Quantikine®

Human CCL21/6Ckine Immunoassay

Catalog Number D6C00

For the quantitative determination of human 6Ckine concentrations in cell 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

Contents	Page
INTRODUCTION	2
PRINCIPLE OF THE ASSAY	3
LIMITATIONS OF THE PROCEDURE	3
MATERIALS PROVIDED	3
STORAGE	4
OTHER SUPPLIES REQUIRED	4
PRECAUTION	4
SAMPLE COLLECTION AND STORAGE	5
REAGENT PREPARATION	5
ASSAY PROCEDURE	6
ASSAY PROCEDURE SUMMARY	7
CALCULATION OF RESULTS	8
TYPICAL DATA	8
TECHNICAL HINTS	9
PRECISION	9
RECOVERY	10
LINEARITY	10
SENSITIVITY	10
CALIBRATION	10
SAMPLE VALUES	11
SPECIFICITY	11
REFERENCES	11

MANUFACTURED AND DISTRIBUTED BY:

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

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

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

TELEPHONE: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems China Co. Ltd.
24A1 Hua Min Empire Plaza
726 West Yan An Road
Shanghai PRC 200050

TELEPHONE: +86 (21) 52380373
FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

6Ckine [also known as CCL21, SLC (secondary lymphoid-tissue chemokine), Exodus-2, or TCA-4 (thymus-derived chemotactic agent-4)] is a CC chemokine. In 1997, three separate groups independently cloned 6Ckine from the GenBank human expressed sequence tag (EST) database (1 - 3).

The full-length cDNA for 6Ckine encodes a highly basic precursor protein of 134 amino acids (aa) with a 23 aa signal peptide (1, 2). The predicted mature protein of 111 aa shares 21 - 33% identity to other human CC chemokines. In addition to the four conserved cysteine residues typically found in other CC chemokines, 6Ckine has a unique carboxyl-terminal extension of 30 aa that contains two additional cysteine residues (2). The gene for 6Ckine maps to human chromosome 9p13 rather than chromosome 17 where the genes of other CC chemokines are commonly clustered (2). The 6Ckine gene is located approximately 100 kb from the gene for MIP-3β.

6Ckine is a high affinity functional ligand for CCR7 that is expressed on T and B lymphocytes (4). It is also a high affinity ligand for the novel CC receptor, CCR10 (5). In addition to these two CC receptors, 6Ckine has also been shown to bind the CXC chemokine receptor CXCR3 in mouse CXCR3-transfected cells (6).

The expression of 6Ckine is predominant in high endothelial venules of lymph nodes and Peyer's patches, in the T cell areas of spleen, lymph nodes and Peyer's patches, and in the lymphatic endothelium of multiple organs (7). 6Ckine was the first chemokine identified having the characteristics required for mediating lymphocyte homing to secondary lymphoid organs. For example, it is capable of inducing integrin-mediated lymphocyte adhesion (8, 9). 6Ckine stimulates $\alpha 4\beta 7$ integrin-mediated adhesion of lymphocytes to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) under both static and flow conditions in *in vitro* adhesion assays (8). Immobilized 6Ckine can also strongly induce $\beta 2$ integrin-mediated binding of naive T lymphocytes and B lymphocytes to ICAM-1 under static conditions (9). The link between expression of 6Ckine and lymphocyte homing is emphasized in a study evaluating 6Ckine-deficient mice. Mice lacking expression of 6Ckine demonstrate defects in both lymphocyte homing and dendritic cell localization (7).

In contrast to most other CC chemokines, 6Ckine is not chemotactic for monocytes or neutrophils. It is a potent and specific chemoattractant for lymphocytes with preferential activity toward naive T cells (7). Recombinant human 6Ckine has been shown to be chemotactic for some human T cell lines, resting peripheral blood lymphocytes, NK cells, freshly isolated B cells and cultured T cells stimulated with PHA and IL-2 (2, 10 - 12). It is also a potent chemoattractant for *in vitro*- and *in vivo*-derived dendritic cells (13 - 15). 6Ckine has been reported to induce chemotaxis of hematopoietic progenitor cells (HPC), yet inhibit HPC colony formation in a dose-dependent manner (16, 17). 6Ckine may also contribute to persistent HIV infection in secondary lymphoid tissues by promoting viral replication in activated T cells (18).

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

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for 6Ckine has been pre-coated onto a microplate. Standards 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 6Ckine is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of 6Ckine bound in the initial step. The color development is stopped and the intensity of the color is measured.

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 fall outside the dynamic range of the assay, dilute the samples with the appropriate 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.
- 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 Quantikine Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

6Ckine Microplate (Part 890927) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against 6Ckine.

6Ckine Conjugate (Part 890928) - 21 mL of polyclonal antibody against 6Ckine conjugated to horseradish peroxidase with preservatives.

6Ckine Standard (Part 890929) - 50 ng of recombinant human 6Ckine in a buffered protein base with preservatives; lyophilized.

Assay Diluent RD1-55 (Part 895066) - 11 mL of a buffered protein base with preservatives.

Calibrator Diluent RD5P Concentrate (Part 895151) - 21 mL of a concentrated buffered protein base with preservatives. *For cell culture supernate samples.*

Calibrator Diluent RD6Z (Part 895466) - 21 mL of diluted animal serum with preservatives. *For serum/plasma samples.*

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895032) - 6 mL of 2 N sulfuric acid.

Plate Covers - 4 adhesive strips.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1-55	
	Calibrator Diluent RD5P (1X)	
	Calibrator Diluent RD6Z	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	Aliquot and store for up to 1 month at ≤ -20° C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
	Microplate Wells	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.*

*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.
- Horizontal orbital shaker (0.12” orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Human 6Ckine Controls (optional; available from R&D Systems, Inc.).
- **Polypropylene** test tubes for dilution.

PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

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.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 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 or heparin 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: *Citrate plasma is not validated for use in this assay. Grossly hemolyzed samples are not suitable for use in this assay.*

REAGENT PREPARATION

Bring all reagents to room temperature before use.

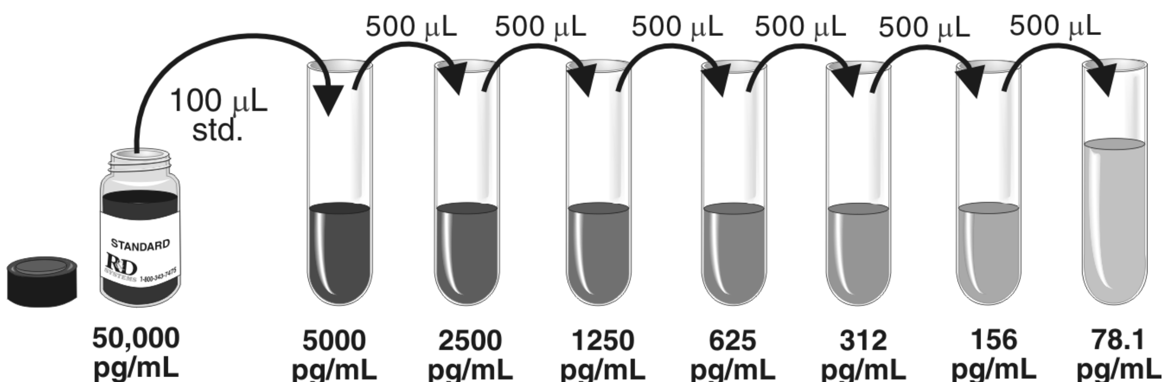
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

Calibrator Diluent RD5P (1X) - Dilute 20 mL of Calibrator Diluent RD5P Concentrate into deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5P (1X).

6Ckine Standard - Reconstitute the 6Ckine Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 50,000 pg/mL. Allow the standard to sit for a minimum of 20 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 μL of Calibrator Diluent RD5P (1X) (*for cell culture supernate samples*) or Calibrator Diluent RD6Z (*for serum/plasma samples*) into the 5000 pg/mL tube. Pipette 500 μL of the appropriate Calibrator Diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 5000 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



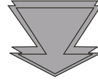
ASSAY PROCEDURE

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

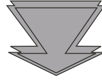
1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-55 to each well.
4. Add 100 µL of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital 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, 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 6Ckine Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. **Protect from light.**
   For Cell Culture Supernate samples: Incubate for 20 minutes at room temperature **on the benchtop.**
   For Serum/Plasma samples: Incubate for 30 minutes at room temperature **on the benchtop.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, 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.

ASSAY PROCEDURE SUMMARY

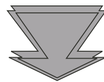
1. Prepare reagents, samples, and standards as instructed.



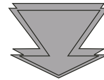
2. Add 100 µL Assay Diluent RD1-55 to each well.



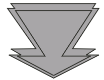
3. Add 100 µL Standard, control, or sample to each well. Incubate 2 hours at RT on the shaker.



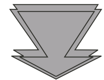
4. Aspirate and wash 4 times.



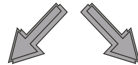
5. Add 200 µL Conjugate to each well. Incubate 2 hours at RT on the shaker.



6. Aspirate and wash 4 times.



7. Add 200 µL Substrate Solution to each well. **Protect from light.**



Serum/Plasma samples
Incubate 30 minutes at RT **on the benchtop.**

Cell Culture Supernate samples
Incubate 20 minutes at RT **on the benchtop.**

8. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 minutes. λ correction 540 or 570 nm

CALCULATION OF RESULTS

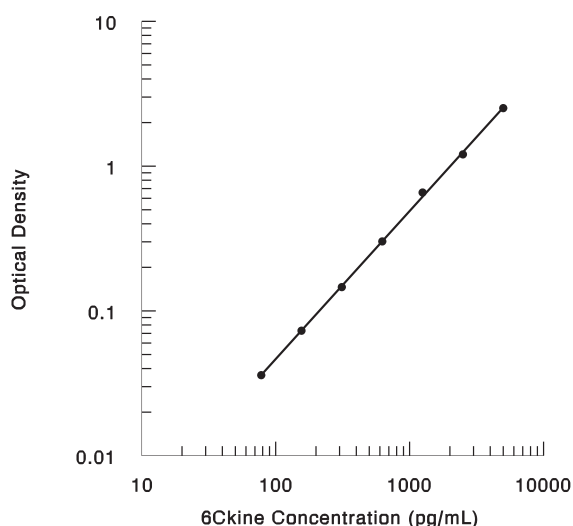
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

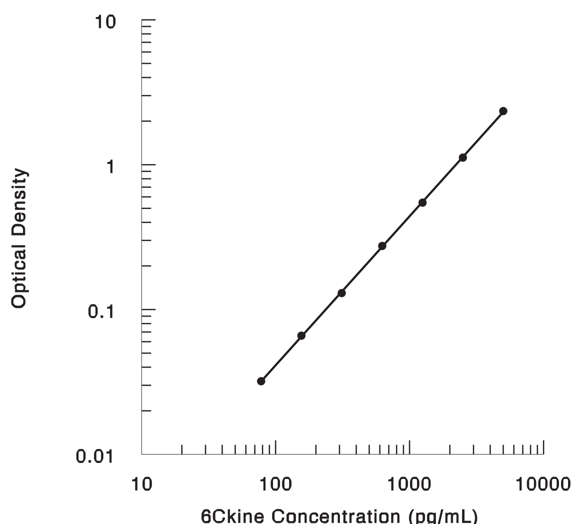
To determine the 6Ckine concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding 6Ckine concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.



pg/mL	O.D.	Average	Corrected
0	0.014 0.014	0.014	—
78.1	0.050 0.049	0.050	0.036
156	0.087 0.087	0.087	0.073
312	0.159 0.160	0.160	0.146
625	0.309 0.322	0.316	0.302
1250	0.695 0.647	0.671	0.657
2500	1.191 1.249	1.220	1.206
5000	2.514 2.546	2.530	2.516



pg/mL	O.D.	Average	Corrected
0	0.013 0.013	0.013	—
78.1	0.045 0.045	0.045	0.032
156	0.078 0.080	0.079	0.066
312	0.146 0.140	0.143	0.130
625	0.287 0.288	0.288	0.275
1250	0.554 0.565	0.560	0.547
2500	1.129 1.136	1.132	1.119
5000	2.367 2.355	2.361	2.348

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.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- 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. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

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.

Serum/Plasma Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	370	1119	3452	368	1086	3688
Standard deviation	12.7	44.3	75.8	34.0	67.9	301
CV (%)	3.4	4.0	2.2	9.2	6.3	8.2

Cell Culture Supernate Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	242	740	2439	277	837	2884
Standard deviation	9.0	30.5	63.3	21.2	43.5	241
CV (%)	3.7	4.1	2.6	7.6	5.2	8.4

RECOVERY

The recovery of 6Ckine spiked to three different levels in samples throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n = 4)	97	89 - 105%
Serum (n = 5)	93	85 - 104%
Heparin plasma (n = 5)	99	89 - 110%
EDTA plasma (n = 5)	97	89 - 108%

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of 6Ckine were diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n = 4)	Serum (n = 5)	Heparin plasma (n = 5)	EDTA plasma (n = 5)
1:2	Average % of Expected	103	97	104	99
	Range (%)	96 - 106	91 - 103	100 - 107	92 - 104
1:4	Average % of Expected	104	101	102	100
	Range (%)	95 - 109	93 - 108	99 - 104	91 - 107
1:8	Average % of Expected	104	102	100	99
	Range (%)	94 - 111	88 - 112	92 - 105	89 - 108
1:16	Average % of Expected	101	99	100	97
	Range (%)	91 - 112	88 - 113	87 - 106	86 - 109

SENSITIVITY

One hundred twenty-seven assays were evaluated and the minimum detectable dose (MDD) of 6Ckine ranged from 2.8 - 33.5 pg/mL. The mean MDD is 9.9 pg/mL.

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

CALIBRATION

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

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of 6Ckine in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)
Serum (n = 62)	195	91 - 371
Heparin plasma (n = 34)	172	104 - 357
EDTA plasma (n = 34)	172	93 - 329

SPECIFICITY

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

Recombinant human:
CTACK
Eotaxin
Eotaxin-2
Eotaxin-3
Eotaxin-3 (aa 24-94)
HCC-1
HCC-4
I-309
MCP-1
MCP-2
MCP-3
MCP-4
MDC
MIP-1α
MIP-1α (70 aa)
MIP-1β
MIP-1δ (68 aa)
MIP-1δ (92 aa)
MIP-3α
MIP-3β
MPIF-1
PARC
RANTES
TARC
TECK

Recombinant mouse:
6Ckine
CTACK
Eotaxin
JE/MCP-1
MARC
MCP-5
MDC
MIP-1α
MIP-1β
MIP-1γ
MIP-3α
MIP-3β
RANTES
TARC
TECK
TCA-3

Recombinant rat:
MIP-3α

REFERENCES

1. Hedrick, J.A. and A. Zlotnik (1997) J. Immunol. **159**:1589.
2. Nagira, M. *et al.* (1997) J. Biol. Chem. **272**:19518.
3. Hromas, R. *et al.* (1997) J. Immunol. **159**:2554.
4. Yoshida, R. *et al.* (1998) J. Biol. Chem. **273**:7118.
5. Gosling, J. *et al.* (2000) J. Immunol. **164**:2851.
6. Soto, H. *et al.* (1998) Proc. Natl. Acad. Sci. USA **95**:8205.
7. Gunn, M.D. *et al.* (1999) J. Exp. Med. **189**:451.
8. Pachynski, R.K. *et al.* (1998) J. Immunol. **161**:952.
9. Tangemann, K. *et al.* (1998) J. Immunol. **161**:6330.
10. Nagira, M. *et al.* (1998) Eur. J. Immunol. **28**:1516.
11. Kim, C.H. *et al.* (1999) Cell. Immunol. **193**:226.
12. Willimann, K. *et al.* (1998) Eur. J. Immunol. **28**:2025.
13. Kellermann, S.A. *et al.* (1999) J. Immunol. **162**:3859.
14. Saeki, H. *et al.* (1999) J. Immunol. **162**:2472.
15. Chan, V.W. *et al.* (1999) Blood **93**:3610.
16. Kim, C.H. and H.E. Broxmeyer (1999) J. Leukoc. Biol. **66**:455.
17. Hromas, R. *et al.* (2000) Blood **95**:1506.
18. Nagira, M. *et al.* (1999) Virology **264**:422.

© 2010 R&D Systems, Inc.