

IsoTag™ AAV – Residual Reagent ELISA

Immunoenzymetric Assay for the Measurement of IsoTag™ AAV — Residual Reagent Catalog # F1035

Intended Use

These kits are intended for use in quantitating IsoTag™ AAV – Residual Reagent. These kits are for **Research and Manufacturing Use Only** in combination with IsoTag™ AAV products only and are not intended for diagnostic use in humans or animals. The F1035 kits incorporate a well-qualified sample treatment method to dissociate the residual from the AAV virus.

Summary and Explanation

This immunoassay method provides sensitivity to the IsoTag™ AAV Residual Reagent to less than 1ng/mL. This kit can be used as a tool to aid in optimal purification process development and in routine quality control of in-process manufacturing as well as final product.

Principle of the Procedure

The IsoTag™ AAV -Residual Reagent assay is a two-site immuno-enzymatic assay. Samples potentially containing residual IsoTag™ AAV Reagent are mixed with sample denaturing buffer to dissociate the biopolymer from the AAV virus. The samples are then reacted simultaneously with a horseradish peroxidase (HRP) enzyme labeled anti-IsoTag™ AAV Reagent antibody (goat polyclonal) in microtiter strips coated with an affinity purified capture antibody (goat polyclonal). The immunological reactions result in the formation of a sandwich complex of solid phase antibody-biopolymer-enzyme labeled antibody. After a wash step to remove any unbound reactants, the strips are then reacted with tetramethylbenzidine (TMB) substrate. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of IsoTag™ AAV - Residual Reagent present in the sample. Accurate quantitation is achieved by comparing the signal of unknowns to IsoTag™ AAV Reagent standards assayed at the same time.

Reagents & Materials Provided

Component	Product#
Anti-IsoTag™ AAV Reagent:HRP Goat polyclonal antibody conjugated to HRP in a protein matrix with preservatives. 1x12mL	F1036
Anti-IsoTag™ AAV Reagent coated microtiter strips 12x8 well strips in a bag with desiccant	F1037
IsoTag™ AAV Reagent Standards IsoTag™ AAV Reagent in a protein matrix with preservatives. Standards at 0, 0.16, 0.31, 0.63, 1.25, 2.5, 5 and 10ng/mL. 1 mL/vial	F1038
Sample Denaturing Buffer Citrate buffer with detergent and preservative. 1x12mL	F1039
Sample Diluent Buffer TBS with bovine albumin and preservative. 1x30mL	I028
Stop Solution 0.5M sulfuric acid. 1x12mL	F006
TMB Substrate 3,3',5,5' Tetramethylbenzidine. 1x12mL	F005
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	F004
Sample Treatment Plate Skirted 96 well PCR plate with adhesive foil seal	F402

Materials & Equipment Required But Not Provided

- Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 650nm. (If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)
- Pipettors - 50µL and 100µL
- Repeating or multichannel pipettor - 100µL
- Microtiter plate rotator (400-600 rpm)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- Reconstituted wash solution is stable until the expiration date of the kit.
- After prolonged storage, you may notice a salt precipitate and/or yellowing of the wash concentrate. These changes will not impact assay performance. To dissolve the precipitate, mix the wash concentrate thoroughly and dilute as directed in the 'Preparation of Reagents' section. Reconstituted wash solution is stable until the expiration date of the kit.

Precautions

- **For Research or Manufacturing use only when used in combination with Isolere Bio Inc. IsoTag™ AAV Reagents.**
- **Stop reagent is 0.5M H₂SO₄. Avoid contact with eyes, skin, and clothing.**
- **This kit should only be used by qualified technicians.**
- **Do not use filtered tips.**
- **Do not push tips all the way to the bottom of the sample treatment plate when loading due to possible precipitate.**

Preparation of Reagents

- Bring all reagents to room temperature.
- Dilute 20x wash concentrate to 1x in 1 liter of distilled water, label with kit lot and expiration date, and store at 4°C.

Procedural Notes

- Complete washing of the plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. The manual wash procedure described below generally provides lower backgrounds, higher specific absorbance, and better precision than automated plate washers.
- When dilutions of samples are required, dilution should be performed in the provided diluent qualified to yield acceptable background and no impurities from IsoTag™ AAV - Residual Reagent. Sample dilution should be performed prior to the sample denaturation step. The diluent should also give acceptable recovery when spiked with known quantities of IsoTag™ AAV - Residual Reagent. Sample Diluent, Cat #I028 is provided and has been qualified for use with this assay. If needed, additional Sample Diluent, Cat #I028 can be purchased. This is the same diluent used to make the kit standards. As your sample is diluted in I028, its matrix begins to approach that of the standards

thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for non-specific binding and acceptable spike and recovery as discussed below.

Limitations

- Before reporting the IsoTag™ AAV - Residual Reagent impurity results, each laboratory should qualify that the kit and assay procedure utilized yield acceptable specificity, accuracy, and precision. A suggested protocol for this qualification can be obtained by contacting our Technical Services Department or at our web site. In general, the most critical qualification experiments involve spike & recovery and dilutional linearity/parallelism.
- Samples in concentrated strong acids can interfere in the assay by lowering the assay pH to below the optimal range of 7.0 to 7.5. The HRP labeled antibody is in a strong buffer designed to neutralize most samples back to the ideal assay pH range.
- Certain sample matrices may interfere in this assay. The standards used in this kit attempt to simulate typical sample protein and matrices. However, the potential exists that the product itself or other components in the sample matrix may result in either positive or negative interference in this assay. High or low pH, detergents, urea, high salt concentrations, and organic solvents are some of the known interference factors. It is advised to test all sample matrices for interference by diluting the 10ng/mL standard 1 part to 3 parts of the matrix containing no or very low levels of the IsoTag™ AAV - Residual Reagent. This diluted standard, when assayed as an unknown, should give recovery in the range of 2ng/mL to 3ng/mL. Consult Cygnus Technologies' Technical Service Department for advice on how to quantitate the assay in problematic matrices.

Sample Treatment

Sample Treatment Protocol

1. **Vortex all reagents before use. Make sample dilutions in sample diluent Cat# I028 and transfer 100µL of sample and kit standards to Sample Treatment Plate (STP, Cat# F402).**
2. **Add 50µL of Sample Denaturing Buffer, Cat# F1039 to each sample and standard well. Mix by pipetting up and down at least 15 times. Use fresh tips for each addition. Be sure to control pipette speed to avoid creating bubbles. Once done mixing, the solution should be opaque in color.**
3. **Incubate on bench for ~5 minutes.**

Assay Protocol

- The protocol specifies use of an orbital microtiter plate shaker for the immunological steps. These can be purchased from most laboratory supply companies. If you do not have such a device, it is possible to incubate the plate without shaking; however, it will be necessary to extend the immunological incubation step in the plate by about one hour in order to achieve comparable results to the shaking protocol. **Do not shake during the 30-minute substrate incubation step as this may result in higher backgrounds and worse precision.**
- Bring all reagents to room temperature.
- Set-up plate spectrophotometer to read dual wavelength at 450nm for the test wavelength and ~650nm for the reference.
- Thorough washing is essential to proper performance of this assay. The manual method described in the assay protocol is preferred for best precision, sensitivity, and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site. In addition, a video demonstration of proper plate washing technique is available in the 'Technical Resources' section of our web site.
- All standards, controls, and samples should be assayed at least in duplicate.
- Maintain a repetitive timing sequence from well to well for all assay steps to ensure that all incubation times are the same for each well.
- Make a work list for each assay to identify the location of each standard, control, and sample.
- It is recommended that your laboratory assay appropriate quality control samples in each run to ensure that all reagents and procedures are correct.
- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Vortex all reagents immediately before use.
2. Pipette 100 μ L of anti-IsoTag™ AAV - Reagent:HRP (#F1036) into each well.
3. Pipette 25 μ L of the denatured standards (#F1038), controls and samples into wells indicated on work list.
4. Cover & incubate on orbital shaker at 400 - 600rpm for 1 hour at room temperature, 24°C \pm 4°C.
5. Dump contents of wells into waste. Blot and gently but firmly tap over absorbent paper to remove most of the residual liquid. Overly aggressive banging of the plate to remove all residual liquid is not necessary and may cause variable dissociation of antibody bound material resulting in lower ODs and worse precision. Fill wells generously to overflowing with diluted wash solution using a squirt bottle (~350 μ L). Dump and tap again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding substrate.
6. Pipette 100 μ L of TMB substrate (#F005).
7. Incubate at room temperature for 30 minutes. DO NOT SHAKE.
8. Pipette 100 μ L of Stop Solution (#F006).
9. Read absorbance at 450/650nm.

Example Data

Well #	Contents	Abs. at 450-650nm	Mean Abs.
H1	Zero Std	0.013	0.012
H2	Zero Std	0.012	
H3	Zero Std	0.013	
G1	0.16ng/mL	0.089	0.090
G2	0.16ng/mL	0.086	
G3	0.16ng/mL	0.094	
F1	0.31ng/mL	0.166	0.172
F2	0.31ng/mL	0.176	
F3	0.31ng/mL	0.174	
E1	0.63ng/mL	0.333	0.336
E2	0.63ng/mL	0.336	
E3	0.63ng/mL	0.340	
D1	1.25ng/mL	0.632	0.641
D2	1.25ng/mL	0.636	
D3	1.25ng/mL	0.656	
C1	2.5ng/mL	1.149	1.162
C2	2.5ng/mL	1.175	
C3	2.5ng/mL	1.163	
B1	5ng/mL	1.994	1.963
B2	5ng/mL	1.943	
B3	5ng/mL	1.953	
A1	10ng/mL	2.903	2.870
A2	10ng/mL	2.813	
A3	10ng/mL	2.894	

Calculation of Results

The standards are used to construct a standard curve with values reported in ng/mL. This data reduction may be performed through computer methods using curve-fitting routines such as point-to-point, cubic spline, or 4 parameter logistic fit. **Do not use linear regression analysis to interpolate values for samples as this may lead to significant inaccuracies!** Data may also be manually reduced by plotting the absorbance values of the standard on the y-axis versus concentration on the x-axis and drawing a smooth point-to-point line. Absorbances of samples are then interpolated from this standard curve.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 0.31 - 10ng/mL. CVs for samples less than 0.31 ng/mL may be greater than 10%.
- It is recommended that each laboratory assay appropriate quality control samples in each run to ensure that all reagents and procedures are correct.

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional analytical criteria as published in the "Phase 2 Qualification Report". This qualification is generic in nature and is intended to supplement but not replace a comprehensive user and sample type qualification that should be performed by each laboratory. At a minimum, each laboratory is urged to perform a spike and recovery study in their sample types. In addition, any of your sample types containing IsoTag™ AAV – Residual Reagent within or above the analytical range of this assay should be evaluated for dilution linearity. Each laboratory and technician should also demonstrate competency in the assay by performing a precision study similar to that described below. A more detailed discussion of recommended user qualification protocols can be obtained by contacting our Technical Services Department or on-line at our web site.

Sensitivity

The lower limit of detection (LOD) is defined as that concentration corresponding to a signal three standard deviations above the mean of the zero standard. LOD is ~0.08 ng/mL.

The lower limit of quantitation (LLOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are less than 20%. The LLOQ is ~0.16 ng/mL.

Specificity/Cross-Reactivity

Cross reactivity to non-HCP components has not been extensively investigated with this kit. You should evaluate components in your samples for positive interferences such as cross reactivity and non-specific binding. Negative interference studies are described below.

Precision

Both intra (n=20 replicates) and inter-assay (n=10 assays) precision were determined on 4 controls with low (~0.2 ng/mL), low-medium (~0.8ng/mL), high-medium (~3 ng/mL), and high concentrations (~7.5 ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	4.6%	6.6%
Low Medium	2.9%	6.3%
High Medium	1.6%	1.8%
High	3.1%	3.4%

Recovery/Interference Studies

Recovery was evaluated by adding known amounts of IsoTag™ AAV Reagent used to make the standards in this kit to the capsid stock solution. All of these samples yielded acceptable recovery defined as between 80-120%. Each user should qualify that their sample matrices yield accurate recovery. Such an experiment can be performed by diluting the 10ng/mL standard provided with this kit into the sample matrix in question as described in the "Limitations" section.

Ordering Information/ Customer Service

Sample Diluent Cat #1028 and Antigen Concentrate can be purchased from *Cygnus Technologies*. To place an order or to obtain additional product information contact *Cygnus Technologies*:

www.cygnustechnologies.com
Cygnus Technologies, LLC
1523 Olde Waterford Way
Leland, NC 28451 USA
Tel: 910-454-9442

Email for all Order inquiries:
orders@cygnustechnologies.com

Email for Technical Support:
techsupport@cygnustechnologies.com