QuantiGlo®

Human IL-6 Immunoassay

Catalog Number Q6000

For the quantitative determination of human interleukin 6 (IL-6) concentrations in cell culture supernates, serum, and plasma.

human
IL-6

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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MANUFACTURED AND DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place N.E.
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 379-6580
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

R&D Systems Europe
19 Barton Lane
Abingdon Science Park
Abingdon, Oxon OX14 3NB
United Kingdom

TELEPHONE: (0)1235 529449
FAX: (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems GmbH
Borsigstrasse 7
65205 Wiesbaden-Nordenstadt
Germany

FREEPHONE: (0)800 909 4455
TELEPHONE: (0)6122 90980
FAX: (0)6122 909819
E-MAIL: infogmbh@RnDSystems.co.uk

INTRODUCTION

Interleukin 6 is a multifunctional protein produced by lymphoid and non-lymphoid cells and by normal and transformed cells, including T cells, monocyte/macrophages, fibroblasts, hepatocytes, vascular endothelial cells, cardiac myxomas, bladder cell carcinomas, myelomas, astrogliomas and glioblastomas. The production of IL-6 in these cells is regulated, either positively or negatively, by a variety of signals including mitogens, antigenic stimulation, lipopolysaccharides, IL-1, TNF, PDGF and viruses. On the basis of its various activities, IL-6 has also been called interferon-β2 (IFN-β2), 26-kDa protein, B-cell stimulatory factor-2 (BSF-2), hybridoma/plasmacytoma growth factor, hepatocyte stimulating factor, cytotoxic T-cell differentiation factor, and macrophage-granulocyte inducing factor 2A (MGI-2A). For reviews on IL-6, see references 1 - 5.

The human IL-6 cDNA sequence predicts a protein of 212 amino acid residues in length with two potential N-glycosylation sites. The hydrophobic N-terminal 28 amino acid residue signal peptide is cleaved to produce a mature protein of 184 amino acids with four cysteine residues and a predicted molecular mass of 21 kDa (6 - 9). The mouse IL-6 cDNA sequence shows a homology of 42% at the amino acid level when compared with the human sequence (10). Sequencing of the genomic DNA for IL-6 indicates that the gene for this factor consists of five exons and four introns. On the basis of sequence similarity and gene structural motif similarity, IL-6 can be grouped in a family of cytokines that also includes OSM, G-CSF, LIF, and CNTF. All of these cytokines are predicted to have a four helix bundle structure similar to that found for growth hormone, suggesting that they all evolved from a common ancestral gene (11 - 13).

The effects of IL-6 on different cells are numerous and varied. The effect on B cells is stimulation of differentiation and antibody secretion (6, 14 - 17). IL-6 also affects T cells, acting as a co-stimulant with sub-optimal concentrations of PHA or Con A to stimulate IL-2 production and IL-2 receptor expression. IL-6 exhibits growth factor activity for mature thymic or peripheral T-cells and reportedly enhances the differentiation of cytotoxic T-cells in the presence of IL-2 or IFN-γ (18 - 20). IL-6 stimulates production of acute phase proteins by hepatocytes (21) and has colony-stimulating activity on hematopoietic stem cells (22, 23). IL-6 has growth factor activities and will stimulate the growth of myeloma/hybridoma/ plasmacytoma cells (24, 25), EBV-transformed B cells (26), keratinocytes and mesangial cells (4, 5). Additional bioactivities attributed to IL-6 include inhibition of the growth and induction of terminal differentiation of M1 myeloid leukemic cells (27), induction of neuronal cell differentiation (28, 29), and induction of the maturation of megakaryocytes (30). Although IL-6 was also discovered as an antiviral activity produced by human diploid fibroblasts, the question of whether or not IL-6 has antiviral activity is controversial.

The various activities of IL-6 described above suggest that this factor will have a major role in the mediation of the inflammatory and immune responses initiated by infection or injury. Although the exact functions of IL-6 *in vivo* are not known, elevated IL-6 levels have been reported to be associated with a variety of diseases, including autoimmune diseases such as arthritis and Castleman's Disease (4, 5, 31, 32), mesangial proliferative glomerulonephritis (4, 5), psoriasis (33), inflammatory bowel disease (34), and malignancies such as plasmacytomas (4), myelomas (2 - 4, 35), lymphomas and leukemias (4, 36), and ovarian cancers (37).

Current methods for the assay of IL-6 are based on its mitogenic effects on appropriate cell lines, such as B9, a mouse B cell hybridoma line, or T1165.85.2.1, a murine plasmacytoma cell line. These bioassays are time-consuming and are not completely specific for IL-6. The QuantiGlo IL-6 Immunoassay is a solid phase chemiluminescent ELISA that specifically measures human IL-6 in less than 5.5 hours. This kit is designed to measure IL-6 levels in serum, plasma, and cell culture supernate. It contains recombinant human IL-6 and antibodies raised against recombinant human IL-6 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural IL-6 showed linear curves that were parallel to the standard curves obtained using the *E. coli*-expressed QuantiGlo kit standards. These results indicate that the QuantiGlo Immunoassay kit can be used to determine relative mass values for natural IL-6. It has been observed in our laboratories that the measurement of IL-6 is insensitive to the addition of the recombinant form of the IL-6 soluble receptor. Therefore it is probable that experimental sample measurements reflect the total amount of IL-6 present, *i.e.,* the total amount of free IL-6 plus the amount of IL-6 initially bound to soluble receptors, if any are present in the samples. High levels of high-affinity autoantibodies to IL-6 in the serum of some normal blood donors have been reported (38, 39). Such autoantibodies would have the potential to interfere with the measurement of IL-6 by immunoassays. In our laboratories, we have been unable to detect high-affinity autoantibodies against IL-6 in the serum of normal donors and have

not seen the interference with measurements of IL-6 in normal serum that such autoantibodies would be expected to produce. While the presence of autoantibodies against IL-6 in serum represents a potential area of concern, it remains to be demonstrated that such antibodies occur with sufficient frequency in normal donors to be of general concern.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate (40) solution is added to the wells and light is produced in proportion to the amount of IL-6 bound in the initial step. A microplate luminometer is used to measure the intensity of the light emitted.

TECHNICAL HINTS

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Variation in pipetting technique, washing technique, luminometers, incubation time or temperature can cause variations in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the QuantiGlo Immunoassay, the possibility of interference cannot be excluded.
- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Relative light units (RLUs) may differ among luminometers. The QuantiGlo IL-6 Immunoassay was optimized using a DYNEX TECHNOLOGIES MLX™ luminometer. Other instruments may require settings to be adjusted.
- Relative light units may vary within the reading window of 20 - 40 minutes.
- Due to limitations of many luminometers, it is recommended that the standards be assayed in duplicate from high to low, beginning with the high standard in wells A_1 and A_2.

REAGENTS

IL-6 Microplate (Part 890515) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against IL-6.

IL-6 Conjugate (Part 890516) - 21 mL of polyclonal antibody against IL-6 conjugated to horseradish peroxidase, with preservative.

IL-6 Standard (Part 890517) - 15 ng of recombinant human IL-6 in a buffered protein base with preservative, lyophilized.

Assay Diluent RD1Y (Part 895122) - 6 mL of a buffered protein base with preservative. Contains a precipitate.

Calibrator Diluent QD6-14 (Part 895220) - 21 mL of a buffered protein base with preservative.

Wash Buffer Concentrate (Part 895222) - 100 mL of a 10-fold concentrated solution of buffered surfactant with preservative.

Substrate A (Part 895224) - 12.5 mL of stabilized enhanced luminol.

Substrate B (Part 895225) - 12.5 mL of stabilized hydrogen peroxide.

Plate Covers - 4 adhesive strips.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="8">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="7">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Calibrator Diluent QD6-14</td></tr>
<tr><td>Assay Diluent RD1Y</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Unmixed Substrate A</td></tr>
<tr><td>Unmixed Substrate B</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*</td></tr>
</table>

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- DYNEX TECHNOLOGIES MLX luminometer set with the following parameters: 1.0 minute lag time; 1 sec/well read time; summation mode; auto gain on; or the equivalent.
- Pipettes and pipette tips.
- 1 liter graduated cylinder.
- Deionized or distilled water.
- Multi-channel pipette, squirt bottle, manifold dispenser, or automated microplate washer.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles. Some samples may require dilution.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Grossly hemolyzed samples are not suitable for measurement of human IL-6 with this assay.*

REAGENT PREPARATION

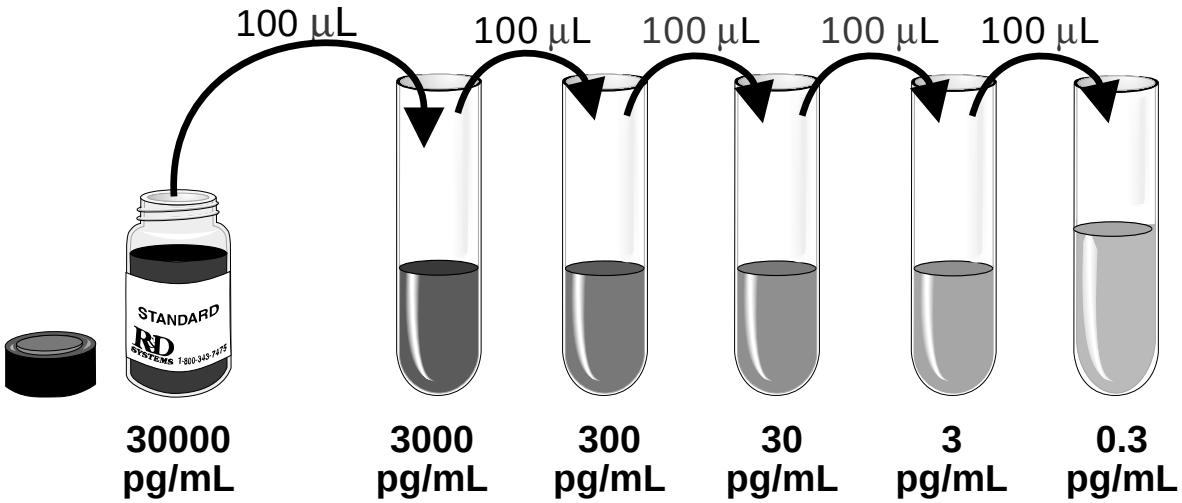
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 100 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 1000 mL of Wash Buffer.

Substrate Solution - Substrates A and B should be mixed together in equal volumes 15 minutes to 4 hours before use. Store in a capped plastic container, protected from light. 200 µL of the resultant mixture is required per well.

Standard - Reconstitute Standard with 0.5 mL of deionized or distilled water. This reconstitution produces a stock solution of 30,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent QD6-14 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 3000 pg/mL standard serves as the high standard. Calibrator Diluent QD6-14 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.

3. Add 50 µL of Assay Diluent RD1Y to each well. Assay Diluent RD1Y contains a precipitate. Mix well before and during its use.

4. Add 150 µL of Standard or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 µL of IL-6 Conjugate to each well. Cover with a new adhesive strip. Incubate for 3 hours at room temperature on the shaker.

   Note: *Prepare Substrate Solution at this time.*

7. Repeat the aspiration/wash as in step 5.

8. Add 200 µL of Substrate Solution to each well. Incubate for 20 - 40 minutes at room temperature **on the benchtop (do not shake).**

9. Determine the RLU of each well using a luminometer set with the following parameters: 1.0 min. lag time; 1 sec/well read time; summation mode; auto gain on.

ASSAY PROCEDURE SUMMARY

1. ☐ Prepare reagents, working standards and samples as instructed.
☐ Return unused components to storage temperature as indicated in the instructions.

2. ☐ Add 50 µL Assay Diluent to each well.

3. ☐ Add 150 µL Standard or sample to each well.
☐ Cover the plate and incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.

4. ☐ Aspirate and wash each well four times.

5. ☐ Add 200 µL Conjugate to each well.
☐ Cover the plate and incubate for 3 hours at room temperature on the shaker.

Note: *Prepare Substrate Solution at this time.*

6. ☐ Aspirate and wash each well four times.

7. ☐ Add 200 µL Substrate Solution to each well.
☐ Incubate 20 - 40 minutes at room temperature **on the benchtop (do not shake)**.

8. ☐ Determine the RLU of each well using a luminometer.

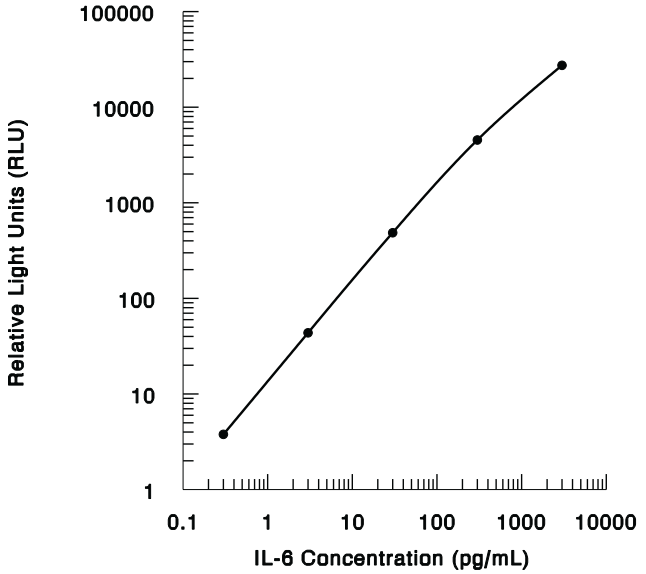
CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard RLU.

Create a standard curve by reducing the data using computer software capable of generating a cubic-spline or quadratic curve fit. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve was generated using a DYNEX TECHNOLOGIES MLX luminometer and is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	(RLU)	Average	Corrected
0	12.84 13.11	12.98	-
0.3	16.57 16.94	16.76	3.78
3.0	56.48 56.84	56.66	43.68
30	491.3 508.3	499.8	486.8
300	4545 4563	4554	4541
3000	27092 27749	27420	27407

PRECISION

Intra-assay Precision (Precision within an assay)
Four samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Four samples of known concentration were tested in forty separate assays to assess inter-assay precision.

	Intra-assay Precision				Inter-assay Precision			
Sample	1	2	3	4	1	2	3	4
n	20	20	20	20	40	40	40	40
Mean (pg/mL)	2.08	9.58	91.9	1001	2.02	9.33	92.9	1057
Standard deviation	0.05	0.24	2.7	34	0.22	0.93	8.6	81
CV (%)	2.4	2.5	2.9	3.4	10.9	10.0	9.2	7.7

RECOVERY

The recovery of natural and recombinant IL-6 spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	102	95 - 114 %
Serum (n=5)	98	90 - 104 %
EDTA plasma (n=5)	96	89 - 109 %
Heparin plasma (n=5)	93	85 - 102 %
Citrate plasma (n=5)	100	92 - 110 %

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of IL-6 in various matrices were diluted with Calibrator Diluent QD6-14 to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=5)	EDTA plasma (n=5)	Heparin plasma (n=5)	Citrate plasma (n=5)
1:2	Average % of Expected	99	102	103	99	102
	Range (%)	96-101	99-106	96-111	91-103	99-109
1:4	Average % of Expected	95	103	104	102	102
	Range (%)	88-103	96-112	97-110	92-111	94-107
1:8	Average % of Expected	96	99	102	100	100
	Range (%)	88-105	92-114	92-112	87-111	90-110
1:16	Average % of Expected	98	99	100	99	98
	Range (%)	88-110	88-115	89-111	86-109	87-108
1:32	Average % of Expected	98	97	99	99	95
	Range (%)	85-112	87-112	93-110	87-114	86-107

SENSITIVITY

The minimum detectable dose of IL-6 is typically less than 0.2 pg/mL.

The minimum detectable dose was determined by adding two standard deviations to the mean RLU of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified, *E. coli*-expressed recombinant human IL-6 produced at R&D Systems.

The NIBSC/WHO International Standard 89/548, which was intended as a potency standard, was evaluated in this kit. This standard is a CHO-derived recombinant human IL-6.

The dose response curve of this international standard parallels the QuantiGlo standard curve. To convert sample values obtained with the QuantiGlo IL-6 kit to equivalent NIBSC 89/548 International Units, use the equation below.

NIBSC (89/548) equivalent value (IU/mL) = 0.131 x QuantiGlo IL-6 value (pg/mL).

SAMPLE VALUES

Serum/plasma - Ninety-nine serum and plasma samples were evaluated for the presence of IL-6 in this assay.

Sample type	% Detectable	Mean of Detectable (pg/mL)	Range (pg/mL)
Serum	97	1.73	ND - 11.82
EDTA plasma	94	1.59	ND - 14.83
Citrate plasma	82	1.60	ND - 13.21
Heparin plasma	92	1.66	ND - 13.20

ND = Non-detectable

Cell culture supernates - Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate and stimulated with 10 μg/mL PHA. Aliquots of the culture supernate were removed on days 1 and 5 and assayed for levels of natural IL-6.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	566	3578
Stimulated	55631	56687

SPECIFICITY

This assay recognizes both natural and recombinant human IL-6. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent QD6-14, and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range rhIL-6 control were assayed for interference. No significant cross-reactivity or interference was observed.

Factors related to or associated with IL-6:

Recombinant human:
IL-6 sR
IL-11
IL-12
CNTF
G-CSF
sgp130
LIF
OSM

Recombinant mouse:
IL-6
LIF

Other factors:

Recombinant human:
IL-1α
IL-1β
IL-1 ra
IL-1 sRI
IL-1 sRII
IL-2
IL-2 sRα
IL-3
IL-3 sRα
IL-4
IL-4 sR
IL-5
IL-5 sRα
IL-5 sRβ
IL-7
IL-8
IL-9
IL-10
IL-13
ANG
AR
β-ECGF
EGF
FGF acidic
FGF basic
FGF-4
FGF-5
FGF-6
GM-CSF
GROα
GROβ
GROγ
HB-EGF
HGF
IFN-γ
IGF-I
IGF-II
KGF
LAP
M-CSF
MCP-1
MIP-1α
MIP-1β
β-NGF
PD-ECGF
PDGF-AA
PDGF-AB
PDGF-BB
PTN
RANTES
SCF
SLPI
TGF-α
TGF-β1
TGF-β2
TGF-β3
TGF-β sRII
TNF-α
TNF-β
sTNF RI
sTNF RII
VEGF

Recombinant mouse:
IL-1α
IL-1β
IL-3
IL-4
IL-5
IL-7
IL-9
IL-10
IL-13
GM-CSF
MIP-1α
MIP-1β
SCF
TNF-α

Other:
bFGF acidic
bFGF basic
hPDGF
pPDGF
hTGF-β1
pTGF-β1
raTGF-β5

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