Avitide AVIPure® — AAV2 Residual Ligand Assay

Immunoenzymetric Assay for the Measurement of Avitide AVIPure® — AAV2 Residual Ligand Catalog # F1000

Intended Use

These kits are intended for use in quantitating the Avitide AVIPure® — AAV2 Ligand. These kits are **for Research and Manufacturing Use Only** and are not intended for diagnostic use in humans or animals. The F1000 kits incorporate a well-qualified sample treatment method to dissociate the ligand from the AAV virus.

Summary and Explanation

AVIPure® — AAV2 Ligand, immobilized on various chromatography media, is used to purify AAV2 virus. Even when covalently attached, the ligand can leach off the chromatography support and co-elute with AAV. Residual AVIPure® — AAV2 Ligand must be minimized to avoid any adverse patient effects. Our immunoassay method provides sensitivity to the AVIPure® — AAV2 Ligand 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 streams as well as final product. The AVIPure® — AAV2 Ligand assay is not designed to detect all currently marketed constructs used for the purification of AAV virus.

Principle of the Procedure

The Avitide AVIPure® — AAV2 Ligand assay is a two-site immuno-enzymatic assay. Samples containing AVIPure® — AAV2 Ligand are mixed with sample denaturing buffer to dissociate the ligand from the AAV virus. The samples are then reacted simultaneously with a horseradish peroxidase (HRP) enzyme labeled anti-AVIPure® antibody (rabbit polyclonal) in microtiter strips coated with an affinity purified capture antibody (rabbit monoclonal). The immunological reactions result in the formation of a sandwich complex of solid phase antibody-Ligand-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 AVIPure® — AAV2 Ligand present in the sample. Accurate quantitation is achieved by comparing the signal of unknowns to AVIPure® — AAV2 Ligand standards assayed at the same time.

Reagents & Materials Provided

Component	Product#
Anti-AVIPure® — AAV2 Ligand: HRP Rabbit polyclonal antibody conjugated to HRP in a protein matrix with preservatives. 1x12mL	F1001
Anti-AVIPure® coated microtiter strips 12x8 well strips in a bag with desiccant	F1002
AVIPure® — AAV2 Ligand Standards AVIPure® — AAV2 Ligand in a protein matrix with preservatives. Standards at 0, 0.16, 0.31, 0.63, 1.25, 2.5, 5 and 10ng/mL. 1mL/vial	F1003
Sample Denaturing Buffer Citrate buffer with detergent and preservative. 1x12mL	F1004R
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)
- Sample Diluent (recommended Cat # I028)
- 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.
- Stop reagent is 0.5M H_2SO_4. Avoid contact with eyes, skin, and clothing.
- This kit should only be used by qualified technicians.

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 a diluent qualified to yield acceptable background and not impurities with AVIPure® — AAV2 Ligand. Sample dilution should be performed prior to the sample denaturation step. The diluent should also give acceptable recovery when spiked with known quantities of AVIPure® — AAV2 Ligand. Sample Diluent, Cat #I028 has been qualified for use with this assay. 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 AVIPure® — AAV2 Ligand 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 and product antibodies 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 1 part of the matrix containing no or very low levels of the AVIPure® — AAV2 Ligand. This diluted standard when assayed as an unknown, should give recovery in the range of 4ng/mL to 6ng/mL. Consult Cygnus Technologies' Technical Service Department for advice on how to quantitate the assay in problematic matrices.

Sample Treatment

Sample Treatment Protocol

1. 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 #F1004R to each sample and standard well. Mix by pipetting up and down ~15 times. Use fresh tips for each addition.

3. Incubate on bench for ~5 minutes.

Assay Protocol

- The protocol specifies use of an approved 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 Help' 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. Pipette 100µL of anti-AVIPure® — AAV2 Ligand: HRP (#F1001) into each well.

2. Pipette 50µL of the denatured standards (#F1003), controls and samples into wells indicated on work list.

3. Cover & incubate on orbital shaker at 400 - 600rpm for 1 hour at room temperature, 24°C ± 4°C.

4. 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 or by pipetting in ~350µL. 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.

5. Pipette 100µL of TMB substrate (#F005).

6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.

7. Pipette 100µL of Stop Solution (#F006).

8. Read absorbance at 450/650nm.

Example Data

Well #	Contents	Abs. at 450-650nm	Mean Abs.
A1	Zero Std	0.021	0.020
A2	Zero Std	0.018	
B1	0.16 ng/mL	0.072	0.069
B2	0.16 ng/mL	0.067	
C1	0.31 ng/mL	0.121	0.120
C2	0.31 ng/mL	0.118	
D1	0.63 ng/mL	0.227	0.221
D2	0.63 ng/mL	0.214	
E1	1.25 ng/mL	0.410	0.400
E2	1.25 ng/mL	0.389	
F1	2.5 ng/mL	0.754	0.748
F2	2.5 ng/mL	0.741	
G1	5 ng/mL	1.499	1.461
G2	5 ng/mL	1.423	
H1	10 ng/mL	2.610	2.585
H2	10 ng/mL	2.561	

Calculation of Results

The standards may be 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 parameters 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 Avitide AVIPure® – AAV2 Residual Ligand 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 ~0.02 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=16 replicates) and inter-assay (n=10 assays) precision were determined on 4 pools with low (~0.20 ng/mL), low-medium (~0.8 ng/mL), 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	3.5 %	5.8 %
Low Medium	2.8 %	2.2 %
Medium	3.0 %	4.1 %
High	4.1 %	3.9 %

Recovery/Interference Studies

Recovery was evaluated by adding known amounts of AVIPure® – AAV2 Ligand 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

Cygnus Technologies also offers kits for the extraction and detection of Host Cell DNA. The following kits are available:

- Residual Host Cell DNA extraction:

  Cat # D100W, DNA Extraction Kit in 96 deep well plate
  Cat # D100T, DNA Extraction Kit in microfuge tubes

To place an order or to obtain additional product information contact *Cygnus Technologies*:

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