GAD67 KITS

PROTOCOL

Part # 63ADK002PEG & 63ADK002PEH

Test size#: 500 tests (63ADK002PEG) and 10,000 tests (63ADK002PEH) - assay volume: 20 µL

Revision: 04-May 2020

Store at: -16°C or below (63ADK002PEG); -16°C or below (63ADK002PEH)

For research use only. Not for use in diagnostic procedures.

ASSAY PRINCIPLE

This kit is intended for the simple and rapid quantification of GAD67 in cell lysates and offers a fast alternative to ELISA.

The detection principle of this kit is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). As shown in Figure 1, GAD67 is detected in a sandwich assay by using anti-GAD67 antibody labeled with Terbium cryptate (donor), and anti-GAD67 antibody labeled with d2 (acceptor).

When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the GAD67 concentration.

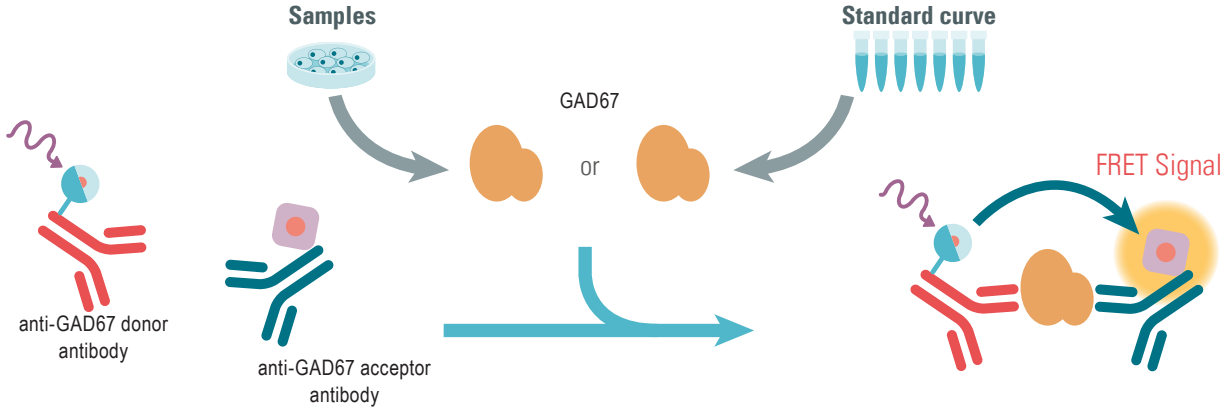
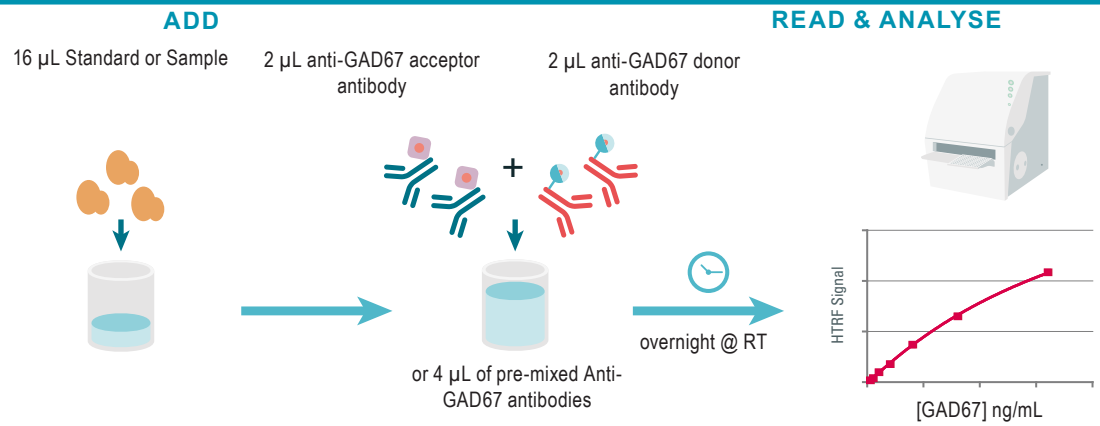


Figure 1: Principle of HTRF GAD67 sandwich assay.

PROTOCOL AT A GLANCE



Make sure to use the set-up for Tb Cryptate.

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MATERIALS PROVIDED:

KIT COMPONENTS	500 TESTS * CAT # 63ADK002PEG	10,000 TESTS * CAT # 63ADK002PEH
GAD67 Standard Frozen	1 vial - 10 µL 100 µg/mL	1 vial - 10 µL 100 µg/mL
GAD67 Tb Cryptate Antibody	1 vial - 20 µL Frozen - 50X	1 vial - 0.4 mL Frozen - 50X
GAD67 d2 Antibody	1 vial - 20 µL Frozen - 50X	1 vial - 0.4 mL Frozen - 50X
Lysis buffer **	Not provided Recommended M-PER	Not provided Recommended M-PER
Detection buffer *** ready-to-use	1 vial 2 mL	1 vial 50 mL

* When used as advised, the two available kit sizes will provide sufficient reagents for 500 tests and 10,000 tests respectively in 20 µL final volume..
Assay volumes can be adjusted proportionally to run the assay in 96 or 1536 well microplates.
** Medium like cell culture medium can be an alternative to the diluent.
*** The Detection buffer is used to prepare working solutions of acceptor and donor reagents.

PURCHASE SEPARATELY:

- HTRF®-Certified Reader. Make sure the setup for Tb Cryptate is used.

For a list of HTRF-compatible readers and set-up recommendations, please visit www.cisbio.com/compatible-readers

- Small volume (SV) detection microplates - .

For more information about microplate recommendations, please visit our website at: cisbio.com/microplates-recommendations

STORAGE AND STABILITY

Reagents

Store the kit at -16°C or below.

Under proper storage conditions, reagents are stable until the expiry date indicated on the label. Detection buffer is shipped frozen, but can be stored at 2-8°C in your premises.

If lyophilized, reconstituted reagents, antibodies, and standard stock solutions may be frozen and thawed only once. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below .

REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare reagents in the specified buffers. The use of an incorrect lysis buffer may affect reagent stability and assay results.
- Thaw the frozen reagents at room temperature, allow them to warm up to room temperature for at least 30 mins before use
- Before use, allow Lysis buffer and Detection buffer to warm up at room temperature and homogenize them with a vortex.
- Antibody solutions must be prepared in individual vials and can be mixed prior to dispensing.
- GAD67 standards (for standard curve) must be prepared in lysis buffer or in the same medium as the samples.

TAKE CARE TO PREPARE STOCK AND WORKING SOLUTIONS ACCORDING TO THE DIRECTIONS FOR THE KIT SIZE YOU HAVE PURCHASED.

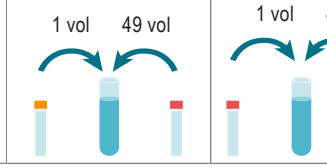
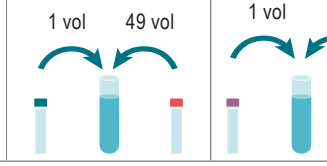
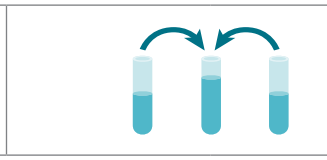
TO PREPARE REAGENT STOCK SOLUTIONS:

500 TESTS KIT - 63ADK002PEG		10,000 TESTS KIT - 63ADK002PEH
Anti-GAD67 Tb Cryptate antibody		
Thaw the GAD67 Tb Cryptate antibody . Mix gently. This 50X stock solution can be frozen and stored at -16°C or below.		Thaw the GAD67 Tb Cryptate antibody . Mix gently. This 50X stock solution can be frozen and stored at -16°C or below.
Anti-GAD67 d2 antibody		
Thaw the GAD67 d2 antibody . Mix gently. This 50X stock solution can be frozen and stored at -16°C or below.		Thaw the GAD67 d2 antibody . Mix gently. This 50X stock solution can be frozen and stored at -16°C or below.
GAD67 Standard		
Thaw the GAD67 Standard in order to obtain a 100 µg/mL stock solution. Mix gently. This stock solution can be frozen and stored at -20°C or below.		Thaw the GAD67 Standard in order to obtain a 100 µg/mL stock solution. Mix gently. This stock solution can be frozen and stored at -20°C or below.
Lysis buffer		
The Lysis buffer is ready-to-use.		The Lysis buffer is ready-to-use.
Detection buffer		
The Detection buffer is ready-to-use.		The Detection buffer is ready-to-use.

TO PREPARE ANTIBODY WORKING SOLUTIONS:

Each well requires 2 µL of GAD67-Tb Cryptate Antibody and 2 µL of GAD67-d2 Antibody.

Prepare the two antibody solutions in separate vials.

500 TESTS KIT - 63ADK002PEG		10,000 TESTS KIT - 63ADK002PEH
GAD67 Eu Cryptate antibody		
Dilute 50-fold the 50X stock solution (thawed reagent) of GAD67 Tb Cryptate antibody with the Detection buffer: add 1 volume of Tb Cryptate antibody stock solution in 49 volumes of Detection buffer (e.g., 20 µL of reconstituted Tb Cryptate antibody stock solution + 980 µL of Detection Buffer).		Dilute 50-fold the 50X stock solution (thawed reagent) of human GAD67 Cryptate antibody stock solution with the Detection buffer : add 1 volume of Tb Cryptate antibody stock solution in 49 volumes of Detection buffer (e.g., 0.4 mL of Tb Cryptate antibody stock solution + 19.6 mL of Detection Buffer).
GAD67 d2 antibody		
Dilute 50-fold the 50X stock solution (thawed reagent) of GAD67 d2 antibody with the Detection buffer: add 1 volume of d2 antibody stock solution in 49 volumes of Detection buffer(e.g., 20 µL of reconstituted d2 antibody stock solution + 980 µL of Detection Buffer).		Dilute 50-fold the 50X stock solution (thawed reagent) of human GAD67 d2 antibody stock solution with the Detection buffer : add 1 volume of d2 antibody stock solution in 49 volumes of Detection buffer (e.g., 0.4 mL of d2 antibody stock solution + 19.6 mL of Detection Buffer).
Antibody mix		
It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2 antibody solution to 1 volume of Tb Cryptate antibody solution (e.g. 1 mL of d2 antibody + 1 mL of Tb Cryptate antibody).		It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2 antibody solution to 1 volume of Tb Cryptate antibody solution (e.g. 1 mL of d2 antibody + 1 mL of Tb Cryptate antibody).

TO PREPARE STANDARD WORKING SOLUTIONS:

- Each well requires 16 µL of standard.
- Dilute the standard stock solution serially with lysis buffer
- In order to check for a potential interference effect from your own assay buffer when using the assay for the first time, we highly recommend the parallel preparation of a standard curve in your own supplemented cell culture medium and in lysis buffer .
- In order to counteract any standard sticking, we recommend changing tips between each dilution.

A recommended standard dilution procedure is listed and illustrated below:

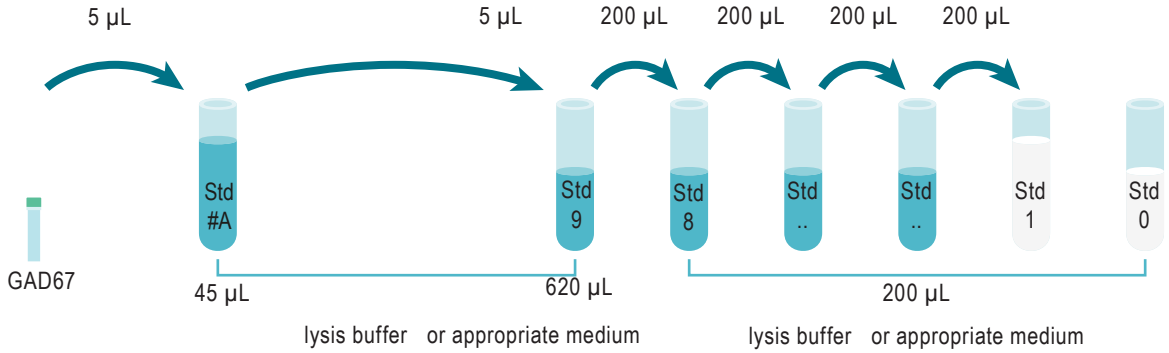
Dilute the standard stock solution 10-fold with lysis buffer; this yields the Intermediate Standard solution #A(10,000 ng/mL). E.g: take 5 µL of standard stock solution and add it to 45 µL of lysis buffer. Mix gently.

Dilute the Intermediate Standard dilution #A 125-fold with lysis buffer to prepare high standard (Std 9): e.g. take 5 µL of Intermediate Standard dilution #A and add it to 620 µL of lysis buffer . Mix gently.

Use the high standard (Std 9) to prepare the standard curve using 1/2 serial dilutions as follows:

- Dispense 200 µL of lysis buffer in each vial from Std 8 to Std 0.
- Add 200 µL of standard to 200 µL of lysis buffer , mix gently and repeat the 1/2 serial dilution to make standard solutions: std8, std7, std6, std5, std4, std3, std2, std1.

This will create 9 standards for the analyte. Std 0 (Negative control) is lysis buffer or appropriate culture medium alone.



STANDARD	SERIAL DILUTIONS	GAD67 WORKING SOLUTIONS (ng/mL)
Standard Stock solution	Thawed stock solution	100,000
Intermediate standard solution #A	5 µL Standard stock solution + 45 µL lysis buffer	10,000
Standard 9	5 µL Intermediate Standard Solution #A + 620 µL lysis buffer	80
Standard 8	200 µL standard 9 + 200 µL lysis buffer	40
Standard 7	200 µL standard 8 + 200 µL lysis buffer	20
Standard 6	200 µL standard 7 + 200 µL lysis buffer	10
Standard 5	200 µL standard 6 + 200 µL lysis buffer	5
Standard 4	200 µL standard 5 + 200 µL lysis buffer	2.5
Standard 3	200 µL standard 4 + 200 µL lysis buffer	1.25
Standard 2	200 µL standard 3 + 200 µL lysis buffer	0.625
Standard 1	200 µL standard 2 + 200 µL lysis buffer	0.313
Standard 0	200 µL lysis buffer	0

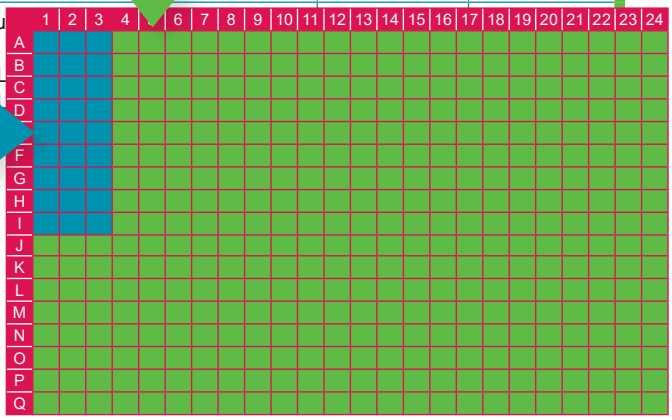
TO PREPARE SAMPLES:

- Each well requires 16 µL of sample.
- Just after their collection, put the samples at 4°C and test them immediately. For later use, samples should be dispensed into disposable plastic vials and stored at -60°C or below. Avoid multiple freeze/thaw cycles.
- We recommend using M-PER lysis buffer or your preferred lysis buffer to perform the lysis of the cells. The assay can be run under a two-plate protocol, where cells are plated and stimulated in the same culture plate, then transferred to the assay plate for the HTRF® detection. This protocol enables the cells' viability and confluence to be monitored. It can also be further streamlined to a one-plate assay protocol where plating, stimulation and detection are performed in a single plate. Cell density, stimulation time, lysis step and other parameters related to the biology are cell-dependent and need to be optimized.
- Samples with a concentration above the highest standard (Std 9) must be diluted lysis buffer
- As Terbium Cryptate is sensitive to phenol red, it is mandatory to use medium without pheneol red (e.g. KRB or HSBC) to run your secretion assay

ASSAY PROTOCOL

	Standard (Std 0 - Std 9)	Samples
Step 1 	Dispense 16 µL of each GAD67 standard (Std 0 - Std 9) into each standard well	Dispense 16 µL of each sample into each sample well
Step 2 	Add 2 µL of GAD67 d2 antibody working solution to all wells	
Step 3 	Add 2 µL of GAD67 Tb Cryptate antibody working solution to all wells	
Step 4 	Seal the plate and incubate overnight @ RT	
Step 5 	Remove the plate sealer and read on an HTRF® compatible reader	

	1	2	3	4	5	6
A	16 µL Std 0 (Negative control) 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well A1	Repeat Well A1	16 µL Sample 1 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well A4	Repeat Well A4
B	16 µL Std 1 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well B1	Repeat Well B1	16 µL Sample 2 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well B4	Repeat Well B4
C	16 µL Std 2 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well C1	Repeat Well C1	16 µL Sample 3 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well C4	Repeat Well C4
D	16 µL Std ... 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well D1	Repeat Well D1	16 µL Sample ... 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well D4	Repeat Well D4
E	16 µLStd ... 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well E1	Repeat Well E1	16 µL Sample ... 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well E4	Repeat Well E4
F	16 µL Std ... 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well F1	Repeat Well F1	16 µL Sample ... 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well F4	Repeat Well F4
G	16 µL Std ... 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well G1	Repeat Well G1	16 µL Sample ... 2 µL GAD67-d2 2 µL GAD67-Tb C	Repeat Well G4	Repeat Well G4
H	16 µL Std ... 2 µL GAD67-d2 2 µL GAD67-Tb Cryptate	Repeat Well H1	Repeat Well H1	16 µ 2 µL		



DATA REDUCTION

1. Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2. Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

3. Calculate the % delta F which reflects the signal to background of the assay. The negative control (Standard 0) plays the role of an internal assay control. Delta F is used for the comparison of day to day runs of the same assay.

$$\text{delta F (\%)} = \frac{\text{Ratio Standard or sample - Ratio Negative Control}}{\text{Ratio Negative Control}} \times 100$$

For more information about data reduction, please visit http://www.cisbio.com/htrf-ratio-and-data-reduction

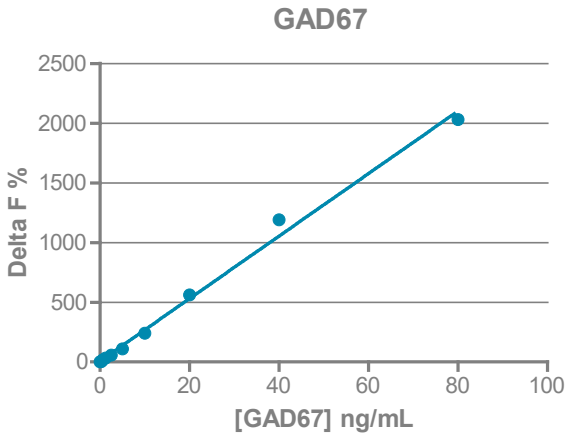
RESULTS

This data must not be substituted for the data obtained in the laboratory and should be considered only as an example.

Results may vary from one HTRF® compatible reader to another.

The assay standard curve is created by plotting delta F% versus the analyte concentration:

	Ratio (1)	CV (2)	Delta F% (3)
Standard 0 - Negative control	1,007	6.3%	-
Standard 1 - 0.313 ng/mL	1,048	1.7%	4%
Standard 2 - 0.625 ng/mL	1,190	6.3%	18%
Standard 3 - 1.25 ng/mL	1,343	5.7%	33%
Standard 4 - 2.5 ng/mL	1,587	2.6%	58%
Standard 5 - 5 ng/mL	2,103	1.4%	109%
Standard 6 - 10 ng/mL	3,434	1.2%	241%
Standard 7 - 20 ng/mL	6,667	0.3%	562%
Standard 8 - 40 ng/mL	12,999	4.0%	1,191%
Standard 9 - 80 ng/mL	21,443	6.9%	2,030%



FOR MORE INFORMATION
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