CHAPTER III

RESULTS

3.1 Preparation of conditioned media

In order to generate conditioned media for supporting of hybridoma growth, culture supernatants were prepared from two cell lines, mouse myeloma and BW5147 thymoma cells. In this study, mouse myeloma and BW 5147 thymoma cell line were first determined for the mycoplasma contamination by PCR. Both cell lines showed no mycoplasma infection (Figure 3.1).

Mouse myeloma and BW5147 thymoma cell lines were then cultured with or without PMA for 18 and 40 hours. Culture supernatants were harvested by centrifugation and filtration. By this study, various culture supernatants (or conditioned media) were obtained and named as was shown in Table 3.1. The obtained culture supernatants were kept at -20^{o}C until used in the further experiments.

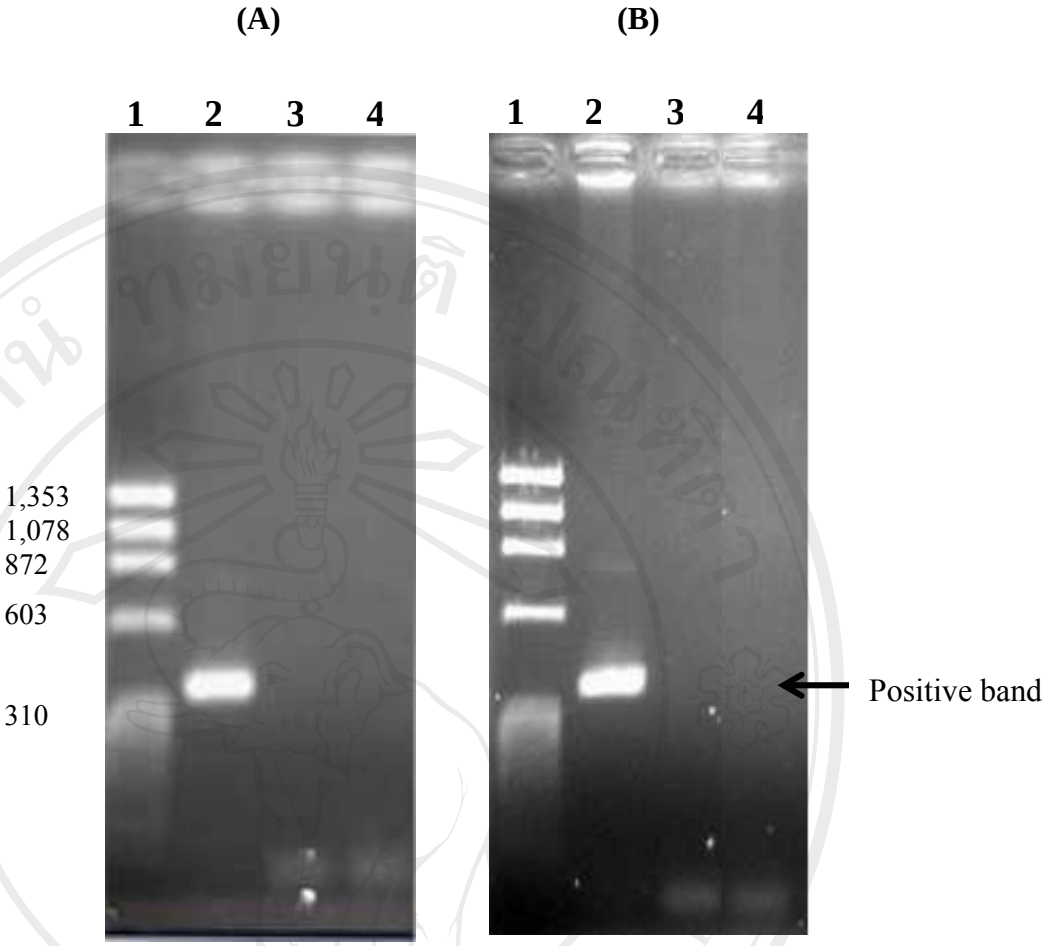


Figure 3.1 Agarose gel electrophoresis of PCR product for determination of mycoplasma infection. Mycoplasma DNA from cell line tested was amplified by PCR using the Ito-Myco F1 and Ito-Myco R1 primers. Lane 1: ØX174 HaeIII digested DNA size markers (bp), lane 2: amplified PCR product of mycoplasma DNA (positive control), lane 3: amplified PCR product of non-mycoplasma DNA template (negative control), lane 4: amplified PCR product using BW5147 thymoma cell (A) or myeloma cell DNA template (B). Sizes of standard DNA markers (bp) are indicated on the left.

Table 3.1 Various culture conditions and culture supernatants (or conditioned media) used in this study

Culture conditions	Supernatant named
Myeloma cells, culture without PMA, incubation time 18 hr.	MM1
Myeloma cells, culture without PMA, incubation time 40 hr.	MM2
Myeloma cells, culture with PMA, incubation time 18 hr.	MM3
Myeloma cells, culture with PMA, incubation time 40 hr.	MM4
BW5147 thymoma cells, culture without PMA, incubation time 18 hr.	BW1
BW5147 thymoma cells, culture without PMA, incubation time 40 hr.	BW2
BW5147 thymoma cells, culture with PMA, incubation time 18 hr.	BW3
BW5147 thymoma cells, culture with PMA, incubation time 40 hr.	BW4

3.2 Study the utilization of the generated conditioned media for hybridoma single cell cloning

3.2.1 Single cell cloning using stable hybridoma line

3.2.1.1 Determination of the appropriate conditioned medium

Since hybridomas often grow poorly or die when they are grown at low cell density, conditioned medium containing appropriate cytokines is always used to support the hybridoma growth in single cell cloning process. To determine which conditioned media produced (Table 3.1) can be appropriate used to support cell growth in hybridoma single cell cloning, a stable hybridoma clone Thal N/B was used in this study. Thal N/B hybridoma cells were subjected for single cell cloning using 10% FCS-IMDM, 10% FCS-IMDM supplemented with 10% commercial conditioned media BM condimed H1 and compared with 10% FCS-IMDM supplemented with 50% of the generated conditioned media. The numbers and size of hybridoma growth were determined after 5, 7, 10, and 14 days of cultivation. Results of three independent experiments are shown in Table 3.2. It was found that culture supernatant obtained from BW5147 thymoma cells without PMA stimulation and incubated for 40 hours (BW2) was the most potent conditioned medium for supporting hybridoma growth.

As shown in Table 3.2, at day 14, with 40 hours non-PMA stimulated BW5147 conditioned medium (BW2) and 40 hours PMA stimulated BW5147 conditioned medium (BW4), the size of the growth hybridomas were scored from small to medium. Whereas, with 18-hours non-PMA stimulated BW5147 conditioned medium (BW1) and 18-hours PMA stimulated BW5147 conditioned medium (BW3), the size of hybridomas were scored very small to small. The results indicated that

supernatants obtained from 40 hours cultivation were better than those of 18 hours cultivation. In comparison to the commercial conditioned medium BM condimed H1, at day 14 of cultivation, the numbers of single clones obtained by using the BW5147 conditioned media were not significantly different from those obtained by using BM condimed H1 (Table 3.2, the last column).

In comparison between BW5147 and myeloma conditioned media, as shown in Table 3.3, the size of hybridomas supplemented with all myeloma conditioned media (MM1-MM4) were scored from very small to small and was similar to those using of 10% FCS-IMDM without any supplement. While using of BW5147 conditioned medium, the size of hybridomas were scored bigger than those using of 10% FCS-IMDM without any supplement (small to medium). Taken together, the results indicated that culture supernatant obtained from culturing of BW5147 cells could support growth of hybridomas better than myeloma cells. Moreover, culture supernatant of un-stimulated BW5147 thymoma cells for 40 hours (BW2) was the most suitable conditioned medium for stable hybridoma single cell cloning. The BW2 condition medium was therefore selected for further study.

Table 3.2 Growth supporting effect of using BW5147 mouse thymoma conditioned media on stable hybridoma Thal N/B single cell cloning.

Experiment I

Thal N/B (1st)	Total[a] (day 5)	Single clone[b] (day 5)	size[c] (day 5)				size[c] (day 7)				size[c] (day 10)				size[c] (day 14)				Single clone[b] (day 14)
			VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	
10% FCS-IMDM	54	30	30	-	-	-	30	-	-	-	26	-	-	-	21	2	-	-	23
50% BW, No PMA, 18 hr.	65	34	34	-	-	-	33	-	-	-	29	2	-	-	15	15	-	-	30
50% BW, No PMA, 40 hr.	63	25	25	-	-	-	25	-	-	-	19	6	-	-	**3**	**16**	**6**	-	25
50% BW, PMA, 18 hr.	56	21	21	-	-	-	21	-	-	-	21	-	-	-	16	5	-	-	21
50% BW, PMA, 40 hr.	59	28	28	-	-	-	27	-	-	-	25	2	-	-	6	18	3	-	27
10% BM condimed H1	49	26	26	-	-	-	28	-	-	-	15	11	-	-	2	13	9	2	26

Experiment II

Thal N/B (2nd)	Total[a] (day 5)	Single clone[b] (day 5)	size[c] (day 5)				size[c] (day 7)				size[c] (day 10)				size[c] (day 14)				Single clone[b] (day 14)
			VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	
10% FCS-IMDM	55	22	22	-	-	-	24	-	-	-	18	-	-	-	10	3	-	-	13
50% BW, No PMA, 18 hr.	66	22	22	-	-	-	22	-	-	-	17	2	-	-	11	6	1	-	18
50% BW, No PMA, 40 hr.	66	30	30	-	-	-	32	-	-	-	27	4	-	-	**12**	**14**	**1**	-	27
50% BW, PMA, 18 hr.	67	28	28	-	-	-	25	-	-	-	22	-	-	-	16	5	-	-	21
50% BW, PMA, 40 hr.	69	18	18	-	-	-	21	-	-	-	15	2	-	-	1	10	5	-	16
10% BM condimed H1	69	26	26	-	-	-	25	1	-	-	19	6	-	-	3	14	4	2	23

Experiment III

Thal N/B (3rd)	Total[a] (day 5)	Single clone[b] (day 5)	size[c] (day 5)				size[c] (day 7)				size[c] (day 10)				size[c] (day 14)				Single clone[b] (day 14)
			VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	
10% FCS-IMDM	55	29	29	-	-	-	29	-	-	-	29	-	-	-	28	1	-	-	29
50% BW, No PMA, 18 hr.	54	25	25	-	-	-	25	-	-	-	19	6	-	-	11	8	6	-	25
50% BW, No PMA, 40 hr.	58	24	24	-	-	-	24	-	-	-	12	12	-	-	**3**	**17**	**4**	-	24
50% BW, PMA, 18 hr.	61	37	37	-	-	-	37	-	-	-	33	2	-	-	20	11	5	-	36
50% BW, PMA, 40 hr.	52	25	25	-	-	-	25	-	-	-	17	8	-	-	3	12	7	1	23
10% BM condimed H1	64	40	40	-	-	-	40	-	-	-	15	22	3	-	8	16	13	2	39

Results of Thal N/B single cell cloning using 10%FCS-IMDM supplemented with or without the indicated condition medium. Three independent experiments were performed. The size and number of hybridomas were determined after 5, 7, 10, and 14 days of cultivation.

[a] Total number of hybridoma growth at day 5 of cultivation.

[b] Number of hybridoma single clone at day 5, day 7, day 10 and day 14 of cultivation..

[c] Size of hybridoma single clone at the indicated days was scored as described in materials and methods. VS; very small, S; small, M; medium, L; large.

Table 3.3 Growth supporting effect of using myeloma conditioned media on stable hybridoma Thal N/B single cell cloning

Experiment I

Thal N/B (1st)	Total[a] (day 7)	Single clone[b] (day 7)	size[c] (day 7)				size[c] (day 14)				Single clone[b] (day 14)
			VS	S	M	L	VS	S	M	L	
10% FCS-IMDM	48	28	18	-	-	-	22	8	1	-	31
50% MM, No PMA, 18 hr.	40	17	17	-	-	-	18	2	-	-	20
50% MM, No PMA, 40 hr.	40	22	22	-	-	-	28	5	-	-	33
50% MM, PMA, 18 hr.	45	27	27	-	-	-	24	1	-	-	25
50% MM, PMA, 40 hr.	42	28	28	-	-	-	24	2	-	-	26
10% BM condimed H1	70	30	28	2	-	-	1	19	7	-	27

Experiment II

Thal N/B (2nd)	Total[a] (day 7)	Single clone[b] (day 7)	size[c] (day 7)				size[c] (day 14)				Single clone[b] (day 14)
			VS	S	M	L	VS	S	M	L	
10% FCS-IMDM	53	36	36	-	-	-	27	2	2	-	31
50% MM, No PMA, 18 hr.	46	29	29	-	-	-	24	2	-	-	26
50% MM, No PMA, 40 hr.	50	31	31	-	-	-	24	2	1	-	27
50% MM, PMA, 18 hr.	45	24	24	-	-	-	22	-	1	-	23
50% MM, PMA, 40 hr.	47	24	24	-	-	-	25	-	2	-	27
10% BM condimed H1	60	36	36	-	-	-	7	20	9	-	36

Results of Thal N/B single cell cloning using 10%FCS-IMDM supplemented with or without the indicated condition medium. Two independent experiments were performed. The size and number of hybridomas were determined after 7 and 14 days of cultivation.

[a] Total number of hybridomas growth at day 7 of cultivation.

[b] Number of single clone hybridomas at day 7 and 14 of cultivation.

[c] Size of single clone hybridomas at the indicated days was scored as described in materials and methods. VS; very small, S; small, M; medium, L; large.

3.2.1.2 Determination of the optimal concentration of the produced conditioned medium

From the previous experiments indicated that culture supernatant obtained from cultivation of un-stimulated BW5147 thymoma cells (BW2) for 40 hours was the most suitable conditioned medium for hybridoma single cell cloning. In this experiment, we further determined the optimal concentration of BW2 condition medium for using in supporting hybridoma growth. Thal N/B hybridomas were subjected for single cell cloning by using various concentrations of BW2 conditioned medium. The results were shown in Table 3.4. The sizes of hybridoma clones in 10% FCS-IMDM supplemented with 10% BW2 conditioned medium were scored from very small to medium at day 14. Whereas, the clones were scored from small to medium in 20% and 50% BW2 supplementation..However, when the numbers of hybridoma clones obtained were compared between 20% and 50% supplementation, the number of medium-sized clones presented in 20% BW2 conditioned medium was higher than 50% BW2 conditioned medium.

These results indicated that 20% BW2 conditioned medium was the optimal concentration for using to support the growth of hybridoma cells.

Table 3.4 Growth supporting effect of using various concentrations of BW5147 mouse thymoma conditioned media on stable hybridoma single cell cloning.

Thal N/B	Total[a] (day 5)	Single clone[b] (day 5)	size[c] (day 5)				size[c] (day 7)				size[c] (day 10)				size[c] (day 14)				Single clone[b] (day 14)
			VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	
10% BW, No PMA, 40 hr.	57	25	25	-	-	-	28	-	-	-	23	8		-	11	15	2	2	30
20% BW, No PMA, 40 hr.	66	34	34	-	-	-	34	-	-	-	17	18	2	-	11	10	10	3	34
50% BW, No PMA, 40 hr.	57	25	25	-	-	-	26	-	-	-	12	12	3	-	4	18	5	-	27
10% BM condimed H1	59	27	27	-	-	-	28	-	-	-	5	14	3	4	1	13	7	4	24

Results of Thal N/B single cell cloning using 10%FCS-IMDM supplemented with various concentrations. .The size and number of hybridomas were determined after 5, 7, 10, and 14 days of cultivation.

[a] Total number of hybridoma growth at day 5 of cultivation.

[b] Number of hybridoma single clone at day 5, day 7, day 10 and day 14 of cultivation.

[c] Size of hybridoma single clone at the indicated days was scored as described in materials and methods. VS; very small, S; small, M; medium, L; large.

3.2.2 Single cell cloning using unstable hybridoma line

From the previous experiments, 20% BW5147 conditioned medium obtained from 40-hours unstimulated cells (BW2) was demonstrated to be the suitable condition for hybridoma single cell cloning of stable hybridoma cells. In this experiment, we studied whether 20% BW2 conditioned medium could also support unstable hybridoma growth. A BALB/c mouse was immunized three times at one-week intervals with OKT3 immunoprecipitated beads. After the third immunization, antibody response in mouse sera was determined by indirect immunofluorescence staining**.** The immunized sera showed positive reactivity at titer of 800 (Figure 3.2).

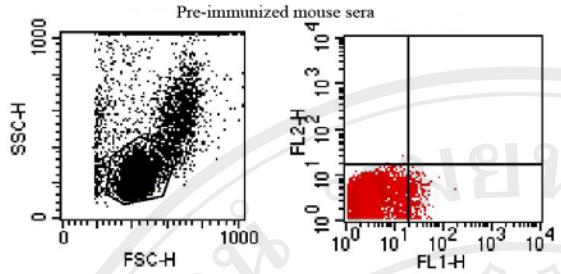
The spleen cells from immunized mouse were then fused with myeloma cells by using standard hybridoma technique. By this fusion, more than 300 hybridoma containing wells were achieved. The culture supernatants from 313 hybridoma containing wells were screened for the presence of antibodies by staining of PBMC surface protein using indirect immunofluorescent technique. By this screening, culture supernatant from a hybridoma containing well showed positive with a lymphocyte sub-population. This positive hybridoma clone was named MT3 and single cell cloning was performed using 10% FCS-IMDM medium supplemented with 10%, 20% and 50% BW5147 conditioned medium (BW2) or 10% commercial BM condimed H1 conditioned medium. As shown in Table 3.5, the size of hybridoma clones in medium supplemented with 10% BW2 conditioned medium was scored from very small to medium at day 14. While, the size of MT3 hybridoma clones using 20% and 50% BW2 conditioned medium were scored from small to medium at day 14. The number of single clones obtained using 20% and 50% BW2 supplementation at day 14 was not different. This result confirmed the previous studies that 20% BW2

conditioned medium is the optimal concentration for using to support the growth of hybridoma cells. The result was indicated that 20% BW2 conditioned medium could support the growth of both stable and un-stable hybridomas.

. The culture supernatants of MT3 single cell cloning obtained using 10%FCS-IMDM supplemented with 20% BW5147 conditioned medium (BW2) and 10% BM condimed H1 (commercial condition medium) were evaluated for the antibodies reactivity. It was found that culture supernatants obtained from using of both conditioned media showed the same positive patterns (Figure 3.3). The results indicated that the produced BW5147 conditioned medium could be used to support the growth of hybridoma without altering the antibody activity.

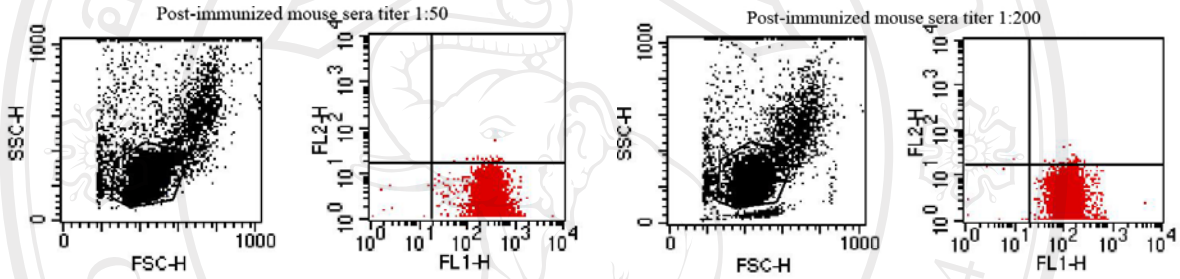
In summary, culture supernatant harvested from BW5147 mouse thymoma after culturing for 40 hours without any stimulant (BW2) can be used as conditioned medium and 20% supplementation is the optimal concentration for supporting the hybridoma growth in single cell cloning. This conditioned medium can be used for single cell cloning of both freshly fused hybridomas and stable hybridoma clones.

Pre-immunized mouse sera



Post-immunized mouse sera

Titer 1:50 **Titer 1:200**



Titer 1:800 **Titer 1:3200**

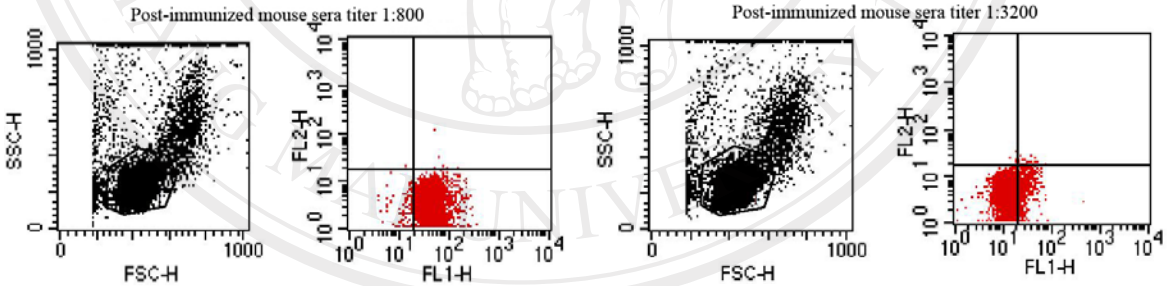


Figure 3.2 Antibody responses of mouse immunized with OKT3 immunoprecipitated beads. The mouse sera were collected at pre-immunization and after the third immunizations and were determined for antibody responses by indirect immunofluorescence staining**.** PBMCs were stained with mouse sera at various dilutions and analyzed by a flow cytometer. Lymphocytes were gated according to their size and granularity and the fluorescence intensity was determined using FL-1 detector.

Table 3.5 Growth supporting effect of using various concentrations of BW5147 mouse thymomas conditioned media on un-stable hybridoma single cell cloning

MT3	Total[a] (day 5)	Single clone[b] (day 5)	size[c] (day 5)				size[c] (day 7)				size[c] (day 10)				size[c] (day 14)				Single clone[b] (day 14)
			VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	
10% BW, No PMA, 40 hr.	62	26	26	-	-	-	26	-	-	-	22	5	-	-	11	6	7	1	26
20% BW, No PMA, 40 hr.	52	25	25	-	-	-	25	-	-	-	21	4	-	-	5	15	2	-	22
50% BW, No PMA, 40 hr.	45	22	22	-	-	-	22	-	-	-	18	5	-	-	7	13	1	-	21
10% BM condimed H1	46	22	22	-	-	-	22	-	-	-	20	2	-	-	9	9	2	1	21

Results of MT3 single cell cloning using in 10%FCS-IMDM supplemented with various concentrations. The size and number of hybridomas were determined after 5, 7, 10, and 14 days of cultivation.

[a] Total number of hybridoma growth at day 5 of cultivation.

[b] Number of hybridoma single clone at day 5 , day 7, day 10, and day 14 of cultivation..

[c] Size of hybridoma single clone at the indicated days was scored as described in materials and methods. VS; very small, S; small, M; medium, L; large.

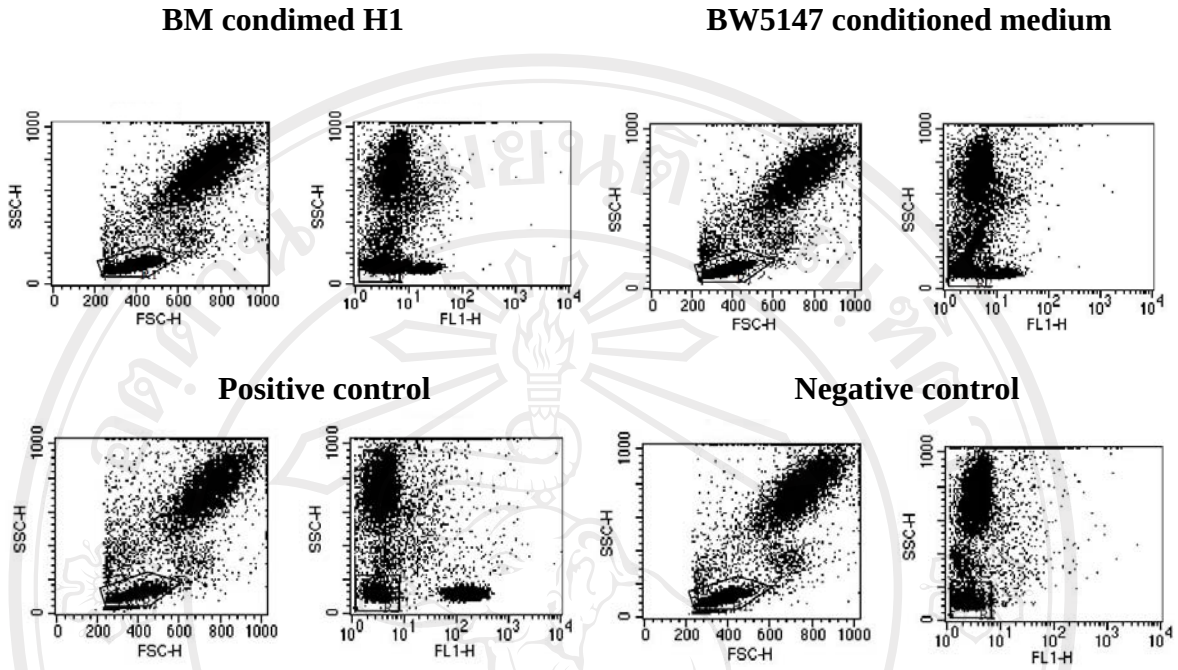


Figure 3.3 Antibody reactivity of culture supernatants of hybridoma clones using the produced BW5147 mouse thymoma condition medium and BM condimed H1 supplementation. The culture supernatants of single cell cloning obtained using 10%FCS-IMDM supplemented with the produced 20% BW5147 conditioned medium and 10% BM condimed H1 were evaluated for antibodies reactivity by lysed whole blood staining.

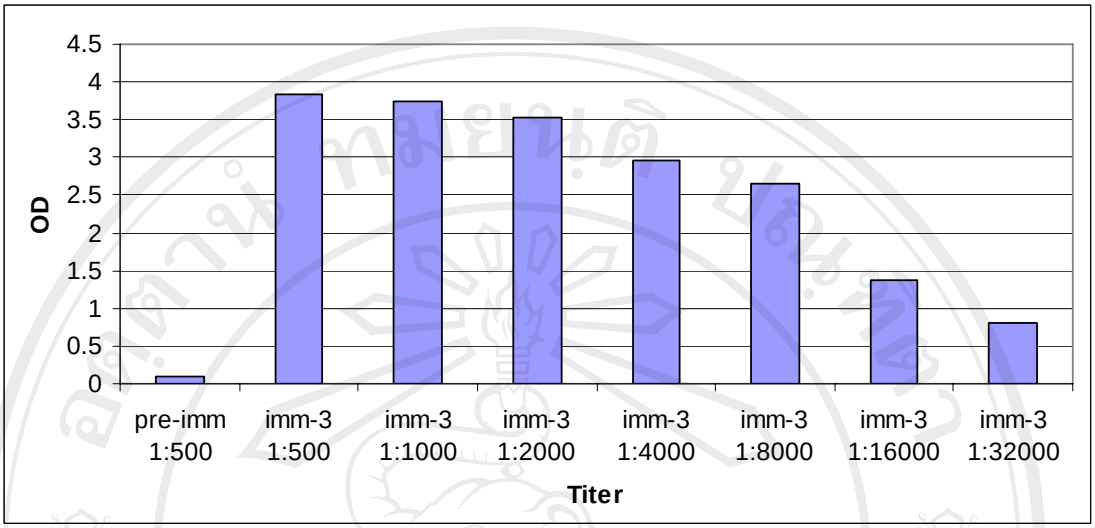
3.3 Utilization of the produced conditioned medium for generation of hybridomas by hybridoma technique

Since the BW5147 conditioned medium was demonstrated to provide growth factors that promote growth of hybridomas. We therefore believed that this conditioned medium can be used as supplement for generation of hybridomas in hybridoma technique. To verify this postulation, generation of hybridomas secreting anti-Hb Portland and anti-Hb A_2 monoclonal antibodies were used as study models.

3.3.1 Mice immunization and antibody responses

BALB/c mice were intraperitoneal immunized with 100 μg of Hb Portland and Hb A_2. After the third immunization, the antibody responses were determined by indirect ELISA technique. The antibodies titers of Hb Portland and Hb A_2 in the immunized mice sera were more than 32,000 after the third immunization (Figure 3.4A and 3.4B, respectively). Both mice were therefore ready for further monoclonal antibody production.

(A)



(B)

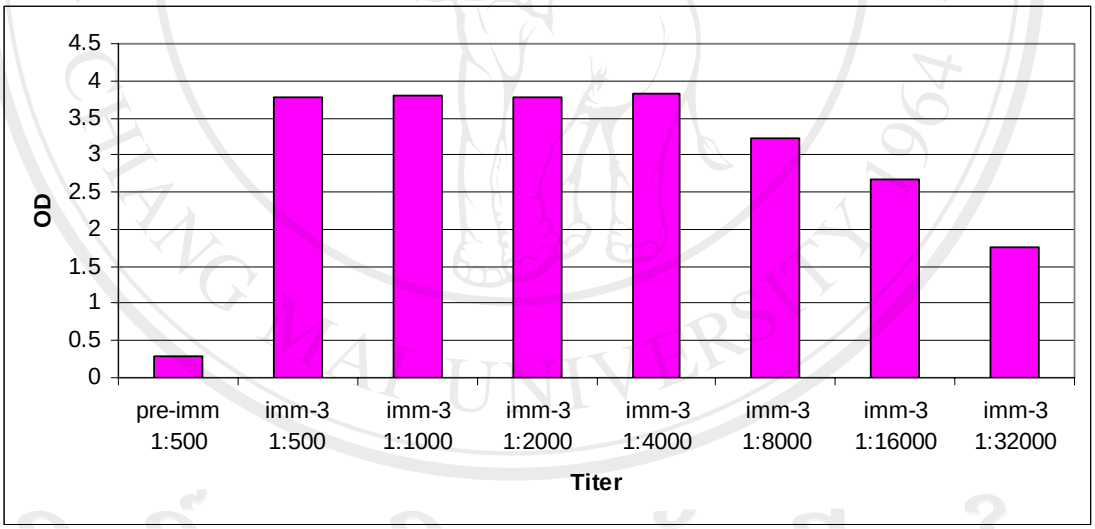


Figure 3.4 Antibody responses in BALB/c mice after immunizations with Hb Portland and Hb A_2. BALB/c mouse was immunized with 100 µg purified Hb Portland (A) or Hb A_2 (B) with Freund's adjuvant. Sera were collected at pre-immunization (pre-imm) and after the third immunization (imm-3) and determined for antibody to Hb Portland or Hb A_2 by indirect ELISA.

3.3.2 Cell fusion

After the third immunization, spleen cells from the immunized mice were fused with myeloma cells using PEG as fusogen. In this study, after cell fusion, HAT and HT medium were prepared using 20% BW5147 conditioned medium (BW2) or 10% BM condimed H1 as supplement. The numbers of hybridoma containing wells obtained from both conditioned media supplementations were determined. The results were shown in Table 3.6. In Hb Portland experiment, the total hydridoma containing wells obtained in BM condimed H1 and BW5147 conditioned medium supplement were 249 and 187 wells, respectively. In Hb A_2 experiment, the total hydridoma containing wells obtained in BM condimed H1 and BW5147 conditioned medium supplement were 275 and 166 wells, respectively. Culture supernatants from hybridoma containing wells were screened for the antibody activity by indirect ELISA. In Hb Portland experiment, 211 culture supernatants from hybridoma containing wells in BM condimed H1 supplement were screened for the antibody activity and 23 of those 211 wells (10.9%) showed positive reactivity. By BW5147 conditioned medium, 128 culture supernatants from hybridoma containing wells were screened and 7 wells (5.5%) showed positive reactivity. In Hb A_2 experiment, 123 culture supernatants from hybridoma containing wells of BM condimed H1 supplement were screened for the antibody activity and 8 wells (6.5%) showed positive reactivity. By BW5147 conditioned medium, 95 culture supernatants from hybridoma containing wells were screened and 9 wells (9.5%) showed positive reactivity. In summary, after antibody screening, by using standard BM condimed H1, 23 hybridomas producing anti-Hb Portland and 8 hybridomas producing anti-HbA_2 were obtained. By using the generated BW5147 conditioned medium, 7 hybridomas producing anti-Hb Portland and 9 hybridomas producing anti-HbA_2 were obtained.

These results indicated that the produced BW5147 conditioned medium could also be used as supplement for generation of hybridoma in hybridoma technique.

Table 3.6 Generation of hybridomas and antibody reactivity screening by using BW5147 conditioned medium and BM condimed H1.

Fusion	Total[a]		Ab-screen[b]		Ab Positive[c]		%Ab Positive[d]	
	BM	BW	BM	BW	BM	BW	BM	BW
Hb Portland	249	187	211	128	23	7	10.90	5.47
Hb A_2	275	166	123	95	8	9	6.50	9.47

[a] Total number of hybridomas growth.

[b] Number of antibody-screening hybridomas.

[c] Number of positive hybridoma wells.

[d] Percentage of hybridoma positive wells in relation to the number of screening hybridoma wells.

3.3.3 Single cell cloning

To further compare between BW5147 conditioned medium and BM condimed H1, four hybridoma positive wells from Hb Portland fusion (No. 65, 95, 233 and 314) were subjected for single cell cloning using BW5147 conditioned medium and BM condimed H1 supplement. After single cell cloning, culture supernatants from hybridoma containing wells were determined for specific antibody by indirect ELISA technique. Hybridoma clone No. 65, 3 of 3 culture supernatants from hybridoma containing wells using BW5147 conditioned medium supplement and 6 of 6 culture supernatants from hybridoma containing wells using BM condimed H1 supplement showed positive (Table 3.7A). Hybridoma clone No. 233 and 314, neither culture supernatants from 6 and 8 hybridoma containing wells using BW5147 conditioned media nor from 3 and 4 hybridoma containing wells using BM condimed H1 showed positive reactivity (Table 3.7B and 3.7C). Hybridoma No. 95, 3 of 5 culture supernatants from hybridoma containing wells using BW5147 conditioned media showed positive reactivity while 1 of 5 culture supernatants from hybridoma containing wells using BM condimed H1 showed positive reactivity (Table 3.7D). The positive wells of hybridoma clone No. 95 were then subjected for the second limiting dilution. After secondary limiting dilution, all culture supernatants from wells containing hybridomas using BW5147 conditioned medium or BM condimed H1 supplement showed positive (Table 3.7E).

Altogether, we concluded that the BW5147 conditioned medium could be used as supplement for generation of hybridomas for monoclonal antibody production by hybridoma technique.

To confirm the above finding, we produced large amount of BW5147 conditioned medium and the produced conditioned medium were then employed in routine hybridoma production and single cell cloning in Dr. Watchara's laboratory. By this employment, the produced conditioned medium were successfully used for production of monoclonal antibodies against human Hb E/A_2, activated platelets and CD99 associated molecules (Table 3.8, 3.9 and 3.10).

For production of monoclonal antibody to Hb E/A_2, mouse was immunized with Hb E and Hb A_2 and the immunized mouse splenocytes were fused with myeloma cells using 20% BW5147 conditioned medium (BW2) and 10% BM condimed H1 supplementation. In this experiment, the final positive hybridoma clones obtained by using BW5147 conditioned medium (BW 2) and commercial BM condimed H1 were not different (Table 3.8).

For production of monoclonal antibody to activated platelets, mouse was immunized with activated platelets and the immunized mouse splenocytes were fused with myeloma cells using 20% BW5147 conditioned medium (BW2) and 10% BM condimed H1 supplementation. In this experiment, the final positive hybrodoma clones obtained by using BW5147 conditioned medium (BW 2) were not significantly lower than those of using commercial BM condimed H1 (Table 3.9).

For production of monoclonal antibody to CD99 associated molecules, mouse was immunized with CD99 associated molecules and the immunized mouse splenocytes were fused with myeloma cells using 20% BW5147 conditioned medium (BW2) and 10% BM condimed H1 supplementation. In this experiment, the final positive hybridoma clones obtained by using BW5147 conditioned medium (BW2) were a bit lower than those of using commercial BM condimed H1 (Table 3.10).

From these results, it is encourage that the “home-made” BW5147 conditioned medium can be utilized for hybridoma technology.

Table 3.7 Single cell cloning of hybridoma produce anti-Hb Portland using BW5147 conditioned medium and BM condimed H1.

(A)

No. 65	Total[a] (day 10)	Single clone[b] (day 10)	size[c] (day 10)				size[c] (day 12)				size[c] (day 15)				size[c] (day 17)				Ab assay[d]	
			VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	Positive	Negative
BM condimed H1	29	29	29	-	-	-	22	7	-	-	12	12	1	-	3	8	6	-	6	0
BW conditioned media	19	19	19	-	-	-	17	2	-	-	7	3	-	-	6	1	3	-	3	0

(B)

No. 233	Total[a] (day 7)	Single clone[b] (day 7)	size[c] (day 7)				size[c] (day 10)				size[c] (day 12)				size[c] (day 15)				size[c] (day 17)				Ab assay[d]	
			VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	Pos	Neg
BM condimed H1	48	37	37	-	-	-	29	1	-	-	23	4	1	-	16	3	4	-	14	2	6	-	0	6
BW conditioned media	26	32	32	-	-	-	28	1	-	-	20	5	-	-	13	4	1	-	12	3	3	-	0	3

(C)

No. 314	Total[a] (day 10)	Single clone[b] (day 7)	size[c] (day 7)				size[c] (day 10)				size[c] (day 12)				size[c] (day 15)				Ab assay[d]	
			VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	Positive	Negative
BM condimed H1	101	40	39	1	-	-	28	7	-	-	27	6	2	-	17	10	6	-	0	8
BW conditioned media	50	33	33	-	-	-	27	-	-	-	21	-	-	-	17	4	-	-	0	4

(D)

No. 95 (1st limit)	Total[a] (day 7)	Single clone[b] (day 7)	size[c] (day 7)				size[c] (day 10)				size[c] (day 12)				size[c] (day 15)				size[c] (day 17)				Ab assay[d]	
			VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	Pos	Neg
BM condimed H1	69	41	41	-	-	-	19	12	-	-	12	12	3	1	7	12	8	1	3	9	16	2	1	4
BW conditioned media	49	43	43	-	-	-	31	9	-	-	20	13	2	-	14	17	2	1	13	15	3	2	3	2

(E)

No. 95 (2nd limit)	Total[a] (day 7)	Single clone[b] (day 7)	size[c] (day 7)				size[c] (day 10)				size[c] (day 15)				Ab assay[d]	
			VS	S	M	L	VS	S	M	L	VS	S	M	L	Pos	Neg
BM condimed H1	155	68	51	17	-	-	11	38	18	-	2	16	47	1	10	0
BW conditioned media	148	52	47	5	-	-	20	23	7	-	7	25	19	1	10	0

The data represent results of single cell cloning of Hb Portland hybridomas positive well containing media with 20%BW conditioned media and BM condimed H1. Five independent experiments were performed.

[a] Total number of hybridoma growth at the indicated day.

[b] Number of hybridoma single clone at the indicated day.

[c] Size of hybridoma single clone at the indicated days.

[d] Number of antibody-screening hybridomas well

Table 3.8 Comparison of using BW5147 conditioned medium and BM condimed H1 in generation of hybridomas produce anti-Hb E/A_2 antibody

Conditioned medium	Total[a]	Ab-screen[b]	Ab Positive[c]
BM condimed H1	**134**	**93**	**2**
BW conditioned media	**104**	**63**	**2**

Mouse was immunized with human hemoglobin E and A_2 for three immunizations and spleen cells were used to generate hybridomas using 20% BW5147 conditioned medium (BW2) and 10% BM condimed H1 supplement.

[a] Total number of hybridoma containing wells.

[b] Number of hybridoma containing wells for antibody screening.

[c] Number of hybridoma positive wells.

Table 3.9 Comparison of using BW5147 conditioned medium and BM condimed H1 in generation of hybridoma produce anti- activated platelet antibody

Conditioned medium	Total[a]	Ab-screen[b]	Ab Positive[c]
BM condimed H1	**28**	**28**	**3**
BW conditioned media	**24**	**13**	**2**

Mouse was immunized with human activated platelets for three immunizations and spleen cells were used to generate hybridomas using 20% BW5147 conditioned medium (BW2) and 10% BM condimed H1 supplement.

[a] Total number of hybridoma containing wells.

[b] Number of hybridoma containing wells for antibody screening.

[c] Number of hybridoma positive wells.

Table 3.10 Comparison of using BW5147 conditioned medium and BM condimed H1 in generation of hybridoma produce anti-CD99 associated molecules.

Conditioned medium	Total[a]	Ab-screen[b]	Ab Positive[c]
BM condimed H1	**146**	**115**	**44**
BW conditioned media	**179**	**125**	**39**

Mouse was immunizied with CD99 associated molecules for three immunizations and spleen cells were used to generate hybridomas using 20% BW5147 conditioned medium (BW2) and 10% BM condimed H1 supplement.

[a] Total number of hybridoma containing wells.

[b] Number of hybridoma containing wells for antibody screening.

[c] Number of hybridoma positive wells.

3.4 Analysis of protein contained in conditioned medium by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

BW5147 conditioned medium, BM condimed H1, and 10% FCS-IMDM were two-folds serial diluted into 3 dilutions, 1:2, 1:4, and 1:8. Then, the proteins were separated by SDS-PAGE using 12.5%, 10%, or 7.5% separating gels and stained with Coomassie Blue (Figure 3.5, 3.6, and 3.7). It was found that the protein bands contained in the three media tested were different.

The results from 12.5% SDS-polyacrylamide gel showed that a protein band of 17 kDa was appeared in BM condimed H1 (Figure 3.5, lane 2), but not in 10%FCS-IMDM and BW5147 conditioned medium (Figure 3.5, lane 3 and 4). Moreover, a protein band at 26 kDa was observed in BM condimed H1 (Figure 3.2B, lane 2) but this band was not observed in 10%FCS-IMDM and was slightly appeared in BW5147 conditioned medium (Figure 3.5, lane 3 and 4).

In 10% SDS-polyacrylamide gel, the appearance of 26 kDa was confirmed as observed in 12.5% SDS-polyacrylamide gel (Figure 3.6).

In an attempt to determine the proteins of molecular weight higher than 50 kDa, the 7.5% SDS-polyacrylamide gel was employed. As shown in Figure 3.7, the proteins which have high molecular weight were not different between the two conditioned media and 10%FCS-IMDM.

Altogether, the results indicated that the proteins contained in the generated BW5147 conditioned medium were different from those of the commercially available BM condimed H1.

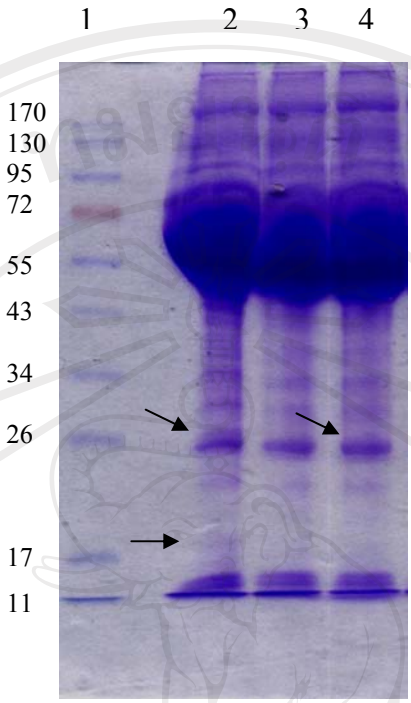


Figure 3.5 Proteins analysis of BW5147 conditioned medium, BM condimed H1 and 10%FCS-IMDM using 12.5% SDS-PAGE. BW5147 conditioned medium, BM condimed H1, and 10%FCS-IMDM were loaded into each well of 12.5% SDS-polyacrylamide gel and performed the electrophoresis. Gels were stained with Coomassie brilliant blue dye. Lane 1: Standard protein markers, Lane 2: BM condimed H1, Lane 3: 10%FCS-IMDM and Lane 4: BW5147 condition medium**.** Arrows indicate the 17 and 26 kDa protein bands.

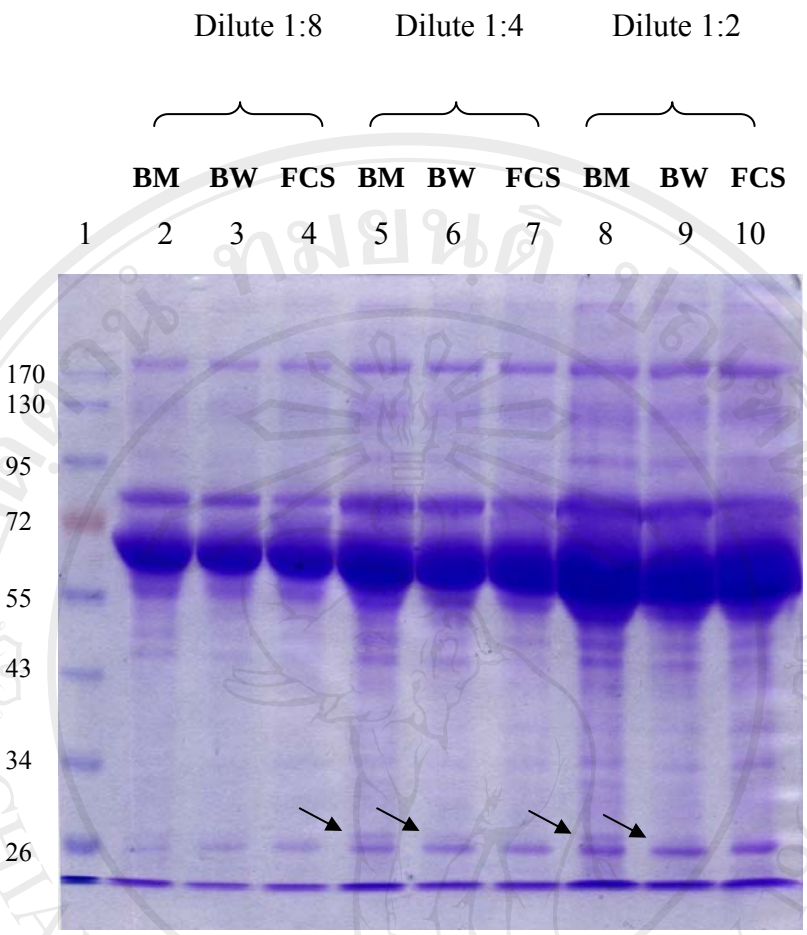


Figure 3.6 Proteins analysis of BW5147 conditioned medium, BM condimed H1 and 10%FCS-IMDM using 10% SDS-PAGE. BW5147 conditioned medium, BM condimed H1 and 10%FCS-IMDM were diluted to 1:2, 1:4 and 1:8 and loaded into each well of 10% SDS-polyacrylamide gel and performed the electrophoresis. Gels were stained with Coomassie brilliant blue dye. Lane 1: Standard protein markers (kDa), Lane 2: BM-condimed H1 dilute 1:8, Lane 3: BW5147 Conditioned media dilute 1:8, Lane 4: 10%FCS-IMDM dilute 1:8, Lane 5: BM condimed H1 dilute 1:4, Lane 6: BW5147 Conditioned media dilute 1:4, Lane 7: 10%FCS-IMDM dilute 1:4, Lane 8: BM-condimed H1 dilute 1:2, Lane 9: BW5147 Conditioned media dilute 1:2, Lane 10: 10%FCS-IMDM dilute 1:2. Arrow indicates the 26 kDa protein bands.

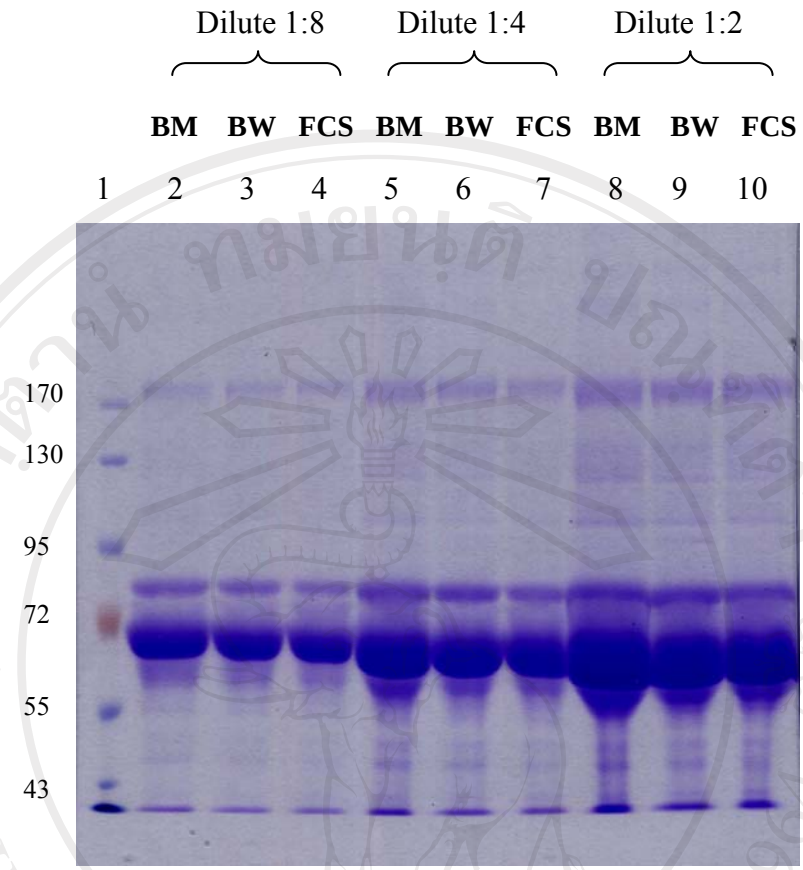


Figure 3.7 Proteins analysis of BW5147 conditioned medium, BM condimed H1 and 10%FCS-IMDM using 7.5% SDS-PAGE. BW5147 conditioned medium, BM condimed H1 and 10%FCS-IMDM were diluted to 1:2, 1:4, and 1:8 and loaded into each well of 7.5% SDS-polyacrylamide gel. Gels were stained with Coomassie brilliant blue dye. Lane 1: Standard protein markers (kDa), Lane 2: BM condimed H1 dilute 1:8, Lane 3: BW5147 conditioned medium dilute 1:8, Lane 4: 10%FCS-IMDM dilute 1:8, Lane 5: BM condimed H1 dilute 1:4, Lane 6: BW5147 conditioned medium dilute 1:4, Lane 7: 10%FCS-IMDM dilute 1:4, Lane 8: BM-condimed H1 dilute 1:2, Lane 9: BW5147 conditioned medium dilute 1:2, Lane 10: 10%FCS-IMDM dilute 1:2.

3.5 Analysis and comparison of cost effectiveness between the produced conditioned medium and commercial reagent

Analysis and comparison of the cost between 100 ml BW5147 conditioned medium and 100 ml BM condimed H1 were shown in Table 3.11. The cost for 100 ml of BW5147 conditioned medium was approximately 1,600 baht. In contrast, 100 ml BM condimed H1 purchased from Roche was 21,000 baht. Although, the preparation of BW5147 conditioned medium requires time and workforce, but the production procedure is so simple and need no special technology and sophisticated equipment. The BW5147 conditioned medium was therefore recommended for replacement of very expensive commercial reagent in resource-limited countries including Thailand.

Table 3.11 Analysis and comparison of cost effectiveness between BW5147 conditioned medium and commercial reagent BM condimed H1

	BW5147 conditioned media	BM condimed H1
Time consumption for preparation of conditioned medium	6 hours	-
Workforce for preparation of conditioned medium (80 baht/hour)	480 baht	-
IMDM medium (0.5 liter)*	230 baht	-
Fetal calf serum (50 ml) *	750 baht	-
Filter membrane 0.1 μm (1 piece)	40 baht	-
Equipment maintainnance	100 baht	-
Cost for 100 ml	1,600 baht	21,000 baht **

* 10%FCS-IMDM is used for maintaining of starting cells and production of conditioned medium.

** BM condimed H1 is the product from Roche Applied Science.