Epigenase™ APOBEC3 Cytidine Deaminase Activity/Inhibition Assay Kit

Base Catalog # P-3140

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The Epigenase™ APOBEC3 Cytidine Deaminase Activity/Inhibition Assay Kit is suitable for measuring the activity/inhibition of total APOBEC3 enzymes using cell extracts or purified APOBEC3 isoforms (A3A, A3B, A3C, A3D, A3F, and A3H) from human and other mammals, in a variety of forms including, but not limited to, cultured cells and fresh/frozen tissues.

Starting Materials: Input materials can be cell extracts or purified APOBEC3 enzymes. The amount of cell extracts for each assay can be 2 µg to 30 µg with an optimal range of 15-20 µg. The amount of purified enzymes can be 10 ng to 1 µg, depending on the type, purity, and catalytic activity of the enzymes.

Internal Control: The APOBEC3 assay standard is provided in this kit for the quantification of APOBEC3 enzyme activity. Because APOBEC3 activity can vary from tissue to tissue and from normal and diseased states, it is advised to run replicate samples to ensure that the signal generated is validated.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the strip wells. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

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KIT CONTENTS

Component	48 Assays Cat. #P-3140-48	96 Assays Cat. #P-3140-96	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	4°C
A3B (APOBEC3 Assay Buffer)	3 ml	6 ml	4°C
BS (Binding Solution)	5 ml	10 ml	RT
A3S (APOBEC3 Assay Standard, 200 µg/ml)*	10 µl	20 µl	–20°C
PPB (Pre-probe Clean Buffer)	10 ml	20 ml	RT
APS (APOBEC3 Probe Solution)	4 ml	8 ml	RT
500X A3P (500X APOBEC3 Probe)	5 µl	10 µl	–20°C
ES (Enhancer Solution)*	8 µl	16 µl	–20°C
DS (Developer Solution)	5 ml	10 ml	4°C
SS (Stop Solution)	3 ml	6 ml	RT
8-Well Sample Strips (With frame)	4	9	4°C
8-Well Standard Strips (Green-Trimmed)	2	3	4°C

** For maximum recovery of the products, centrifuge the original vial prior to opening the cap.*

SHIPPING & STORAGE

The kit is shipped in two parts: the first part at ambient room temperature, and the second part on frozen ice packs at 4°C. Upon receipt: (1) Store **A3S**, **500X A3P**, **ES**, at –20°C away from light; (2) Store **WB**, **A3B**, **DS**, **8-Well Sample Strips and 8-Well Standard Strips** at 4°C away from light; (3) Store remaining components (**BS, PPB, APS** and **SS**) at room temperature away from light.

***Note**: Check if **WB** (10X Wash Buffer) contains salt precipitates before use. If so, warm at room temperature or 37°C and shake the buffer until the salts are re-dissolved.*

All components of the kit are stable for 6 months from the date of shipment, when stored properly.

MATERIALS REQUIRED BUT NOT SUPPLIED

- ☐ Adjustable pipette or multiple-channel pipette
- ☐ Multiple-channel pipette reservoirs
- ☐ Aerosol resistant pipette tips
- ☐ Microplate reader capable of reading absorbance at 450 nm
- ☐ 1.5 ml microcentrifuge tubes
- ☐ Incubator for 37°C incubation
- ☐ Distilled water
- ☐ Cell extracts or purified enzymes
- ☐ Parafilm M or aluminum foil

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of Epigenase™ APOBEC3 Cytidine Deaminase Activity/Inhibition Assay Kit is tested against predetermined specifications to ensure consistent product quality. EpigenTek guarantees the performance of all products in the manner described in our product instructions.

Product Warranty: If this product does not meet your expectations, simply contact our technical support unit or your regional distributor. We also encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.

Product Updates: EpigenTek reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice. Be sure to use the latest User Guide for this kit which can be accessed online at www.epigentek.com/datasheet.

Usage Limitation: The Epigenase™ APOBEC3 Cytidine Deaminase Activity/Inhibition Assay Kit is for research use only and is not intended for diagnostic or therapeutic application

Intellectual Property: The Epigenase™ APOBEC3 Cytidine Deaminase Activity/Inhibition Assay Kit and methods of use contain proprietary technologies by EpigenTek.

A BRIEF OVERVIEW

The APOBEC3 cytosine deaminases (Apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3) are a DNA/RNA editing family, which includes 7 subtypes: APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3E, APOBEC3F, APOBEC3G, and APOBEC3H. APOBEC3s are found exclusively in mammals and can catalyze the removal of an amino group from the cytosine (C) base to form uridine (U) in single-strand DNA or RNA. The genetic alterations by APOBEC3s will change transcription and mRNA processing, and they actively participate in various biological processes.

APOBEC3s are part of the innate immune system against various DNA or RNA viruses to protect the integrity of cells. Through promoting the deamination of cytosine to uracil on ssDNA/RNA, APOBEC3s generate mutations in virus genomes to inhibit virus replication. For example, a C-to-U mutation caused by APOBEC3s in the SARS-CoV-2 genomes was reported to be a dominant mutation (>50%). While APOBEC3 confers its intrinsic host resistance to infection to various viruses, including the SARS-CoV-2 virus, it may also cause potential evolutionary consequences for viruses.

APOBEC3s also contribute significantly to DNA mutagenesis in cancer. As a major driver of cancer evolution, APOBEC3 proteins are one of the most predominant causes of genomic mutations detected in a variety of tumors. APOBEC3 mutation signatures can be seen in more than 50% of various cancers, thus increasing diversity in tumors and thereby promoting disease progression and resistance to therapies.

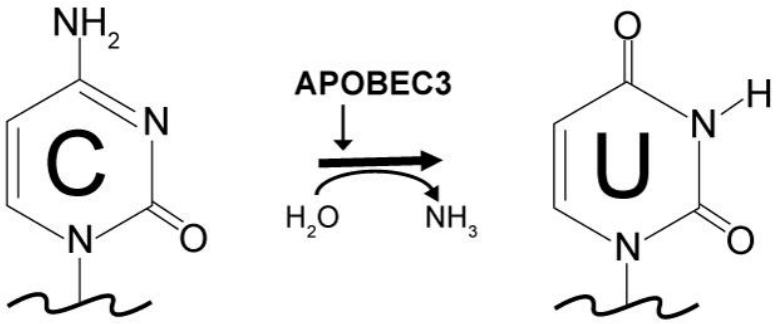

Fig 1. Cytosine deamination reaction catalyzed by APOBEC3

Thus, detection of APOBEC3 enzyme activity and inhibition would be important in benefiting virus infection control, cancer diagnostics, and developing new target-based cancer therapeutics. The currently used methods for detecting APOBEC3 activity are mainly based on nuclear magnetic resonance (NMR), gel electrophoresis, or droplet digital PCR. These methods need specific instruments and are complicated, laborious, and time-consuming. To address this issue, EpigenTek developed and offers the Epigenase™ APOBEC3 Cytidine Deaminase Activity/Inhibition Assay Kit. The kit has the following advantages and features:

- Colorimetric assay with easy-to-follow steps that can be finished within 4 hours.
- Directly measures total APOBEC3 activity via a straightforward detection of APOBEC3-converted end products.
- Innovative kit composition enables background signals to be extremely low, allowing the assay to be simple, accurate, reliable, and consistent.
- Both cell/tissue extracts and purified APOBEC3 enzymes can be used for detecting APOBEC3 inhibitor effects *in vivo* and *in vitro*.
- Novel assay principle allows for high sensitivity with a detection limit as low as 10 ng of purified APOBEC3 enzymes.
- C-to-U converted standard is included for quantifying the specific activity of APOBEC3s.
- Strip microplate format for manual or high throughput analysis (96 assays).

PRINCIPLE & PROCEDURE

In this assay, a substrate is pre-coated onto microplate wells. Active APOBEC3s bind to the substrate and convert cytosine to uracil products. The APOBEC3-converted products can be recognized with a specific probe. The ratio or amount of uracil products, which is proportional to enzyme activity, can then be colorimetrically measured by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. The activity of the APOBEC3 enzyme is, in turn, proportional to the optical density intensity measured.

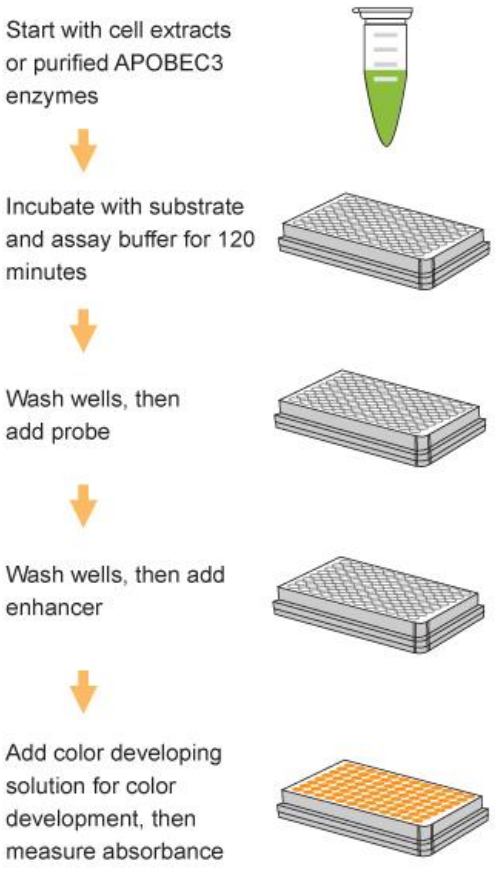

Fig. 2. Schematic procedure of Epigenase™ APOBEC3 Cytidine Deaminase Activity/Inhibition Assay Kit.

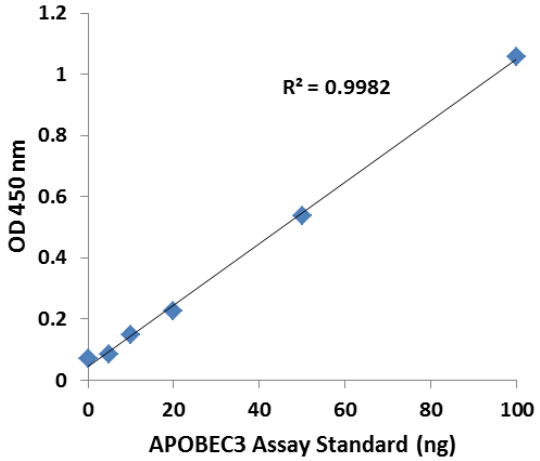

Fig. 3. Illustrated standard curve generated with the APOBEC assay standard.

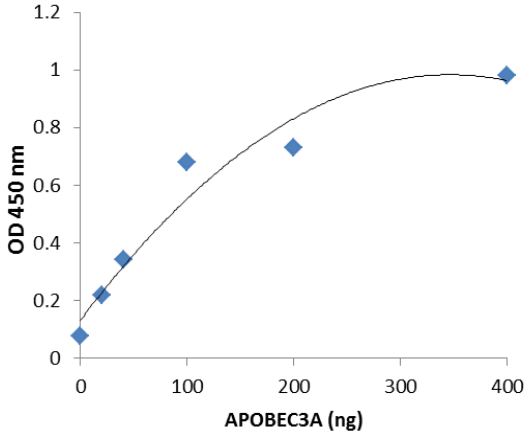

Fig. 4. APOBEC3 activity achieved by using recombinant APOBEC3A with the Epigenase™ APOBEC3 Cytidine Deaminase Activity/Inhibition Assay Kit. Recombinant human APOBEC3A enzyme was added at different concentrations.
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ASSAY PROTOCOL

For the best results, please read the protocol in its entirety prior to starting your experiment.

Starting Materials

Input Amount: The amount of cell extracts for each assay can be 2 µg to 30 µg with an optimal range of 15-20 µg. The amount of purified enzymes can be 10 ng to 1 µg, depending on the type, purity, and catalytic activity of the enzymes.

Cell Extraction: You can use your method of choice for preparing cell extracts. EpigenTek offers a whole-cell extraction kit (Cat # OP-0003) optimized for use with this kit.

Storage of Cell Extract or Purified APOBEC3 Proteins: Using freshly prepared cell extract is the best. Otherwise, the cell extract or purified APOBEC3 enzymes should be stored in aliquots at –80°C until use.

1. Buffer Solution & Preparation

a. Prepare **Diluted WB** (1X Wash Buffer):

48-Assay Kit: Add 13 ml of **WB** (10X Wash Buffer) to 117 ml of distilled water and adjust pH to 7.2-7.5.

96-Assay Kit: Add 26 ml of **WB** (10X Wash Buffer) to 234 ml of distilled water and adjust pH to 7.2-7.5.

This **Diluted WB** (1X Wash Buffer) can now be stored at 4°C for up to six months.

b. Prepare **Diluted ES** (Enhancer Solution):

Dilute **ES** (Enhancer Solution) with **Diluted WB** (1X Wash Buffer) at a ratio of 1:2000 (i.e., add 1 µl of **ES** to 2000 µl of **Diluted WB**). About 50 µl of **Diluted ES** will be required for each assay well.

c. Prepare **Diluted A3S (**APOBEC3 Assay Standard Solution):

Suggested Standard Curve Preparation: First, dilute **A3S** (APOBEC3 Assay Standard Solution) with **PPB** (Pre-probe Clean Buffer) to 100 ng/µl by adding 6 µl of **A3S** to 6 µl of **PPB**. Then, further prepare 6 concentrations by combining the 100 ng/µl **Diluted A3S** with **PPB** into final concentrations of 1, 2, 5, 10, 20, 50, and 100 ng/µl according to the following dilution chart:

Tube	A3S (100 ng/µl)	PPB	Resulting A3S Concentration
1	1.0 µl	99.0 µl	1 ng/µl
2	1.0 µl	49.0 µl	2 ng/µl
3	1.0 µl	19.0 µl	5 ng/µl
4	1.0 µl	9.0 µl	10 ng/µl
5	1.0 µl	4.0 µl	20 ng/µl
6	2.0 µl	2.0 µl	50 ng/µl
7	4.0 µl	0.0 µl	100 ng/µl

***Note**: Keep each of diluted solutions except **Diluted WB** (1X Wash Buffer) on ice until use. Any remaining diluted solutions other than **Diluted WB** should be discarded if not used within the same day.*

2. Enzymatic Reaction

a. Predetermine the number of strip wells required for your experiment. Carefully remove un-needed strip wells from the plate frame and place them back in the bag (seal the bag tightly and store at 4°C).

b. For Standard Blank: Add 100 µl of **BS (**Binding Solution) to standard blank well of **8-well Standard Strips** (Green-Trimmed) and then add 2 µl of **PPB** (Pre-probe Clean Buffer).

c. For Standard Curve: Add 100 µl of **BS (**Binding Solution) to standard curve well of **8-well Standard Strips** (Green-Trimmed), and then add 2 µl of diluted **A3S** (APOBEC3 Assay Standard) at different concentrations.

***Note**: For the standard curve, add 2 µl of **Diluted A3S** at concentrations of 1 to 100 ng/µl (see the chart in Step 1c). The final concentrations should be 2, 4, 10, 20, 40, 100, and 200 ng per well.*

d. For Sample Blank: Add 45 µl of **A3B (**APOBEC3 Assay Buffer) to sample blank wells of **8-well Sample Stripes,** and then add 5 µl of buffer used for cell extraction

e. For Sample Without Inhibitor: Add 45 µl of **A3B** (APOBEC3 Assay Buffer) to sample wells of **8-well Sample Stripes,** and then add 5 µl of cell extracts or purified APOBEC3 enzymes.

f. For Sample With Inhibitor: Add 40 µl of **A3B (**APOBEC3 Assay Buffer) to sample wells of **8-well Sample Stripes,** and then add 5 µl of inhibitors and 5 µl of cell extracts or purified APOBEC3 enzymes.

***Note:** (1) follow the designated wells depicted in Table 1 under "Suggested Strip Well Setup" below. 2) The concentration of inhibitor to be added into the sample wells can be varied (1 µM to 1000 µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with **A3B** (APOBEC3 Assay Buffer) at a 1:10 ratio (i.e., add 0.5 µl of inhibitor to 4.5 µl of **A3B**) so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less.*

g. Mix solution by gently tilting from side to side or shaking the plate several times. Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 120 min.

***Note**: (1) The incubation time may depend on intrinsic APOBEC3 activity. However, in general, 120 min incubation is suitable for active purified APOBEC3 enzyme.*

h. Remove the solution from each well and wash each well two times with 150 µl of the **Diluted WB** (1X Wash Buffer) each time.

i. Wash each well one time with 150 µl of the **PPB (**Pre-probe Clean Buffer).

3. Probe Binding & Signal Enhancing

a. Dilute **500X A3P** (500X APOBEC3 Probe) to **1X A3P** by adding 1 µl of the **500X A3P** to each 500 µl of **APS (**APOBEC3 Probe Solution). Mix well and add 50 µl of the **1X A3P** to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 60 min.

b. Remove the **1X A3P** solution from each well.

c. Wash each well two times with 150 µl of the **Diluted WB (**1X Wash Buffer) each time.

d. Add 50 µl of the **Diluted ES** (Enhancer Solution) to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

e. Remove the **Diluted ES** solution from each well.

f. Wash each well four times with 150 µl of the **Diluted WB** (1X Wash Buffer) each time.

Note: *Ensure any residual wash buffer in the wells is thoroughly removed at each wash step. The wash can be carried out by simply pipetting the wash buffer into the wells and then pipetting the buffer out from the wells (discard the buffer).*

4. Signal Detection

a. Add 100 µl of **DS** (Developer Solution) to each well and incubate at room temperature for 1 to 10 min away from light. Begin monitoring color changes in the sample wells and control wells. The **DS** solution will turn blue in the presence of sufficient C-to-U products.

b. Add 100 µl of **SS** (Stop Solution) to each well to stop enzyme reaction when the color in the positive control wells turns medium blue. The color will change to yellow after adding **SS,** and the absorbance should be read on a microplate reader within 2 to 10 min at 450 nm with an optional reference wavelength of 655 nm.

Note: *(1) Most microplate readers have the capability to carry out dual-wavelength analysis and will automatically subtract reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, the plate can be read twice – once at 450 nm and once at 655 nm. Then manually subtract the 655 nm ODs from 450 nm ODs; (2) If the stripwell microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

5. APOBEC3 Activity Calculation

a. Calculate the average duplicate readings for sample wells and sample blank wells.

b. Calculate APOBEC3 activity or inhibition using the following formulas:

For simple calculation:

$$\textit{APOBEC3 Activity (OD/min/mg)} = \frac{\textit{(Sample OD – Sample Blank OD)}}{\textit{(Protein Amount (µg)* x min**)}} \textbf{ x } \textit{1000}$$

* Protein amount added into the reaction at step 2e.
** Incubation time at step 2g (in minutes).

Example calculation:

Average OD450 of sample is 0.27
Average OD450 of blank is 0.05
Protein amount is 5 µg
Incubation time is 120 min

$$\text{APOBEC3 activity} = \frac{(0.27 - 0.05)}{(5 \times 120)} \times 1000 = 0.3 \text{ OD/min/mg}$$

For accurate or specific activity calculation:

First, generate a standard curve and plot the OD values versus the amount of **A3S** at each concentration point. Then determine the slope as OD/ng using linear regression (*Microsoft Excel's* linear regression or slope functions are suitable for such calculation) and the most linear part (include at least 4 concentration points) of the standard curve for optimal slope calculation. Now calculate the amount of APOBEC3-converted product using the following formulas:

$$\textit{C-to-U product (ng)} = \frac{\textit{(Sample OD – Sample Blank OD)}}{\textit{Slope}}$$

$$\textit{APOBEC3 Activity (ng/min/mg)} = \frac{\textit{C-to U Product (ng)}}{\textit{(Protein Amount (µg)* x min**)}} \textbf{ x } \textit{1000}$$

* Protein amount added into the reaction at step 2e.
** Incubation time at step 2g (in minutes).

For inhibition calculation:

$$\textit{Inhibition \%} = \left[1 - \frac{\textit{Inhibitor Sample OD – Sample Blank}}{\textit{No Inhibitor Sample OD – Sample Blank}} \right] \textbf{ x } \textit{100\%}$$

SUGGESTED STRIP WELL SETUP

Table 1. The suggested strip-well plate setup for standard curve preparation in a 48-assay format (in a 96-assay format, Strips 7 to 12 can be configured as Sample). The controls and samples can be measured in duplicate.

Well #	Strip 1 (Green Trimmed)	Strip 2 (Green Trimmed)	Strip 3	Strip 4	Strip 5	Strip 6
A	Standard Blank	Standard Blank	Sample Blank	Sample Blank	Sample	Sample
B	A3S 1 ng/µl	A3S 1 ng/µl	Sample	Sample	Sample	Sample
C	A3S 2 ng/µl	A3S 2 ng/µl	Sample	Sample	Sample	Sample
D	A3S 5 ng/µl	A3S 5 ng/µl	Sample	Sample	Sample	Sample
E	A3S 10 ng/µl	A3S 10 ng/µl	Sample	Sample	Sample	Sample
F	A3S 20 ng/µl	A3S 20 ng/µl	Sample	Sample	Sample	Sample
G	A3S 50 ng/µl	A3S 50 ng/µl	Sample	Sample	Sample	Sample
H	A3S 100 ng/µl	A3S 100 ng/µl	Sample	Sample	Sample	Sample

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal or weak signal in both the standard and sample wells	Reagents are added incorrectly.	Check if reagents are added in the proper order with the right amount, and if any steps in the protocol may have been omitted by mistake.
	Probe is not properly diluted	Ensure that (1) The probe is properly diluted and added into the wells; (2) the wells are completely covered with sufficient probe solution.
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol are followed correctly.
	Incorrect absorbance reading.	Check if the appropriate absorbance wavelength (450 nm filter) is used.
	Kit was not stored or handled properly.	Ensure all components of the kit were stored at the appropriate temperatures and the cap is tightly closed after each opening or use.
No signal or weak signal in only the standard curve wells	The standard amount is insufficiently added to the well in Step 2c.	Ensure a sufficient amount of standard is added.
	The standard is degraded due to improper storage conditions.	Follow the Shipping & Storage guidance of this User Guide for storage of **A3S** (APOBEC3 Assay Standard).
High background present in the blank wells	Insufficient washing of wells.	Check if washing at each step is performed according to the protocol.
	Contaminated by sample or standard.	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips.
	Over development of color.	Decrease the development time in Step 4a before adding **SS** (Stop Solution) in Step 4b.
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified.	Ensure your protocol is suitable for APOBEC3 protein extraction. For the best results, it is advised to use EpigenTek's Whole Cell Extraction Kit (Cat. No. OP-0003). Also, use fresh cells or tissues for protein extraction, as frozen cells or tissues could lose enzyme activity.
	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of purified enzymes or cell extracts is used as indicated in Steps 2e and 2f. The sample can be titrated to determine the optimal amount to use in the assay.

	Sample was not stored properly or has been stored for too long.	Ensure sample is stored in aliquots at –80°C, with no more than 6 weeks for cell extracts and 6 months for purified enzymes. Avoid repeated freezing/thawing.
	Little or no activity of APOBEC3 contained in the sample.	This problem may be a result of many factors. If the affecting factors cannot be determined, use new or re-prepared cell extracts or purified enzymes.
Uneven color development	Insufficient wash of the wells.	Ensure the wells are washed according to the guidance of washing and residue washing buffer is removed as much as possible.
	Delayed color development or delayed stopping of color development in the wells.	Ensure color development and stop solutions are added sequentially and consistent with the order you added the other reagents (e.g., from well A to H or from well 1 to 12).

RELATED PRODUCTS

Cell Extract Preparation
OP-0003 EpiQuik™ Whole Cell Extraction Kit