CHAPTER IV

RESULTS

1. Results of Preparation of Viral Antigens

1.1 Preparation of cell culture

HeLa and Vero cells were grown in MEM. Suspended cells became a completed monolayer in 24 h at 37°C. After inoculation, the infected monolayer showed 3^+ to 4^+ CPE. At which point the antigen was collected. This study found that the amount of 10 bottles of cell culture yielded 1.5 ml of antigen.

1.2 Preparation of stock virus

Herpes simplex virus was propagated in HeLa and Vero cells. One hundred ml of each stock virus pool was prepared and divided in small aliquots and kept at -70°C until used.

1.3 Titration of stock virus

Titer of the virus was determined by plaque forming assay. The titers in Vero and HeLa cells were 1.3×10^7 and 2.0×10^7 PFU/ml, respectively.

1.4 Comparision in the methods of viral inactivation

For safety precaution, the infections viruses

were inactivated prior to antigen preparation used for coating the ELISA plate. Three methods of viral inactivation were carried out with the use of UV lamp, 0.1-1% formalin, and heat. As shown in Table 1, UV-light could not completely inactivate virus in the duration ranging from 0 to 75 min. On the other hand, the inactivation of virus by formalin, cannot detect CPE. Heat inactivation at 50°C, however, completely inactivated virus by 15 to 60 min (Table 2).

1.5 Selection of antigen

1.5.1. Comparison of HSV antigen preparations in minimal essential medium and phosphate buffer saline

HSV was propagated in Vero cells for preparing two kinds of antigens. The first antigen was prepared in MEM without serum. The second antigen was prepared in PBS. Antigens prepared from both methods were then tested by ELISA using commercial antiserum. In Table 3 showed that HSV antigens prepared in PBS was positive with commercial HSV antiserum while antigens prepared in MEM without serum showed negative result.

1.5.2 Comparison of HSV antigen preparations in HeLa and Vero cells

HSV was propagated in HeLa and Vero cells. The viral antigen was subsequently prepared in PBS. Three sera used in this test was previously tested

by CF and IFA, and were designated as strong-positive serum (CF 1:32, IFA 4^{+}), weak-positive serum (CF < 1:4, IFA 1^{+}), and negative serum (CF <1:4, FA -). As shown in Table 4, antigen prepared from HeLa cells was positive by ELISA with optical density higher than that prepared from Vero cells.

2. Determination of Factors Affecting the ELISA System

2.1 Optimal conditions of ELISA assay for the detection of HSV-antibody

Several variable conditions in the ELISA system were examined in order to obtain the optimal conditions for the detection of IgG antibody to HSV as shown in Table 5.

2.1.1 Determination the optimal concentration of antigen.

Preliminary experiments were carried out in order to determine the optimal antigen concentration to discriminate between HSV-positive and negative sera. Viral and control antigen concentrations of 20, 40, 80, and 160 µg protein/ml were tested with known positive - and negative sera. No significant difference in reactivity was found when antigen concentrations varied between 80 and 160 µg/ml, as shown in Figure 8. An antigen concentration of 80 µg protein/ml was therefore chosen for further work.

2.1.2 Determination for optimal dilution of antibody.

In Table 5, antigen concentations of 80 µg/ml was coated onto the wells of microtiterplate. The dilutions of sera were 1:50, 1:100, and 1:200 in PBS-Tween-albumin. HSV antigen reacted with the serum dilution of 1:50 gave the highest absorbance while control antigen also showed the high absorbance value (Data was not shown). On the other hand, serum dilution 1:100 gave high absorbance value whereas, control antigen gave low absorbance value. In this study, therefore, serum dilution of 1:100 was used for screening test of HSV-antibody in human serum.

2.1.3 Determination for the optimal dilution of conjugate

An optimal dilution of enzyme - conjugated rabbit antibody to human IgG is essential for minimizing nonspecific binding of the conjugate. Therefore, the optimal dilution of conjugate was determined by titration before use. Microtiterplate coated with antigen of HSV and Hela antigen were prepared as previously described and were allowed to react with 1:100 dilution of serum known to be positive for HSV. After appropriate washings, the wells were incubated with

various dilutions of conjugate (1:1,200, 1:2,400, 1:4,800, and 1:9,600). The assay was then carried out as described, and the appropriate dilution of conjugate was determined (Fig. 9). The use of a 1:2,400 dilution of conjugate resulted in optimal specific activity, therefore, this dilution was selected for further use.

2.2 Determination for optimum of time and temperature for antigen-coating

Polystyrene microtiter plate was coated with HSV and control antigens. In this study, the antigen of 80 μg protein/ml was coated. The incubation period and temperature were varied from 4 $^{\circ}$C overnight, room temperature overnight, 37 $^{\circ}$C for 2 h, 6 h and overnight (Table 6). The results showed that the highest absorbance value was at 4 $^{\circ}$C overnight for temperature and time in coating antigen.

2.3 Determination for optimum of time-course for:

2.3.1 HSV antigen and antibody reaction

The effects of time on the antigen-antibody reactions in ELISA procedure were investigated for the maximal binding. The dilution 1:100 of known positive and negative sera to HSV were incubated at 37 $^{\circ}$C for 30, 60, 90, and 120 min (Fig. 10). The conjugate was added and further incubated at 37 $^{\circ}$C for 1 h. Finally, color was developed by an incubation with substrate at

room temperature for 30 min. The results indicated that activity increased with increasing time to a maximum at 90 min of the serum incubation.

2.3.2 Antibody and conjugate reaction

Maximal binding of both primary- (serum) and secondary (conjugate) antibody is essential to attain high levels of sensitivity. Accordingly, the assay of 1:100 dilution of a positive antiserum to HSV were incubated with the antigen at 37 $^\circ$C for 90 min. The reaction of bound serum and conjugate was incubated at 37 $^\circ$C for 15, 30, 60, 90, and 120 min. As shown in Fig. 11, maximum absorbance appeared during incubation period for the primary antiserum and the conjugate was at 37 $^\circ$C for 30 min.

2.3.3 Color development

The utilization of substrate by well bound enzyme was dependent upon both the quantity of enzyme presented and the length of the reaction-period. The reaction-time of added substrate must be long enough so that one can recognize various amount of bound enzyme, yet, short enough to avoid depletion of substrate. Fig. 12 represented the effect on the variations of substrate reaction-times on the ELISA specific activity of a positive antiserum to HSV. The amount of enzyme bound to well coated by virus and control antigen was shown to be

increased with the increase of the time of substrate reaction. Therefore, a substrate reaction time of 30 min at room temperature (in the dark) was chosen because it gave a considerably high level of sensitivity.

The optimal conditions of ELISA test for the detection of HSV antibody were summarized in Table 7.

3. Standardization of the Assay

3.1 Precision analysis of ELISA test

3.1.1 A within-plate precision was determined by using three sera; HSV-strong-positive control, HSV-positive commercial antibody, and negative control. Precision for each serum was calculated by running 20 replicates in one microtiter plate. As shown in Table 8, in a within plate precision analysis, the coefficient of variation (CV) ranging from 2.89% to 4.46%.

3.1.2 A between-plate precision (reproducibility) was determined by the same antibody, analysed on separate microtiter plates. The reproducibility varied with coefficient of variation ranging from 9.56 to 13.58% (Table 8).

3.2 Specificity of HSV-antigen in ELISA test

The specificity of HSV antigen in ELISA was tested by reacting with antibody of other viruses in Herpesviridae (CMV and VZV). As shown in Table 9, no cross reactivity of HSV by both antisera was observed.

4. Study of the Sensitivity and Specificity of the ELISA (local-made) by Comparing to ELISA Kit and IFA

4.1 Comparison between ELISA (local-made) and ELISA kit

After standardization of various ELISA parameters, 92 sera were tested by ELISA (local-made) for antibody to HSV. These results were then compared to those of ELISA kit (Table 10). Of the 92 sera tested, 67 were positive for antibody to HSV by ELISA kit. Sixty-two of these 67 were also positive by ELISA (local-made). Five of thirty sera negative for antibody to HSV by ELISA (local-made) was, on the other hand, positive by ELISA kit. Therefore, the sensitivity and specificity of the indirect ELISA were 92.5% and 100%, respectively, and efficiency, positive predictive value and negative predictive value were 94.6%, 100% and 83.3%, respectively, as compared with ELISA kit.

4.2 Comparison between ELISA (local-made) and IFA

Immunofluorescent antibody technic (IFA) was used in the clinical diagnosis for detection of HSV antibody at Chulalongkorn Hospital. In this study, the ELISA (local-made) was also compared with IFA. A total of 92 sera tested by ELISA (local-made) as described above were subjected to determine by IFA. As shown in Table 11, 60 of 62 sera positive by ELISA (local-made) were also

positive by IFA. The remaining 32 sera were negative.
Therefore, the sensitivity and specificity of the ELISA
(local-made) were 100% and 93.75%, respectively, and
efficiency, positive predictive value, and negative
predictive value were 97.82%, 96.77%, and 100%,
respectively, as compared with IFA.

5. Preservation and Stability of HSV-Antigen for ELISA

This antigen was preserved by various conditions
as follows : freezing at -20 $^{\circ}$C, lyophilization (kept at
4 $^{\circ}$C), coating on the plate (kept at -20 $^{\circ}$C), and coating on
plate (washed before kept at -20 $^{\circ}$C). The stability of
antigens was studied from the duration of 1 to 6 months
(1, 2, 3, and 6 months). In this study, total of 40 sera
tested which were 32 positive and 8 negative sera. In the
period of preservation for 6 months, thirty-eight sera (31
positive- and 7 negative sera) gave the same results as
original reports before storage, only two sera in which
the results differed from the previous ones and shown
in Table 12.

Table 1. Inactivation of HSV by ultraviolet light.

Time of inactivation (min)	Results (%CPE within 7 days)
0	100
10	70
20	50
30	0
40	10
50	0
60	10
75	0

Table 2. Inactivation of HSV by heat.

Temperature ($^\circ$C)	Time (min)	Number of plaques (PFU/ml)
4	0	2.0×10^{7}
30	60	2.0×10^{5}
40	60	8.0×10^{4}
50	15	0
50	30	0
50	45	0
50	60	0
60	60	0

Table 3.   Comparison of HSV-antigen preparations in MEM
           and PBS

Antigen conc. (μg/ml)	Antibody dilutions	Δ O.D.[⊙] of Antigens were prepared in			
		MEM without serum		PBS	
		HSV*	Neg.**	HSV*	Neg.**
40	1:100	0.121	0.059	0.233	0.132
	1:200	0.063	ND	0.211	ND
	1:400	0.057	ND	0.030	ND
	1:800	0.050	ND	0.014	ND

⊙ Δ O.D. = O.D. of HSV-antigen – O.D. of control antigen

* Commercial positive HSV serum

** Negative control serum

Table 4. Comparison of HSV-antigen preparations from HeLa
and Vero cells

Cell-Types	Concentration of antigen (µg/ml)	Δ O.D. of tested sera *		
		positive	weak-positive	negative
Vero	40	0.333	0.072	-0.035
HeLa	40	1.098	0.351	0.016

* Δ O.D. = O.D. of HSV-antigen - O.D. of control antigen

Table 5. Checkerboard titration to determine optimal conditions

Concentrations of antigen (μg/ml)	Tested sera positive dilutions			weak-positive dilutions			Dilutions of conjugated
	1:50	1:100	1:200	1:50	1:100	1:200	
20	0.874*	0.912	0.834	0.182	0.160	0.125	1:1200
	0.880	0.794	0.734	0.221	0.213	0.177	1:2400
	0.630	0.585	0.534	0.184	0.168	0.132	1:4800
	0.384	0.357	0.371	0.131	0.121	0.101	1:9600
40	1.052	0.938	0.817	0.226	0.183	0.149	1:1200
	0.994	0.941	0.852	0.267	0.251	0.209	1:2400
	0.752	0.714	0.646	0.233	0.207	0.172	1:4800
	0.460	0.448	0.416	0.164	0.152	0.132	1:9600
80	1.106	1.083	0.941	0.203	0.220	0.189	1:1200
	1.155	1.046	0.910	0.249	0.269	0.229	1:2400
	0.816	0.719	0.650	0.210	0.198	0.193	1:4800
	0.525	0.465	0.418	0.156	0.148	0.124	1:9600
160	1.145	1.118	0.937	0.179	0.211	0.171	1:1200
	1.172	1.030	0.912	0.231	0.247	0.216	1:2400
	0.848	0.768	0.648	0.158	0.195	0.173	1:4800
	0.550	0.478	0.431	0.128	0.270	0.118	1:9600

* Δ O.D. = O.D. of HSV-antigen - O.D. of control antigen

(O.D. of negative sera were less than 0.047.)

Table 6. Optimal conditions of time and temperature in the coating of antigen

Conditions	ΔO.D. * (mean $\pm$ SD)
1. 4 $^{\circ}$C and overnight incubation	1.201 $\pm$ 0.070
2. Room temperature and overnight incubation	1.126 $\pm$ 0.071
3. 37 $^{\circ}$C and 2 h incubation	1.002 $\pm$ 0.028
4. 37 $^{\circ}$C and 6 h incubation	1.055 $\pm$ 0.019
5. 37 $^{\circ}$C and overnight incubation	1.105 $\pm$ 0.093

* ΔO.D. = O.D. of HSV-antigen - O.D. of cell control

Table 7. Optimal conditions of ELISA test for the
         detection of HSV antibody

Steps of ELISA test	Concentration or dilution	Temperature ($^\circ$C)	Reaction time (min)
Coating of HSV-antigen	80 μg/ml	4 $^\circ$C	overnight
Reacting with tested sera	1:100	37 $^\circ$C	90
Reacting with conjugate	1:2,400	37 $^\circ$C	30
Color development	340 μg/ml [*]	RT [**]	30

[*] O-phenylenediamine substrate concentration

[**] room temperature

Table 8. Precision analysis of ELISA for detection of HSV antibody

	Δ O.D. of tested sera		
	Strong positive	Weak positive	Negative
Within-plate (n = 20)			
Mean	1.230	0.283	0.158
SD	0.036	0.013	0.006
% CV	2.89	4.46	4.0
Between-plate (n = 20)			
Mean	1.169	0.273	0.164
SD	0.112	0.033	0.022
% CV	9.56	12.12	13.58

Table 9. Specificity test of HSV-antigen in ELISA

Dilutions of antibody	Positive control sera against (mean of $\triangle$ O.D. $\pm$ SD)		
	HSV	VZV	CMV
1:5	0.460 ±0.016	0.053 ±0.016	0.016 ±0.006
1:25	0.422 ±0.014	0.060 ±0.006	0.011 ±0.003
1:50	0.275 ±0.009	0.021 ±0.003	0.009 ±0.003
1:100	0.245 ±0.003	0.013 ±0.003	0.004 ±0.002

Table 10. Comparison between ELISA (local-made) and ELISA kit

	ELISA kit		
	Positive	Negative	Total
* ELISA (local-made)			
Positive	62	0	62
Negative	5	25	30
Total	67	25	92

* Sensitivity = 92.5 %

  Specificity = 100 %

  Efficiency = 94.6 %

  Positive predictive value = 100 %

  Negative predictive value = 83.3 %

Table 11. Comparison between ELISA (local-made) and IFA

	IFA (routine assay)		
	Positive	Negative	Total
* ELISA (local-made)			
Positive	60	2	62
Negative	0	30	30
Total	60	32	92

* Sensitivity = 100 %

  Specificity = 93.75 %

  Efficiency = 97.82 %

  Positive predictive value = 96.77 %

  Negative predictive value = 100 %

Table 12. Preservation and stability of HSV-antigen for ELISA by:

12.1 Freezing at $-20\,^{\circ}C$

No.	Period of time (month)				No.	Period of time (month)			
	1	2	3	6		1	2	3	6
1	1.584	1.063	1.362	1.276	21	0.696	0.572	0.783	0.706
2	1.407	1.053	1.312	1.182	22	0.686	0.689	0.785	0.903
3	1.394	0.936	1.207	1.071	23	0.565	0.539	0.633	0.649
4	1.327	0.977	1.212	1.150	24	0.547	0.437	0.510	0.523
5	1.326	0.976	1.220	1.085	25	0.546	0.460	0.574	0.713
6	1.263	1.015	1.321	1.230	26	0.512	0.535	0.651	0.721
7	1.237	0.920	1.163	1.125	27	0.506	0.503	0.561	0.526
8	1.165	0.949	1.209	1.293	28	0.485	0.509	0.571	0.551
9	1.160	0.889	1.156	1.044	29	0.456	0.452	0.496	0.467
10	1.145	0.857	1.053	1.033	30	0.419	0.327	0.352	0.406
11	1.041	0.835	1.086	0.964	31	0.284	0.298	0.260	0.270
12	1.028	0.899	1.176	1.086	32	0.222	0.204	0.267	0.212
13	0.995	0.879	0.994	1.050	33	0.180	0.141	0.162	0.153
14	0.958	0.761	0.876	0.799	34	0.067	0	0	0
15	0.930	0.833	0.960	0.863	35	0.048	0	0.007	0
16	0.837	0.707	0.904	0.975	36	0.023	0.002	0	0
17	0.831	0.732	0.887	0.810	37	0.022	0.008	0.005	0
18	0.802	0.564	0.588	0.696	38	0.016	0.005	0	0
19	0.793	0.723	0.795	0.745	39	0.015	0.008	0	0
20	0.717	0.649	0.867	0.834	40	0.010	0.005	0	0

There is no different result between 2, 3, and 6 months and 1 month as judged by positive (O.D.$\geqslant$0.2), and negative (O.D.$<$0.2)

12.2   Lyophilization

No.	Period of time (month) 1	2	3	6	No.	Period of time (month) 1	2	3	6
1	ND	1.208	1.355	1.322	21	0.821	0.683	0.838	0.830
2	ND	1.159	1.269	1.225	22	ND	0.737	0.813	1.056
3	1.502	1.084	1.241	1.176	23	ND	0.536	0.509	0.755
4	ND	1.169	1.221	1.274	24	0.532	0.477	0.466	0.613
5	1.583	1,099	1.126	1.178	25	ND	0.475	0.550	0.840
6	1.542	1.240	1.336	1.311	26	ND	0.595	0.706	0.863
7	ND	1.073	1.184	1.219	27	0.581	0.494	0.498	0.590
8	ND	1.112	1.297	1.421	28	0.571	0.562	0.560	0.654
9	ND	1.009	1.126	1.127	29	0.507	0.467	0.454	0.607
10	ND	0.994	1.068	1.148	30	ND	0.356	0.277	0.495
11	1.297	0.993	1.092	1.087	31	ND	0.290	0.232	0.327
12	1.413	1.133	1.116	1.182	32	0.271	0.239	0.214	0.292
13	ND	0.956	1.014	1.171	33	ND	0.183	0.121	0.261*
14	1.063	0.823	0.829	0.946	34	ND	0.030	0.038	0
15	1.207	0.975	1.019	0.972	35	0.006	0.015	0	0.016
16	ND	0.878	0.895	1.098	36	0	0.008	0.014	0.013
17	0.976	0.768	0.796	0.882	37	0.014	0.031	0.012	0.056
18	ND	0.571	0.580	0.766	38	ND	0.028	0	0.035
19	0.940	0.824	0.795	0.891	39	0	0.003	0.012	0.028
20	ND	0.755	0.866	1.014	40	0.008	0.010	0.008	0.041

ND = Not determined
There is no different result comparing 1, 2, 3, and 6
months to 1 month in table 12.1 except 6 months (No. 33)

12.3   Coating on plate and kept at $-20\,^{\circ}C$

No.	Period of time (month)				No.	Period of time (month)			
	1	2	3	6		1	2	3	6
1	1.261	1.391	1.231	1.619	21	0.907	0.611	0.731	1.038
2	1.404	1.504	1.192	1.535	22	0.922	1.103	0.783	0.975
3	1.334	0.880	1.073	1.470	23	0.772	0.819	0.706	0.880
4	1.278	1.379	1.151	1.490	24	0.622	0.482	0.512	0.616
5	1.372	1.024	1.150	1.571	25	0.812	0.901	0.720	0.904
6	1.489	1.109	1.174	1.519	26	0.751	0.842	0.580	0.798
7	0.961	1.308	1.048	1.334	27	0.701	0.505	0.572	0.682
8	1.312	1.466	1.169	1.437	28	0.763	0.466	0.685	0.791
9	1.174	1.387	1.081	1.482	29	0.677	0.453	0.590	0.969
10	1.008	1.252	1.044	1.354	30	0.643	0.670	0.439	0.503
11	1.218	0.857	0.936	1.397	31	0.435	0.372	0.300	0.385
12	1.421	0.999	1.085	1.418	32	0.308	0.228	0.291	0.361
13	1.093	1.161	0.962	1.257	33	0.378	0.357	0.267	0.365
14	1.128	0.745	0.874	1.139	34	0.020	0	0.022	0.036
15	1.178	0.851	0.943	1.258	35	0.012	0.013	0	0.016
16	0.922	1.133	0.938	1.055	36	0	0	0	0
17	1.170	0.723	0.871	1.117	37	0.038	0.015	0.010	0.026
18	0.745	0.825	0.648	0.751	38	0.015	0.017	0.008	0.018
19	1.037	0.686	0.799	1.122	39	0.034	0.004	0	0.007
20	0.917	1.104	0.876	1.135	40	0	0.005	0	0.013

There is no different result comparing 1, 2, 3, and 6
months and 1 month in table 12.1 except 1, 2, 3, and 6
months (No. 33).

12.4 Coating on plate and washed before kept at -20 °C

No.	Period of time (month)				No.	Period of time (month)			
	1	2	3	6		1	2	3	6
1	1.121	1.310	1.105	1.417	21	0.520	0.574	0.588	0.705
2	1.231	1.345	1.029	1.256	22	0.624	0.734	0.664	0.705
3	0.933	0.855	0.911	1.075	23	0.538	0.622	0.518	0.557
4	1.080	1.181	1.030	1.148	24	0.403	0.365	0.371	0.362
5	1.098	0.995	0.980	1.220	25	0.465	0.612	0.536	0.529
6	1.123	1.058	1.018	1.264	26	0.560	0.626	0.540	0.569
7	0.881	1.184	0.904	0.979	27	0.478	0.432	0.438	0.432
8	1.080	1.182	1.040	1.115	28	0.495	0.419	0.459	0.448
9	1.006	1.169	0.971	1.042	29	0.398	0.429	0.395	0.454
10	0.852	1.047	0.900	1.056	30	0.402	0.457	0.341	0.344
11	0.873	0.822	0.855	1.021	31	0.231	0.297	0.255	0.214
12	0.913	0.909	0.921	1.081	32	0.159	0.198	0.190	0.182
13	0.861	0.920	0.796	0.841	33	0.174	0.197	0.178	0.171
14	0.820	0.659	0.653	0.794	34	0.023	0.002	0.022	0
15	0.865	0.750	0.758	0.900	35	0	0.004	0	0
16	0.743	0.832	0.757	0.752	36	0	0	0	0
17	0.718	0.649	0.702	0.725	37	0	0.002	0.005	0.023
18	0.588	0.649	0.512	0.553	38	0	0.003	0	0
19	0.739	0.622	0.639	0.720	39	0	0.003	0	0.027
20	0.627	0.864	0.702	0.784	40	0	0	0.001	0

There is no different result between 1, 2, 3, and 6 months
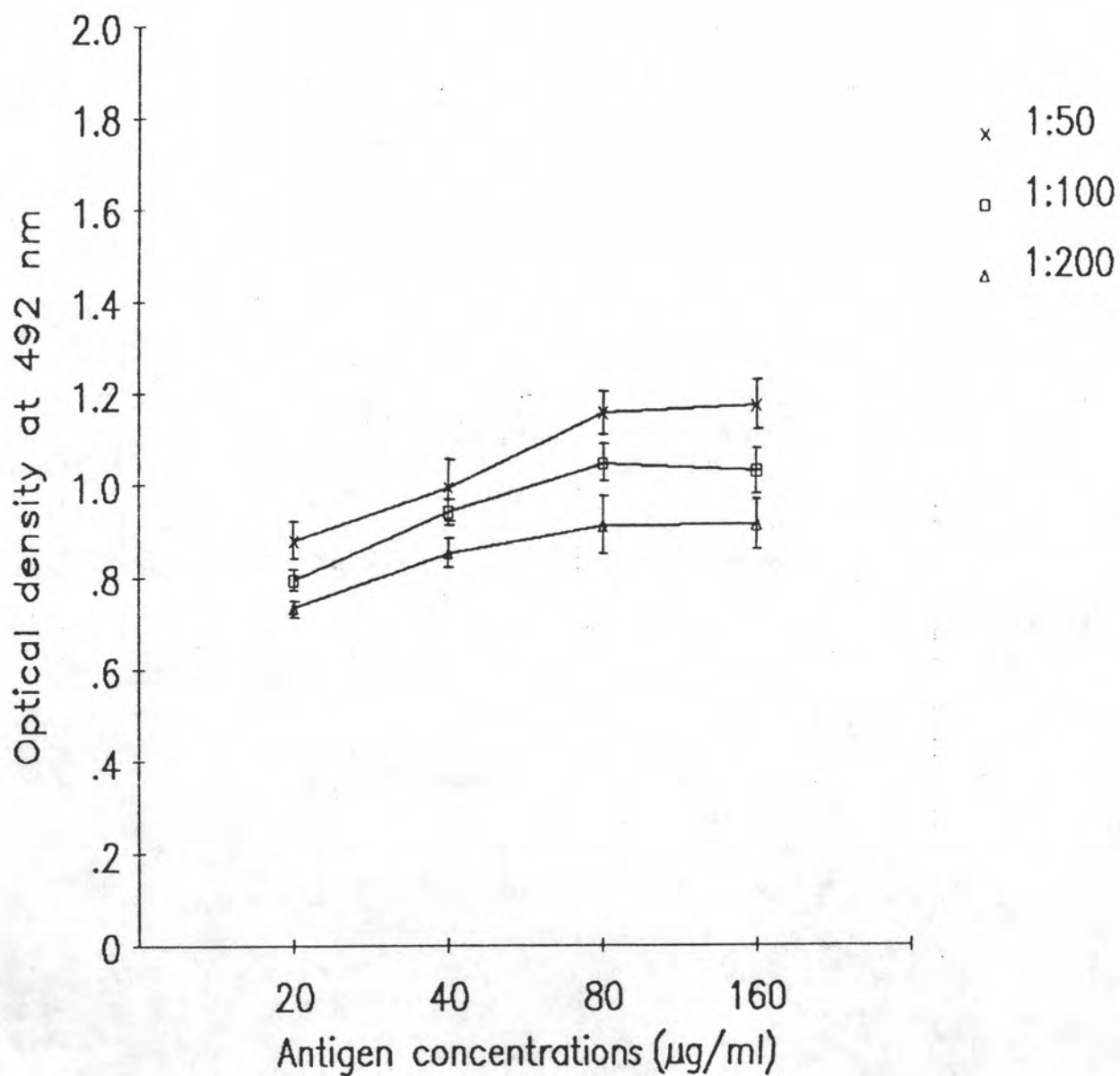and 1 month in table 12.1 except 1, 2, 3, and 6 months
(NO.32).

Fig. 8 Determination for the optimal concentration of antigen. Three dilutions of positive antibody (1:50, 1:100, 1:200) were tested.
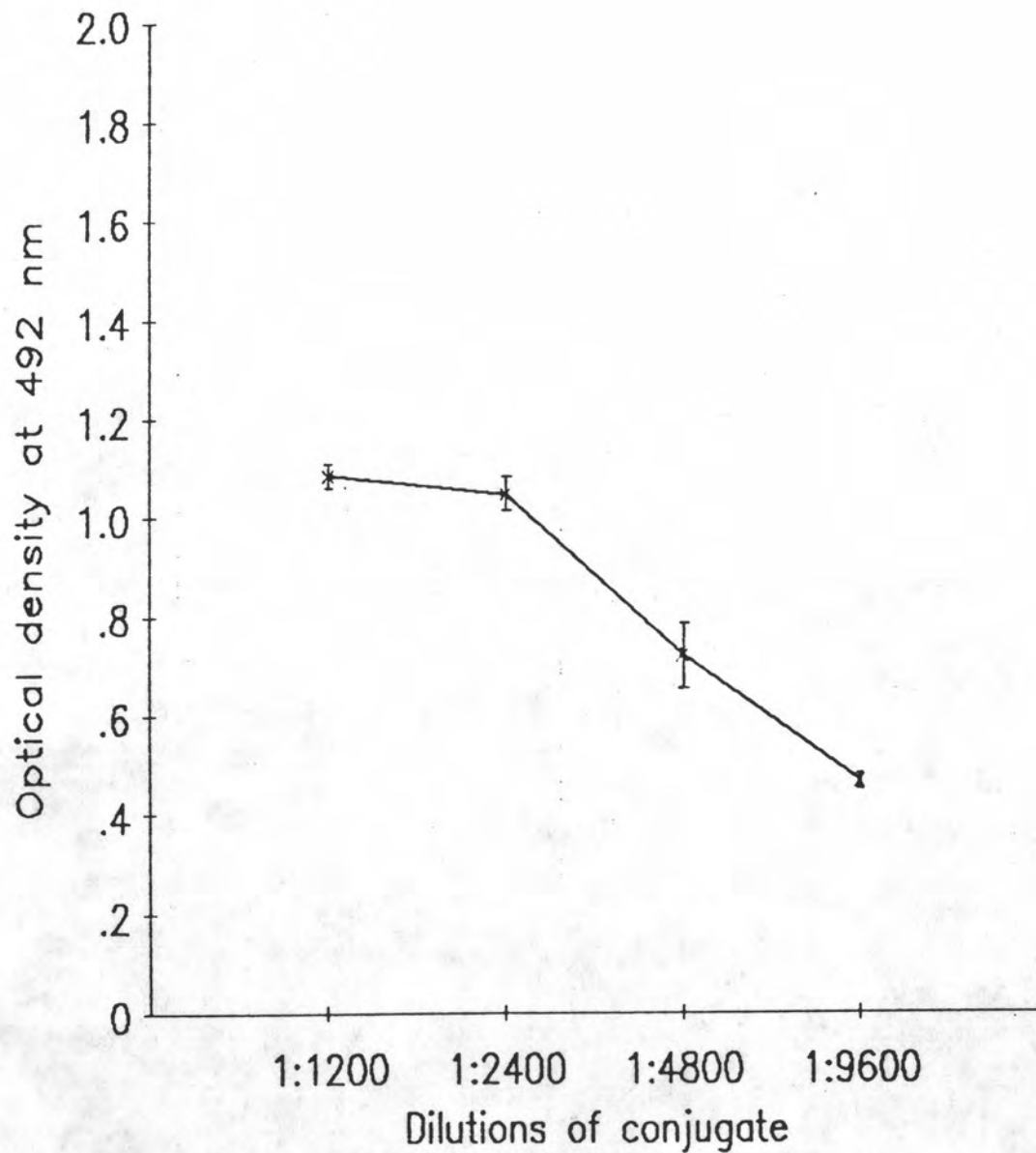
Fig. 9 Determination for the optimal dilution of conjugate. The concentration of antigen was 80 µg/ml and the dilution of antibody was 1:100. Following antigen-antibody reaction, they were incubated with the given dilutions of conjugate.
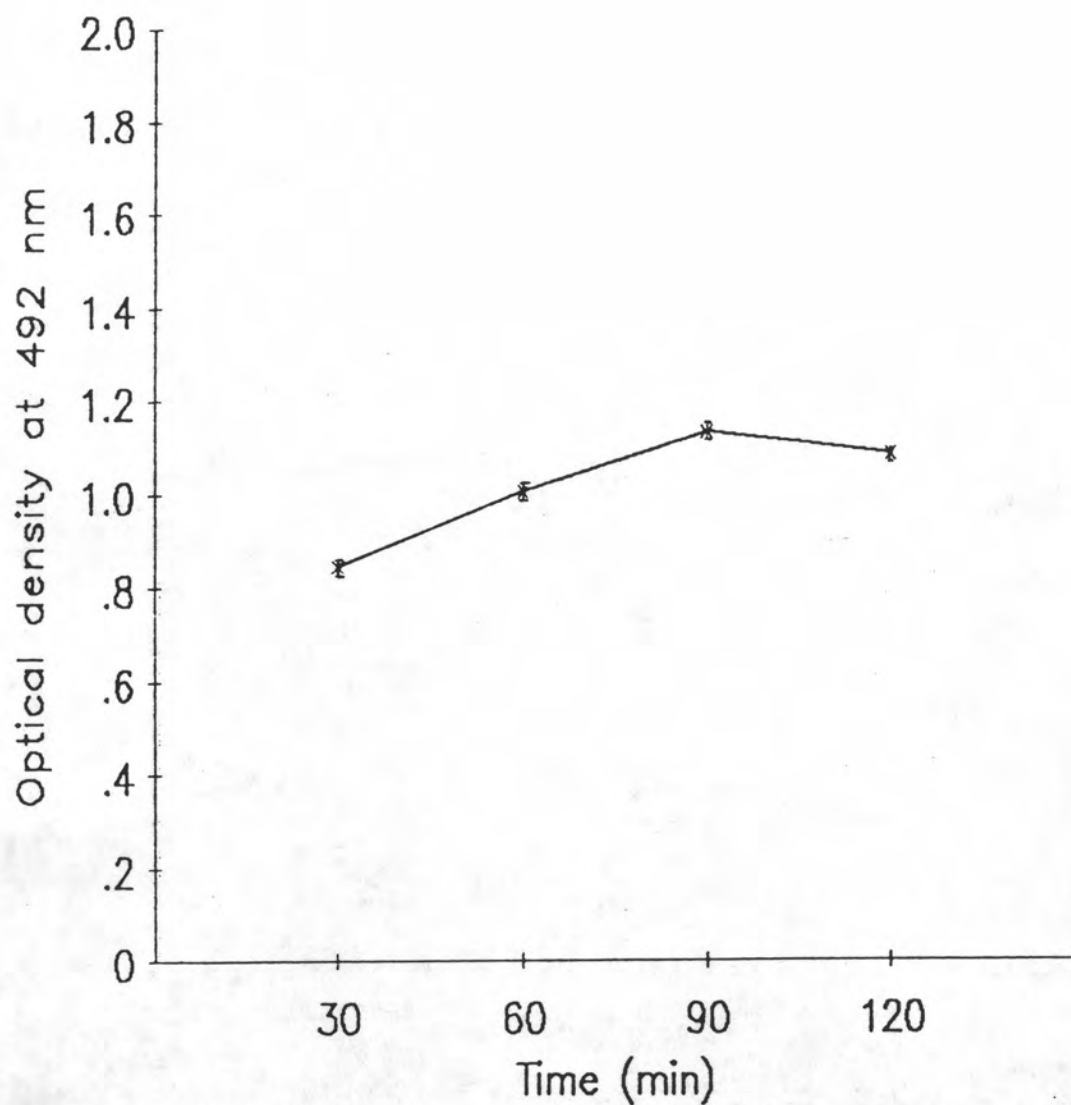
Fig. 10 Optimum of time for antigen and antibody reaction. Eighty µg/ml of antigen was coated at 4 °C overnight. The dilution of antibody 1:100 reacted with antigen at the time given, followed by reacted with 1:2,400 conjugate at 37 °C for 1 h.
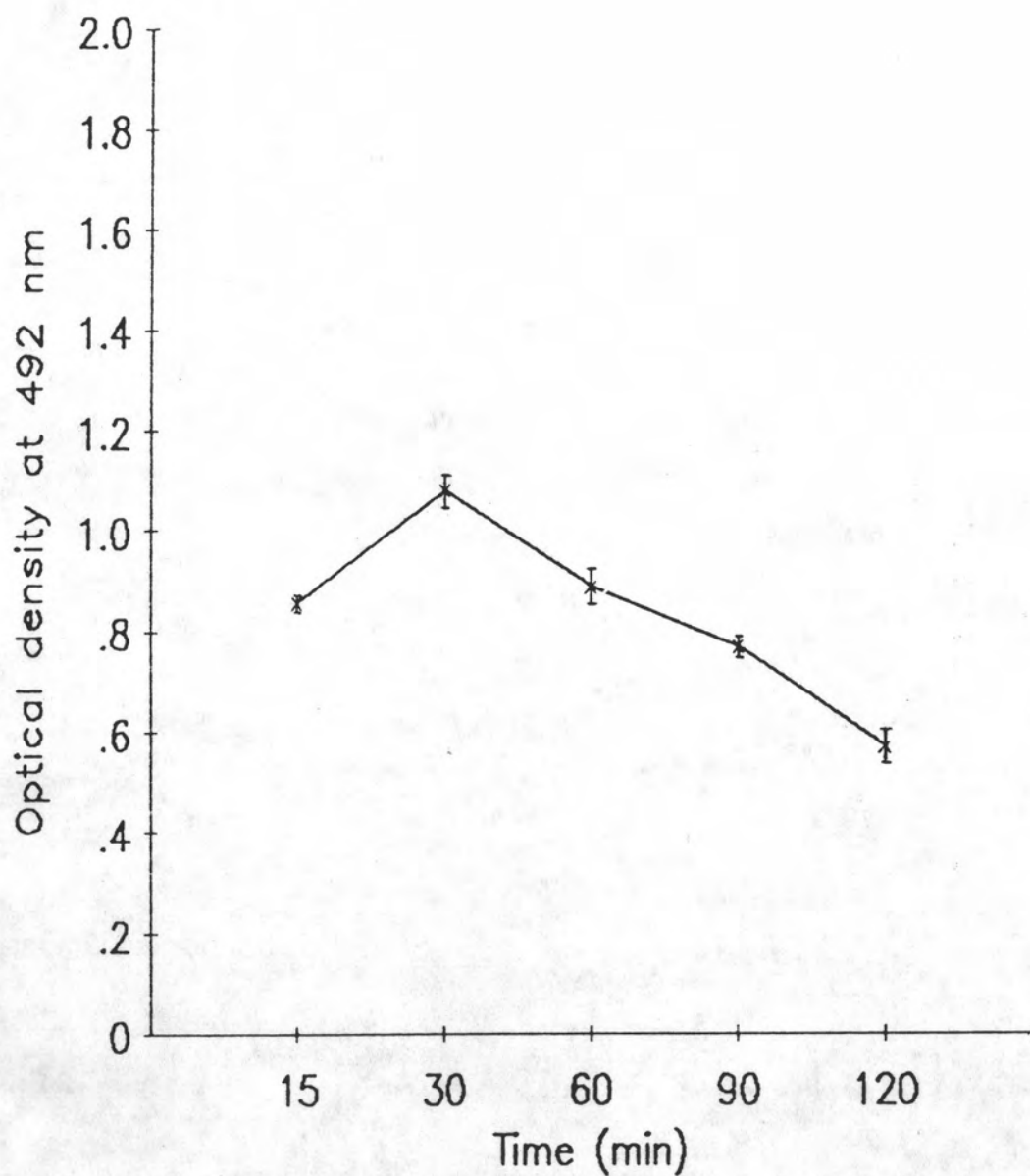
Fig. 11 Optimum of time for antibody and conjugate

reaction. Eighty µg/ml of antigen was coated at

4 °C overnight. The antibody dilution of 1:100 was
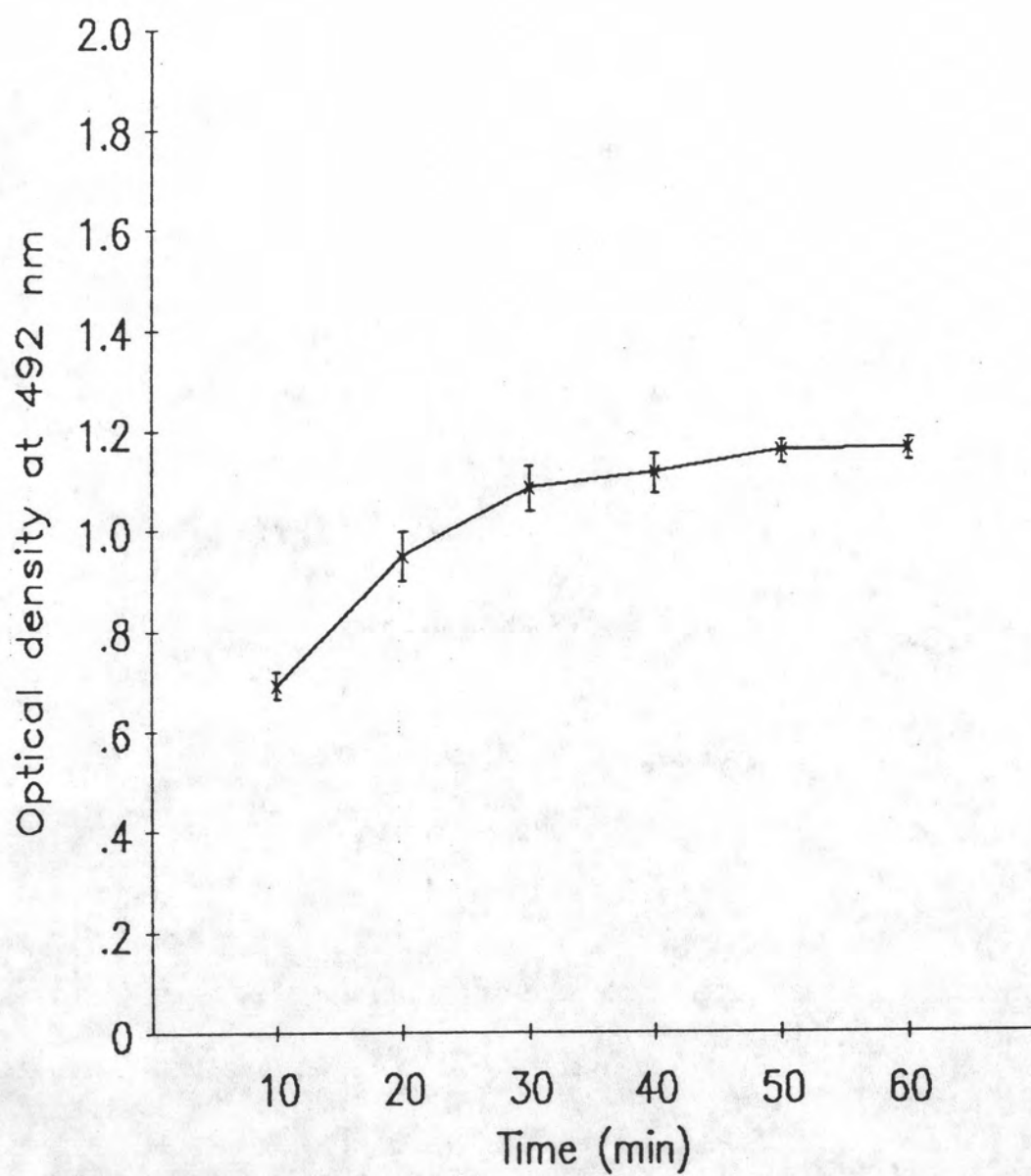
incubated at 37 °C for 90 °C.

Fig. 12 Determination of time course for color development.