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14th June, 2000

Preparation of CMG14-12 culture supernatant

(CMG14-12: M-CSF producing cell line)

Media and reagents:

D-10 (normal media)

	Stock	Volume
DMEM (Sigma D5796)	1 X	500 ml
10% HIFBS	100%	56 ml
1 X PenStrep (GibcoBRL 15140-122)	100 X	5.6 ml

D-10G (selection media)

	Stock	Volume
DMEM (Sigma D5796)	1 X	500 ml
10% HIFBS	100%	56 ml
400 μg/ml G418(GibcoBRL 10131-027)	50 mg/ml	4.5 ml
1 X PenStrep	100 X	5.6 ml

α-10 (preparation media)

	Stock	Volume
α-MEM (Gibco 12571)	1 X	500 ml
10% HIFBS	100%	56 ml
0.1 X PenStrep	100 X	5.6 ml

Trypsin/EDTA (0.05% Trypsin/0.02% EDTA)

0.5% Trypsin/0.2% EDTA (GibcoBRL 15400-054)
(10-times diluted with PBS)

PBS

Culture dishes:

ϕ10 cm dish (Corning Coster 430167)
ϕ15 cm dish (Falcon 353025)
Nunclon TripleFlask (Nunc 132867)

(1) Introductions for the M-CSF producing cell line

Name of cell line: CMG14-12
Parental cell line: Ltk$^-$ (ATCC CCL 1.3)
Establishment of CMG14-12:

Mouse M-CSF cDNA was cloned into the mammalian expression vector, pCXN2 (Niwa et al., Gene, 108 1991 p193-200) which is driven under the control of chicken □-actin promoter and CMV enhancer. The resulting M-CSF expression plasmid was transfected into Ltk$^-$ cells. Transfectants were selected by 400 μg/ml G418. The limiting dilution was performed and selected M-CSF high producing cell lines by M-CSF bioassay. One of them, named CMG14-12, was established. This cell line produces 1-2 μg/ml M-CSF in the 2 days culture supernatant. This culture supernatant was examined the colony forming activity using mouse bone marrow cell culture and had 3.4-3.6 X 10^5 units/ml while that of parental Ltk- cells was 4.0 X 10^3 units/ml.

(2) Instructions for culture of CMG14-12 cell line

1.Thaw quickly frozen cells at 37℃

Add 1 ml of cells into 9 ml D-10 media and mix

Spin at 1200 rpm for 5 min

Suspend cells into 20 ml D-10 media

Seed cells into two ϕ10cm dishes (10 ml/dish)

Incubate at 37 C for one day

2. On next day, change media to 10 ml D-10G media per plate

Incubate at 37℃ until sub-confluent

(Do not grow at over confluent)

3. After sub-confluent, wash cells with PBS twice

Add 2 ml of Trypsin/EDTA per dish

Leave at RT for 5 min and tap dish occasionally

Add 6 ml D-10 media in one dish, suspend cells by pipetting, transfer cells to the other dish and re-suspend

Transfer cells into a 15 ml or 50 ml conical tube

Spin cells and re-suspend cells into 10 ml D-10 media

(Change media every 3 days if necessary)

Count cell number

(Number of cells should be 4 - 8 X 10^6 per □10cm plate at sub-confluent.)

4. Split 1:5 into ϕ10cm dishes with D-10G media

(1 X 10^6 cells/ϕ10cm dish or 2.5 X 10^6 cells/ϕ15cm dish should be better)

5. Freeze cells at 2 – 6 X 10^6 cells/tube(1ml)

*** For the maintenance of CMG14-12 cells, cells should be cultured in the selection media (D-10G). I strongly recommend to make a lot of frozen stocks as soon as possible and to examine M-CSF bioactivity of your culture supernatant using M-CSF dependent cell line (M-NFS-60 etc.) or mouse whole bone marrow cells, and recombinant mouse M-CSF (R&D) for the control.**

(3) Cell expansion and large scale prep.

You can get culture supernatant from following 3 different dishes. It depends on your necessarily how much you want.
You can get the M-CSF high titer supernatant at least 5 times of 2 days culture from 1 dish.

Items	ϕ10 cm	ϕ15 cm	TripleFlask
Area (cm^2)	55 cm^2	148 cm^2	500 cm^2
Media (ml)	20 ml	50 ml	200 ml
Needed cell # / dish	$2X10^6$	$5X10^6$	$2X10^7$
Final vol.(X5)/ dish	100 ml	250 ml	1000 ml
Final vol.(X5)/10 dishes	1 L	2.5 L	10 L

1. Prepare several ϕ10cm or ϕ15cm dishes at sub-confluent
2. Harvest cells with Trypsin/EDTA (see (2).3)
   Suspend cells into α -10 media
   Count cell number
   Adjust at 1 X 10^5/ml with α -10 media
   Seed cells on appropriate plates or flasks
   Culture cells until confluent
   (It will take for 3 days)
3. After confluent, change media (20, 50 or 200 ml α-10 per ϕ10cm, ϕ15cm dishes or TripleFlask)
   Incubate for 2 days
4. Filtrate culture supernatant and store at 4℃ before M-CSF bioassay (the 1st round of sup.)
5. Add fresh media to cells in dishes or flask
   Incubate for 2 days, filtrate culture supernatant and store at 4℃ before bioassay (the 2nd round of sup.)
6. Prepare culture supernatant (next 3rd to 10th) of as same as above (see (3).3, 4 and 5)
7. After performing M-CSF bioassay (next session), good

batches of bottles should be equally divided into new bottles and stored at –20℃ for long term storage

(4) M-CSF bioassay for CMG14-12 culture supernatant

Cell line: mouse myelogenous leukemia M-CSF dependent cell line, M-NFS-60 (ATCC CRL-1838)

Bone marrow (BM) cells: mouse whole bone marrow cells, Mouse strain: C57BL/6 or ddY

Media and Reagents:

R-10

	Stock	Volume
RPMI1640 (Sigma R8758)	1 X	500 ml
55 μM 2-ME(GibcoBRL 21985-023)	1000 X	0.56 ml
10% HIFBS	100%	56 ml
1 X PenStrep	100 X	5.6 ml

R-10M (complete media)

R-10 media		50 ml
20 ng/ml M-CSF	50 μg/ml	20 μl
(or CMG sup	(1~2 μg/ml)	0.5 ml)

MTT solution: 5 mg/ml (dissolve 50 mg MTT into 10 ml PBS, filtrate 0.2 μm filter, divide 2 ml each and store at –20℃)
MTT:(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)(Sigma M2128)

IPA/HCl: isopropyl-OH/0.04N HCl (mix with 500 ml isopropyl-OH and 1.73 ml of HCl)

rmM-CSF: R&D Cat# 416-ML

PBS

A-1. Preparation of M-CSF dependent cell line

1. **Culture 20 ~ 40 ml M-NFS-60 cells (< 1 X 10^6 cells/ml) in R-10M media (cells should not be over-confluent because M-CSF non-dependent cells can sometimes be grown)**
2. **Wash cells with cold PBS twice**
   Re-suspend cells with 10 ml R-10 media
3. **Count cell number**
4. **Adjust at 2 X 10^5 cells/ml with 10 ml R-10 media and chill on ice until use**

A-2. Preparation of mouse bone marrow cells

1. **Take mouse long bone**
2. **Flush marrow in α-0 media**
3. **Wash cells with R-10 media**
4. **Count cell number**
5. **Adjust at 2 X 10^6 cells/ml with 10 ml R-10 media and chill on ice until use**

B. Dilution of M-CSF (recombinant and sup.)

1. **rmM-CSF:200 ng/ml with R-10 media (mix 4 μl of 50 μg/ml rmM-CSF with 1 ml R-10 media and store**

at 4℃ for 1 month)

2. Sup. (the 1st to 10th): 10-times dilution (mix 100 μl each sup. with 900 μl R-10 media and store at 4℃)
3. Mixed sup.: mix 100 μl 10-times diluted each sup.(from the 1st to 10th)

C. Preparation of 96-well-plate

1. Add 100 μl of rmM-CSF (200 ng/ml) into A-1 of 96-well-plate, 100 μl of 10-times diluted mixed sup. into A-2 and 100 μl of 10-times diluted each sup.(the 1st to 10th) into A-3 to A-12
2. Add 50 μl R-10 media into all other wells
3. Using a 12-pipete, set 50 μl and dilute serially from A to H in a 96-well-plate

D. Addition of cells

1. Add 50 μl of cells (1 X 10^4 M-NFS-60 cells or 1 X 10^5 BM cells) into all wells containing diluted M-CSF
2. Incubate at 37 C for 2 – 3 days until confluent on A-1
3. Add 10 μl of MTT solution into each well and incubate at 37℃ for 4 hours
4. Add 150 μl of IPA/HCl into each well and mix well by vigorously pipetting

5. **Measure the plate at OD_{570}**
6. **Make dose-response curve of rmM-CSF**
7. **Calculate the M-CSF concentration of each sup.**

Takeshita S, Kaji K, Kudo A. Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J Bone Miner Res. 2000 15:1477-88.