Quantikine® ELISA

Mouse CCL21/6Ckine Immunoassay

Catalog Number M6C00

For the quantitative determination of mouse 6Ckine concentrations in tissue culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

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USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

CCL21, also known as 6Ckine, TCA-4, SLC, Exodus-2, and A21, is a 12 kDa homeostatic chemokine that plays an important role in adaptive immune responses and inflammation (1, 2). Unlike other CC chemokines, mouse CCL21 has a 36 amino acid (aa) C-terminal extension which mediates its attachment to carbohydrate structures and extracellular matrix components (3, 4). Mature mouse CCL21 shares 71% and 84% aa sequence identity with human and rat CCL21, respectively (5-7). In mice, three genes encode CCL21, with B and C forms being identical and the A form carrying serine at aa 65 instead of leucine. Both human and mouse CCL21 signal through the chemokine receptor CCR7, while mouse CCL21 additionally can signal through CXCR3 (8). CCL21 is constitutively presented on initial lymphatic vessels, high endothelial venules (HEV), and lymph node dendritic cells (DC) (9-11). Immobilized CCL21 promotes the docking of DC to lymphatic vessels and the retention of T cells by lymph node DC, resulting in T cell priming for activation (9, 10). DC interaction with the anchored chemokine can induce CCL21 cleavage and release of an 8 kDa fragment that lacks the C-terminal extension (11). During chronic inflammation or tissue damage, CCL21 is upregulated on local vascular endothelial cells, macrophages, T cells, and neurons (12-15). In these settings, it promotes fibrosis, inflammatory cytokine production, and neuropathic pain (13-16). The soluble chemokine is elevated in rheumatoid arthritis synovial fluid and in the serum of coronary artery disease patients (12, 14). CCL21 has been shown to exert either angiogenic or angiostatic effects (12, 17, 18). These effects, in combination with the ability of CCL21 to attract immune suppressor cells (Treg and MDSC) to a tumor site can have positive or negative effects on tumor progression (18-20).

The Quantikine® Mouse CCL21/6Ckine Immunoassay is a 4.5 hour solid-phase ELISA designed to measure mouse 6Ckine in tissue culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant mouse 6Ckine and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant mouse 6Ckine. Results obtained using natural mouse 6Ckine showed dose response curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse 6Ckine.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse 6Ckine has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any 6Ckine present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse 6Ckine is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of 6Ckine bound in the initial step. The sample values are then read off the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- 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.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse 6Ckine Microplate	898297	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse 6Ckine.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Mouse 6Ckine Standard	898299	2 vials of recombinant mouse 6Ckine in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard and control for each assay. Discard after use.
Mouse 6Ckine Control	898300	2 vials of recombinant mouse 6Ckine in a buffered protein base with preservatives, lyophilized. The assay value of the control should be within the range specified on the label.	
Mouse 6Ckine Conjugate	898298	12 mL of a polyclonal antibody specific for mouse 6Ckine conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1S	895137	11 mL of a buffered protein solution with preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:4 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of diluted hydrochloric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- **Polypropylene** test tubes for dilution of standards and samples.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Tissue Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 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 or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Serum and plasma samples require a 20-fold dilution prior to assay. A suggested 20-fold dilution is 10 µL of sample + 190 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse 6Ckine Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

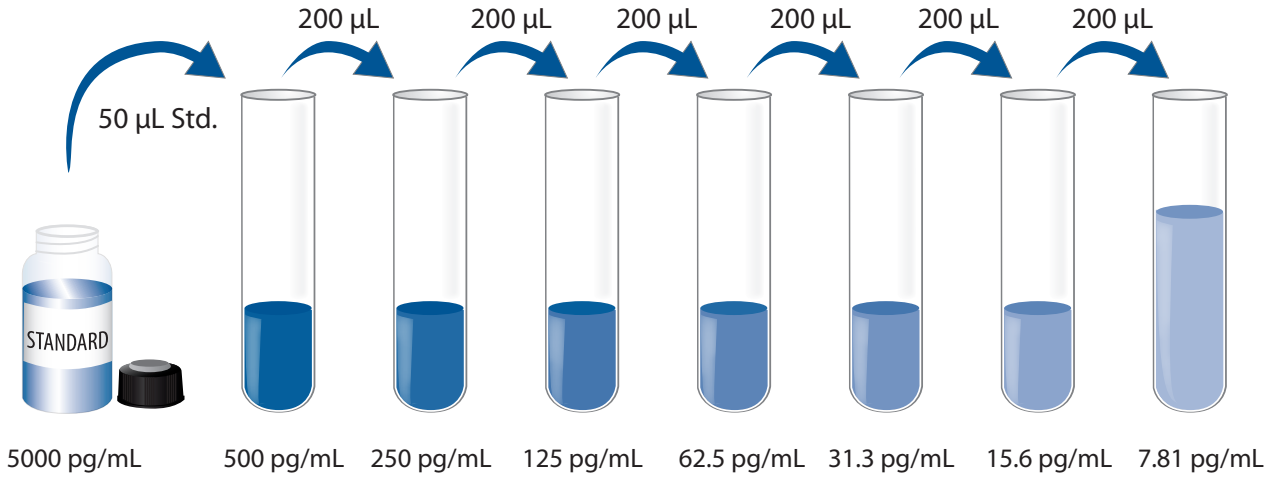
Calibrator Diluent RD5-26 (diluted 1:4) - Add 10 mL of Calibrator Diluent RD5-26 Concentrate to 30 mL of deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5-26 (diluted 1:4).

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Mouse 6Ckine Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse 6Ckine Standard with deionized or distilled water. Do not substitute other diluents. This reconstitution produces a stock solution of 5000 pg/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Note: *Do not use rocker.*

Use polypropylene tubes. Pipette 450 µL of Calibrator Diluent RD5-26 (diluted 1:4) into the 500 pg/mL tube. Pipette 200 uL into remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The 500 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:4) 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, control, and standards be assayed in duplicate.

1. Prepare all reagents, standard dilutions, control, 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, and reseal.
3. Add 50 µL of Assay Diluent RD1S to each well.
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
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, 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 100 µL of Mouse 6Ckine Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

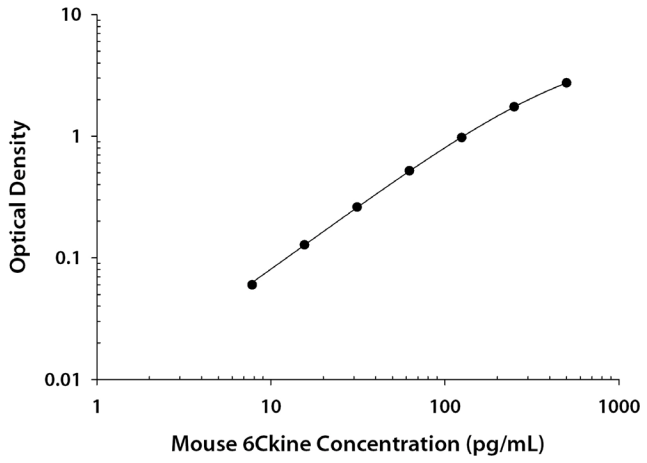
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the mouse 6Ckine concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.005 0.005	0.005	—
7.81	0.064 0.066	0.065	0.060
15.6	0.133 0.133	0.133	0.128
31.3	0.265 0.269	0.267	0.262
62.5	0.523 0.525	0.524	0.519
125	0.971 0.989	0.980	0.975
250	1.713 1.779	1.746	1.741
500	2.744 2.748	2.746	2.741

PRECISION

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

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	70.4	145	315	70.1	138	295
Standard deviation	2.96	4.30	13.9	4.79	8.72	15.8
CV (%)	4.2	3.0	4.4	6.8	6.3	5.4

RECOVERY

The recovery of mouse 6Ckine spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Tissue culture media (n=4)	96	88-104%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of mouse 6Ckine were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Tissue culture supernates (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	96	98	100	96
	Range (%)	90-99	97-98	96-105	94-99
1:4	Average % of Expected	96	99	102	98
	Range (%)	88-102	98-100	99-106	94-103
1:8	Average % of Expected	98	99	107	99
	Range (%)	90-102	94-101	104-110	95-105
1:16	Average % of Expected	94	98	107	93
	Range (%)	90-97	94-103	106-108	92-96

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Twenty-four assays were evaluated and the minimum detectable dose (MDD) of mouse 6Ckine ranged from 0.088-0.489 pg/mL. The mean MDD was 0.220 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant mouse 6Ckine produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse 6Ckine in this assay.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=10)	2611	1891-4916	1016
EDTA plasma (n=5)	2228	1950-2516	275
Heparin plasma (n=5)	2545	1720-3340	663

Tissue Culture Supernates - Kidneys from mice were rinsed with PBS, cut into 1-2 mm pieces, and homogenized with a tissue homogenizer in PBS. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 2 days. An aliquot of the tissue culture supernate was removed, assayed for mouse 6Ckine, and measured 207 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse 6Ckine.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range mouse 6Ckine control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

CCL2/JE
CCL3/MIP-1α
CCL4/MIP-1β
CCL5/RANTES
CCL6/C10
CCL11/I-TAC
CCL12/MCP-5
CCL17/TARC
CCL19/MIP-3β
CCL22/MDC

Recombinant rat:

CCL21/6Ckine
CCR7

Recombinant human:

CCL21/6Ckine

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