BHARATHIDASAN UNIVERSITY

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Programme: M.Sc., Biomedical Science

Course Title : Drug Discovery and Assay Development

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Unit-IV

Bioassay and HTS

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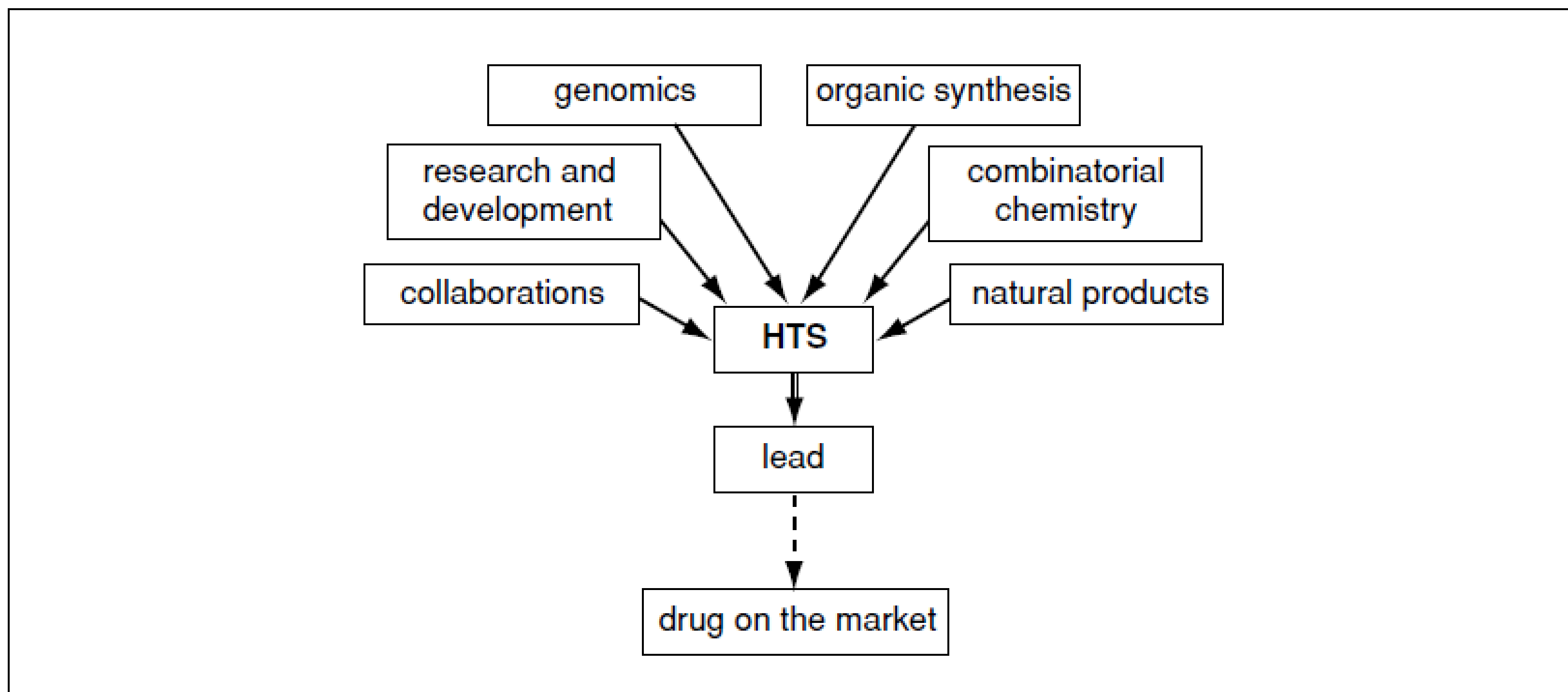


Figure 9.4.1 High-throughput screening (HTS) is one of the key methodologies used to find active compounds. HTS receives its input from numerous scientific disciplines and operational groups.

Figure 9.4.3 Liquid compound storage system with a capacity of more than 10 million deep-well sample tubes or 1.6 million microtiter plates. Robotic access enables automatic sample retrieval. (Photo reprinted with permission from REMP AG).

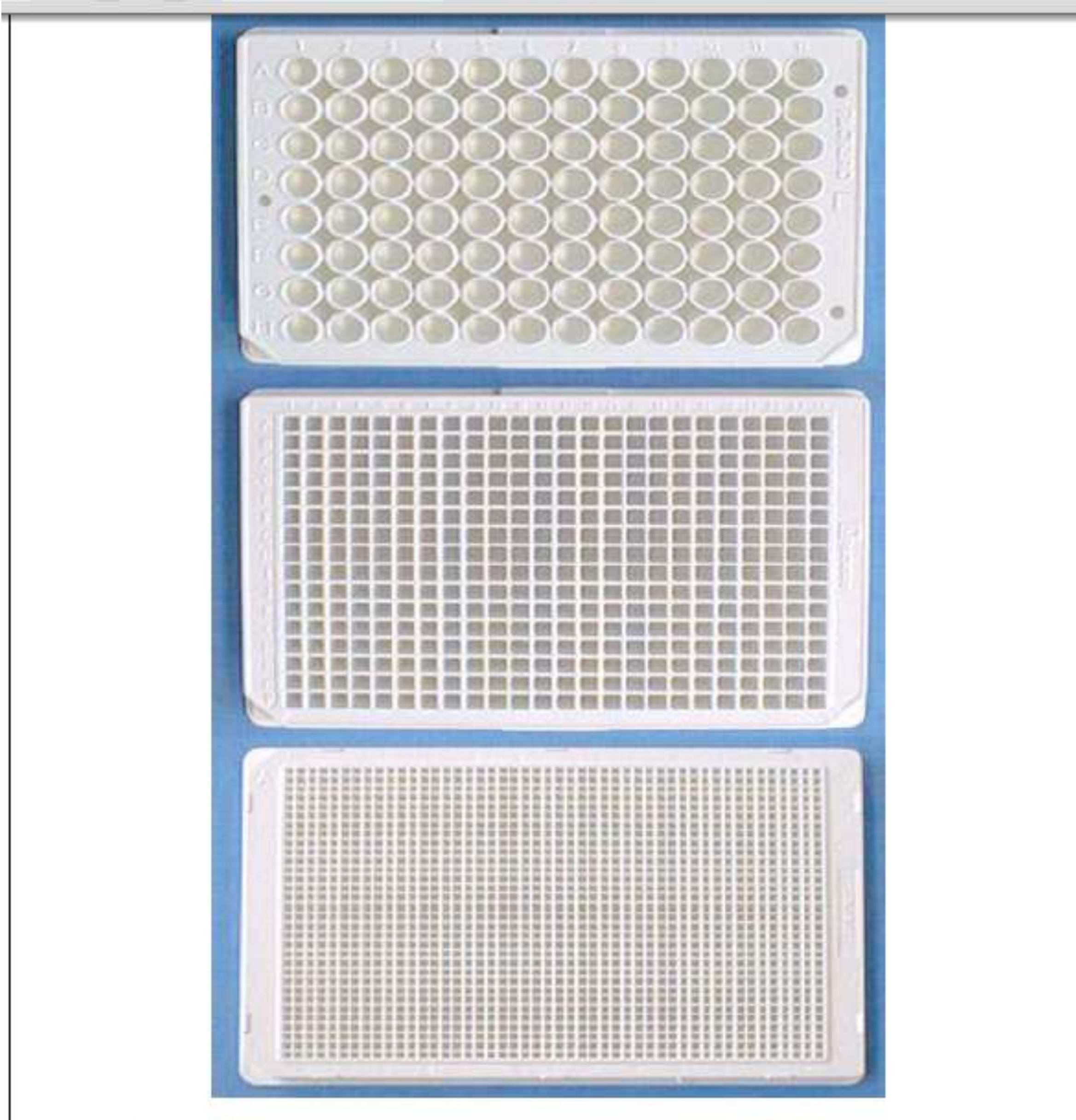


Figure 9.4.4 Three types of microtiter plates commonly used in HTS. Top: 96-well; middle: 384-well; bottom: 1536-well. All plates have the same standardized footprint.

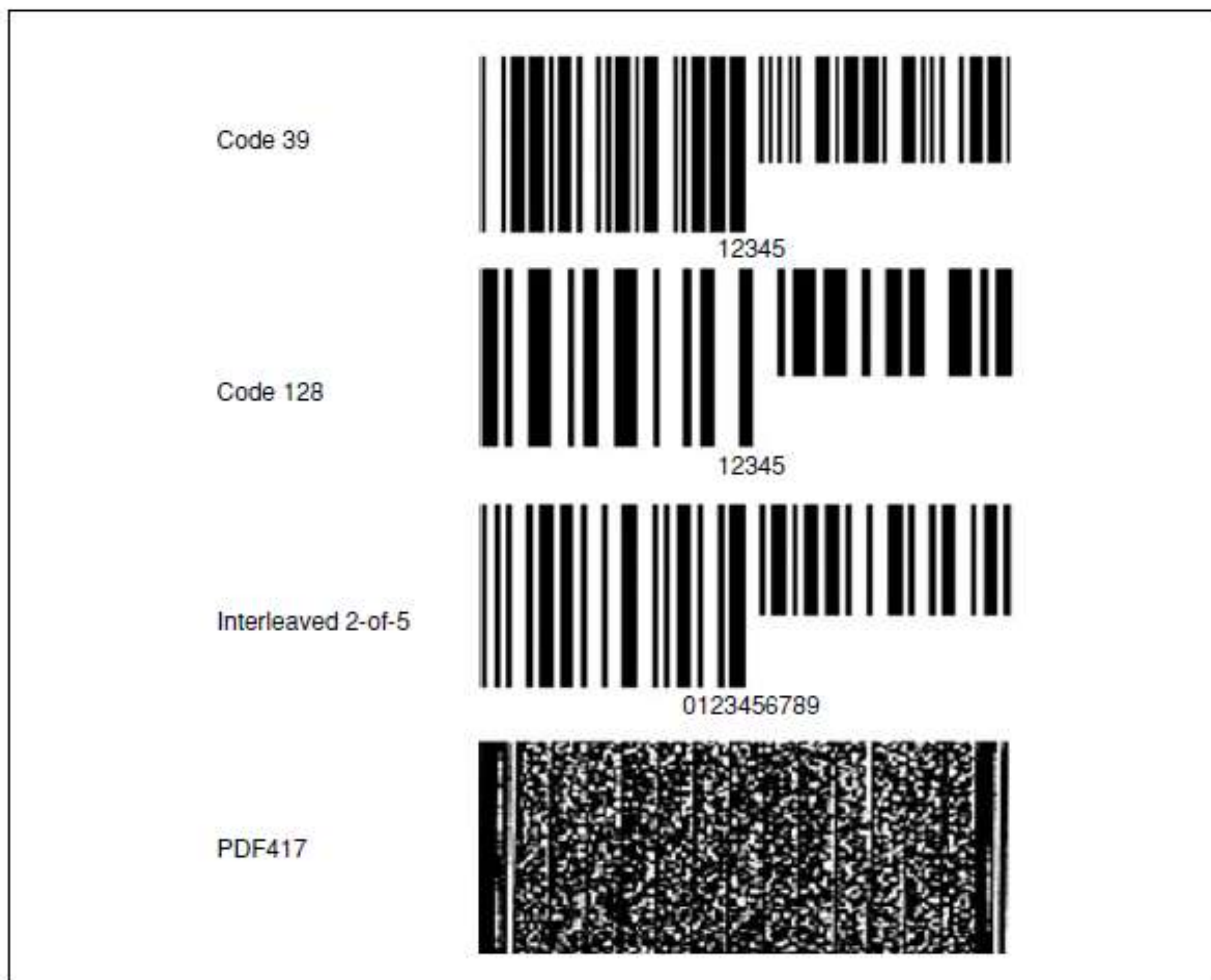


Figure 9.4.5 Typical types of bar codes used for sample and plate identification in HTS.

HTS

- The aim of HTS is to identify bioactive molecule from large compound collection and further development of active compounds to leads.

Types

- Biochemical - and cell-based assays.
- Depends on nature of target and assay feasibilities
- Assay optimization and validation

Fig. 11.1 Sequential steps involved in the high-throughput screening

- Identification of competent drug target.
- Target validation

(Biochemical assays and animal model Exp)

- Compounds modulating target identified.
- Assay development to screen modulators.
- Modulators -dose-dependent target modulation -lead compounds.
- Common pharmacophore can be developed from lead compounds showing common chemical properties.
- Structural activity relationship can be accessed
- Molecular descriptors can be optimized to improve selectivity and drug likeness of lead compounds; Lead optimization.
- Potential candidates for drug development.

(Animal models, and clinical trials)

Biochemical assays

- Enzyme inhibition
- Receptor-ligand binding assays.
- Evaluates **binding affinity or specific binding** of compounds against biological target in an artificial environment.
- Purification is not possible - biological targets.
- Cell-specific responses against small molecules.

Cell-based assays

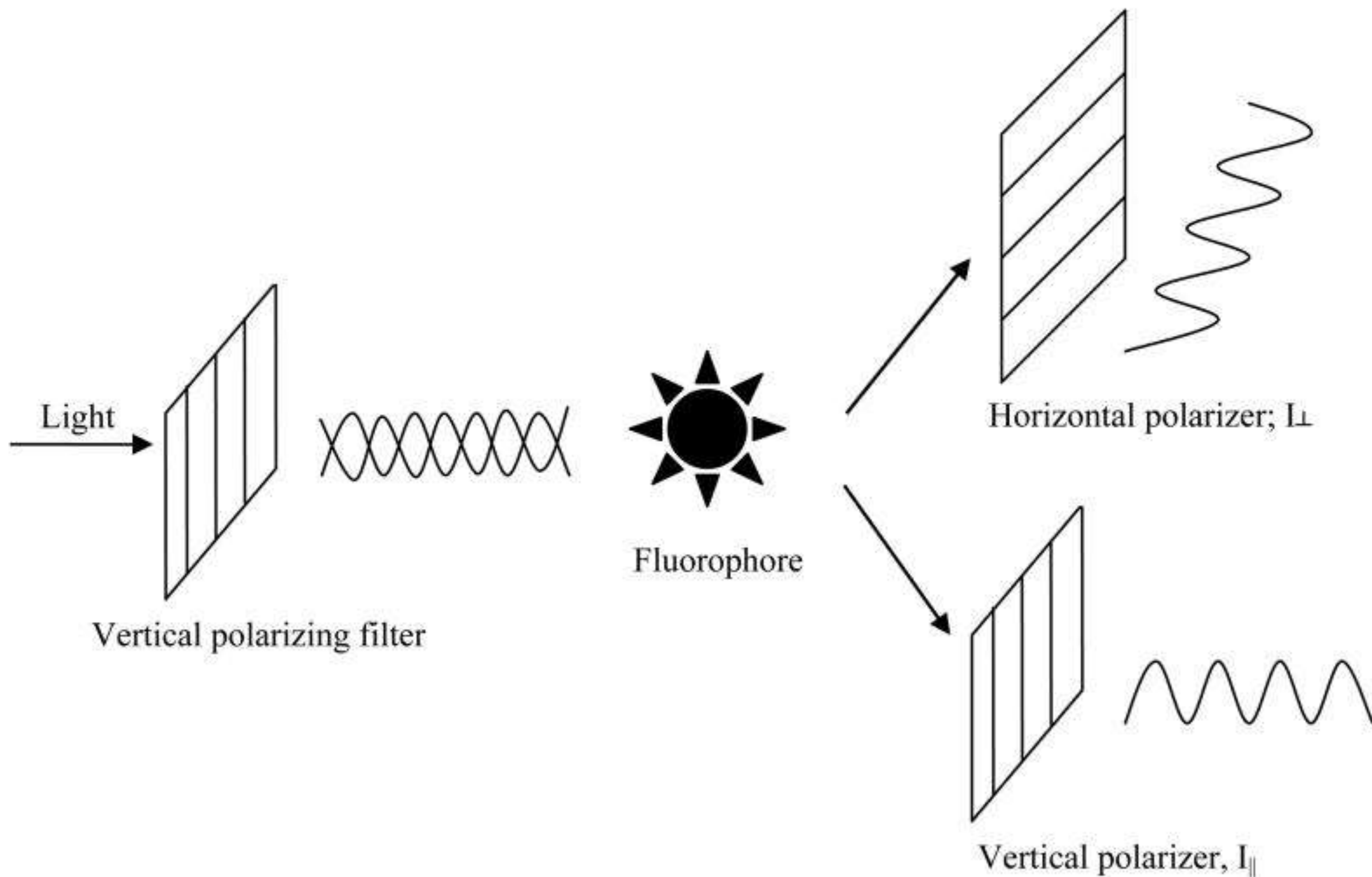
- Monitor drug effects on biochemical pathways or more specific targets
- Provide more biologically relevant microenvironment
- Provide direct information about cell permeability of compounds
- Acute cytotoxicity of compounds.

Detection Methods in HTS

- Readouts used in biochemical assay for HTS
- Optical, including absorbance, fluorescence, luminescence, and scintillation

Fluorescence Polarization or Anisotropy

- It is a solution-based homogeneous technique
- Quantitative analysis of molecular interactions and enzymatic activities.
- Polarized light to excite molecules in solution.
- Free fluorescent molecule shows fast tumbling- low polarization value.
- Receptor-bound fluorophore that tumbles slowly - high polarization value



A fluorophore is excited with light that is linearly polarized by passing through an excitation polarizing filter; the polarized fluorescence is measured through an emission polarizer either parallel or perpendicular to the exciting light's plane of polarization.

- It has been adapted to almost every protein class like GPCRs, nuclear receptors, and enzymes.
- It can be used for the analysis of molecular interactions studies **protein-ligand, protein-protein, and protein-DNA binding events.**
- It is also used for monitoring enzymatic reaction progress

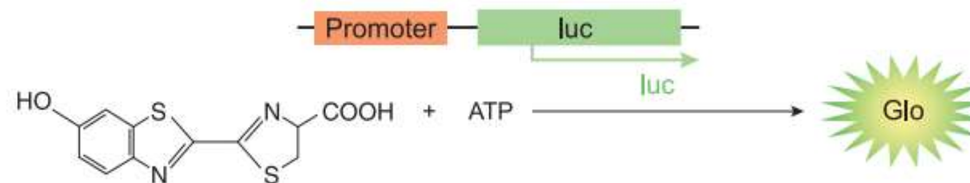
Fluorescence Resonance Energy Transfer (FRET)

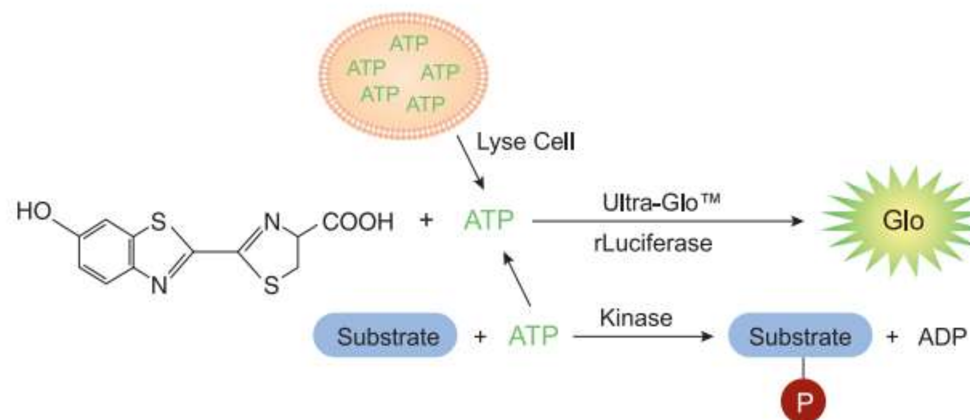
- It is an energy transfer between a fluorophore donor and a suitable fluorophore energy acceptor
- The absorption spectrum of the acceptor should overlap with the emission spectrum of the donor.
- Two fluorophores are in close molecular proximity
- In cell-based HTS, genetically incorporated fluorescent indicators are used to understand signal transduction dependent on protein phosphorylation

Bioluminescence

- ATP derived from enzyme-coupled reaction
- Detected by a reporter enzyme (luciferase from firefly Photinus pyralis) that acts on luciferin substrate to create a luminescent output.
- Free from compound interference
- 1000 times sharper than equivalent FBA
- Access the activity of cytochromes P450, proteases, and monoamine oxidases

- Luciferase can be mutated to develop multiple luminescent sensors in an assay.
- Dual luciferase reporter assays with different kinetics or emission maxima and substrate specificities has been used for identification of activities specific to the **signaling pathway of interest**
- Luciferase reporters can be clumped with other detection formats, green fluorescent protein (GFP) reporter with β-galactosidase or alamarBlue to estimate **cytotoxicity.**





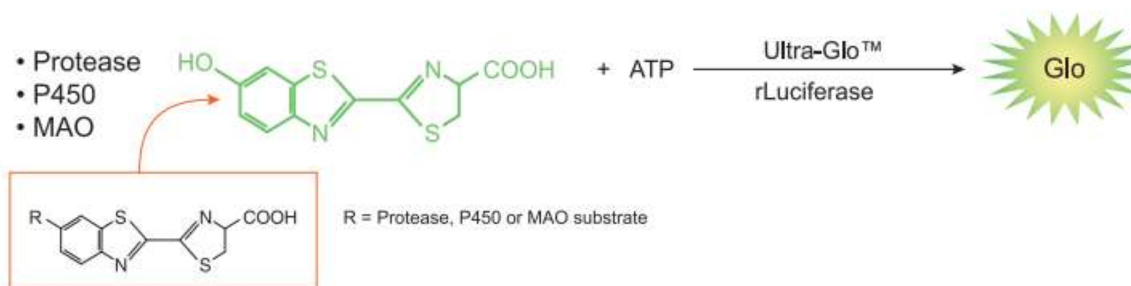


FIG. 3. Bioluminescent assays for HTS. luc, firefly luciferase gene; Ultra-Glo™ rLuciferase, stable recombinant firefly luciferase (Promega, Madison, WI); MAO, monoamine oxidase.

Bioluminescence Resonance Energy Transfer (BRET)

- It is a hybrid system involving the donor and acceptor
- Donor is a luminescent molecule excited by the enzyme
- Acceptor - fluorescent protein like a GFP or YFP
- Enzyme as an excitation source eliminates interferences from auto-fluorescent compounds and inner filter effects.
- Study of GPCRs by investigating receptor oligomerization or activation

- Disassociation-Enhanced Lanthanide Immunoassay (DELFIA)
- Homogenous Time-Resolved Fluorescence (HTRF)
- Homogenous Time-Resolved Fluorescence (HTRF)
- Alpha (Amplified Luminescent Proximity Assay) Screen

- Biochemical assays - to find compounds that interact with an isolated target in vitro environment.
- Cell-based assays assess phenotypic effect of compounds on the cell.
- Measure G protein- coupled receptor (GPCR) and ion channel function
- Confocal imaging platforms for rapid cellular and subcellular imaging

In Vitro Enzyme-Based HTS Assays

- Recognition of accurate enzyme and substrate forms,
- Purification methods
- Precise measurement of kinetic parameter
- Characterization of cofactors
- Choice of detection technology
- Mode of action.

- HTS enzymes have been developed using detection of substrate consumption, product formation, and enzyme-ligand binding.
- Development of enzyme based HTS begins with demonstration of catalytic activity on substrate in vitro environment.

Components of Enzyme-Based Assays

Enzyme

- In vitro biochemical assays use enzyme **isolated from cell.**
- Absence of **native in vivo conditions** can significantly affect enzyme activity and stability.
- Enzymes may be expressed as **truncated variants**, or it may be expressed in alternatively tagged species.
- These artificial conditions may give compounds irrelevant in physiological conditions or **miss compounds** showing activity in physiological conditions.

- Impure enzyme may lead to aberrant result.
- Mass spectrometry can be used to analyze enzyme purity.
- Enzymatic purity analysis is done by analyzing **IC50 curves** of known inhibitor or by determination of Michaelis-Menten parameters and comparing with previous results
- Vehicle-carrying compound may impair enzyme function.
- Vehicle sensitivity is estimated by **titrating the vehicle in different concentrations against enzyme** and analyzing its activity.
- Besides, poor interaction of enzyme - decrease of effective enzyme concentration.
- This can be prevented by **adding BSA** or small amount of detergent in the reaction buffer.

Substrate

- Concentration of substrates comparative to their Km will have influence on inhibitor type identified.
- In the case of competitive inhibitor, substrate concentration **above Km value** decreases the capability of inhibitor to bind enzyme active site.
- In uncompetitive inhibitor, substrate concentration **relative to Km value** improves the binding.
- Allosteric (noncompetitive) inhibitors bind independent of the substrate molecule.

Cofactors

- Enzymes require cofactors for **structural integrity or to assist in the enzyme reaction.**
- The enzyme may be purified with cofactor, or additional cofactor may need to be added to maximize enzymatic activity.
- The affinity of the cofactor will also influence whether a compound that competes with cofactor binding can be identified.
- The amount required depends on the level of activity needed

- Stability of a cofactor needs to be considered for the time and environment that the cofactor will be exposed to during an HTS run.
- For example, some cofactors are **light sensitive** (iron guanylylpyridinol), while others **can change redox state** in common buffers without reducing agents (iron salts).
- The timing of these modifications must be considered and tested to assure compatibility with the HTS process.

Buffers

- In vitro biochemical assays are performed at near physiological pH in an attempt to mimic the intracellular environment of the native enzyme.
- For cytosolic proteins, pH=7.4 can be maintained by a number of buffers including Tris, HEPES, MOPS, and sodium or potassium phosphate buffers.

- Components of reaction mixture can interact poorly with certain buffers resulting in suboptimal assay conditions and affecting the reproducibility of an assay.
- Free amine group in the tris buffer react with enzymes and/or substrates, altering the equilibrium of the system

Receptor-Ligand Binding-Based HTS Assays

- Radioactive method and fluorescent-based methods.
- Radioactive methods like a filtration and scintillation proximity assay (SPA)
- Both these methods use radiolabeled ligand and membrane.
- Reagents used for binding assays should have high specific activity, indicated by radioactivity/ molecule of ligand, and its unit is Curies per millimole.

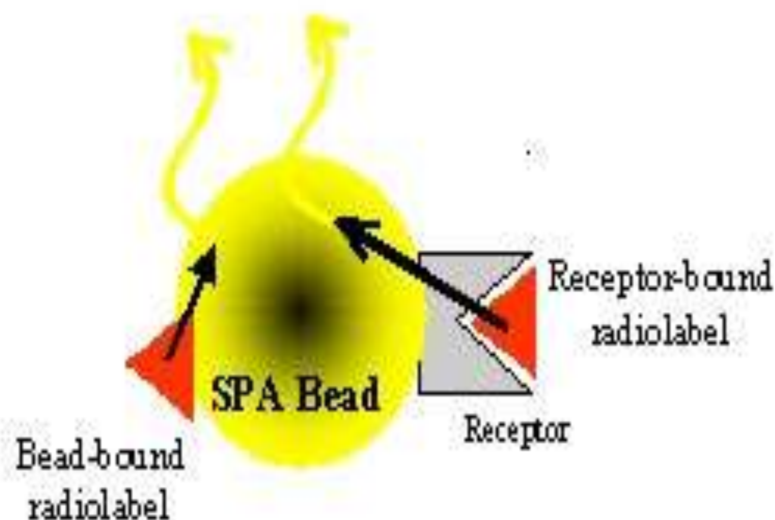
- Nonspecific binding of reagents used in binding assays can be reduced by coating filters with bovine serum albumin (BSA).
- Radiochemical purity of ligand should be above 90%.
- The purity of radiolabeled ligand decreases with time.

- Highly selective ligand will provide more reliable result.
- 125I-labeled ligands - stable for 1–2 months
- Tritated ligands have 3–6 months of stability.
- Beta energy released by 3H
- 125I releases both gamma and beta energy.
- For flash plate assay 125I will be ideal candidate.
- 3 H- or 125I-labeled ligands -SPA

Assay Formats

SPA scintillation proximity assay (SPA)

- Homogenous assay, which can be run in 96- or 384-well format.
- Cell membranes are attached to SPA beads.
- When radio ligand binds to receptor, the proximate radio ligand transfers beta energy to scintillant in the bead and produces a signal that can be measured using microplate scintillation counter.

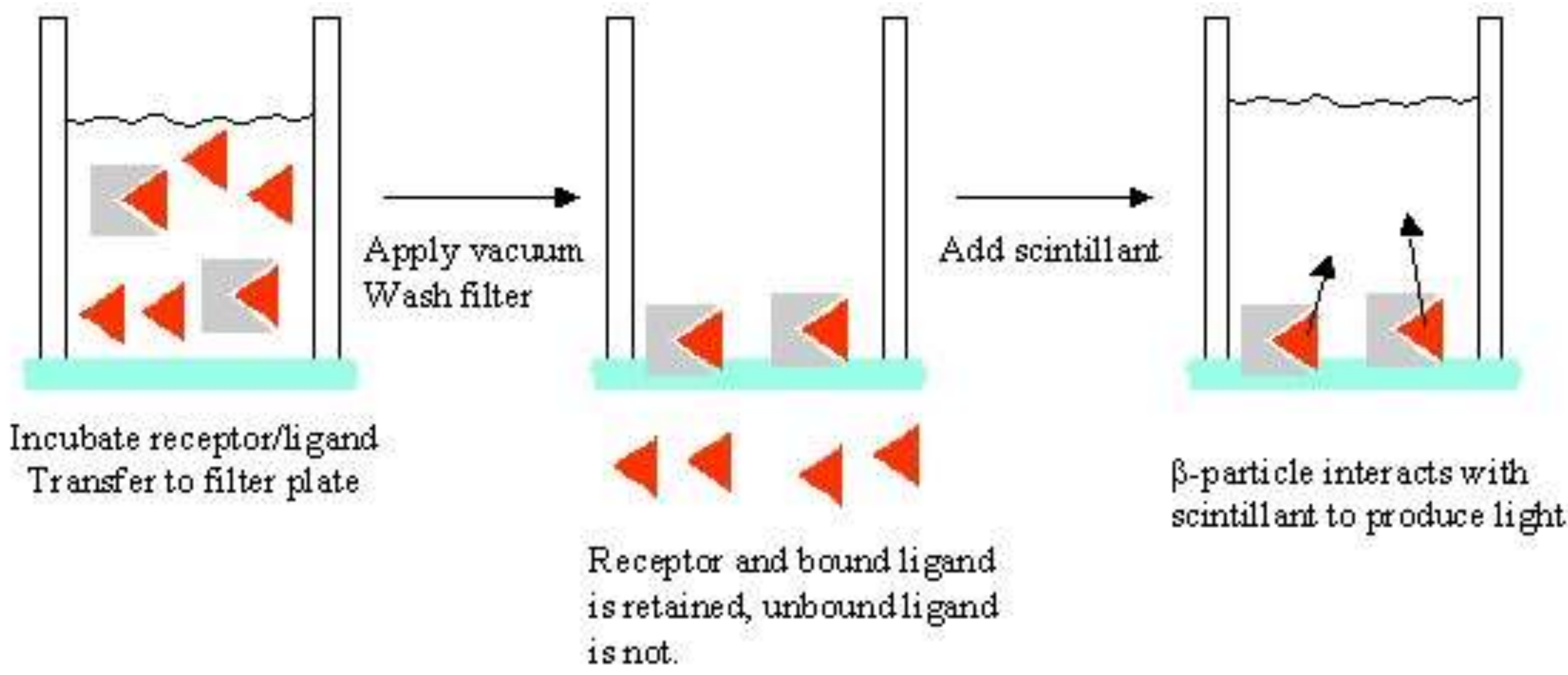


Non-specific binding to SPA Bead

Non-proximity effects

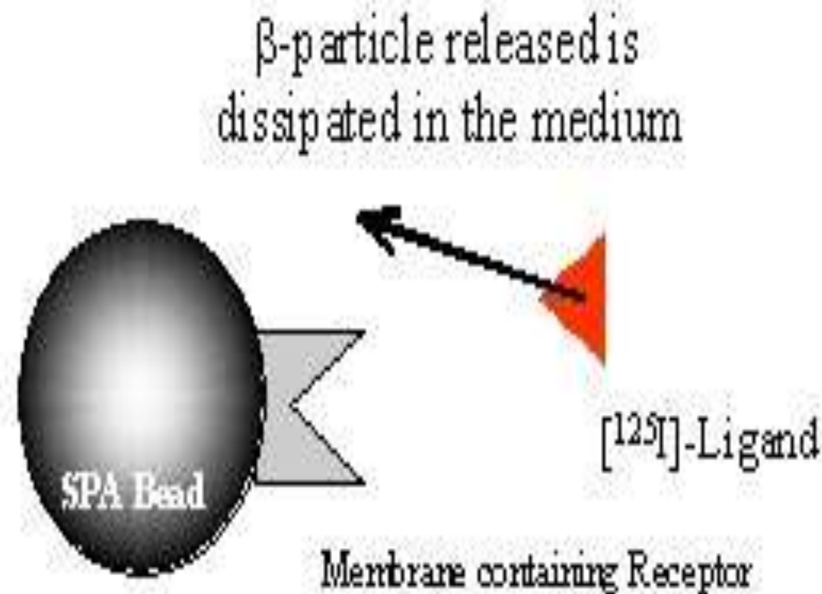
Filtration Ligand Binding Assay

- Separation of free radio ligand and radio ligand bound to the receptor is required for measurement.
- FBA is carried out first in assay plate.
- After adding and incubating assay components, unbound ligand is removed by applying vacuum, while the bound ligand remains attached to the filter.
- The liquid scintillation cocktail or liquid scintillator is added to dried filter.
- Result is measured using microplate scintillation counter.

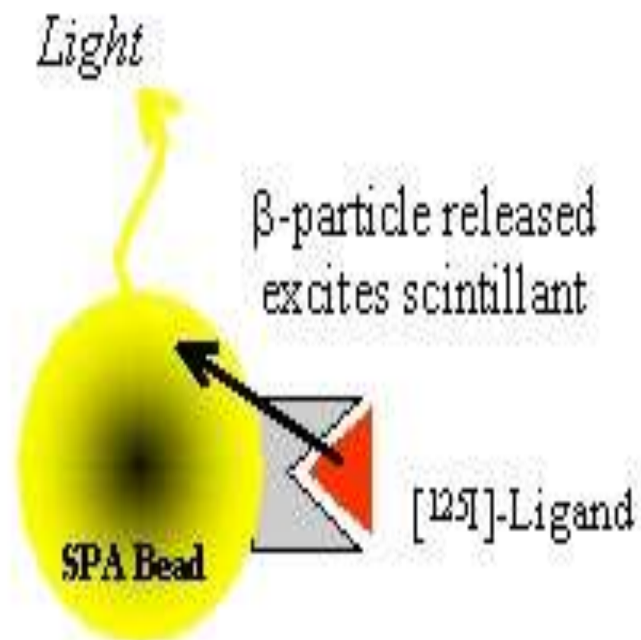

Apply vacuum
Wash filter
Add scintillant
Incubate receptor/ligand
Transfer to filter plate
Receptor and bound ligand
is retained, unbound ligand
is not.
β-particle interacts with
scintillant to produce light

Flash Plate Ligand Binding Assays

- Target receptor or receptor membrane is bound to wall of flash plate.
- Energy from radio ligand interacts with scintillator bound in the wall of flash plate, producing signal.
- Unbound ligand far from plate can't produce signal, which distinguishes bound and unbound ligand



Unbound Radioligand



Bound Radioligand

Acknowledgement

- The Presentation is being used for educational and non commercial Purposes
- Thanks are due to all the original contributors and entities whose pictures were used in the creation of this presentation.