PHERAstar: A Next Generation Multimode Plate Reader for Sophisticated HTS Applications

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Introduction

High-Throughput Screening (HTS) is an approach to drug discovery that has gained widespread popularity over the last ten years. Due to the need to process thousands of assays per day, HTS has revolved around the technologies of multiple-well microplates and robotic processing. Cost reduction and increases in throughput are the primary driving force within most HTS groups, necessitating the change to higher-density, lower-volume microplates.

The PHERAstar, BMG LABTECH's new multimode microplate reader, combines the rapid plate reading necessary for HTS with enhanced performance and sensitivity needed to read small liquid volumes along with the flexibility to tackle the most demanding assays.

This new microplate reader is dedicated to reading all leading HTS detection modes (fluorescence intensity, time-resolved fluorescence, fluorescence polarization, luminescence and absorbance) in all formats up to 1536. The detection system is a lens based dual channel photomultiplier configuration that features simultaneous dual emission detection. This system offers minimal read times and allows ratiometric assays to be carried out at the highest possible level of performance.

The PHERAstar has been applied to a number of assays, including protein-protein binding, ligand-receptor binding, enzyme activity assays, molecular biological applications and second messenger quantification. The performance and features of the PHERAstar are presented in this poster with commercially available HTS kits such as HTRF®, AlphaScreen®, and PolarScreen™.

Materials and Methods

The following different HTS kits have been performed on the PHERAstar according to their particular application. The measurement techniques involved are time-resolved fluorescence, fluorescence polarization, and AlphaScreen® in various plate formats up to 1536-wells.

The time-resolved fluorescence mode was explored using the HTRF® reader control kit (#62RCLPEA, Cisbio, France) that is designed for the calibration of HTRF® compatible readers.[1] Results were obtained in black 96-well half area plates (#3694, Costar®, USA), 384-well small-volume (SV) plates (#784076, Greiner Bio-One, Germany) and 1536-well plates (#782076, Greiner Bio-One, Germany) according to the kit protocol. The incubation took place overnight at room temperature and the final assay volumes were 100 µL in the 96-well plate, 20 µL in the 384-well plate and 7 µL in the 1536-well plate. The kit is based on a Tumor Necrosis Factor alpha (TNFα) assay and may be used also for reader validations. Therefore, a comparison of the PHERAstar with the dedicated time-resolved fluorescence reader, the RUBYstar, was performed in 96-well and 384-well formats. On the flashlamp-based PHERAstar the assays were run with 200 or 400 flashes per well and on the laser-based RUBYstar the assays were performed with 20 flashes. The FRET donor europium cryptate was excited at 337 nm and emission was simultaneously read at 620 nm and 665 nm on both readers.

In fluorescence polarization mode, the Far Red PolarScreen™ assay (# PV3327, Invitrogen, USA) for Tyrosine Kinase (Csk) was used in low-volume 384 (LV384)-well plates (#3676, Corning®, USA) with a final assay volume of 20 µL according to the kit protocol.[2] The Far Red fluorophore was excited with 200 flashes at 610 nm and the emission was detected in both polarization planes simultaneously at 670 nm.

Two assays were performed in AlphaScreen® mode on the PHERAstar, the P-Tyr-100 (Phosphotyrosine) assay kit (#6760620, PerkinElmer, USA) and cAMP assay kit (#6760625, PerkinElmer, USA). All assays were performed in accordance with the kit protocols in white 384-well SV plates (#784075, Greiner Bio-One, Germany) with a final assay volume of either 17 µL or 25 µL.[3] The AlphaScreen® beads were excited with 400 flashes at 680 nm and the emission was detected simultaneously in the range of 520 nm to 620 nm. To avoid evaporation all plates were sealed with microplate sealers (#77400-05, BMG LABTECH, UK) during incubation or storage and this sealer was not removed during reading of the microplate.

Results and Discussion

Homogeneous time-resolved fluorescence (HTRF®) TNFα immunoassay:

Cisbio's HTRF® assays employ fluorescent Eu^{3+} cryptates (donor) and XL665 (acceptor) in homogeneous time-resolved FRET-based assays. Upon excitation, when the two entities come into close proximity, FRET can occur and XL665 re-emits a specific long-lived fluorescence at 665 nm, in addition to the donor emission at 620 nm.
Tumor Necrosis Factor alpha (TNFα), a 17 kDa cytokine, is an important mediator secreted by activated macrophages and monocytes with a large spectrum of antiviral immunoregulation, metabolic and inflammatory properties. This factor is cytotoxic for some tumor cell lines *in vitro* and causes the necrosis of certain tumors *in vivo*. TNFα acts via binding to specific cell surface receptors.
The HTRF® TNFα assay is a single step double-site immunometric assay involving two MABs conjugated either with europium cryptate or to XL665. The HTRF® TNFα assay principle is shown in figure 1.

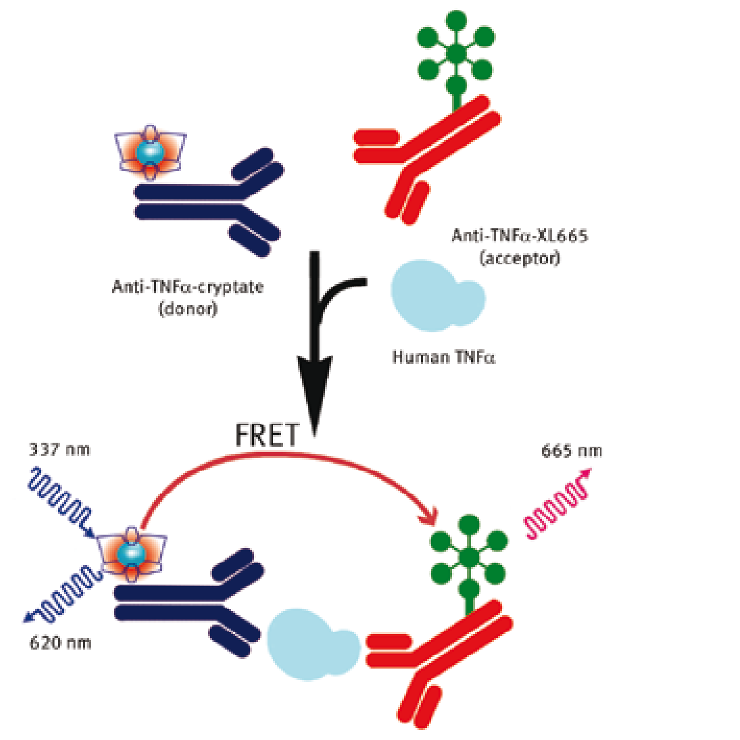

Fig. 1: Assay principle of the homogeneous time-resolved fluorescence immunoassay for TNFα. The assay is run using straightforward „mix & measure" detection.

Under its final configuration, free TNFα from calibrators or samples is sandwiched by mouse MAb IPM2-Eu cryptate (IPM2-K) and mouse MAb IPM3-XL665 (IPM3-XL665) conjugates. The FRET signal generated by the simultaneous binding of the two conjugates is proportional to the amount of TNFα present in the sample. Both 665 nm and 620 nm signals were measured simultaneously on the PHERAstar. Under routine use, the 665 nm / 620 nm fluorescence ratio (US patent 5,527,684) eliminates most interference from the medium.[4] For a direct comparison of the PHERAstar with the RUBYstar, the microplates were measured on both readers and the results are shown in figure 2.

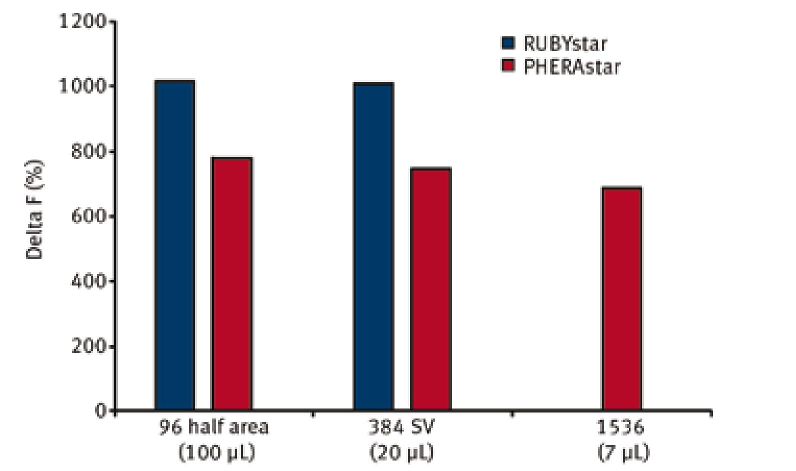

Fig. 2: Direct comparison of the PHERAstar and the RUBYstar in different plate formats using the HTRF® reader comparison kit (high calibrator, n = 8) based on a TNFα immunoassay.

Delta F is a value calculated from the 665 nm / 620 nm ratios which enables the data to be normalized with respect to between-assay variations. In addition, delta F is reader independent and can be used for indicating and comparing the quality of a reader.[5] A total of five PHERAstars and two RUBYstars were compared to obtain these results. When comparing the PHERAstar to the RUBYstar, the PHERAstar shows approximately 20% less signal than the RUBYstar in terms of delta F in 96- and 384-well plates. However, the %CVs and signal separation obtained with the PHERAstar still results in an excellent delta F value and good HTRF® performance.

The results from the multimode PHERAstar reader proved that HTRF® assay miniaturization (100 µL to 7 µL) in plate formats up to 1536, has no significant influence on the excellent reader sensitivity, dynamic range, or %CVs (2%). In addition, we have shown that the already acknowledged "HTRF® compatible" reader PHERAstar can produce high quality data for HTRF® technology assays and is comparable to the dedicated RUBYstar ("the gold standard" reader for HTRF® assays) in terms of sensitivity. Due to the fact that the RUBYstar is designed for plate formats up to 384-wells, a direct comparison in 1536-well format was not possible. However, the data shows that the PHERAstar is capable of producing excellent results for HTRF®, even in 1536-well mode, with no reduction in assay sensitivity.

Fluorescence Polarization Far Red PolarScreen™ Kinase Assay:

Invitrogen's new Far Red PolarScreen™ assays employ a proprietary Far Red fluorophore in homogeneous fluorescence polarization assays. The fluorophore is highly water soluble and, unlike cyanine-based fluorophores, has a fluorescence lifetime that allows for large polarization shifts between free and bound tracer.
Protein kinases (PKs) are a diverse group of enzymes involved in many areas of cell signalling. These include cell growth and proliferation and neural functions. The keen interest in PKs arises from their role in regulating biological mechanisms. Through phosphorylation, PKs participate in many cellular signal transduction processes. Furthermore, defects in these pathways have been implicated in numerous human diseases including cancer, inflammation and diabetes. Research focused on kinase activity could ultimately identify targets that can be used to develop new pharmaceutical agents to treat many of these diseases.

Fluorescence Polarization (FP) is a powerful tool with applications in kinase research: FP kinase assays are homogeneous and are amenable to miniaturization, and therefore quite useful in HTS applications. Because FP reports a molecule's tumbling rate, the polarization value relates directly to the molecular volume of the fluorescent molecule. An increase in molecular volume will slow a fluorescent molecule's tumbling rate and yield a high polarization value. Conversely, a decrease in molecular volume will increase the fluorescent molecule's tumbling rate and yield a lower polarization value.

In a Far Red FP kinase assay, kinase, substrate, and ATP are allowed to react in the presence of library compounds. After the reaction is complete, antibody and Far Red labelled tracer are added. The antibody can associate with either the labelled tracer (resulting in a high FP value) or the kinase-produced phosphorylated substrate (resulting in a lower FP value). The amount of antibody that binds to the tracer is inversely related to the amount of phosphorylated product present, and in this manner kinase activity can be detected and measured by a decrease in FP value. Thus, library compounds that inhibit the reaction are identified as wells that have a high polarization value.

To demonstrate the use of the PHERAstar with Invitrogen's PolarScreen™ Far Red kinase assay, a tyrosine kinase (Csk) titration curve (n = 3) was performed, in the concentration range of 0.5 pg/mL to 2 µg/mL Csk, as shown in figure 3. During the fluorescence polarization measurement the Far Red fluorophore was excited at 610 nm and the emission was detected at 670 nm in both polarization planes simultaneously.

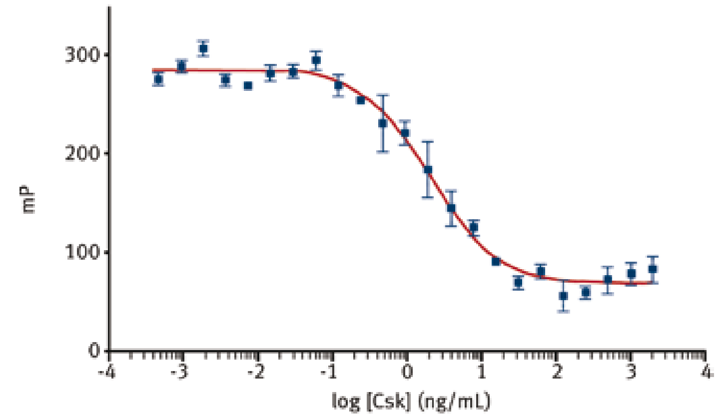

Fig.3: Tyrosine kinase (Csk) titration with the PolarScreen™ Far Red detection kit on the PHERAstar.

Through this specific PolarScreen™ FP Far Red kinase assay example, we have shown that the PHERAstar can produce high quality fluorescence polarization data amenable to screening efforts. Far Red kinase assays can be used for both high-throughput screening, as well as further investigation of potential leads to determine accurate EC_{50} values.

AlphaScreen® assays:

PerkinElmer's homogeneous time-resolved AlphaScreen® assays rely on the use of "Donor" and "Acceptor" beads. When a biomolecular interaction brings the beads into proximity, a cascade of chemical reactions is initiated to produce a greatly amplified luminescence signal in the range of 520 nm – 620 nm, well below the excitation wavelength of 680 nm and allowing good signal separation.

AlphaScreen® Phosphotyrosine (P-Tyr-100) assay – detection assay
The AlphaScreen® P-Tyr-100 assay is based on a sandwich assay principle. After tyrosine kinase phosphorylation, a biotinylated polypeptide substrate is sandwiched between streptavidin coated Donor beads and anti-phosphotyrosine antibody P-Tyr-100 conjugated Acceptor beads. Phosphorylation of the peptide by the tyrosine kinase results in an increase of the luminescence signal.

To demonstrate the functionality of the AlphaScreen® assays and the performance on the PHERAstar, a titration curve (n = 3) with biotinylated phosphorylated polypeptide (b-LCK-P) was performed as shown in figure 4.

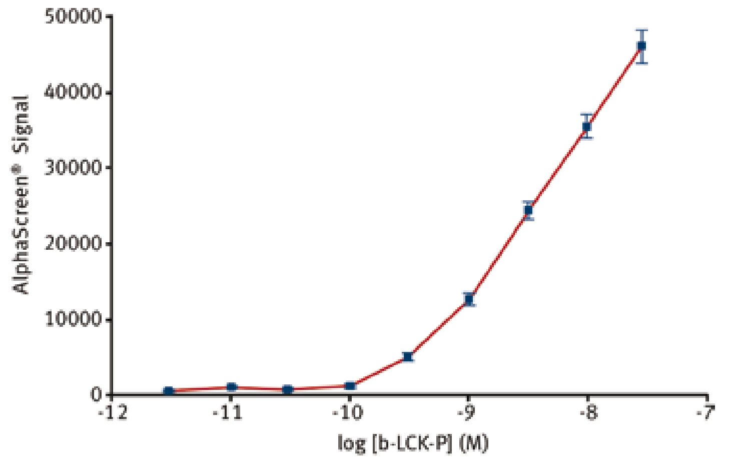

Fig. 4: A typical biotinylated-LCK-P AlphaScreen® titration curve on the PHERAstar.

Plates were read on the PHERAstar after 1 hour incubation at room temperature with an integration time of 0.5 second per well. The concentration of b-LCK-P was in the range of 3 pM to 30 nM and the final assay volume 17 µL per well. The results of the assay very closely corresponded to the titration curve published in the kit protocol.[3]

In order to show the well to well variation, the same assay has been performed with 48 replicates at a single b-LCK-P concentration (10 nM) and a control (without b-LCK-P) as shown in figure 5.

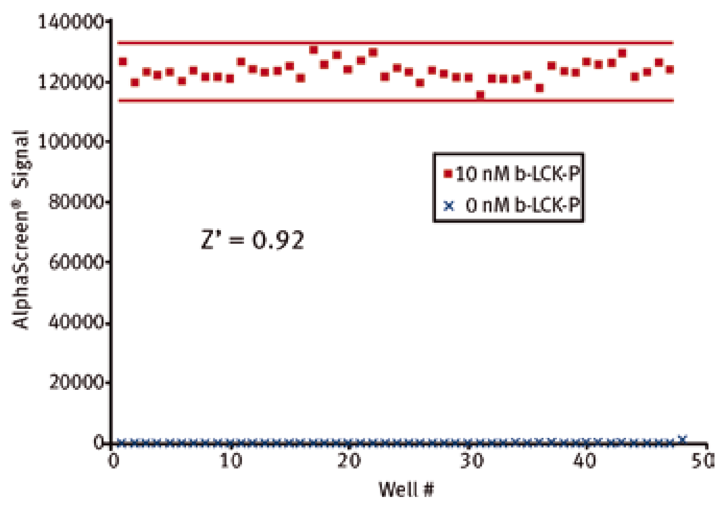

Fig. 5: A representative Z'-factor value of 0.92 was generated with 48 replicates in a 384-well format for the AlphaScreen® assay.

As a characteristic parameter for the quality of the assay, a Z'-factor value of 0.92 was calculated, which represents excellent assay performance in a 384-well SV plate with 17 µL final assay volume. Z'-factor scores between 0.5 and 1 indicate a highly robust screening assay and in addition reflect the high quality of the instrumentation.[6]

AlphaScreen® cAMP assay – competition assay
Cyclic AMP (cAMP) is one of the most important intracellular mediators. Its concentration in cells may be regulated upon binding of many hormones to their receptor. In many instances of G-protein-coupled receptor (GPCR) activation, a major class of target in the drug discovery process, adenylate cyclase is stimulated to convert ATP to cAMP. Cyclic AMP is then involved in regulatory processes such as protein kinase activation or ion channel gating. The determination of cAMP is therefore an essential tool for monitoring both agonist and antagonist activities on GPCRs.
Detection of cAMP with AlphaScreen® is based on the competition between cAMP produced by cells and a biotinylated cAMP probe that is sandwiched by streptavidin-Donor and anti-cAMP antibody conjugated Acceptor beads. The beads are brought into proximity and a signal is detected. A decrease in signal is observed with an increase in intracellular cAMP produced. In the absence of intracellular cAMP a maximum signal is detected.

To demonstrate the functionality of the AlphaScreen® assays and the performance of the PHERAstar, a titration curve (n = 3) with cAMP was performed as shown in figure 6.

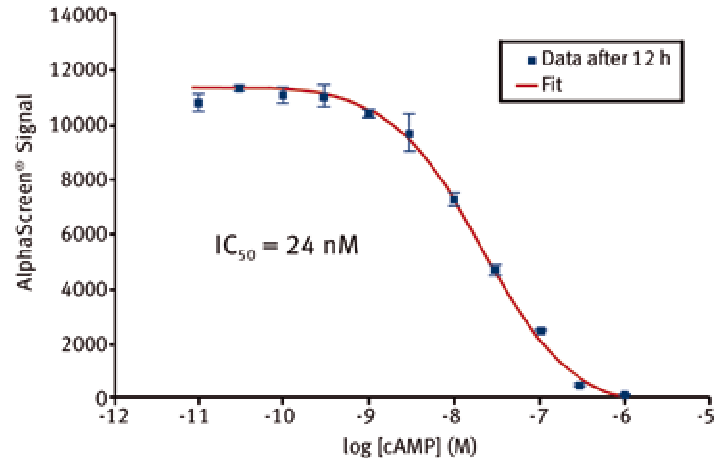

Fig. 6: A typical cAMP AlphaScreen® titration curve on the PHERAstar.

Final assay volume was 25 µL per well in a small volume 384-well plate and after 12 hours incubation at room temperature the plate was read on PHERAstar using an integration time of 0.5 second per well. The concentration of cAMP was in the range of 1 µM to 10 pM and the cAMP titration curve reveals after 12 hours incubation a high S/B = 82 value. The BMG LABTECH's evaluation software, including a 4-parameter-fit function, was used for curve fit and IC_{50} determination. The calculated IC_{50} = 24 nM value complies with the value reported in the literature.[7]

Through these experimental evaluations we have shown that the PHERAstar can produce high quality data for the AlphaScreen® assay technology, more importantly, these results have shown that laser-based instruments are not the only instruments capable of reading AlphaScreen® with results suitable for screening. In fact, while performing these evaluations we discovered that laser-based instruments "bleach" the beads during a single read making rapid re-readings of questionable results impossible. With the PHERAstar it is possible to re-read a plate several times without loss in absolute signal or sensitivity as shown in figure 7.

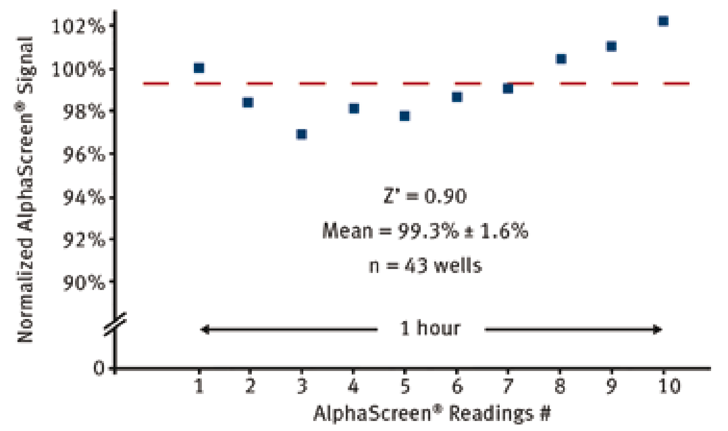

Fig. 7: Even after ten readings of the same AlphaScreen® plate on the PHERAstar, absolute signal and sensitivity remain constant. There is no "bleaching" effect detectable.

Ten consecutive PHERAstar measurements were performed within one hour in a 384-well SV plate containing 43 replicates of the assay. The signal of the first AlphaScreen® measurement was normalized to 100% and the following nine readings show a constant signal with a mean value of 99.3% ± 1.6%. The robustness of the data is indicated by a Z'-factor value of 0.90.

Conclusion

The discovery of new leads through HTS is based on the ability to precisely measure biomolecular interactions and find successful detection strategies that are compatible with miniaturized HTS. Furthermore, the application of the time-resolved fluorescence mode in HTRF® assays, the shift to "red" fluorophores in PolarScreen™ FP assays, and the use of AlphaScreen® assays have been recognized as useful strategies to overcome interference due to autofluorescence, light scatter due to precipitated compounds, or inherent detection mode noise.
The PHERAstar multimode reader has a wide range of possible applications for HTS needs.
As shown in time-resolved fluorescence mode, the "HTRF® compatible" PHERAstar allows straightforward assay miniaturization to plate formats up to 1536 with no influence on the excellent sensitivity of the reader. This reduces the consumption of expensive reagents and provides significant cost savings.
The PHERAstar also shows great performance in fluorescence polarization mode as demonstrated with the new homogeneous Far Red PolarScreen™ assay for tyrosine kinase in 384-well SV plate format. In AlphaScreen® mode the tyrosine kinase and the cAMP assay were tested with the PHERAstar resulting in excellent performance and read times in 384-well SV plate format.

The criteria used in selecting an HTS instrument should be cost, sensitivity, speed, flexibility, ease of use, and reliability. The PHERAstar (figure 8) fulfils all these criteria and the results in this poster prove that the PHERAstar is a robust and versatile multimode reader with excellent performance for HTS needs.

Fig.8: The new multimode microplate reader PHERAstar is optimized to perform HTS assays in plate formats up to 1536-wells.

References:

1) HTRF® Reader Control Kit Protocol #62RCLPEA, Cisbio, France.
2) PolarScreen™ Tyrosine Kinase Assay Kit, Far Red Protocol #PV3327, Invitrogen, USA.
3) AlphaScreen® Phosphotyrosine (P-Tyr-100) Assay Kit Protocol #6760620 and cAMP Assay Kit Protocol # 6760625, PerkinElmer, USA.
4) Degorce, F. *et al.*, HTRF® TNFα Kit Application Note 3, Cisbio, France.
5) Liu, J. *et al.*, (2004) *J. Biol. Chem.* **279**, 15824-30.
6) Zhang, J. *et al.* (1999) *J. Biomol. Screen.* **4**. 67-73.
7) Illy, C. *et al.*, Sci. Poster (#P01047), SBS 9th Annual Conference, (2003).

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