

Comparing Quantitative Viability Bioassays: An Evaluation of MTT, alamarBlue™, and Guava® ViaCount® Methods

Krista McCutcheon and David Fei, Department of Analytical Sciences,
Genentech Inc., South San Francisco, CA 94080-4990

ABSTRACT

Viability assays are crucial tools in the development of many drugs, particularly of cytotoxic agents. To best characterize potential compounds, viability assays must ideally be able to reproducibly and accurately report small percentage changes in viability, provide statistically significant quantification of biological activity, and account for changes in cell number during the assay. An analysis of cell number interference on MTT, alamarBlue™, and Guava ViaCount® viability assays was performed in the development of a quantitative CD40 ligation assay. MTT and alamarBlue assays both showed significantly large statistical variations in viability measurements due to changes in cell numbers. Guava ViaCount's ease of use and high reproducibility provide significant advantages over current viability or cytotoxicity assays where changing cell numbers can be problematic.

INTRODUCTION

The investigation of cytotoxic therapeutic agents to treat malignant cells or other disorders of cell proliferation is a very active field of drug development. Viability assays that have the statistical power to quantitate biological activity, differentiate the potency of compounds and indicate stability are the most valuable. For this purpose a sensitive, reproducible, accurate, and dose-dependent cell-based assay is required.

A number of different methods are currently used to measure cell viability by indirect means of proliferation and metabolism (e.g., tetrazolium dyes such as MTT¹, ³[H]-thymidine, or BrdU uptake², and alamarBlue³). Both MTT and alamarBlue can be carried out in a 96-well, high-throughput format and have been used in quantitative cytotoxicity assays.^{1,3} A critical assumption used in indirect assays of viability, is that cell number will decrease in a reproducible manner, inversely and linearly proportional to the dose of the drug. This assumption would fail in cases where cell number is highly variable over the time course of the assay. For instance: if a small number of responsive cells are masked by a majority of proliferating cells; if the drug affects cellular aggregation or adhesion (properties which also are known to indirectly affect cell growth); if the drug has a nonlinear effects on cell number (e.g., by causing apoptosis or changing the cell cycle); or if the target cell line itself has irregular growth properties. Changing target cell lines or obtaining separate cell number data (e.g., using protein assays, coulter

counters, TruCount™ beads or a haemocytometer), for each well in an assay, would often not be feasible. Guava ViaCount offers a unique alternative to measuring viability, enumerating both viable and non-viable cell populations, at the same time, in one sample. Dual labeling with two dyes of different fluorescent wavelengths and membrane permeability, are used to discriminate the viable and non-viable cell populations. One dye, PM2 (permeable to all cells) and the other, PM1 (permeable only to cells with a compromised cell membrane) enable viability data to be expressed as a percentage of total cell number in each sample well of the assay. Using Guava ViaCount, the direct measurement of non-viable cells, and the normalization of each data point to total cell number, corrects for inter-well variability introduced by cell number, improving intra- and inter-assay precision.

We report the impact of cell number interference on the MTT, alamarBlue, and Guava ViaCount assays, measured during the development of a quantitative bioassay for a humanized monoclonal antibody directed against human CD40. On malignant B cells, ligation of CD40 can result in direct inhibition of cell growth with or without apoptosis. The outcome and extent of CD40 activation on cells is complex, and may be regulated by cell phenotype, the strength of the CD40 signal, and the presence of co-factors.⁴⁻⁶ Both the pleiotropic anti-CD40 drug activity and the irregular growth properties of the malignant B-cell line used in the assays contributed to cell number interference in the MTT and alamarBlue assays. We demonstrate how the ability of the Guava ViaCount assay to normalize viability to cell number offered a major improvement in the monoclonal anti-CD40 bioassay variability. The relative ease of the assay and our statistical results suggest that a viability assay using the Guava PCA and Guava ViaCount reagent may provide a significant advantage over other bioassays in current use, particularly in cases where intra- and inter-assay cell number is a problem.

MATERIALS AND METHODS

Reagents

Humanized anti-CD40 monoclonal antibody (IgG1) is a Genentech reagent (South San Francisco, CA, USA). Human IgG1 κ was obtained from Sigma (St. Louis, MO, USA). Anti-human Fc γ , used for cross-linking

the humanized antibodies to the culture plates, was obtained from Jackson ImmunoResearch (Westgrove, PA, USA). The goat anti-human IgG-FITC conjugate, used for FACS analysis, was purchased from BD PharMingen (San Diego, CA, USA).

Guava ViaCount reagent was purchased from Guava Technologies (Hayward, CA, USA), MTT reagent was purchased from ATCC (Rockville, MD, USA), and alamarBlue was purchased from Trek Diagnostic Systems, (Westlake, OH, USA).

Cell culture and characterization of Raji cell line

The Raji human cell line was obtained from ATCC (Rockville, MD, USA), and was grown in culture media conditions recommended by ATCC with 100 μ g/mL penicillin/streptomycin. The cells were analyzed for homogeneity and uniformity of CD40 expression by flow cytometry. One million cells in growth media were centrifuged at 1,200 \times g and re-suspended in 0.25 mL block buffer (1% BSA, PBS) for 1 hour on ice. Block buffer was removed and 0.25 mL of 10 μ g/mL anti-CD40 monoclonal antibody or isotype-matched human IgG1 κ in block buffer was added for 1 hour at ambient temperature. The cells were washed 3 times in PBS, followed by adding a 1:10 dilution of goat anti-human IgG-FITC in block buffer for 30 minutes at ambient temperature. The stained cells were washed three times in PBS, resuspended in 0.4 mL of PBS, and analyzed by flow cytometry on a BD FACSCaliber (Becton Dickinson, San Jose, CA).

Cell morphology was examined under a Nikon Diaphot 200, inverted light microscope, using a Phase I, DL 40 \times magnification lens.

Cell-based viability assays

A dilution series of anti-CD40 monoclonal antibody was cross-linked to high protein binding plates through the constant region of the heavy chain. Unbound anti-CD40 monoclonal antibody was removed by washing, and 50,000 Raji cells were added to each well. After 72 hours, the cells in each well were mixed, and aliquots were removed for staining with the MTT, alamarBlue, or Guava ViaCount reagents.

High protein binding, 96-well, Costar plates were coated with 0.1 mL, 30 μ g/mL, goat anti-human Fc γ

Application Note: Comparing Quantitative Viability Bioassays

in 50 mM sodium bicarbonate, pH 9.6, overnight in a humidified 5% CO₂, 37 °C incubator. Unbound goat anti-human Fcγ was removed with two 0.2 mL washes using sterile PBS. Coated plates were blocked with 0.3 mL, 0.5% BSA, PBS for 1 hour at ambient temperature. Blocking solution was removed and the plate washed once with 0.3 mL PBS. A dilution series of anti-CD40 monoclonal antibody (4 to 0.002 µg/mL) was prepared in PBS, and 0.1 mL applied on the plate with gentle agitation for 4 hours at ambient temperature. Unbound antibody was removed, and the plate was washed twice with 0.2 mL PBS. Raji cells were seeded into growth medium (RPMI 1640, 10% FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 100 µg/mL penicillin/streptomycin) at less than 1 x 10⁶ cells/mL, and grown in a humidified 5% CO₂, 37 °C incubator for 24 hours before the assays were performed. Raji cells grew in reversible cell aggregates, and to maintain responsiveness in the assay, the cells were routinely split before the density exceeded 1 x 10⁶ cells/mL. On the assay day, a suspension of 0.25 x 10⁶ cells/mL of Raji cells was prepared in assay media (growth medium, containing 2% FBS), and 0.2 mL was added to each well to give 0.5 x 10⁵ cells/well. It was necessary to assay the cells in medium with reduced serum (1-5%). Cells assayed in 10% serum did not respond well in the assay. The cells are incubated with the Fc-cross-linked anti-CD40 monoclonal antibody for 72 hours in a humidified 5% CO₂, 37 °C incubator. Raji cells typically grow in suspension. However, to make sure we assayed a population of cells representative of each well, we pipetted up and down in the wells 10 times before transferring cells for viability assays.

The alamarBlue assays were performed by adding 50 µL of alamarBlue to 50-100µL cells for 2–16 hours in a humidified 5% CO₂, 37 °C incubator. Fluorescence was measured using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. alamarBlue acts as an oxidation-reduction indicator that fluoresces in response to the chemical reduction of growth medium resulting from cell growth. Signal is quantified at 590 nm with a fluorometer.

MTT assays were performed as described in the product manual using 50 µL of cells. After 6 hours in a humidified 5% CO₂, 37 °C incubator, detergent was added at RT and the signal measured from 6–24 hours. The yellow tetra-

zolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the detergent. The color is then quantified by spectrophotometric means (570 nm).

Guava ViaCount assays were performed by transferring 50 µL of cells in media into microtitre tubes containing 150 µL of Guava ViaCount reagent. After incubation for 5 minutes at room temperature in the dark, viability and cell count measurements were taken using the Guava Personal Cell Analysis System (PCA™). Viability data was expressed as a percentage of total cell number.

Data Analysis

Percent viability (Guava ViaCount) or optical density (alamarBlue or MTT) was plotted on the y-axis against a logarithmic scale of anti-CD40 monoclonal antibody concentration (µg/mL) on the x-axis. Kaleidograph software was used to fit a 4-parameter logistic curve, according to the equation:

$$y = [(m_1 - m_4) / (1 + (m_0 / m_3)^{m_2})] + m_4$$

where m_1 = the estimated response at zero dose (minimum); m_2 = curvature parameter; m_3 = the response at 50% maximum response (IC₅₀); and m_4 = the estimated response at infinite dose (maximum).

RESULTS

Anti-CD40 monoclonal antibody was observed to have multiple effects on the Raji cell line. In addition to decreasing cell viability, dose-dependent effects on cell number, homotypic aggregation, and cell spreading were also observed (Figure 1). Each effect had its own dose-dependence and time course of activity in response to the monoclonal anti-CD40. The contribution of cell number to assay variability was found to be significant, and normalization of viability data to cell number was essential to developing a reproducible assay. The cell number data (Figure 1A) fit poorly to a 4-parameter curve (correlation term, R = 0.88), and the coefficient of variation (CV) of individual points across the anti-CD40 antibody concentration range, was unacceptably high (8 - 30%).

A comparison of typical results from alamarBlue, MTT and Guava ViaCount assays is shown in Figure 2. Across the dose-response curves, the intra-assay variability

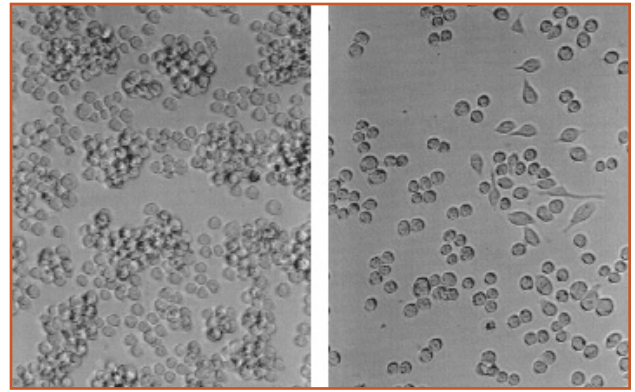
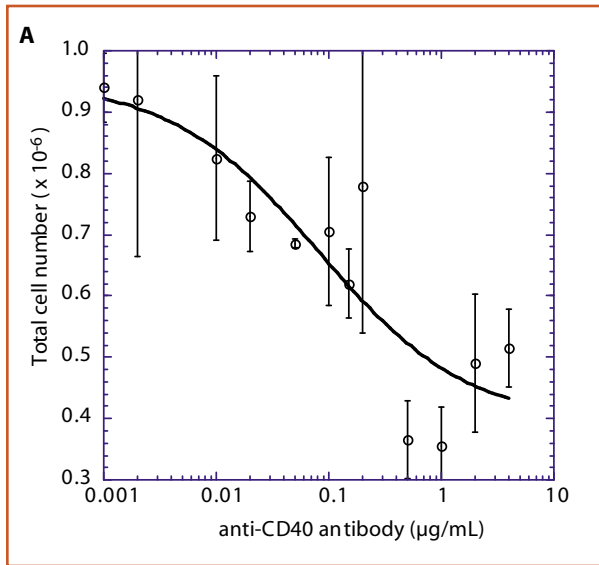


FIGURE 1: Pleiotropic effects of the humanized anti-CD40 monoclonal antibody on Raji cells. Raji cells were incubated with humanized anti-CD40 monoclonal antibody for 72hrs. Samples were either assayed by Guava ViaCount (A), or examined under an inverted microscope at 40x magnification (B, 1 µg/mL anti-CD40 antibody is shown). The effect of the drug on cell number, cell aggregation, and cell spreading are illustrated.

was 3–57% in the alamarBlue assay, 8–30% in the MTT assay, and 1–5% in the Guava ViaCount assay. Furthermore, alamarBlue and MTT assays on some days were unable to detect any change in viability. Guava ViaCount showed an inter-assay variability of 4–12% over 10 assays.

A viability assay for the humanized anti-CD40 monoclonal antibody was qualified using Guava ViaCount. An inhibition curve with sensitivity over 1.5 log units was developed on Raji cells from 0.01 to 2 µg/mL anti-CD40 monoclonal antibody. The mean 50% inhibitory concentration from ten independent assays, with cells from passages 10 to 22, was 0.21 ± 0.03 µg/mL (with a coefficient of variance of 12.3%). Precision was evaluated by creating three controls at the high (2 µg/mL), mid (0.3 µg/mL), and low (0.1 µg/mL) dose points of an inhibition curve. From 12 replicates in a plate, the percent viability of low, mid, and high controls was $57.8 \pm 2.26\%$ (CV= 3.9%), $45.2 \pm 4.53\%$ (CV= 10.4%) and 32.8 ± 1.97 (CV = 6.0%), respectively. An intra-sample CV of 2.9% was obtained in the Guava ViaCount assay when the mid control sample was measured repeatedly six times. The stability of the Guava ViaCount signal was tested using a row of 12 mid control samples measured after 10 minutes and one hour. The coefficient of variation between the 10 minute and 1 hour samples was less than 4.5% and the measurements showed no time-dependent drift. Using the entire 96-well plate, the intra-plate variation,

determined using an average IC_{50} dose, was less than 5%. Accuracy was tested by measuring the recovery of a sample prepared at 75% of the average IC_{50} dose. The percent viability of the recovery sample was compared to 75% of the IC_{50} value obtained from the dose-response curve in the same assay. The percent viability of the recovery sample was 103% of the expected value.

DISCUSSION

The Raji cell line used in these assays appeared homogeneous by FACS analysis: side scatter and forward scatter parameters showed a morphologically similar population; 100% of the live cells were consistently CD40+; and the level of CD40 expression was uniform (not shown). Despite this apparent cell uniformity, anti-CD40 monoclonal antibody was observed to not only decrease cell viability but also to have dose-dependent and irregular effects on cell number and morphology. Changes in input cell number and assay media, sub-cloning the cell line, and synchronizing the cell cycle, were all unable to improve the assay variability. As a control, purified human IgG1κ was tested in the dose range of the assay and showed no effect on Raji cells. The observed morphological changes in Raji cells in response to anti-CD40 antibody in our assays have been reported in related studies.⁷⁻¹³

Cross-linking of the anti-CD40 monoclonal antibody via Fc has been found to optimize the inhibitory signal,

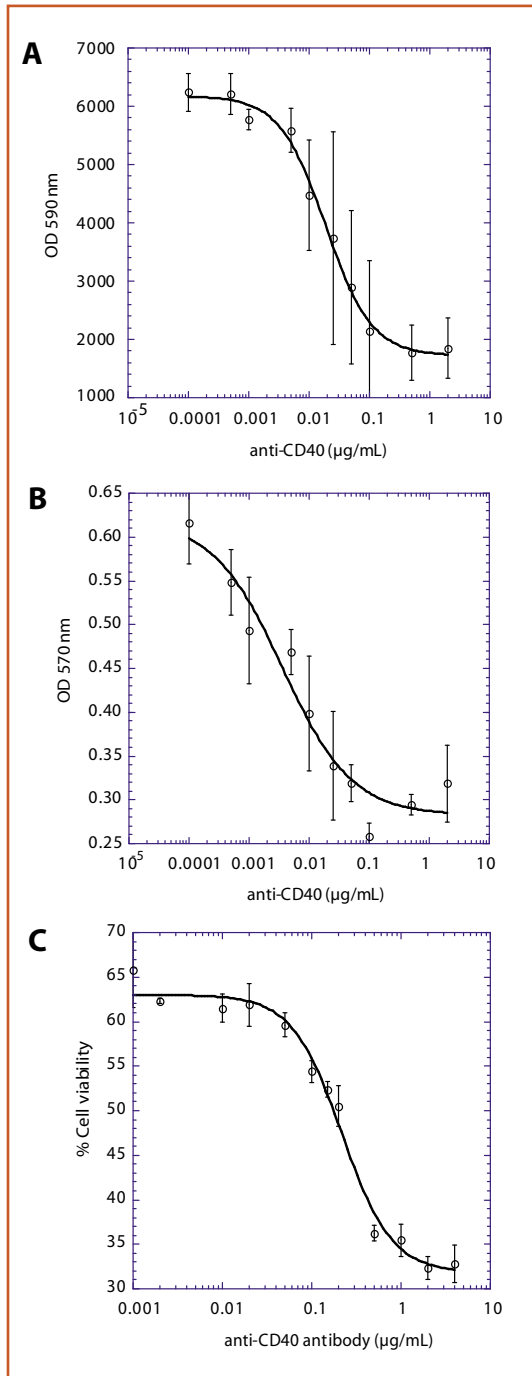


FIGURE 2: Dose-dependent inhibition of Raji cell viability by humanized anti-CD40 monoclonal antibody. Raji cells were incubated with humanized anti-CD40 monoclonal antibody for 72 hours. alamarBlue (A), MTT (B), or Guava ViaCount (C), from duplicate samples within one plate are shown, demonstrating variability within an assay.

possibly by facilitating the functional multimerization of CD40 at the cell surface.^{14,15} Examination of the wells under the microscope showed no evidence that the dose-dependent decrease in cell numbers or viability measured in these assays was caused by a proportional increase in cells being irreversibly captured by the anti-CD40 monoclonal antibody coat. The mechanism underlying the reduced cell numbers in this assay was examined but our data was inconclusive. Anti-CD40 antibody has been observed in other studies to inhibit cell growth, with or without apoptosis.⁴⁻⁶

Flow cytometry analysis to assess viability and determine chemotherapeutic IC_{50} values on human leukaemic cell lines has previously been found to be comparable to the MTT assay.¹⁶ The ability of the Guava PCA to use smaller sample volumes, fewer events, and 10-fold lower cell numbers than traditional flow cytometers is an advantage when cell culture volume and time is valuable. Furthermore, we found normalizing viability to cell number increased the confidence of IC_{50} determinations. This normalizing was facilitated by the Guava ViaCount software, which reports absolute cell counts and concentration for all samples. For this purpose, the Guava ViaCount method is much simpler than other methods, such as MTT or alamarBlue, that require a second assay to normalize data to cell number (e.g., coulter counters, TruCount beads, or a hemocytometer).

When used on the Guava PCA-96 system, the Guava ViaCount assay permits high throughput data acquisition directly from 96-well plates, creating a simple way to screen compounds or antibodies for cytotoxicity. Our data support that the Guava ViaCount assay is able to generate a sensitive, reproducible, and accurate quantitation of the anti-CD40 monoclonal antibody's biological activity. This assay will be a valuable tool in the development of assays measuring the drug's potency and stability. Other drug-cell line combinations may benefit from this type of analysis, particularly when there is a need to isolate the activity of a drug on cell viability from its non-linear effects on cell number.

To summarize:

- Guava ViaCount is significantly more reproducible than MTT and alamarBlue assays, particularly when cell numbers fluctuate during an experiment.
- Direct measurements of absolute cell numbers make Guava ViaCount simpler to use than MTT or alamarBlue, both of which require a second cell counting assay in conjunction.

ACKNOWLEDGEMENTS

We thank Lori O'Connell and Leonard Presta for engineering the humanized anti-CD40 antibody used in this study. We also thank Eleanor Canova-Davis and Maureen Beresini for helpful comments and advice.

REFERENCES

1. van de Loosdrecht A.A., R.H. Beelen, G.J. Ossenkoppele, M.G Broekhoven, and M.M Langenhuijsen (1994) A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *J Immunol Methods* 174(1-2):311-20.
2. Porstmann T., T. Ternynck, and S. Avrameas (1985) Quantitation of 5-bromo-2-deoxyuridine incorporation into DNA: an enzyme immunoassay for the assessment of the lymphoid cell proliferative response. *J Immunol Methods* 82(1):169-79.
3. Gazzano-Santoro H., P. Ralph, T.C. Ryskamp, A.B. Chen and V.R. Mukku (1997) A non-radioactive complement-dependent cytotoxicity assay for anti-CD20 monoclonal antibody. *J Immunol Methods* 202:163-71.
4. Kehry M.R. CD40-mediated signaling in B Cells. (1996) Balancing cell survival, growth and death. *J Immunol* 156: 2345-48.
5. Costello R.T., J.A. Gastaut, and D. Olive (1999). What is the real role of CD40 in cancer immunotherapy? *Immunology Today* 20(11):488-93.
6. Ziebold J.L.I, J. Hixon, A. Boyd, and W.L. Murphy (2000) Differential effects of CD40 stimulation on normal and neoplastic cell growth. *Archivum Immunologiae et Therapiae Experimentalis* 48:225-33.
7. Zhou Z., J. Wang , Y. Wang, Y. Qiu, J. Pan, W. Xie, L. Jiang, B. Klein, and X. Zhang (1999) An agonist anti-human CD40 monoclonal antibody that induces dendritic cell formation and maturation and inhibits proliferation of a myeloma cell line. *Hybridoma* 18(6):471-78.
8. Aldinucci D., D. Poletto, P. Nanni, M. Degan, M. Rupolo, A. Pinto, and V. Gattei (2002) CD40L induces proliferation, self-renewal, rescue from apoptosis and production of cytokines by CD40-expressing AML blasts. *Exp Hematol* 30(11): 1283-92.
9. Wagner N., P. Engel, and T.F. Tedder (1993) Regulation of the tyrosine kinase-dependent adhesion pathway in human lymphocytes through CD45. *J Immunol* 150(11):4887-99.
10. Pellat-Deceunynck C., M. Amiot, N. Robillard, J. Wijdenes, and R. Bataille (1996) CD11a-CD18 and CD102 interactions mediate human myeloma cell growth and arrest induced by CD40 stimulation. *Cancer Res* 56(8):1909-16.
11. Zetter B.R. (1993) Adhesion molecules in tumor metastasis. *Semin Cancer Biol* 4(4):219-29.
12. Hirokawa M., J. Kuroki, A. Kitabayashi, and A.B. Miura (1996) Transmembrane signaling through CD80 (B7-1) induces growth arrest and cell spreading of human B lymphocytes accompanied by protein tyrosine phosphorylation. *Immunology Letters* 50: 95-8
13. Lebakken C.S., and A.C. Rapraeger (1996) Syndecan-1 mediates cell spreading in transfected human lymphoblastoid (Raji) cells. *JCB* 132: 1209-21
14. Funakoshi S., D.L. Longo, and W. Murphy (1996) Differential in vitro and in vivo antitumor effects mediated by anti-CD40 and anti-CD20 monoclonal antibodies against human B-cell lymphomas. *J Immunotherapy* 19(2):93-101.
15. Parry S.L., M.J. Holman, J. Hasbold, and G.G.B.Klaus. 1994. Plastic-immobilized anti- μ or anti- δ antibodies induce apoptosis in mature murine B lymphocytes. *Eur J Immunol* 24: 974-9.
16. Gupta M., S. Naik, C.M. Pandey, and S. Dabadghao (2002) Drug sensitivity assay for leukaemic cells by flow cytometry. *Indian J Med Res* 115; 260-4.

MTT is a trademark of ATCC. alamarBlue is a trademark of Trek Diagnostic Systems. FACSCalibur is a trademark of Becton Dickinson.



Guava Technologies®

USA & World

Guava Technologies, Inc.
25801 Industrial Blvd.
Hayward, CA 94545 USA
Tel: 866.448.2827
www.guavatechnologies.com
info@guavatechnologies.com

European Office

Guava Technologies
Guava House
Drope Rd.
St. Georges Super Ely
Cardiff CF5 6EP UK
Tel: +44 1446 760112
Fax: +44 1446 761015
info@guavatechnologies.com

© 2004 Guava Technologies Inc.
All rights reserved. Guava and
ViaCount are registered trade-
marks and PCA is a trademark of
Guava Technologies, Inc.