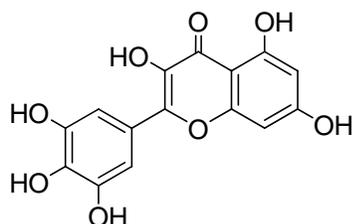


Project: Inhibitors of DNA Polymerase III Holoenzyme System, Primase

Probe: 3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-4H-chromen-4-one



SID: 11111487

Probe Synonyms: myricetin, Cannabiscetin, Myricetol, Myricitin, LOPAC M-6760
Internal ID: NCGC00015697, NCGC00094083

PubChem Bioassay Identifiers (AID):

SID	AC50 (μM)	Anti-target	Selectivity*
11111487	30	Clamp Loader	20
11111487	30	Core Polymerase	9.2

*Selectivity = antitarget IC50/target IC50

Assigned Assay Grant #: MH077636-01

Screening Center Name: NIH Chemical Genomics Center
Principal Investigator of Screening Center: Christopher Austin

Assay Submitter & Institution: Charles McHenry, University of Colorado
Assay or Pathway Target: Inhibitors of *E. coli* DNA Polymerase III Holoenzyme, primase.

Assay provider information

Specific Aim: The overall goals of this proposal are two-fold. We will explore the DNA replication apparatus as a target for antibacterial discovery and, with the access to the Molecular Libraries Screening Centers Network (MLSCN) compound collection and high-throughput screening (HTS) resources, develop a set of reagents to permit development of 'chemical genetic' approaches to study DNA replication in model Gram (-) and Gram (+) organisms. Such studies should provide a prototype for application of the technology developed to eukaryotic systems. To enable this, we have and will propose a robust assay suitable for HTS against the DNA replication pathway of *E. coli*. Ongoing studies will implement specificity assays to permit elimination of non-specific compounds and counterscreens against the eukaryotic replication apparatus, to permit identification of compounds that are specific against bacterial targets. These goals will be accomplished by pursuit of the following specific aims:



1. Transfer and run the HTS assay for the DNA polymerase III Holoenzyme (Pol III HE) from the representative Gram (-) model organism *E. coli*. We have developed a 384-well plate format HTS assay for the DNA polymerase III holoenzyme elongation system from *E. coli* for the discovery of small molecule inhibitors. We would like to test this screen against the MLSCN compound collection.

2. Prioritize compounds identified via HTS of the MLSCN library for further chemical optimization and development. We will develop and run a panel of specificity assays to screen out apparent inhibitors that act by non-discriminate mechanisms. A panel of deconvolution assays will also be developed and run to identify the precise biochemical target among the many present in our system. We will also implement *in vitro* microbiological assays in order to determine which compounds act at the cellular level and have the potential to be developed as novel antibacterials.

Significance: Bacterial infections continue to represent a major worldwide health hazard. Infections range from the relatively innocuous to the very serious, and potentially lethal, infections in immunocompromised patients. With the recent emergence of numerous, clinically important, drug-resistant bacteria including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, an emergency is becoming apparent. Clearly, novel targets provided by essential enzymes are needed for discovery of new compounds extant in existing compound collections. In response, many companies focus on defined biochemical targets with the intent of detecting small molecules that might be missed in whole cell assays, because they cannot achieve the necessary cellular concentrations, either due to permeability barriers or rapid metabolic inactivation. *In vitro* assays can detect small molecule inhibitors of defined targets that would not be detectable in whole cell systems (1). This is not to say that whole cell assays do not have a place in drug discovery, when imaginatively applied. But, we believe, given the difficulty of obtaining direct readouts for DNA replication in live cells in a HTS formats and the complexity of the replication target, that our approach offers significant advantages.

The process of DNA replication is central to the propagation of all bacteria. To date, no commercially available antibacterial targets any of the enzymes that make up the central replication system in bacteria. The DNA replication machinery of infectious organisms therefore stands as an unexplored target for drug development efforts and presents a significant opportunity. Replication has not previously been targeted in large part because its complexity poses a formidable technological barrier to setting up HTS assays. In addition, since activity of many individual subunits depends on proper association with other components of the replication apparatus, target-based assays using single subunits are generally not feasible. A substantial portion of the system must therefore be reconstituted for drug screening. In this proposal, we present a plan to develop and adapt assays where protein components of the bacterial replication apparatus work together in complex DNA replication reactions. The Pol III HE of bacteria contains approximately 10 subunits that undergo marked changes in protein-protein association during each of nearly 20 identified kinetic steps of the complex processive reaction they catalyze (2-5). Since all of these proteins must function together, the ca. 100 distinct interactions and catalytic events all provide targets for antibacterial action, and thus provide a multiple of the proteins present as targets. We have developed a simple fluorescence-based assay (6), and have adapted this format to a high throughput screen. Such assays are expected to be profoundly more efficient compared to traditional screening assays using single target enzymes, since activities of each of the proteins that comprise the holoenzyme are targeted simultaneously.



We anticipate this will increase the number and the quality of hits obtained from screening the MLSCN collection, thereby providing a richer variety of inhibitors for further development and, in turn, appreciably increasing the odds of successfully identifying effective and novel antibacterials.

Rationale: Early work in McHenry's laboratory established the feasibility of assaying for double-stranded DNA production of a reconstituted *E. coli* DNA Pol III holoenzyme system (2-6), consisting of DNA Polymerase III - ($\alpha\epsilon\theta$) $2\tau3\delta\delta'\chi\psi$, β subunit - processivity factor, SSB - single-stranded DNA binding protein, and Primase. Inhibition of polymerase activity was screened by measuring the production of double-stranded DNA via PicoGreen fluorescence increase. A fully-automated qHTS experiment (7) was performed against a collection of 71,028 compounds tested as 7- to 15-point concentration series at 4 μ L reaction volume in 1536-well plate format. Actives identified from the screen were subjected to confirmatory experiments using the screening assay and subsequently against the individual targets in deconvolution assays.

Screening center information

Assay Implementation and Screening

PubChem Bioassay Names:

AID: 603 qHTS Assay for Inhibitors of DNA Polymerase III Holoenzyme System

List of PubChem bioassay identifiers generated for this screening project (AIDs):

AID	Target	Concentration	Bioassay type
603	Polymerase III Holoenzyme	57 μ M - 3 nM ^a	Primary qHTS

^aSeven point concentration-titration series was used covering the indicated range; 1:5 dilutions.

Primary Assay Description as defined in PubChem:

Overview:

E. coli DNA polymerase III holoenzyme complex was assayed for DNA production by fluorescent detection of the double-stranded DNA product with PicoGreen dye. The holoenzyme complex was reconstituted from the following purified protein components: DNA Polymerase III* - ($\alpha\epsilon\theta$) $2\tau3\delta\delta'\chi\psi$, β subunit - processivity factor, SSB - single-stranded DNA binding protein, and Primase. Single-stranded, circular M13Gori was used as a substrate with dNTPs and NTPs also supplied to the substrate reagent. Compounds from the NCGC collection were incubated with the holoenzyme mixture for 15 min at room temperature to allow binding to occur, and then the reaction was started by the addition of substrate. After 30 min incubation at room temperature, PicoGreen solution containing EDTA was added to stop the reaction and to visualize the double-stranded DNA produced. Reaction progress was measured in a ViewLux CCD imager in fluorescence intensity mode by using 480 nm excitation and 540 nm emission filter set.

Assay Principle, Miniaturization, and Optimization: Using a protocol for the fluorescence-based assay of *E. coli* Pol III HE as a starting point (6), we optimized an assay in a form



appropriate for automated HTS. Optimal assay conditions were determined by varying pH and titrating buffer components including salt, divalent cation (Mg^{2+}), reducing agents, detergents, and glycerol; additionally, DMSO tolerance was verified during the 384-well optimization stage in the assay provider's laboratory (data not shown). *E. coli* DNA polymerase III holoenzyme complex was assayed for DNA production by fluorescent detection of the double-stranded DNA product with PicoGreen dye (6). The holoenzyme complex was reconstituted from the following purified protein components: DNA Polymerase III* - ($\alpha\epsilon\theta$) $2\tau 3\delta\delta'\chi\psi$ (core), β subunit (processivity factor), SSB (single-stranded DNA binding protein), and DNA G primase. Single-stranded, circular M13Gori DNA was used as a template, with dNTPs and NTPs also included in the substrate reagent. The assay was miniaturized to 1536-well format by volume reduction and appropriate adjustment of stock concentrations of enzymes and substrates to reflect the volumes being combined. For example, the assay was started by the dispense of the reconstituted holoenzyme mix at 4/3 of its final concentration to account for the reaction volume increase from 3 to 4 μ L upon substrate addition, while the substrate mix was delivered as 4x concentrated solution to account for its dilution. The above preparations of reagents were tested and found to be stable overnight at 4 $^{\circ}$ C, as needed for the execution of an uninterrupted fully automated screen on the Kalypsys robotic system. Additionally, to capture potential interference from autofluorescent compounds and library members capable of producing strong fluorescent signal upon intercalation into the newly-synthesized DNA, two pre-reads were included in the screening protocol prior to the PicoGreen addition and final measurement steps.

Center Summary of the Primary Screen:

Buffer. 50 mM Hepes-KOH, pH 7.5, 5 % Glycerol, 0.02% NP40, 30 mM potassium glutamate, 7 mM Magnesium Acetate, 16 μ M TCEP.

Reagents/Controls.

Buffer in columns 3 and 4 as negative control (no enzyme).

Substrate solution: 5 μ M each dATP, dGTP, dCTP and TTP, 50 μ M each GTP, CTP, UTP and 200 μ M ATP, 1.4 nM M13gori (final concentrations).

Enzyme: 154 nM SSB, 2 nM Primase, 1 nM Beta, 0.35 nM Pol III (final concentrations).

PicoGreen/EDTA: 10 mM Tris-HCl, 10 mM EDTA, 5 μ M PicoGreen (final concentrations).

Control titration: ChemBridge 5805060 (from 10 mM, then 1:2, in duplicate) was pin-transferred to upper half of column 2.

Assay Steps.

Three μ L of holoenzyme mixture (154 nM SSB, 2 nM Primase, 1 nM Beta, 0.35 nM Pol III (final concentrations)) were dispensed to 1536-well Greiner black solid bottom plates. Compounds and controls (23 nL) were transferred via Kalypsys PinTool (V&P Scientific, Palo Alto, CA). The plates were incubated for 15 min at room temperature, and then 1 μ L of substrate solution (100x Stock: 0.5 mM dATP, dGTP, dCTP and TTP, 5 mM GTP, CTP, UTP and 20 mM ATP, M13gori (1.4 nM final concentration)) was added to start the reaction. After room temperature incubation for 35 minutes, 1 μ L of PicoGreen solution (4 μ M PicoGreen in TE buffer (10 mM Tris-HCl, 10 mM EDTA)) was added to each well and the plates were read 480 nm excitation and 540 nm emission using ViewLux High-throughput CCD imager (Perkin-Elmer, Wellesley, MA) and fluorescence protocol settings. During dispense, the reagent bottles were kept submerged into 4 $^{\circ}$ C recirculating chiller bath to minimize degradation. All screening operations were performed under reduced lighting on a fully integrated robotic system (Kalypsys, San Diego, CA) containing one RX-130 and two

RX-90 anthropomorphic robotic arms (Staubli, Duncan, SC). Library plates were screened starting from the lowest and proceeding to the highest concentration. Vehicle-only plates, with DMSO being pin-transferred to the entire column 5-48 compound area, were inserted uniformly at the rate of approximately one plate for every 50 library plates in order to monitor for and record any shifts in the background.

Table 1. *E. coli* DNA Polymerase III Holoenzyme qHTS protocol.

Step	Parameter	Value	Description
1	Reagent	3 μ L	Enzyme and control solutions
2	Library Compounds	23 nL	57 μ M to 0.7 nM titration series
3	Controls	23 nL	Intraplate titration
4	Incubation Time	15 min	Compound interaction with targets
5	Assay Readout	490/540 nm	ViewLux fluorescence intensity read1
6	Reagent	1 μ L	Substrate
7	Incubation Time	35 min	Enzymatic Reaction
8	Assay Readout	490/540 nm	ViewLux fluorescence intensity read2
9	Reagent	4 μ L	Pico Green
10	Assay Readout	490/540 nm	ViewLux fluorescence intensity read3

Step Notes

- 1 Black solid bottom plates, single-tip dispense, enzyme in columns 1, 2, 5-48, no-enzyme in columns 3 and 4.
- 2 Pintool transfer of library to columns 5-48.
- 3 Pintool transfer of control titration to upper half of column 2.
- 4 Room temperature incubation in auxiliary hotel.
- 5 Pre-read to note highly fluorescent library compounds.
- 6 Addition of nucleotides and ssDNA template.
- 7 Room temperature incubation in auxiliary hotel.
- 8 Pre-read to note fluorescent intercallators.
- 9 Addition of Pico Green as dsDNA detection reagent and EDTA as stopping reagent.
- 10 Endpoint measurement of reaction progress.

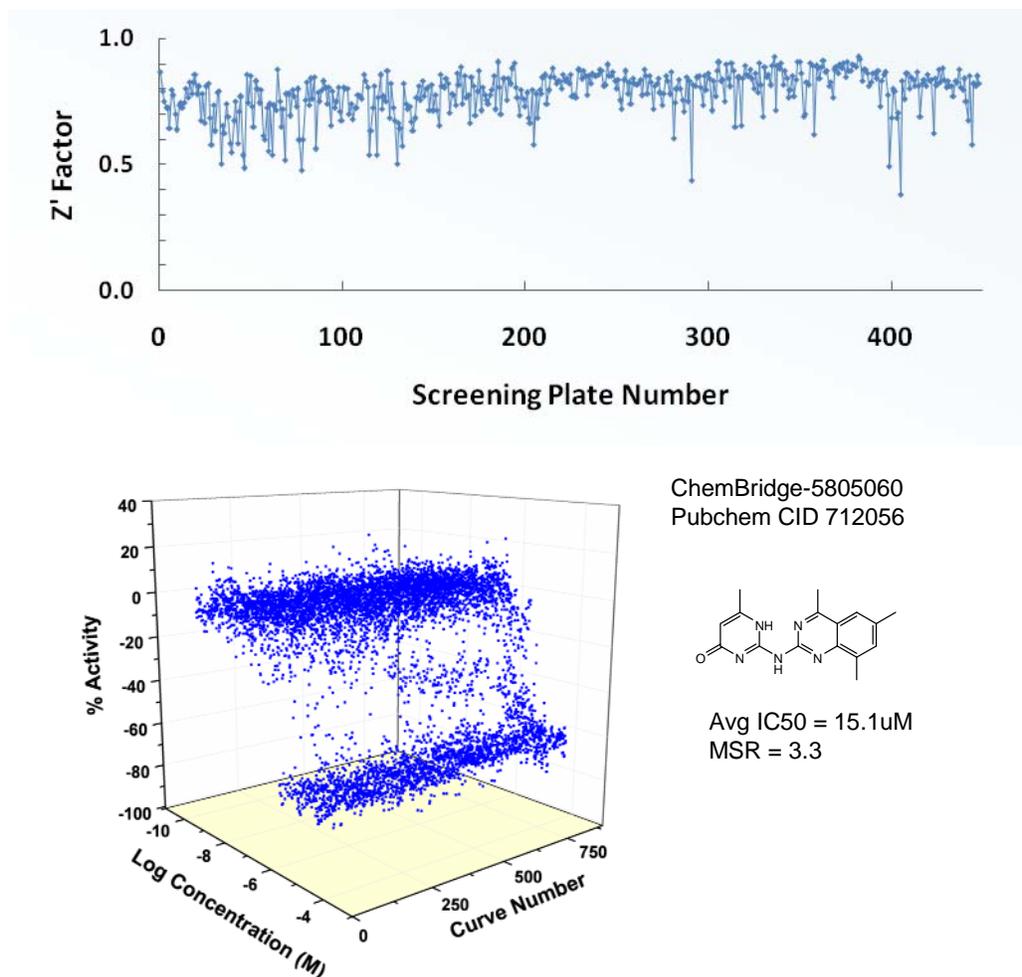


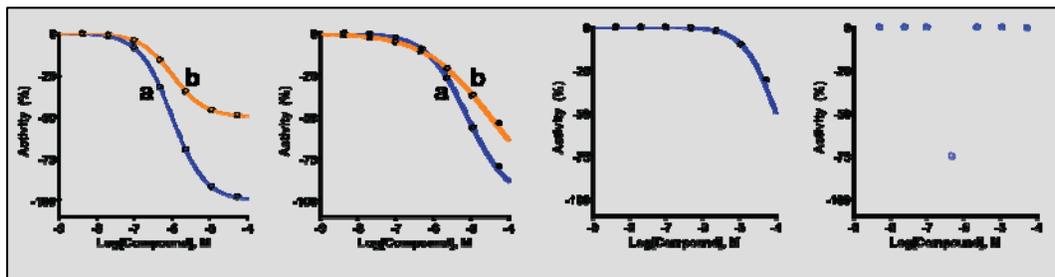
Figure 1. qHTS Performance: Z' trend (A) and intraplate control titrations (duplicate curves per plate) (B) as a function of plate number.

qHTS Summary of Assay results:

In total, 448 assay plates (equivalent to 604,202 samples), corresponding to 438 compound library plates and 10 DMSO-only plates, were screened in one uninterrupted sequence. Dispenser malfunction resulting in excessive bubble formation necessitated the rescreen of 182 plates, and since the issue was noted in real time, the problematic plates were scheduled for re-screening immediately after the end of the main run. In this manner, the re-screened series were tested using the same batch of reagents as last series of the main screen. The assay performed robustly, yielding an average Z' value of 0.78 and an average

signal-to-background of 6.3. Overall, the Z' factor remained flat with the screen progression, with minor shifts tracking the introduction of new batches of the two enzymes (Figure 1). The intraplate control titration was stable throughout the screen progression, resulting in average IC_{50} of 15 μ M and mean significant ratio of 3.3 (Figure 1). Each library compound was tested at a minimum of seven concentrations, ranging from 57 μ M to 2.9 nM, and for each well, 3 data points (2 prereads and one product detection read) were collected.

Identification of Active Clusters: Following the qHTS, the concentration response curve (CRC) data were subjected to a classification scheme to rank the quality of the CRCs as described previously (7). Briefly, CRCs are placed into four classes. Class 1 contains complete CRCs showing both upper and lower asymptotes and r^2 values > 0.9 . Class 2 contains incomplete CRCs lacking the lower asymptote and shows r^2 values greater than 0.9. Class 3 curves are of the lowest confidence because they are defined by a single concentration point where the minimal acceptable activity is set at 3 SD of the mean activity calculated as described above. Finally, class 4 contains compounds that do not show any CRCs and are therefore classified as inactive. For this assay, compounds that showed 'activation' were regarded as active due to fluorescence and were thus filtered out. Remaining compounds that showed signal decrease were considered apparent inhibitors of the assay. Once this active set of compounds was identified, hierarchical agglomerative clustering with a 0.7 Tanimoto cutoff was performed by using Leadscape (Leadscape Inc., Columbus, OH) fingerprints. For each cluster, maximal common substructures (MCS) were extracted, a manual step of MCS trimming was performed to create a list of scaffolds, and any overlapping scaffolds were abridged to a canonical set. Each scaffold was then represented as a precise definition to indicate descriptors such as the number of attachment points or the ring size variability. All filters were then relaxed to include the entire negative (class 4) assay data.



Scheme 1: Example classification scheme for assignment of resulting curve-fit data into classes. Curves fitting the following classification criteria are then used to establish SAR: Class 1 curves display two asymptotes, an inflection point, and $r^2 \geq 0.9$; subclasses 1a vs. 1b are differentiated by full ($>80\%$) vs. partial ($\leq 80\%$) response. Class 2 curves display a single left-hand asymptote and inflection point; subclasses 2a and 2b are differentiated by a max response and r^2 , $>80\%$ and >0.9 or $<80\%$ and <0.9 , respectively. Class 3 curves have a single left-hand asymptote, no inflection point, and a response $>3SD$ the mean activity of the sample field. Class 4 defines those samples showing no activity across the concentration range.

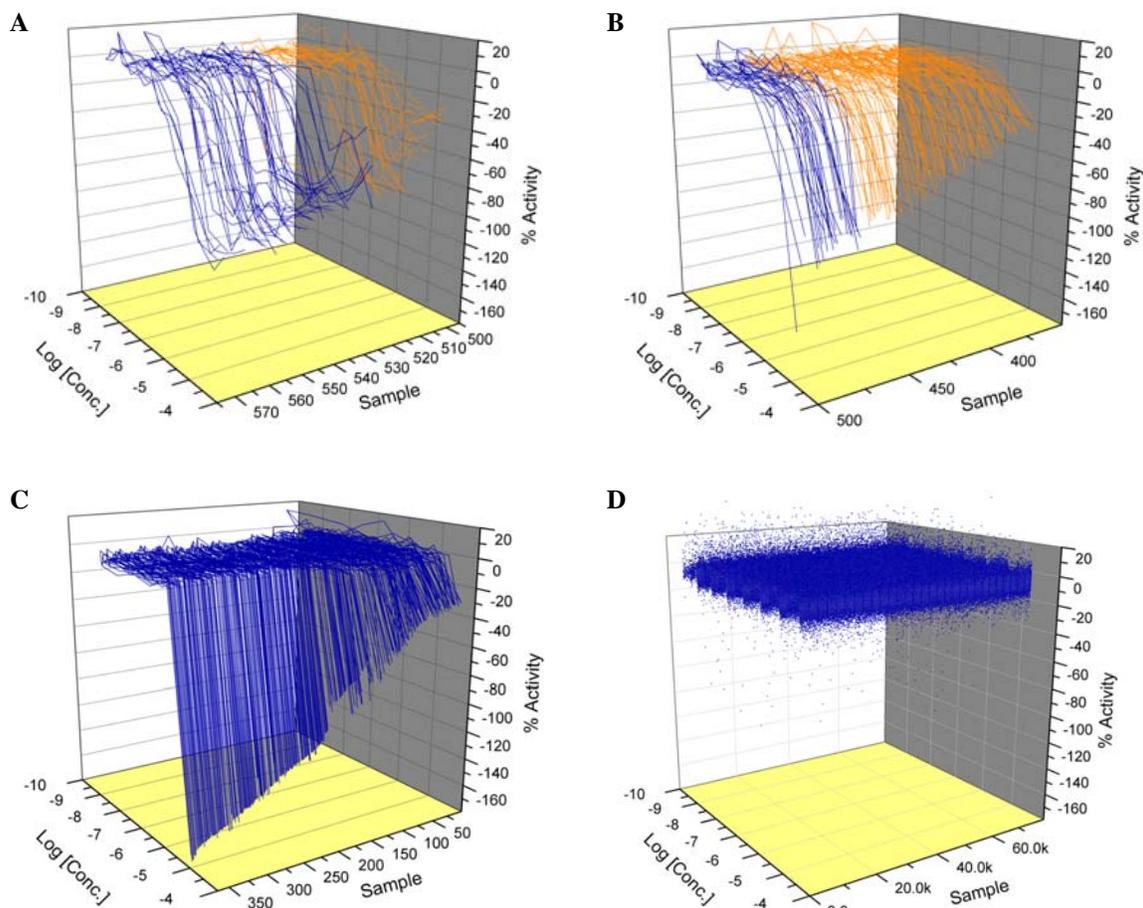


Figure 2. Inhibitors from DNA polymerase III qHTS data (AID 603) binned into curve classifications 1-4 based on curve-fitting classification criteria. A) Class 1a (navy) and class 1b (orange) curves. B) Class 2a (navy) and class 2b (orange) curves. C) Class 3 curves (navy). D) Data with no apparent dose-response, curve class 4 (navy points).

Confirmatory Testing. Thirteen compounds were re-sourced and tested in the primary screening assay as 24-point titrations in 1536-well plates. Further, the same samples were split and aliquots of the 10 mM stock solutions were shipped to the assay provider. Drs. Garry Dallmann and Charles McHenry retested the samples in the holoenzyme screening assay and further subjected them to a panel of target deconvolution assays as detailed in the next section (Figure 3). Summary of the confirmatory and secondary testing is shown in Figure 3.

Probe Characterization

Prior Art: No specific inhibitors for the *E. coli* polymerase core enzyme and primase have been previously reported.

Target Deconvolution Assays. The Primary *E. coli* DNA Polymerase III HTS assay can be broken down into 3 different reaction stages (Priming, Initiation complex formation and

Elongation). Assays for each stage have been developed and used to determine the mechanism of action of inhibitors of the Holoenzyme. **A. Primase Assay.** The assay measures the synthesis of an RNA primer by *dnaG* Primase on SSB-coated M13Gori Phage DNA. **B. Clamp-Loader ATPase Assay.** The assay measures the hydrolysis of ATP concomitant with the loading of a β processivity clamp on a model primer-template. **C. Gap-Filling DNA polymerase Assay.** The assay measures the distributive DNA synthesis of DNA Polymerase III Core (*aeq*) on DNase I activated Calf Thymus DNA.

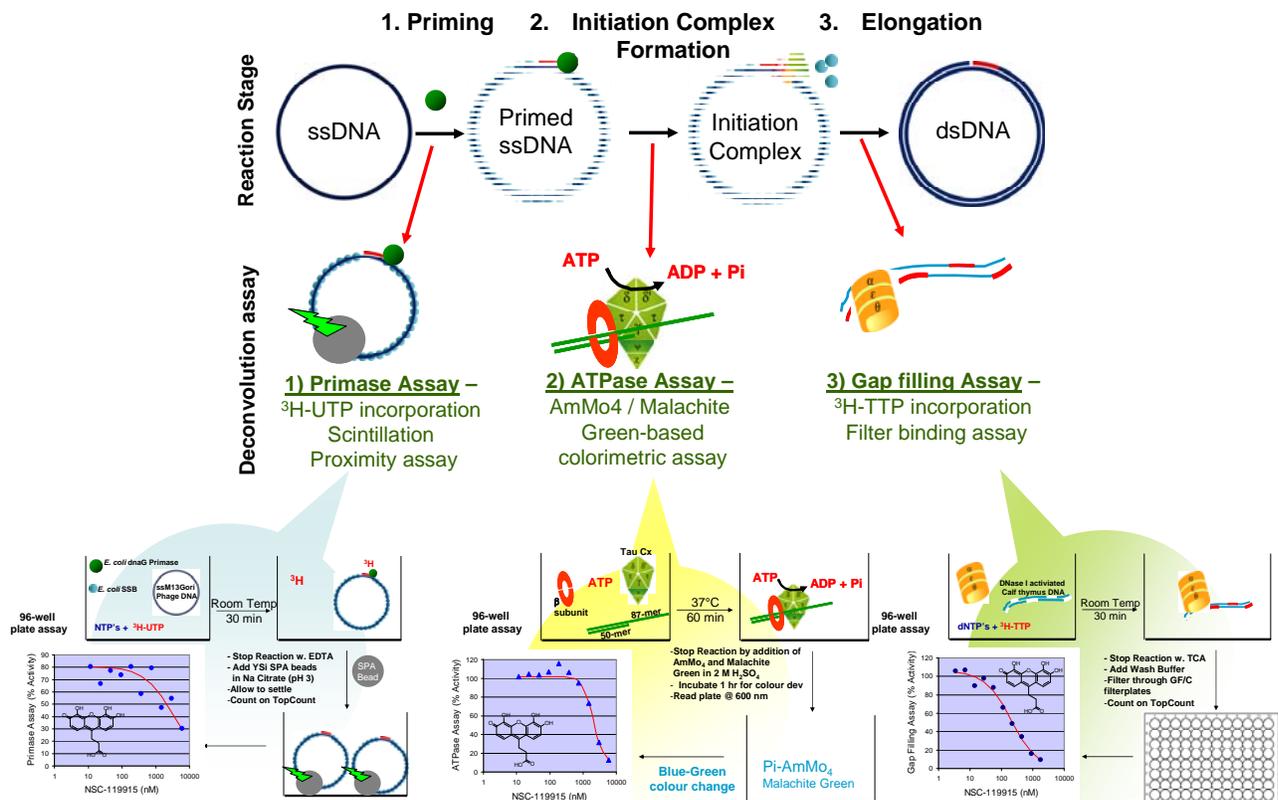
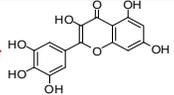
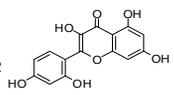
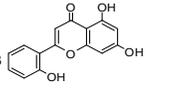
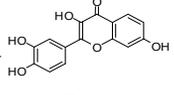
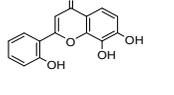


Figure 3. Target deconvolution Assays. Shown is the multistep process leading to a synthesis of a new DNA strand starting from a single-stranded template and NTPs/dNTPs via the catalytic action of the holoenzyme complex. Below, the three distinct steps of the process: creation of short RNA primer (primase), the initiation of DNMA Synthesis (clamp loader), and the processive strand extension (polymerase core) are indicated, along with the respective assays used to address the specific reaction components.

Table 2. Summary of confirmatory and target deconvolution experiments.

Pubchem SID NCGC ID	STRUCTURE	Holoenzyme IC50 (M)	Polymerase core IC50 (M)	Core / Holoenzyme ratio	Clamp Loader IC50 (M)	Clamp Loader / Holoenzyme ratio	Primase IC50 (M)	Primase / Holoenzyme Ratio	LogP	Compound Source	
CID: 5281672 NCGC00015697 PROBE		3.00E-05	2.77E-04	9	>	6.00E-04	20	3.00E-05	1	1.72	SigmaAldrich Lopac-M-6760
CID: 5281670 NCGC00015672		4.11E-05	2.12E-05	1	>	6.00E-04	15	2.40E-05	1	2.01	SigmaAldrich Lopac-M-4008
CID: 5322064 NCGC00017193		inactive	>	1.50E-04		6.00E-04		3.94E-05		2.42	Timtec TNP00057
CID: 5281614 NCGC00017344		4.68E-05	2.12E-04	5	>	6.00E-04	13	8.75E-05	2	2.31	Timtec TNP00284
CID: 676309 NCGC00017191		5.60E-05	2.77E-04	5	>	6.00E-04	11	1.00E-04	2	2.42	Timtec TNP00055

Compound preparation: Compound is prepared in DMSO at 10 mM stock concentration.

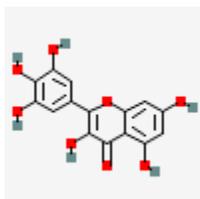
Project related direct publications:

See references 2, 3 for further information.

Known probe properties:

Molecular Weight	318.2351 [g/mol]
Molecular Formula	C ₁₅ H ₁₀ O ₈
XLogP	0.1
H-Bond Donor	6
H-Bond Acceptor	8
Rotatable Bond Count	1
Tautomer Count	516
Exact Mass	318.037567
MonoIsotopic Mass	318.037567
Topological Polar Surface Area	148
Heavy Atom Count	23
Formal Charge	0

Properties of probe compounds as reported in PubChem:



myricetin; Cannabiscetin; Myricetol ...

IUPAC: 3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one

MW: 318.235100 g/mol | MF: C₁₅H₁₀O₈

Tested in 164 BioAssays; BioActivity Analysis 

Active in 23 BioAssays

Canonical SMILES: C1=C(C=C(C(=C1O)O)O)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O

InChI: 1/C15H10O8/c16-6-3-7(17)11-10(4-6)23-15(14(22)13(11)21)5-1-8(18)12(20)9(19)2-5/h1-4,16-20,22H

Has this compound been provided to the MLSMR:

No. This compound is in the Sigma-Aldrich catalog.

Probe availability: Compound is readily available from Sigma-Aldrich, catalog number M-6760.

Appendices: N/A

Bibliography:

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- (2) Sdfsad Marians, K.J., Hiasa, H., Kim, D.R. and McHenry, C.S. (1998) Role of the core DNA polymerase III subunits at the replication fork. Alpha is the only subunit required for processive replication. *Journal of Biological Chemistry*, **273**, 2452-2457.
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- (7) Inglese, J. et al. (2006) Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc Natl Acad Sci U S A* **103** (31), 11473-11478