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1. INTRODUCTION

GTI, conducted stability testing on the proposed ARMWG reference material (VR-1516, lot # 001502). The material was evaluated for short-term stability at room temperature and 4ºC, exposure to three freeze thaw cycles and stability of the product when the shipping package is exposed to extreme temperature conditions.

2. STUDY DESIGN

2.1. Short Term Stability

Short term studies were conducted at five time points, 4 hr, 8 hr, 24 hr, 72 hr and 168 hr as specified in RFP11. Samples were stored at room temperature and at 2-8º C for the duration of the studies. Samples were tested at each time point for strength by both OD_{260} (RFP 8) and Anion Exchange Chromatography (QC-24), infectivity by both NAS $TCID_{50}$ (RFP 9) and Hexon-FACS (QC-23) and the pH (EQU-41) and appearance of the samples were recorded.

2.2. Freeze Thaw Stability

Freeze thaw studies were conducted as specified in RFP11. Six vials were entered into the study. On day 1, two vials were thawed and labeled three freeze thaws. On day 2, four vials were thawed, the two from day one and two new vials; the two new vials were labeled two freeze thaws. On day 3, all six vials were thawed and the two new vials were labeled one freeze thaw. All of the samples were analyzed for strength by OD_{260} (RFP 8) and Anion-Exchange Chromatography (QC-24), infectivity by NAS $TCID_{50}$ (RFP 9) and Hexon-FACS (QC-23), and the pH (EQU-41) and appearance of the samples was recorded.

2.3. Shipping Study

Shipping simulation was conducted as specified in RFP 11. The shipper has the following dimensions: outer dimension 23” high x 14” wide x 15” long. The walls of the shipper are 3” thick. The inner dimensions are 19” high x 7” wide x 8” long. The foam lid closure was 4”thick. Two sample vials and eight additional vials with buffer were wrapped in bubble rap and inserted into the primary container. Two thermocouples were inserted into the primary container and the container was sealed. The shipper was packed with 12.4kg of dry ice, two thermocouples were inserted into the secondary container and the container was sealed. Two additional thermocouples were attached to the outside of the secondary container. The container was exposed to 40ºC for two days and then to 50ºC for one additional day. Samples were analyzed for strength by Anion Exchange Chromatography (QC-24), appearance, pH (EQU-41), infectivity (RFP 9) and infectivity by Hexon-FACS (QC-23).

3. EQUIPMENT AND REAGENTS

3.1. General Equipment

Equipment Number	Calibration Due
00100 (Forma Scientific Incubator)	3/2002
000343 (Forma Scientific Incubator)	9/2002
000344 (Forma Scientific Incubator)	9/2002
00240 (-80ºC Revco Freezer)	11/2002
00110 (2-8ºC Imperial Refrigerator)	3/2002

3.2. OD_{260}

3.2.1. Equipment

Equipment Number	Calibration Due
GN-234 (Beckman DU 640 spectrophotometer	3/02

3.2.2. Reagents

Reagent	Manufacturer	Part #	Lot #	Expiration Date
Water, ultra-pure	Gibco/Invitrogen	10977-015	1114505	11/14/02
1M Tris-HCL pH 8.0	Gibco/Invitrogen	15568-025	3057021	11/14/02
5M NaCl	BioWhittaker	16-008Y	1M0552	11/14/02
Glycerin	Gibco/Invitrogen	15514-011	1089168	5/23/02
10% SDS	Gibco/Invitrogen	15553-035	1101066	11/14/02
Hellmanex II	Hellma Worldwide	320.001	13226	01/25/03
Methanol	Fisher	A457-4	005219	10/13/02

3.3. Anion Exchange HPLC

3.3.1. Equipment

Equipment Number	Calibration Due
AI-11 (Agilent HPLC 1100)	7/18/02
01463 (Mettler Balance, PG503)	10/02

3.3.2. Reagents

Reagent	Manufacturer	Part #	Lot #	Expiration Date
HEPES (sodium salt)	Sigma	H-3784	89H54101	10/29/03
HEPES (Acid)	Sigma	H-4034	81K5418	11/02/03
NaCl	Fisher	S271-1	016089	20 Aug 03
10 N NaOH	Fisher	SS255-1	007474-24	Dec 02
pH Buffer 7	Fisher	SB-107-500	012832-24	4/03
pH Buffer 10	Fisher	SB-141-500	012837-24	4/03
TGN Buffer	Sigma	CR1059	91K2335	9/03

3.4. Hexon-FACS

3.4.1. Equipment

Equipment Number	Calibration Due
01712 (BD Facscalibur Flow Cytometer)	03/2002

3.4.2. Immunostaining reagents

Reagent	Manufacturer	Part #	Lot #	Expiration Date
Trypsin-Versene	BioWhittaker	17-161E	012588	2/26/02
Paraformaldehyde 4%	Polyscientific	S2334	49884	8/2/02
Versene(EDTA)	BioWhittaker	12-711A	013705	2/14/02
Trypan Blue	BioWhittaker	17-942E	OM1354	8/16/02
DPBS	BioWhittaker	17-512F	012695	2/14/02
Tween 20	J.T. Baker	X 251-07	N37638	2/16/02
Mouse Anti-Adenovirus, FITC Monoclonal Antibody	CHEMICON	MAB-8052-F-K	RM 126	8/02

3.4.3. Media reagents

Medium	Reagent	Manufacturer	Part #	Lot #	Expiration Date
10% Richters	Richter's CM	BioWhittaker	04-648F	OM0607	4/02
	FBS	Hyclone	RM054	AGH6842	8/02
5% Richters	Richter's CM	BioWhittaker	04-648F	OM0607	4/02
	FBS	Hyclone	RM054	AGH6842	8/02
	IM HEPES	Gibco	15630-080	1108178	7/03

Medium	Reagent	Manufacturer	Part #	Lot #	Expiration Date
	L-Glutamine	BioWhittaker	17-905C	011571	7/02

3.5. pH

Equipment Number	Calibration Due
(Corning pH meter 440)	3/02

3.6. NAS $TCID_{50}$

3.6.1. Media reagents

Medium	Reagent	Manufacturer	Part #	Lot #	Expiration Date
DMEM High Glucose 10%FBS	DMEM	GIBCO	N/A	1114456	1/1/02
	FBS	Hyclone	RM054	AGH6842	8/02
DMEM Low Glucose 10%FBS	DMEM	GIBCO	N/A	1114456	1/1/02
	L-glutamine	BioWhittaker	17-905C	011571	7/02
	FBS	Hyclone	RM054	AGH6842	8/02

4. RESULTS

4.1. Short Term Study

Table 4.1-1 Summary of Short Term Study Results

	Condition	Test Result				
		4hr	8hr	24hr	72hr	168hr
OD_{260} (vp/ml)	RT	5.806E+11	5.902E+11	5.530E+11	5.560E+11	5.407E+11
AX-HPLC (vp/ml)		5.46E+11	5.35E+11	5.53E+11	N/A	4.47E+11
Hexon-FACS (IU/ml)		1.01E+10	ND	4.49E+09	5.94E+09	5.94E+09
pH		7.91	7.94	7.89	7.99	7.98
Appearance		Clear colorless	ND	ND	ND	Clear colorless
NAS $TCID_{50}$ (IU/ml)		2.14E+11	6.13E+10	1.38E+11	5.58E+10	*1.23E+11
OD_{260} (vp/ml)	2-8°C	5.931E+11	5.747E+11	5.568E+11	5.680E+11	5.720E+11
AX-HPLC (vp/ml)		5.23E+11	5.85E+11	5.47E+11	N/A	4.72E+11
Hexon-FACS (IU/ml)		1.04E+10	ND	5.61E+09	6.43E+09	6.95E+09
pH		7.92	7.93	7.95	8.01	7.97
Appearance		Clear colorless	ND	ND	ND	Clear colorless
NAS $TCID_{50}$ (IU/ml)		**2.26E+11	1.40E+11	1.27E+11	6.82E+10	7.75E+10

ND – Not Determined

N/A – Not Applicable
*Only assay B used due to contamination in assay A.
** Plate A, dilutions 1.13E+09 through 1.28E+10 not used due to contamination.

4.1.1. OD_{260}

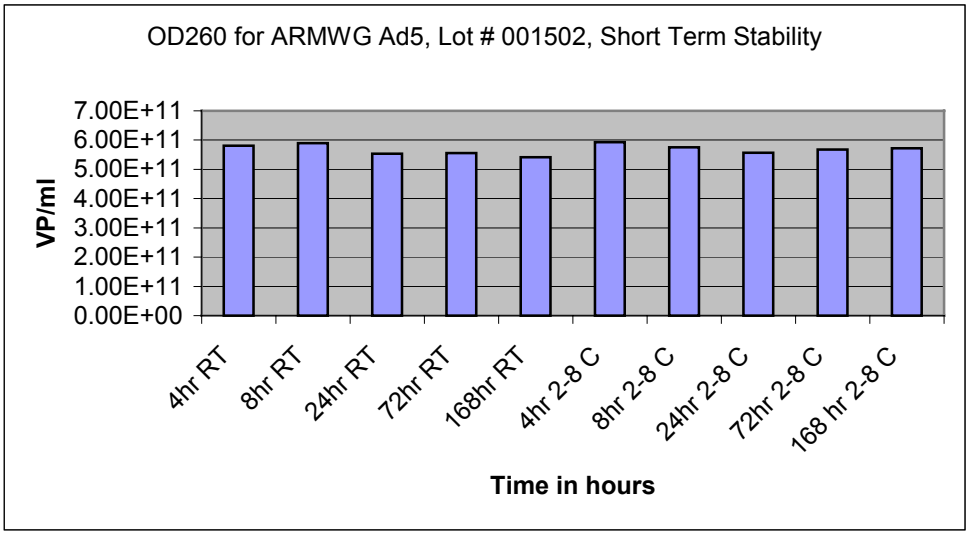
The OD_{260} determination was performed following the protocol in RFP 8.

For the samples stored at room temperature conditions, the percent difference over the range is 9% and the %CV of all of the values is 3.64%.

The results obtained for the samples stored at 2-8ºC shows percent difference over the range of values of 6.5% and a %CV of 2.30%. See summary of data evaluation below.

Condition	OD_{260} Evaluation			
	Average	SD	%CV	Range
Room Temperature	5.641E+11	2.06E+10	3.64	5.407E+11-5.902E+11
2-8ºC	5.729E+11	1.32E+10	2.30	5.568E+11-5.931E+11

Figure 4.1-1 OD_{260} versus Time



4.1.2. Anion Exchange HPLC

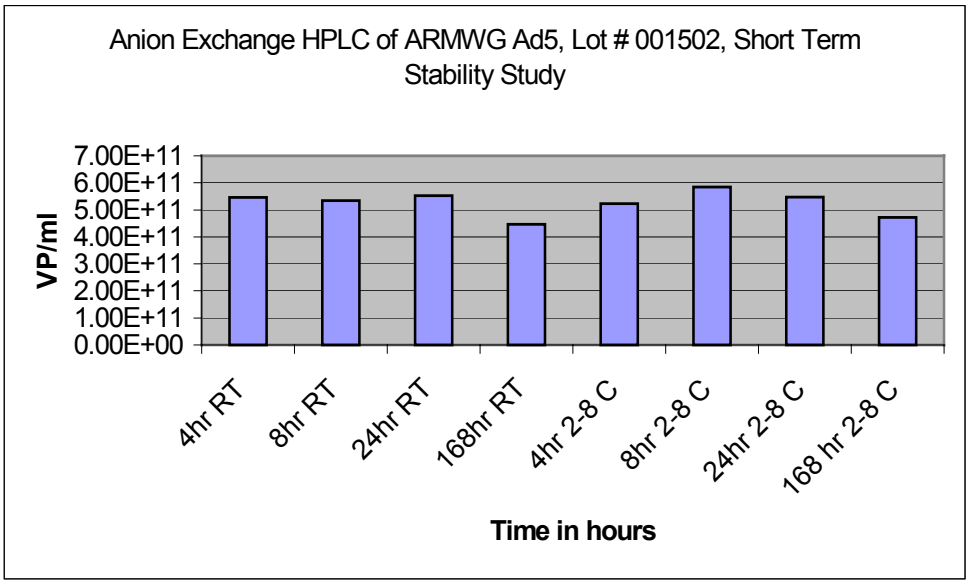
Anion exchange chromatography was performed following GTI's in-house procedure. An in-house reference standard of wild type Ad 5 (Lot No.: TCA 349) was used to create a calibration curve with five points, from 5.5E11 vp/ml to 5.5E9 vp/ml. The correlation coefficient for the curve was 1.00. Dilutions of sample were prepared in TE buffer at a dilution of 1:25. See chromatograms for details. Quantitation of the samples was performed using linear regression analysis.

The results obtained for the samples stored at room temperature indicate a 24% difference over the range of values obtained in 7 days. The %CV is 9.49%. No three day results were obtained due to mechanical failure in the HPLC equipment.

The results obtained for the samples stored at 2-8ºC show a 24% difference over the range of values obtained in 7 days. The %CV is 8.90%. No three day results were obtained due to a malfunction in the HPLC equipment.

Condition	AX-HPLC Evaluation			
	Average	SD	%CV	Range
Room Temperature	5.20E+11	4.94E+10	9.49	4.47E+11-5.53E+11
2-8ºC	5.32E+11	4.73E+10	8.90	4.72E+11-5.85E+11

Figure 4.1-2 AX-HPLC versus Time



4.1.3. Hexon FACS

The determination of infectious particles by Hexon-FACS was performed according to GTI's in-house procedure. S8 cells between passage 3 and 10 were seeded in 6-well plates. The cells used in each experiment were ≥ 80% confluent with a viability of > 90%. Three fold serial dilutions of the sample were made in the range 1E-3 to 5.65E-9. Four uninfected wells were used as negative controls. Samples were infected and

incubated for 24 hours prior to determining the infectious titer. The concentration of infectious particles was determined in the following way:

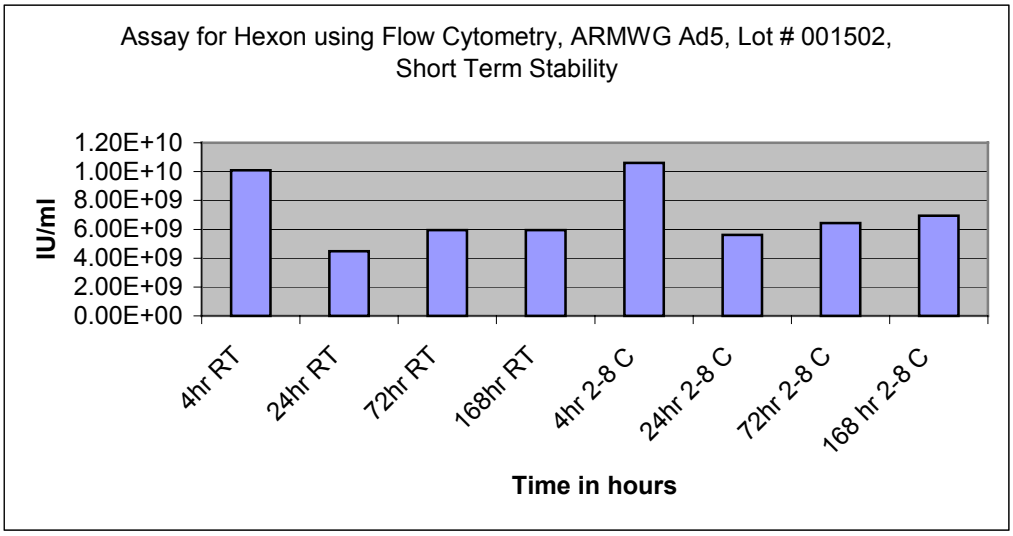
0.5 (50% infection) x 5E5 (No. of cells per well)/EC50 (from GraphPad Prism)

The results obtained for the samples stored at room temperature exhibit a 225% difference across the range. The %CV obtained for the range of values was 37%. The biggest change was between the 4 hour and 24 hour time points, and then the results stayed consistent thereafter. See summary below.

The results obtained for the samples stored at 2-8ºC show a 185% difference across the range and a %CV of 28.7%. The largest change was between the 4 hour and 24 hour time points, and then the results stayed consistent thereafter. See summary below.

Condition	Hexon-FACS Evaluation			
	Average	SD	%CV	Range
Room Temperature	6.62E+09	2.42E+09	36.6	4.49E+09-1.01E+10
2-8ºC	7.35E+09	2.11E+09	28.7	5.61E+09-1.04E+10

Figure 4.1-3 Hexon-FACS versus Time



4.1.4. pH

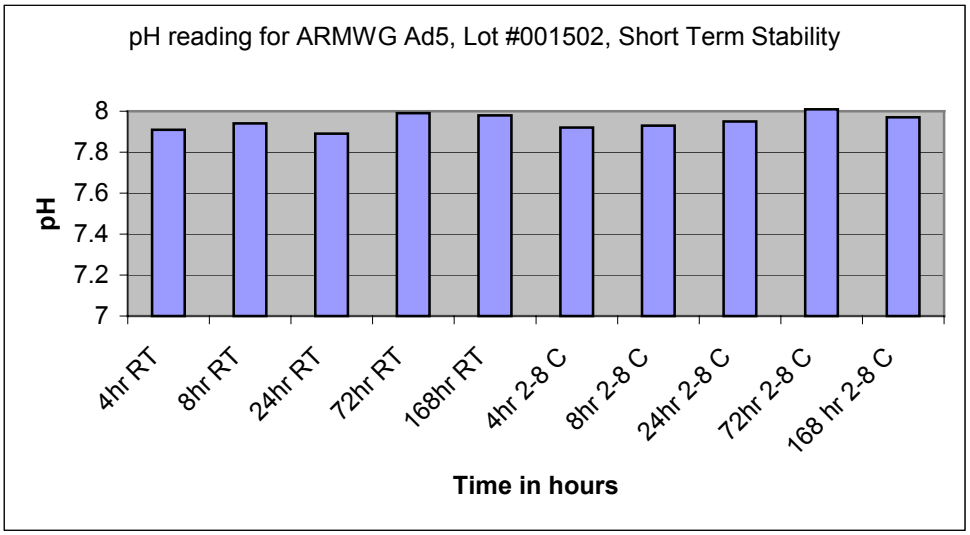
pH readings were performed using GTI's in-house procedure utilizing a Corning model pH meter with combination electrode and automatic temperature compensation. Prior to determining pH readings the electrode was calibrated using two pH buffers, 7 and 10, and

the slope was > 95% in each case. Results obtained for the samples stored at room temperature show a 1.3% difference over the range. The %CV over the range of values obtained at each time point was 0.54%.

The result for the samples stored at 2-8ºC show a difference of 1.1% across the range. The % CV obtained utilizing the data from each time point was 0.45%.

Condition	pH Evaluation			
	Average	SD	%CV	Range
Room Temperature	7.94	0.043	0.54	7.89-7.99
2-8ºC	7.96	0.036	0.45	7.92-8.01

Figure 4.1-4 pH versus Time



4.1.5. NAS $TCID_{50}$

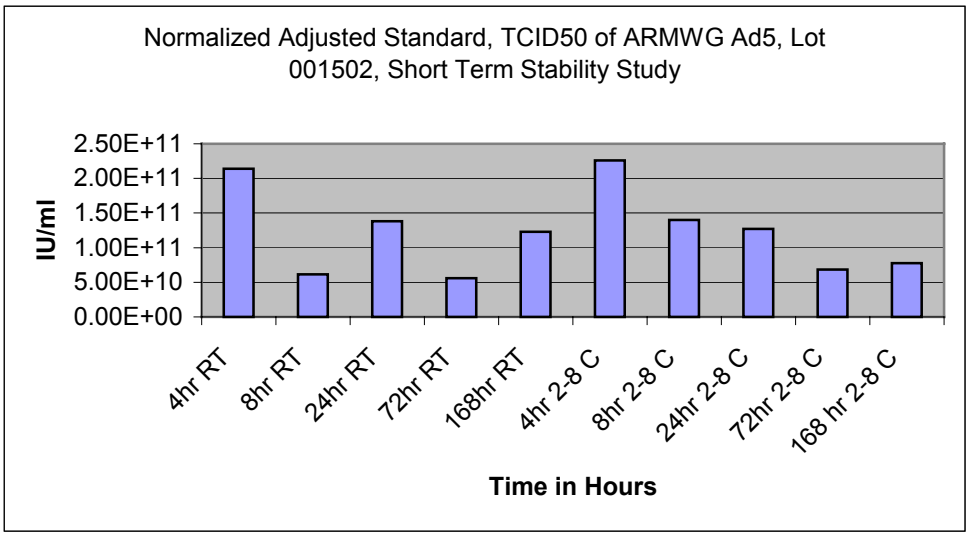
The end point infectivity assay was performed according to RFP 9. HEK 293 cells were <80% confluent during culturing and were 97% viable.

The results obtained for the samples stored at room temperature show a 384% difference across the range of values obtained. The %CV obtained for all values was 54.6%. A number of plates showed unusual trends for the dilutions of virus. As a result, a number of these unusually high values were eliminated from the calculations (see revised Excel data sheets).

The results obtained for the samples stored at 2-8ºC show a 331% difference across the range with a %CV of 49.3%. A number of data points were removed from the calculations due to inconsistent trends. These points are marked with an "X" in the No. of positive wells column of the Excel spreadsheet. The results obtained for Assay A of the day 7 sample gave inconsistent results. The second, third and fourth dilutions had 9, 3, and 3 positive wells respectively. These results are highly implausible and hence were not included in the calculations.

Condition	NAS $TCID_{50}$ Evaluation			
	Average	SD	%CV	Range
Room Temperature	1.18E+11	6.47E+10	54.6	5.58E+10-2.14E+11
2-8ºC	1.28E+11	6.30E+10	49.3	6.82E+10-2.26E+11

Figure 4.1-5 NAS $TCID_{50}$ versus Time



4.2. Freeze Thaw Study

Table 4.2-1 Summary of Freeze Thaw Study Results

Test	FT-1	FT-2	FT-3
OD_{260} (VP/ml)	5.868E+11	5.786E+11	5.775E+11
AX-HPLC (VP/ml)	6.25E+11	6.66E+11	6.24E+11
Hexon-FACS (IU/ml)	9.33E+09	8.43E+09	8.46E+09
Appearance	Clear colorless liquid	Clear colorless liquid	Clear colorless liquid
pH	7.94	7.94	7.94
NAS $TCID_{50}$ (IU/ml)	1.89E+11	5.48E+10	1.44E+11

4.2.1. Appearance

All of the vials used for the study contained a clear colorless liquid.

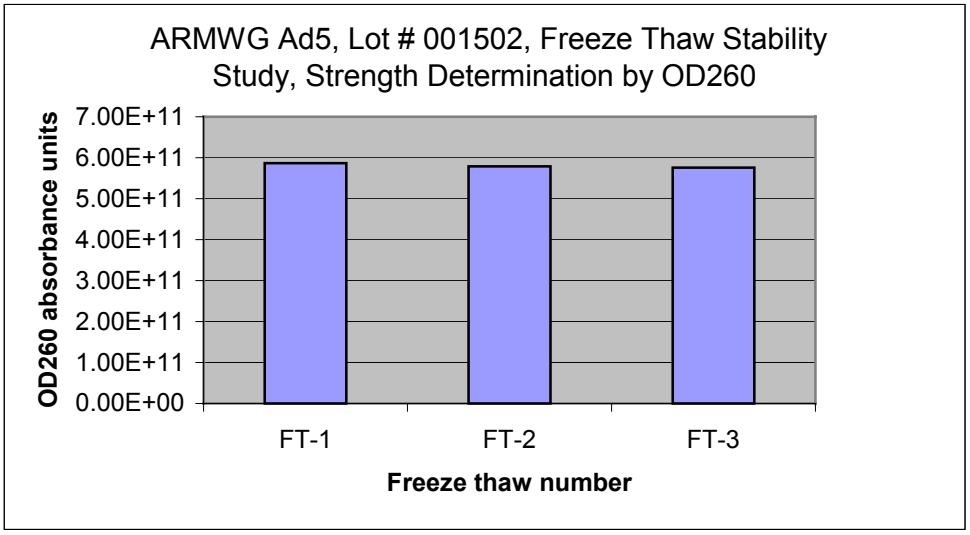
4.2.2. OD_{260}

The OD_{260} determination was performed following the protocol in RFP 8.

A 1.9% difference was obtained across the range of values and a %CV of 0.98%. See summary below.

OD_{260} Evaluation	Average	SD	%CV	Range
FT-1 to FT-3	5.81E+11	5.69E+09	0.98	5.76E+11-5.87E+11

Figure 4.2-1 OD_{260} versus Freeze Thaw



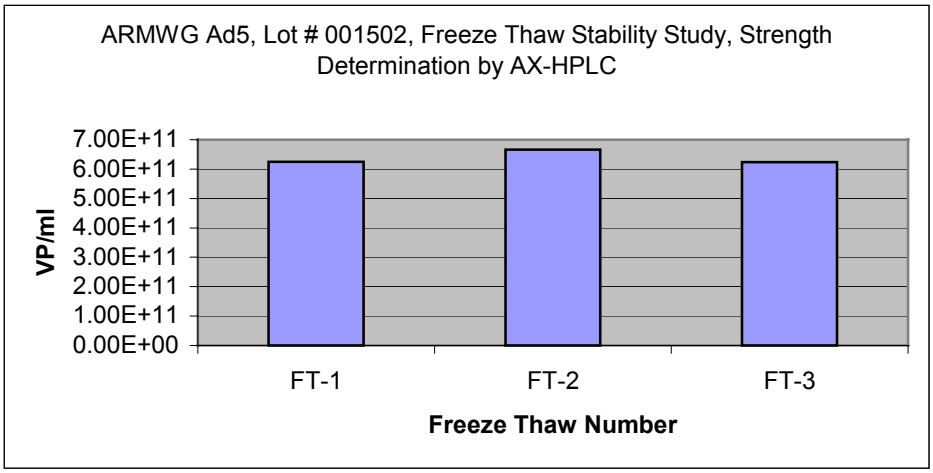
4.2.3. Anion Exchange HPLC

Anion exchange chromatography was performed following GTI's in-house procedure. An in-house reference standard of wild type Ad 5 (Lot No.: TCA 349) was used to create a calibration curve with five points, from 5.5E11 to 5.5E9. The correlation coefficient for the curve was 1.00. Dilutions of sample were prepared in TE buffer at a dilution of 1:50. See chromatograms for details. Quantitation of the samples was performed using linear regression analysis.

The percent difference across the range was 6.7% and the %CV across the range was 3.75%. See summary below.

AX-HPLC Evaluation	Average	SD	%CV	Range
FT-1 to FT-3	6.38E+11	2.40E+10	3.75	6.24E+11-6.66E+11

Figure 4.2-3 AX-HPLC versus Freeze Thaw



4.2.4. Hexon FACS

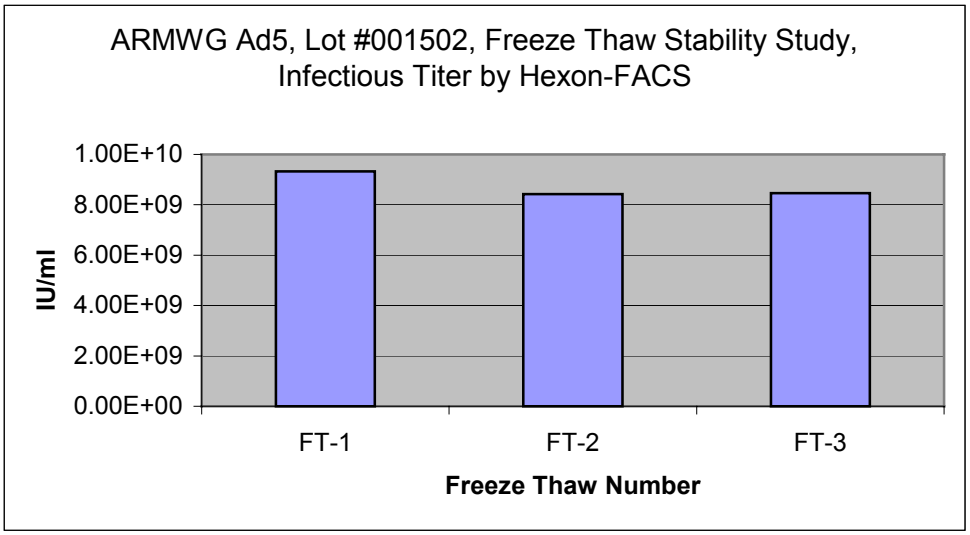
The determination of infectious particles by Hexon-FACS was performed according to GTI's in-house procedure. S8 cells within the passage range 3 through 10 were used. The cells used in each experiment were ≥80% confluent and a viability of >90% was obtained. Three fold serial dilutions of the sample were made in the range 1E-3 to 5.65E-9. Four uninfected wells were used as negatives. Samples were infected and incubated for 24 hours prior to determining the infectious titer. The concentration of infectious particles was determined in the following way:

0.5 (50% infection) x 5E5 (No. of cells per well)/EC50 (from GraphPad Prism)

The percent difference across the range was 10.7% and the %CV of values across the range was 5.85%.

Hexon-FACS Evaluation	Average	SD	%CV	Range
FT-1 to FT-3	8.74E+09	5.11E+08	5.85	8.43E+09-9.33E+09

Figure 4.2-3 Hexon-FACS versus Freeze Thaw



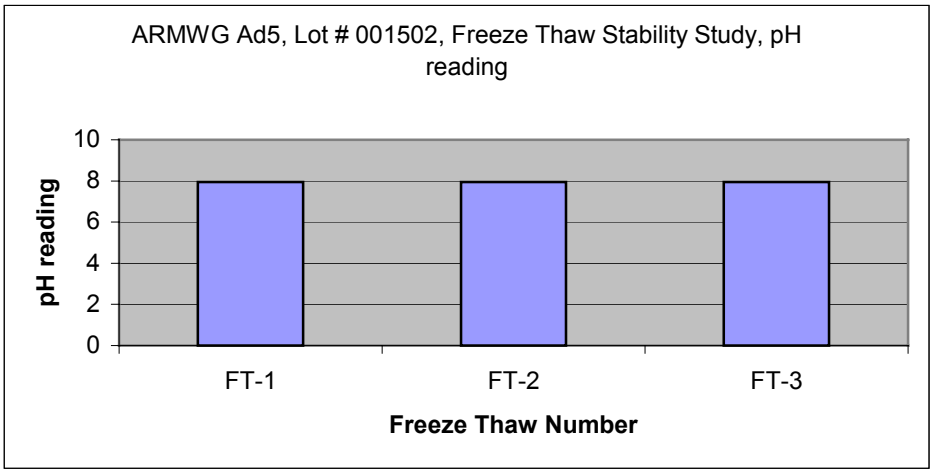
4.2.5. pH

pH was determined after the electrode was standardized using two buffers of known pH, buffer 7 and buffer 10 with a slope >95%.

pH Evaluation	Average	SD	%CV	Range
FT-1 to FT-3	7.94E+00	0.00E+00	0.00	0

The results obtained for each freeze thaw sample was identical.

Figure 4.2-4 pH versus Freeze Thaw

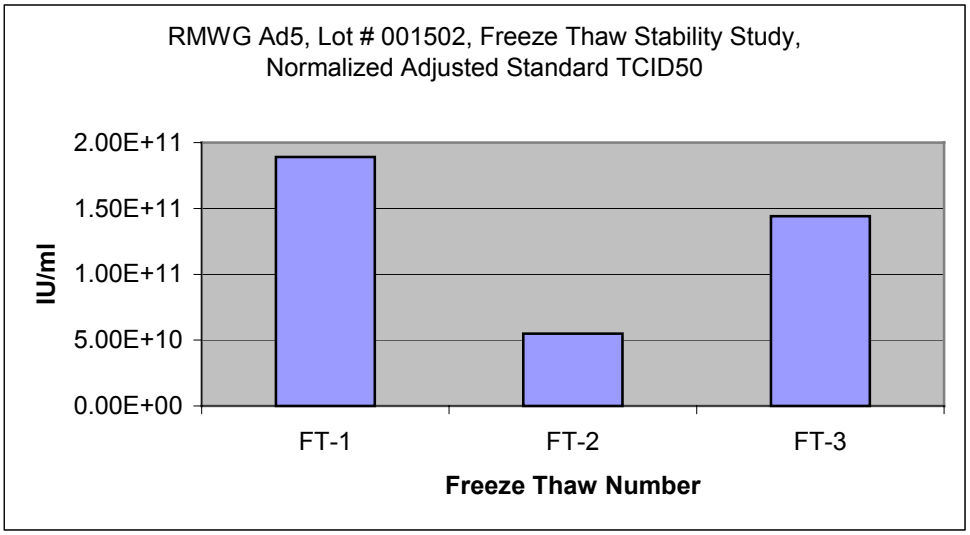


4.2.6. NAS $TCID_{50}$

The end point infectivity assay was performed according to RFP 9. HEK 293 cells were < 80% confluent during culturing and were 95% viable. The results obtained were found to be unreliable due to exceedingly high standard deviations and inconsistent particle response to dilutions. The plausible cause for the aberrant results is the seeding density of 40,000 cells per well (see Conclusions, section 5.2.5). The results reported below are for information only, data from the Hexon-FACS assay should be used for the freeze thaw study.

NAS $TCID_{50}$ Evaluation	Average	SD	%CV	Range
FT-1 to FT-3	1.29E+11	6.83E+10	52.8	5.48E+10-1.44E+11

Figure 4.2-5 NAS $TCID_{50}$ versus Freeze Thaw



4.3. Shipping Study

The shipping study was conducted as specified in RFP11. The entire shipping study was repeated due to the aberrant result obtained for the infectivity assay by NAS $TCID_{50}$. The Hexon-FACS assay was also added as one of the evaluation criterion. The results obtained are shown below. The results agree very favorably with the results obtained for the 4 hour sample at 2-8ºC.

Test	Shipping # 1	Shipping # 2 repeat
OD_{260} (vp/ml)	ND	ND
AX-HPLC (vp/ml)	6.70E+11	**6.56E+11
Hexon-FACS (IU/ml)	N/A	9.02E+09
Appearance	Clear colorless liquid	Clear colorless liquid
pH	7.96	8.00
NAS $TCID_{50}$ (IU/ml)	*8.45E+10	1.10E+11

ND-Not Determined

* Contamination observed for assay B, dilution 2E+08.

** A four point calibration curve was used due to the absence of a quantifiable peak at the lowest standard dilution. The sample concentration was within the calibration range.

5. CONCLUSIONS

5.1. Short Term Study

5.1.1. OD_{260}

The results obtained for strength by OD_{260} for both the room temperature and 2-8ºC storage conditions over a seven day period showed negligible change. The %CV's of 3.63% and 2.29% are within the inter-assay variability for the method.

5.1.2. Anion Exchange HPLC

The difference in strength observed between the 4 hour and 168 hr samples stored at room temperature is 22% and the %CV for all of the data points is 9.49%. The chromatograms from the 168 hr samples also exhibit an extra peak at approximately 2.8 minutes.

The results obtained for the strength of the product stored at 2-8ºC over 7 days shows a decrease of 10.9% between the 4 hour sample and the 168 hour (7 day) sample. The %CV obtained with all of the time points was 8.9%. The 168 hr samples show an extraneous peak at approximately 2.8 minutes.

5.1.3. Hexon-FACS

The largest observable change in strength over time as determined by hexon-FACS was between the 4 hour samples and the 24 hour samples for both storage conditions. No significant change was observed from the 24 hour samples through the 168 hour samples. For the time points from 24 hours through 168 hours, the %CV for samples stored at 2-8ºC was 10.7% and for samples at room temperature 15.3%.

5.1.4. pH

The results obtained for pH at all time points for both temperature conditions indicate negligible change.

5.1.5. NAS $TCID_{50}$

A 53.8% decline in biological activity was observed at room temperature between the 4 hours and 168 hours samples. An 85.62% decline in biological activity was observed at 2-8ºC between 4 hours and 168 hours. See also section 5.2.5.

5.2. Freeze Thaw Study

5.2.1. OD_{260}

No significant change was observed from the data generated from freeze thaw sample one through sample three. There is a 1.4% decrease in strength between freeze thaw one and two and a 0.5% decrease in strength between freeze thaw two and three. The net change is 1.9% from freeze thaw one through three. The %CV obtained for all of the freeze thaw results was 0.98%.

5.2.2. Anion Exchange HPLC

No significant change was observed for the three freeze thaw samples analyzed by HPLC. The %CV obtained utilizing all three values was 3.75%.

5.2.3. Hexon-FACS

A 9.6% drop in biological activity was noticed between freeze thaw one and freeze thaw two and a 0.35% increase between freeze thaw two and three. The %CV obtained with all of the values was 5.85%.

5.2.4. pH

Identical results were obtained for each freeze thaw sample.

5.2.5. NAS $TCID_{50}$

The results obtained for the infectivity assay by NAS $TCID_{50}$ are un-interpretable. Immediately prior to the initiation of the study, the ARMWG changed the required seeding density from 10,000 cells/well to 40,000 cells/well. The analysis of the freeze thaw samples was performed using 40,000 cells/well. Significant variability in the results was obtained with 40,000 cells/well. Separate results from our laboratory indicates that 10,000 cells/well yields results with much higher precision.

5.3. Shipping Study

A comparison of the results of the shipping study to the results obtained for freeze thaw number one, and the 4 hour results at 2-8ºC is shown below.

Test	Study			Average	SD	%CV
	Shipping	4hr, 2-8ºC	Freeze Thaw # 1			
OD_{260} (vp/ml)	ND	5.806E+11	5.868E+11	5.837E+11	4.38E+09	7.51E-01
AX-HPLC (vp/ml)	6.56E+11	5.23E+11	6.25E+11	6.01E+11	6.96E+10	1.16E+01
Hexon-FACS (IU/ml)	9.02E+09	1.04E+10	9.33E+09	9.69E+09	8.18E+08	8.45E+00
pH	8.00	7.92	7.94	7.95E+00	4.16E-02	5.23E-01
NAS $TCID_{50}$	1.10E+11	2.26E+11	N/A	1.68E+11	8.20E+10	4.88E+01

ND-Not Determined

N/A-Not Applicable