KinaseRa

Instruction manual

ARC-Lum Protein Kinase Assay Kit

Catalogue code: BAS-LUM590

The **ARC-Lum Protein Kinase Assay Kit** is used for a homogenous, high-throughput **binding/displacement assay** for screening/characterization of inhibitors of basophilic protein kinases (AGC group), *e.g.,* **PKA, ROCK.**

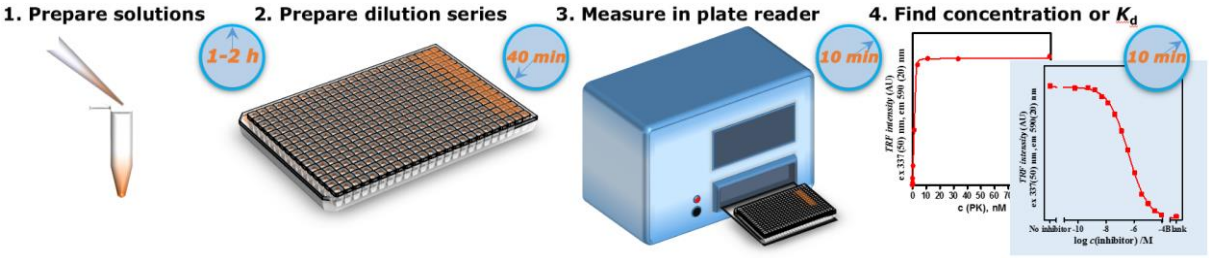

Table 1. ARC-Lum Protein Kinase Assay Kit (BAS-LUM590)

Application	Determination of K_d values for both ATP- and substrate-competitive protein kinase inhibitors
Targets	Basophilic protein kinases (AGC group)
Currently tested for	**PKA, ROCK I, ROCK II, AKT3, PKG I, PKC, MSK1, etc.** (Tab. 2) Easy assay adaption for the analysis of other basophilic kinases
Detection principle	Time-gated measurement of luminescence intensity(TGL) Ex: 337 nm/ Em: 590 nm [1]
Assay setup	Homogeneous, 1-step, mix & read; 384-well low-volume format
Assay volume	10 - 25 µl
Probe	BAS-LUM590 probe

Cat. No.	Amount
BAS-LUM590	1 Kit (384 assay points)

For *in vitro* use only

Storage: Upon arrival open the package and store the reagent pouch at -20°C, the rest of the package can be stored at ambient temperature.

Quality guaranteed for 6 months since the date of purchase

Kit contents

2x1.1 mL 10x Assay Buffer

50 µL BAS-LUM590 probe (20 µM in DMSO)

2x solid DTT (1x for 5000 µl 1x assay buffer)

1 Film-covered 384-well low-volume microtiter plate

1 Transparent lid

To be provided by the user

Protein kinase

Cofactors if necessary

Shaking thermostat

Fluorescence plate reader with TRF (time-resolved fluorescence) or TF-FRET (time-resolved FRET) capabilities (excitation at 337 nm and emission at 590 or 615 nm). Tested on PHERAstar plus (BMG Labtech), Synergy Neo (BioTech) and Cytation 5 (BioTech) platereaders.

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1. Description

The **ARC-Lum Protein Kinase Assay Kit** is for a homogenous, high-throughput **binding/displacement assay** designed for determination of the concentration of active form of the protein kinase and screening/characterization of inhibitors of basophilic protein kinases (AGC group) (Tab. 1).

The principle of the assay relies on the competitive displacement of the photoluminescent probe (BAS-LUM590) from its complex with the protein kinase by the inhibitor (Fig. 1) [1]. BAS-LUM590 probe possesses high affinity towards basophilic kinases (Tab. 2) and enables the **characterization of both ATP- and substrate competitive inhibitors** due to its unique bisubstrate character (simultaneous association with both binding sites of the protein kinase).

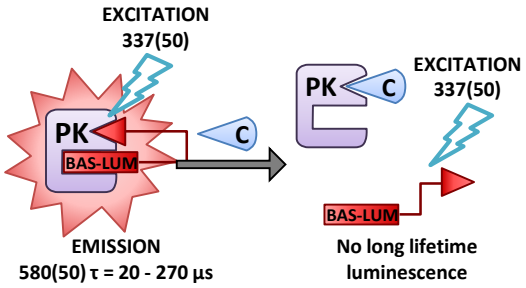

Figure 1. Principle of ARC-Lum Protein Kinase Assay Kit

BAS-LUM590 probe associates with the protein kinase by simultaneous binding to both its ATP and substrate binding sites (bisubstrate character) resulting in protein-induced microsecond-scale lifetime luminescence signal at 590 nm upon pulse excitation with near-UV light (300-360 nm). The displacement of BAS-LUM590 probe by a competitive inhibitor (C) leads to the loss of luminescence signal that is detected upon excitation with a microplate reader (read-out: time-gated luminescence (TGL) intensity)[1]. The luminescence signal is not affected by the presence of the free BAS-LUM590 probe. The detected signal is therefore directly proportional to the amount of the BAS-LUM590:Protein Kinase complex in the solution.

Since the luminescence signal is not substantially affected by the luminescence of free BAS-LUM590 probe, high concentrations of BAS-LUM590 probe (up to 100 nM) can be used in the assay for the determination of binding affinities of compounds that under conventional assay conditions demonstrate tight binding properties (*e.g.*, K_i values of Staurosporine towards several kinases are lower than 1 nM and cannot be precisely determined with assays based on the inhibition of substrate phosphorylation).

2. Calculation of K_d values

Unbound BAS-LUM590 probe does not significantly contribute to the time-gated luminescence (TGL) signal and can therefore be used at high concentrations (up to 100 nM) which

a) supports the use of the **Cheng-Prusoff equation (Eq.1)** for the simplified calculation of K_d values from displacement curves
b) results in protein concentration independent experimental data.

$$K_d = \frac{IC_{50}}{1+{}^{[L_t]}/_{K_D}}$$ (**Eq.1)**, where

- ***K*$_D$** is the dissociation constant of the BAS-LUM590:kinase complex,
- $K_D = \frac{[\text{BAS−LUM590}][\text{kinase}]}{[\text{BAS−LUM590:kinase}]}$
- ***K*$_d$** is the dissociation constant of the inhibitor:kinase complex, $K_d = \frac{[\text{inhibitor}][\text{kinase}]}{[\text{inhibitor:kinase}]}$
- ***IC*$_{50}$** is the concentration of the inhibitor that displaces 50% of BAS-LUM590 probe from BAS-LUM590:kinase complex
- ***L*$_t$** is the total concentration of BAS-LUM590 probe

3. Experimental set-up

3.1 Instrument settings

ARC-Lum Protein Kinase Assay can be performed using instruments and instrument settings that are suitable for europium-based TRF, TR-FRET measurements [excitation 337(50) nm (or similar), emission 615(10) or similar, delay 100 µs, gate 100 µs]. Greater signal intensity will be obtained if 590(50) nm (or similar) emission filter is used. Depending on the instrument, the delay time could be reduced to obtain greater luminescence intensity and better S/N ratio.

3.2 Preparation of 1x Assay Buffer

Prepare 5 mL of fresh 1x Assay Buffer (sufficient for 8 inhibitors) by diluting the 10x Assay Buffer:

- Dissolve one batch of provided DTT in 550 µl 10x Assay Buffer
- Transfer 500 µl of the prepared DTT solution in 10x Assay Buffer to a centrifuge tube with a capacity of at least 5 mL
- Add 4500 µL deionized water (e.g. Milli-Q Quality)*

* reduce the volume of water accordingly if cofactors and/or activators will be added (Tab.2)

Please note: *1x Assay buffer is active for 18 hours and should not be used beyond that time. Always prepare fresh buffer and do not store the 1x Assay Buffer!*

4. Choosing the optimal concentration of BAS-LUM590 probe for the assay

The optimal concentration of the BAS-LUM590 probe used in the assay depends on the affinity of the probe towards the protein kinase, but also on the affinity of the kinase towards the inhibitors that are about to be tested. The usable concentration range for several protein kinases is provided in Table 2. As a general rule if the K_d of an inhibitor is expected to fall in the same range as the affinity (or it has higher affinity) of BAS-LUM590 probe, the application of higher concentrations of the probe is mandatory. If the affinity of the inhibitor is expected to be several orders of magnitude weaker, lower concentrations of the probe should be used to obtain a full inhibition curve.

Table 2. Recommended concentration ranges of the BAS-LUM590 probe

Protein Kinase	Cofactors	BAS-LUM590 Probe concentration range [nM][1]	Active kinase concentration [nM]	Approx. K_D, [nM]
PKAcα	-	2-100	1	0.02
ROCK II	-	2-100	1	0.2
MSK1	-	2-100	1	0.1
Akt3	-	15-100	1	5
PKCη	PKC mix[1]	5-100	2	1.1
PKCδ	PKC mix[1]	5-100	2	0.77
PKCβ1	PKC mix[1]	5-100	2	1.3
PKG Iα	1 µM cGMP	10-100	2	2.7

[1] 0.2 mM $CaCl_2$, 10 µg/mL DAG (diacylglycerol), 50 µg/mL PS (phosphatidylserine), 0.015% Triton X-100

5. Determination of the Optimal Protein Kinase Concentration

This assay is tested for several protein kinases (Tabel 2: Overview of recommended final protein kinase concentrations). However, the following procedure is mandatory since protein kinase activity may vary in different preparations.

This is a simplified approach and sufficient for the procedure of choosing the optimal concentration of the kinase for the inhibition assay. For the determination of the K_D value of the protein kinase:BAS-LUM590 probe complex or the concentration of the active form of the protein kinase, please refer to: www.kinasera.com/assay_support

Please note: *Centrifugation of all solutions before dispensing them into the wells is recommended.*

a. Prepare 30 µl of 125 nM solution of protein kinase in 1x assay buffer
    - Note that the prepared concentration is 1.25-fold higher than the final concentration in the well (Tab. 3)
    - *Lower protein kinase concentrations can be used, however the starting concentration of protein kinase should be at least 20 times the concentration (active kinase concentration) provided in Table 2.*
b. Dispense 16 µl of 1x assay buffer into wells **(#1B to #1N)** of a single column of the 384-well plate
c. Dispense 24 µl of 125 nM protein kinase into well **#1A**.
    - Prepare a titration series by transferring 8 µl of protein kinase sample from well #1A into #1B, mix thoroughly by careful pipetting. Transfer 8 µl from #1B to #1C and continue stepwise until #1L. After mixing thoroughly in #1L, discard the tip with 8 µl solution.
d. Dispense 20 µL of 1x assay buffer into wells **#1O and #1P** of the column (for blank correction)
e. Prepare 60 µl of 5x BAS-LUM590 probe solution in 1x assay buffer, which is 5x the optimal concentration of the BAS-LUM590 probe chosen at (step 4)
    - e.g. for a final concentration of 20 nM BAS-LUM590 probe in the assay, prepare 100 nM working solution by first diluting the 20 µM stock solution 10-fold in 1x assay buffer and then diluting the resulting 2 µM BAS-LUM590 probe solution 20-fold in 1x assay buffer
f. Add 4 µL of 5x BAS-LUM590 probe working solution into **wells #1A to #1N**.
g. Incubate the plate on a shaking thermostat for 15 minutes at 30°C and 400 rpm.
h. Measure the time-gated luminescence intensity values of each well with a fluorescent plate reader
    - Use well #1M or #1N for the adjustment of instrument settings
    - Use wells #1O and #1P for blank correction.
i. Plot luminescence intensity values against the concentration of the kinase.

Table 3. Final concentrations of the BAS-LUM590 probe and protein kinase (final volume: 20 µl per well)

Well #	Protein conc.	BAS-LUM590 probe conc.
1A	100 nM	20 nM
1B	33.3 nM	20 nM
1C	11.1 nM	20 nM
1D	3.7 nM	20 nM
1E	1.2 nM	20 nM
1F	412 pM	20 nM
1G	137 pM	20 nM
1H	45.7 pM	20 nM
1I	15.2 pM	20 nM
1J	5.1 pM	20 nM
1K	1.7 pM	20 nM
1L	0.6 pM	20 nM
1M	-	20 nM
1N	-	20 nM
1O	-	-
1P	-	-

The optimal kinase concentration equals to the concentration that gives **at least a 10 times greater luminescence signal** than the free BAS-LUM590 probe (average of wells #1M to #1N).

6. Determination of IC_{50} and K_d values of inhibitors

Please note: *Centrifugation of all solutions before dispensing them into the wells is recommended.*

a. Dispense 16 µl of 1x assay buffer to one column per one inhibitor of the 384-well plate **(wells #B to #O)**

b. Prepare a dilution of each inhibitor in 1x assay buffer (the starting concentrations should be 1.25-fold higher than the desired final well concentrations as BAS-LUM590 probe and protein kinase will be added).
   - *E.g. if the highest concentration of the inhibitor in the dilution series should be 1 mM, prepare 1.25 mM solution in 1x assay buffer*

   - Dispense 24 µl of each inhibitor into #A in separate columns.
   - Prepare a serial dilution by transferring 8 µl from #A into #B, mix thoroughly by careful pipetting and continue until row #M for each inhibitor.

c. Dispense 20 µL of 1x assay buffer into **well #P** of the column (for blank correction)

d. Prepare a 5x **Master Mix** of the kinase and BAS-LUM590 probe in 1x assay buffer:

   - Use the optimal kinase concentration determined earlier (step 5).
     - Example: For a final kinase concentration of 2 nM and an BAS-LUM590 probe concentration of 20 nM prepare a solution of 10 nM kinase and 100 nM BAS-LUM590 probe)
   - Volume: 56 µL per inhibitor and extra for pipetting loss

e. Add 4 µl of the 5x Master Mix to each **well #A to #N**.
   - This leads to a final well volume of 20 µl

f. Prepare 5x BAS-LUM590 probe in 1x assay buffer
   - Volume: 4 µl per inhibitor and extra for pipetting loss.

g. Add 4 µl of 5x BAS-LUM590 probe solution to **well #O**.

h. Incubate the plate on a shaking thermostat for 15 minutes at 30°C and 400 rpm

i. Determine the time-gated luminescence intensity values of each well with a fluorescence plate reader
   - Use well #O for the adjustment of instrument settings
   - Use wells #P for blank correction.

j. Plot the obtained luminescence intensity values against log *C* (inhibitor) and analyze the curves using sigmoidal dose-response (variable slope) function.

k. The K_D values of competitive inhibitors can be calculated using Eq. 1.

Selected references

[1] Enkvist *et al.* (2011) Protein-Induced Long Lifetime Luminescence of Nonmetal Probes. *ACS chemical biology* **6**:1052.

[2] Vaasa *et al.* (2009) High-affinity bisubstrate probe for fluorescence anisotropy binding/displacement assays with protein kinases PKA and ROCK. *Analytical Biochemistry* **385(1)**:85.

[3] Lagovina *et al.* (2012) Conjugates of 5-isoquinolinesulfonylamides and oligo-D-arginine possess high affinity and selectivity towards Rho kinase (ROCK). *Bioorganic & Medicinal Chemistry Letters* **22(10)**:3425