

Instruction manual

ARC-Fluo Protein Kinase Assay Kit

Catalogue code: BAS-FLUO590

The **ARC-Fluo 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, ROCK1, ROCK2, AKT3, PKG I, MSK1**.

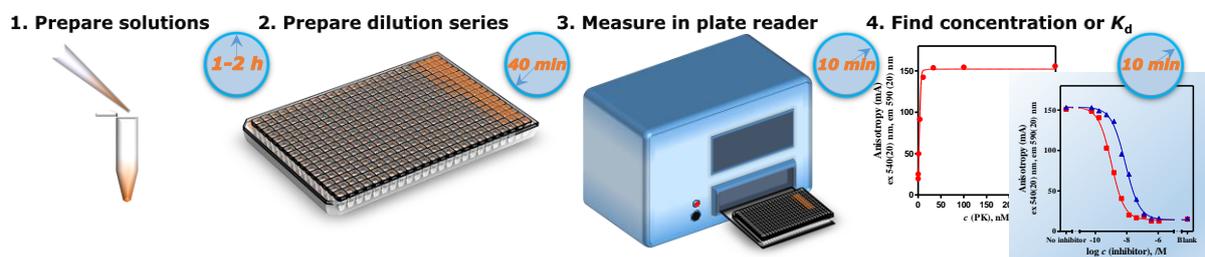


Table 1. ARC-Fluo Protein Kinase Assay Kit (BAS-FLUO590)

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	Fluorescence anisotropy or polarization Ex: 540 nm/ Em: 590 nm [1]
Assay setup	Homogeneous, 1-step, mix & read; 384-well low-volume format
Assay volume	10 - 25 μ l
Probe	BAS-FLUO590 probe

Cat. No.	Amount
BAS-FLUO590	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-FLUO590 probe (1 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

High sensitivity Fluorescence Polarization Plate-Reader (< 5 mP standard deviation at 1 nM Fluorescein), e.g., PHERAStar FS or comparable, with excitation at 540 nm and emission at 590 nm. Tested on PHERAstar plus (BMG Labtech), Synergy Neo (BioTech) and Cytation 5 (BioTech) platereaders.

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1. Description

The **ARC-Fluo 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 fluorescent probe (BAS-FLUO590) from its complex with the protein kinase by the inhibitor (Fig. 1) [1]. BAS-FLUO590 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).

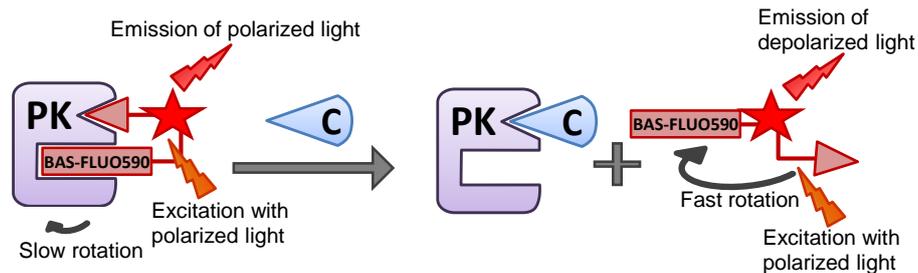


Figure 1. Principle of ARC-Fluo Protein Kinase Assay Kit

The fluorescent probe, BAS-FLUO590 probe, has low molecular weight (MW < 2000) and possesses low value of fluorescence anisotropy (polarization) in solution. When BAS-FLUO590 probe binds to the kinase, a high molecular weight complex is formed (MW > 40000) which leads to increase of anisotropy of the solution. An inhibitory compound competes with BAS-FLUO590 for binding to the kinase and displaces the probe from the complex resulting in decrease of fluorescence anisotropy.

2. Calculation of K_i values

When $K_i > K_D$, where

- K_D is the dissociation constant of the BAS-FLUO590:kinase complex, $K_D = \frac{[\text{BAS-FLUO590}][\text{kinase}]}{[\text{BAS-FLUO590:kinase}]}$
- K_i is the dissociation constant of the inhibitor:kinase complex, $K_i = \frac{[\text{inhibitor}][\text{kinase}]}{[\text{inhibitor:kinase}]}$

The IC_{50} values for the inhibitors are linearly proportional to the K_i values [2]. Comparison of IC_{50} values for a compound under evaluation (X) and a reference compound (Y) with previously established K_i -value enables the determination of the dissociation constant K_i for X from the comparison of IC_{50} -values for these 2 compounds without knowing the concentration of the kinase in the assay

$$K_i(X) = \frac{IC_{50}(X)}{IC_{50}(Y)} \times K_i(Y) \quad (\text{Eq.1}), \text{ where}$$

- IC_{50} is the concentration of the inhibitor that displaces 50% of BAS-FLUO590 probe from BAS-FLUO590:kinase complex

3. Experimental set-up

3.1 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!

3.2 Preparation of the BAS-FLUO590 probe stock solution

Thaw the BAS-FLUO590 probe (1 μM in DMSO) at room temperature and dilute 10-fold in 1x assay buffer.

The resulting 100 nM solution can be stored at 4°C in the dark for one day.

4. 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-FLUO590 probe complex or the concentration of the active form of the protein kinase, please refer to: www.kinase.com/assay_support

Table 2. Recommended concentrations of the BAS-FLUO590 probe and protein kinases in the measurement solution

Protein Kinase	Cofactors	BAS-FLUO590 Probe concentration [nM]	Active kinase concentration [nM]	Approx. K_D , [nM]
PKA α	-	2	2	0.02
ROCK II	-	2	3	0.2
MSK1	-	2	3	0.1
Akt3	-	2	10	5
PKC η	PKC mix ¹	2	3	1.1
PKC δ	PKC mix ¹	2	3	0.77
PKC β 1	PKC mix ¹	2	3	1.3
PKG I α	1 μ M cGMP	2	10	2.7

¹ 0.2 mM CaCl₂, 10 μ g/mL DAG (diacylglycerol), 50 μ g/mL PS (phosphatidylserine), 0.015% Triton X-100

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

- a. Prepare 30 μ l 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)
- b. Dispense 16 μ l of 1x assay buffer into wells (**#1B to #1N**) of a single column of the 384-well plate
- c. Dispense 24 μ l of 125 nM protein kinase into well **#1A**
 - Prepare a titration series by transferring 8 μ l of protein kinase sample from well #1A into #1B, mix thoroughly by careful pipetting. Transfer 8 μ l from #1B to #1C and continue stepwise until #1L. After mixing thoroughly in #1L, discard the tip with 8 μ l solution
- d. Dispense 20 μ l of 1x assay buffer into wells **#1O and #1P** of the column (for blank correction)
- e. Prepare 60 μ l of 5x BAS-FLUO590 probe working solution in 1x assay buffer
 - mix 6 μ l of 100 nM BAS-FLUO590 with 54 μ l of 1x assay buffer
- f. Add 4 μ l of 5x BAS-FLUO590 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 fluorescence anisotropy values of each well with a fluorescent plate reader

- Use well #1M or #1N for the adjustment of instrument settings (the polarization value of free BAS-FLUO590 probe should be set to 0.02)
- Use wells #1O and #1P for blank correction

Plot fluorescence anisotropy values against the concentration of the kinase. Fit the curve to the hyperbolic function

Table 3. Final concentrations of the BAS-FLUO590 probe and protein kinase (final volume: 20 μ l per well)

Well #	Protein conc.	BAS-FLUO590 probe conc.
1A	100 nM	2 nM
1B	33.3 nM	2 nM
1C	11.1 nM	2 nM
1D	3.7 nM	2 nM
1E	1.2 nM	2 nM
1F	412 pM	2 nM
1G	137 pM	2 nM
1H	45.7 pM	2 nM
1I	15.2 pM	2 nM
1J	5.1 pM	2 nM
1K	1.7 pM	2 nM
1L	0.6 pM	2 nM
1M	-	2 nM
1N	-	2 nM
1O	-	-
1P	-	-

The optimal kinase concentration for the inhibition assay equals the concentration that gives

80% of the maximal anisotropy signal.

5. 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 μ l 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-FLUO590 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 μ l of each inhibitor into #A in separate columns
 - Prepare a serial dilution by transferring 8 μ l from #A into #B, mix thoroughly by careful pipetting and continue until row #M for each inhibitor
- c. Dispense 20 μ L of 1x assay buffer into **well #P** of the column (for blank correction)
- d. Prepare a 5x **Master Mix** of the kinase and BAS-FLUO590 probe in 1x assay buffer:
 - Use the optimal kinase concentration determined earlier (step 4)
 - Example: For a final kinase concentration of 3 nM and BAS-FLUO590 probe concentration of 2 nM prepare a solution of 15 nM kinase and 10 nM BAS-FLUO590 probe)
 - Volume: 56 μ L per inhibitor and extra for pipetting loss
- e. Add 4 μ l of the 5x Master Mix to each **well #A to #N**
 - This leads to a final well volume of 20 μ l
- f. Prepare 5x BAS-FLUO590 probe in 1x assay buffer
 - Volume: 4 μ l per inhibitor and extra for pipetting loss
- g. Add 4 μ l of 5x BAS-FLUO590 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 anisotropy 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 anisotropy 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] 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.
- [2] Huang *et al.* (2003) Fluorescence Polarization Competition Assay: The Range of Resolvable Inhibitor Potency Is Limited by the Affinity of the Fluorescent Ligand. *J. Biomolecular Screening* 8:34.