers an additional useful and ready resource for FISH studies. With robotics, it is possible to automate the various stages of slide processing. In addition, the amount of specimens and the position

Figure 1. Standard slide template for 20 specimens. This template is generated using the Microsoft's Paintbrush software of a slide with a grid pattern as shown. Twenty such slide patterns can be fitted onto an A4 paper to allow serial spotting of duplicates. Standard $25 \times 75\text{-mm}$ slides are placed over the template patterns, with the edges of the slide in the specimen area carefully aligned to act as a guide for sample application.

Figure 2. FISH of uncultured human lymphoblasts and archival cytogenetic samples. (A) FISH of uncultured human lymphoblasts directly prepared from liquid nitrogen storage using a chromosome 11-specific, α -satellite probe (Oncor, Gaithersburg, MD, USA). The arrow points to a metaphase cell with tightly bunched chromosomes. Two strong chromosome 11 signals are detected on this cell and on the other interphase nuclei. (B) FISH on a clinical cytogenetic specimen that has been kept under methanol:acetic acid fixatives for 8 years using a chromosome 10-specific, centromeric α -satellite probe, pZ10-1.3 (double arrows) and a 80-kb, 10q25-specific, genomic probe, E8 (single arrows) (1). (C) FISH on archival clinical cytogenetic specimens using a chromosome 10-specific paint probe (COATASOME® 10; Oncor) showing 2 distinct domains at interphase and the corresponding specific painting signals on metaphase chromosome 10. All probes described were digoxigenin-labeled and detected by a single round of incubation with anti-digoxigenin-fluorescein, Fab fragment (Boehringer Mannheim Pty. Ltd., Castle Hill, NSW, Australia). Nuclei and chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Boehringer Mannheim). Gray-scale images were captured with a cooled charged-coupled device (CCD) camera (Photometrics, Tucson, AZ, USA) by Axioskop® Epifluorescence Microscope (Carl Zeiss Pty. Ltd., Carnegie, VIC, Australia) equipped with a $100\times$ objectives and the appropriate filter sets. Images were merged and processed with the IP Lab Spectrum software Version 2.5.5. (Signal Analytics, Vienna, VA, USA) running on a Macintosh® PowerMac® (Apple Computer, Cupertino, CA, USA) personal computer system. Background was reduced using a threshold algorithm according to the manufacturer's recommendations.

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of application can be more precisely controlled, and therefore, a higher density of spotting should be achievable. This work represents a useful step towards incorporating FISH as a robust screening procedure in diagnostic laboratories and for genome research.

REFERENCES

1. **Barry, A.E., E.V. Howman, M.R. Cancilla, R. Saffery and K.H.A. Choo.** Sequence analysis of an 80-kb human neocentromere. *Hum. Mol. Genet.* (In press).
2. **Choo, K.H.A.** 1994. *In Situ* Hybridization Protocols. Humana Press, Totowa.
3. **Gosden, J. and G. Scopes.** 1996. Uncultured blood samples can be labeled by PRINS and ready for chromosome enumeration analysis 1 h after collection. *BioTechniques* 21:88-91.
4. **Kuo, W.-L., H. Tenjin, R. Segraves, D. Pinkel, M.S. Golbus and J. Gray.** 1991. Detection of aneuploidy involving chromosomes 13, 18, and 21, by fluorescence *in situ* hybridization (FISH) to interphase and metaphase amniocytes. *Am. J. Hum. Genet.* 49:112-119.
5. **Phelan, M.C.** 1997. Techniques for mammalian cell culture, p. A.3F.1-A.3F.11. In F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman and K. Struhl (Eds.), *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.
6. **Pinkel, D., T. Sraume and J.W. Gray.** 1986. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proc. Natl. Acad. Sci. USA* 83:2934-2938.

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