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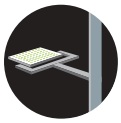
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C L I P R™
Chemiluminescent Plate Reader

Homogeneous, Chemiluminescence-Based Assays for Receptor Binding, Cellular cAMP, Tyrosine Kinase and Caspase-3 Using the CLIPR™ Chemiluminescence Imaging Plate Reader

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INTRODUCTION

We have applied commercially available homogeneous, *in vitro* assays called HitHunter™ to high throughput screening using chemiluminescence detection. These assays are available from DiscoveRx and can be miniaturized to 384- and 1536-well microtiter plates, with format-independent, simultaneous detection of all wells on the Molecular Devices CLIPR™ imaging plate reader. The homogeneous assay technology uses complementation of inactive fragments of *E. coli* ß-galactosidase to monitor a variety of biochemical reactions, with no separation or wash steps.

HitHunter™ assays for human progesterone receptor, cellular cAMP, tyrosine kinase and the proteolytic enzyme caspase-3 were developed using the enzyme fragment complementation method. All assays were performed at room temperature in microtiter plates. Formation of ß-galactosidase was measured using a dioxetane galactoside substrate. The resulting chemiluminescence was measured on the CLIPR system in ≤ 30 seconds per plate. Similar signal intensities and signal-to-noise ratios were observed using 384- and 1536-well microtiter plates.

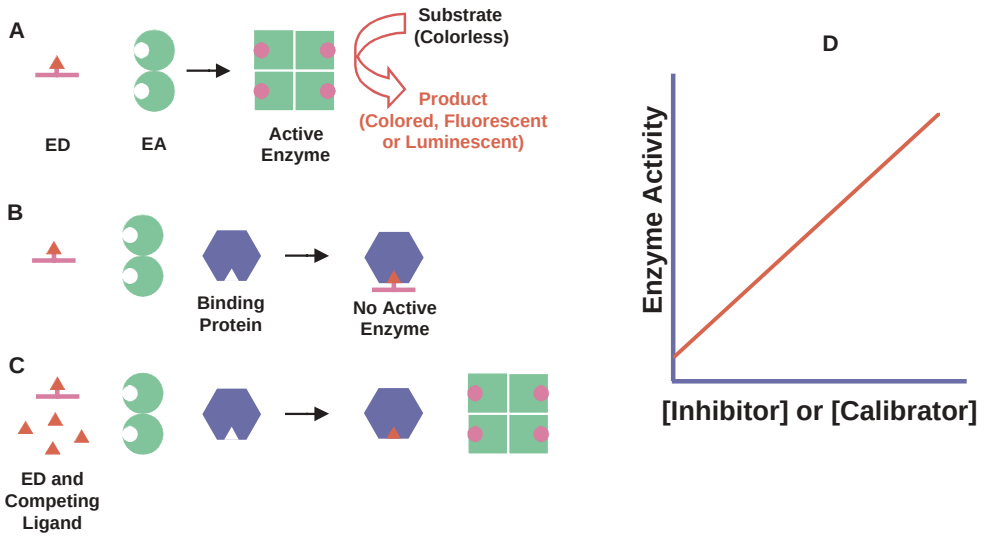
The combination of the CLIPR system's CCD camera-based detection system and chemiluminescence-based homogeneous assays using the enzyme fragment complementation technology should be ideally suited to ultra-high throughput screening, enabling miniaturization and rapid measurement of each plate.

Molecular Devices

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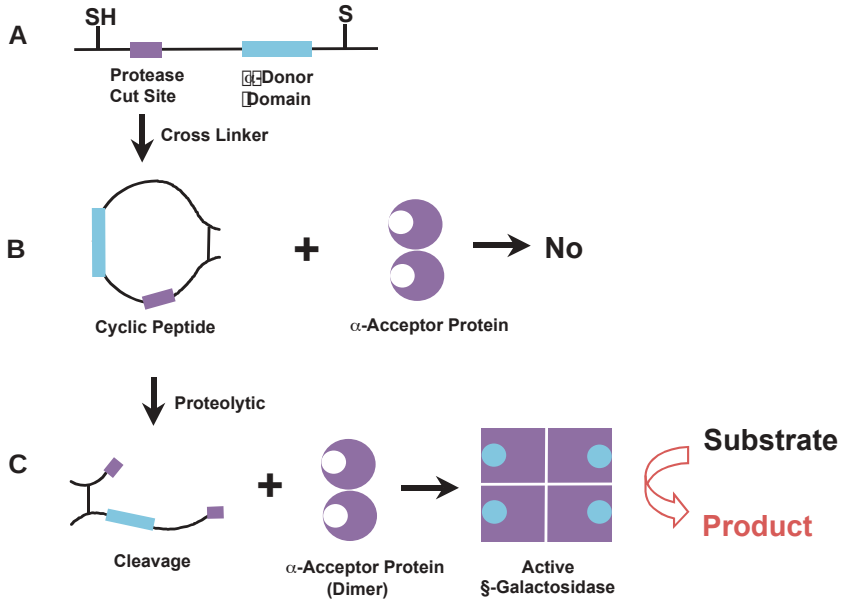
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PRINCIPLE OF ENZYME FRAGMENT COMPLEMEN-TATION TECHNOLOGY



Principle of enzyme fragment complementation technology. (A) Incubation of ED (Enzyme Donor) or ED –ligand with EA (Enzyme Acceptor) results in formation of active, tetrameric ß-galactosidase, which can be measured by hydrolysis of chromogenic, fluorogenic, or luminescent substrates. (B) Binding of a binding protein (e.g., antibody, receptor, or binding factor) to the ligand prevents formation of active enzyme. (C) In the presence of unbound ligand or ligand analog, there is competition for binding sites, leading to an increase of free ED-ligand and of active enzyme. (D) The amount of enzyme activity is directly proportional to the concentration of the competing ligand and its relative affinity for the binding protein.

PRINCIPLE OF HITHUNTER™ PROTEASE CASPASE-3 ASSAY



Principle of HitHunter™ protease caspase-3 assay. (A) A linear peptide is constructed to contain an ED domain and a specific protease cleavage site, with cycteine residues near the amino and carboxyl termini. (B) Cross-linking of cysteine residues using a homobifunctional linker results in a cyclic peptide that has virtually no complementation activity. (C) Incubation of the cyclic peptide with specific protease results in cleavage to form a linear, cross-linked peptide that can complement with EA to form active enzyme.

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Assay Protocol (384-Well)

1. Add 10 µl hPR cytosol, 5 µl competing drug: incubate for 15 minutes at room temperature.
2. Add 25 µl ED-progesterone conjugate: incubate for 30 minutes at room temperature.
3. Add 25 µl EA reagent, 25 µl substrate; incubate for 30 minutes at room temperature.
4. Measure chemiluminescence for 20 seconds on Molecular Devices' CLIPR™ imaging plate reader.

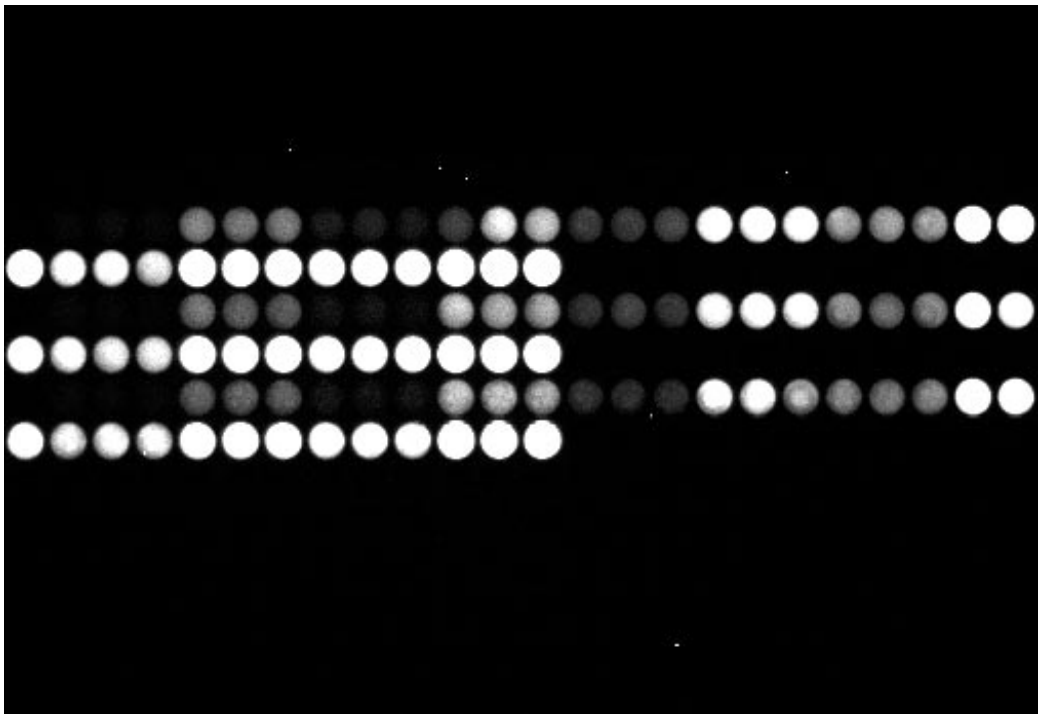


Figure 1. CLIPR image of the HitHunter™ progesterone receptor assay performed in a 384-well plate. Three receptor cytosols (each cytosol in two rows) were assayed with various concentrations of ED-progesterone with and without 10 µM progesterone. From left to right, ED-progesterone conjugates were used at 0 (blank, single well), 0.13, 0.26, 0.51, 0.77, 1.02, and 1.54 nM (triplicate each concentration).

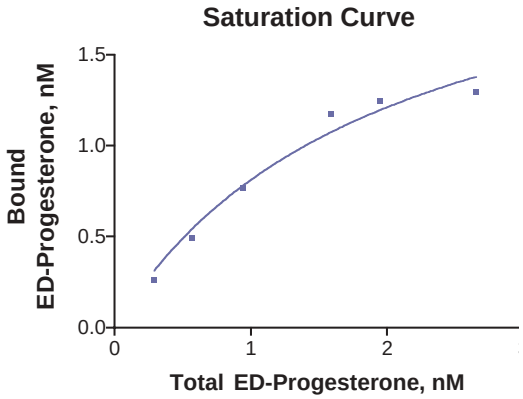


Figure 2. Binding of progesterone to the human progesterone receptor. Results were plotted from the image data of one cytosol preparation, top two rows in Figure 1.

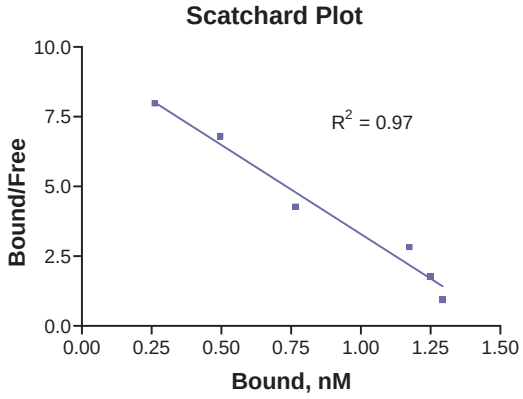


Figure 3. Scatchard plot of progesterone binding (data from Figure 2). The binding affinity was estimated as 0.16 nM and the total binding sites as 1.5 nM.

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Assay Protocol (384-Well)

1. Add 12 µl cell lysate or calibrator, 4 µl antibody; incubate for 60 minutes at room temperature.
2. Add 10 µl EA; incubate for 60 minutes at room temperature.
3. Add 10 µl substrate; incubate for 60 minutes at room temperature.
4. Measure chemiluminescence for 20 seconds on Molecular Devices' CLIPR™ imaging plate reader.

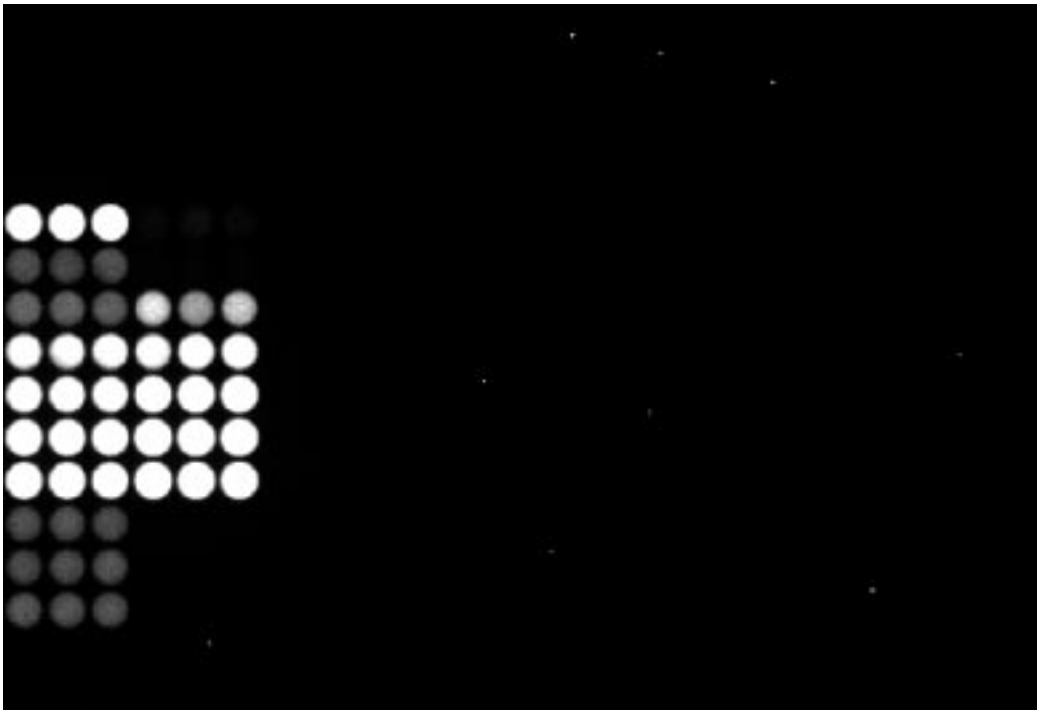


Figure 4. CLIPR image of HitHunter™ cAMP assay performed in a 384-well plate. The calibrators at 0, 0.1, 1, 10, 100, and 1000 pmole/well were located from top down at the second to seventh row (triplicates from left to right).

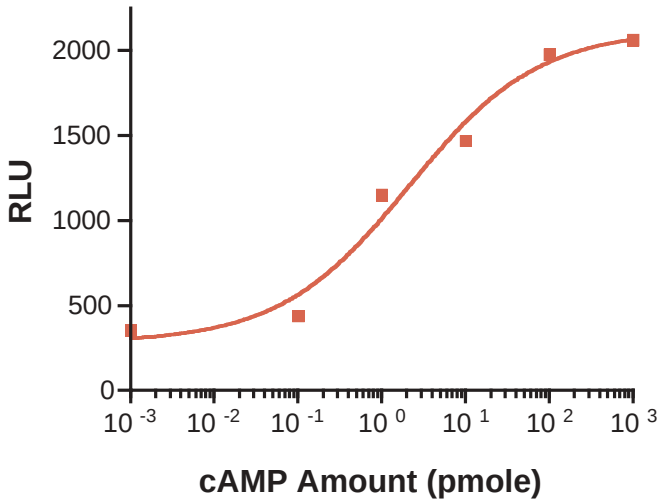


Figure 5. The calibration curve of cAMP assay, results taken from the image data.

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TYROSINE KINASE ASSAY (IRK AND SRC)

Assay Protocol (384-Well)

1. Add 5 µl assay buffer or drug candidate, 5 µl enzyme, 10 µl enzyme substrate; incubate for 2 hours at room temperature.
2. Add 5 µl antibody; incubate for 30 minutes at room temperature.
3. Add 5 µl ED conjugate; incubate for 30 minutes at room temperature.
4. Add 5 µl EA, 5 µl substrate; incubate for 60 minutes at room temperature
5. Measure chemiluminescence for 20 seconds on Molecular Devices' CLIPR™ imaging plate reader.

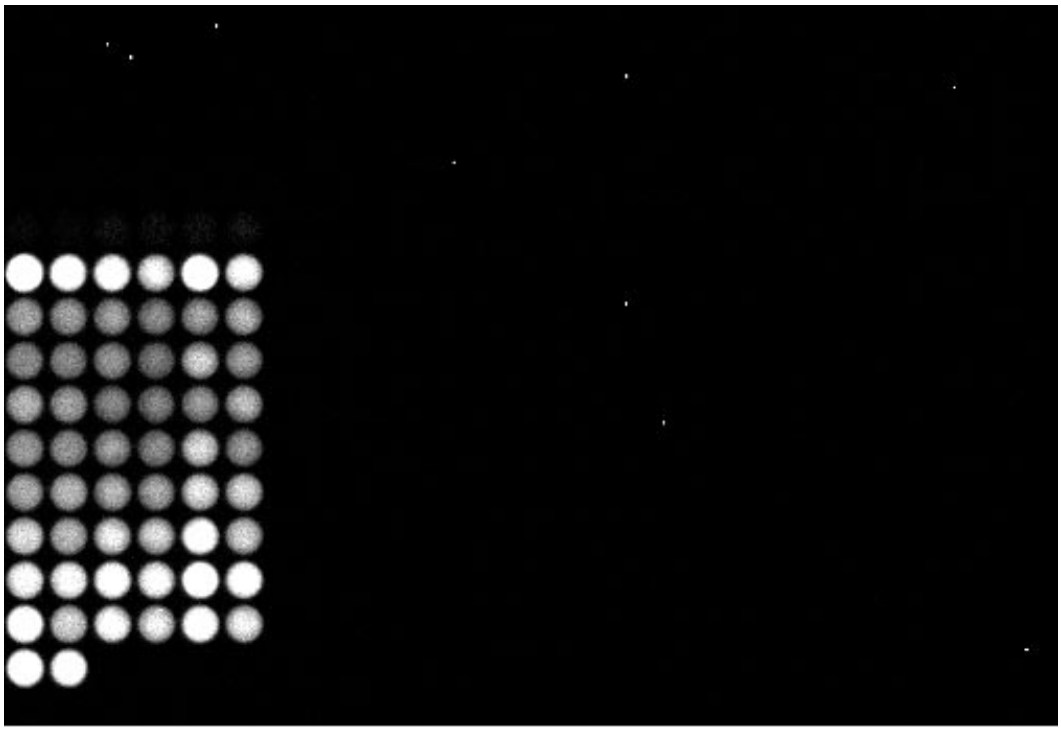


Figure 6. CLIPR image of the tyrosine kinase (SRC) HitHunter™ assay performed in a 384-well plate. SRC kinase was used at 0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 units from top down the third to tenth row in duplicate (far left two columns).

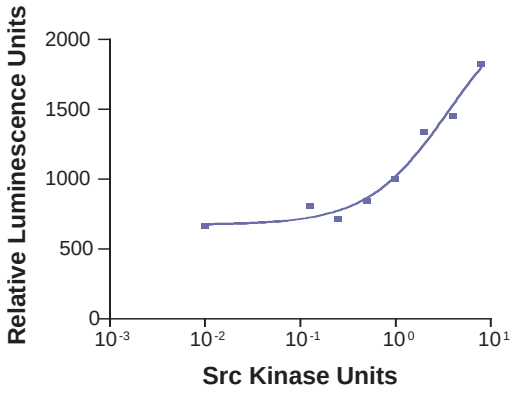


Figure 7. Enzyme reaction curve of Src kinase. Results were plotted from the image data as shown in figure 6.

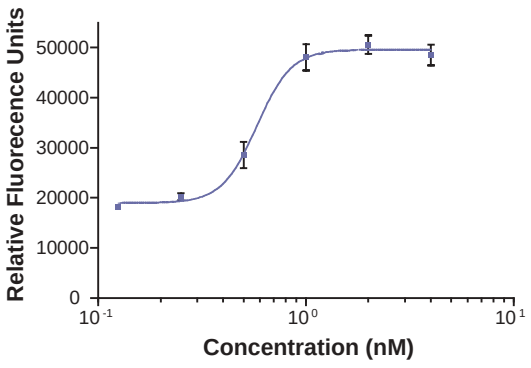


Figure 8. Enzyme reaction curve of Insulin Receptor Kinase (IRK) (image data not shown).

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PROTEASE CASPASE-3 ASSAY PROTOCOL

Assay Protocol (96-Well)

1. Add 12 µl enzyme, 12 µl cSED-DEVD; incubate for 60 minutes at room temperature.
2. Add 40 µl EA; incubate for 15 minutes at room temperature.
3. Add 40 µl substrate; incubate for 15 minutes at room temperature.
4. Measure chemiluminescence for 20 seconds on Molecular Devices' CLIPR™ imaging plate reader.

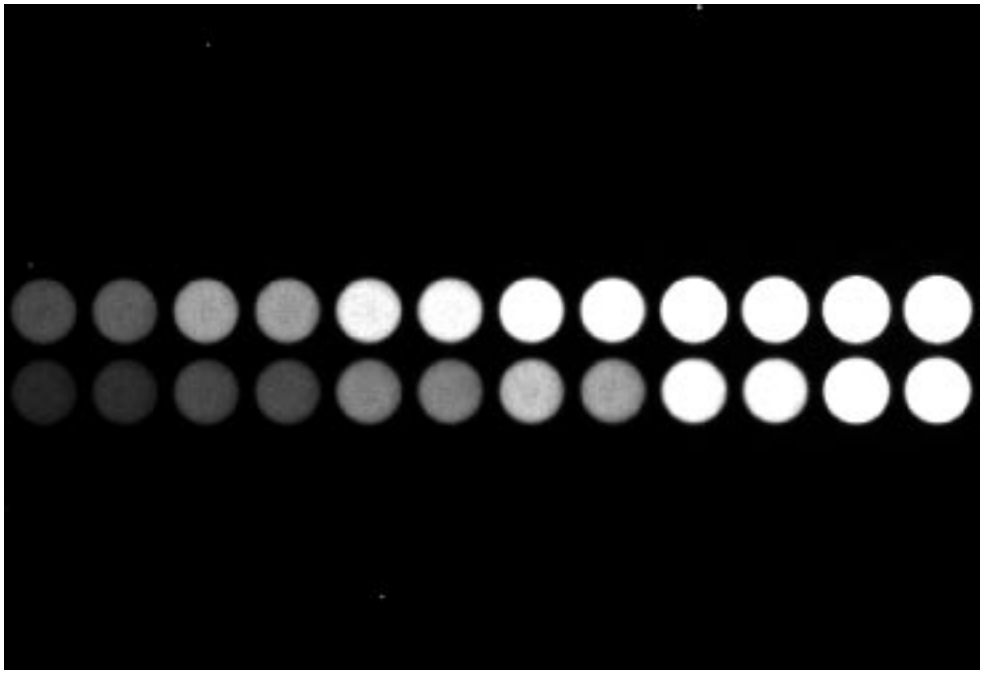


Figure 9. CLIPR image of caspase-3 assay performed in a 96-well plate. Two levels of Cyclic ED were used; top row at 5 nmole/well and bottom row at 2 nmole/well. Caspase-3 concentrations were at 0, 1, 2, 4, 8, and 16 pg/well (duplicate) from left to right.

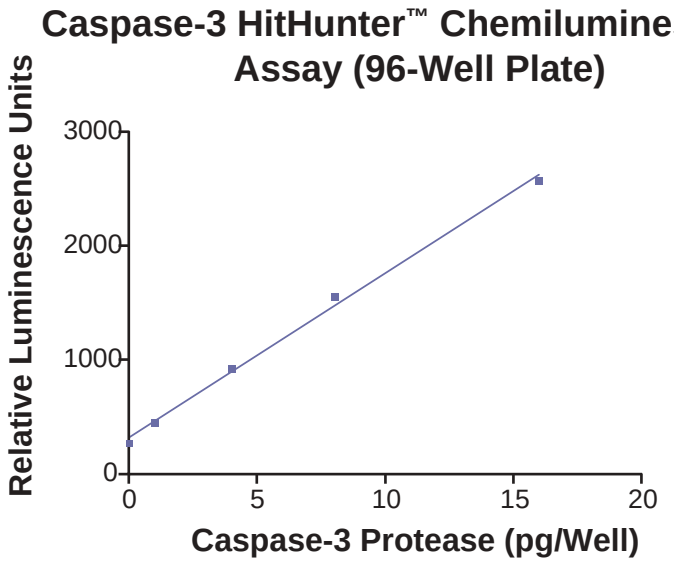


Figure 10. Caspase-3 enzymatic reaction curve performed in 96-well plate. Data were plotted from results as shown in Figure 9.

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Assay Protocol (384-Well)

1. Add 6 µl enzyme, 6 µl cSED-DEVD; incubate for 60 minutes at room temperature.
2. Add 20 µl EA; incubate for 15 minutes at room temperature.
3. Add 20 µl substrate; incubate for 15 minutes at room temperature.
4. Measure chemiluminescence for 20 seconds on Molecular Devices' CLIPR™ imaging plate reader..

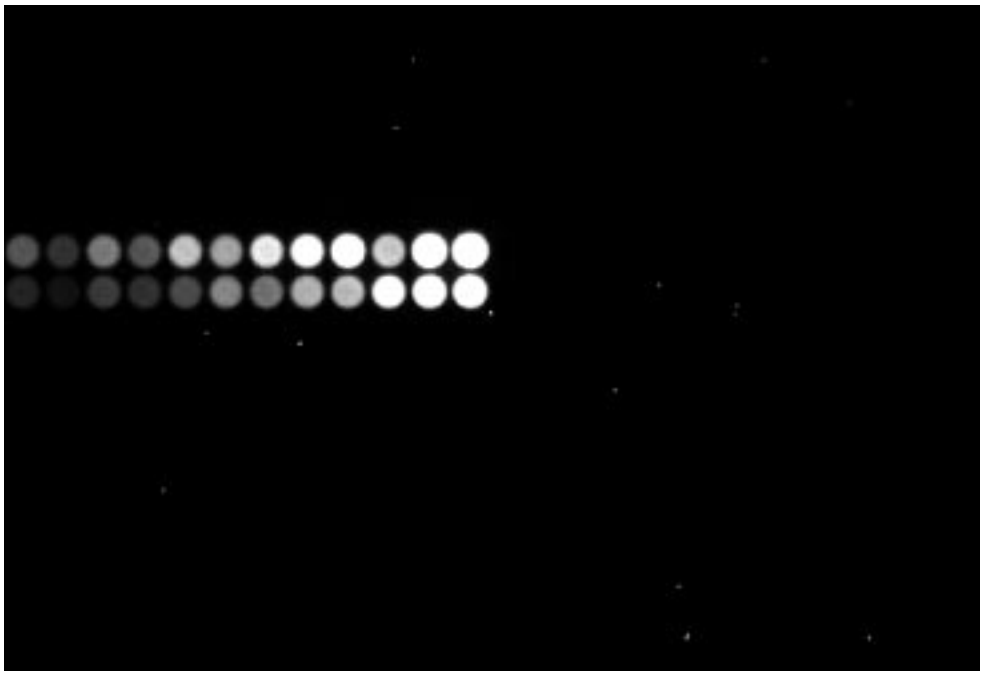


Figure 11. CLIPR image of caspase-3 assay performed in a 384-well plate. Well assignments of caspase-3 were the same as in Figure 9. Caspase-3 concentrations were 0, 0.5, 1, 2, 4, 8 pg/well from left to right.

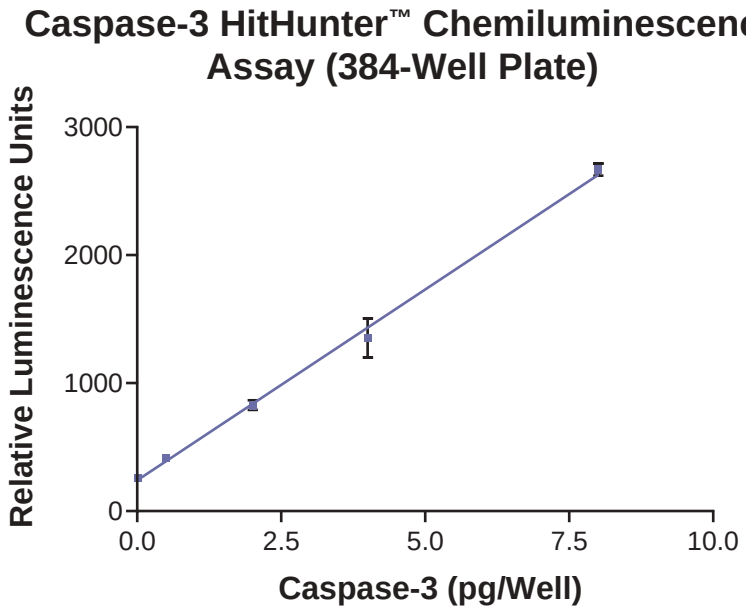


Figure 12. Caspase-3 enzymatic reaction curve performed in a 384-well plate.

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Assay Protocol (1536-Well)

The final reaction in the 96-well plate was transferred into a 1536-well plate (5 µl/well) and imaged for 20 seconds on Molecular Devices' CLIPR™ imaging plate reader.

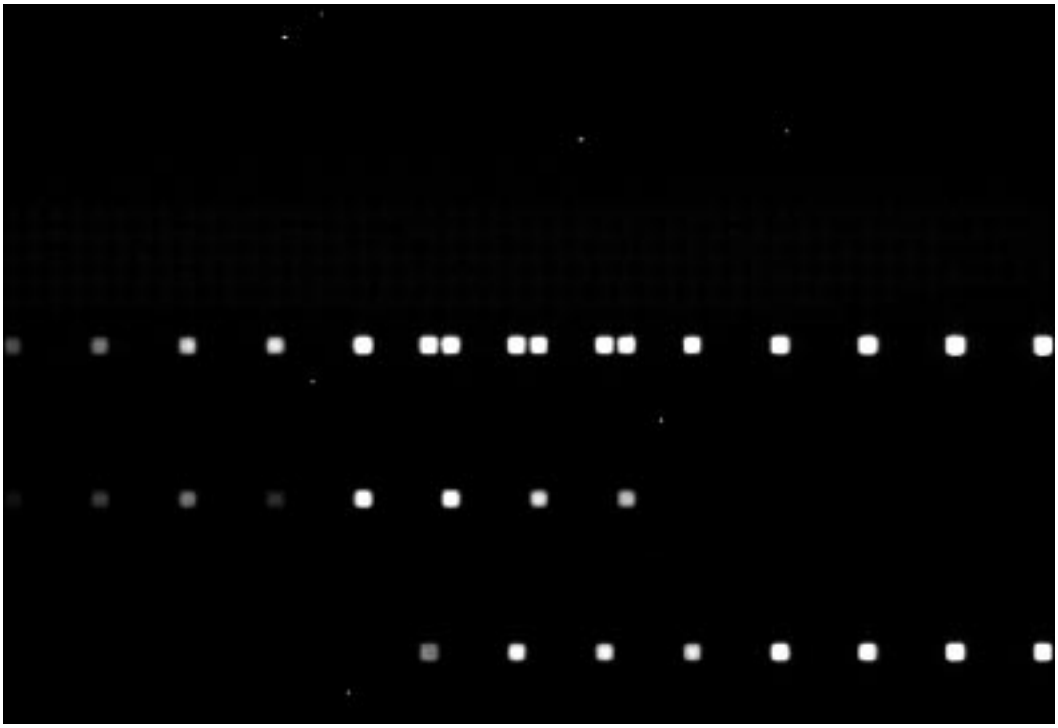


Figure 13. CLIPR image of caspase-3 assay in a 1536-well plate. Well assignments were the same as in Figure 9. Reaction mixtures were transferred twice using multi-channel pipette (8 tips) into one single row and into two separate rows. Caspase-3 concentrations were 0, 0.05, 0.1, 0.2, 0.4, 0.8 pg/well from left to right.

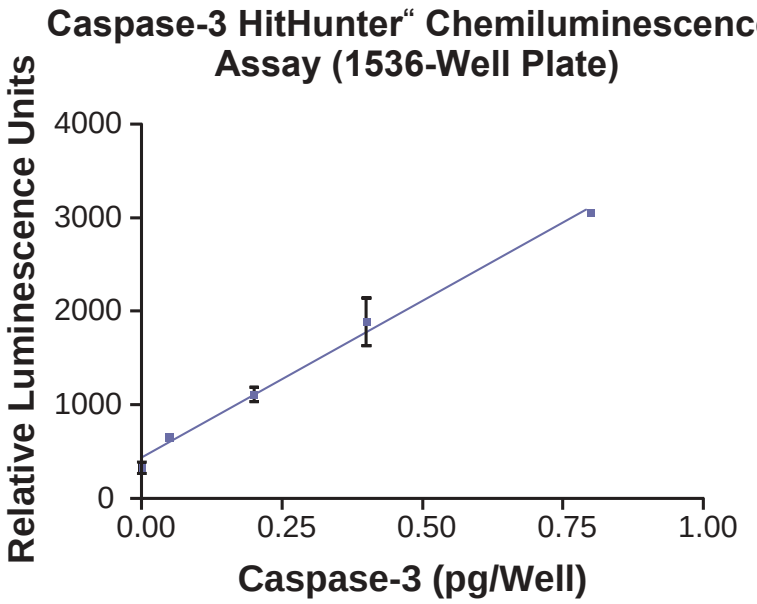


Figure 14. Caspase-3 enzymatic reaction curve performed in a 1536-well plate.

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CONCLUSIONS

1. Homogeneous assays for progesterone receptor, cAMP, tyrosine kinase, and caspase-3 have been applied to Molecular Devices CLIPR™ imaging microplate reader.

2. These assays have been miniaturized to 384- and 1536-well format without losing assay characteristics.

3. The assay gives positive signal response to drug hits and minimizes false positives.

4. Measurement of luminescence signal has been achieved within 30 seconds per plate for 96-, 384- or 1536-well plate.

5. All assays were performed and measured at room temperature.

6. Each assay gives high sensitivity of detection.

7. DiscoveRx HitHunter™ homogeneous assays are applicable to drug screening for enzyme, cAMP, protease caspase-3 and cytosol progesterone receptor.

8. The combination of the CLIPR imaging plate reader detection system with fast read time and DiscoveRx chemiluminescence homogeneous assays with high detection sensitivity is ideally suited to high throughput screening.

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