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Enzo Life Sciences

Enabling Discovery in Life Science®

Calcineurin Cellular Activity Assay Kit

A complete colorimetric assay for measuring cellular calcineurin (PP2B) phosphatase activity.

Instruction Manual
BML-AK816

For research use only

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✦ CALCINEURIN CELLULAR ACTIVITY ASSAY KIT - BML-AK816✦

BACKGROUND

Calcineurin (CaN) is the neuronal form of the widely distributed Ca^{2+}/calmodulin-dependent Ser/Thr protein phosphatase 2B (PP-2B). CaN is a heterodimer consisting of a catalytic A subunit (57-61 kDa) and a regulatory B subunit (19 kDa). The catalytic A subunit is composed of four functional domains: the catalytic core with sequence homology to PP-1 and PP-2A, binding sites for both calmodulin and CaN B-regulatory subunit, and a C-terminal autoinhibitory domain.

The *Calcineurin Cellular Activity Assay Kit* is a complete colorimetric assay kit for measuring cellular calcineurin (PP2B) phosphatase activity. It employs a convenient 96-well microtiter-plate format with all reagents necessary for measuring calcineurin (PP2B) phosphatase activity in tissue/cellular extracts PLUS, human recombinant calcineurin[5] is included as a positive control! The RII phosphopeptide substrate, supplied with this kit, is the most efficient and outstanding peptide substrate known for calcineurin[1,2]. The detection of free-phosphate released is based on the classic Malachite green assay[3,4] and offers the following advantages: Non-radioactive; convenient 1-step detection; excellent sensitivity.

Refs: 1. A. Enz *et al. Anal. Biochem.* 1994 **216** 147
2. A. Donella-Deana *et al. Eur. J. Biochem.* 1994 **219** 109
3. B. Martin *et al. J. Biol. Chem.* 1985 **260** 14932
4. K.W. Harder *et al. Biochem. J.* 1994 **298** 395
5. A. Mondragon *et al. Biochemistry* 1997 **36** 4934

ALSO AVAILABLE SEPARATELY…

PRODUCT	CATALOG #
Calcineurin Phosphatase Assay Kit	BML-AK804
BIOMOL GREEN™ Reagent 250 ml bottle	BML-AK111
Calcineurin (human, recombinant)	BML-SE163
RII phosphopeptide substrate (0.5 mg)	BML-P160
Cyclophilin A (human, recombinant)	BML-SE105
Cyclosporin A	BML-A195
CaN Autoinhibitory Peptide CN412	BML-PR104
Additional Desalting Column and Resin	BML-KI100

PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT ENZO LIFE SCIENCES TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

COMPONENTS

NOTE ON STORAGE: *Store all components except the microtiter plate at -70°C for the highest stability. The calcineurin enzyme component BML-SE163-9090 must be handled particularly carefully in order to retain maximal enzymatic activity. Thaw it quickly in a RT water bath or by rubbing between fingers, then immediately store on an ice bath. The remaining unused enzyme should be quickly refrozen by placing at -70°C. To minimize the number of freeze/thaw cycles, aliquot the calcineurin into separate tubes and store at -70°C.*

BML-SE163-9090 CALCINEURIN ENZYME (human, recombinant)
FORM: 50 U/µl in 50 mM Tris, pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$, 5 mM DTT, 0.025% NP-40, 0.5 mM $CaCl_2$. 1 U=1 pmol/min @30°C.
STORAGE: -70°C; AVOID FREEZE/THAW CYCLES!
QUANTITY: 500 U

BML-SE325-9090 CALMODULIN (human, recombinant)
FORM: 25 µM in dH_2O
STORAGE: -70°C
QUANTITY: 100 µl

BML-P160-9090 SUBSTRATE (RII phosphopeptide, sequence Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-pSer-Val-Ala-Ala-Glu; MW=2192.0)
FORM: 1.5 mg net peptide/vial
STORAGE: -20 or -70°C
QUANTITY: 1 x 1.5 mg

BML-KI128 2X ASSAY BUFFER
(100 mM Tris, pH 7.5, 200 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40, 1 mM $CaCl_2$)
FORM: Liquid in screw-cap plastic bottle.
STORAGE: -70°C
QUANTITY: 20 ml

BML-KI136 2X EGTA BUFFER
(100 mM Tris, pH 7.5, 20 mM EGTA, 200 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40)
FORM: Screw-cap microfuge tube.
STORAGE: -70°C
QUANTITY: 1 ml

BML-KI135 LYSIS BUFFER
(50 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.2% NP-40)
FORM: Liquid in screw-cap plastic bottles. (white cap)
STORAGE: -70°C
QUANTITY: 2 X 40 ml

BML-KI103 PROTEASE INHIBITORS COCKTAIL
FORM: Tablet
STORAGE: 4°C or colder
QUANTITY: 2 tablets (1 tablet per 10 ml Lysis Buffer BML-KI135)

BML-AK111-9090 *BIOMOL GREEN*™ REAGENT
FORM: Liquid in screw-cap plastic bottle.
STORAGE: 4°C. Long-term at -70°C.
QUANTITY: 20 ml

BML-KI132 PHOSPHATE STANDARD
FORM: 80 µM in dH_2O
STORAGE: -20 or -70°C
QUANTITY: 0.5 ml

BML-EI181-9090 OKADAIC ACID
HARMFUL! AVOID ALL FORMS OF CONTACT.
FORM: 5 µM in 2X Assay Buffer (BML-KI128)
STORAGE: -70°C.
QUANTITY: 325 µl

BML-KI100 DESALTING COLUMN AND RESIN
FORM: 5 ml polypropylene disposable column and P6 DG desalting resin
STORAGE: RT. After rehydration store at 4°C.
QUANTITY: 1 column and 1 g of resin

BML-KI101 1/2 VOLUME MICROPLATES
STORAGE: RT
QUANTITY: 2 plates

OTHER MATERIALS REQUIRED

Microplate reader capable of measuring A_{620} to ≥3-decimal accuracy.
Centrifuge capable of 100k x g RCF.
Swing bucket centrifuge capable of 800 x g RCF.
Pipetman capable of pipetting 5-100 µl accurately.
Multi-channel Pipetman capable of pipetting 100 µl (optional).
Ice bucket to keep reagents cold until use.
16g needle/syringe
TBS buffer, 100 ml (20 mM Tris, pH 7.2, 150 mM NaCl)
15 and 50 ml conical centrifuge tubes
Biological test material (e.g.: tissue, cells)

EXPERIMENTAL METHODS

NOTE ON HANDLING: *Hold all samples on ice bath until use, unless otherwise noted.*
PRECAUTIONS: *The BIOMOL GREEN™ reagent is a highly sensitive phosphate detection solution. Free phosphate present on labware and in reagent solutions will greatly increase the background absorbance of the assay. This is detected visually as a change in color from yellow to green. Detergents used to clean labware may contain high levels of phosphate. Use caution by either rinsing labware with dH_2O or employ unused plasticware. Do not use phosphate buffered saline (PBS) for any tissue/cell rinses -use TBS (Tris buffered saline)!*

PREPARATION OF TISSUE/CELL EXTRACTS

To prepare a tissue/cell extract for calcineurin activity assay:
Calcineurin activity is highly dependent on the experimental conditions and the cell/tissue source. Therefore, the amount of material required for an assay should be determined empirically by the user. Typically, between 0.5-5 µg of total protein or 5,000 and 50,000 cells per assay will provide sufficient signal for detection.
NOTE ON BIOLOGICAL SAMPLE MATERIAL: *The following procedures have been tested for rat and mouse brain tissue. Other tissue or cell culture samples employed may require adjustment to this protocol for satisfactory results.*

1. Add protease inhibitor tablets to lysis buffer (BML-KI135) immediately before use (1 tablet/10 ml buffer). Vortex.
2. Obtain tissue, if fresh, excise quickly.
3. Rinse tissue quickly in ice-cold TBS and shake-off/blot excess wetness.
4. Weigh the tissue in centrifuge tube.
5. Add lysis buffer (BML-KI135) with protease inhibitors to tissue. Use 0.33-0.5 ml per gram of tissue.
6. Loosely break up cells by passing them through a 16g needle. Avoid air bubbles.
7. Optional: Sediment at 100-200k x g in centrifuge at 4°C for 45 min. Save supernatant=HSS (high-speed supernatant). Please note that this step will sediment the nucleus and any associated nuclear calcineurin.
8. Freeze immediately at -70°C.

REMOVAL OF FREE PHOSPHATE FROM EXTRACTS

To desalt tissue samples by gel filtration:
NOTE: *This procedure is intended to remove excess phosphate and nucleotides (which are slowly hydrolyzed to release free phosphate in the presence of the BIOMOL GREEN™ reagent) in the high speed supernatant (HSS) extract.*

1. Rehydrate Desalting Column Resin (BML-KI100) in a 50 ml conical tube by adding 20 ml of phosphate free dH_2O and vortexing briefly. Allow to set for 4 hours at RT or overnight at 4°C.
2. Decant the dH_2O carefully, then add fresh dH_2O at a 1:1 ratio to the rehydrated resin (BML-KI100) (~10 ml).
3. Add rehydrated resin (BML-KI100) to the Chromatography Column (BML-KI100) to obtain a 5 ml settled-bed volume (~5.5 cm bed height). Remove tip from column and allow dH_2O to drain by gravity.
4. Equilibrate column by adding 8 ml of lysis buffer (BML-KI135) (without protease inhibitors) and allow to drain by gravity.
5. Place column in a 15 ml centrifuge tube. Centrifuge at 800 x g for 3 min at 4°C to displace column buffer. Discard flow-through buffer.
6. Place column in a clean 15 ml centrifuge tube.
7. Add up to 350 µl HSS sample, from above, to column.
8. Centrifuge at 800 x g for 3 min. Save extract flow-through. This is the desalted cell lysate material to be tested for calcineurin activity, below.
9. Freeze sample immediately at -70°C.

TIP: *The effective removal of phosphate/nucleotides from the extract should be tested qualitatively by adding 100 µl BIOMOL GREEN™ reagent to 1 µl extract, and a separate sample of 1 µl dH_2O. If no phosphate/nucleotides are present, both samples should remain yellow in color over a time period of 30 min @ RT. The development of a visible green color indicates phosphate contamination, which must be eliminated from the samples before proceeding further!*

BIOMOL GREEN™ PHOSPHATASE ASSAY

To prepare reagents for the assay:

1. Thaw all kit components and hold on ice bath, except *BIOMOL GREEN™* reagent at RT.
2. Add calmodulin to the 2x assay buffer: Dilute calmodulin (BML-SE325-9090) 1/50 in 2X assay buffer (BML-KI128) to required quantity (25 µl are required per assay well). For example, add 20 µl to 980 µl 2X assay buffer.
3. Reconstitute substrate (RII phosphopeptide, BML-P160) with dH_20 to 0.75 mM (1.64 mg/ml): Add 915 µl dH_20 per 1.5 mg vial (10 µl are needed per assay well).

To prepare phosphate standard curve sample wells:

1. Prepare 1 ml of 1X assay buffer (dilute 500 µl of 2X assay buffer with 500 µl of dH_2O)
2. Perform 1:1 serial dilutions of phosphate standard and an assay buffer blank. Concentrations of 40, 20, 10, 5, 2.5, 1.25 and 0.625 µM correspond to 2, 1, 0.5, 0.25, 0.125, 0.063 and 0.031 nmol PO_4 (see Table 1):
   a) Add 50 µl of assay buffer (BML-KI128) to wells A1, and A2 (2nmol PO_4 standards)
   b) Add 50 µl of 1X assay buffer (prepared in step 1 above) to wells B1-H1 and wells B2-H2. (remaining standard concentrations)
   c) Add 50 µl of 80 µM phosphate standard to wells A1 and A2 of assay plate. Mix thoroughly by pippetting up and down several times.
   d) Remove 50 µl from well A1 and add it to well B1. Mix thoroughly by pipetting up and down several times.
   e) Remove 50 µl from well B1 and add it to well C1.

f) Mix thoroughly and repeat for wells D1-G1. At well G1, remove 50 µl and discard. DO NOT PROCEED TO WELL H1 (assay buffer blank). Final volume=50 µl.
g) Repeat serial dilution for the wells in column 2 (standard curve duplicates)

To prepare calcineurin activity assay sample wells:
(See Tables 1 & 2):
Background (no substrate):
{Control for background phosphate/interfering substances}
1. Add 20 µl dH_2O to appropriate wells.
2. Add 25 µl 2X assay buffer (BML-KI128) w/ calmodulin to each well.

Total phosphatase activity wells:
{Total phosphatase activity in the extract }
1. Add 10 µl dH_2O to each well.
2. Add 25 µl 2X assay buffer (BML-KI128) w/ calmodulin to each well.

EGTA buffer (Ca^{2+}/CaM free):
{Total activity less PP2B (calcineurin)}
1. Add 10 µl dH_2O to each well.
2. Add 25 µl 2X EGTA buffer (BML-KI136) to each well.

OA (okadaic acid):
{Total activity less PP1 & PP2A}
1. Add 5 µl dH_2O to each well.
2. Add 25 µl 2X assay buffer (BML-KI128) w/ calmodulin to each well.
2+. Add 5 µl okadaic acid (BML-EI181-9090; 5 µM)

OA + EGTA:
{Total activity less PP1, PP2A & PP2B}
1. Add 5 µl dH_2O to each well.
2. Add 25 µl 2X EGTA buffer (BML-KI136) to each well.
2+. Add 5 µl okadaic acid (BML-EI181-9090; 5 µM)

Positive control (calcineurin enzyme):
{Purified CaN enzyme positive control}
1. Add 10 µl dH_2O to each well.
2. Add 25 µl 2X assay buffer (BML-KI128) w/ calmodulin to each well.

Add phosphopeptide substrate:
3. Add 10 µl phosphopeptide substrate BML-P160 to each well of the calcineurin samples except the "Background" control. DO NOT ADD SUBSTRATE TO THE PHOSPHATE STANDARD CURVE SAMPLES!
4. Equilibrate microtiter plate to reaction temperature (e.g.: 30°C) for 10 min.

To initiate calcineurin assay:
5. Add 5 µl extract or diluted calcineurin (dilute to 8 U/µl prior to use) to appropriate wells. For sample extract wells, it may be necessary to dilute the HSS tissue extract (e.g.: 1/5-1/10 in lysis buffer BML-KI135). For calcineurin "Positive control" add 5 µl BML-SE163-9090 (40 U/well).
6. Incubate plate at reaction temperature for desired duration (e.g.: 30 min@30°C).

To terminate reactions:
7. After incubating wells for desired duration, terminate reactions by adding 100 µl *BIOMOL GREEN*™ reagent (BML-AK111-9090) to ALL samples including the phosphate standard curve.
8. Allow color to develop 20-30 minutes, making sure all wells spend approximately the same time with the reagent before reading on microplate reader.
9. Read OD_{620nm} on microplate reader.
10. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells!

TABLE 1. EXAMPLE OF MICROTITER PLATE SAMPLES.

Sample†	Std Curve	Extract/CaN Samples
Well #	1,2	3,4
A	**2 nmol PO_4**	***Background***
B	**1**	***Total***
C	**0.5**	***EGTA buffer***
D	**0.25**	***OA (okadaic acid)***
E	**0.125**	***OA + EGTA***
F	**0.063**	***Positive Control***
G	**0.031**	
H	**0**	

† For highest accuracy, perform all samples in duplicate.

TABLE 2. TYPICAL ASSAY COMPONENTS

	H_2O	2X Assay Buffer w/CaM	2X EGTA Buffer	OA	Substrate (0.75 mM)	Extract/ CaN (dilute to 8 U/µl)
Background	20 µl	25 µl	0	0	0	5 µl[a]
Total	10 µl	25 µl	0	0	10 µl	5 µl[a]
EGTA buffer	10 µl	0	25 µl	0	10 µl	5 µl[a]
OA (okadaic acid)	5 µl	25 µl	0	5 µl	10 µl	5 µl[a]
OA + EGTA	5 µl	0	25µl	5 µl	10 µl	5 µl[a]
Positive Control	10 µl	25 µl	0	0	10 µl	5 µl[b]

a Add cellular extract (HSS)
b Add calcineurin enzyme (SE163-9090)

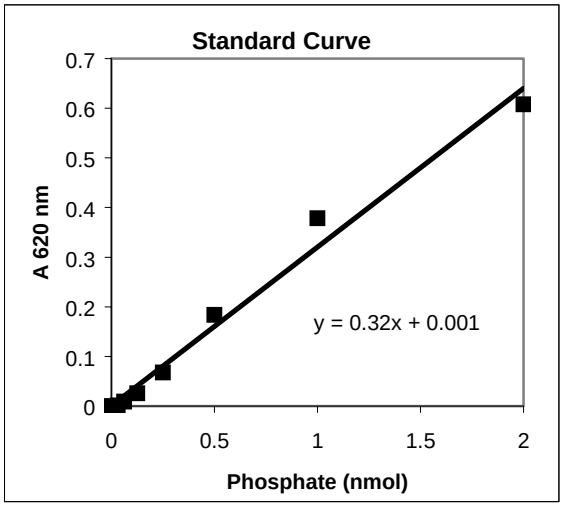
DATA ANALYSIS

Phosphate (PO_4) Standard Curve

1. Plot standard curve data as OD_{620nm} versus nmol PO_4 (see Figure 1).
2. Obtain a line-fit to the data using an appropriate routine.
3. Use the slope and Y-intercept to calculate amount of phosphate released for the experimental data (see below).

NOTE: *For highest accuracy, a standard curve must be performed for each new set of assay data. This will normalize for variations in free phosphate in samples, time of incubation with the BIOMOL GREEN™ reagent, and other experimental factors.*

FIGURE 1. *BIOMOL GREEN*™ PHOSPHATE STANDARD CURVE



Conversion of OD620nm to Amount Phosphate Released

1. Convert OD_{620nm} data into the amount of phosphate released using the standard curve line-fit data, from above:

Phosphate released = $(OD_{620nm} - Y_{int})$/slope

EXAMPLE:
Std curve slope=0.3 OD_{620nm}/nmol phosphate
Std curve Yint=0.001 OD_{620nm}
Sample OD=0.4

Phosphate released=(0.4-0.001)/0.3 = 1.33 nmol

Data Reduction to Determine Calcineurin Phosphatase

PRECAUTION: *The procedures for data analysis which follow are intended only as a guideline. The individual user must determine the suitability of this analysis for their particular experimental protocol. Additional controls and other samples may be appropriate for accurate analysis.*

ANALYSIS DESCRIPTION: *This assay uses the RII phosphopeptide, the best known substrate for CaN (PP2B). Nonetheless, in cellular extracts, the phospho-group is cleaved by other competing phosphatases. Thus, a series of conditions must be employed to discriminate between the contribution of other phosphatases. CaN requires calcium for its activity, thus the "EGTA buffer" sample represents total phosphatase activity less CaN. Okadaic acid (OA) at 100 and 500 nM is known to completely inhibit PP1 and PP2A, while it has no effect on CaN (see Figure 3). Finally, OA + EGTA buffer inhibits PP1, PP2A and PP2B, but not PP2C. Thus, for a given biological system, the analysis of these samples allows the quantification of calcineurin (PP2B) activity in a cellular extract. These methods have been described in the literature. Additional experimental modulation of the cell extracts may be desirable. Inhibitors of calcineurin, calmodulin and calcium ion modulators may be appropriate.*

Suggested reading
Minireview:
C.B. Klee *et al. J. Biol. Chem.* 1998 **273** 13367
Brain cellular extracts:
P.M. Stemmer *et al. FEBS Lett.* 1995 **374** 237
X. Wang *et al. Nature* 1996 **383** 434
Human recombinant CaN:
A. Mondragon *et al. Biochem*. 1997 **36** 4934
CaN Assays:
D.A. Fruman *et al. Methods: A Companion to Meth. Enzymol.* 1996 **9** 146

1. Substract the "Background" phosphate released from each sample except the "Positive control".
2. Plot a graph analogous to Figure 2, below. Use either OD_{620nm} or phosphate released for the Y-axis.
3. Determine the contribution of calcineurin:

eq. 1 CaN (PP2B) = Total - EGTA buffer

or

eq. 2 CaN (PP2B) = OA - (OA + EGTA)

Eq. 1 is a conventional method to report CaN activity. However, the user must determine the most appropriate analysis for their specific experimental goal.

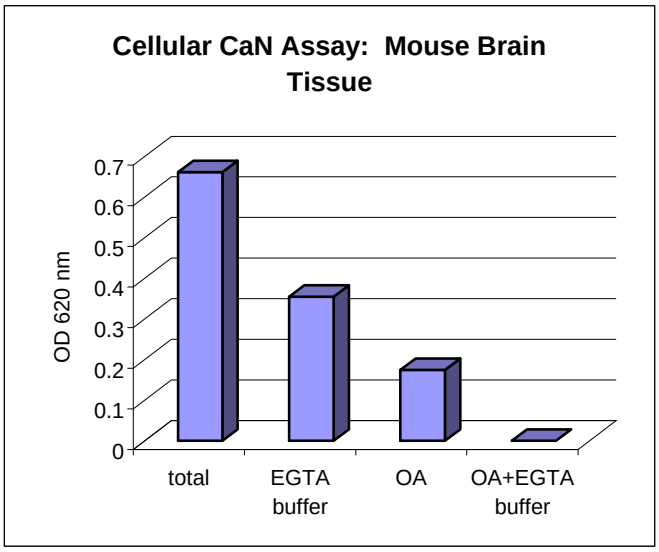


FIGURE 2. CELLULAR CaN ASSAY USING MOUSE BRAIN
Phosphatase activity from a freshly prepared mouse brain extract. Prior to gel filtration the extract was diluted 1:1 in lysis buffer containing protease inhibitors. After gel filtration and prior to the phosphatase assay, the extract was diluted 1/25 in lysis buffer. Well contents were as in Table 2. The reaction was incubated for 30 min @ 30°C.

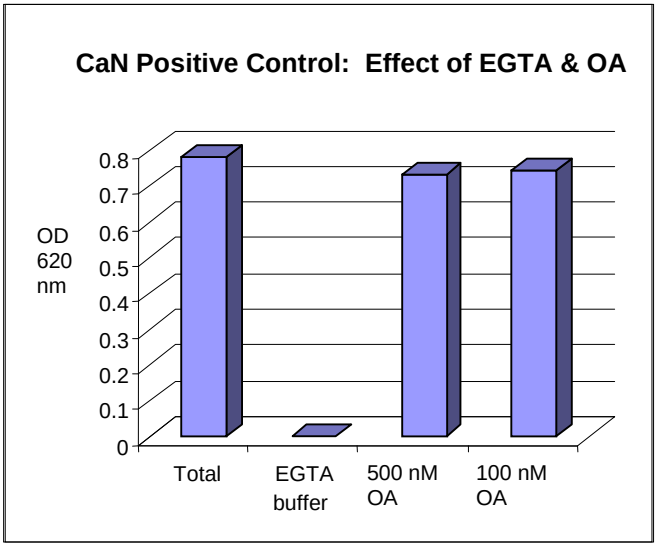


FIGURE 3. CaN POSITIVE CONTROL
Purified calcineurin (BML-SE163-9090) positive control. The CaN was incubated 1 hr @ 30°C under various buffer conditions. The results demonstrate that CaN activity is inhibited by the EGTA buffer, but not inhibited by 100 nM or 500 nM concentrations of okadaic acid.

Literature Citations of Calcineurin Assay Kits

B. Mehul *et al. J. Biol. Chem.* 2000 **275** 12841
T. Taigen *et al. Proc. Natl. Acad. Sci*. 2000 **97** 1196
G. Mallert *et al. Cell* 2001 **104** 675
M. Ichida and T. Finkel *J. Biol. Chem.* 2001 **276** 3524
O. Bueno *et al. Proc. Natl. Acad. Sci*. 2002 **99** 4586
K.J. Sellar *et al. Anal. Biochem.* 2006 **358** 104

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