
Cytokine Neutralization, *In Vitro*

Research Use Only

Introduction

Antibodies that block binding of cytokines to their specific receptors and neutralize their effects are critical in studies of cytokine function. The following four protocols describe *in vitro* bioassays using neutralizing anti-mouse and anti-human cytokine antibodies.

[Protocol A: Antibody Neutralization of Cytokine-Induced Proliferation of Indicator Cell Lines](#)

[Protocol B: Antibody Neutralization of TNF- \$\alpha\$ -Induced Killing of L929 Cell Line](#)

[Protocol C: Antibody Neutralization of IFN- \$\gamma\$ -Protection from Viral Infection of L929 and A549 Cell Lines](#)

[Protocol D: Antibody Neutralization of Cytokine-Induced Cytokine Production](#)

In general, the cytokine bioassay protocols are modified to pre-incubate the cytokine of interest with the specific neutralizing antibody prior to addition to the responding cells. This prevents cytokine binding to its receptor on the responding cells, thereby inhibiting the cytokine effect. The chart below summarizes the general optimized experimental conditions for the neutralization assays using indicator cell lines. When working with other cell types, cytokine concentrations, neutralizing antibody concentrations and incubation times may need to be determined by the investigator.

Protocol A: Antibody Neutralization of Cytokine-Induced Proliferation of Indicator Cell Lines

Materials

- Indicator cell line (see [Quick Guide Chart](#) for a given cytokine)
- Culture Medium (RPMI supplemented with 10% FBS)
- Assay Medium (RPMI supplemented with 10% FBS)
- 96-well flat-bottom culture plate (Costar Cat. No. 3595)
- MTT solution (Sigma Cat. No. M5655) 5mg/ml stock in PBS kept at room temperature (protect from light)
- MTT Lysing solution 20% SDS/50% DMF

Instruments

- Pipettes and pipettors
- Humidified incubator
- 96-well micro test spectrophotometer

Experiment Duration

- 24-48 hour incubation (see [Quick Guide Chart](#))
- 1 hour assay preparation

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Experimental Procedure

1. Add 50 μ L/well of Assay Medium to each well of the 96-well assay plate.
2. Dilute Functional Grade (FG) anti-cytokine antibody by 2-fold serial dilution in the assay plate from row 3 to 12. Leave rows 1 and 2 empty (control rows).
3. Add 50 μ L/well of samples and recombinant cytokine (see [Quick Guide Chart](#) for concentration) to the assay plate from row 2 to row 12 (row 2 will be indicator cells and cytokine). Leave row 1 empty (cells alone).
4. Incubate the plate at 37°C, 5% CO₂ in a humidified incubator for 2 hours.
5. Wash indicator cells 3 times with RPMI-1640 and resuspend in Assay Medium at a density of 2-3.5x10⁵ cells/mL (see [Quick Guide Chart](#)).
6. Add 100 μ L of cell suspension to each well.
7. Incubate cells for 24-48 hours (see [Quick Guide Chart](#)) at 37°C, 5% CO₂ in a humidified incubator.
8. Add 10 μ L/well of 5 mg/mL MTT solution to the plate and incubate for 4 hours.
9. Add 50 μ L/well of MTT Lysing Solution to the plate and incubate overnight.
10. Read plate at 570-650 nm.
11. Graph standard curve and analyze data.

Protocol B: Antibody Neutralization of TNF- α -Induced Killing of L929 Cell Line

Materials

- L929 mouse fibroblast line (ATCC Cat. No. CCL-1)
- Culture Medium (RPMI supplemented with 10% FBS)
- Assay Medium (RPMI supplemented with 2% FBS)
- 96-well flat-bottom culture plate (Costar Cat. No. 3595)
- Actinomycin D, 500 μ g/mL stock aliquot kept at minus 80°C (protect from light)
- MTT solution (Sigma Cat. No. M5655) 5 mg/mL stock in PBS kept at room temperature (protect from light)
- MTT Lysing solution, 20% SDS/50% DMF

Instruments

- Pipettes and pipettors
- Humidified incubator
- 96-well micro test spectrophotometer

Experiment Duration

- 48 hour incubation (see [Quick Guide Chart](#))
- 1 hour assay preparation

Experimental Procedure

1. Prepare L929 cell suspension at a density of 3.5x10⁵/mL in Assay Medium. Add 100 μ L/well to the 96-well assay plate and incubate overnight at 37°C, 5% CO₂ in a humidified incubator.
2. Add 50 μ L/well of Assay Medium to each well of another dilution plate.

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3. Dilute Functional Grade (FG) anti-cytokine antibody by 2-fold serial dilution in the plate from row 3 to 12. Leave rows 1 and 2 empty (control rows).
4. Add 50 µL/well of samples and recombinant cytokine (see [Quick Guide Chart](#) for concentration) to the dilution plate from row 2 to row 12 (row 2 will be indicator cells and cytokine). Leave row 1 empty (cells alone).
5. Incubate the dilution plate at 37°C, 5% CO₂ in a humidified incubator for 2 hours.
6. Transfer 50 µL/well of the Ab/Ag mixed solution to the corresponding well of the assay plate containing cells.
7. Prepare a 4 µg/mL working solution of the Actinomycin D by diluting the 500 µg/mL stock 125X in the Assay Medium. Keep Actinomycin D solution protected from light. Add 50 µL of this working solution of Actinomycin D to each well.
8. Incubate plate for 24 hours at 37°C, 5% CO₂ in a humidified incubator.
9. Add 10 µL/well of 5 mg/mL MTT solution to each well and incubate for 4 hours.
10. Add 50 µL of MTT Lysing solution to each well and incubate overnight.
11. Read plate at 570-650 nm.
12. Graph standard curve and analyze data.

Protocol C: Antibody Neutralization of IFN-γ-Protection from Viral Infection of L929 and A549 Cell Lines

Materials

- L929 mouse fibroblast line (ATCC Cat. No. CCL-1) or A549 human lung carcinoma (ATCC Cat. No. CCL-185)
- Culture Medium (RPMI supplemented with 10% FBS)
- Assay Medium (RPMI supplemented with 2% FBS)
- 96-well flat-bottom culture plate (Costar Cat. No. 3595)
- EMC virus, 10⁷ pfu/mL aliquots stock kept at minus 80°C
- MTT solution (Sigma Cat. No. M5655) 5 mg/mL stock in PBS kept at room temperature (protect from light)
- MTT Lysing solution, 20% SDS/50% DMF

Instruments

- Pipettes and pipettors
- Humidified incubator
- 96-well micro test spectrophotometer

Experiment Duration

- 24 hour incubation (see [Quick Guide Chart](#))
- 1 hour assay preparation

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Experimental Procedure

1. Prepare L929 or A549 cell suspension at a density of 3.5×10^5 /mL in Assay Medium. Add 100 μ L/well to the 96-well assay plate and incubate over night at 37°C, 5% CO₂ in a humidified incubator.
2. Add 50 μ L/well of Assay Medium to each well of another dilution plate.
3. Dilute Functional Grade (FG) anti-cytokine antibody by 2-fold serial dilution in the plate from row 3 to 12. Leave rows 1 and 2 empty (control rows).
4. Add 50 μ L/well of samples and recombinant cytokine (see [Quick Guide Chart](#) for concentration) to the dilution plate from row 2 to row 12 (row 2 will be indicator cells and cytokine). Leave row 1 empty (cells alone).
5. Incubate the dilution plate at 37°C, 5% CO₂ in a humidified incubator for 2 hours.
6. Transfer 50 μ L/well of the Ab/Ag mixed solution to the corresponding well of the assay plate containing cells.
7. Incubate the assay plate for 6 hours.
8. Aspirate supernatant from the wells carefully and add 100 μ L/well of EMC virus solution at a density of $1-4 \times 10^4$ pfu/mL (see [Quick Guide Chart](#) for human IFN- γ : 1/250 dilution of stock of 10^7 pfu/mL, for mouse IFN- γ , 1/1000 dilution of stock of 10^7 pfu/mL).
9. Incubate plate for 40 hours at 37°C, 5% CO₂ in a humidified incubator.
10. Add 10 μ L of 5 mg/mL MTT solution to each well and incubate for 4 hours.
11. Add 50 μ L of MTT Lysing solution to each well and incubate overnight.
12. Read plate at 570-650 nm.
13. Graph standard curve and analyze data.

Protocol D: Antibody Neutralization of Cytokine-Induced Cytokine Production

Materials

- Indicator cell line (see [Quick Guide Chart](#) for a given cytokine)
- Culture Medium (RPMI-1640 supplemented with 10% FBS)
- 96-well flat-bottom culture plate (Costar Cat. No. 3595)
- Cytokine ELISA pair or Ready-SET-Go! Reagent Set

Instruments

- Pipettes and pipettors
- Humidified incubator
- 96-well micro test spectrophotometer
- ELISA plate washer

Experiment Duration

- 24-48 hour incubation (see [Quick Guide Chart](#))
- 1 hour assay preparation

Experimental Procedure

1. Add 50 μ L of Culture Medium to each well of the 96-well assay plate.
2. Dilute Functional Grade (FG) anti-cytokine antibody by 2-fold serial dilution in the plate from row 3 to 12. Leave rows 1 and 2 empty (control rows).

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3. Add 50 μ L/well of samples and recombinant cytokine (see [Quick Guide Chart](#) for concentration) to the dilution plate from row 2 to row 12 (row 2 will be indicator cells and cytokine). Leave row 1 empty (cells alone).
4. Wash indicator cells 3 times with RPMI-1640 and resuspend in Culture Medium at a density of $2\text{--}3.5 \times 10^5$ cells/mL (see [Quick Guide Chart](#)).
5. Add 100 μ L of cell suspension to each well.
6. Incubate cells for 24-48 hours (see [Quick Guide Chart](#)) at 37°C, 5% CO₂ in a humidified incubator.
7. Harvest supernatants for determination of cytokine expression by ELISA.
8. Follow ELISA protocol for target cytokine of interest.

Quick Guide: Neutralization Assay Quick Guide Chart

The following tables summarize the conditions and reagents needed for [Mouse](#) and [Human](#) cytokine neutralization assays.

Mouse Neutralization Assay Quick Guide Chart					
Specificity	Clone	Indicator	Cytokine Conc. (ng/mL)	Ab Top Conc. (ug/mL)	ND ₅₀ (ng/mL)
GM-CSF	MP1-22E9	MC/9	1	10	1250
IFN- γ	R4-6A2	L929	1	1	15
IFN- γ	XMG1.2	L929	1	1	15
IL-1 α	ALF-161	D10	4.0	1	31.25
IL-1 β	B122	D10	4.0	10	125
IL-2	JES6-1A12	CTLL-2	1	1	250
IL-4	11B11	CTLL-2	1	1	125
IL-5	TRFK5	MC/9	1	1	60
IL-6	MP5-20F3	B9	0.01	1	30
IL-10	JES5-2A5	MC/9	1	1	4
IL-12 / IL-23 p40	C17.8	splenocytes	1	1	62
IL-13	eBio1316H	TF-1	7.5	10	40
IL-15	AIO.3	CTLL-2	50	10	390
IL-17A	eBioMM17F3	NIH/3T3	5.0	1	8
IL-21	FFA21	B9	0.5	50	200-500
IL-22	IL22JOP	COLO205	0.2	1	10-40
IL-23	G23-8	splenocytes	0.1	10	1250
TNF- α	MP6-XT22	L929	1	10	600
TNF- α	MP6-XT3	L929	1	10	600

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TNF-α	TN3-19	L929	1	10	150
TSLP	eBio28F12	Nag8/7	0.5	0.1	2-8

ND₅₀: 50% neutralizing dose of antibody for given cytokine concentration

Table 1: Mouse Neutralization Assay Quick Guide Chart

Human Neutralization Assay Quick Guide Chart

Specificity	Clone	Indicator	Cytokine Conc. (ng/mL)	Ab Top Conc. (ug/mL)	ND ₅₀ (ng/mL)
IFN-β	A1	A549	.05	100	3-12x10 ³
IFN-γ	NIB42	A549	1	100	5000
IL-1β	CRM56	D10	1	10	2500
IL-4	MP4-25D2	TF-1	1	1	30
IL-5	JES1-5A10	TF-1	1	1	15
IL-5	TRFK5	MC/9	1	1	60
IL-6	MQ2-13A5	B9	0.05	0.05	0.8
IL-10	JES3-9D7	MC/9	5	1	60
IL-12 / IL-23 p40	C8.6	hPBMC	1	1	60
IL-13	PVM13-1	TF-1	5	1	62
IL-15	ct2nu	CTLL-2	0.5	0.125	0.5-5
IL-17A	eBio64CAP17	NHDF	5	1	≤ 63
IL-22	IL22JOP	COLO205	0.2	1	10-40
TNF-α	MAb1	L929	1	1	60

ND₅₀: 50% neutralizing dose of antibody for given cytokine concentration

Table 2: Human Neutralization Assay Quick Guide Chart