
Cytokine Bioassays

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Introduction

The biological activity of cytokines is commonly measured by cellular proliferation of primary cell cultures or in vitro adapted cell lines that are dependent and/or responsive to a specific growth factor. Other aspects of biological activity of cytokines include induction of further cytokine secretion, induction of killing, antiviral activity, degranulation, cytotoxicity, chemotaxis, and promotion of colony formation. In vitro assays to measure all of these activities are available. Here, we discuss general protocols for setting up bioassays to measure:

[Protocol A: Cytokine-induced proliferation](#)

[Protocol B: Cytokine-induced killing](#)

[Protocol C: Protection against viral effects](#)

[Protocol D: Cytokine-induced cytokine production](#)

General Notes

Many of the available indicator cell lines are responsive to more than one cytokine (e.g., CTLL2 cells respond to IL-2, IL-4, IL-15) and several cytokines share signaling receptors, so this should be taken into account during experimental design. Neutralizing antibodies for one particular cytokine are useful to demonstrate origin of response and attribute the activity to a particular cytokine. The dependence and responsiveness of cell lines should be carefully ascertained since continuous culture of cells may alter their sensitivity to growth factors. Bacterial endotoxins are effective inducers of some cytokines; hence, their presence in the bioassay cultures should be prevented. In order to obtain statistically significant values, samples should be performed in triplicate wells.

Useful websites

Cytokine Research Institute (<http://www.cytokineresearch.org/>)

Cytokine Research Institute is a website maintained by Justin Hoopes at Utah State. This website is to provide general information about the role cytokine play in disease pathogenesis.

Cytokine Reference (<http://apresslp.gvpi.net/apcyto/lpext.dll?f=templates&fn=main-h.htm&2.0>)

Cytokine Reference is a collection of information regarding cytokines and other mediators of immune response.

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Protocol A: Cytokine-induced proliferation

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)
- 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

Experimental Procedure

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

Protocol B: 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)

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- 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

Experimental Procedure

1. Prepare L929 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 overnight at 37°C, 5% CO₂ in a humidified incubator.
2. Dilute samples and standard (see [Quick Guide Chart](#) for standard range) by 2-fold serial dilution in the Assay Medium in 100 μ L/well in another 96-well plate from row 2 to 12. Leave row 1 empty.
3. Prepare a 4 μ g/mL working solution of the Actinomycin D by diluting the 500 μ g/mL stock 125 times in the Assay Medium. Keep Actinomycin D solution protected from light. Add 50 μ L of this working solution of Actinomycin D to each well.
4. Transfer 50 μ L of titrated samples and standard to the corresponding wells on the assay plate.
5. Incubate plate for 24 hrs at 37°C, 5% CO₂ in a humidified incubator.
6. Add 10 μ L/well of 5 mg/mL MTT solution to each well and incubate for 4 hours.
7. Add 50 μ L of MTT Lysing Solution to each well and incubate overnight.
8. Read plate at 570-650 nm. Graph standard curve and analyze data.

Protocol C: IFN- γ Protection from Viral Infection of 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, 10^7 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

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- 96-well micro test spectrophotometer

Experiment Duration

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

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 plate and incubate overnight at 37°C, 5% CO₂ in a humidified incubator.
2. Dilute samples and standard (see [Quick Guide Chart](#) for standard range) by 2-fold serial dilution in the assay plate from row 2 to 12. Leave row 1 empty. Incubate the assay plate for 6 hours.
3. 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).
4. Incubate plate for 40 hours at 37°C, 5% CO₂ in a humidified incubator.
5. Add 10 μ L of 5 mg/mL MTT solution to each well and incubate for 4 hours.
6. Add 50 μ L of MTT Lysing Solution to each well and incubate overnight.
7. Read plate at 570-650 nm. Graph standard curve and analyze data.

Protocol D: 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

Experiment Duration

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

Experimental Procedure

1. Add 100 μ L of Culture Medium to each well of the 96-well assay plate.
2. Dilute samples and standard (see [Quick Guide Chart](#) for standard range) by 2-fold serial dilution in the assay plate from row 2 to 12. Leave row 1 empty.
3. Wash indicator cells 3 times with RPMI-1640 and resuspend in Culture Medium at a density of $2-3.5 \times 10^5$ cells/mL (see [Quick Guide Chart](#)).

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4. Add 100 μ L of cell suspension to each well.
5. Incubate cells for 24-48 hours (see [Quick Guide Chart](#)) at 37°C, 5% CO₂ in a humidified incubator.
6. Harvest supernatants for determination of cytokine expression by ELISA.
7. Follow ELISA protocol for target cytokine of interest.

Quick Guide: Cytokine Bioassays

The following tables summarize the conditions and reagents needed for [Mouse](#), [Human](#) and [Rat](#) cytokine bioassays.

Mouse Cytokine Bioassay Quick Guide									
Cytokine	Indicator Cells	Cell Density (x10 ⁵)	Feeder Cytokine	Assay Medium FBS(%)	Incubation Time (hours)	Specific Activity U/mg	Cytokine Top Conc. (ng/mL)	ED50 (pg/mL)	Read-Out/Comments
CCL5 (RANTES)	CHO-mCCR5	10		10	6		50	5-10x10 ³	Chemotaxis assay
Chemerin	CHO-mCMKLR	10			6		5-150	5-10x10 ⁴	Transmigration assay
Flt3 Ligand	M1 cells	3		10	48		2000	5-25x10 ⁴	IL-6 production, 1 ng/mL rmLIF co-stim
G-CSF	M-NFS-60	2	rhM-CSF (10 ng/mL)	10	48		5	15-60	Proliferation
GM-CSF	MC/9 (ATCC CRL-8306)	2	mIL-3 (25 U/mL)	10	48	10 ⁷	10	100-300	Proliferation
HGF	mIMCD-3	1		2	96	5x10 ⁴	100	6-20x10 ³	Proliferation
HMGB1	RAW264.7	3.5		10	24	3x10 ²	10 ⁵	3-25x10 ⁶	TNF α production; with 10 ug/mL of Polymycin B
IFN α 2	L929 (ATCC CCL-1)	3.5		2	40	10 ⁷	10	100	EMCV cell protection assay
IFN α 4	L929 (ATCC CCL-1)	3.5		2	40	2x10 ⁷	0.1	75	EMCV cell protection assay
IFN- γ	L929 (ATCC CCL-1)	3.5		2	40	10 ⁷	10	100	EMCV cell protection assay
IL-1 α	D10 (ATCC TIB-224)	2	IL-1 α (10 pg/mL) + 10% rat ConA supe	10	48	10 ⁸	1	10	Proliferation

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Cytokine	Indicator Cells	Cell Density (x10 ⁵)	Feeder Cytokine	Assay Medium FBS(%)	Incubation Time (hours)	Specific Activity U/mg	Cytokine Top Conc. (ng/mL)	ED50 (pg/mL)	Read-Out/Comments
IL-1β	D10 (ATCC TIB-224)	3	mIL-1α (10 pg/mL)	10	48	10 ⁸	1	10	Proliferation
IL-2	CTLL-2 (ATCC TIB-214)	2	hIL-2 (100 U/mL)	10	24	10 ⁷	10	80-300	Proliferation
IL-3	MC/9 (ATCC CRL-8306), NFS-60	2	mGM-CSF	10	48	10 ⁷	10	80-300	Proliferation
IL-4	CTLL-2 (ATCC TIB-214)	2	hIL-2 (100 U/mL)	10	24	10 ⁷	10	100-300	Proliferation
IL-5	TF-1 (ATCC CRL-2003)	2	hGM-CSF (100 U/mL)	10	48	10 ⁷	10	150-625	Proliferation
IL-6	B9	2	hIL-6 (50 pg/mL)	10	48	10 ⁸	0.1	3-12	Proliferation; Starve cells for 48 hrs prior
IL-7	hPBMC	2		10	48	2x10 ⁶	100	300-1800	Proliferation; PHA (5 ug/mL) as costimulator
IL-9	TS-1	2	rmIL-9 (100 ng/mL)	10	72		50	250-2000	Proliferation
IL-10	MC/9 (ATCC CRL-8306)	2	mGM-CSF	10	48	3x10 ⁶	10	300	Proliferation; mIL-4 (5 pg/mL) as costimulator
IL-12 p70	splenocytes	50		10	24	3x10 ⁶	10	300	mIFN-γ production
IL-13	TF-1 (ATCC CRL-2003)	2	hGM-CSF (100 U/mL)	10	48	3x10 ⁵	100	3,800	Proliferation
IL-15	CTLL-2 (ATCC TIB-214)	2	rmIL-2 (100 ng/mL)	10	48	10 ⁴	1000	6-12x10 ⁴	Proliferation
IL-15R/IL-15	CTLL-2 (ATCC TIB-214)	2	rmIL-2 (100 ng/mL)	10	48		250	1-4x10 ³	Proliferation
IL-17A	NIH/3T3 (ATCC CRL-1658)	5		10	24	3x10 ⁵	100	250-2500	IL-6 production
IL-17A/F	NIH/3T3 (ATCC CRL-1658)	5		10	24	10 ⁵	1000	0.8-3x10 ⁴	IL-6 production

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Cytokine	Indicator Cells	Cell Density (x10 ⁵)	Feeder Cytokine	Assay Medium FBS(%)	Incubation Time (hours)	Specific Activity U/mg	Cytokine Top Conc. (ng/mL)	ED50 (pg/mL)	Read-Out/Comments
IL-17F	NIH/3T3 (ATCC CRL-1658)	5		10	24	5x10 ²	10 ⁴	1.25-2.5x10 ³	IL-6 production
IL-19	RAW264.7	3		10	48		10 ⁵	12.5-50x10 ⁶	IL-6 production
IL-21	B9	2		10	48		10	100-300	Proliferation
IL-22	Colo205	10		10	24	10 ⁷	1	50-100	IL-10 production
IL-23	splenocytes	50		10	24	10 ⁷	10	100	mIL-17 production; PMA (5 ng/mL) as costimulator
IL-27	splenocytes	10		10	72	2x10 ⁴	100	3000	Inhibition of IL-2 secretion; immobilized @CD3, soluble @CD28 as costimulators
IL-28	HEPG2	3.5		10	40	10 ⁷	500	5-50x10 ³	EMCV cell protection assay
IL-33	D10.G4.1	2		10	72	10 ⁷	1	30-100	Proliferation
LIF	M1	3		10	48		50	200-800	IL-6 production
M-CSF	M-NFS-60	2	rhM-CSF (10 ng/mL)	10	48		250	0.5-10x10 ³	Proliferation
SCF	MC/9 (ATCC CRL-8306)	2	mGM-CSF (100 ng/mL)		72	6x10 ⁴	500	1.5-6.25x10 ⁴	Proliferation
Sonic Hedgehog (SHH)	C3H/10T1/2	3		10 (heat inactivated FBS)	7 days		2.5x10 ⁴	500-3000	Alkaline phosphatase induction
TNF-α	L929 (ATCC CCL-1)	3.5		2	24	2x10 ⁸	1.0	5.0	L929 cytotoxicity assay; Actinomycin D (2 ug/mL)
TSLP	Nag8/7	2	rmIL-7 (10 ng/mL)	10	48		20	100-300	Proliferation
VEGF	Human umbilical vein endothelial cells	2	rhVEGF (100 U/mL)	10	72	10 ⁵	10 ³	8x10 ³	Proliferation

HGF: hepatocyte growth factor
ED₅₀: 50% effective dose of cytokine (1 U/mL observed activity)

Table 1: Mouse Cytokine Bioassay Quick Guide

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Human Cytokine Bioassay Quick Guide									
Cytokine	Indicator Cells	Cell Density (x10 ⁵)	Feeder Cytokine	Assay Medium FBS(%)	Incubation Time (hours)	Specific Activity U/mg	Cytokine Top Conc. (ng/mL)	ED50 (pg/mL)	Read-Out/Comments
ActivinA	MPC-11	2		10	72		50	750-3000	Inhibition of proliferation
BMP-2	C3H/10T1/2	3		10 (heat inactivated serum)	1 week		5000	0.25-1x10 ⁶	Alkaline phosphatase induction
CCL5 (RANTES)	CHO-mCCR5	10		10	6		50	1000-5000	Chemotaxis
FGF8	Balb/c-3T3	1		10	72		50	0.3-1.5x10 ⁴	Proliferation; 1 ug/mL Heparin co-stim
FGFa	Balb/c-3T3	1		10	72		25	100-1000	Proliferation; 10 ug/mL Heparin co-stim
FGFb	Balb/c-3T3	1		10	72		10	100-1250	Proliferation
Flt3-L	M1	3		10	48		250	0.5-1.5x10 ⁴	IL-6 production; 1 ng/mL rmLIF co-stim
G-CSF	M-NFS-60	2	rhM-CSF (10 ng/mL)	10	48		1	10-50	Proliferation
GM-CSF	TF-1 (ATCC CRL-2003)	2	hGM-CSF (100 U/mL)	10	48	10 ⁷	10	100	Proliferation
HGF	4MBr-5 (ATCC CCL-208)	2	rmEGF (30 ng/mL)	10	96	2x10 ⁴	10 ³	5x10 ⁴	Proliferation
IFN-α1	A549 (ATCC CCL-185)	3.5		2	40	3x10 ⁶	10	300	A549/EMCV cell protection assay
IFN-α2	A549 (ATCC CCL-185)	3.5		2	40	3x10 ⁷	0.5	5-10	A549/EMCV cell protection assay
IFN-γ	A549 (ATCC CCL-185)	3.5		2	40	10 ⁷	10	40	A549/EMCV cell protection assay
IL-1α	D10 (ATCC TIB-224)	3	mIL-1α (10 pg/mL)	10	48	5x10 ⁸	1	31	Proliferation
IL-1β	D10 (ATCC TIB-224)	2	IL-1α (10 pg/mL) + 10% rat ConA supe	10	48	10 ⁷	10	100	Proliferation
IL-2	CTLL-2 (ATCC TIB-214)	2	hIL-2 (100 U/mL)	10	24	10 ⁷	10	100-300	Proliferation

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Cytokine	Indicator Cells	Cell Density (x10 ⁵)	Feeder Cytokine	Assay Medium FBS(%)	Incubation Time (hours)	Specific Activity U/mg	Cytokine Top Conc. (ng/mL)	ED50 (pg/mL)	Read-Out/Comments
IL-3	TF-1 (ATCC CRL-2003)	2	hGM-CSF (2 ng/mL)	10	48	5x10 ⁶	10	150-600	Proliferation
IL-4	TF-1 (ATCC CRL-2003)	2	hGM-CSF (100 U/mL)	10	48	10 ⁶	10	100	Proliferation
IL-5	TF-1 (ATCC CRL-2003)	2	hGM-CSF (100 U/mL)	10	48	10 ⁷	10	100	Proliferation
IL-6	TF-1 (ATCC CRL-2003)	2	hGM-CSF (100 U/mL)	10	48	10 ⁷	10	100	Proliferation
IL-7	Human PHA-treated PBMCs (3day)	5		10	48	5x10 ⁵	100	2,000	Proliferation
IL-8	Human Neutrophils								
IL-10	MC/9 (ATCC CRL-8306)	2	mCM-CSF	10	48	10 ⁶	10	10 ³	Proliferation
IL-12 p70	hPBMC (PHA blast)	5		10	48	3x10 ⁶	10	40-80	Proliferation
IL-13	TF-1 (ATCC CRL-2003)	2	hGM-CSF (100 U/mL)	10	48	10 ⁶	10	1-2.5x10 ³	Proliferation
IL-15	CTLL-2 (ATCC TIB-214)	3	IL-2 (100 U/mL)	10	48	10 ⁷	1	100	Proliferation
IL-17A	NHDF	3		10	48	3x10 ⁵	100	3,000	IL-6 production
IL-17A/F	NIH/3T3	5		10	24		10 ⁴	2x10 ⁴	IL-6 production
IL-17F	NIH/3T3	5		10	24		10 ⁴	1.5x10 ⁶	IL-6 production

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IL-19	hPBMC	50		10	24		1000	2.5-5x10 ⁵	IL-6 production
IL-21	B9	2	hIL-6 (50 pg/mL)	10	48	10 ⁵	100	10-50	Proliferation
IL-22	Colo205	1		10	24	10 ⁷	1	100	IL-10 production
IL-23	Mouse splenocytes	50		10	24	2x10 ⁵	100	2-4x10 ³	mIL-17 production; costim w/ TPA (5 ng/mL)
IL-26	COLO206	3		10	48		5000	6-60x10 ⁴	IL-10 production
IL-27	Mouse splenocytes	10		10	72	2x10 ⁴	1000	6.2-12.5x10 ⁴	Inhibition of IL-2 secretion
IL-28B	HepG2	3		10	40		100	1-5x10 ³	EMCV cell protection assay
IL-29	HepG2	3.5			40		10	300	EMCV cell protection assay
IL-31	U-87			10	36		5		STAT3 activation
IL-33	D10.G4.1	2		10	72	10 ⁶	10	300	Proliferation
M-CSF	M-NFS-60	2	rhM-CSF (10 ng/mL)	10	48		250	0.5-10x10 ³	Proliferation
SCF	TF-1 (ATCC CRL-2003)	2	hGM-CSF (100 U/mL)	10	48	5x10 ⁵	100	2,000	Proliferation
TGF-β1	HT-2 (CRL-1841)	2	rmIL-2 (100 U/mL)	10	48	10 ⁷	10	40	Inhibition of IL-4 induced proliferation
TGF-β2	CTLL-2	2	IL-2 (100 ng/mL)	10	72		50	300-1500	Inhibition of IL-4 induced proliferation
TGF-β3	CTLL-2	2	IL-2 (100 ng/mL)	10	72		5	30-125	Inhibition of IL-4 induced proliferation
TNF-α	L929 (ATCC CCL-1)	3.5		2	24	2x10 ⁸	10	40-156	L929 cytotoxicity assay; Actinomycin D (2 ug/mL)

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TSLP	hPBMC	10		10	24		10 ⁴	<=10 ⁴	hTARC induction
VEGF	HUVEC	2	rhVEGF (100 U/mL)	10	72	10 ⁵	1000	8,000	Proliferation

HGF: hepatocyte growth factor

ED₅₀: 50% effective dose of cytokine (1 U/mL observed activity)

Table 2: Human Cytokine Bioassay Quick Guide

Rat Cytokine Bioassay Quick Guide

Cytokine	Indicator Cells	Cell Density (x10 ⁵)	Feeder Cytokine	Assay Medium FBS(%)	Incubation Time (hours)	Specific Activity U/mg	Cytokine Top Conc. (ng/mL)	ED50 (pg/mL)	Read-Out/Comments
IFN-γ	L929	3.5		2	40	10 ⁷	10	100	EMCV cell protection assay
IL-1β	D10 (ATCC TIB-224)	2	mIL-1α (10 pg/mL)	10	48	10 ⁸	10	156-312	Proliferation
IL-2	CTLL-2 (ATCC TIB-214)	2	hIL-2 (100 U/mL)	10	24-48	2.5x10 ⁶	10	400	Proliferation
IL-4	splenocytes	2		10	48		100	1.5-3x10 ³	Proliferation
IL-17A	NIH/3T3	3		10	48		2.5x10 ⁵	1-5x10 ⁴	Mouse IL-6 production
IL-17AF	NIH/3T3	3		10	48		250	1-10x10 ⁴	Mouse IL-6 production
IL-17F	NIH/3T3	3		10	48		2.5x10 ⁴	1-5x10 ⁶	Mouse IL-6 production
TNF-α	L929 (ATCC CCL-1)	3.5		2	24	2x10 ⁸	1	5	L929 cytotoxicity assay; Actinomycin D (2 ug/mL)

ED₅₀: 50% effective dose of cytokine (1 U/mL observed activity)

Table 3: Rat Cytokine Bioassay Quick Guide

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