

Strategies for combining high throughput screens with orthogonal read-outs for hit validation against viral protein-protein interaction targets

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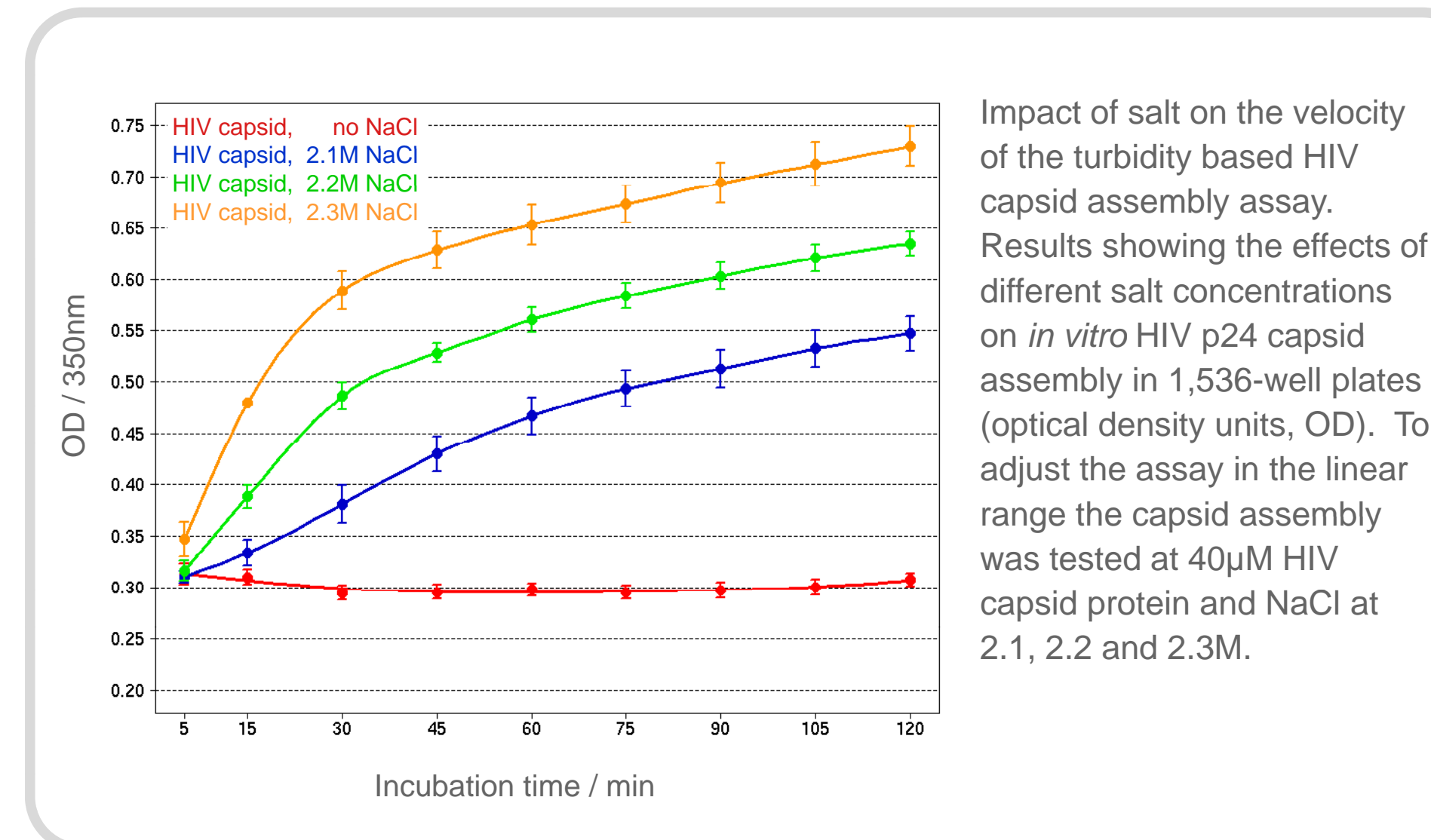
Introduction

Evotec is a global service provider along the drug discovery value chain progressing new chemical entities identified during high-throughput screening (HTS) to clinical candidate nomination. Besides other target classes Evotec has key expertise in protein-protein interaction targets which still present challenges for assay development and subsequent transfer to HTS. This presentation describes progress toward protein-protein interaction targets with a specific emphasis on the identification of novel early stage drug candidates interfering with viral replication. Results of two case studies with HTS against viral capsid assembly assays for HBV and HIV are discussed. General strategies for combining HTS with orthogonal and biophysical assay tools for hit compound validation are presented.

Virus assembly is a particularly attractive candidate for antiviral intervention because viral structures are formed by multiple, relatively weak non-covalent interactions and represent essential building blocks of all virions. In addition, high resolution capsid and capsid-inhibitor crystal structures are available and provide the opportunity for rationale drug design. Recently, small molecule inhibitors have been described that interfere with HBV (HAP-1, Deres *et al.*, Science 299, pp893-896) and HIV (CAP-1, Kelly *et al.*, J. Mol. Biol. 373, pp355-366) capsid formation and thus viral infectivity.

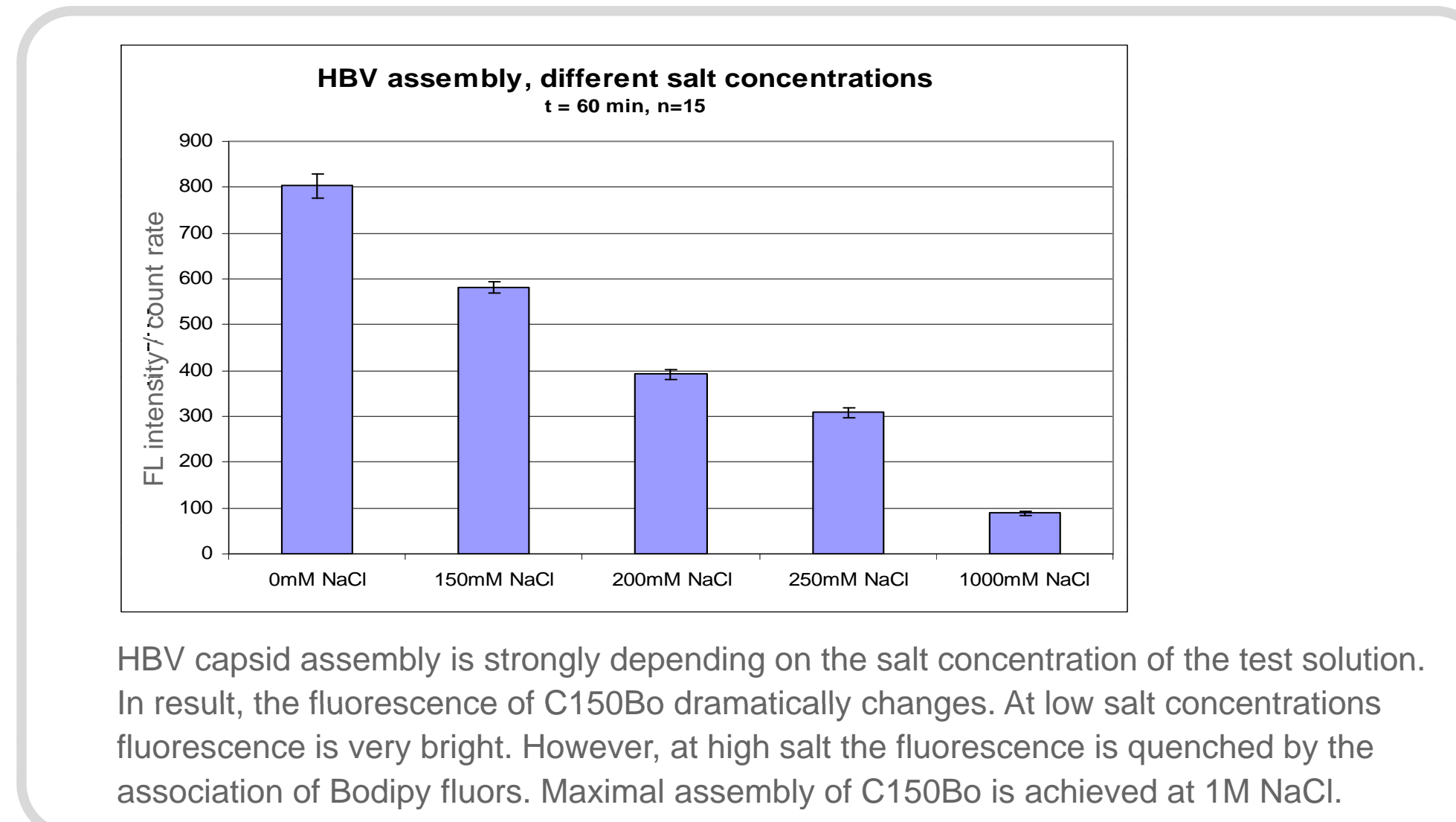
The goal of this drug discovery program was to identify small molecules affecting the assembly process of HBV and HIV capsid protein and that can be developed to an antiviral drug. The strategy applied to identify such inhibitors was to screen a focused set comprising about 55,000 compounds of Evotec's lead and fragment-like libraries as well as compounds derived from a virtual screen.

Adjustment of HIV capsid assembly assay



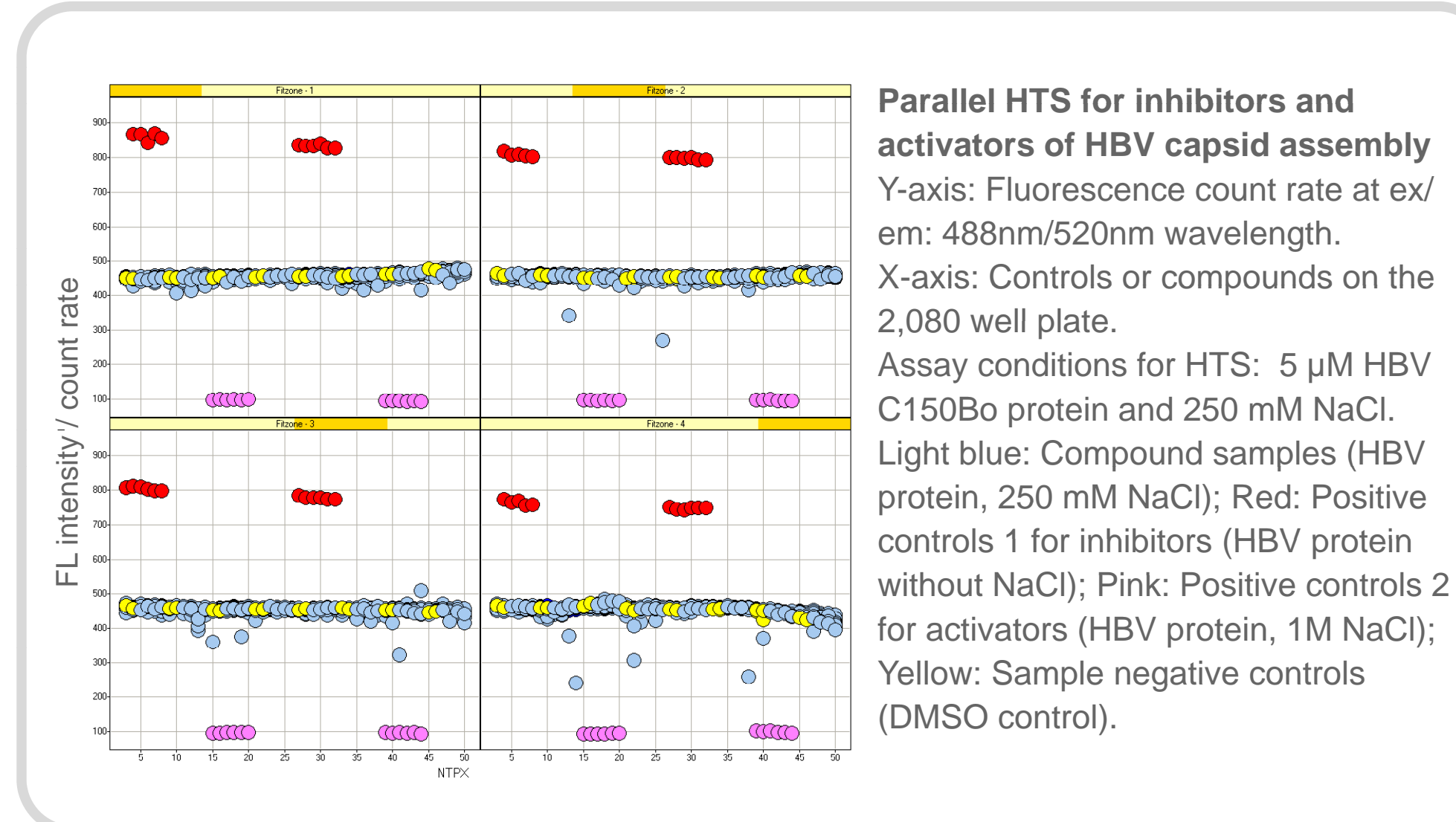
The primary assay applied to screen for HIV capsid assembly inhibitors was based on a turbidimetric readout (Tang *et al.*, J. Mol. Biol. 327, pp1013–1020). Recombinant full length HIV-1 p24 capsid protein (residues 1-231) was expressed in *E.coli* and applied during HTS screening for capsid assembly inhibitors.

Adjustment of HBV capsid assembly assay



To monitor the aggregation of HBV capsid protein during HTS a homogeneous fluorescence based assay was developed according to a published protocol (Zlotnick *et al.*, Nature Protocols 2, pp490-498). For this purpose, recombinant HBV capsid domain comprising residues 1-150 was expressed in *E.coli* and modified with a Bodipy dye at a C-terminal cysteine (C150Bo). In this assay capsid assembly leads to self quenching of the labelled C150Bo capsid protein and thus to low fluorescence intensity of the sample.

Performance of HBV capsid assembly during HTS

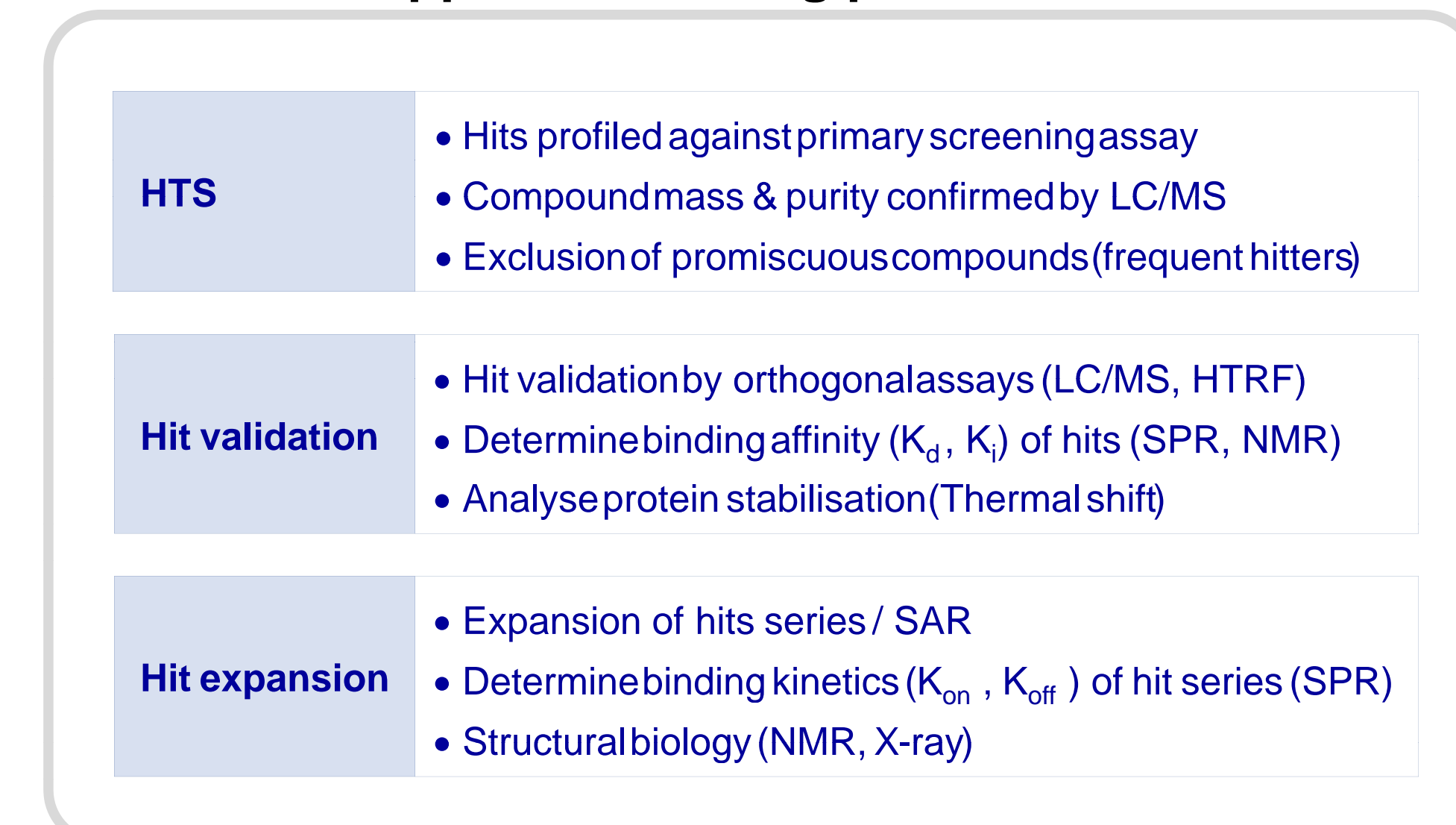


The fluorescent HBV capsid assembly assay was miniaturised to a 2,080-well assay plate format (1µl/well) and adapted to Evotec's screening device for HTS (EVOscreen™ Mark II). Compounds were reformatted to one 2,080 plate, which is subdivided into 4 control lines and 4 compound areas. In the miniaturised format the assay shows excellent statistical robustness with typical Z' values of 0.7.

Results of HTS runs against HIV and HBV capsid assembly

| | HIV capsid | HBV capsid |
|-----------------------|---------------|--------------|
| Assay read-out: | Turbidimetric | Fluorescence |
| Plate format: | 1,536 wells | 2,080 wells |
| Assay volume: | 6µl/well | 1µl/well |
| Performance (Z') | 0.68 | 0.7 |
| Library size: | 55,000 | 55,000 |
| Primary hits: | 883 | 706 |
| Confirmed hits: | 554 | 461 |
| Hit series/singleton: | 23/40 | 25/27 |
| Overlapping hits: | 5 | |

Flowchart of applied screening process



Screening for novel chemical matter within large compound libraries typically is performed in a three stage process. Primary screening of diverse or target-focused libraries ideally includes compound profiling against an orthogonal assay readout. In result, artefact hits are sorted out and an initial SAR can be projected. Next phase is validation of hit compounds by help of various biophysical technologies finally followed by expansion of the SAR and initiation of structural biology studies (X-ray, *in-silico* models).

Biophysical technologies for hit compound validation



This summary outline shows a collection of the most relevant biophysical technologies routinely applied in drug discovery for hit compound validation.

Summary

HIV and HBV capsids are interesting targets for small molecule drug discovery. For both viral proteins capsid assembly assays have been established, miniaturised and employed in two sequential HTS runs of 55,000 member compound libraries each. Besides showing excellent statistical assay performance in both screens a large number of target selective hit compound have been identified. Many of the confirmed hit compounds show clear structure-activity relationship. In a next step, a representative number of the hit compounds will be re-synthesised and validated by help of biophysical technologies like e.g. SPR or NMR and cell-based viral replication assays.