

Fluorescence-polarization based RNA synthesis assay

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- HTS-compatible assay to identify compounds targeting influenza virus polymerase (FluPol)
- Suited for enzymatic characterization of FluPol and mechanism of action-studies
- Reliable detection is provided by the CLARIOstar's FP detection system

Introduction

Influenza virus is a major threat to global public health. Essential to virus propagation is the viral RNA-dependent RNA polymerase (FluPol) which amplifies the viral genome and transcribes mRNAs coding for viral proteins. FluPol, a ~260 kDa multifunctional heterotrimeric complex, is an attractive target for anti-influenza drug discovery (1). Here, we present a novel fluorescence polarization assay that directly reads out RNA synthesis by FluPol (2). Moreover, the assay is applicable for measuring RNA synthesis in general and is compatible with high throughput screening.

Assay Principle

The fluorescence polarization (FP) of fluorescently-labelled nucleic acids is sensitive to the conformation of the fluorophore's environment. Specifically, the FP of FAM-labelled single-stranded RNA increases when it becomes double-stranded (Figure 1). Using an 18 nucleotide 5'-end FAM-labelled single-stranded "template" RNA, the highest signal amplitude (ΔFP_{obs}) is observed when adding 18 nt complementary RNA (black), resulting in a perfect 18 bp dsRNA. Increasing the single-stranded environment of the fluorophore by shortening the complementary RNA to 16 nt (yellow) or 14 nt (purple), respectively, reduced ΔFP_{obs} max. Active-site titration of the perfectly

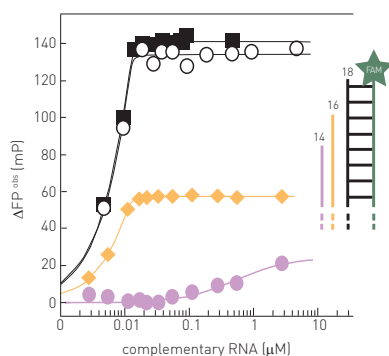


Fig. 1: Assay Principle
Observed Fluorescence Polarization is proportional to and most sensitive for a full-length RNA complementary to a 18 nt FAM-labelled single-stranded "template" RNA. Constant 0.015 μ M "template" RNA [5'-(FAM-Ex-5)-UUAUACCUUGCUUCUGCU-3'] interacting with 18 nt complementary RNA (black), 16 nt complementary RNA (yellow) or 14 nt non-perfectly complementary RNA (purple; 5'-pAGUAGUAACAAG-3'). The FP-signal of synthetic 18 nt complementary RNA and the labelled "template" RNA interacting was not affected by 0.02 μ M FluPol (black squares). Modified from [2].

complementary RNAs of 18 nt (black) and 16 nt (yellow) was observed, while the interaction of the non-perfectly complementary 14 nt RNA (purple) would correspond to a K_D of ~0.5 μ M. Note that changing the RNA sequence or the hybridization strength may affect ΔFP_{obs} max and shift the detection limit where the FP-signal is directly proportional to the amount of product RNA. The 5'-end FAM-labelled 18 nt "template" RNA used here actually corresponds to the influenza virus promoter 3' RNA and this serves as template for RNA synthesis by FluPol in biochemical assays *in vitro* [2]. This will produce a complementary 18 nt product RNA at a certain rate, which if it hybridizes to the FAM-labelled template will produce a change in its FP-signal. Before recording the FP-signal of the enzyme-catalyzed RNA synthesis, the reaction needs to be quenched (e.g. by high salt) to dissociate FluPol and RNAs while allowing RNA-RNA-interactions (Figure 2). With this "trick", the contributions to the observed FP-signal of FluPol and the FAM-labelled template RNA interacting are removed and the detected FP-signal is directly proportional to the ratio of product RNA over template RNA (Figure 1).

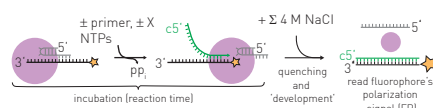


Fig. 2: Enzymatic RNA synthesis assay - Workflow
Influenza polymerase (FluPol, purple sphere) activated by 5' RNA and bound to the 3' template RNA (labelled by FAM-Ex-5 at its 5' end) is incubated with NTPs and optionally a primer or generally a molecule X whose effect on RNA synthesis is to be monitored. The reactions are quenched by addition of 4 M NaCl which perturbs polymerase-RNA-interactions but permits RNA-RNA-interactions. Fluorescence polarization [FP] is directly proportional to the ratio of full-length product RNA over labelled template RNA. Modified from [2].

Materials & Methods

- Oligonucleotides (IBA Lifesciences)
- Primer [5'-(N7MeGppp)AAUCUUAUAAUAG-3'], 2'F-2'dNTPs (Trilink Biotechnologies)
- NTPs (Sigma)
- FluPol (influenza B polymerase; Reich *et al.*, 2014)
- 384 well plates (Greiner, #781076)
- CLARIOstar® (BMG LABTECH)

RNA synthesis was performed as described. Briefly, RNA synthesis was initiated by supplementing 0.25 μ M FluPol (influenza B polymerase; bound to v5' RNA nts 1-14) with 0.15 μ M FAM-labelled template RNA [5'-(FAM-Ex-5)UUAUACCUUGCUUCUGCU-3'], 0.5 μ M primer and 25 μ M NTPs (each) in assay buffer (50 mM HEPES/NaOH, 150 mM NaCl, 10% (v/v) glycerol, 5 mM $MgCl_2$, 2 mM

TCEP, 1% [v/v] DMSO, pH = 7.4). RNA synthesis proceeded in polypropylene reaction tubes and at indicated times aliquots of 10 μ l were transferred to 80 μ l of quenching solution (4.5 M NaCl) before the FP-signal was recorded in 384 microplates using the CLARIOstar® (BMG LABTECH). Dr. J. Timmins (IBS Grenoble) is acknowledged for access to the microplate reader.

Instrument Settings

Optic settings	Fluorescence Polarization, endpoint	
	Filters	Ex: 482-16 Dichroic: LP504 Em: 530-40
	Focus and gain	adjusted before measurement
	Target mP	35
	Flashes	50 per well
General settings	Settling time	0.1 s

Results & Discussion

With the described RNA synthesis assay, influenza polymerase can be enzymatically characterized *in vitro*, e.g. regarding promoter RNA requirements, primer efficiency, K_M (NTPs), turnover numbers, initiation strategies or inhibition [2]. Kinetics of RNA synthesis are most informative and report on the amount of product and the rate it is produced. Figure 3 shows complete kinetics of RNA synthesis catalyzed by FluPol (primed with capped RNA) at 25 μ M NTPs (black) and the effect of increasing concentrations of the inhibitor 2'F-2'dCTP [X; purple (8 μ M) to dark red (8.3 mM)]. Progress curves are fitted double-exponentially according to a pseudo-first order rate law (solid lines) enabling the RNA synthesis rate constants k (min^{-1}) to be derived.

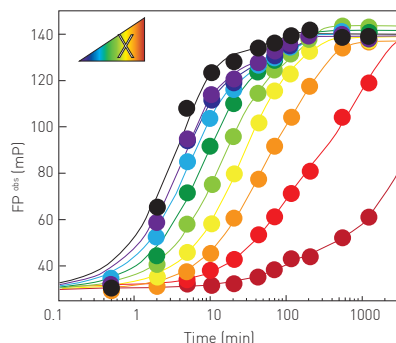


Fig. 3: RNA synthesis kinetics (and inhibition)

RNA synthesis kinetics of FluPol at 25 μ M NTPs (each) and 24 °C. The NTP-analogue 2'F-2'dCTP [X; purple blue (8 μ M) to dark red (8.3 mM)] impeded the uninhibited RNA synthesis reaction (black) in a concentration-dependent manner. Modified from [2].

The rate constants corresponding to the prominent, fast RNA synthesis phase are shown in Figure 4, open black circles and yielded an IC_{50} (2'F-2'dCTP) of 0.2 mM. When quenching the synthesis reaction at a defined reaction time (within the linear range), the assay becomes compatible with high throughput screening campaigns. Figure 4 shows dose-response curves of NTP-analogues at conditions as in Figure 3 but allowing RNA synthesis to proceed for 5 minutes only and yielded e.g. IC_{50} (2'F-2'dCTP) = 0.2 mM (Figure 4, black squares), in agreement with the IC_{50} -value determined from complete kinetics (Figure 4, black open circles).

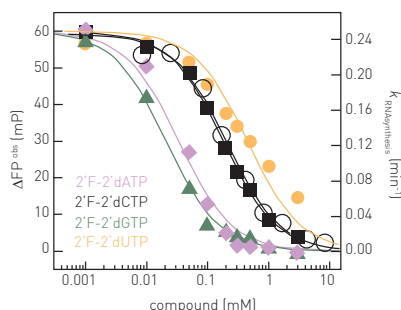


Fig. 4: Inhibition of RNA synthesis in dose-response
Characterization of NTP-analogues inhibiting FluPol catalysed RNA synthesis in HTS-compatible mode. Here, FP-signals were recorded after 5 minutes reaction time and yielded IC_{50} -values of 0.03 mM, 0.2 mM, 0.02 mM and 0.45 mM for 2'F-2'dATP (purple diamonds), 2'F-2'dCTP (black squares), 2'F-2'dGTP (green triangles) and 2'F-2'dUTP (yellow circles), respectively. For comparison, a similar IC_{50} -value of 0.2 mM for 2'F-2'dCTP is determined by recording and fitting complete RNA synthesis kinetics (see Figure 3) and analysing the observed rate constants (black open circles) Modified from [2].

Conclusion

We developed a simple *in vitro* RNA synthesis assay that utilizes fluorescence-polarization changes of FAM-labelled model template RNAs associated with FluPol catalyzed product RNA formation. The assay is high throughput compatible and can easily be performed in 384 well microplates. It reliably reports on compounds inhibiting RNA synthesis, readily can be miniaturized to 0.2 pmole/reaction of recombinant (active) FluPol and might provide an attractive choice for drug discovery campaigns.

References

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- Reich *et al.*, Nucleic Acids Research 2017 Apr 7;45(6):3353-3368. doi: 10.1093/nar/gkx043

