KinaseRa

Instruction manual

ARC(CK2)-Fluo Protein Kinase CK2 Assay Kit

Catalogue code: CK2-FLUO590

The **ARC(CK2)-Fluo Protein Kinase Assay Kit** is used for a homogenous, high-throughput **binding/displacement assay** for screening/characterization of inhibitors of the protein kinase CK2**.**

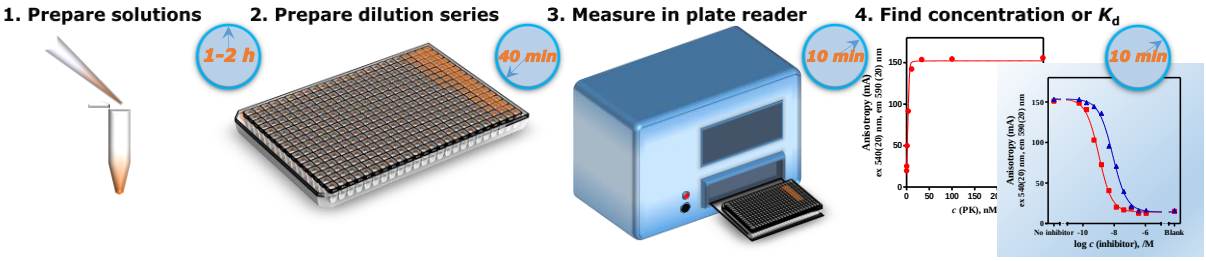

Table 1. ARC(CK2)-Fluo Protein Kinase CK2 Assay Kit (CK2-FLUO590)

Application	Determination of K_d values for both ATP- and substrate-competitive protein kinase CK2 inhibitors
Targets	CK2 (both the free CK2α catalytic subunit and CK2 holoenzyme)
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 µl
Probe	CK2-FLUO590 probe

Cat. No.	Amount
CK2-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

30 µL CK2-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

A Fluorescence Polarization Plate-Reader possessing high sensitivity (< 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(CK2)-Fluo590 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 protein kinase CK2 (Tab. 1).

The principle of the assay relies on the competitive displacement of the fluorescent probe [CK2-FLUO590] from its complex with the protein kinase by the inhibitor (Fig. 1) [1]. CK2-FLUO590 probe possesses high affinity towards CK2α (K_D = 0.4 nM) [2], it is binding with high affinity to both the free CK2α catalytic subunit and CK2 holoenzyme 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).

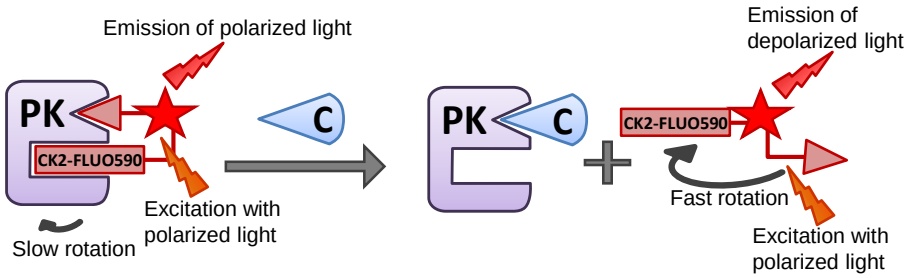
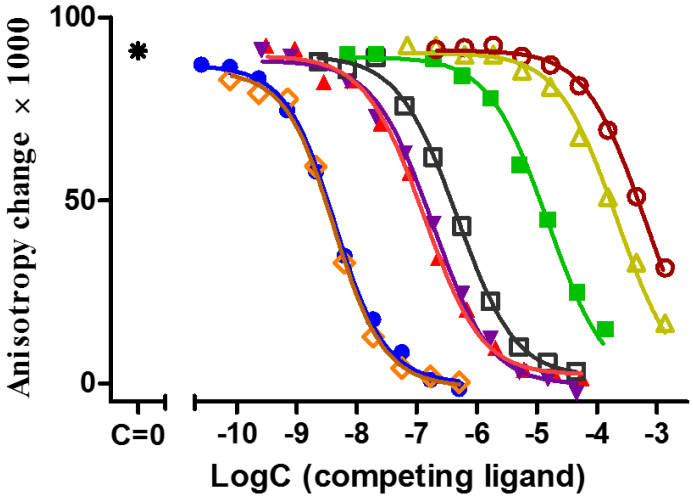


Figure 1. Principle of ARC(CK2)-Fluo Protein Kinase Assay Kit

The fluorescent probe CK2-FLUO590 probe has low molecular weight (MW < 2000) and possesses low value of fluorescence anisotropy (polarization) in solution. When CK2-FLUO590 probe binds to the protein kinase CK2, a high molecular weight complex is formed (MW > 40000) which leads to increase of anisotropy of the solution. An inhibitory compound competes with CK2-FLUO590 for binding to the kinase and displaces the probe from the complex resulting in decrease of fluorescence anisotropy.



Competing ligand	K_d (nM)
ARC-1502	0.4
CX-4945	0.4
ARC-1183	16
ARC-1154	22
ARC-1155	51
K68	1800
ATP + Mg^{2+}	24000
GTP + Mg^{2+}	73000

Figure 2. Displacement of the probe CK2-FLUO590 from the complex with CK2α1–335 by inhibitors or nucleotides ATP and GTP.

2. Calculation of K_d values

When $K_i > K_D$, where

- ***K_D*** is the dissociation constant of the CK2-FLUO590:kinase complex, $K_D = \frac{[\text{CK2}-\text{FLUO590}][\text{kinase}]}{[\text{CK2}-\text{FLUO590:kinase}]}$
- ***K_i*** is the dissociation constant of the inhibitor:kinase complex, $K_\text{i} = \frac{[\text{inhibitor}][\text{kinase}]}{[\text{inhibitor:kinase}]}$

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

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

- ***IC_{50}*** is the concentration of the inhibitor that displaces 50% of CK2-FLUO590 probe from CK2-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 CK2-FLUO590 probe stock solution

Thaw the CK2-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 both the free CK2α catalytic subunit and CK2 holoenzyme. The recommended concentrations can be found in Table 2. 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:CK2-FLUO590 probe complex or the concentration of the active form of the protein kinase, please refer to: www.kinasera.com/assay_support

Table 2. Recommended concentrations of the CK2-FLUO590 probe and protein kinases in the measurement solution

Protein Kinase	CK2-FLUO590 probe concentration [nM]	Active kinase concentration [nM]	Approx. K_D, [nM]
CK2α	2	3	0.4

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)
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 CK2-FLUO590 probe working solution in 1x assay buffer
    - mix 6 µl of 100 nM CK2-FLUO590 probe with 54 µl of 1x assay buffer
f. Add 4 µL of 5x CK2-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 CK2-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 CK2-FLUO590 probe and protein kinase (final volume: 20 µl per well)

Well #	Protein conc.	CK2-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 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 CK2-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 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 CK2-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 CK2-FLUO590 concentration of 2 nM prepare a solution of 15 nM kinase and 10 nM CK2-FLUO590 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 CK2-FLUO590 probe in 1x assay buffer
   - Volume: 4 µl per inhibitor and extra for pipetting loss

g. Add 4 µl of 5x CK2-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] E. Enkvist *et al*. (2012) A subnanomolar fluorescent probe for protein kinase CK2 interaction studies. Organic & Biomolecular Chemistry, **10(43)**:8645.

[3] 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.