Innova Biosciences
first for Antibody labeling

GTPase assay kit

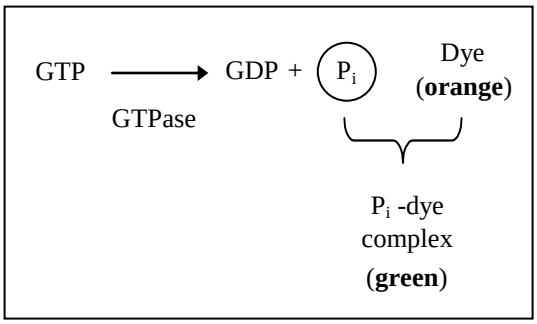
Product codes: 602-0120, 602-0121, 602-0122

Release 004

1. INTRODUCTION

The GTPase colorimetric assay kit employs a 96-well plate format with all reagents necessary for measuring GTPase activity. The kit contains specially purified P_i-free GTP to ensure the lowest possible background signals. It also contains P_iColorLock™ Gold reagent (an improved malachite green formulation) with additives to prevent background signals arising out of non-enzymatic GTP hydrolysis. Assays can be read anywhere in the wavelength range 590-660nm.

Fig 1. Principle of the GTPase assay kit:



2. INSTRUCTIONS

2.1. Components in the 2-plate GTP assay kit
(5-plate kit amounts in brackets)

Store at 4°C:

1 x 10ml of P_iColorLock™ Gold*	(1 x 25ml)
1 x 0.25ml of Accelerator	(1 x 0.5ml)
1 x 5ml of Stabiliser	(1 x 10ml)
1 x 1.5ml of 0.1M $MgCl_2$	(2 x 1.5ml)
1 x 5ml of 0.5M Tris pH 7.5	(1 x 10ml)
1 x 5ml of 0.1mM P_i standard	(1 x 10ml)

Store at -70°C:

2 x 1ml specially purified GTP	(5 x 1ml)

Store at room temperature:

2 x 96-well plates	(5 plates)

*Exercise caution – this reagent contains 5M HCl. Wear gloves and safety glasses.

2.2. Overview of GTP colorimetric assay

(a) Make substrate/buffer mix.

(b) Make 'Gold mix' by adding Accelerator to P_iColorLock™ Gold.

(c) Set up assays with GTPase (not supplied); 100µl enzyme plus 100µl substrate/buffer mix.

(d) Add 50µl of Gold mix to stop reactions.

(e) After 2 minutes, add 20µl of Stabiliser. Mix the solution in the wells by gently pipetting up and down with the pipette volume set above 100µl.

(f) After 30 min, read the plate at a wavelength in the range 590-660nm.

2.3. Preparation of reagents

2.3.1. GTP substrate

GTP should be thawed by warming between the fingers or by immersion in cold water. Store the substrate on wet ice until required. For best results treat the GTP vials as single use. Surplus GTP stock can be re-frozen in aliquots and stored at -70°C but this may result in higher background due to degradation.

2.3.2. Substrate/buffer (SB) mix

The assay kit is supplied with 0.5M Tris assay buffer pH 7.4. However, you may use any other non-phosphate-containing buffer if you wish. Most GTPases will require a metal ion cofactor. $MgCl_2$ (0.1M) is supplied with the kit but you can substitute your own metal ion, or include other metal ions, if required.

Table 1 (below) shows the volumes required to make up SB mix for selected numbers of wells. These volumes result in final buffer and Mg^{2+} concentrations (i.e. after your enzyme has been

added) of 50mM and 2.5mM, respectively. If you require concentrations that are different to those suggested, simply alter the amount of water added in Table 1 to compensate. The final concentration of purified GTP is 0.5mM.

Table 1. Preparation of SB mix

No. of Wells	0.5M Buffer (µl)	0.1M $MgCl_2$ (µl)	10mM GTP (µl)	Water (µl)
1	20	5	10	65
25	500	125	250	1625
50	1000	250	500	3250
75	1500	375	750	4875
100	2000	500	1000	6500
150	3000	750	1500	9750
200	4000	1000	2000	13000
250	5000	1250	2500	16250
500	10000	2500	5000	32500

2.3.3. Gold mix

Prepare 'Gold mix' shortly before the reagent is required by adding 1/100 vol. of Accelerator to P_iColorLock™ Gold (see Table 2 below).

Table 2. Volumes needed to make Gold mix

No. of Wells	P_iColorLock™ Gold (µl)	Accelerator (µl)
1	50	0.5
25	1250	12.5
50	2500	25
75	3750	37.5
100	5000	50
150	7500	75
200	10000	100
250	12500	125
500	25000	250

2.4. Important considerations

2.4.1. Checking for free P_i in enzyme preps

Free P_i will cause a high background. To check if your enzyme sample contains free P_i, make up the solutions shown in Table 3. Set up duplicate wells (200µl/well) and add 50µl of Gold mix. Two minutes later add 20µl of Stabiliser. Solutions 1 & 4 should change colour from dull yellow into golden yellow over a period of 5-10 minutes. Solutions 2 & 3 should give a strong green colour as soon as the Gold mix is added. After 30 minutes, read the plate at 635nm (max. absorbance) or use a wavelength in the range 590-660nm (which will give at least 80% of the maximum signal). Solution 4 should give <0.15 absorbance units. If sample 1 gives a similar value (<0.2) P_i contamination is minimal. Solutions 2 & 3 will give >1.0 absorbance units.

Significant amounts of free P_i in the enzyme may be eliminated by dialysis or desalting. Alternatively, P_iBind™ resin may be used (see '4. Related products').

Table 3. Checking for P_i contamination

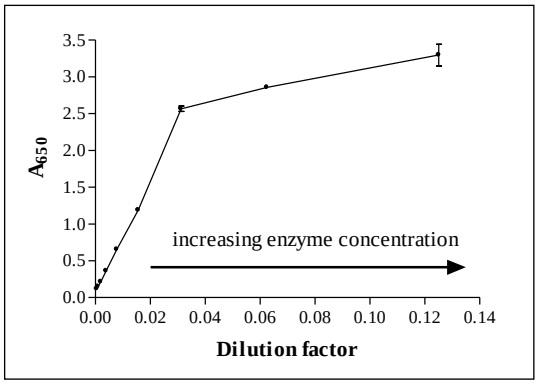
Solution	Enzyme (µl)	Water (µl)	0.1mM P_i (µl)
1	100	100	0
2	100	60	40
3	0	160	40
4	0	200	0

2.4.2. Absorbance versus amount of enzyme

It is important for quantitative work to ensure that absorbance versus enzyme concentration is linear. Most assays are linear if substrate hydrolysis is less than 15%. By fixing the assay time (e.g. 30 min) and temperature (e.g. 25°C) the degree of hydrolysis may be controlled simply by using a suitable dilution of enzyme.

Fig 2 below shows an illustrative plot of absorbance versus enzyme concentration.

Fig 2. Absorbance versus amount of enzyme



In this example, the assay is linear up to OD 2.5. A dilution factor of 0.02 would be ideal for assay work, as this gives a large signal (~1.5) and lies in the middle of the linear range. Calculations based on results for dilution factors between 0.04

and 0.12 (i.e. the region with reduced slope) will clearly underestimate the true level of enzyme activity. Appendix I gives a simple equation for calculating enzyme activity values.

2.4.3. Assay time

While almost any time can be used, it is usual to select a time between 15 and 60 min. If the assay is short, it is important to ensure that the reagents have equilibrated to the correct temperature before the assay is set up (see below).

2.4.4. Absorbance versus time

A linear relationship should be seen. Make sure that the enzyme and substrate have equilibrated to the required assay temperature before they are combined in the plate, otherwise there will be a lag while the reagents warm up in the first few minutes of the assay. The calculated amount of P_i generated per minute (which is used to determine activity) may be underestimated in this situation.

2.4.5. Compatible substances

Table 4 lists chemicals that are often used in enzyme assays, with the expected type of interference (if any) for the stated concentrations.

Table 4. Effects of some common assay components in the GTPase assay

Component	Conc.*	Effect
NaCl	250mM	None
KCl	250mM	None
$MgCl_2$	25mM	None
DTT	0.25mM	Slight signal loss
βME	0.5mM	None
Tris	25mM	None
HEPES	25mM	None
MES	25mM	None
MOPS	25mM	None
BSA	0.1mg/ml	None
BSA	1mg/ml	Risk of precipitation
DMSO	2.5%	None
Detergents	0.03%	See footnote**

*The stated values refer to concentrations in the assay samples **before** the addition of Gold mix.

**Very low concentrations of detergent (0.002-0.005%) may cause precipitation. If a detergent is needed in the assay, use at least 0.03%. Tween 20 is the preferred choice. SDS should be avoided, but since the Gold mix is very acidic there is no need to use SDS if the purpose is simply to stop the reaction.

2.5. Standard curves

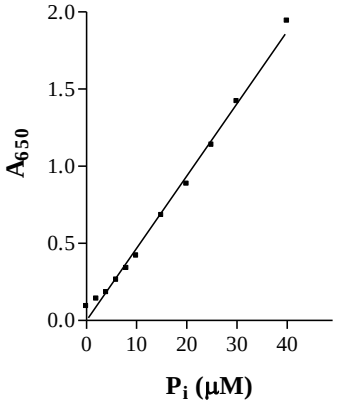
A standard curve is required if you wish to calculate enzyme activity. Prepare a set of P_i standards using the 0.1mM P_i stock (see Table 5). Set up duplicate wells containing 200µl of each standard and add 50µl of Gold mix. Two minutes later add 20µl of Stabiliser, and mix the wells gently. After 30 minutes, read the plate. Subtract the blank values (i.e. for sample 12) and plot absorbance versus concentration of P_i.

Table 5. Phosphate standards

Tube	0.1mM P_i standard (µl)	Water (µl)	Concentration of P_i (µM)
1	500	500	50
2	450	550	45
3	400	600	40
4	350	650	35
5	300	700	30
6	250	750	25
7	200	800	20
8	150	850	15
9	100	900	10
10	50	950	5
11	25	975	2.5
12	0	1000	0

A typical standard curve is shown in Fig 3. The absorbance value for the assay samples is used to determine µM P_i from the intercept on the x-axis of the standard curve.

Fig 3. Standard curve for P_i



3. Shelf life

The Gold mix *cannot* be stored for long periods; make up only what you will use on the day.

4. Related products

303-0125 P_iColorLock™ Gold

The detection reagent from this kit is available separately and can be used to assay any P_i-generating enzyme.

501-0015 P_iBind™ resin

P_iBind™ resin can be used to eliminate P_i from water, buffers and protein samples.

5. Troubleshooting/FAQ

Q1. Why do assays go non-linear at high enzyme concentrations?

The rate of reaction is dependent on the concentration of substrate. If a large fraction of the substrate is utilised (i.e. at high enzyme concentrations) there may be a reduction in the rate. Hydrolysis of 5-15% of the substrate is usually fine.

Q2. Do I need to subtract blanks from the standard curve?

No, but we recommend it. The blank is the value obtained in the absence of P_i, and the value is ~0.1. Thus, if the OD for a sample is 1.0, the signal due to P_i released by the GTPase is 0.9. Remember, if you subtract blanks from your standard curve, you must also subtract blanks from your assay data (but see Q3).

Q3. What controls do I need?

You can simply omit the enzyme and replace with enzyme diluent. However, a more rigorous approach is to *include* the enzyme in the control, but change the order of addition so that the Gold mix is added to the substrate *before* the enzyme. This control should be set up when the other assay wells are being stopped, so that all wells receive the Gold mix at the same time. The advantage of this approach is that subtraction of a single control value corrects for all free P_i, whatever its source. Moreover, this operation subtracts the blank value too, thus the resulting value can be used to determine P_i from the blank-subtracted standard curve.

Q4. Why am I getting high backgrounds when there is no free P_i in my enzyme or substrate?

Did you remember to add the Stabiliser? It is essential that the Stabiliser is added to prevent high backgrounds caused by non-enzymatic hydrolysis of GTP.

Q5. I know my sample has activity but all my wells are yellow. Why is this?

The most likely explanation is that the Stabiliser has been added with, or immediately following, the Gold mix. Make sure that the Stabiliser is added two minutes after the Gold mix.

Technical support team

Tel: +44(0)1223 496175
Email:
technical.enquiries@innovabiosciences.com

Appendix I

Calculation of enzyme activity

One unit is the amount of enzyme that catalyses the reaction of 1 μmol of substrate per minute. The activity (units/ml) of your *undiluted* enzyme sample is given by the following equation:

Activity = (AxC)/500B

where, A = concentration of P_i (μM) determined from the standard curve
B = assay time in minutes
C = reciprocal of the enzyme dilution factor*

*Note: the value for 'C' *must* be 1 or >1. For example, if the enzyme is diluted 1/100 prior to the addition of the 100 μl enzyme sample to the substrate, C =100).

The above equation is valid *only* if the assay is set up as indicated in the protocol (i.e. the assay volume is 200μl, comprising 100μl of enzyme and 100μl of substrate mix). Note: the 1:1 dilution that occurs upon addition of the enzyme to the substrate has been factored into the above equation, so the only dilution factor you need to consider is that of the enzyme before the enzyme is added to the plate.