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## 🌐 Cell culture of RAW264.7 cells

Sijia Liao<sup>1</sup>, Lisa Börmel<sup>1</sup>, Maria Wallert<sup>1</sup>, Stefan Lorkowski<sup>1</sup>

<sup>1</sup>Institute of Nutritional Science, Friedrich Schiller University Jena



Sijia Liao

Friedrich Schiller University Jena, Germany

### ABSTRACT

RAW264.7 cells are a macrophage-like cell line that was derived from a male BALB/c mouse ascites tumor induced by Abelson Leukemia Virus (A-MuLV) [1]. Due to its rapid proliferation and ease of handling, the RAW264.7 cell line is widely used to study immune function, inflammation, and cancer biology. The cells have a doubling time of about 11 to 30 hours. The growth media should be refreshed every 2 to 3 days but not exceeding 4 days. RAW264.7 cells can perform phagocytosis and pinocytosis, which are essential functions of macrophages. In addition, RAW264.7 M0 macrophages can differentiate into M1 or M2 macrophages upon respective stimuli. Hence, stimulated RAW264.7 cells have also been widely used as an *in vitro* inflammatory model for screening the effectivity of anti-inflammatory compounds and the investigation of the underlying molecular mechanism.

### PROTOCOL REFERENCES

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- [3] Hartley JW, Evans LH, Green KY, Naghashfar Z, Macias AR, Zerfall PM, Ward JM. Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. *Retrovirology* 2008; 4(5):1. DOI: [10.1186/1742-4690-5-1](https://doi.org/10.1186/1742-4690-5-1). PMID: 18177500.

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We use this protocol and it's working

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## MATERIALS

### Equipment:

- Personal protective equipment (sterile gloves, laboratory coat, safety visor etc.)
- Water bath set to 37 °C
- Microbiological safety cabinet at the appropriate containment level
- Incubator at 37 °C and 5 % (v/v) CO<sub>2</sub> atmosphere
- Centrifuge
- Inverted microscope
- Neubauer counting chamber and cover slips or cell counter
- Freeze container (e.g. Mr. Frosty, Nalgene)
- - 80°C freezer
- Liquid nitrogen tank

### Materials:

- Cell culture flasks: 25 and 150 cm<sup>2</sup>
- Sterile serological pipette
- Sterile Pasteur pipette
- Sterile filter tips
- Sterile reaction tubes (15 and 50 ml)
- Sterile cell scraper
- Sterile cryotubes (2 ml)

### Chemicals:

- Dulbecco's Modified Eagle's Medium (4500 mg/L glucose, sterile, suitable for cell culture) pre-warmed to 37 °C
- Fetal bovine serum (Merck/Sigma Aldrich, Cat. No. S0615)
- L-Glutamine–penicillin–streptomycin solution (L-glutamine 200 mM, streptomycin 10 mg/mL, penicillin 10,000 units)
- Sterile dimethyl sulfoxide (DMSO)
- 2-propanol

## SAFETY WARNINGS



Biosafety level 2 applies to the use of the RAW264.7 cell line, as it polyploid murine leukemia virus (MLV) have been detected in the cell culture supernatant of these cells, in addition to replication-competent, ecotropic MLV [2,3].

## Thaw RAW264.7 cells

- 1 Transfer the cryotube with the cell suspension from the liquid nitrogen tank to the cell culture on ice.  
  
**Note:** When handling with liquid nitrogen, wear protective goggles, gloves and a gown, otherwise cold burn may occur.
- 2 Thaw the cells immediately in a 37 °C water bath until no more ice chunks are visible.  
  
**Note:** This procedure and the next steps must be performed quickly because the cryoprotectant dimethyl sulfoxide (DMSO) is cytotoxic above 4 °C.
- 3 Mix the thawed 1 ml cell suspension with 14 ml pre-warmed high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS) and 0.1 mg/ml glutamine–penicillin–streptomycin (PSG) solution in a 15 ml tube and resuspend well but gently.
- 4 Centrifuge the cell suspension for 10 min with 300 x g at room temperature and carefully aspirate the supernatant with a sterile pasteur pipette.
- 5 Resuspend the cell pellet in 5 ml fresh high-glucose DMEM (containing 10 % (v/v) FBS and 0.1 mg/ml PSG) and transfer the cell suspension to a 25 cm<sup>2</sup> cell culture flask and place it in a 37 °C incubator with 5 % (v/v) CO<sub>2</sub> atmosphere for growth.
- 6 Leave the cells in the incubator and observe the cell growth every two days. Once the cells reach 60 to 80 % confluency, remove the supernatant and detach the adherent cells in 5 ml fresh high-glucose DMEM (containing 10 % (v/v) FBS and 0.1 mg/ml PSG) using a cell scraper.
- 7 Fill the cell suspension with fresh pre-warmed high-glucose DMEM (containing 10 % (v/v) FBS and 0.1 mg/ml PSG) to a total volume of 50 ml and transfer the whole cell suspension into a 150 cm<sup>2</sup> cell culture flask and put the flask in a 37 °C incubator with a 5 % (v/v) CO<sub>2</sub> atmosphere.

## Passaging RAW264.7 cells

- 8 Control the cells using an inverted microscope to assess the degree of confluency, check for morphological changes, and confirm the absence of bacterial and fungal contamination.  
  
**Note:** Elongated cells indicate activation, non-activated cells have a round shape.
- 9 For a 150 cm<sup>2</sup> cell culture flask, transfer the whole used DMEM in a tube (50 ml) and centrifuge at 400 x g for 5 min at room temperature.  
  
**Note:** To avoid contamination, all steps onwards should be performed in a sterile environment.
- 10 Add 10 ml fresh high-glucose DMEM (containing 10 % (v/v) FBS and 0.1 mg/ml PSG) to the cell monolayer and aspirate the supernatant.  
  
**Note:** High-glucose DMEM (containing 10 % (v/v) FBS and 0.1 mg/ml PSG) should be pre-warmed to 37 °C. During this procedure, the cells should be placed on a 37 °C pre-heated thermoplate.
- 11 Add an additional 10 ml fresh high-glucose DMEM (containing 10 % (v/v) FBS and 0.1 mg/ml PSG), detach cells carefully but with sufficient pressure with a cell scraper, and transfer the whole cell suspension into a new tube.
- 12 Label the cell culture flask with the date and the number of passaging.  
  
**Note:** Under normal conditions, one cell culture flask can be used for 10 to 20 cell passages. If significantly more cells are observed in the supernatant (indicating dead cells) during the morphological evaluation, the cell culture flask should be replaced with a new one accordingly.
- 13 To passage the cells, the cell suspension is diluted (at 80 % confluency choose a 1:5 dilution with high-glucose DMEM (containing 10 % (v/v) FBS and 0.1 mg/ml PSG), referring to total volume of 10 ml.
- 14 Transfer the defined amount (e.g. 2 ml at a 1:5 dilution) back into the culture flask and add 28 ml fresh high-glucose DMEM (containing 10 % (v/v) FBS and 0.1 mg/ml PSG) and 15 ml used DMEM (about a third of the old medium is required for the subculturing of RAW264.7 cells).  
  
**Note:** Used medium from the subculture contains important hormones and growth factors expressed by RAW264.7 cells that help the cells to proliferate better.
- 15 When subcultured cells reach 60 to 80 % confluency (normally 2 to 3 days), repeat the cell passaging as outlined above.

## Seeding RAW264.7 cells for subsequent experiments

**16** Use the remaining cell suspension from the subculture for seeding cells for further experiments.

**17** Use the Neubauer counting chamber (or an automated cell counter) to determine the cell number per milliliter cell suspension.

**Note:** If using a Neubauer counting chamber, a total of 10  $\mu$ l cell suspension should be pipetted under the cover slip. Usually, a 1:10 to 1:50 dilution of the suspension of RAW264.7 cells is needed to count the cell number using a microscope.

**18** Prepare the required volume for seeding the cell suspension with fresh high-glucose DMEM (containing 0.1 mg/ml PSG) and used DMEM; the final cell suspension for seeding should contain two thirds fresh medium and one third old medium.

One example for the calculation:

A	B
Determined cell number of suspension	$5 \times 10^6$ cells per ml
Total volume for seeding	5 ml
Required cell number for seeding	$2 \times 10^6$ cells in 5 ml
Calculation:	
Required cell suspension	0.40 ml
Fresh high-glucose DMEM (containing 0.1 mg/ml PSG) to add	2.93 ml
Used culture medium to add	1.67 ml

**19** Transfer the prepared cell suspension into culture plates or cell culture flasks as required.

**Note:** Depending on the experiment (e.g. with regard to the duration of incubation) and the research question, it may be useful or even necessary to adjust the cell density.

A	B	C	D	E	F	G
	25 cm <sup>2</sup> -flask	6-well plate	12-well plate	24-well plate	48-well plate	96-well plate
Cell number/flask or well	2 x 10 <sup>6</sup>	0.8 x 10 <sup>6</sup>	0.3 x 10 <sup>6</sup>	0.15 x 10 <sup>6</sup>	0.08 x 10 <sup>6</sup>	0.03 x 10 <sup>6</sup>
Volume/flask or well	5 ml	2 ml	1 ml	0.5 ml	0.3 ml	0.2 ml

- 20** Label the cell culture vessel with the date and passage number of the cell culture. Put the seeded cells back into the 37 °C incubator with a 5 % (v/v) CO<sub>2</sub> atmosphere and allow the cells to settle for 24 h.

### Freeze RAW264.7 cells

- 21** Prepare freeze container: Fill the freeze container (e.g. Mr. Frosty, Nalgene) with 2-propanol and cool it at 4 °C. Cooling in 2-propanol ensures a slow and gentle temperature decrease. A cooling rate of -1°C/min is the optimal rate for cell preservation.
- 22** Prepare freezing medium: high-glucose DMEM containing 20 % (v/v) FBS, 0.1 mg/ml PSG and 10 % (v/v) sterile DMSO.
- 23** Count the cells with a Neubauer counting chamber or a cell counter, calculate the number of needed cryotubes and freezing medium (see the section "Seeding RAW264.7 cells for subsequent experiments"). Adjust cell concentration to 1x10<sup>7</sup> cells/ml.
- 24** **Note:** Cells should be kept on ice from now on.
- Distribute 1 ml of the cell suspension in freezing medium into each cryotube. Place the cryotube into the freeze container and subsequently put the freeze container into a - 80 °C freezer for cooling down overnight. On the next day, place the frozen cryotubes into liquid nitrogen for long term storage.