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HTRF® Transcreener® ADP 20,000 tests

For in vitro research use only
Storage temperature : 2-8°C

www.htrf.com

HTRF® package insert General information

Document reference : 62ADPPEC rev05 (January 2009)

Packaging details :

		384-well low volume plate (20 µL)
62ADPPEC	HTRF® TRANSCREENER® ADP	20 ,000 tests

1. Assay description and intended use

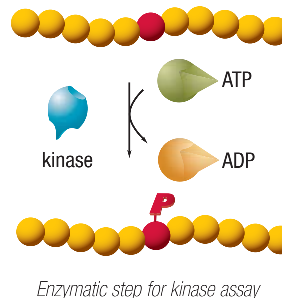
HTRF® TRANSCREENER® ADP is a generic method for detection of ADP.

The assay can be used for any enzyme class that produces ADP (Kinases, ATPases,..). Kinases catalyze the transfer of a phosphate group from ATP to a protein, peptide, lipid, or small molecule substrate. As well as this phosphorylated substrate, the reaction generates ADP. For ATPases, a phosphate group from ATP is transferred to a water molecule. The HTRF® TRANSCREENER® ADP assay applies to both kinase and ATPase activity assessment. The assay involves the two steps described below:

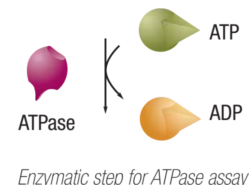
1.1. Enzymatic step:

For KINASES: During this step, the substrate is incubated with the kinase. ATP is added to start the enzymatic reaction and the kinase phosphorylates the substrate.

For ATPases: During this step the ATPase will convert the ATP into ADP and inorganic phosphate.



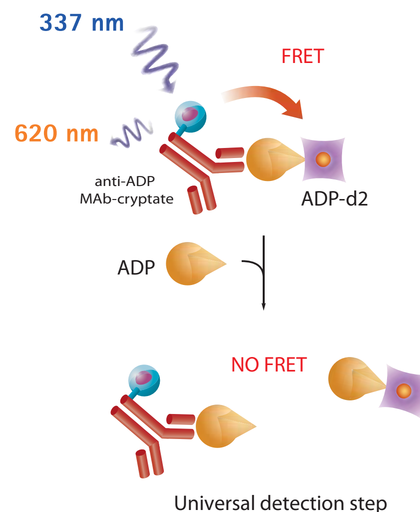
Enzymatic step for kinase assay



Enzymatic step for ATPase assay

1.2. Universal detection step:

The detection method is based on an antibody specific to ADP labeled with Eu^{3+}-Cryptate, which competes with both native ADP and d2-coupled ADP. The resulting TR-FRET signal is inversely proportional to the concentration of ADP in the calibrator or in the sample. The anti-ADP antibody labeled with Eu^{3+}-Cryptate and the ADP-d2 are added with EDTA, in order to stop the kinase activity.



Universal detection step

2. Reagent description and storage conditions - Preparation of stock and working solutions

Supplied reagents	Reagent reconstitution (stock solutions) *Storage conditions*		Working solutions *Storage conditions*
ADP calibrator 1 vial, lyophilized	Reconstitute with distilled water (refer to product label) to obtain a 1 mM stock solution *ADP stock solution can be stored 3 days at 2-8°C or dispensed into single use aliquots and stored at -20°C.*	⇨	See calibration curve preparation for further dilution
ADP-d2 1 vial, lyophilized Anti-ADP Cryptate 1 vial, lyophilized	Reconstitute each vial with 5 ml of distilled water. Mix gently *Conjugate stock solutions are not stable at 2-8°C. They can be dispensed into single use aliquots and stored at -20°C.*	⇨	For each vial dilute 1 volume of reconstituted reagent in 19 volumes of HTRF® TRANSCREENER® ADP detection buffer (1/20 dilution). Mix gently *Conjugate working solutions can be stored 3 days at 2-8°C or dispensed into single use aliquots and stored at -20°C.*

HTRF® TRANSCREENER® ADP enzymatic buffer 5X HEPES 250mM pH7.0, NaN3 0.1%, BSA0.05%	1 vial of 50 mL (liquid 5X)
HTRF® TRANSCREENER® ADP detection buffer Buffer supplemented with BSA 0.1%, KF 0.4M, EDTA 60mM	1 vial of 200 mL

All supplied reagents must be stored at +4°C until the expiration date printed on the product label.

3. Additional material required (not provided)

Enzymatic buffer supplements: The enzymatic buffer must be supplemented with any components required by the kinase or ATPase of interest.

	Recommended Supplier*	Stock solution to prepare
ATP	Sigma # A7699	1 mM in distilled water
Sodium Orthovanadate**	Sigma # S6508	
DTT	Sigma # D0632	100 mM in distilled water
MgCl2	Sigma # M1028	1 M (ready to use)
MnCl2	Sigma # M1787	1 M (ready to use)

***** Suppliers' names are indicative***

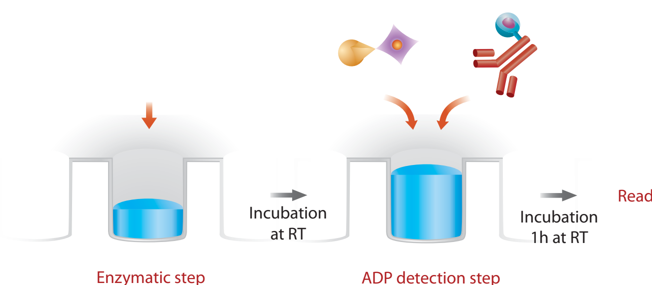
***** Or other phosphatase inhibitors***

Storage: Stock solutions of ATP and DTT should be aliquoted and stored at -20°C for additional use.

4. Assay protocol for 384 well low-volume plate (20µL)

Add assay components (working solutions) in the following order:

ATPase assay	Kinase assay	ADP detection
4 µL compounds (or enzymatic buffer)* + 2 µL ATP + 4 µL ATPase	4 µL compounds (or enzymatic buffer)* + 2 µL Substrate + 2 µL Kinase + 2 µL ATP	5 µL ADP-d2 in EDTA + 5 µL anti-ADP-cryptate in EDTA



The enzymatic reaction (Step 1) is stopped by the addition of the ADP detection reagents which contain EDTA (step 2). The incubation period for the enzymatic step is optimized depending on the enzyme.

For a 384w low-volume plate, we recommend the 10 µL for the enzymatic step and the 10 µL for the detection step for a final assay volume of 20 µL. For a 96 half-well plate (100 µL), each addition volume is simply multiplied by 5.

***** For low volume compound addition, adjust volume to 4 µl with 1x enzymatic buffer-Keep DMSO < 2% in the enzymatic step.***

5. Preparation of the working solutions

The working solutions are prepared from stock solutions by following the instructions :

	Buffer to prepare
Enzymatic buffer	Prepare "enzymatic buffer 1X" by diluting 1 volume of Transcreener® ADP enzymatic buffer 5X with 4 volumes of distilled water and any supplements required by the enzyme of interest, i.e. phosphatase inhibitors, DTT, MgCl2, MnCl2, etc…
Detection buffer	Ready to use Transcreener® ADP detection buffer

	Working solutions to prepare
Compounds	Dilute compound stock solution with enzymatic buffer to prepare a working solution which has 2.5X the required final concentration for the enzymatic step .
Substrate	Dilute the substrate stock solution with enzymatic buffer to prepare a working solution which has 5X the required final concentration for the enzymatic step
Enzyme	Dilute the kinase stock solution with enzymatic buffer to prepare a working solution .
ADP	Dilute the ADP stock solution with enzymatic buffer to prepare a working solution (refer to standard curve preparation in § 6).
ATP	Dilute the ATP stock solution with enzymatic buffer to prepare a working solution (refer to standard curve preparation in § 6).
ADP-d2	Ready to use working solution
Anti ADP-Cryptate	Ready to use working solution

Precautions:

- It is recommended to prepare the required amount of enzymatic buffer 1X (supplemented with ions, components required by the enzyme of interest) just before use, as DTT is stable only one day at 2-8°C once diluted in the enzymatic buffer .

- ATP, substrate and enzyme working solutions must be kept in an ice bath for the time of the experiment (to avoid degradation). They can not be stored and must be used immediatly.

- HTRF® detection reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the detection reagents will impair the assay quality.

6. ADP/ATP Standard Curve preparations

The Standard curves mimic a kinase/ATPase reaction (as ADP is produced, ATP is depleted). The adenosine total concentration should remain constant throughout the range studied i.e. [ATP]+[ADP].

Standard curves should be performed with the concentration of ATP used for the assay.

Standard curves are given as examples for 2 different ATP concentrations: ATP 100 µM and ATP 10 µM.

It is recommended not to use concentrations of ATP above 100 µM. If the enzyme requires to work above 100 µM, please contact our technical support.

ADP standard max solutions are prepared respectively at ADP 100 µM and ADP 10µM then diluted in ATP solutions as shown below.

Standard Curve for ATP 100 µM

	Standard preparation				Working concentrations			
					ADP (µM)	/	ATP (µM)	% ATP Conversion
Standard 7	100 µl ADP 100 µM				100	/	0	100
Standard 6	50 µL	Standard 7	+	100 µl ATP 100 µM	33.33	/	66.67	33.33
Standard 5	50 µL	Standard 6	+	100 µl ATP 100 µM	11.11	/	88.89	11.11
Standard 4	50 µL	Standard 5	+	100 µl ATP 100 µM	3.70	/	96.30	3.70
Standard 3	50 µL	Standard 4	+	100 µl ATP 100 µM	1.23	/	98.77	1.23
Standard 2	50 µL	Standard 3	+	100 µl ATP 100 µM	0.41	/	99.59	0.41
Standard 1	50 µL	Standard 2	+	100 µl ATP 100 µM	0.14	/	99.86	0.14
Standard 0	100 µl ATP 100 µM				0	/	100	0

Standard Curve for ATP 10 µM

	Standard preparation				Working concentrations			
					ADP (µM)	/	ATP (µM)	% ATP Conversion
Standard 6	100 µl ADP 10 µM				10	/	0	100
Standard 5	50 µL	Standard 6	+	100 µl ATP 10 µM	3.33	/	6.67	33.33
Standard 4	50 µL	Standard 5	+	100 µl ATP 10 µM	1.11	/	8.89	11.11
Standard 3	50 µL	Standard 4	+	100 µl ATP 10 µM	0.37	/	9.63	3.70
Standard 2	50 µL	Standard 3	+	100 µl ATP 10 µM	0.12	/	9.88	1.23
Standard 1	50 µL	Standard 2	+	100 µl ATP 10 µM	0.04	/	9.96	0.4
Standard 0	100 µl ATP 10 µM				0	/	10	0

7. Detailed protocol

The enzyme assay is performed as described below, using different controls:

Negative Control (Std 0): 0% ATP Conversion (No Enzyme). This control consists of the ADP antibody-Cryptate and ADP-d2 in the presence of enzymatic reaction components (without enzyme) and ATP (0% ADP). This control defines the upper limit of the assay window. Appropriate negative control must be prepared for each ATP concentration tested.

Buffer control: used to make sure that buffers are not contaminated by Cryptate and do not generate any background fluorescence.

Cryptate control: used to check the Cryptate signal at 620 nm and for the calculation of delta F. This control defines the lower limit of the assay window.

	Standard Curve	KINASE assay		ATPase assay		Blank controls	
Enzymatic step (10 µL)	Standard	Negative control	Sample	Negative control	Sample	Cryptate Control	Buffer Control
Standard	10 µL						
Compounds (or enzymatic buffer)		4 µL	4 µL	4 µL	4 µL	10 µL enzymatic buffer	10 µL enzymatic buffer
Substrate		2 µL	2 µL				
Kinase / ATPase		2 µL enzymatic buffer	2µL	4 µL enzymatic buffer	4 µL		
ATP		2 µL	2 µL	2 µL	2 µL		

Seal plate and incubate at room temperature (RT) or 37°C

Detection step (10µL)							
ADP-d2	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL detection buffer	5µL detection buffer
anti-ADP Cryptate	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL detection buffer

Seal plate and incubate 1h at RT

Remove plate sealer and read on an HTRF® compatible reader*

* More information at http://www.htrf.com/technology/htrfmeasurement/compatible_readers/

8. Data reduction

The tables below present the results obtained with the standard protocol in 20µL. Results are calculated from the 665nm/620nm ratio and expressed in Delta F. Two examples of standard curves for 100µM and 10µM of ADP/ATP are given in the tables below.

The fluorescence is measured at 620 nm (Cryptate) and 665 nm (d2).

A ratio is calculated (665/620) for each well.

Results are expressed as follows (readout on PHERAstar Plus reader from BMG LABTECH):

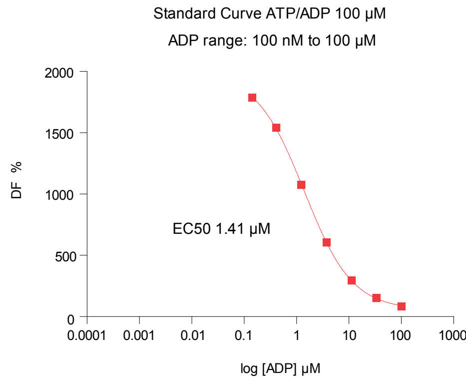
100 µM ADP/ATP Standard Curve Data

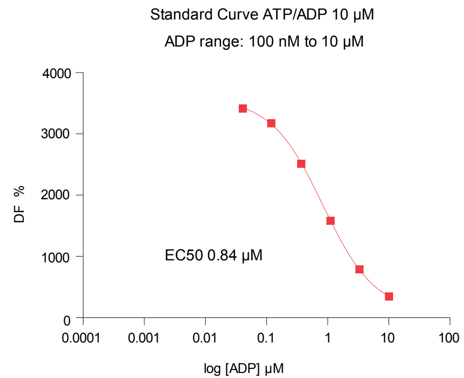
	% ATP Conversion	ADP (µM) / ATP (µM) working concentrations	A (665nm)	B (620nm)	ratio (1)	Mean ratio (2)	CV% (3)	Delta F% (4)	1-(dF/dFmax) (5)
Blank (Cryptate control)			1443 1319	28031 25785	515 512	513	0.4		
Standard 0	0	0 µM / 100 µM	28253 29603	26895 26238	10505 11282	10894	5.0	2023	
Standard 1	0.14	0.14 µM / 99.86 µM	23995 26282	24858 27055	9653 9714	9684	0.4	1787	12%
Standard 2	0.41	0.41 µM / 99.59 µM	23796 22232	27318 27256	8711 8157	8434	4.6	1543	24%
Standard 3	1.23	1.23 µM / 98.77 µM	16211 17393	26587 29106	6097 5976	6037	1.4	1076	47%
Standard 4	3.70	3.7 µM / 96.3 µM	8771 10641	24479 29027	3583 3666	3624	1.6	606	70%
Standard 5	11.11	11.1 µM / 88.9 µM	5055 5812	24747 28680	2043 2026	2035	0.6	296	85%
Standard 6	33.33	33.3 µM / 66.7 µM	3690 3551	27177 28692	1358 1238	1298	6.5	153	92%
Standard 7	100	100 µM / 0 µM	2296 2501	24598 26149	933 956	945	1.7	84	96%

10 µM ADP/ATP Standard Curve Data

	% ATP Conversion	ADP (µM) / ATP (µM) working concentrations	A (665nm)	B (620nm)	ratio (1)	Mean ratio (2)	CV% (3)	Delta F% (4)	1-(dF/dFmax) (5)
Blank (Cryptate control)			1249 1196	22647 22593	552 529	540	2.9		
Standard 0	0	0 µM / 10 µM	37945 33962	18674 16901	20320 20095	20207	0.8	3639	
Standard 1	0.4	0.04µM / 9.96 µM	37857 35845	19950 18823	18976 19043	19010	0.3	3417	6%
Standard 2	1.23	0.12 µM / 9.88 µM	34250 34001	19222 19351	17818 17571	17694	1.0	3174	13%
Standard 3	3.70	0.37 µM / 9.63 µM	31518 29293	22213 20794	14189 14087	14138	0.5	2516	31%
Standard 4	11.11	1.11 µM / 8.89 µM	19535 19044	21053 21313	9279 8935	9107	2.07	1585	56%
Standard 5	33.33	3.33 µM / 6.67 µM	10188 10704	20953 22356	4862 4788	4825	1.1	793	78%
Standard 6	100	10 µM / 0 µM	5653 4940	23375 20323	2418 2431	2425	0.4	349	90%

1. Ratio = (665 nm/620 nm) x 104
2 . Mean ratio = Σ ratio / 2 for n=2
3. CV%= (Std deviation/Mean ratio) x 100
4. Delta F% = (ratio - ratioBlank) / ratioBlank x 100
Delta Fmax = Delta F of the negative control (standard)
5. In order to obtain positive modulation, data can be expressed as follows: 1-(Delta Fsample / Delta Fmax)





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