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TITLE:

Nucleoside Triphosphate Hydrolases Assay in *Toxoplasma gondii* and *Neospora caninum* for High-Throughput Screening Using a Robot Arm.

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SUMMARY:

Toxoplasma gondii and *Neospora caninum* infections are found in humans and animals and lead

to serious health issues. The two parasites share similar nucleoside triphosphate hydrolases and play important roles in propagation and survival. We established a high-standard assay of the enzymes requiring robot arm usage.

ABSTRACT:

Protozoan parasites infect humans and many warm-blooded animals. *Toxoplasma gondii*, a major protozoan parasite, is commonly found in HIV-positive patients, organ transplant recipients and pregnant women, resulting in the severe health condition, Toxoplasmosis. Another major protozoan, *Neospora caninum*, which bears many similarities to *Toxoplasma gondii,* causes serious diseases in animals, as does Encephalomyelitis and Myositis-Polyradiculitis in dogs and cows, resulting in stillborn calves. All these exhibited similar nucleoside triphosphate hydrolases (NTPase). *Neospora caninum* has a NcNTPase, while *Toxoplasma gondii* has a TgNTPase-I. The enzymes are thought to play crucial roles in propagation and survival. In order to establish compounds and/or extracts preventing protozoan infection, we targeted these enzymes for drug discovery. The next step was to establish a novel, highly sensitive, and highly accurate assay by combining a conventional biochemical enzyme assay with a fluorescent assay to determine ADP content. We also validated that the novel assay fulfills the criteria to carry out high-throughput screening (HTS) in the two protozoan enzymes. We performed HTS, identified 19 compounds and six extracts from two synthetic compound libraries and an extract library derived from marine bacteria, respectively. In this study, a detailed explanation has been introduced on how to carry out HTS, including information about the preparation of reagents, devices, robot arm, etc.

INTRODUCTION:

Robotics have been established as sophisticated and powerful tools for achieving significant breakthroughs in various fields beyond industry and fabrication engineering, such as biochemistry, molecular biology, and clinical research, and notably HTS[1–3]. *Toxoplasma gondii* is a major parasite and a single-cell parasitic eukaryote[4] that causes serious health issues in humans[5] and many homeothermic animals[4], resulting in infections leading to Toxoplasmosis, a particularly severe condition in AIDS patients[6], organ transplant recipients[7], and pregnant women[8]. *Neospora caninum* belonging to *Phylum Apicomplexa*[9] mainly infects dogs and cows[6], which results in Encephalomyelitis and Myositis-Polyradiculitis in dogs[10,11] and abortion in cows[12,13]. Further, *Neospora caninum* exhibits morphological and phylogenetical close similarities of *Toxoplasma gondii*[9,14]. Additionally, they have a nucleoside triphosphate hydrolase (NTPase; EC3.6.1.15)[14]. The enzymes are quite different from conventional ecto-ATPase[14]. These parasites generate a considerable amount of NTPase proteins, 2%–8% of the total protein and play an important role as dormant enzymes in their tachyzoite stage[15]. It should be noted that in dense secretory granules, these are condensed[16] and secreted into the parasitophorous vacuole[16]. As a biochemical enzymatic character, NTPase is activated by dithiothreitol[17]. It is predicted that the inducers such as the dithiol compound, an unidentified enzyme such as dithiol-disulfide oxidoreductase, and another exhibit the same nature. They have not yet been identified in parasites. However, the enzyme does play an important role in releasing tachyzoite from infected host cells[17].

Toxoplasma gondii has two NTPase isoforms[18]: type I enzyme TgNTPase-I, and type II enzyme TgNTPase-II[18]. The former preferentially utilizes triphosphate nucleosides as a substrate[18]. The latter hydrolyzes both triphosphate and diphosphate nucleosides[18]. The homology is 97% in amino acid levels[18]. *Neospora caninum* also has an orthologue of TgNTPase-I named NcNTPase[19]. The homology is 73% in amino acid levels[19]. Prof. Asai and Prof. Harada generated recombinants of both the NTPase using *E. coli.* and changed the constitutively active mutants of these as previously reported[20]. They kindly gifted the two active mutants. Both enzymes can convert ATP to ADP *in vitro*[20]. Very recently, we measured the activity of NTPase using ADP content hydrolyzed by the enzymes. Finally, we succeeded in establishing the high-standard assay through the process of determining ADP content with a combination of fluorescence and enzymatic reaction as previously reported[21,22]. We also did high-throughput screening (HTS)[22].

This study introduces detailed procedures of a novel high-accuracy and dynamic-range assay[21] and a detailed explanation on how to prepare reagents to measure the enzyme activity and develop fluorescent intensity using a robot arm for HTS.

PROTOCOL:

1. Expression and purification of recombinant TgNTPase-I and NcNTPase

1.1. Prepare the expression plasmid and introduce it to the *E. coli.* strain BL21.

NOTE: Detailed information on constructs and procedures is shown in a previous report[14]. In this study, both TgNTPase-I and NcNTPase constitutively active mutants were kindly gifted by Prof. Asai and Prof. Harada.

2. Preparation and placement of biofluorescent reaction solution onto the plate

2.1. Prepare 2x biofluorescent reaction solution as described in **Table 1** for 2 mL of master mix for a 400-well assay in 384-well format.

2.2. Prepare stock solutions using distilled water (DW) for each indicated concentration with the exception of Resazurin and N-ethylmaleimide. Dissolve the remaining two reagents with DMSO in the appropriate concentration. Then, store the reagents at -30 °C until the time to conduct the experiments.

2.3. Add 15 µL of 2x biofluorescent reaction solution to each of the 368 wells (384 well format).

NOTE: The first plate serves as a reservoir to have enough of the reagent to conduct experiments twice.

3. Put test compounds or extracts onto the bottom of each well in the assay plate.

3.1. Put 0.5 µL of each compound onto the bottom of the plate using a robot arm from the library mother plate, including test compounds.

3.2. Add 0.5 µL of DMSO to both negative and positive control.

4. Preparation of enzyme reaction mixture and the beginning of the reaction.

4.1. Prepare enzyme reaction solution as given in **Table 2**.

4.2. Add NTPase (Final concentration is 0.0002 µg/mL) to 50 mL of the enzyme reaction mixture, mix quickly and transfer to a plastic reservoir.

4.3. Proceed to simultaneously inject 4.5 µL into each well except for the negative control line using the robot arm.

4.4. Add the same amount of the enzyme reaction mixture with PBS instead of the enzyme preceded by the simultaneous injection.

4.5. Place the plate in an incubator at 37 °C for 10 min.

5. Conduct real-time measurement of enzymatic activities using a microplate reader

5.1. After 10 min of incubation, simultaneously add 5 µL of 2x biofluorescent reaction solution to each well immediately using the robot arm.

5.2. Temporarily stop the robot arm to hold the solution in each tip over the location where the plate is to be placed.

5.3. Once the assay plate is in place, restart the robot arm.

NOTE: To avoid saturation of the generated ADP, immediately measure the fluorescent intensity.

5.4. Quickly spin down (200 x *g* for 1 min) the plate, and place the plate in the plate reader.

5.5. Start the real-time measurement of fluorescence at 540 nm/590 nm (excitation/emission) every minute for 1 h.

6. Analysis of results

6.1. Calculate values as the mean ± standard error of the mean (SEM) from the indicated and replicated samples in each experimental group; replicate experiments to ensure consistency.

6.2. Perform statistical significance using a Student's *t*-test.

6.3. Calculate values as statistically significant if the *P* values are $P < 0.05$.

6.4. Calculate Signal-to-Background ratio (S/B), Signal-to-Noise ratio (S/N) and Z'-factor using the following formulae:

S/B = Average ligand/Average vehicle

S/N = (Average ligand – Average vehicle)/Standard deviation vehicle

Z'-factor = 1 - (3 x Standard deviation ligand + Standard deviation vehicle)/(Average ligand - Average vehicle)

6.5. Ensure that S/B, S/N, and Z'-factor are more than 3, 10 and 0.5, respectively.

NOTE: Confirm whether each experiment meets the general criterion sufficient to do HTS as stated in previous reports[23,24].

REPRESENTATIVE RESULTS:

A principle of the assay is summarized in **Supplementary Figure 1** and based on a previous report[12,18]. The assay was designed in a 384-well format, as shown in **Figure 1**. The far-right and left lines were avoided on the plate. The two lines next to the far left and right lines were then used as negative control and positive control with or without the enzyme, respectively (n = 16). This allowed for the 320 compounds on the plate to be easily examined. **Figure 1B** indicates the plate, container, robot arm, its tips and microplate reader used in this study. The procedures used in this study are shown in **Figure 1C**. Initially, in order to determine the fluorescent intensities, 2x biofluorescent reaction solution was prepared and aliquoted (5 µL) to every well except for the far right and left lines (352 wells). Following this, 0.5 µL of 320 test compounds were transferred to the 384-well assay plate. We then prepared an enzyme reaction mixture according to the protocol. At the same time, we re-aliquoted 4.5 µL of the solution with the enzyme to every well except for the negative control. After the enzyme reaction mixture was added, the plate was incubated at 37 °C for 10 min. When the incubation was finished, an equal amount of the enzyme reaction mix was added to every well and flashed. And finally, the fluorescent intensity was measured every minute for 1 h by a microplate reader.

This allows monitoring fluorescent intensities in a high dynamic range and real-time (**Figure 2A**) using a microplate reader. Initially, we added the step of stopping the enzyme reaction by 0.1 N HCl and allowed the reaction to develop fluorescence. The speed of the enzyme reaction is too fast to measure the correct activity at the inappropriate time point due to the saturation of the reaction. Omitting this step allows for obtaining fluorescent intensity in all wells every minute for 1 h without stopping the reaction (**Figure 2B**). When the data clearly indicates the linear range for all samples, the samples can be effectively compared (**Figure 2B**). The red dashed line indicates that negative control is stable during measurement (**Figure 2A,B**). We validated that two extracts from marine bacteria have an inhibitory effect on the enzyme (**Figure 2**, Blue and Yellow dashed lines).

Finally, we were able to obtain serial dilutions of the four extracts ranging from 1 to 1,000,

3,000, and 10,000 dilutions at an appropriate time point, which is the linear range (**Figure 3**). Two extracts significantly inhibited the enzyme activity in a concentration-dependent manner (**Figure 3**). The remaining two extracts did not have a significant effect in a concentration-dependent manner, although extract #2 did have a significant inhibitory effect on the enzyme at only a 1 to 1,000 dilution rate. In this assay, the S/B, S/N, and Z' factor were 7.06, 100.5, and 0.89, respectively.

FIGURE AND TABLE LEGENDS:

Figure 1: Design for HTS assay on protozoan NTPase activity. (**A**) Example of a 384-well format. Yellow indicates test samples, #1–320. Light green indicates positive control, including DMSO and NTPase. Orange indicates negative control, including DMSO and PBS. Gray indicates blank. (**B**) The photos of the plates, devices, and tips in this study. The upper-left panel shows the plate used as an assay plate or a 2x biofluorescent reaction solution container. The upper-right panel shows a container for a 50 mL enzyme reaction mixture. The lower left panel shows tips for the robot arm. The lower middle panel shows the robot arm. The lower-right panel shows a microplate reader. (**C**) Schematic chart indicates brief procedures for HTS.

Figure 2: Representative results analyzed using a microplate reader. (**A**) The monitor measures fluorescent intensity in every well every min for 1 h. (**B**) The plots show that the sigmoid curves of every well were automatically described by the microplate reader wizard. The red dashed lines indicate samples of the negative control. The blue and yellow dashed lines indicate samples treated with extract #3 and #1, respectively.

Figure 3: NTPase activity. Extracts #1 and #3 inhibited NTPase activity in a concentration-dependent manner. The data are expressed as means ± SEM ($n = 16$, * $P < 0.05$).

Table 1: Biofluorescent reaction solution. Volume and final concentrations of the constituents used to prepare the biofluorescent reaction solution.

Table 2: Enzyme reaction solution. Volume and final concentrations of the constituents used to prepare the enzyme reaction solution.

Supplementary Figure 1: Assay principle. Blue dashed circle indicates that NTPase hydrolyzes ATP to ADP in 'Enzyme reaction mixture'. Red dashed circle indicates that ADP drives the reaction changing Resazurin to Resorufin in 'Biofluorescent reaction solution'.

DISCUSSION:

We succeeded in establishing a novel high-dynamic range and -accuracy assay with a combination of a classical enzyme assay and a fluorescent assay for ADP, which is the end product through ATPase, including Tg and Nc ATPase[22]. In order to carry out HTS, it is important that the assay has better values of S/B, S/N, and Z' factor than a classical enzyme assay[15,22]. Additionally, omitting the step of stopping the enzyme reaction with an acid such as HCl and using real-time measuring of fluorescence in all wells every minute for 1 h, results in easier handling and minimal misleading, allowing for a good comparison of the data with an

inappropriate time point, which is saturated among samples. The speed of the enzyme reaction is too fast to measure the correct activity at the inappropriate time point due to the saturation of the reaction.

In this paper, we introduced an efficient method of preparing the reagents, enzymes, enzyme reaction mixture, 2x biofluorescent reaction solution, described how to effectively use a robot arm and microplate reader, and provided the details of all the procedures. Note that in addition to ATPase, the novel approach to this assay utilizes kinase and other enzyme assays *via* monitoring ADP content[21], which can be utilized by scientists performing basic research and drug discovery in both biological and basic medical research. This experiment makes it feasible to conduct drug screening for a large number of test compounds available today and at a very reasonable cost of 2 USD per sample[21]. If the number of compounds is 20,000, the total cost is 40,000 USD. Thus, this technique is advantageous for scientists to carry out drug screening.

From the standpoint of clinical diagnostics, our two protozoan enzymes are increasingly being used, as a feasible blood marker, in the treatment of acute toxoplasmosis, and its diagnostic rate is 93% by ELISA, concurring with the Sabin-Feldman dye test titer, a well-established serological test for toxoplasmosis[25]. Although we have not conclusively confirmed the active enzyme in the blood of infected patients, the high-sensitive assay for protozoan enzymes introduced in this study can be greatly instrumental specifically in clinical diagnostics of toxoplasmosis and affect cutting edge advances in basic biological and biomedical fields, drug discovery, and general clinical diagnostics.

In conclusion, this novel approach achieved significant improvements in high-standard assay by omitting stop enzymatic reactions and by performing real-time measurement of fluorescent intensities in every well. The very important step of injecting reagents to carry out enzyme reactions and fluorescent development in every well is automatic and simultaneous. Additionally, real-time measurement is also very important to avoid false-negative results so that there are no different fluorescent intensities among the samples due to saturation. To date, no errors have been found in these methods, no improvements shown necessary, and the protocol is relatively easy to follow. In comparison to conventional NTPase assay, the throughput, accuracy, and dynamic range of this technique are quite remarkable. The only limitation is that we have not yet miniaturized the assay from the 384 to 1536-well format. If available, the running cost can be much more reasonable in the 1536-well format. From the viewpoint of NTPase, the active form of NTPase can only be measured with this assay. If necessary, a step to make the enzyme active can be added. However, this assay is eminent and easy to achieve as compared to conventional low-throughput and dynamic-range assays. Moreover, the running cost is very reasonable and thus helpful in carrying out drug screening even in the case of a large number of test compounds. This assay can be greatly instrumental, specifically in clinical diagnostics of toxoplasmosis, and will lead to cutting-edge advances in basic biological and biomedical fields, drug discovery, and general clinical diagnostics. Our introduction and demonstration of this assay process, including the method of preparing reagents and handling devices effectively, is highly beneficial to research science and can be applied to the handling of devices for other enzymatic assays *via* measuring ADP content.

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DISCLOSURES:

The authors have no financial interest in this study.

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Figure 1

Figure 1

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A			1	17	33	49	65	81	97	113	129	145	161	177	193	209	225	241	257	273	289	305		
B			2	18	34	50	66	82	98	114	130	146	162	178	194	210	226	242	258	274	290	306		
C			3	19	35	51	67	83	99	115	131	147	163	179	195	211	227	243	259	275	291	307		
D			4	20	36	52	68	84	100	116	132	148	164	180	196	212	228	244	260	276	292	308		
E			5	21	37	53	69	85	101	117	133	149	165	181	197	213	229	245	261	277	293	309		
F			6	22	38	54	70	86	102	118	134	150	166	182	198	214	230	246	262	278	294	310		
G			7	23	39	55	71	87	103	119	135	151	167	183	199	215	231	247	263	279	295	311		
H			8	24	40	56	72	88	104	120	136	152	168	184	200	216	232	248	264	280	296	312		
I			9	25	41	57	73	89	105	121	137	153	169	185	201	217	233	249	265	281	297	313		
J			10	26	42	58	74	90	106	122	138	154	170	186	202	218	234	250	266	282	298	314		
K			11	27	43	59	75	91	107	123	139	155	171	187	203	219	235	251	267	283	299	315		
L			12	28	44	60	76	92	108	124	140	156	172	188	204	220	236	252	268	284	300	316		
M			13	29	45	61	77	93	109	125	141	157	173	189	205	221	237	253	269	285	301	317		
N			14	30	46	62	78	94	110	126	142	158	174	190	206	222	238	254	270	286	302	318		
O			15	31	47	63	79	95	111	127	143	159	175	191	207	223	239	255	271	287	303	319		
P			16	32	48	64	80	96	112	128	144	160	176	192	208	224	240	256	272	288	304	320		

Test samples

Positive control (DMSO and enzyme)

Negative control (DMSO and PBS)

Blank

B

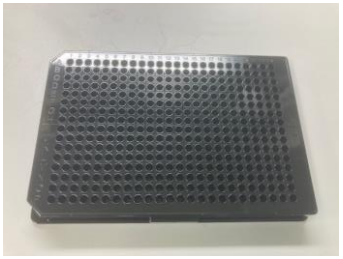
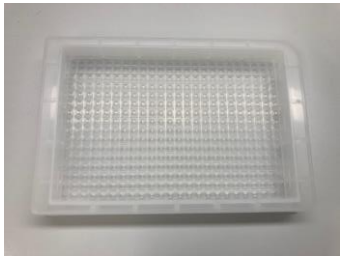
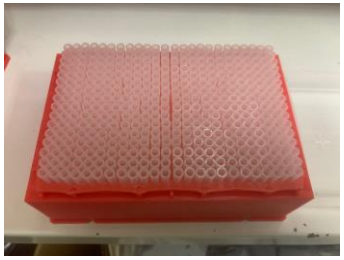


Plate for assay and container for 2x biofluorescent reaction solution

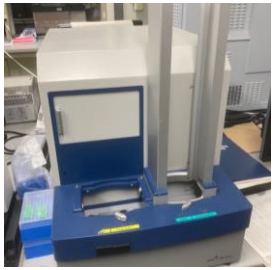


Container for enzyme reaction mixture



Disposable tips for Robot arm

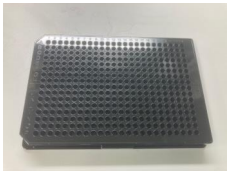
Robot arm



Microplate reader

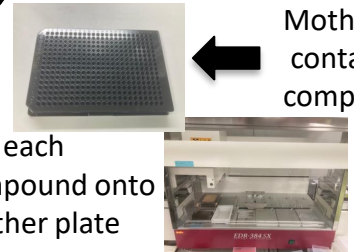
C

Prepared 2x biofluorescent reaction solution and re-aliqouted 320 wells



Add 2x reaction to each well

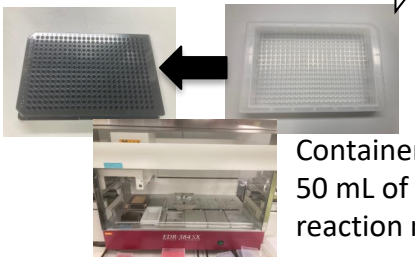
Test compounds were put onto assay plate from mother plate by robot arm



Add each Compound onto another plate

Mother plate containing compounds

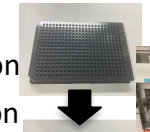
Simultaneously injected enzyme reaction mixture to every wells except for negative control



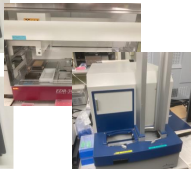
Container filled with 50 mL of enzyme reaction mixture

Put the assay plate in the incubator at 37 degrees for 10 min

Prepared plate containing 2x reaction solution



Mix 2x reaction solution to the assay plate



Simultaneously injected 2x biofluorescent reaction solution to every wells and then, started measuring every min for 1 h

Figure 2

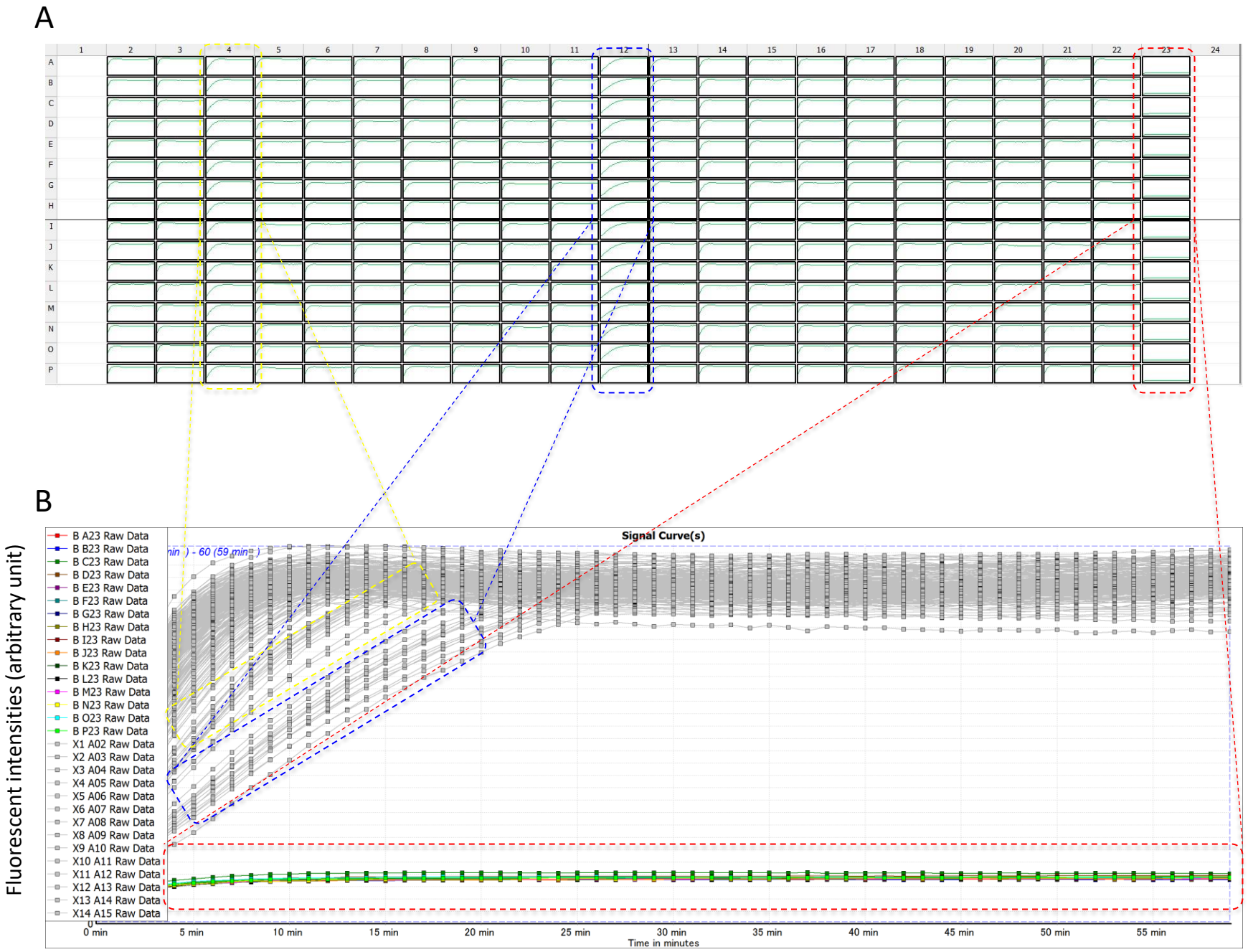


Figure 3

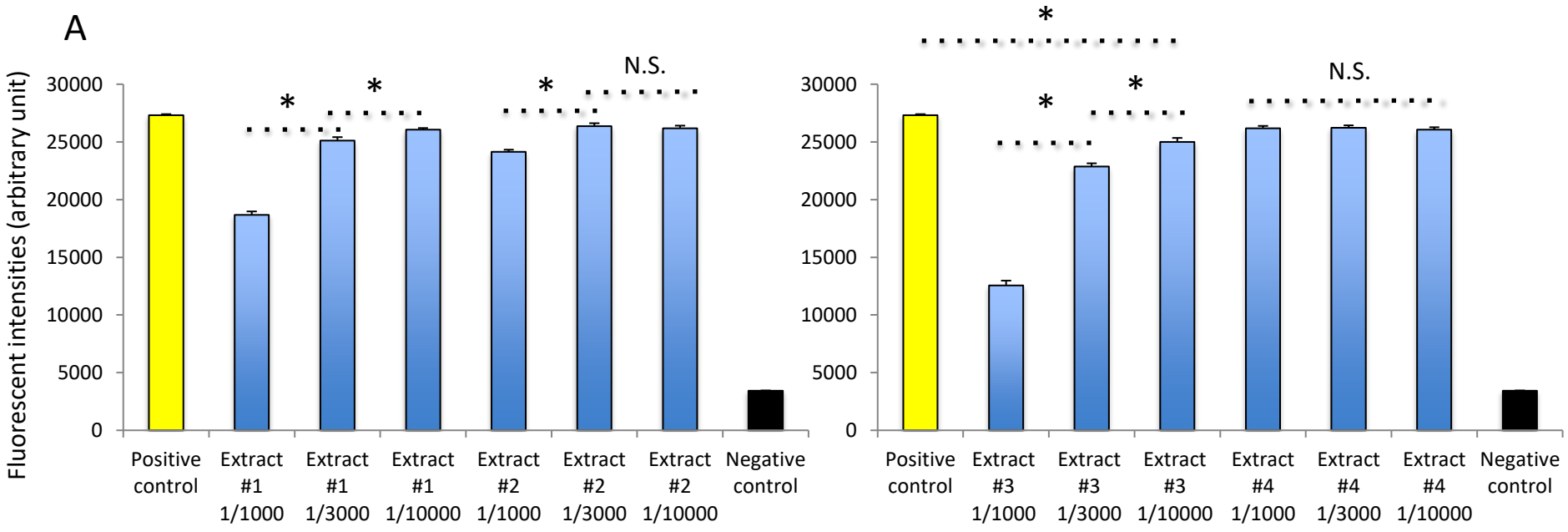


Table 1

Constituents	Volume	Final Concentration
Distilled water (DW)	1420 µL	
Tris-HCl (500 mM, pH 7.5), $MgCl_2$ (50 mM)	400 µL	100 mM and 10 mM
Glucose (200 mM)	20 µL	2 mM
ADP-hexokinase (200 U/mL)	20 µL	2 U/mL
Glucose-6-phosphate dehydrogenase (200 U/mL)	20 µL	2 U/mL
Diaphorase I (200 U/mL)	20 µL	2 U/mL
NADP (20 mM)	20 µL	200 µM
Resazurin in DMSO (20 mM)	10 µL	100 µM
BSA (1%)	20 µL	0.01%
Triton X-100 (10%)	10 µL	0.05%
N-ethylmaleimide in DMSO (1 M)	40 µL	20 mM

Table 2

Constituents	Volume	Final Concentration
HEPES-KOH (500 mM, pH 7.5)	5 mL	50 mM
Mg $(CH_3COO)_2$ (60 mM)	5 mL	6 mM
ATP (100 mM)	500 µL	1 mM
Distilled Water (DW)	39.5 mL	

Comments on Jove editors,

Dear Prof. Lyer,

Thank you so much for your comments on our manuscript (JoVE62874R3).
I am returning herewith the above manuscript, which had been revised according to reviewer's comments. We would be happy if the revised manuscript is reviewed again and is accepted for publication in Journal of Visualized Experiments.

Sincerely yours,

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Editor

1. We changed our manuscript format to fit the journal standard and responded rebuttal comments on each comment.
2. We carefully improved the manuscript to ensure that there are no spelling or grammar issues.
3. We rewrote the final part of discussion as given below.
4. In conclusion, our novel approach achieved significant improvements in high-standard assay by omitting stop enzymatic reactions and by performing real-time measurement of fluorescent intensities in every well. The very important step of injecting reagents to carry out enzyme reactions and fluorescent development in every well is automatic and

simultaneous. Additionally, real-time measurement is also very important to avoid false negative results so that there are no different fluorescent intensities among the samples due to saturation. To date, no errors in our methods have been found, no improvements shown necessary, and the protocol is relatively easy to follow. In comparison to conventional NTPase assay, our throughput, accuracy and dynamic range are quit remarkable. Our only limitation is that we haven't yet miniaturized the assay from 384 to 1536-well format. If available, the running cost can be much more reasonable in 1536-well format. From the view point of NTPase, the active form NTPase can be only measured with this assay. If necessary, a step to make the enzyme active can be added. However, this assay is eminent and easy to achieve as compared to conventional low-throughput and -dynamic range assay Moreover, the running cost is very reasonable and thus, helpful in carrying out drug screening even in the case of a large number of test compounds. This assay can be greatly instrumental, specifically in clinical diagnostics of toxoplasmosis, and will lead to cutting edge advances in basic biological and biomedical fields, drug discovery, and general clinical diagnostics.Our introduction and demonstration of this assay process including the method of preparing reagents and handling devices effectively is highly beneficial to research science, and can be applied to the handling of devices for other enzymatic assay via measuring ADP content.

5. We sent psd of figures to you.
6. We improved the description of figure according to your comments. Then, I sent pptx file of the figure.
7. We improved the duplication of references and checked any other duplication.
8. We improved Biotec in Table of Materials. Biotec is correct.

Supplemental Figure 1