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# A Comparison of ALPHAScreen, TR-FRET, and TRF as Assay Methods for FXR Nuclear Receptors

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## ABSTRACT

New developments in detection technologies are providing a variety of biomolecular screening strategies from which to choose. Consequently, we performed a detailed analysis of both separation-based and non-separation-based formats for screening nuclear receptor ligands. In this study, time-resolved fluorescence resonance energy transfer (TR-FRET), ALPHAScreen, and time-resolved fluorescence (TRF) assays were optimized and compared with respect to sensitivity, reproducibility, and miniaturization capability. The results showed that the ALPHAScreen system had the best sensitivity and dynamic range. The TRF assay was more time consuming because of the number of wash steps necessary. The TR-FRET assay had less interwell variation, most likely because of ratiometric measurement. Both the ALPHAScreen and the TR-FRET assays were miniaturized to 8- $\mu$ l volumes. Of the photomultiplier tube-based readers, the ALPHAScreen reader (ALPHAQuest) presented the advantage of faster reading times through simultaneous reading with four photomultiplier tubes.

## INTRODUCTION

THE NUCLEAR BILE ACID receptor FXR (farnesoid X receptor) is a key regulator of cholesterol homeostasis. FXR serves as a molecular sensor for cholesterol metabolites; it binds to DNA and regulates the transcription of genes involved in the metabolism and transport of cholesterol, thus controlling the balance of lipids essential for health.<sup>1</sup> Previous reports have studied these receptors using a homogeneous time-resolved fluorescence assay (commercially available as LANCE™ [Wallac Oy, Turku, Finland])<sup>1,2</sup> based on the principles of time-resolved fluorescence resonance energy transfer (TR-FRET), first described in 1988 by Morrison.<sup>3</sup> In a version of the standard TR-FRET assay, bile acid receptors were measured by the association of coactivator-derived peptide with the receptor. This interaction is stimulated in the presence of ligands, such as chenodeoxycholic acid (CDCA). When excited at a wavelength of 320 nm, the association of a europium chelate-labeled receptor with an allophycocyanin (APC)-labeled peptide results in transfer of energy to APC, leading to maximal emission at a wavelength of 650 nm. This energy transfer system is time-gated in order to reduce short-lived fluorescent background.<sup>1,2</sup> Other prior versions of the FXR assay include an enzyme-linked im-

munosorbent assay, which measures binding of the receptor to a biotinylated coactivator peptide immobilized on a microplate.<sup>1</sup>

More recently, ALPHAScreen technology, first described in 1994 by Ullman and based on the principle of luminescent oxygen channeling,<sup>4,5</sup> has become commercially available. ALPHAScreen is a bead-based, nonradioactive *amplified luminescent proximity homogeneous assay*. In this assay, a donor and an acceptor pair of 250-nm-diameter reagent-coated polystyrene microbeads are brought into proximity by a biomolecular interaction of binding partners immobilized to these beads. Excitation of the assay mixture with a high-intensity laser at 680 nm induces the formation of singlet oxygen at the surface of the donor bead, following conversion of ambient oxygen to a more excited singlet state by a photosensitizer present in the donor bead. The singlet oxygen molecules can diffuse up to 200 nm, and, if an acceptor bead is in proximity, can react with a thioxene derivative present in this bead, generating chemiluminescence at 370 nm that further activates the fluorophores contained in the same bead. The fluorophores subsequently emit light at 520–620 nm.<sup>5–7</sup> The donor bead generates about 60,000 singlet oxygen molecules, resulting in an amplified signal. Because the signal is very long lived, with a half-life in the second range, the detection system can be time-gated, thus elimi-

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<sup>2</sup>BioSignal-Packard, Montreal, Quebec, Canada.

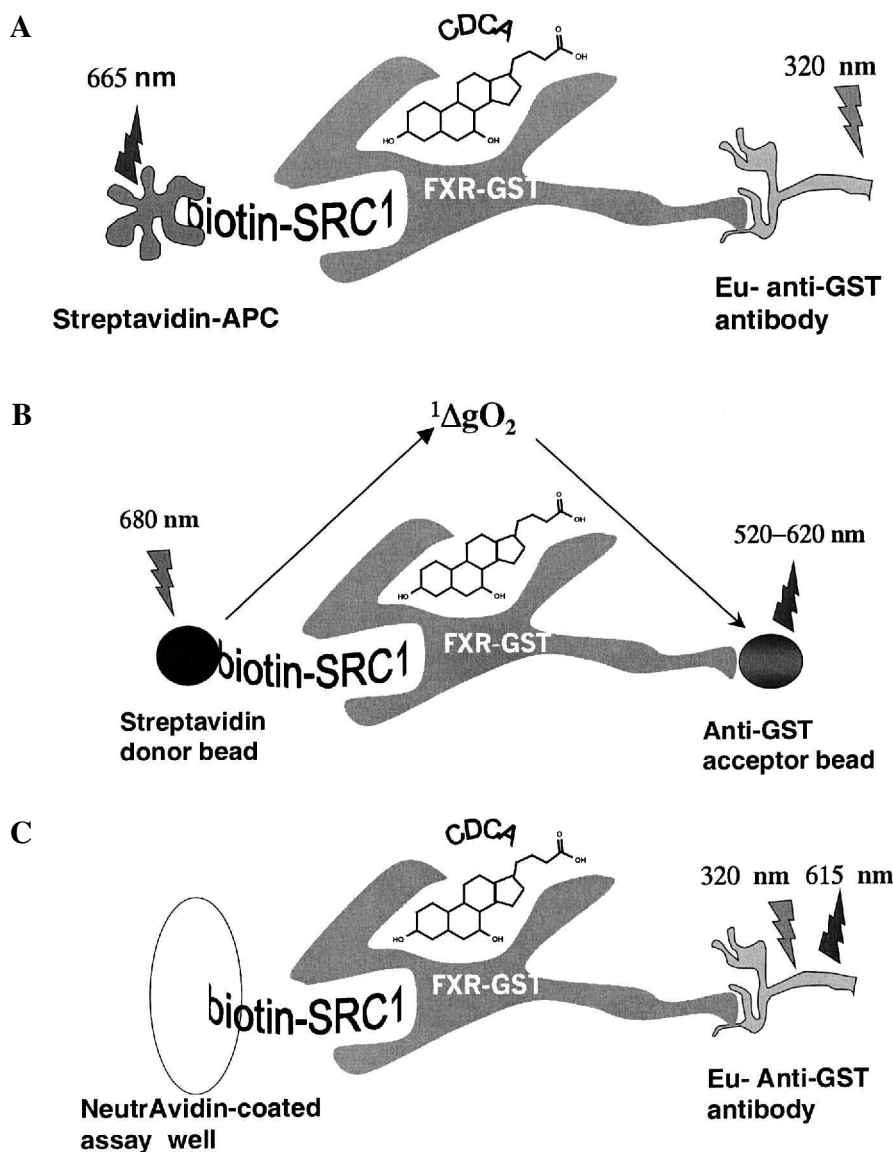
nating short-lived background (the ALPHAScreen signal is measured with a delay between illumination and detection of 20 ms). Furthermore, the detection wavelength is shorter than the excitation wavelength, thus further reducing the potential for fluorescence interference. The sensitivity of the assay derives from the very low background fluorescence.<sup>5</sup> The larger diffusion distance of the singlet oxygen enables the detection of binding distance up to 200 nm, whereas TR-FRET is limited to 9 nm.<sup>8</sup>

New approaches in the field of molecular genetics for identifying drug targets, along with improved methods for synthesis of chemical libraries, have created a need for increased sensitivity and throughput in screening campaigns. In the field of

HTS, there are often various choices available for measuring a particular biomolecular interaction, and the trend has been toward homogeneous assays in which separation steps are eliminated. In this report, we have used FXR as a model system for comparison of a TR-FRET format, a microplate binding format using time-resolved fluorescence (TRF), and a newer format based on ALPHAScreen.

## MATERIALS AND METHODS

APC-labeled streptavidin was from Prozyme (San Leandro, CA) (Phycolink Streptavidin APC, PJ25S, lot 896015, 2.9



**FIG. 1.** Schematic diagram of FXR assay formats. The bile acid CDCA is needed to induce a complex between the FXR–GST and the coactivator-derived peptide SRC-1. (A) TR-FRET format, where an energy transfer between europium chelate and allophycocyanin (APC) occurs. (B) ALPHAScreen format, where the excitation of a donor bead at 680 nm produces singlet oxygen, thus diffusing to an acceptor bead and undergoing a chemiluminescent reaction. (C) TRF-based plate-binding assay. A binding reaction occurs in a plate coated with NeutrAvidin. The complex is captured via a biotin–NeutrAvidin interaction, the plate is washed to separate unbound reagents, and GST is detected with a europium chelate–labeled antibody, the europium being released and detected by the addition of an enhancement solution.

mg/ml); LANCE Eu-W1024-labeled anti-glutathione-*S*-transferase (GST) antibody, and DELFIA® Eu-N1-labeled anti-GST antibody were purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA). The oxysterol agonists and antagonists CDCA and lithocholate (LCA) were purchased from Sigma Chemical Co. (St. Louis, MO). Streptavidin-coated ALPHAScreen donor beads and anti-GST acceptor beads were provided by BioSignal-Packard (Montreal). NeutrAvidin™ was purchased from Pierce Chemical Company (Rockford, IL).

The rat FXR ligand binding domain was expressed in *Escherichia coli* as a GST fusion protein. Briefly, recombinant strain DH5 $\alpha$  transformed with pGEX4T rat FXR (amino acids 215 through 469) vector was grown in 1.5 L of Luria's broth to O.D.<sub>600</sub> = 0.5 and induced with 0.8 mM isopropyl-thiogalactoside (GIBCO, Bethesda, MD). The cells were harvested by centrifugation and lysed in 12 ml of 20 mM Tris (pH 7.4), 150 mM NaCl, and 0.1% Triton X-100 (containing 2 mg/ml lysozyme and 1 protease inhibitor tablet [Roche Diagnostics Corporation, Indianapolis, IN]) by two cycles of freeze-thawing in liquid nitrogen. The homogenates were then pelleted, and the supernatants were purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ) affinity chromatography and dialyzed against triethanolamine-buffered saline (pH 8.0) to which glycerol was added to 10%, yielding a 10  $\mu$ M solution with 95% purity as analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein concentrations were determined by Bradford assays using bovine serum albumin (BSA) and rabbit immunoglobulin G as a standard.

A 26-amino-acid biotinylated peptide derived from the coactivator SRC1 (a coactivator of FXR)<sup>2</sup> was synthesized using standard methods and purified by high-performance liquid chromatography. The stock solutions were stored at 5 mM in Tris ethylenediaminetetraacetic acid (pH 8.0) with 1 mM dithiothreitol (DTT) at  $-80^{\circ}\text{C}$ .

All assays were performed in an aqueous assay buffer of 50 mM Tris (pH 7.4), 50 mM KCl, 1.0 mM DTT, and 0.1% BSA. The 384-well assays were performed in 30- $\mu$ l volumes in Costar® (Corning Incorporated, Acton, MA) 384-well black polystyrene assay plates for TR-FRET (model 3710), NUNC™ (Nalge Nunc International, Naperville, IL) white assay plates for ALPHAScreen (model 264572), or NUNC MaxiSorp™ 384-well black polystyrene plates for TRF (model 460518). The 1536-well assays were performed in 8- $\mu$ l volumes in Greiner

(Greiner America, Inc., Lake Mary, FL) black polystyrene plates for TR-FRET (model 782072) and Greiner white polystyrene plates for ALPHAScreen (model 782075).

### Liquid dispensing

For 384-well assays, the assay reagents were added using a Multidrop 384™ (LabSystems Oy, Helsinki, Finland) reagent dispenser. The 1536-well assays were dispensed using the PlateMate™ Plus automated liquid dispenser (Matrix Technologies, Hudson, NH). Dose–response curves were generated by hand pipetting into 384-well plates and, where indicated, transferring to 1536-well assay plates with standard gel-loading pipet tips.

### TR-FRET assay

A 15- $\mu$ l solution of CDCA in assay buffer was added to a 15- $\mu$ l mixture of LANCE Eu-W1024-labeled anti-GST antibody, GST–FXR, biotin–SRC1, and streptavidin–APC. The incubation times and reagent concentrations are presented in the figure legends. Compounds or test reagents were added first as 3  $\mu$ l of a 10  $\times$  stock solution in assay buffer and allowed to preincubate for 30 min with the binding mixture prior to the addition of CDCA. This preincubation step, although unnecessary, was included to allow the inhibitors a longer period to bind to the receptor than the CDCA. The 1536-well, 8- $\mu$ l assays were performed by proportional scale-down of assay volumes from the 384-well, 30- $\mu$ l format.

The 384-well assays were read in a Victor<sup>2</sup>™ model 1420 (Wallac Oy) optical microplate reader. Two readings per well were taken with instrument settings as follows:

- Reading 1 (for time-gated energy transfer from europium to APC), 320-nm excitation filter 7.5-nm bandwidth, 650-nm emission with 7.5-nm bandwidth, counting delay of 75  $\mu$ s, counting window of 100  $\mu$ s
- Reading 2 (for europium time-gated fluorescence), 320-nm excitation filter 10-nm bandwidth, 615-nm emission filter 10-nm bandwidth, counting delay of 400  $\mu$ s, counting window of 400  $\mu$ s

For both readings, the flash energy was 175 units, the light integration capacitor was set at 1, the light integration reference

TABLE 1. OPTIMIZATION OF FXR ASSAY DETECTOR REAGENTS IN 384-WELL FORMAT<sup>a</sup>

Assay type	Assay reagent	Tested concentration range	S:B ratio	Fixed as optimal	S:B at optimal
TRF	NeutrAvidin	0.1–10 $\mu$ g/ml	0.4–14	2.5 $\mu$ g/ml	14
	Eu-anti-GST antibody	0.1–5 nM			
TR-FRET	Streptavidin–allophycocyanin	0.1–10 nM	2.2–17.2	2 nM	17.2
	Eu-anti-GST antibody	0.05–5 nM	2.2–17.2	1 nM	17.2
ALPHAScreen	Streptavidin donor beads	2–20 $\mu$ g/ml	20–170	2 $\mu$ g/ml <sup>b</sup>	170
	Anti-GST antibody acceptor beads	2–20 $\mu$ g/ml	20–170	10 $\mu$ g/ml <sup>b</sup>	170

<sup>a</sup>A range of concentrations of each detector reagent was tested and the signal:background (S:B) ratio was defined in the absence or presence of 50  $\mu$ M CDCA ( $K_d \approx 30$  M). Initial conditions for the TR-FRET and AlphaScreen were 10 M SRC1–biotin and 1 nM FXR–GST. For TRF assays, the FXR–GST was 1 nM and the SRC1–biotin was coated at 25 nM. The average value of triplicates is presented.

<sup>b</sup>A total of 20  $\mu$ g/ml of beads is coupled to approximately 20 nM anti-GST antibody or 20 nM streptavidin.

level was 151, the aperture was normal, the beam size was normal, and the counting cycle was set at 1000 ms.

The 1536-well assays were read in a Tecan Ultra optical microplate reader (Tecan Inc., Research Triangle Park, NC). Two readings per well were taken with instrument settings as follows:

- Reading 1 (for time-gated energy transfer from europium to APC), 340-nm excitation with 35-nm bandwidth (different than Victor filter because of commercial availability), 670-nm emission filter with 25-nm bandwidth, counting delay of 75  $\mu$ s, counting window of 100  $\mu$ s
- Reading 2 (for europium time-gated fluorescence), 340-nm excitation filter with 35-nm bandwidth, 612-nm emission filter with 10-nm bandwidth, counting delay of 400  $\mu$ s, counting window of 400  $\mu$ s

For both readings, the gain was set for automatic optimization, the Z height settings were automatic, and there were 10 flashes per reading cycle. The results were expressed as ratio of (APC counts/europium counts)  $\times$  1000.

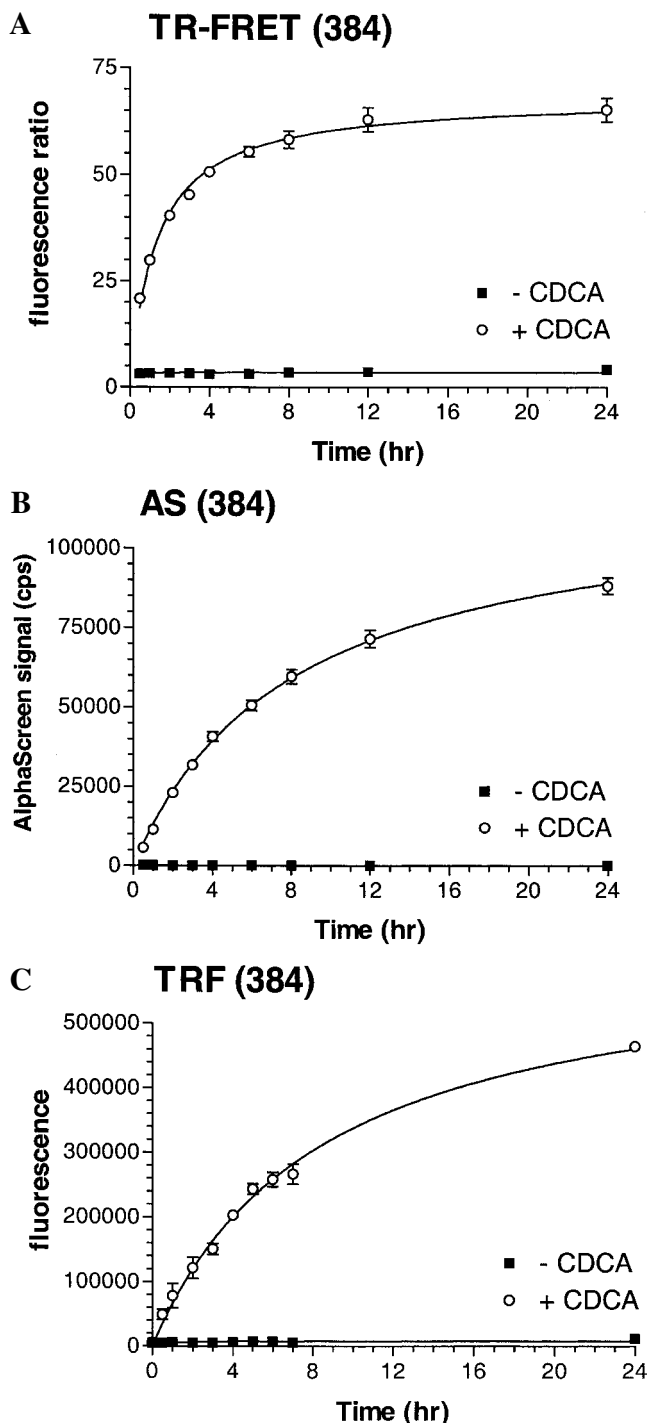
#### ALPHAScreen assay

Assays (25  $\mu$ l) were performed under the same conditions as the TR-FRET assay with the following exceptions:

1. Assays were performed under subdued lighting (high levels of ambient light can increase nonspecific chemiluminescence).
2. Anti-GST acceptor beads and streptavidin donor beads were used instead of anti-GST Eu and streptavidin-APC.
3. The acceptor beads were added to the assay with the FXR-GST and the SRC1-biotin.
4. The donor beads were added with the CDCA.
5. The assay plates were read on an ALPHAQuest™ model  $\alpha$ Q optical plate reader (Packard Biosciences) set at 1 s/well.

#### TRF plate-binding assay

NUNC Maxisorp 384-well black plates (model 460518) were coated with 30  $\mu$ l of various concentrations of NeutrAvidin in 50 mM bicarbonate (pH 9.6), 150 mM NaCl, and 0.02 mg/mL NaN<sub>3</sub>. The assay plates were incubated at 4°C overnight. Coating solution was removed by manual inversion and shaking, and plates were incubated with 50  $\mu$ l of a blocking solution of 4% BSA in phosphate-buffered saline for 2 h at room temperature. This solution was removed by three washes in 50 mM TRIS-buffered saline with 0.05% Tween 20 (pH 8.0) (TBST). The washes were performed using a Skatron Embla 384-well plate washer (Molecular Devices, Sunnyvale, CA) with a 100- $\mu$ l dispense volume, 5-s soak period, and 1-s aspirate time. Test compounds (3  $\mu$ l) or controls were then added prior to the addition of various concentrations of SRC1-biotin peptide and various concentrations of GST-FXR in 20  $\mu$ l of assay buffer (these parameters were optimized by varying both peptide and protein reagents). The mixture was incubated for 30 min at room temperature (this incubation was not necessary, but was included to allow the inhibitors a longer period to bind to the receptor than the CDCA), and then supplemented with a solution of CDCA (10  $\mu$ l) in assay buffer.



**FIG. 2.** Time course of binding reaction. All assays (triplicate) were performed at optimal concentration of detector reagents (see Table 1). Aliquots of 50  $\mu$ M CDCA were added at time zero to a reaction buffer containing 10 nM SRC1-biotin and 1 nM FXR-GST for TR-FRET and ALPHAScreen (AS). For TRF, the plates were coated with 100 nM SRC1-biotin and the [FXR-GST] was 0.5 nM. (A) TR-FRET assay. (B) ALPHAScreen assay. (C) TRF plate-binding assay.



The plates were incubated for 2 h at room temperature. Then 10  $\mu$ l/well of a 1 nM DELFIA Eu-N1-labeled anti-GST antibody was added and the solution was incubated for 1 h at room temperature and washed 3 times in TBST as described above. Enhancement solution (30  $\mu$ l of Wallac solution #1244-105) was added and mixed, and reading was performed in a Victor<sup>2</sup> optical microplate reader set at 340-nm excitation and 615-nm emission with a 400- $\mu$ s delay time and 400- $\mu$ s counting time.

Curve fitting was done with nonlinear regression according to a one-site binding model using Prism<sup>®</sup> software (GraphPad Software, Inc., San Diego, CA). Z values were calculated according to Zhang et al.<sup>9</sup>

## RESULTS

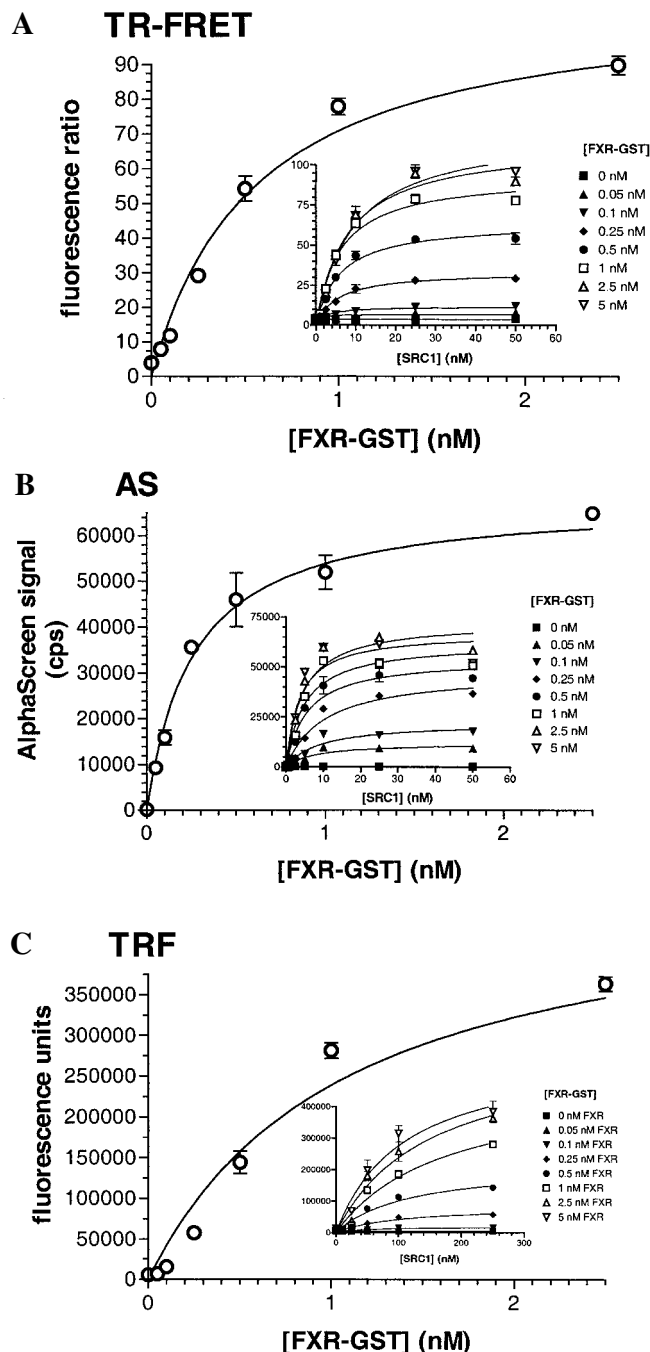
A high-throughput assay for measuring antagonists or agonists of rat FXR was optimized in three different formats—TR-FRET, TRF plate-binding, and ALPHAScreen—which varied only in the detection reagents and optical plate-reading system used. These assay schemes are illustrated in Figure 1. The similarity of protein and peptide reagents in the three formats permitted a comparison of assay performance with regard to sensitivity, reproducibility, and reagent requirements. Dose-response curves, reagent titrations, binding kinetics, and assay performance in 384-well and 1536-well plates were examined.

Assay optimization is a complex process because of the number of variables one must work with. We sought to optimize each assay independently in order to achieve the best possible sensitivity, reproducibility, and dynamic range. In order to determine suitable concentrations of detector reagents for a comparison, the detector reagents were varied, with fixed concentrations of the SRC1-biotin peptide and FXR-GST, both in the absence or presence of the agonist, CDCA. The concentrations were set at the point where the ratio between the CDCA-stimulated and the unstimulated signal was greatest. The results for 30- $\mu$ l assays are presented in Table 1. Generally, detector reagents were optimized to the nanomolar range, roughly equivalent to the concentration of SRC1-biotin and FXR-GST in the assay. Throughout this study, we used these concentrations of detector reagents in both 384-well and 1536-well formats.

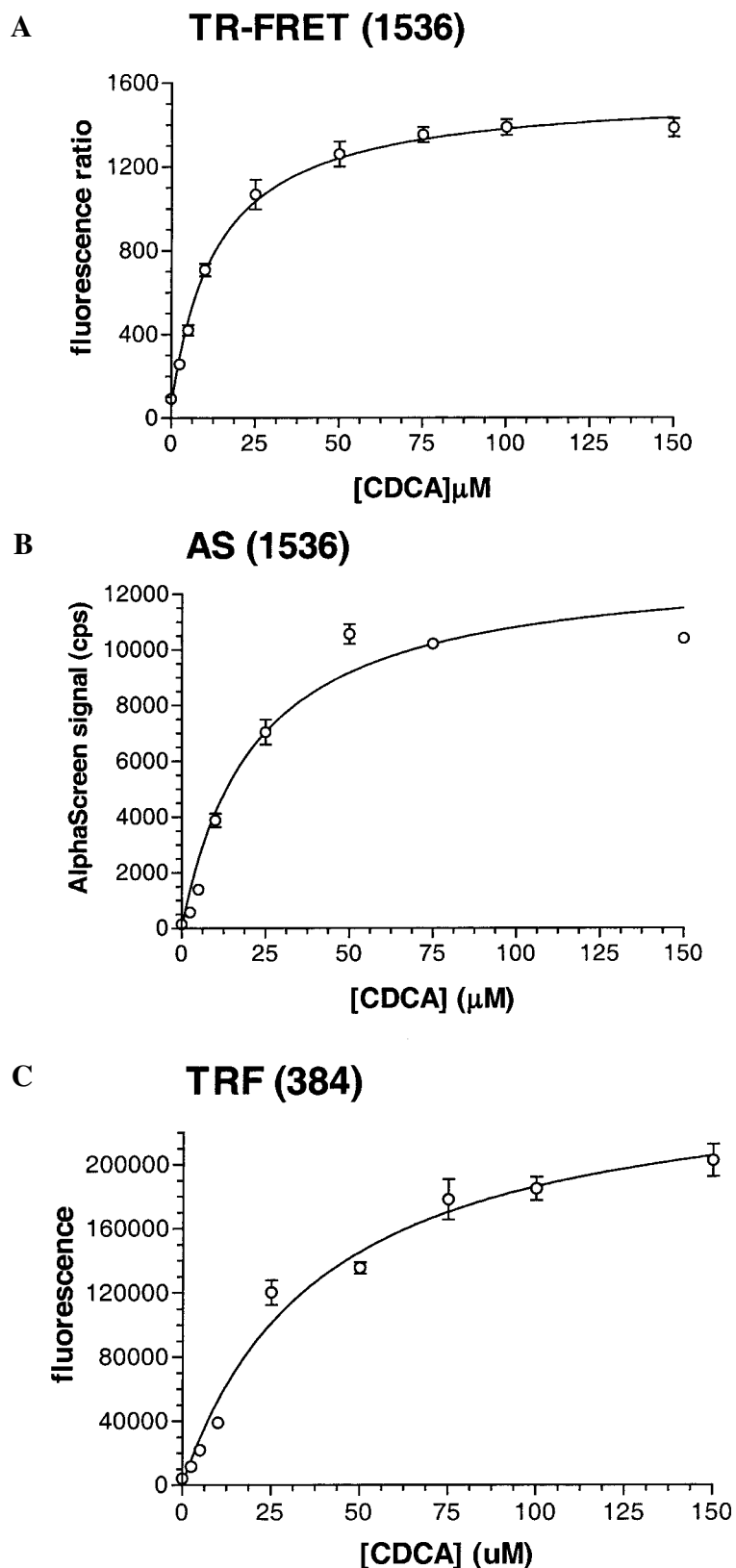
The kinetics of complex formation was measured, and is shown in Figure 2. The TRF and ALPHAScreen assays had a slower rate of complex formation than the TR-FRET assays. This phenomenon might have been due to the protein and peptide reagents being coupled to a solid phase, thus resulting in slower effective diffusion, or steric effects. The screening assay was run generally within 3 h without a quencher because, over the course of the plate-reading period (2–8 min in 384-well plates), there was little intrawell variation. Generally, the reading window, as defined by the ratio between CDCA-stimulated and unstimulated signal, was greatest for the ALPHAScreen. For example, the results from Figure 2 show that, even at a minimal time of 30 min, this ratio for ALPHAScreen was 30, for TR-FRET it was 7, and for TRF it was 9.

In order to compare the sensitivity and dynamic range of the three assays with respect to biotin-SRC1 and FXR-GST, these reagents were varied at a fixed concentration of detec-

tor reagents and CDCA. The results are shown in Figure 3. Of the three assays, ALPHAScreen had the greatest sensitivity and dynamic range (Fig. 3B). For example, at saturating concentrations of SRC1-biotin, the ALPHAScreen assay had



**FIG. 3.** SRC1-biotin and FXR-GST dose-response curves. Quadruplicate assays in 30- $\mu$ l volumes were measured in 384-well plates containing 50  $\mu$ M CDCA, varied concentrations of biotin-SRC1, and varied concentrations of FXR-GST. The detector reagents were fixed as shown in Table 1 and the binding reaction time was 2.5 h. (A) TR-FRET assay. (B) ALPHAScreen assay. (C) TRF plate-binding assay.



**FIG. 4.** CDCA dose-response curves for assays in 1536-well Greiner plates. **(A)** TR-FRET format: quadruplicate assays in 8- $\mu$ l volumes contained 25 nM biotin-SRC1 and 2.5 nM FXR-GST.  $K_{d(app)} = 13 \mu\text{M}$ . **(B)** ALPHAScreen format: quadruplicate assays in 8- $\mu$ l volumes contained 25 nM biotin-SRC1 and 2.5 nM FXR-GST.  $K_{d(app)} = 21 \mu\text{M}$ . **(C)** TRF plate-binding assay in 40- $\mu$ l volumes (384-well format). [FXR-GST] = 0.5 nM. Plate coated with 100 nM SRC1-biotin.  $K_{d(app)} = 30\text{--}47 \mu\text{M}$ .

an incremental increase in response to FXR–GST between 0.05 and 2.5 nM FXR–GST. With TR-FRET, this incremental response ranged between 0.1 and 2.5 nM, and with TRF, this range was between 0.25 and 2.5 nM. The TRF plate-binding assay was the least sensitive of the assays (Fig. 3C), and a much larger amount of the peptide, SRC1–biotin, was required in order to coat the microplate. Also, the micromolar affinity of CDCA most likely resulted in much of the complex being removed from the plate during the wash step. For the TRF assay, the use of streptavidin instead of NeutrAvidin resulted in significant increase in nonspecific binding (data not shown).

The TR-FRET assay showed an acceptable dynamic range and generally showed very low standard deviations (Fig. 3A), probably because of the ratiometric measurement, which would normalize for pipetting error. However, at 0.05 nM, FXR–GST was undetectable in the TR-FRET assay while still yielding a 10:1 reading window in the ALPHAScreen format. Titrating down the detector reagents below 1 nM in the TR-FRET assay did not significantly increase the sensitivity.

All three assay formats produced a dose-dependent response to the agonist CDCA (Fig. 4). The  $EC_{50}$  concentrations ranged from 13 to 34  $\mu$ M, which is consistent with previously reported values of 10–20  $\mu$ M<sup>1</sup> and 4.5  $\mu$ M.<sup>2</sup> In addition, both the TR-FRET and ALPHAScreen performed well in 8- $\mu$ l volumes, read in 1536-well Greiner plates (Fig. 4A and 4B). The extremely large reading window (signal:background ratio up to 70) in the ALPHAScreen format should provide enough dynamic range for further miniaturization with the appropriate liquid handling apparatus.

Table 2 presents a summary of our results with the three assay formats. All three assays performed generally well in the 384-well format, with measurements of dimethylsulfoxide (DMSO) sensitivity (the point at which DMSO had a statistically detectable effect on activity) being acceptable, with typical screening concentrations being less than 1%. The TRF as-

say was less sensitive to the known inhibitor lithocholic acid because of an unusual CDCA-independent activation effect at the 10  $\mu$ M range (data not shown). We do not as yet have an explanation for this, but it is possible that this phenomenon is due to the amphipathic nature of lithocholic acid. We did not pursue adapting the TRF assay to a 1536-well format, because of the necessity of washing steps. The minimal levels of detection were measured by titrating down the SRC1–biotin and FXR–GST to the point where the CDCA-stimulated signal was no longer significantly above the unstimulated signal in quadruplicate values. In the ALPHAScreen assay, we were able to detect at least a 2-fold signal:background ratio even at the lowest concentrations of FXR–GST (0.05 nM) and SRC1–biotin (0.1 nM) tested. At these concentrations, the TRF and TR-FRET assays were unable to detect any CDCA-dependent signal.

## DISCUSSION

The ability to measure precisely a biomolecular interaction is central for the discovery of new leads through HTS. The technologies for accomplishing this have become increasingly diverse to the point that we now have many choices. Among the various criteria used in making these decisions are cost, sensitivity, speed, ease, and reliability. The data presented herein suggest that the sensitivity, dynamic range, and plate reading time of ALPHAScreen will be useful for reducing screening time, easing the optimization process, and facilitating miniaturization of nuclear receptor–coactivator assays.

Mix-and-read assays are often preferred in HTS campaigns because wash assays are time consuming. Generally, the TRF plate-binding assay did not perform as well for the measurement of FXR ligands. Its limitations were in the measurement of the inhibitor, LCA, and the many dispensing and wash steps.

The TR-FRET format is simpler with respect to the number

TABLE 2. COMPARISON OF ASSAY PARAMETERS FOR ALPHASCREEN, TR-FRET, AND TRF<sup>a</sup>

	ALPHAScreen	TR-FRET	TRF
$EC_{50}$ ( $\mu$ M) chenodeoxycholic acid ( $n = 3$ )	21 $\pm$ 2	13 $\pm$ 3	43 $\pm$ 4
$IC_{50}$ ( $\mu$ M) lithocholic acid ( $n = 3$ )	73	77	121 <sup>b</sup>
Signal:background 384 (1536) <sup>c</sup>	350 (70) <sup>d</sup>	22 (14) <sup>d</sup>	16 <sup>e</sup>
%CV, 384 (1536) <sup>c,f</sup>	5.2 (6.5)	2.1 (3.8)	8.9
$Z'$ , 384 (1536) <sup>c,f</sup>	0.8 (0.7)	0.9 (0.8)	0.6
Plate read time (min), 384 (1536)	2.3 (9.2) <sup>g</sup>	13.5 (54)	8
Sensitivity to DMSO (maximum %)	3–4	7	5
Number of dispensing (washing) steps	3 (0)	3 (0)	7 (3)
Theoretical proximity limits	200 nm	7 nm	NA
Minimal level of detection FXR	<0.05 nM	0.1–0.25 nM	0.25 nM
Minimal level of detection SRC1	<0.1 nM	1 nM	~12 nM

<sup>a</sup>Summary of TRF, TR-FRET, and ALPHAScreen assay performance for the measurement of FXR–GST ligands.  $Z'$  values and %CV values were calculated from at least three full plate dispenses as described in the *Materials and Methods* section. The sensitivity to DMSO was determined in quadruplicate values and was the DMSO concentration that decreased the signal to a level below 1 standard deviation from the mean of the 0% DMSO control.

<sup>b</sup>A slight CDCA-independent activation was observed, which was subtracted from the final  $IC_{50}$  calculation.

<sup>c</sup>Maximal response to CDCA/no CDCA, with 1536-well assay done in 8- $\mu$ l volumes and 384-well assay done in 30  $\mu$ l volumes, with optimal detector reagents shown in Table 1.

<sup>d</sup>Performed with 25 nM SRC1–biotin and 2.5 nM FXR–GST with or without 50 M CDCA.

<sup>e</sup>Performed with 0.5 nM FXR–GST and plate coating at 100 nM SRC1–biotin.

<sup>f</sup>Using a Titertek Multidrop for 384-well assays and a Matrix PlateMate Plus for 1536-well assays. Three full plates were dispensed, with 16 controls (no CDCA) per 384-well plate and 48 controls (no CDCA) for 1536-well plates.

<sup>g</sup>Set at 1 s/well, this parameter can be reduced.



of reagent addition steps, and has the advantage of being ratiometric, thus smoothing errors in reagent addition and presumably eliminating artifacts caused by inner filter effects.<sup>8</sup> However, we have found that some compounds can affect the fluorescence ratio by quenching the time-gated emission at 615 nm. Also, the TR-FRET assay had a limited dynamic range at fixed detector reagent concentrations, as compared to the ALPHAScreen assay. The slower plate-reading times in the TR-FRET assay could have been improved by the use of imaging-based plate readers; however, these are generally far more costly than the photomultiplier tube-based systems.

Because of its increased sensitivity, decreased plate reading time, and increased proximity limits, the ALPHAScreen system was an excellent alternative to TR-FRET for the measurement of CDCA-induced FXR–SRC interactions. The large signal:background ratio and increased sensitivity in the ALPHAScreen assay have the potential to provide a significant reduction in the quantities of FXR–GST and SRC1–biotin required for screening. For the ALPHAScreen format, acceptable reading windows could have been obtained with 5-fold less of these reagents as compared to the TRF and TR-FRET assays (see Fig. 3). The reading time of 9.2 min for a 1536-well plate and 2.3 min for a 384-well plate served to significantly increase the efficiency of the HTS process. The ALPHAScreen system is generally applicable over a wide variety of biomolecular targets and can supplant solid-support binding assays in many applications.

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