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## **A high-throughput fluorescence polarization assay to discover inhibitors of arenavirus and coronavirus exoribonucleases**

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1 A high-throughput fluorescence polarization assay to discover inhibitors of  
2 arenavirus and coronavirus exoribonucleases.

3

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13

14 Abstract

15 Viral exoribonucleases are uncommon in the world of RNA viruses. To date, this activity has been identified  
16 only in the *Arenaviridae* and the *Coronaviridae* families. These exoribonucleases play important but  
17 different roles in both families: for mammarenaviruses the exoribonuclease is involved in the suppression of  
18 the host immune response whereas for coronaviruses, exoribonuclease is both involved in a proofreading  
19 mechanism ensuring the genetic stability of viral genomes and participating to evasion of the host innate  
20 immunity. Because of their key roles, they constitute attractive targets for drug development. Here we  
21 present a high-throughput assay using fluorescence polarization to assess the viral exoribonuclease activity  
22 and its inhibition. We validate the assay using three different viral enzymes from SARS-CoV-2, lymphocytic  
23 choriomeningitis and Machupo viruses. The method is sensitive, robust, amenable to miniaturization (384  
24 well plates) and allowed us to validate the proof-of-concept of the assay by screening a small focused  
25 compounds library (23 metal chelators). We also determined the IC<sub>50</sub> of one inhibitor common to the three  
26 viruses.

27

28 Keywords

29 Phosphohydrolase activity, *Arenaviridae*, *Coronaviridae*, exoribonuclease activity, fluorescence  
30 polarization, screening, inhibitors, IC<sub>50</sub>.

31

32 Highlights

- 33 • *Arenaviridae* and *Coronaviridae* viral families share an exoribonuclease activity of common  
34 evolutionary origin
- 35 • *Arenaviridae* and *Coronaviridae* exoribonuclease is an attractive target for drug development
- 36 • We present a high-throughput assay in 384 well-plates for the screening of inhibitors using fluores-  
37 cence polarization
- 38 • We validated the assay by screening of a focused library of 23 metal chelators against SARS-CoV-2,  
39 Lymphocytic Choriomeningitis virus and Machupo virus exoribonucleases
- 40 • We determined the IC<sub>50</sub> by fluorescence polarization of one inhibitor common to the three viruses.

41

42 1. Introduction

43

44 RNA viruses code for a polyprotein, forming the replication/transcription complex (RTC) that orcherstrates  
45 viral replication. In this complex process, specific viral enzymatic activities are only found in some RNA  
46 viruses families. This is the case for the 3'-5'-exoribonuclease (ExoN) activity identified in *Arenaviridae* and  
47 *Coronaviridae* viral families, although these viruses display very different genome organization and  
48 replication/transcription strategies. Indeed, arenaviruses have an ambisense bi-segmented negative single-  
49 stranded RNA genome whereas the coronavirus genome is composed of a positive single-stranded RNA  
50 (Ferron et al., 2017; Knipe and Howley, 2013, Ogando et al., 2019). The ExoN activity identified in these  
51 two viral families are involved in different processes (Hastie et al., 2011; Snijder et al., 2003). Indeed, the  
52 ExoN carried by arenavirus nucleoprotein (NP) is likely involved in the suppression of the host innate  
53 immune response (Martínez-Sobrido et al., 2009, 2007, 2006) while the ExoN carried by the nsp14 protein  
54 of coronaviruses allows optimization of the replication fidelity of the viral genome (Bouvet et al., 2012;  
55 Ferron et al., 2018) as well as evasion of the host innate immunity (Becares et al., 2016; Lei et al., 2020).

56 The arenavirus and coronavirus ExoN belong to the same DEDD superfamily (DED/EDh subfamily), are  
57 structurally similar (Zuo and Deutscher, 2001) and are characterized by the presence of four conserved acidic  
58 residues (Asp and Glu) (Bouvet et al., 2012; Yekwa et al., 2019). The catalytic residues are essential for the  
59 binding of two metal cations involved in the RNA hydrolysis mechanism (Steitz and Steitz, 1993), always  
60 proceeding from the 3'- to the 5'-direction.

61 Because of its biological significance to the viral life cycle and role in infectious processes, arenavirus and  
62 coronavirus ExoN is an attractive target for drug development (Papageorgiou et al., 2020; Subissi et al.,  
63 2014). Currently, there are almost no FDA-approved antivirals against these viruses, despite the loss of  
64 human lives during the short-lived SARS-CoV outbreak in 2002 and the continuing MERS epidemic or  
65 regular outbreaks of LASSA virus in West Africa causing several thousand case fatalities. Today, the  
66 situation is different, the world is paralyzed by a global SARS-CoV-2 pandemic and this event revealed the  
67 urgency to act to develop potent antivirals, in support of a vaccine approach.

68 While millions of molecules are available in countless compounds libraries, drug development efforts are  
69 generally restricted by the limitations of validated therapeutic targets or available potent high-throughput  
70 screening assays. For mammarenavirus and coronavirus ExoN assay making use of radiolabeled substrate are  
71 available but barely adapted to HT screening (Bouvet et al., 2012; Ferron et al., 2018; Yekwa et al., 2019,  
72 2017; Baddock et al., 2020; Saramago et al., 2021)).

73 Here we present a method using fluorescence polarization (FP) to assess the ExoN activity and its inhibition.  
74 The method relies on the hydrolysis of a fluorescent RNA substrate into smaller fragments which translates  
75 in a modification of the FP signal which is measured and recorded. The FP signal is altered proportionally to  
76 the size of the fluorescent RNA probe. We validate the method on three different viral enzymes of interest  
77 from SARS-CoV-2, Lymphocytic Choriomeningitis and Machupo viruses. The method is sensitive, robust,  
78 amenable to miniaturization (384 well plates) and allowed us to screen a focused library of 23 metal  
79 chelators over the three targeted viral ExoN, validating the proof-of-concept of the assay. We also  
80 determined the  $IC_{50}$  of one inhibitor common to the three viruses.

81

## 82 2. Materials and methods

83

### 84 2.1 Products and reagents.

85 Raltegravir (**1**) and Baloxavir (**5**) were purchased from Carbosynth. Aurintricarboxylic acid (**11**) was  
86 purchased from Acros Organics. Excepting for the compounds **8**, **13**, **14**, **15** and **24**, the other compounds  
87 have been described previously (Saez-Ayala et al., 2019, 2018). All compounds were resuspended in 100 %  
88 DMSO at 20 mM and stored at - 20 °C. The 22-mer RNA 5'-UGACGGCCCGGAAAACCGGGCC-3'  
89 containing FAM dye at the 5'-end (5'-FAM-RNA) was purchased from Microsynth AG (Switzerland).

90

## 91 *2.2 Protein expression and purification.*

92 The LCMV, MACV, ExoN plasmids used for this study were described in (Yekwa et al., 2019). The protein  
93 production method is detailed in the supplementary data file (see also figure S1 for elution profiles). For the  
94 expression of the SARS-CoV-2 nsp10 and nsp14, the synthetic genes were purchased from Twist (USA) and  
95 were cloned with a hexahistidine tag in the N-terminus of the protein. *E. coli* DE3 cells were transformed  
96 with the corresponding expression vectors. Bacteria were grown in TB medium with the corresponding  
97 antibiotic and protein expression was induced by addition of IPTG to a final concentration of 500 µM for  
98 nsp10 and 50 µM for nsp14 when the OD<sub>600nm</sub> of the culture reached a value of 0.5. The induction was  
99 performed during 16 h at 17°C and 200 rpm. Bacterial cell pellets were frozen and resuspended in lysis  
100 buffer (50 mM HEPES, pH 7.5 and 300 mM NaCl) supplemented with 1 mM PMSF, 10 mM imidazole, 10  
101 µg/ml DNase I, 0.25 mg/ml of lysozyme and 0.5% Triton X-100. After sonication and clarification, proteins  
102 were purified by two steps of chromatography. The first step consisted of an IMAC (Ni Resin). The lysate  
103 was passed through the column and washed with lysis buffer supplemented with 20 mM imidazole. The  
104 protein was eluted with lysis buffer supplemented with 250 mM imidazole. Protein fractions were then  
105 loaded on a HiLoad 16/60 Superdex 200 gel filtration column (GE Healthcare), and eluted with 10 mM  
106 HEPES, pH 7.5, 300 mM NaCl, 1 mM DTT and 5% glycerol. The fraction containing the pure protein, as  
107 examined by SDS-PAGE and coomassie staining, were pooled and concentrated in the gel filtration buffer,  
108 aliquoted in small volumes, flash frozen in liquid nitrogen and stored at -80°C.

109

## 110 *2.3 Set-up of exonuclease activity conditions based on fluorescent polarization assay*

### 111 *2.3.1 Optimisation of 5'-FAM-RNA concentration and SARS-CoV-2, LCMV or MACV ExoN ratio*

112 Reactions were performed in 20 µl total volume in a buffer containing 40 mM Tris (pH 8), 5 mM DTT and 2  
113 or 5 mM MnCl<sub>2</sub>. The concentration of 5'-FAM-RNA tested varied from 50 to 1000 nM. For each 5'-FAM-

114 RNA concentration (50, 100, 250, 500 and 1000 nM), a range of concentration of ExoN (SARS-CoV-2,  
115 LCMV or MACV) was tested from a ratio of 5-fold less enzyme up to 10-fold more enzyme (10 nM to 10  
116  $\mu$ M) than 5'-FAM-RNA. For SARS-CoV-2 ExoN, the molar ratio of nsp14:nsp10 complex in the reactions  
117 was always kept at 1:4, as optimized previously (Bouvet et al., 2012). The reaction started by the addition of  
118 5'-FAM-RNA and the fluorescence polarization (FP) was read in the Pherastar FSX (BMG Labtech) using  
119 the 480 nm excitation and 520 nm emission filter, during 30 minutes at 25°C, every 30 seconds. The gain  
120 was set up using the negative control which contained the fluorescent RNA in the reaction buffer with metal  
121 ion and in the presence of heat-denatured nuclease. Other negative controls tested used consisted in the  
122 replacement of  $MnCl_2$  by  $CaCl_2$  or, depletion of the metal ion in the reaction mix and depletion of the  
123 enzyme. After 30 minutes, the reactions were stopped by the addition of an equal volume of loading buffer  
124 (8M urea containing 10 mM EDTA) and the digestion products were then loaded in 7 M urea containing  
125 20% (wt/vol) polyacrylamide gels (acrylamide/bisacrylamide ratio 19:1) buffered with TBE and visualized  
126 using a Fluorescent Image Analyzer Typhoon (GE Healthcare).

127

### 128 *2.3.2 Optimisation of temperature, metal ion nature and concentration requirement*

129 To find the optimal temperature, metal ion nature and reagent concentration, reactions were performed in the  
130 buffer mentioned above. The 5'-FAM-RNA concentration was kept at 100 nM and ExoN concentration  
131 ranged from 100 nM to 1600 nM. The activity was tested in the presence of  $MnCl_2$  or  $MgCl_2$  at three  
132 different concentrations 1, 2 or 5 mM. The FP signal was recorded as mentioned previously either at 25°C or  
133 at 37°C. After finishing the recording of the FP signal, an aliquot of the sample was examined in urea PAGE  
134 as mentioned above.

135

### 136 *2.3.3 Time course of ExoN assay*

137 Reactions of 20  $\mu$ l total volume were performed in the buffer mentioned above, with 2 mM  $MnCl_2$  for  
138 SARS-CoV-2 ExoN and 5 mM for LCMV or MACV ExoN. The 5'-FAM-RNA concentration was fixed at  
139 100 nM. The SARS-CoV-2 nsp14/nsp10 ExoN complex concentration was ranged from 200 nM to 1  $\mu$ M  
140 and pre-incubation at RT during 5 minutes was performed to allow the complex formation. The LCMV and  
141 MACV ExoN concentration was ranged from 100 nM to 1600 nM. The reaction started by the addition of 5'-  
142 FAM-RNA and The FP signal was recorded as mentioned previously. After finishing the recording of the FP

143 signal, an aliquot of the sample was examined in urea PAGE as mentioned above. The assay was done in  
144 triplicate for each ExoN.

145

#### 146 *2.4 High throughput screening and IC<sub>50</sub> determination.*

147

##### 148 *2.4.1 HTS assay based on fluorescent polarization assay*

149 The screening at 5 μM and 20 μM inhibitor concentration was performed in 384-wells with flat bottom Nunc  
150 plates, in 20 μL total volume reaction. Reactions were performed in a buffer containing 40 mM Tris (pH 8),  
151 5 mM DTT, with 2 mM MnCl<sub>2</sub> for SARS-CoV-2 and 5 mM MnCl<sub>2</sub> for LCMV or MACV ExoN. The 5'-  
152 FAM-RNA concentration was fixed at 100 nM. The ExoN concentration was fixed at 400 nM. Compounds  
153 (**1-23**) were added to the reaction with final concentration of 5 μM and 20 μM in 5% DMSO. Reactions were  
154 initiated by addition of the 5'-FAM-RNA using a multichannel pipette. 48 positive controls (the reaction mix  
155 with 5% DMSO, with ExoN and without compound) have been deposited randomly in the 384-well plate. 48  
156 negative controls (the reaction mixture with 5% DMSO, without or with ExoN that has been heat denatured,  
157 and without compound), have been deposited randomly in the 384-well plate. The reaction started by the  
158 addition of 5'-FAM-RNA and the FP signal was recorded as mentioned previously. To calculate the  
159 percentage of inhibition, a time correction was applied for the delayed initiation of the reaction due to the use  
160 of a multichannel pipette. The percentage of inhibition at a given time was calculated as follows :

$$\text{Inhibition\%} = \frac{100 \times (\text{FP} - \text{average of positive controls})}{(\text{average negative controls} - \text{average positive controls})}$$

161 where FP correspond to the fluorescent polarization signal of a compound.

162 The time selected for doing this calculation was the time when the signal of the positive control reached the  
163 plateau (30 minutes). The Z' factor for the assay was calculated using the following equation:

$$Z' = 1 - \frac{3 \times (\text{SD of negative controls} + \text{SD of positive controls})}{(\text{mean of negative controls} - \text{mean of positive controls})}$$

164 where SD is the standard deviation. 48 positive controls and 48 negative controls were considered to  
165 calculate the Z' factor.

166

##### 167 *2.4.2 IC<sub>50</sub> determination of compound **II** based on fluorescent polarization assay*

168 Reactions were performed in a buffer containing 40 mM Tris (pH 8), 5 mM DTT, with 2 mM MnCl<sub>2</sub> for  
169 SARS-CoV-2 ExoN and 5 mM for LCMV or MACV ExoN. The 5'-FAM-RNA concentration was fixed at  
170 100 nM. The ExoN concentration was fixed at 400 nM. The compound **11** concentration varied from 0.2 to  
171 12.5 μM for LCMV ExoN, from 0.4 to 25 μM for MACV ExoN and from 0.5 to 16 μM for SARS-CoV-2  
172 ExoN nsp14/nsp10 complex. The reaction started by the addition of 5'-FAM-RNA and the FP signal was  
173 recorded as mentioned previously. The percentage of inhibition was calculated as indicated in the previous  
174 section. The curves of percentage of inhibition respect to the inhibitor concentration in a logarithmic scale  
175 were fitted in Graphpad Prism software using a four parameters equation. The assays were done in triplicate.

176

### 177 3. Results and discussion

178

179 Fluorescence polarization (FP) is a reliable and sensitive tool for monitoring enzymatic reaction  
180 progress, by determining the difference of polarization signals during reaction (Zhang et al., 2012). FP -  
181 based assays in HTS studies have been used in drug discovery (Lea and Simeonov, 2011; Uri and Nonga,  
182 2020, Baughman et al., 2012; Liu et al., 2014)). The FP signal recorded is proportional to the molecular  
183 weight (MW) of a fluorescent molecule (Kwok, 2002; Latif et al., 2001). We decided to apply this method to  
184 viral ExoN activity, by monitoring the size of a fluorescent labeled RNA probe which is altered in the course  
185 of the nuclease activity, reflecting the enzymatic activity (Liu et al., 2014; Zhang et al., 2012). Because  
186 several factors can change the FP- monitoring success, the assay was first optimized.

187

#### 188 *3.1 Determination of optimized experimental conditions of the SARS-CoV-2 nsp14/nsp10 complex, LCMV* 189 *and MACV ExoN activity based on FP assay*

190

191 To set-up a HTS assay for the SARS-CoV-2, LCMV and MACV ExoN activity, we explored 5'-FAM-RNA  
192 substrate(s) and enzyme(s) concentration, metal ion co-factors, temperature and reaction duration. RNA  
193 substrate and optimal conditions for arenavirus and coronavirus ExoN activity are already described (Bouvet  
194 et al., 2012; Saramago et al., 2021; Yekwa et al., 2019, 2017). A 22mer RNA that forms stable hairpin in its  
195 3' end has been reported to be a valuable substrate for both arenavirus and coronavirus ExoN. Moreover, for  
196 SARS-CoV-2 nsp14 ExoN, nsp10 was added in the reaction (ratio 1:4 of nsp14:nsp10) as nsp10 was



197 previously demonstrated to stimulate > 35-fold the nsp14 ExoN activity (Bouvet et al., 2012; Saramago et  
198 al., 2021).

199 We first determined the smallest 5'-FAM-RNA concentration that provides sufficient FP signal,  
200 concomitantly with the set-up of the ratio of ExoN and 5'-FAM-RNA to obtain a reproducible and stable FP  
201 signal. For each 5'-FAM-RNA concentration tested (50 to 1000 nM), single turn over (STO) conditions  
202 (excess of enzyme respect to substrate) and also multiple turn over (MTO) conditions (excess of substrate  
203 respect to enzyme) were tested. Because the RNA substrate is degraded during the ExoN activity, FP value is  
204 altered proportionally. A significant change in the FP signal due to the hydrolysis of the 5'-FAM-RNA was  
205 observed only under STO conditions. 100 nM concentration of 5'-FAM-RNA was selected because it was the  
206 smallest concentration of substrate providing a reproducible and stable FP signal.

207 We optimized the positive and negative controls of ExoN activity. Several combinations were tested  
208 (Supplementary figure S2): 5'-FAM-RNA in reaction buffer with metal ions, 5'-FAM-RNA in reaction buffer  
209 with ExoN and without metal ions, 5'-FAM-RNA in reaction buffer with ions and denaturated ExoN and 5'-  
210 FAM-RNA in reaction buffer with CaCl<sub>2</sub> instead of MnCl<sub>2</sub>. For all the negative controls tested, there was no  
211 variation between the initial and final FP values after 30 min incubation, indicating the absence of  
212 degradation of the fluorescent probe. However, we observe that the initial FP values are lower without metal  
213 ions in the reaction buffer (Supplementary figure S2), or in the presence of EDTA 10 mM (data not shown).  
214 This results is in agreement with the study of Liu *et al* (Liu et al., 2014), who carried out the experiments to  
215 investigate the influence of metal ions in FP assays, and reported that cations concentration can affect the FP  
216 signal by altering the mobility of the fluorophore through stabilizing the RNA secondary structure. Thus, for  
217 the rest of the study, the negative controls were prepared by mixing 5'-FAM-RNA in reaction buffer with  
218 ions and without ExoN.

219 Regarding the nature of the metal ion, MnCl<sub>2</sub> and MgCl<sub>2</sub> were tested. An increased activity is observed in the  
220 presence of MnCl<sub>2</sub> for both arenaviruses and SARS-CoV-2 ExoN (data not shown). The concentration of  
221 MnCl<sub>2</sub> that shows the highest reduction in FP signal is 5 mM for arenaviruses as previously reported (Yekwa  
222 et al., 2019, 2017) and 2 mM for SARS-CoV-2 as recently reported (Baddock et al., 2020; Saramago et al.,  
223 2021). Finally the temperature selected to perform the assay is 25°C since the incubation at 37°C leads to a  
224 significative evaporation of the sample that reduces the stability and reproducibility of the FP signal.

225

### 226 3.2 Assessing the SARS-CoV-2, LCMV and MACV ExoN activity by FP

227

228 While the 5'-FAM-RNA substrate was fixed at 100 nM, which was the lowest concentration fairly detected  
229 by the sensitivity of the method, different concentrations of ExoN were tested (100 nM to 1600 nM). The FP  
230 signal change was measured during 30 min. The FP curves and digestion products examined in urea PAGE  
231 after FP reading, are gathered in figure 1.

232 The negative controls (figure 1, brown curves, in panels A, B and C) present flattened curves, as expected.  
233 Remarkably, early in the reaction a small increase in the initial FP value is observed, which could correspond  
234 to the time required for the formation of the catalytic complex between the ExoN, the catalytic cations and  
235 the 5'-FAM-RNA substrate. This is more visible for the SARS-CoV-2 ExoN, which requires the interaction  
236 of nsp14 and nsp10 forming the active complex. This increase of fluorescence occurs for all FP curves but is  
237 visible mainly on the FP curves corresponding to the lowest ExoN concentrations when the formation of the  
238 pre-hydrolytic complex presumably takes the longest time.

239 For all tested ExoN, excepting for the lowest used concentration, we observe a reduction of the FP signal  
240 with time. The decrease of the FP value is correlated with the hydrolysis of the 5'-FAM-RNA substrate by  
241 the ExoN, as confirmed on the urea PAGE images (figure 1, panels D, E and F). The difference between  
242 initial and final FP value after 30 min is proportional to the number of nucleotides removed from the 5'-  
243 FAM-RNA.

244 By increasing the ExoN concentration, we observe an increase in the FP curves slope but the FP signals  
245 reach a plateau which might be related to the inability to remove any extra nucleotide after a certain point.

246 The method efficiency was validated by the difference of FP values between the negative control (figure 1,  
247 brown curves, in panels A, B and C) and the FP value obtained at one concentration after 30 min reaction.  
248 This difference was selected as significant enough, with  $\Delta FP \pm 30$  units of mP.

249 To validate this method and use it as screening assay, we decided to use a molar ratio 1:4 between the 5'-  
250 FAM-RNA substrate (100 nM) and ExoN (400 nM). With the optimized duration, temperature and  
251 experimental conditions, this ratio gives the more stable and reproducible FP signals, correlated with specific  
252 5'-FAM-RNA fragments.

253

### 254 3.3 Screening of a focused library against the SARS-CoV-2, LCMV and MACV ExoN using FP

255 Because ExoN activity is metal dependent, we tested a focused library of 23 metal chelators that we have  
256 developed previously (Saez-Ayala et al., 2019, 2018), in order to demonstrate the robustness of our FP  
257 method. Two screenings were performed using 5  $\mu$ M and 20  $\mu$ M of compounds in 5% DMSO in a single  
258 assay. The robustness of the assay for HTS was calculated using 48 negative and 48 positive controls. The  
259 lowest Z' value is 0.68 indicating that the assay is reliable (see supplementary data figure S3). The  
260 percentages of inhibition at 5  $\mu$ M and 20  $\mu$ M for each ExoN, extracted from FP curves (see supplementary  
261 data figure S4), are gathered in supplementary figure S5 and figure 2 (panels A, B and C), respectively. The  
262 degree of digestion of the RNA substrate was also controlled by fragments separation on urea-PAGE at the  
263 end of the reaction for compounds **4**, **11**, **13**, **18** and **23** (figure 2, panel D).

264 We identified compound **11** which inhibits 100% of the different ExoN activity at 20  $\mu$ M and 78%, 76% and  
265 100% at 5  $\mu$ M, respectively against LCMV, MACV and SARS-CoV-2 ExoN. Compound **11** which is  
266 Aurintricarboxylic Acid (ATA) was included as positive control as it has been previously described as  
267 nuclease inhibitor (Huang et al., 2016), acting as non-specific metal chelator (Sharma et al., 2000). We also  
268 identified inhibitors showing more specific inhibition profile. Compound **4** is active against SARS-CoV-2  
269 with 61% inhibition at 20  $\mu$ M (less than 10% inhibition of LCMV ExoN) while compound **23** is specific to  
270 LCMV ExoN with 71% inhibition (less than 10% inhibition of MACV ExoN). Compounds **13** and **18** are  
271 potent inhibitors of SARS-CoV-2 ExoN with more 90% and weak inhibitors of LCMV and MACV ExoN  
272 (less than 20% inhibition). The FP method allowed the identification of potent ExoN inhibitors as  
273 compounds **4**, **11**, **13**, **18** and **23**, and the absence of RNA degradation was confirmed by urea PAGE analysis  
274 (figure 2 - panel D) used as an orthogonal validation assay.

275

#### 276 *3.4 Assessing IC<sub>50</sub> measurement of compound 11 by FP*

277 Compound **11** displaying the highest inhibition, at both 5 and 20  $\mu$ M, was used as model for IC<sub>50</sub>  
278 determination. The FP curves, the digestion products examined in urea PAGE after FP reading and dose-  
279 response curves obtained by extraction of FP data are gathered in figure 3. The IC<sub>50</sub> values were determined  
280 by hill plot curve fitting (see materials and methods section). The IC<sub>50</sub> values for LCMV, MACV and SARS-  
281 CoV-2 ExoN were  $3.61 \pm 0.20 \mu$ M,  $3.46 \pm 0.29 \mu$ M and  $1.64 \pm 0.17 \mu$ M, respectively. Notably, these values

282 correlated perfectly with the 80% inhibition obtained by screening at 5  $\mu$ M, confirming the robustness and  
283 reproducibility of the assay. The FP signal correlates also with the degree of digestion observed by gel.

284

285

#### 286 4. Conclusion

287 The use of fluorescence polarization assay for inhibitors identification was investigated and we prove that  
288 this technique is reliable and sensitive to monitor nuclease activity.

289 Our work presents the development of a viral ExoN HTS assay in 384-well plates. Its most valuable feature  
290 is that allows a reliable and rapid identification of ExoN inhibitors limiting the ExoN activity of viral  
291 enzymes belonging to arenavirus (LCMV and MACV) and coronavirus (SARS-CoV-2).

292 These results make fluorescence polarization assay an important tool in the screening of compounds libraries  
293 to discover antivirals.

294

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304

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306

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411 Legend to figures

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413 Figure 1. ExoN activity measured by FP for LCMV (panel A), MACV (panel B) and SARS-CoV-2 (panel  
414 C). The FP signal variation is recorded with time during 30 min, each 30 seconds, at 25°C. The 5'-FAM-  
415 RNA substrate concentration used is 100 nM and the ExoN concentration tested ranges 100 nM to 1600 nM.  
416 The data represents the average and SEM of three independent experiments. The bottom panels illustrate a  
417 representative image of the digestion products analyzed in urea PAGE, of the ExoN activity for LCMV  
418 (panel D), MACV (panel E) and SARS-CoV-2 (panel F), after recording the FP signal. An aliquot of the  
419 sample was loaded into a urea-PAGE 20% and scanned in a fluorescence imager.

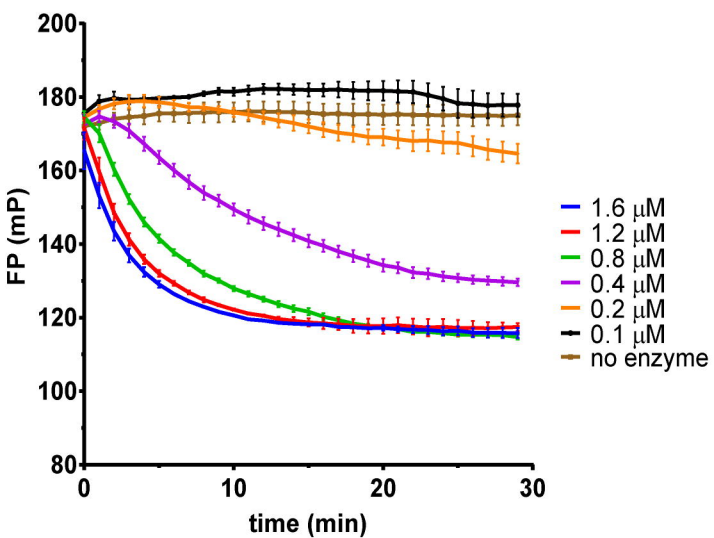
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421 Figure 2. Screening of focused library of metal chelators (23) against LCMV (panel A), MACV (panel B)  
422 and SARS-CoV-2 (panel C) ExoN followed by FP. Orthogonal validation assay by analysis of the  
423 fluorescent RNA substrate by urea PAGE (panel D) The bars show the % of inhibition of the ExoN activity  
424 as described in materials and methods. For the screening conditions 100 nM 5'-FAM-RNA, 400 nM ExoN  
425 and 20  $\mu$ M of inhibitor were used.

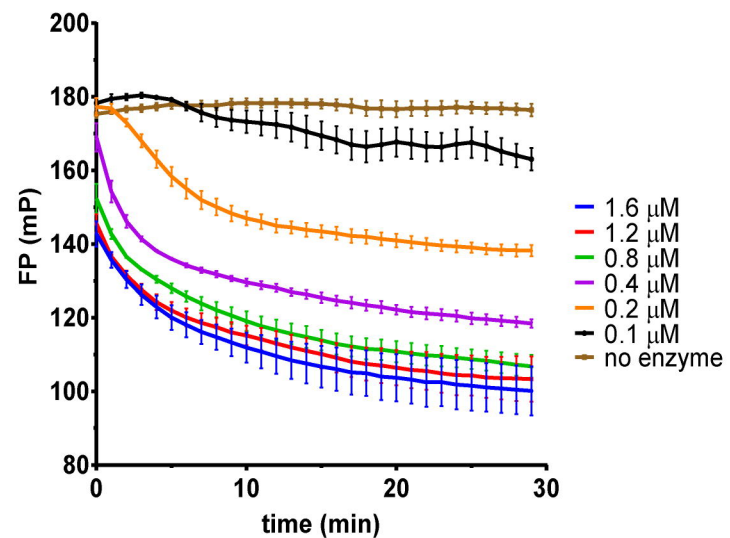
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427 Figure 3. IC<sub>50</sub> measurement of compound **11** by FP on LCMV, MACV and SARS-CoV-2 ExoN. In A, B and  
428 C the FP signal variation is recorded with time during 30 min, each 30 seconds, at 25°C. The 5'-FAM-RNA  
429 substrate and LCMV (panel A), MACV (panel B) and SARS-CoV-2 (panel C) ExoN concentration were  
430 respectively 100 nM and 400 nM. The data represents the average and SEM of three independent  
431 experiments. In the middle panels it is shown a representative image of the digestion products of the LCMV  
432 (panel D), MACV (panel E) and SARS-CoV-2 (panel F) ExoN in presence of the different concentrations of  
433 compound **11**, visualized in urea AGE after recording the FP signal. In the bottom panels it is shown the  
434 dose-response curves obtained by extraction of FP data, the IC<sub>50</sub> values for compound **11** with LCMV (panel  
435 G), MACV (panel H) and SARS-CoV-2 (panel I). The data represents the average and SEM of three  
436 independent experiments.

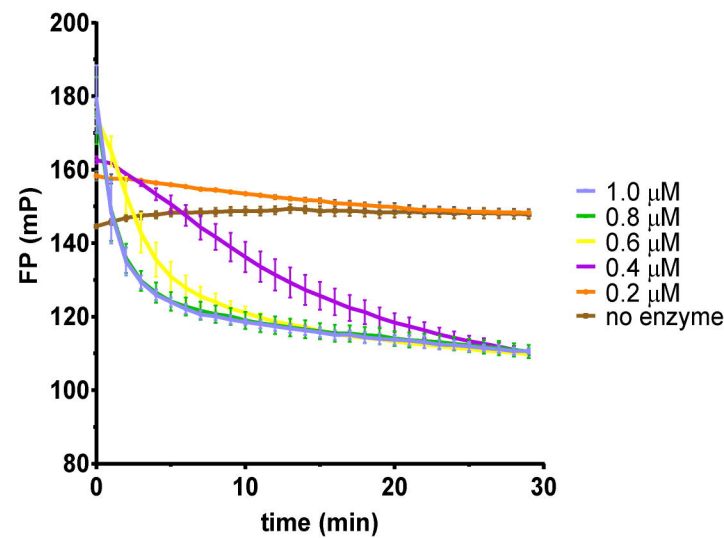
A)



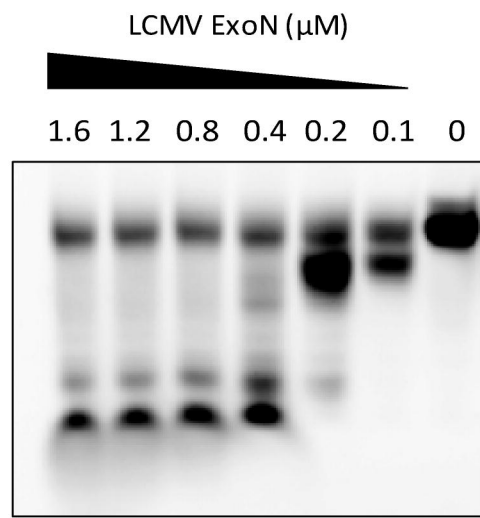
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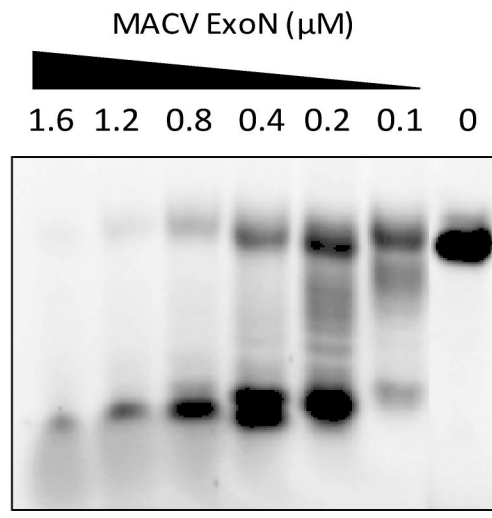
C)



D)



E)



F)

