Protein-Protein interaction approaches to virology with HTRF assays

Abstract

Viruses rely heavily on interactions with their host to sustain their life cycle. For this reason, several steps of this cycle have long been considered desirable targets for assay development and drug discovery.

Initially, research was successful at generating peptide and antibody inhibitors for several critical interactions in viral life cycles, most often deriving those peptides from one of the target interaction partners. Small molecule inhibitors remained more challenging to access yet more desirable. In recent years, new assay formats have taken on this challenge, resulting in promising advances and potential inhibitors identifications.

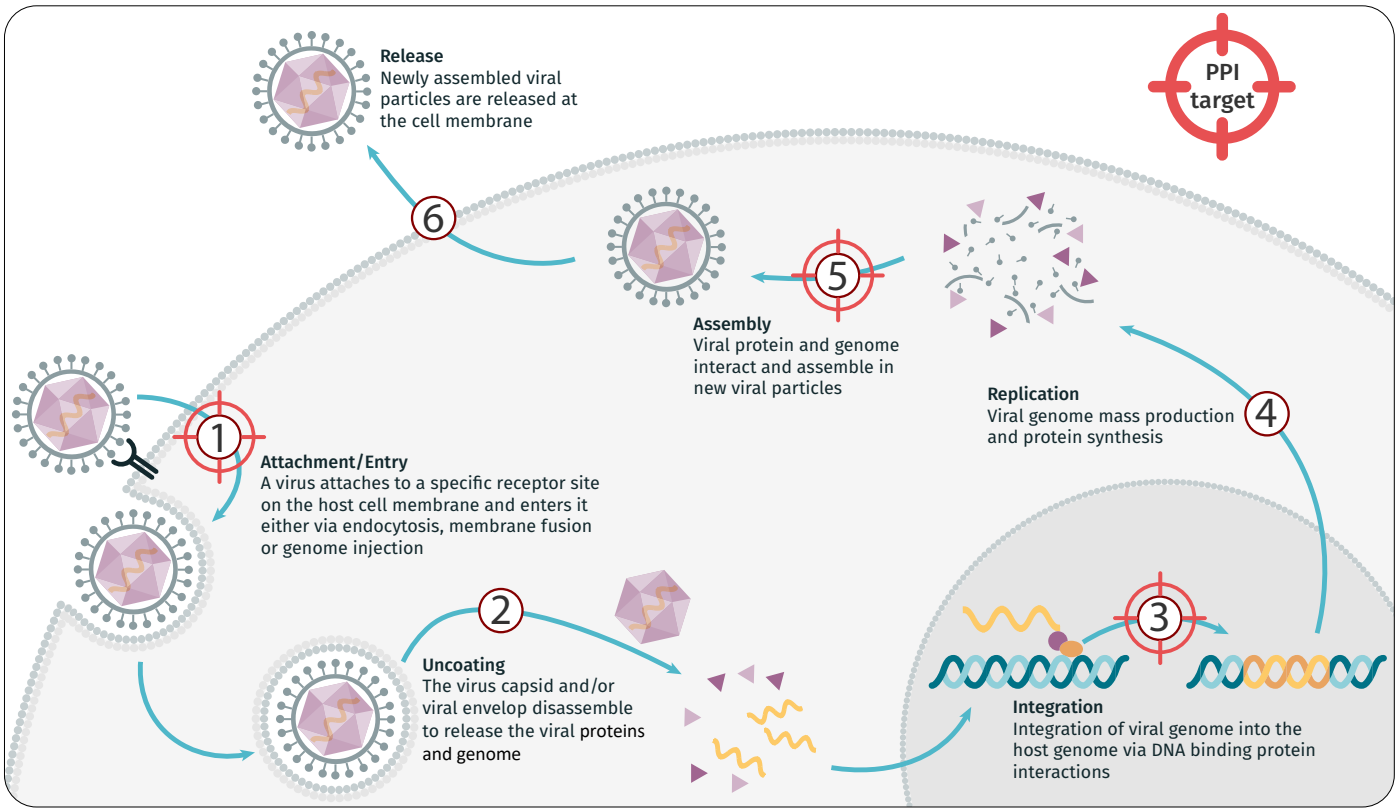

Figure 1: Viral life cycle

Attachment

Newton, P. et al. (2012) Journal of Biomolecular Screening 18(3):237-46

Viruses recognize and infect a host cell through surface receptor interactions. Such interactions are potential targets to prevent virus infection from the start and contain propagation to other cells.

An example of these interactions is the attachment process of rhinoviruses (HRV), where the virus binds to the cell surface protein ICAM-1. In 2012, Newton *et al* from Medimmune investigated this relationship and developed an HTRF HTS assay suitable for attachment inhibitor screening. The resulting assay used live viruses (HRV16) for increased relevance (Newton *et al,* 2012).Following validation, the assay was implemented in a single-point HTS format and used to screen 3344 antibody fragment chain samples (scFv), 52 of which were hits (50% inhibitions). The assay was then used to further investigate hits for dose-response profiles, along with their corresponding complete IgG equivalents (2 examples in fig 3).

Along with the identification of novel potential HRV-ICAM-1 binding inhibitors, the results of this study include a new homogeneous HTS assay format for virus-host cell receptor interaction inhibitor screening. The format is fully compatible with live viruses and may have a wider application in other virus-cell receptor interactions.

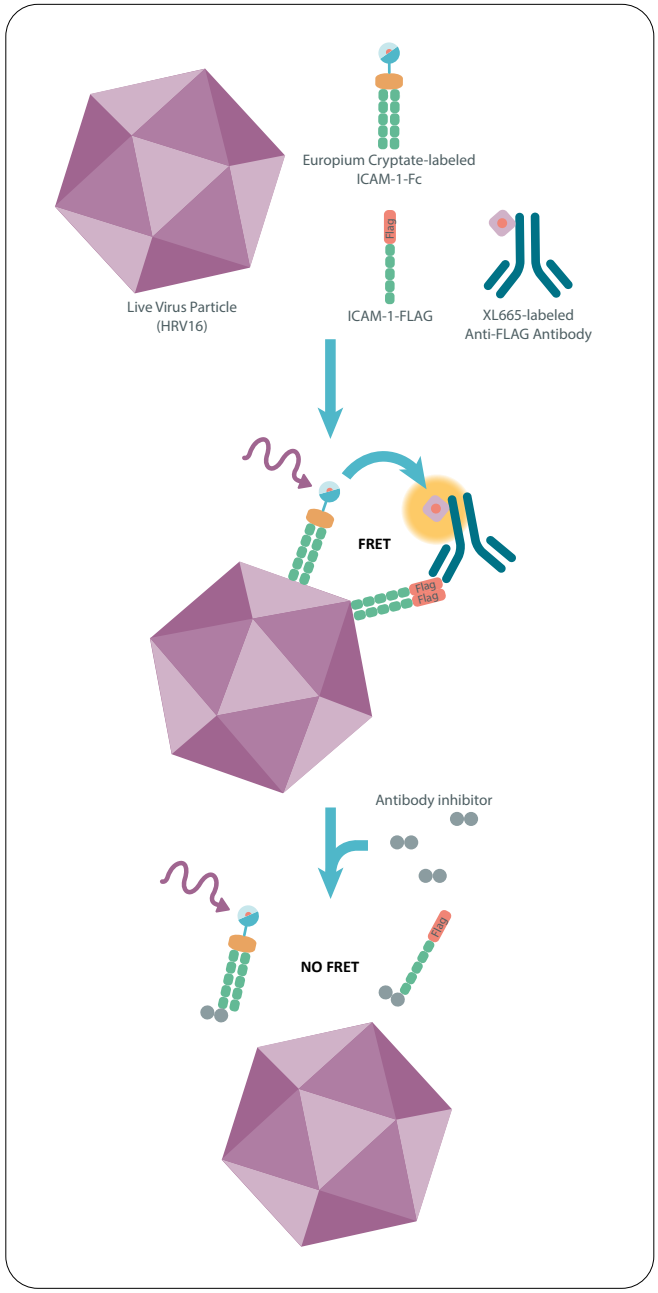

Figure 2: Assay format – ICAM1-Fc and ICAM1 labeled with Europium cryptate (donor) and Flag peptide respectively are incubated with live HRV particles and anti-Flag antibodies coupled with XL665 (acceptor). The virus-ICAM1/ICAM1-Fc binding brings the Europium cryptate (donor) and XL665 (acceptor) in proximity and triggers FRET. The addition of anti-ICAM1 antibodies creates inhibits HRV/ICAM1 binding and shuts off FRET.

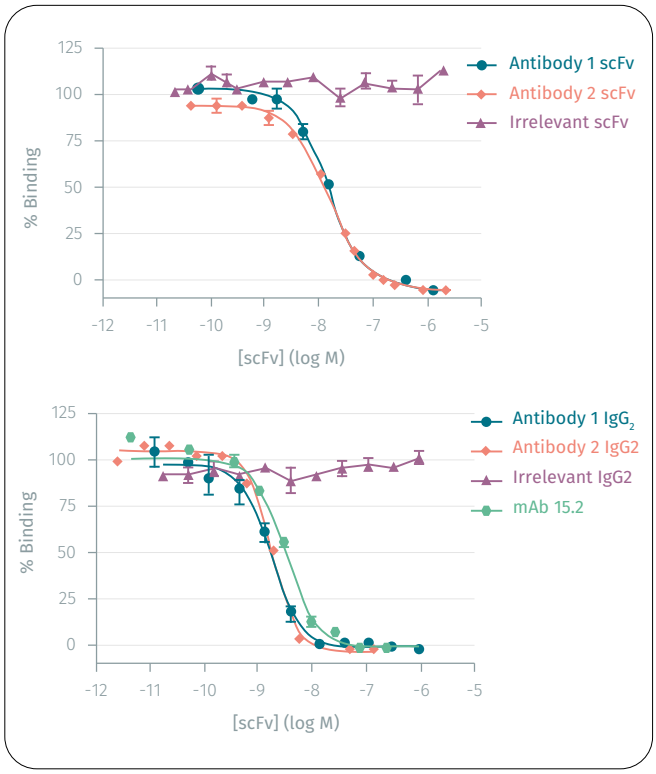

Figure 3: Dose-response analysis of 2 antibody scFV and their corresponding complete IgG – The addition of scFv and IgG gradually inhibits the HRV particle binding to ICAM1.

Integration

Tsiang M, et al. (2009) J Biol Chem 284(48):33580-99

Viruses often take advantage of DNA-binding proteins when going through a genome integration. Disrupting the interaction between a virus' integrase proteins and their DNA-binding host cell partner is an opportunity to rupture their life cycle.

In the case of HIV-1, the integration of reverse transcripted genomes depends on an interaction of dimerized Integrase with the Lens Epithelium Derived Growth factor of its host cell (LEDGF). In 2009, Tsiang *et al* from Gilead developed two HTRF based assays that allowed for the first kinetic characterization (equilibrium and kinetic constants) of these HIV-1 Integrase dimerization and Integrase Dimer/LEDGF interaction. This opened opportunities for competing format screens looking for HIV-1 integration inhibitors (Tsiang *et al*, screens looking for HIV-1 integration inhibitorsscreens looking for HIV-1 integration inhibitors (Tsiang *et al*, 2009).

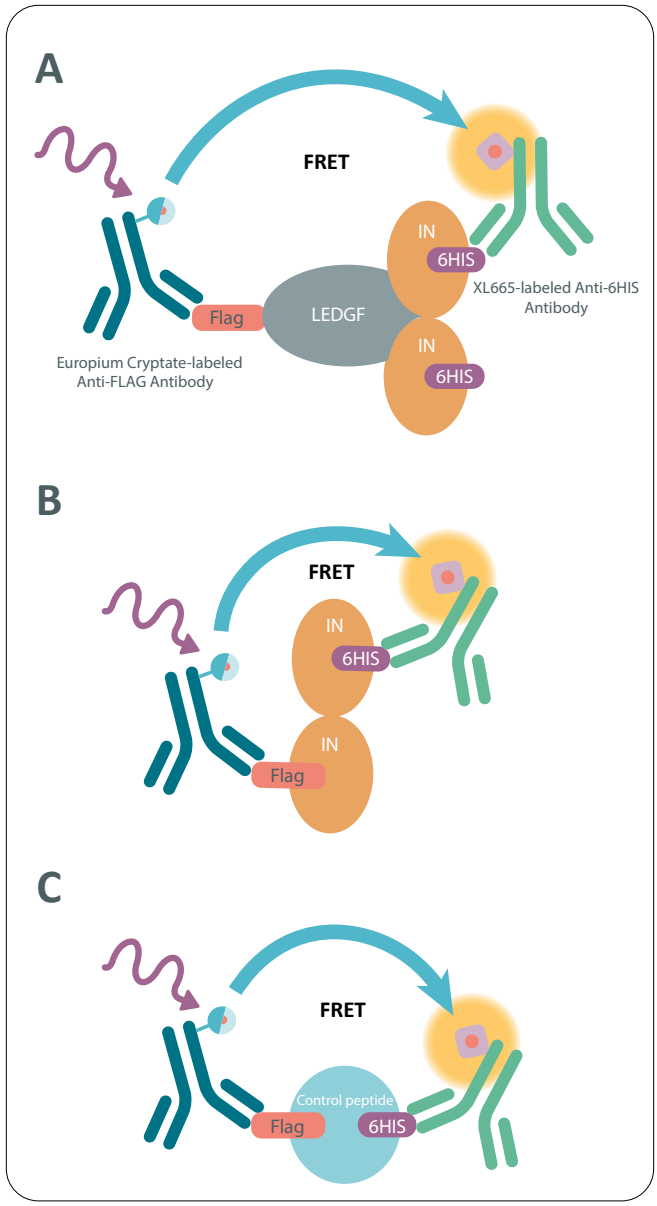

Figure 4: Assay format – Anti-Flag and Anti-6HIS antibodies are coupled with Eu+ cryptate (donor) and XL665 (acceptor) respectively. (A) LEDGF and Integrase (IN) are labeled with Flag peptide and 6HIS respectively to monitor the interaction of Integrase dimer and LEDGF. (B) Two populations of Integrase are labeled with Flag peptide and 6HIS respectively to monitor the Integrase Dimer dimerization. (C) A control peptide is designed with and N-terminal 6HIS tag connected to a C-terminal Flag tag to identify potential positive inhibitors that would impair antibodies binding.

After validation, the determination of Kd, Kon, Koff and Ki of Integrase dimerization and Integrase dimer/LEDGF interaction led the authors to conclude a significantly tighter nature of the Integrase dimerization over the Integrase/LEDGF interaction.

The researchers went a step further and used this assay to investigate peptides and small molecules as potential inhibitors of the Integrase Dimer/LEDGF interaction (table 1).

Table 1: IC50 of various inhibitors tested

Inhibitor	IC50
Tag-free LEDGF	91 ± 7 nM
MBP-IBD (Maltose Binding Protein fused to LEDGF Integrase Binding Domain)	565 ± 9 nM
MBP-IBD mutated on previously identified hot spot I365 and D366	10200 ± 1500 nM
LEDGF Integrase Binding Domain derived peptide #1 (354-378)	2.02 ± 0.05 nM
LEDGF Integrase Binding Domain derived peptide #2 (360-370)	11.9 ± 0.6 nM
LEDGF Integrase Binding Domain derived peptide #3 (400-413)	514 ± 109 nM
Tetraphenylarsonium chloride (TPAsCl), known to co-crystallize at the LEGDF binding pocket of HIV-1 integrase	400 ± 7 nM

The results further validated the assay and allowed for distinguishing the inhibitory potency of different inhibition approaches, as well as characterizing the inhibitory potency of slightly different mutated inhibitors.

The IN-LEDGF HTRF assay described in this publication has been continuously validated and used in various publications since then, such as Kessl *et al* (2012), Carcelli *et al* (2017) or Zhao *et al* (2018).

Assembly

Kota S, et al. (2010) Assay Drug Dev Technol 8(1):96-105
Thenin-Houssier S, et al. (2010) Antimicrob Agents Chemoter 60(4):2195-2208

Viral capsids are structured in repetitive subunits interacting in geometric patterns (Prasad & Schmid *et al,* 2013). For drug discovery purposes, attempts have been made to modulate the interaction of capsid proteins and prevent them from encapsulating viral nucleic material, thus erasing a virus' ability to propagate.

A recent success is the work of Kota *et al* which proposed an HTRF assay approach to screen LOPAC and CMLD-BU libraries (3520 compounds total) for Hepatitis C Virus (HCV) assembly inhibitors (2010). They focused on HCV's most conserved capsid protein Core, whose dimerization is critical for the virus assembly (McLauchlan, 2000) (Kota, 2009).

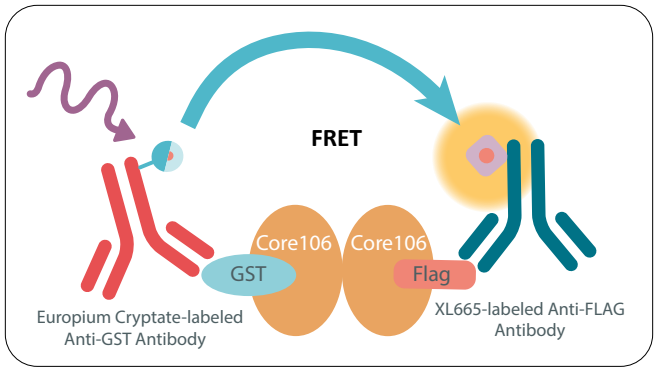

Figure 5: Assay format – Core proteins are tagged on the N-terminal domain 106 last amino acids with GST and Flag peptide respectively. Anti-GST and Anti-Flag antibodies are coupled with Eu+ cryptate (donor) and XL665 (acceptor) respectively. The dimerization of Core proteins via their 106 N-terminal domains brings the donor and acceptor-coupled antibodies in proximity and triggers FRET. The addition of tag-free Core106 domain creates competition and shuts off FRET.

The assay the authors developed displayed a range from 1.7 to 3.0 and checked for robustness with Z values scattering from 0.56 to 0.72. Investigations around experimental conditions and assay formats showed a decrease in false positives when adding detergent (preventing protein aggregation) but failed to notice differences in performance between direct coupling of fluorophore and fluorophore-coupled commercially available antibodies.

The authors also found their HTRF assay format to be HTS compatible with the ability to identify small molecule inhibitors with higher potency than co-derived peptides obtained from other approaches in a previous publication (Kota S, 2009).

The screening of the CMLD -BU library (2240 compounds) resulted in 10 hits and a Z factor of 0.52. One of these hits (SL201) was further investigated for a dose-response profile and exhibited an IC50 of 9.3 mM. It was determined to have an inhibitory effect on HCV production in culture but only after viral infection was established, still showing potential for the virus life cycle inhibition.

Thenin-Houssier *et al* have adapted the same assay format and TR-FRET based approach to capsid proteins (CA) of HIV-1. They successfully identified the small molecule Ebselen as a potential inhibitor of the virus assembly, more precisely inhibiting CA dimerization on its C-Terminal Domain (CTD) during the virus assembly. (Thenin-Houssier S, 2016).

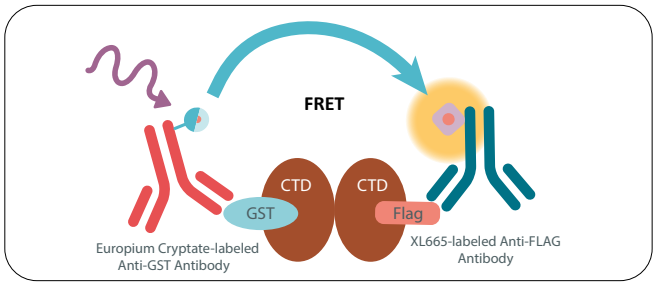

Figure 6: Assay format – CA proteins C-Terminal Domains (CTD) are tagged with GST and Flag peptide respectively. Anti-GST and Anti-Flag antibodies are coupled with Eu+ cryptate (donor) and XL665 (acceptor) respectively. The dimerization of CTDs brings the donor and acceptor-coupled antibodies in proximity and triggers FRET.

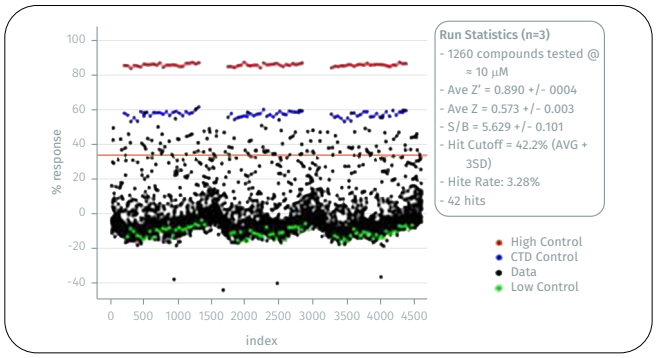

Figure 7: Screening of LOPAC and CMLD-BU compound libraries - % of inhibition obtained for all compounds.

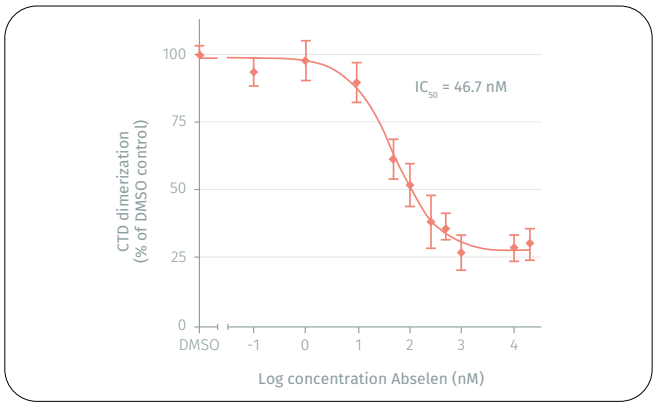

Figure 8: Dose-response analysis of Ebselen – The addition of Ebselen inhibits the CA protein C-Terminal Domain dimerization with an IC50 of 46.7nM.

Conclusion

While the research for viral PPI inhibitors has focused on designing interaction partner-derived peptides and antibodies, screening technologies including HTRF have now reached a satisfactory level of relevance, robustness and ease to make HTS assays an easy and reliable way to investigate small molecule-like drugs. These claims are supported by various publications that have demonstrated the relevance of HTRF approaches to perform research and propose different assay formats suited for tackling viruses in several steps of their life cycle.

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Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
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