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Cryopreserved Cell Monolayers for Rapid Detection of Herpes Simplex Virus and Influenza Virus

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Cryopreserved cell monolayers are a new cell culture technology intended to ensure the availability of cells in the laboratory for virus detection. Two cryopreserved cell monolayers, ELVIS for the detection of herpes simplex virus (HSV) and R-Mix for the detection of influenza virus, were evaluated. The results indicated that fresh and cryopreserved cell monolayers are comparable in sensitivity for the detection of HSV and influenza virus. The cells retain the same level of sensitivity for up to 4 months at −80°C.

A diagnostic virology laboratory generally receives fresh cells from commercial sources once or twice a week. Receipts of commercially prepared cells can be compromised by shipping delays, mishandling of packages, and exposure to temperature stress, which may lead to suboptimal performance. Additionally, because these shipments are estimated standing orders, shortage or overage is common. Finally, the quality of cell monolayers is variable from lot to lot and difficult to control and standardize. These fundamental shortcomings of commercially prepared cells are generally accepted by clinical virology laboratories because the only functional alternative, i.e., preparing one's own cells each week, is generally impractical for technical and/or economic reasons.

Recently, Diagnostic Hybrids Inc. (DHI, Athens, Ohio) developed a cryopreservation method that addresses the practical issues cited above. In this study, we compare two sensitive cell culture systems, ELVIS cells for the rapid detection of herpes simplex virus (HSV) (5, 7) and R-Mix (mixture of A549 and mink lung) cells for the detection of influenza viruses A and B (1, 2, 3, 4, 6), in both frozen and nonfrozen monolayer formats, to determine whether frozen monolayers can match the virus detection performance of fresh, commercially prepared monolayers.

The cryopreserved ELVIS and R-Mix cell monolayers (ready cell frozen monolayers [RCFM]) were provided on a glass coverslip in shell vials by DHI. RCFM were shipped on dry ice and transferred quickly to storage in a −80°C chest freezer. The nonfrozen cell equivalents were commercially produced and shipped by express courier, as is routinely done. Prior to inoculation with clinical specimens, cryopreserved ELVIS RCFM and R-Mix RCFM vials were removed from the −80°C freezer and placed in an empty 24-well cluster plate. The plate was gently placed in a 35 to 37°C water bath such that the water level was just high enough to flood the plate. The vials were incubated for 4 min (±15 s) without any agitation. The thawed vials were gently removed from the water bath, and the freeze medium was immediately removed from the vials by gentle aspiration.

For ELVIS RCFM, 1 ml of ELVIS replacement medium (DHI) was added to each vial and 0.2 ml of clinical specimen was inoculated into both RCFM and fresh cells. All shell vials were centrifuged at 700 × *g* for 60 min at room temperature and incubated at 35 to 37°C for 20 to 24 h. ELVIS cells were fixed and stained for HSV detection and typing by using the ELVIS HSV ID/typing test system (DHI) as previously described (4) and according to the manufacturer's instructions. The staining buffer is a mixture of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), which detects the HSV-induced production of the β-galactosidase reporter gene; two HSV type 2 (HSV-2)-specific mouse immunoglobulin G monoclonal antibodies (MAbs) conjugated with fluorescein isothiocyanate (FITC); and two HSV type 1 (HSV-1)-specific unlabeled MAbs. After being stained for 60 min, the ELVIS monolayer was examined under a light microscope for the appearance of blue cells, indicating an HSV-positive specimen. The coverslips with blue cells were subsequently examined under a fluorescence microscope. If specific fluorescent cells were observed, the specimen was designated HSV-2 positive. If fluorescent cells were not observed, the cells were washed with phosphate-buffered saline to remove the mounting fluid and stained with FITC-conjugated anti-mouse immunoglobulin G for examination to confirm the presence of HSV-1. As controls, HSV-1- and HSV-2-positive and -negative specimens were used to infect cells of both preparations and were processed in the same manner as the clinical specimens to ensure the quality of the blue cells and fluorescent cells used for HSV-1 and HSV-2 typing.

For R-Mix RCFM, the vials were rinsed two times with 1 ml of R-Mix serum-free replacement medium with trypsin to facilitate the efficient removal of freeze medium containing a high level of protein. Additionally, the thawed and washed R-Mix RCFM were incubated at 35 to 37°C for 4 h, the medium was removed again to eliminate the protein residue in the freeze solution, and the same, fresh medium was added prior to inoculation with 0.2 ml of clinical specimen. If not used immediately, the thawed cells retained a similar level of sensitivity for 2 days (the number of days tested). Fresh cells were treated identically by washing out the shipping medium and

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TABLE 1. Comparison of ELVIS fresh cells and ELVIS RCFM for the detection of HSV

HSV type	Sample	No. of positive infected blue cells/shell vial[a]	
		Fresh	RCFM
1	1	TNTC	TNTC
	2	TNTC	TNTC
	3	9	7
	4	90	73
	5	0	0
	6	TNTC	TNTC
	7	12	10
	8	TNTC	TNTC
	9	57	65
	10	186	136
	11	TNTC	TNTC
	12	38	30
	13	TNTC	TNTC
2	1	4	1
	2	12	16
	3	TNTC	TNTC
	4	TNTC	TNTC
	5	4	7
	6	43	20
	7	TNTC	TNTC
	8	43	50
	9	2	4
	10	20	24
	11	TNTC	TNTC
	12	5	6

[a] TNTC, too numerous to count.

incubating the cells for 4 h prior to inoculation with the clinical specimen.

Twenty-five HSV-positive specimens (13 for HSV-1 and 12 for HSV-2) and 25 negative specimens, as determined by previous culturing with H&V mix cells (DHI) and antibody confirmation by the MicroTrak HSV-1/HSV-2 culture identification/typing test (Syva Co., San Jose, Calif.), were used in this study as frozen specimens. Twenty-four specimens were identified as HSV positive by use of both ELVIS fresh cells and ELVIS RCFM. The intensity of blue cells and the specific fluorescent-antibody signal were indistinguishable between the two different cell culture formats (data not shown). The number of positive blue cells is shown in Table 1. In almost half of the specimens, the number of infected blue cells exceeded counting capacity, but the numbers of blue cells were approximately equal in both cell culture formats. One specimen identified originally as containing HSV-1 could not be recultured in either ELVIS format after freezing and thawing. This specimen was originally a very-low-titer specimen and may have lost the infectious virus after freezing and thawing. The number of positive blue cells recovered from ELVIS fresh cells infected with HSV-1 was highly correlated with that recovered from RCFM (Pearson's r^2, >0.98; P, 0.0001), and a paired Student t test (t, 1.35; P, 0.2) revealed no difference. A similar analysis applied to the 12 HSV-2-positive specimens (Pearson's r^2, >0.98; P, 0.0001) (t, 0.2; P, 0.85) disclosed no difference. The 25 negative specimens were found negative upon retesting.

To reflect a real clinical setting, 146 clinical samples for which HSV culturing was requested were compared in ELVIS fresh cells and ELVIS RCFM. These specimens included 76 from genital sites, 44 from skin, 19 from the mouth, and 7 from the throat. The specimens were collected in M4-RT viral transport medium (Micro Test, Inc., Liburn, Ga.). All specimens were processed, inoculated, and identified as described above. Of these 146 specimens, 43 were found positive for HSV, including 23 HSV-1- and 20 HSV-2-positive specimens. ELVIS fresh cells detected 22 HSV-1-positive and 20 HSV-2-positive specimens, while ELVIS RCFM detected 23 HSV-1- and 19 HSV-2-positive specimens. The amounts of blue cells were very similar even for specimens with only a few positive blue cells. However, ELVIS fresh cells failed to identify one HSV-1-positive specimen which had a positive result on ELVIS RCFM. At the same time, ELVIS RCFM failed to identify one HSV-2-positive specimen which had a positive result on ELVIS fresh cells. ELVIS fresh cells and ELVIS RCFM were strikingly similar for the detection of HSV in clinical specimens.

Twenty influenza virus A-positive and 5 influenza virus B-positive clinical specimens from the 1999 and 2000 influenza virus seasons, previously identified by direct antigen detection with a direct immunofluorescence assay (Chemicon International, Temecula, Calif.), were retrieved from the −80°C freezer and inoculated into shell vials of both R-Mix fresh cells and R-Mix RCFM. Both preparations of cell monolayers were intact and healthy. All shell vials were centrifuged at $700 \times g$ for 60 min at room temperature and incubated for 20 to 24 h; samples were stained with FITC-labeled influenza virus A- or B-specific MAbs (DHI) for 30 min and examined by using a fluorescence microscope. The results showed that all specimens were positive in both cell formats, and the numbers of positive fluorescent cells were quite similar, except that the majority of influenza virus B-positive specimens showed slightly more positive results on R-Mix RCFM (data not shown).

To further analyze the sensitivity of R-Mix fresh cells and R-Mix RCFM for the detection of influenza viruses A and B, eight influenza virus A-positive and eight influenza virus B-positive original patient specimens were prepared as three fourfold serial dilutions and inoculated into two vials of each cell format. The inoculated cells were processed as described above. As shown in Table 2, analysis of variance with Dunnett's t tests, with stratification for the three dilutions used, indicated that the numbers of immunofluorescence-positive cells recovered from R-Mix fresh cells and R-Mix RCFM each exposed to eight samples containing influenza virus A did not differ significantly. Specifically, the titers measured in the R-Mix fresh cells were highly correlated with those measured in R-Mix RCFM (all Pearson's r^2 values, >0.88; all P values, <0.01), and a paired t test analysis disclosed no difference (t, 0.772; P, 0.44) between the two preparations of infected cells. A similar analysis applied to eight different samples containing influenza virus B (Table 2) revealed that although the results were highly correlated (r^2, 0.90; P, <0.01), consistently fewer positive cells were recovered from R-Mix fresh cells than from R-Mix RCFM. Consequently, parametric or nonparametric paired analyses disclosed a significantly higher number of positive cells for samples plated on R-Mix RCFM than for samples plated on R-Mix fresh cells (t, 2.48; P, <0.05) (U′, 1,823; P, <0.025). These results suggested that R-Mix fresh cells and R-Mix RCFM were comparable in sensitivity for the detection of influenza viruses A and B.

To assess the stability of ELVIS RCFM and R-Mix RCFM

TABLE 2. Comparison of R-Mix fresh cells and R-Mix RCFM for the detection of influenza virus

Influenza virus	Sample	Cells	No. of immunofluorescence-positive cells/shell vial at the following dilution of original sample[a]:		
			1:4	1:16	1:64
A	1	RCFM	5, 7	5, 0	1, 1
		Fresh	5, 6	2, 0	0, 0
	2	RCFM	192, 193	45, 41	14, 19
		Fresh	219, 217	61, 68	12, 14
	3	RCFM	12, 6	1, 0	0, 2
		Fresh	17, 6	0, 0	0, 0
	4	RCFM	5, 6	2, 1	0, 0
		Fresh	11, 12	4, 3	2, 1
	5	RCFM	23, 20	11, 10	2, 4
		Fresh	69, 68	30, 23	6, 4
	6	RCFM	15, 16	5, 2	2, 3
		Fresh	18, 19	8, 10	2, 4
	7	RCFM	10, 11	4, 6	2, 1
		Fresh	12, 11	6, 7	3, 3
	8	RCFM	8, 6	1, 0	0, 0
		Fresh	10, 8	2, 1	0, 0
B	1	RCFM	51, 49	21, 19	6, 8
		Fresh	36, 31	17, 17	6, 7
	2	RCFM	90, 86	20, 21	4, 5
		Fresh	56, 54	9, 11	4, 4
	3	RCFM	128, 120	22, 21	6, 8
		Fresh	124, 129	19, 20	6, 7
	4	RCFM	186, 138	47, 46	14, 13
		Fresh	148, 113	14, 20	3, 2
	5	RCFM	46, 45	23, 19	4, 8
		Fresh	38, 29	11, 13	2, 4
	6	RCFM	35, 31	7, 7	2, 4
		Fresh	36, 30	9, 10	4, 2
	7	RCFM	70, 69	39, 45	13, 16
		Fresh	79, 75	45, 51	9, 11
	8	RCFM	64, 71	19, 19	9, 6
		Fresh	79, 70	24, 20	8, 9

[a] Results from duplicate shell vials for each sample are shown.

stored at −80°C, duplicate shell vials of both fresh cells and RCFM were inoculated with previously tested specimens having known concentrations (to generate significant counts) of HSV-1 and HSV-2 (ELVIS) and influenza viruses A and B (R-Mix). Due to different periods of storage, three lots of cells were used for comparison. Thus, shell vials with similar densities of cell monolayers were selected prior to specimen inoculation. The numbers of positive cells were counted after overnight incubation and staining as described above. The results (Table 3) showed that both ELVIS RCFM and R-Mix RCFM retained high sensitivities for up to 4 months. Interestingly, influenza virus B produced more positive cells in R-Mix RCFM than in R-Mix fresh cells, consistent with the results of the sensitivity analysis (Table 2).

This study, while small, represents the first published demonstration that cryopreserved cells can perform substantially the same as commercially prepared fresh cells for virus detection. For the detection of HSV, both fresh and frozen clinical samples were compared in ELVIS fresh cells and ELVIS RCMF. The strikingly similar results suggested that they were comparable for both HSV-1 and HSV-2. The comparison of R-Mix fresh cells and R-Mix RCFM for the detection of influenza viruses A and B was done with frozen specimens and dilutions because of the nature of the influenza virus season and because fresh specimens were difficult to obtain routinely. The data showed that R-Mix fresh cells and R-Mix RCFM were comparable in sensitivity for the detection of influenza virus or might even have been better for the detection of influenza virus B. After proper recovery from the frozen state by use of a simple and rapid thawing protocol, the cells maintained their healthy morphology and were indistinguishable from their freshly prepared counterparts. Taken together, the results obtained with ELVIS cells and R-Mix cells strengthen the notion that cryopreserved cells can dramatically change how cell cultures are used not only by clinical virology laboratories but also by small microbiology laboratories, where having small volumes of frozen cells on hand could represent a cost-effective way to perform selected virology tests.

TABLE 3. Stability of cryopreserved cells (RCFM) for the detection of viruses

Cell type[a]	No. of cells/shell vial positive for[b]:			
	HSV-1	HSV-2	FluA	FluB
Fresh	19, 21	5, 7	45, 61	14, 22
RCFM 3m	18, 22	7, 11	36, 27	38, 20
RCFM 4m	16, 18	4, 8	52, 49	50, 101

[a] RCFM 3m, after 3 months at −80°C; RCFM 4m, after 4 months at −80°C.
[b] Number of infected blue cells positive for HSV and number of infected immunofluorescent cells positive for FluA and FluB. Results from duplicate shell vials for each cell type are shown.

Cryopreserved cells have the following attractive benefits. (i) They are stable for up to 4 months under proper storage conditions. (ii) They are always on hand for variable volumes of test requests. (iii) A supply in the form of a large shipment reduces interlot variations over time. (iv) They allow time for evaluation prior to clinical use. (v) All specimens are processed by using cells aged for the same period of time, i.e., less than 1 day postthawing. (vi) Tighter inventory control is possible because of the use of only the number of cell monolayers needed. (vii) Finally, they eliminate the need, cost, and exposure to various shipping stresses associated with the receipt of weekly shipments.

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