RayBio® Mouse Cytokine Mouse Cytokine Antibody Array C1

*For the semi-quantitative detection of 22 mouse proteins in serum, plasma, cell culture media and other liquid samples types*

Cat# AAM-CYT-1-2 (2 Sample Kit)
Cat# AAM-CYT-1-4 (4 Sample Kit)
Cat# AAM-CYT-1-8 (8 Sample Kit)

*Please read manual carefully before starting experiment*

Neuromics
5325 West 74th Street, Suite 8
Edina, MN 55438
Phone: 952-374-6161
Fax: 612-677-3976
Email: pshuster@neuromics.com
Website: www.neuromics.com

Manufactured for Neuromics by RayBiotech, Inc.
Table of Contents
I. Introduction ................................................................................................. 3
   A. References .................................................................................................. 3
II. Here’s how it works ..................................................................................... 4
III. Materials Provided ..................................................................................... 4
IV. Additional Materials Required .................................................................. 5
V. Overview and General Considerations ....................................................... 5
   A. Preparation of Samples ........................................................................... 5
   B. Handling Array Membranes .................................................................... 5
   C. Incubation ................................................................................................. 5
VI. Protocol ....................................................................................................... 6
   A. Blocking ..................................................................................................... 6
   B. Sample Incubation .................................................................................... 6
   C. First Wash .................................................................................................. 6
   D. *Biotinylated Antibody Cocktail Incubation* ............................................ 6
   E. Second Wash .............................................................................................. 6
   F. HRP-Streptavidin Incubation .................................................................... 7
   G. Third Wash ................................................................................................ 7
   H. Chemiluminescence Detection ................................................................ 7
   I. Storage ....................................................................................................... 7
VII. TYPICAL RESULTS: .................................................................................... 8
    Typical results obtained with RayBio® C-Series Antibody Arrays ................. 8
VIII. INTERPRETATION OF RESULTS ............................................................ 8
    A. Control Spots .......................................................................................... 8
    B. Data Extraction ....................................................................................... 8
    C. Data Analysis .......................................................................................... 9
IX. ARRAY MAP .................................................................................................. 10
X. Troubleshooting guide .................................................................................. 11
I. Introduction

New techniques such as cDNA microarrays have enabled us to analyze global gene expression1-3. However, almost all cell functions are executed by proteins, which cannot be studied simply through DNA and RNA techniques. Experimental analysis clearly shows disparity can exist between the relative expression levels of mRNA and their corresponding proteins4. Therefore, analysis of the proteomic profile is critical.

The conventional approach to analyzing multiple protein expression levels has been to use 2-D SDS-PAGE coupled with mass spectrometry5,6. However, these methods are slow, expensive, labor-intensive and require specialized equipment7. Thus, effective study of multiple protein expression levels can be complicated, costly and time-consuming. Moreover, these traditional methods of proteomics are not sensitive enough to detect most cytokines (typically at pg/ml concentrations).

Cytokines, broadly defined as secreted cell–cell signaling proteins distinct from classic hormones or neurotransmitters, play important roles in inflammation, innate immunity, apoptosis, angiogenesis, cell growth and differentiation7. They are involved in most disease processes, including cancer, obesity and inflammatory and cardiac diseases.

Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool to study cell signaling pathways. Regulation of cellular processes by cytokines is a complex, dynamic process, often involving multiple proteins. Positive and negative feedback loops, pleiotrophic effects and redundant functions, spatial and temporal expression of or synergistic interactions between multiple cytokines, even regulation via release of soluble forms of membrane-bound receptors, all are common mechanisms modulating the effects of cytokine signaling8-14. As such, unraveling the role of individual cytokines in physiologic or pathologic processes generally requires consideration and detection of multiple cytokines rather than of a single cytokine.

RayBio®/Neuromics’ C-Series Antibody Arrays have several advantages over detection of cytokines using single-target ELISA kits:

1. More Data, Same or Less Sample: Antibody arrays provide high-content screening using about the same sample volume as traditional ELISA.
2. Global View of Cytokine Expression: Antibody array screening improves the chances for discovering key factors, disease mechanisms, or biomarkers related to cytokine signaling.
3. Similar (sometimes better) Sensitivity: As little as 4 pg/ml of MCP-1 can be detected using the C-Series array format. In contrast, our similar MCP-1 ELISA assay has a sensitivity of 40 pg/ml of MCP-1.
4. Increased Range of Detection: ELISA assays typically detect a concentration range of 100- to 1000-fold, however, RayBiotech arrays can detect IL-2 at concentrations of 25 to 250,000 pg/ml, a range of 10,000-fold.
5. Better Precision: As determined by densitometry, the inter-array Coefficient of Variation (CV) of spot signal intensities is 5-10%, comparing favorably with ELISA testing (CV = 10-15%).

A. References

1. LPS induces the interaction of a transcription factor, LPS-induced TNF-a factor, and STAT6 (B) with effects on multiple cytokines. Tang X, Marciano DL, Leeman SE, Amar S. PNAS. April 5, 2005 vol. 102 no. 14 5132-5137
3. Synergistic increases in intracellular Ca(2+), and the release of MCP-1, RANTES, and IL-6 by astrocytes treated with opiates and HIV-1 Tat. GLIA. 2005 Apr 15;50(2):91-106.

www.neuromics.com

Neuromics Antibodies • 5325 West 78th Street, Suite 8 • Edina, MN 55438
phone 952-374-6161 • fax 612-677-3976 • e-mail pshuster@neuromics.com-V1-05-201-
II. Here’s how it works

III. Materials Provided

Upon receipt, all components of the RayBio® Human Cytokine Antibody Array kit should be stored at -20°C to -80°C. At -20°C to -80°C the kit will retain complete activity for up to 6 months. Once thawed, the array membranes and Blocking Buffer should be kept at -20°C and all other component should be stored at 4°C. After thawing the reagents, the kit must be used within three months, and please use the kit within six months of purchase.
### COMPARTMENT

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>AAM-CYT-1-2</th>
<th>AAM-CYT-4</th>
<th>AAM-CYT-1-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Cytokine Array C6 and C7</td>
<td>2 membranes per array (4 total)</td>
<td>4 membranes per array (8 total)</td>
<td>8 membranes per array (16 total)</td>
</tr>
<tr>
<td>Blocking Buffer</td>
<td>1 vial (25 ml)</td>
<td>2 vials (25 ml)</td>
<td>4 vials (25 ml)</td>
</tr>
<tr>
<td>Biotinylated Antibody Cocktail</td>
<td>1 vial per array (2 total)</td>
<td>2 vials per array (4 total)</td>
<td>4 vials per array (8 total)</td>
</tr>
<tr>
<td>1,000X HRP Streptavidin Concentrate</td>
<td>1 vial (50 μl)</td>
<td>2 vials (50 μl/ea)</td>
<td></td>
</tr>
<tr>
<td>20X Wash Buffer I Concentrate</td>
<td>1 vial (10 ml)</td>
<td>1 vial (20 ml)</td>
<td>2 vials (20 ml/ea)</td>
</tr>
<tr>
<td>20X Wash Buffer II Concentrate</td>
<td>1 vial (10 ml)</td>
<td>1 vial (20 ml)</td>
<td>2 vials (20 ml/ea)</td>
</tr>
<tr>
<td>2X Cell Lysis Buffer Concentrate</td>
<td>1 vial (10 ml)</td>
<td>1 vial (16 ml)</td>
<td>2 vials (16 ml/ea)</td>
</tr>
<tr>
<td>Detection Buffer C</td>
<td>1 vial (1.5 ml)</td>
<td>1 vial (2.5 ml)</td>
<td>2 vials (2.5 ml/ea)</td>
</tr>
<tr>
<td>Detection Buffer D</td>
<td>1 vial (1.5 ml)</td>
<td>1 vial (2.5 ml)</td>
<td>2 vials (2.5 ml/ea)</td>
</tr>
<tr>
<td>8-Well Incubation Tray w/ Lid</td>
<td>1 tray</td>
<td>2 trays</td>
<td></td>
</tr>
</tbody>
</table>

**Other Kit Components:** Plastic Sheets, Array Map Template, User Manual

### IV. Additional Materials Required

- Small plastic boxes or containers
- Orbital shaker
- Plastic sheet protector or SaranWrap
- Kodak X-Omat AR film (REF 165 1454) and film processor or Chemiluminescence imaging system

### V. Overview and General Considerations

#### A. Preparation of Samples

- Use serum-free conditioned media if possible.
- If serum-containing media is required, use an uncultured media aliquot as a negative control sample, since many types of sera contain cytokines.
- For cell lysates and tissue lysates, we recommend using RayBio® Cell Lysis Buffer to extract proteins from cell or tissue (e.g. using homogenizer). Dilute 2X RayBio® Cell Lysis Buffer with H2O (we recommend adding proteinase inhibitors to Cell Lysis Buffer before use). After extraction, spin the sample down and save the supernatant for your experiment. Determine protein concentration.
- We recommend using per membrane:
  - 1 ml of Conditioned media (undiluted), or
  - 1 ml of 2-fold to 5-fold diluted sera or plasma, or
  - 50-500 μg of total protein for cell lysates and tissue lysates (use
  - ~200-250 μg of total protein for first experiment)

**Note:** Dilute the lysate at least 10 fold with 1 X blocking buffer. The amount of sample used depends on the abundance of cytokines. More of the sample can be used if the signals are too weak. If the signals are too strong, the sample can be diluted further. If you experience high background, you may further dilute your sample.

#### B. Handling Array Membranes

- Always use forceps to handle membranes, and grip the membranes by the edges only.
- Never allow the array membranes to dry during experiments.

#### C. Incubation

- Completely cover the membranes with sample or buffer during incubation, and cover the eight-well tray with lid to avoid drying.
• Avoid foaming during incubation steps.
• Perform all incubation and wash steps under gentle rotation.
• Several incubation steps such as step 2 (blocking), step 3 (sample incubation), step 8 (biotin-Ab incubation) and step 11 (HRPstreptavidin incubation) may be done at 4°C for overnight, but make sure to cover the 8 well plate tightly to prevent evaporation.

VI. Protocol

**NOTE:** Prepare all reagents and samples immediately prior to use. See Sections V and VII. **ALL** incubations and washes must be performed under gentle rotation/rocking (~0.5-1 cycle/sec)

1. Remove the kit from storage and allow the components to equilibrate to room temperature (RT).
2. Carefully remove the Antibody Arrays (ITEM 1) from the plastic packaging and place each membrane (printed side up) into a well of the Incubation Tray (ITEM 10). One membrane per well.

**NOTE:** The antibody printed side is marked by a dash (-) or number (#) in the upper left corner.

A. Blocking
3. Pipette 2 ml of Blocking Buffer (ITEM 2) into each well and incubate for 30 minutes at RT.
4. Aspirate blocking buffer from each well with a pipette.

B. Sample Incubation
5. Pipette 1 ml of diluted or undiluted sample into each well and incubate for 1.5 to 5 hours at RT OR overnight at 4 °C.

**NOTE:** Longer incubations can help maximize the spot signal intensities. However, doing so can also increase the background response so complete liquid removal and washing is critical.
6. Aspirate samples from each well with a pipette.

C. First Wash

**NOTE:** The 20X Wash Buffer Concentrates I and II (ITEM 5 and 6) must be diluted 20-fold before use. See Section VII for details.
7. **Wash Buffer I Wash:** Pipette 2 ml of **1X** Wash Buffer I into each well and incubate for 5 minutes at RT. Repeat this 2 more times for a total of 3 washes using fresh buffer and aspirating out the buffer completely each time.
8. **Wash Buffer II Wash:** Pipette 2 ml of **1X** Wash Buffer II into each well and incubate for 5 minutes at RT. Repeat this 1 more time for a total of 2 washes using fresh buffer and aspirating out the buffer completely each time.

D. Biotinylated Antibody Cocktail Incubation

**NOTE:** The Biotinylated Antibody Cocktail (ITEM 3) must be prepared before use. See Section VII for details.
9. Pipette 1 ml of the **prepared** Biotinylated Antibody Cocktail into each well and incubate for 1.5 to 2 hours at RT OR overnight at 4°C.
10. Aspirate biotinylated antibody cocktail from each well.

E. Second Wash
12. Wash membranes as directed in Steps 7 and 8.
F. HRP-Streptavidin Incubation

**NOTE:** The 1,000X HRP-Streptavidin Concentrate (ITEM 4) must be diluted before use. See Section VII for details.

- Pipette 2 ml of 1X HRP-Streptavidin into each well and incubate for 2 hours at RT OR overnight at 4°C.
- Aspirate HRP-Streptavidin from each well.

G. Third Wash

- Wash membranes as directed in Steps 7 and 8.

H. Chemiluminescence Detection

**NOTE:** Do not allow membranes to dry out during detection.

- Transfer the membranes, printed side up, onto a sheet of chromatography paper, tissue paper, or blotting paper lying on a flat surface (such as a benchtop).
- Remove any excess wash buffer by blotting the membrane edges with another piece of paper.
- Transfer and place the membranes, printed side up, onto a plastic sheet (provided) lying on a flat surface.

**NOTE:** Multiple membranes can be placed next to each other and fit onto a single plastic sheet. Use additional plastics sheets if necessary.

- Into a single clean tube, pipette equal volumes (1:1) of Detection Buffer C (ITEM 8) and Detection Buffer D (ITEM 9). Mix well with a pipette.

**EXAMPLE:** 250 μl of Detection Buffer C + 250 μl of Detection Buffer D = 500 μl (enough for 1 membrane)

Gently pipette 500 μl of the Detection Buffer mixture onto each membrane and incubate for 2 minutes at RT (DO NOT ROCK OR SHAKE). Immediately afterwards, proceed to Step 20.

**NOTE:** Exposure should ideally start within 5 minutes after finishing Step 19 and completed within 10-15 minutes as chemiluminescence signals will fade over time. If necessary, the signals can usually be restored by repeating washing, HRP-Streptavidin and Detection Buffers incubations (Steps 11-19).

- Place another plastic sheet on top of the membranes by starting at one end and gently "rolling" the flexible plastic sheet across the surface to the opposite end to smooth out any air bubbles. The membranes should now be "sandwiched" between two plastic sheets.

**NOTE:** Avoid "sliding" the top plastic sheet along the membranes’ printed surface. If using X-ray film, do not use a top plastic sheet so that the membranes can be directly exposed to the film.

- Transfer the sandwiched membranes to the chemiluminescence imaging system such as a CCD camera (recommended) and expose.

**NOTE:** Optimal exposure times will vary so performing multiple exposure times is strongly recommended. See Section VI for additional details.

I. Storage

- To store, without direct pressure, gently sandwich the membranes between 2 plastic sheets (if not already), tape the sheets together or use plastic wrap to secure them, and store at ≤ -20 °C for future reference.
VII. TYPICAL RESULTS:

Typical results obtained with RayBio® C-Series Antibody Arrays

Sample-1  Sample-2  Control

The preceding figures present typical images obtained with RayBio® C-Series Antibody Arrays. These membranes were probed with conditioned media from two different cell lines. Membranes were exposed with Kodak X-Omat® film at room temperature for 1 minute.

Note the strong signals of the Positive Control spots in the upper left and lower right corners. (See below for further details on the control spots.)

The signal intensity for each antigen-specific antibody spot is proportional to the relative concentration of the antigen in that sample. Comparison of signal intensities for individual antigen-specific antibody spots between and among array images can be used to determine relative differences in expression levels of each analyte sample-to-sample or group-to-group.

The signals also can be detected and quantitated by using a chemiluminescence imaging device. The RayBio® Analysis Tool is a program specifically designed for analysis of RayBio® Cytokine Antibody Arrays. This tool will not only assist in compiling and organizing your data, but also reduces your calculations to a “copy and paste.” Call or e-mail Pete Shuster (612-801-1007 or pshuster@neuromics.com) for ordering information.

VIII. INTERPRETATION OF RESULTS

A. Control Spots

Positive Control Spots (POS) – controlled amount of biotinylated antibody printed onto the array. Used for normalization and to orientate the arrays.

Negative Control Spots (NEG) – buffer printed (no antibodies) used to measure the baseline responses. Used for determining the level of non-specific binding of the samples.

Blank Spots (BLANK) – nothing is printed here. Used to measure the background response.

B. Data Extraction

Visual comparison of array images may be sufficient to see differences in relative protein expression. However, most researchers will want to perform numerical comparisons of the signal intensities (or more precisely, signal densities), using 2-D densitometry. Gel/Blot documentation systems and other chemiluminescent or phosphorescent detection systems are usually sold as a package with compatible densitometry software.
Any densitometry software should be sufficient to obtain spot signal densities from your scanned images. One such software program, ImageJ, is available for free from the NIH website along with an array plug-in.

We suggest using the following guidelines when extracting densitometry data from our array images:

- For each array membrane, identify a single exposure that exhibits a high signal to noise ratio (strong spot signals and low background response). Strong Positive Control Spot signals but not too strong that they are “bleeding” into one another is ideal. The exposure time does not need to be identical for each array, but Positive Control signals on each array image should have similar intensities.
- Measure the density of each spot using a circle that is roughly the size of one of the largest spots. Be sure to use the same extraction circle dimensions (area, size, and shape) for measuring the signal densities on every array for which you wish to compare the results.
- For each spot, use the summed signal density across the entire circle (ie, total signal density per unit area)

C. Data Analysis

**NOTE:** RayBiotech offers Microsoft® Excel-based Analysis Software Tools for each array kit for automatic analysis. Please visit the website at www.raybiotech.com or contact us for ordering information.

Once the raw numerical densitometry data is extracted, the background must be subtracted and the data normalized to the Positive Control signals to analyze.

Background Subtraction: Select values which you believe best represent the background. If the background is fairly even throughout the membrane, the Negative Control Spots (NEG) and/or Blank Spots (BLANK) should be similar and are accurate for this purpose.

Positive Control Normalization: The amount of biotinylated antibody printed for each Positive Control Spot is consistent from array to array. As such, the intensity of these Positive Control signals can be used to normalize signal responses for comparison of results across multiple arrays, much like housekeeping genes and proteins are used to normalize results of PCR gels and Western Blots, respectively.

To normalize array data, one array is defined as “Reference Array” to which the other arrays are normalized to. The choice of the Reference Array is arbitrary.

**NOTE:** The RayBio® Analysis Software Tools always designate Array 1/Sample 1 as the Reference Array.

Next, the simple algorithm below can be used to calculate and determine the signal fold expression between like analytes.

\[
X(Ny) = X(y) \times \frac{P1}{P(y)}
\]

Where:
- \(P1\) = mean signal density of Positive Control spots on reference array
- \(P(y)\) = mean signal density of Positive Control spots on Array "y"
- \(X(y)\) = mean signal density for spot "X" on Array for sample "y"
- \(X(Ny)\) = normalized signal intensity for spot "X" on Array "y"

[www.neuromics.com](http://www.neuromics.com)
For example:
Let’s determine the relative expression for IL-6 on two different arrays (Arrays 1 and 2). Let’s assume that the duplicate signals for the IL-6 spots on each array are identical (or that the signal intensity used in the following calculation is the mean of the two duplicates spots). Also assume the following:

P1 = 2500
P2 = 2700
IL-6 (1) = 300
IL-6 (2) = 455

Then IL-6(N2) = 455 * 2500/2700 = 421.30

The fold increase of IL-6(N2) vs IL-6(1) = 421.3/300 = 1.40-fold increase or a 40% increase in the signal intensity of IL-6 in Array 2 vs. Array 1.

IX. ARRAY MAP

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>G-CSF</td>
<td>GM-CSF</td>
<td>IL-2</td>
<td>IL-3</td>
</tr>
<tr>
<td>2</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>G-CSF</td>
<td>GM-CSF</td>
<td>IL-2</td>
<td>IL-3</td>
</tr>
<tr>
<td>3</td>
<td>IL-4</td>
<td>IL-5</td>
<td>IL-6</td>
<td>IL-9</td>
<td>IL-10</td>
<td>IL-12p40/70</td>
<td>IL-12p70</td>
<td>IL-13</td>
</tr>
<tr>
<td>4</td>
<td>IL-4</td>
<td>IL-5</td>
<td>IL-6</td>
<td>IL-9</td>
<td>IL-10</td>
<td>IL-12p40/70</td>
<td>IL-12p70</td>
<td>IL-13</td>
</tr>
<tr>
<td>5</td>
<td>IL-17</td>
<td>IFN gamma</td>
<td>MCP-1</td>
<td>MCP-5</td>
<td>RANTES</td>
<td>SCF</td>
<td>sTNFRI</td>
<td>TNF alpha</td>
</tr>
<tr>
<td>6</td>
<td>IL-17</td>
<td>IFN gamma</td>
<td>MCP-1</td>
<td>MCP-5</td>
<td>RANTES</td>
<td>SCF</td>
<td>sTNFRI</td>
<td>TNF alpha</td>
</tr>
<tr>
<td>7</td>
<td>THPO</td>
<td>VEGF</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>POS</td>
</tr>
<tr>
<td>8</td>
<td>THPO</td>
<td>VEGF</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>BLANK</td>
<td>POS</td>
</tr>
</tbody>
</table>

POS = Positive Control Spot
NEG = Negative Control Spot
BLANK = Blank Spot
# Troubleshooting guide

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak signal or no signal</td>
<td>1. Taking too much time for Detection.</td>
<td>1. The whole Detection process must be completed in 30 min.</td>
</tr>
<tr>
<td></td>
<td>2. Film developer does not work properly.</td>
<td>2. Fix film developer.</td>
</tr>
<tr>
<td></td>
<td>3. Did not mix HRP-streptavidin well before use.</td>
<td>3. Mix tube containing 1,000X HRP-Conjugated Streptavidin well before use since precipitation may form during storage.</td>
</tr>
<tr>
<td></td>
<td>4. Sample is too dilute.</td>
<td>4. Increase sample volume, (e.g. using undilute sample) or using more cells (e.g. seed 2 million cells. After 1 or 2 days, change complete medium with low serum medium and collect conditioned medium 2 day later. Use about 1 to 2 ml of conditioned medium for experiment).</td>
</tr>
<tr>
<td></td>
<td>5. Other.</td>
<td>1. Reduce blocking concentration by diluting in 1X Wash Buffer II.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Slightly increase HRP concentrations.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Slightly increase biotin-antibody concentrations.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Expose film for overnight to detect weak signal.</td>
</tr>
<tr>
<td></td>
<td>2. Membranes were not completely covered by solution.</td>
<td>2. Completely cover membranes with solution.</td>
</tr>
<tr>
<td>High background</td>
<td>1. Exposure to x-ray file is too long.</td>
<td>1. Decrease exposure time.</td>
</tr>
<tr>
<td></td>
<td>2. Membranes were allowed to dry out during experiment.</td>
<td>2. Completely cover membranes with solution during experiment.</td>
</tr>
<tr>
<td></td>
<td>3. Sample is too concentrated.</td>
<td>3. Use more diluted sample.</td>
</tr>
</tbody>
</table>
Notes:
RayBio® is the trademark of RayBiotech, Inc. Cytokine protein arrays are RayBiotech patent-pending technology.

This product is intended for research only and is not to be used for clinical diagnosis. Our products may not be resold, modified for resale, or used to manufacture commercial products without written approval by RayBiotech, Inc.

Under no circumstances shall Neuromics or RayBiotech be liable for any damages arising out of the use of the materials.

Products are guaranteed for three months from the date of purchase when handled and stored properly. In the event of any defect in quality or merchantability, Neuromics' liability to buyer for any claim relating to products shall be limited to replacement or refund of the purchase price.

Kodak X-OmatTM is the trademark of Eastman Kodak Company.