Ready to use cell-based assay kits can be cost effective tools in drug discovery: Be aware of potential pitfalls

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OBJECTIVE

Optimization of experimental conditions when using a cryopreserved cell-based assay kit for potency determination.

INTRODUCTION

GLP-1R is a G-protein coupled receptor present on pancreatic beta cells. It plays a key role in controlling the glucose-insulin balance. A reliable method for evaluation of binding of potential drug candidates to GLP-1R is needed in drug development for Type-2 diabetes. Commercially available cryopreserved cell-based assay kits provide an attractive option. These kits are easy to use and essentially eliminate the huge cost of building and maintaining a live cell culture facility. We evaluated the consistency of performance of   a kit using the following parameters: (a) preparation dependence of disassociation constant (Kd); (b) Use of freshly thawed versus previously thawed cells, and (c) variability of incubation time.

METHOD

- A commercial cell-based binding assay kit from CisBio Bioassays (Bedford, MA) using homogeneous time-resolved fluorescence HTRF Tag-lite® technology was used.
- Acceptor and donor components of the HTRF system include:
  - Donor: HEK293 Cells expressing hGLP-1R that are labeled with the fusion tag SNAP-Lumi4 Tb.
  - Acceptor: Fluorescent labeled Exendin4-red (GLP-1R ligand).
- When acceptor and donors bind and come in close proximity a time-resolved fluorescence resonance energy transfer (TR-FRET) occurs.
- After 340nm excitation, the emissions from a donor (620nm) and acceptor (665nm) are measured and the ratio of the two are calculated. Ratio = [665nm/620nm]x10^4
- First, optimum concentration of Donor (Kd) is determined from the dose-response curve and then the Michaelis-Menten equation was used to calculate the Kd.
- The Acceptor (ligand) is used at constant concentration (i.e. Kd) with varying concentration of the test compounds. The dose-response curve that is generated is used to compute the inhibition constant (Ki) for the test compound.
- The signal is measured using an HTRF cartridge in an i3 SpectraMax. (Molecular Devices). Data are processed using SOFTMax Pro vs. 6.4.
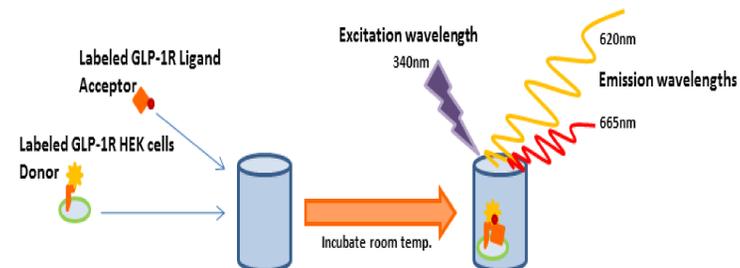
RESULTS

Fig. 1 Determining Disassociation Constant of GLP-1R Ligand

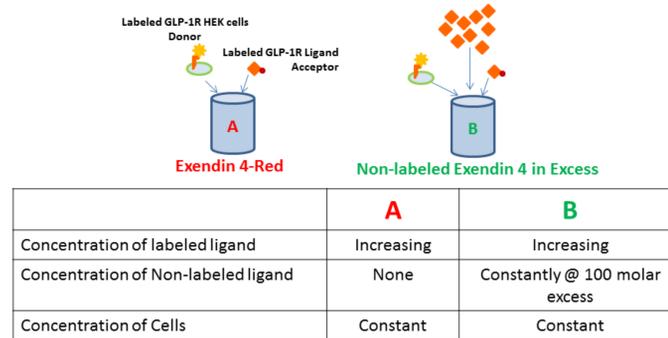|	A	B
Concentration of labeled ligand	Increasing	Increasing
Concentration of Non-labeled ligand	None	Constantly @ 100 molar excess
Concentration of Cells	Constant	Constant

Figure 1 (A) Varying concentrations of Fluorescent ligand (acceptor) are added to wells containing a fixed number of labeled cells (donor) to obtain the dose-response curve. (B) To account for non-specific binding at each point the dose-response curve is determined in the presence of unlabeled ligand in 100 molar excess. Dose-response curve for the specific binding is constructed after the HTRF signal of (B) is subtracted from (A). The disassociation constant (Kd) is determined by using the Michaelis-Menten equation.

Fig. 2 Determination of Kd Maintains High Stability

A – Initial Kd

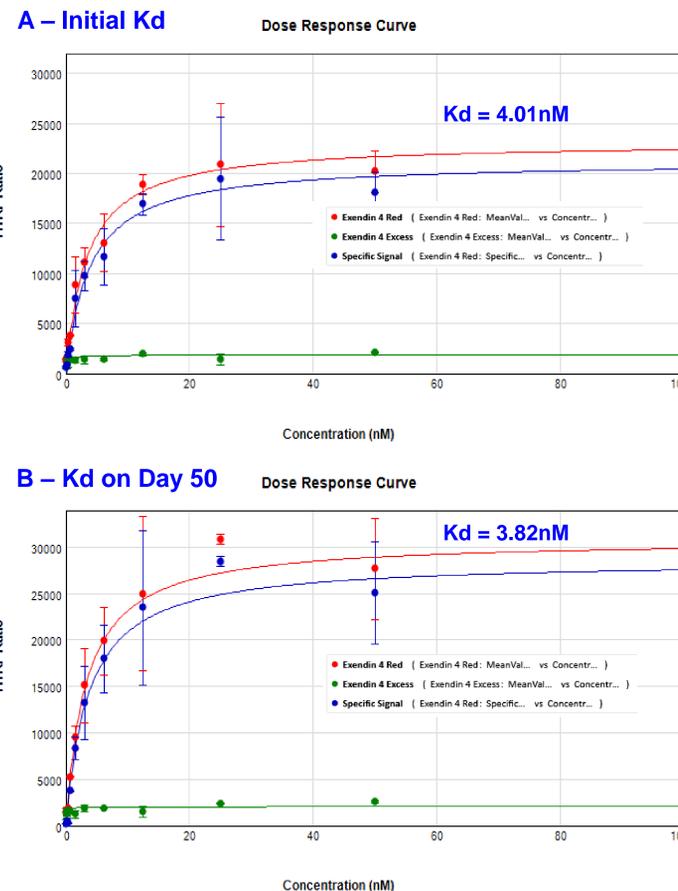Kd = 4.01nM

B – Kd on Day 50

Kd = 3.82nM

Figure 2. Two independent runs of Kd determination for the GLP-1R Labeled ligand performed almost 2 months apart shows high fidelity. (A) The specific signal curve (in blue) gave a Kd of 4.01nM at day 1. (B) using 2-month old cells, the specific signal curve (in blue) gave a Kd of 3.82nM.

RESULTS (CONT.)

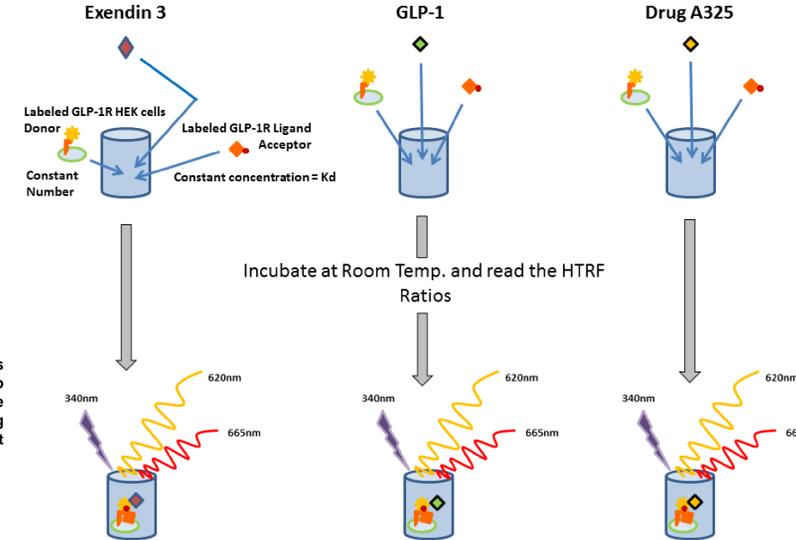### Fig. 3 Positive Inhibition of GLP-1R Agonists (test compound)

Figure 3. Dose response curves are obtained by varying the concentrations of the positive controls (Exendin 3 and GLP-1) and test compound (Drug A325) while keeping a fixed amount of labeled cells (donor) / well and labeled ligand (acceptor) at Kd concentration. The inhibition in response (HTRF ratio) is used to compute inhibition constant (Ki)

Fig. 4 Positive Inhibition Using Freshly Thawed Cells

A

20 minutes

Exendin 3 Ki = 11.4nM
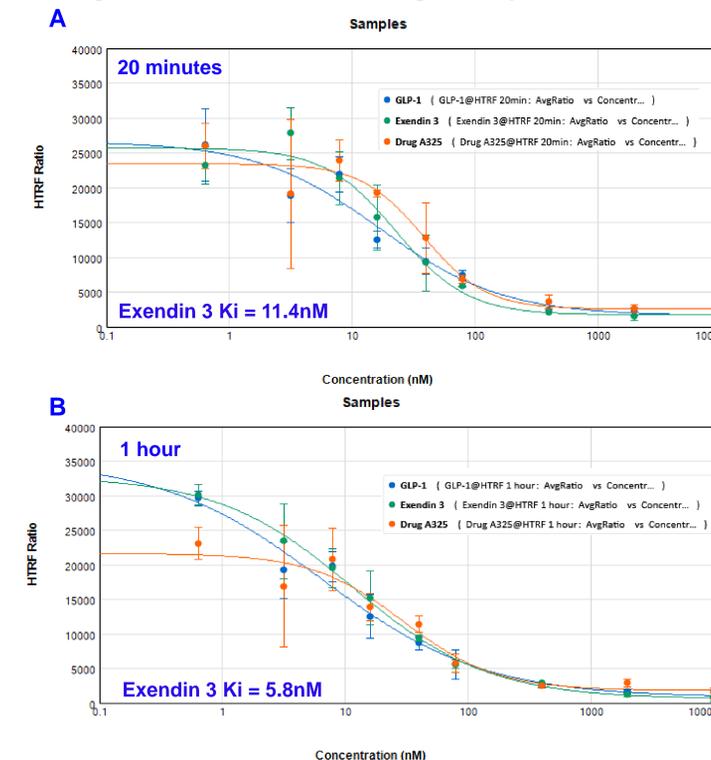

B

1 hour

Exendin 3 Ki = 5.8nM

Figure 4. Freshly thawed cells used in determining the Ki of known agonists. A) At 20 minutes incubation the Ki of Exendin-3 was 11.4nM while B) after 1 hour of incubation the Ki was calculated to be 5.8nM. The previously published results for Exendin-3 assigns it a Ki of 11.0nM. Further the graph at 2 hours showed an uncoupling of the curve fit at the edges of the concentration curve (data not shown).

RESULTS (CONT.)

Fig. 5 Positive Inhibition Using Cells 24hr Post Thaw

A

30 minutes

Exendin 3 Ki = 37.2nM

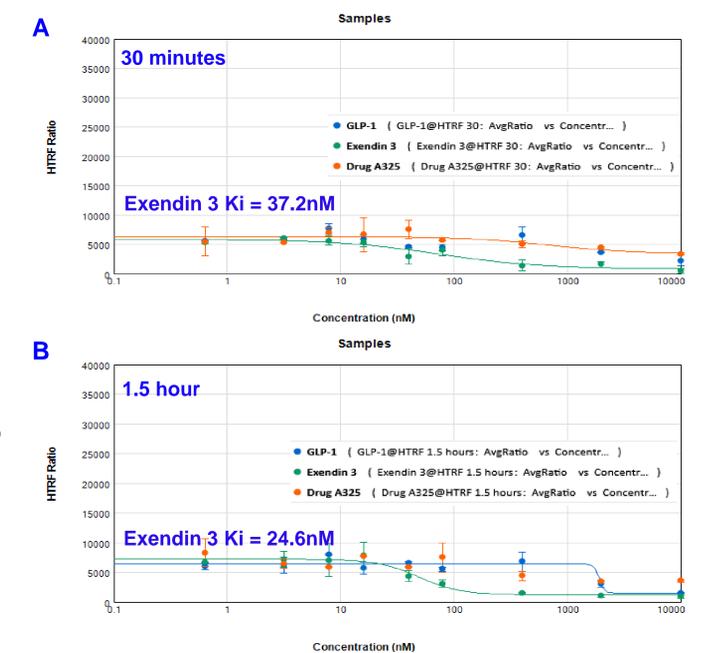B

1.5 hour

Exendin 3 Ki = 24.6nM

Figure 5. Thawed cells used after 24 hours A) At 30 minutes the Ki of Exendin-3 was 37.2nM while B) after 1.5 hours the Ki was 24.6nM. In Addition there is a decrease of HTRF ratio signal and increase in variability.

SUMMARY

- Kd of the labeled Exendin 4 (acceptor) for GLP-1R from two different preps were essentially unchanged (4.0nM Vs. 3.8nM).
- Ki values determined with freshly thawed cells were closer to previously published results and less variable during the experiment (Ki 5.8nM to 11.4nM).
- Cells should be used on the same day they were thawed.
- Shorter incubation times provided better reproducibility. Inhibition curves were more robust after only 20 minutes to 1hr (Figure 4 B) compared to 120mins (not shown).

CONCLUSION

- Attempting to save on expensive kits by using thawed cryopreserved cells after overnight suspension introduces serious anomalies in the results. However, eliminating the need to re-test the Kd within every experiment greatly reduces the consumption of prepared cells.
- Decreased incubation times suggested by the more robust inhibitions curves after only an hour incubation should also increase workflow efficiency and decrease cost of screening for potential Type-2 Diabetes therapeutics.

REFERENCES

- Rapid Screening of a Cell-based Assay for GLP-1 Receptor Using a Natural Product Library. B. Larson, P. Banks, BioTek Instruments, Inc.; N. Pierre, S. Graham, J. Tardieu, F. Degorce, Cisbio US. Application Note BioTek. 2012

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