GLP REPORT

TEST FACILITY:

NAMSA
6750 Wales Road
Northwood, OH 43619

SPONSOR:

Paul Tiege
ViRexx Medical Corporation
8223 Roper Road NW
Edmonton, Alberta, T6E 6S4
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CONFIDENTIAL

STUDY TITLE:

ASTM Hemolysis

TEST ARTICLE:

Occlusin™ 505 Artificial Embolization Device

IDENTIFICATION NO.:

Batch: FL288

TABLE OF CONTENTS Page

Summary 3

Statement of GLP Compliance 4

1. Introduction 5
2. Materials 5
3. Test System 6
4. Animal Management 6
5. Preparation of Standards and Controls 7
6. Methods 8
7. Evaluation and Statistical Analysis 9
8. Results 9
9. Conclusion 10
10. Quality Assurance 10
11. Proposed Dates 10
12. Records 10
13. References 10
14. Protocol Changes 10

Statement of Quality Assurance Activities 11

Summary

The test article, Occlusin™ 505 Artificial Embolization Device, Batch: FL288, was evaluated based on ASTM F756-00, Standard Practice for Assessment of Hemolytic Properties of Materials and also per the requirements of ISO 10993-4:2002, Biological Evaluation of Medical Devices - Part 4: Selection of Tests for Interactions With Blood. Blood was obtained from three rabbits, pooled, and diluted for use in this study. Diluted rabbit blood was added to triplicate tubes of the test article in calcium and magnesium-free phosphate buffered saline (CMF-PBS) and triplicate tubes of the CMF-PBS test article extract. These combinations were evaluated to determine whether direct contact with the test article or an extract of the test article would cause *in vitro* red blood cell hemolysis. Negative and positive controls were prepared in the same manner as the test article. Each tube was inverted gently to uniformly mix the contents with the blood. The tubes were then maintained for 3 hours at 37°C with periodic inversions. Following incubation, suspensions were mixed gently and centrifuged. The resulting supernatant was added to hemoglobin reagent. The absorbances of the solutions were spectrophotometrically measured at a wavelength of 540 nm.

Under the conditions of this study, the mean hemolytic index for the test article in CMF-PBS was 1%, and the mean hemolytic index for the CMF-PBS test article extract was 0%. The direct contact of the test article was nonhemolytic and the test article extract was nonhemolytic. The negative and positive controls performed as anticipated.

Study and Supervisory Personnel:

Shelly L. Nielsen, B.S.
Heatherbea L. Weirich, B.S.
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Heather A. Huseman, B.S.

Approved by:

Michelle E. Longstreet, B.S.
Study Director

7-17-07
Date Completed

Statement of GLP Compliance

This study was conducted in accordance with the provisions of the FDA Good Laboratory Practice (GLP) Regulations (21 CFR, Part 58).

There were no deviations from the protocol, standard operating procedures or the GLP Regulations which were judged to have had any significant impact on the validity or interpretation of the data.

All laboratory data has been accurately recorded and verified, as indicated by the signature below.

Study Director: Michelle E. Longstreet

Michelle E. Longstreet, B.S.

7-17-07

Date

1. Introduction

Purpose
The test article identified below was evaluated to determine whether the test article would cause hemolysis *in vitro* by direct contact or extraction per ASTM F756-00, Standard Practice for Assessment of Hemolytic Properties of Materials and also per the requirements of ISO 10993-4:2002, Biological Evaluation of Medical Devices - Part 4: Selection of Tests for Interactions With Blood. Hemolysis testing of medical device materials has been used historically to measure blood compatibility.

Dates
The test article was received on June 7, 2007. The test was performed on July 2, 2007.

GLP Compliance
The study initiated by protocol signature on June 11, 2007, was conducted in accordance with the provisions of the FDA Good Laboratory Practice (GLP) Regulations, 21 CFR 58. A Statement of Quality Assurance Activities was issued with this report.

2. Materials

The test article provided by the sponsor was identified and handled as follows:

Test Article:	Occlusin™ 505 Artificial Embolization Device
Identification No.:	Batch: FL288
Stability Testing:	In progress (per sponsor)
Expiration Date:	Stable for duration of intended testing (per sponsor)
Strength, Purity and Composition:	The sponsor elects not to provide this information to NAMSA and takes full responsibility for this data and can supply this information if requested to do so.
Physical Description of Test Article:	Glass vials containing white beads
Storage Conditions:	Refrigerated. The sample was stored at room temperature for one day prior to pour, which was acceptable per sponsor.
Vehicle:	Calcium and magnesium-free phosphate buffered saline (CMF-PBS)
Test Article Preparation:	The test article was prepared based on the sponsor supplied surface area of 44 cm^2 per sample. One sample was included in each preparation. Based on the USP ratio of 120 cm^2:20 ml, triplicate 44.0 cm^2 portions (one vial) of the test article were covered with 7.3 ml of CMF-PBS to be tested as direct contact. A 7.3 ml portion of the extract was added to the original container in order to remove the test article from the original container. The test article and vehicle were then transferred to a sterile vial for testing. For the extraction method, two samples were included in each of the three preparations. The test article was prepared based on the sponsor supplied surface area of 44 cm^2 per sample. 88.0 cm^2 portions of the test article were covered with 15 ml of CMF-PBS for each preparation. 7.5 ml were added to each vial and the test article and extract vehicle were combined and transferred to one sterile vial These preparations were extracted with agitation at 37°C for 72 hours.
Negative Control Preparation:	High density polyethylene (HDPE) was used as the negative control. Based on the USP ratio of 60 cm^2:20 ml, triplicate 21.0 cm^2 portions of HDPE were prepared and covered with 7.0 ml of CMF-PBS to be tested as direct contact.

Based on the USP ratio of 60 cm^2:20 ml, triplicate 30.8 cm^2 portions of HDPE were prepared and covered with 10 ml of CMF-PBS. These preparations were subjected to the extraction conditions previously described for the test article.

Positive Control Preparation: Sterile Water for Injection (SWFI) was used for the positive control

Triplicate 7.0 ml portions of SWFI were prepared to be tested as direct contact.

Triplicate 10 ml portions of SWFI were prepared and subjected to the extraction conditions previously described for the test article.

Blank Preparation: CMF-PBS was used as the blank.

Triplicate 7.0 ml portions of CMF-PBS were prepared to be tested direct contact.

Triplicate 10 ml portions of CMF-PBS were subjected to the extraction conditions previously described for the test article

Condition of Extracts:
Test: clear
Negative Control: clear
Positive Control: clear
Blank: clear

3. Test System

Test System

Species:	Rabbit (*Oryctolagus cuniculus*)
Breed:	New Zealand White
Source:	Myrtle's Rabbitry Inc.
Sex:	Male
Body Weight Range:	2.6 kg to 3.0 kg
Estimated Date of Birth:	March 19, 2007 and April 2, 2007
Acclimation Period:	Minimum 5 days
Number of Animals:	Three
Identification Method:	Ear tag

Justification of Test System
Hemolysis testing of medical device materials has historically been used to measure blood compatibility *in vitro*. Whole blood samples for use in this test were collected from the rabbits into EDTA vacuum tubes.

4. Animal Management

Husbandry:	Conditions conformed to Standard Operating Procedures that are based on the "Guide for the Care and Use of Laboratory Animals."
Food:	A commercially available rabbit feed was provided daily.
Water:	Potable water was provided *ad libitum* through species appropriate water containers or delivered through an automatic watering system.
Contaminants:	Reasonably expected contaminants in feed or water supplies did not have the potential to influence the outcome of this test.
Housing:	Animals were individually housed in stainless steel suspended cages identified by a card indicating the animal number, sex, and blood draw date.
Environment:	The room temperature was monitored daily. The temperature range for the room was within a range of 61-72°F.
	The room humidity was monitored daily. The humidity range for the room was 30-70%.

	The light cycle was controlled using an automatic timer (12 hours light, 12 hours dark).
Accreditation:	NAMSA is an AAALAC International accredited facility and is registered with the United States Department of Agriculture. Additionally, NAMSA maintains an approved Animal Welfare Assurance on file with the National Institutes of Health, Office for Laboratory Animal Welfare.
Personnel:	Associates involved were appropriately qualified and trained.
Selection:	Only healthy animals were selected. To reduce the number of animals used for testing, and to comply with the directives of the NAMSA IACUC, the rabbits (54759, 54832, 54736) used as blood donors for this study were used previously in an unrelated, test model. Any previously evaluated test or control articles did not cause a response in the animals. Complete history of animal usage is traceable in laboratory records.
Sedation, Analgesia or Anesthesia:	Sedation, analgesia or anesthesia was necessary during the routine course of this procedure.
Veterinary Care:	In the unlikely event that an animal became injured, ill, or moribund, care was conducted in accordance with current veterinary medical practice. If warranted for humane reasons, euthanasia was conducted in accordance with the current report of the American Veterinary Medical Association's Panel on Euthanasia. The objective of the study will be given due consideration in any decision and the study sponsor will be advised.
IACUC:	This procedure has been approved by NAMSA Institutional Animal Care and Use Committees (IACUC), and is reviewed at least annually by the same committees. Any significant changes to this procedure were approved by the IACUC prior to conduct.

5. Preparation of Standards and Controls

Dilution Factors for Calculations

Drabkin's reagent was used as the hemoglobin reagent throughout the study. Throughout the course of the study, several dilutions of the whole blood or the blood plasma were conducted. To account for these in the calculations, the following dilution factors (DF) were used:

Plasma Hemoglobin Determination:

750 µl of plasma added to 750 µl of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume plasma}} = \frac{1500\ \mu\text{l solution}}{750\ \mu\text{l plasma}} = 2$$

Whole Blood Hemoglobin Determination:

20 µl of whole blood added to 5 ml of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume blood}} = \frac{5.02\ \text{ml solution}}{0.02\ \text{ml blood}} = 251$$

Diluted Blood Hemoglobin Determination:

400 µl diluted blood added to 5 ml of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume diluted blood}} = \frac{5.4\ \text{ml solution}}{0.4\ \text{ml diluted blood}} = 13.5$$

Sample Hemoglobin Determination:

1.0 ml of Supernatant added to 1.0 ml of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume supernatant}} = \frac{2.0\ \text{ml solution}}{1.0\ \text{ml supernatant}} = 2$$

Total Hemoglobin Concentration in each tube:

1.0 ml of diluted blood added to 7.0 ml CMF-PBS

$$DF = \frac{\text{Final volume blood/PBS - CMF}}{\text{Volume diluted blood}} = \frac{8.0\ \text{ml}}{1.0\ \text{ml}} = 8$$

Standards Preparation
The Human Hemoglobin Standard was dissolved in hemoglobin reagent. The reconstituted standard was tested at the following concentrations: 1.44, 0.800, 0.600, 0.300, 0.150, 0.0750, 0.0375, and 0.0188 mg/ml. The absorbances of the concentrations were read against a hemoglobin reagent blank in a spectrophotometer set at a wavelength of 540 nm. Using the information obtained from the absorbance readings and concentrations, a standard curve was generated.

Plasma Hemoglobin Determination
A 3 ml aliquot of the anticoagulated pooled rabbit blood was centrifuged at 700-800 Xg for 15 minutes. A 750 µl portion of the plasma (supernatant) was added to 750 µl of hemoglobin reagent. The solution was allowed to stand for 15 minutes at room temperature and the absorbance was read at 540 nm. The plasma hemoglobin concentration of the blood sample was calculated from the prepared standard curve. If the plasma hemoglobin was greater than 2 mg/ml, the blood was not used for the study.

Blood Hemoglobin Determination
Duplicate 20 µl portions of well-mixed, pooled whole blood (plasma hemoglobin ≤ 2 mg/ml) were added to 5.0 ml aliquots of hemoglobin reagent. These solutions were allowed to stand for 15 minutes at room temperature and then the absorbances were read at 540 nm. The whole blood hemoglobin concentration was calculated from the prepared standard curve.

The hemoglobin concentration of the pooled blood sample was adjusted to 10 ± 1 mg/ml by diluting with an appropriate amount of CMF-PBS. The hemoglobin concentration was confirmed by taking 400 µl of the well-mixed, diluted blood and adding it to 5.0 ml of hemoglobin reagent in triplicate. The solutions were allowed to stand at room temperature for 15 minutes and the absorbances were read at 540 nm. The diluted blood hemoglobin concentration of the sample was calculated from the prepared standard curve.

6. Methods

Clot-free blood samples were collected from each rabbit (numbers 54759, 54832, 54736) into EDTA vacuum tubes on the same day as the test was performed. The blood collected from each rabbit was pooled into a borosilicate screw cap tube and mixed gently to prevent mechanical hemolysis.

The pooled rabbit blood was diluted with CMF-PBS to a total hemoglobin concentration of 10 ± 1 mg/ml. Based on a ratio of 1.0 ml diluted blood to 7.0 ml vehicle, the following tubes were prepared:

Direct Contact
1.0 ml of diluted blood and the test article in 7.3 ml CMF-PBS.
1.0 ml of diluted blood and the negative control in 7.0 ml CMF-PBS
1.0 ml of diluted blood and 7.0 ml of SWFI as the positive control
1.0 ml of diluted blood and 7.0 ml CMF-PBS (blank)
Extraction
1.0 ml of diluted blood and 7.0 ml of a test article CMF-PBS extract
1.0 ml of diluted blood and 7.0 ml of a negative control CMF-PBS extract
1.0 ml of diluted blood and 7.0 ml of a positive control SWFI extract
1.0 ml of diluted blood and 7.0 ml of a blank CMF-PBS extract.

The tubes were capped, inverted gently to mix the contents, and then maintained for 3 hours at 37°C with periodic inversions. Following incubation, the blood-CMF-PBS mixtures were transferred to separate disposable centrifuge tubes. These tubes were centrifuged for 15 minutes at 700-800Xg. A 1.0 ml aliquot of each test article, negative control, positive control, and blank supernatant was added to individual 1.0 ml portions of Drabkin's reagent and allowed to stand for 15 minutes at room temperature. The absorbance of each test article, negative control, positive control, and blank solution was measured at 540 nm. The hemoglobin concentration of each test article, negative control, positive control and blank solution was then calculated from the standard curve. The blank corrected percent hemolysis was calculated for each test article and the negative and positive controls as follows:

$$\text{Blank Corrected \% Hemolysis} = \frac{\text{ABS (Sample)} - \text{ABS (Blank)}}{(0.844)\,\text{ABS (Diluted Blood)} - \text{ABS (Blank)}} \times 100$$

ABS = Absorbance

7. Evaluation and Statistical Analysis

The mean blank corrected % hemolysis was calculated by averaging the blank corrected % hemolysis values determined for each of the triplicate test samples. This value is reported to the nearest 1%. The standard deviation for the replicates was also determined.

An average hemolytic index of the triplicate test samples was also calculated compared to the negative control. A hemolytic index of 2% or less was considered to be nonhemolytic. A hemolytic grade was assigned based on the following scoring scheme:

Hemolytic Index	Hemolytic Grade
0 - 2%	Nonhemolytic
2 - 5%	Slightly Hemolytic
> 5%	Hemolytic

For the suitability of the system to be confirmed, the negative control must have had a blank corrected % hemolysis value < 2% and the positive control must have had a blank corrected % hemolysis value of ≥ 8%. If either of these values were not within the acceptable range, the test was repeated with fresh rabbit blood.

8. Results

The values obtained in this study are summarized below:

TEST AND CONTROL DIRECT CONTACT SAMPLES

Sample	ABS 1*	ABS 2*	ABS 3*	Mean Blank Corrected % Hemolysis	Standard Deviation	Mean Concentration (mg/ml)	Hemolytic Index†
Test Article	0.016	0.015	0.016	1.7	0.1	0.05	1
Negative Control	0.011	0.014	0.013	1.1	0.3	0.04	
Positive Control	0.414	0.436	0.435	94.9	2.8	1.31	
Blank	0.007	0.009	0.008	1.8	0.2		

TEST AND CONTROL EXTRACT SAMPLES

Sample	ABS 1*	ABS 2*	ABS 3*	Mean Blank Corrected % Hemolysis	Standard Deviation	Mean Concentration (mg/ml)	Hemolytic Index†
Test Article	0.005	0.005	0.005	0	0.0	0.02	0
Negative Control	0.005	0.005	0.007	0.1	0.3	0.02	
Positive Control	0.440	0.443	0.443	98.0	0.4	1.36	
Blank	0.004	0.005	0.006	1.1	0.2		

= Not Applicable

* Absorbance readings (ABS) for three replicates

†Hemolytic Index calculated as follows:

Test article mean blank corrected % hemolysis – Negative control mean blank corrected % hemolysis

9. Conclusion

Under the conditions of this study, the mean hemolytic index for the test article in CMF-PBS was 1%, and the mean hemolytic index for the CMF-PBS test article extract was 0%. The test article in direct contact was nonhemolytic and the test article extract was nonhemolytic. The negative and positive controls performed as anticipated.

Results and conclusions apply only to the test article tested. Any extrapolation of these data to other samples is the sponsor's responsibility. All procedures were conducted in conformance with good manufacturing practices and ISO 13485:2003.

10. Quality Assurance

Inspections were conducted at intervals adequate to assure the integrity of the study in conformance with 21 CFR 58.35(b)(3). The final report was reviewed for conformance to Section 58.185, Subpart J, of the GLP Regulations. A Statement of Quality Assurance Activities is provided with this final report.

11. Proposed Dates

The study dates were finalized by the study director following receipt of the sponsor approved protocol and appropriate material for the study. Initiation of the study was the date on which the study director signed the GLP protocol. Projected dates for starting the study (first treatment) and for the completion of the study (final report release) were provided to the sponsor (or representative of the sponsor).

12. Records

All raw data pertaining to this study and a copy of the final report are to be retained in designated NAMSA archive files.

13. References

21 CFR 58 (GLP Regulations).

ASTM F756-00, Standard Practice for Assessment of Hemolytic Properties of Materials.

ISO 10993-4 (2002) Biological evaluation of medical devices - Part 4: Selection of tests for interactions with blood.

Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, National Academy of Sciences (Washington: National Academy Press, 1996).

OLAW, Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH Publication).

United States Code of Federal Regulation (CFR) 9: The Animal Welfare Act.

14. Protocol Changes

Any necessary changes to the protocol after sponsor approval or study initiation were documented and approved by the study director as protocol amendments. Copies were distributed to the sponsor, the raw data file, and the NAMSA Quality Assurance department.

Statement of Quality Assurance Activities

Phase Inspected	Auditor	Date
Test	R. J. Spino	July 2, 2007
Final Report Review	J. A. McClure	July 17, 2007

Reports to Management and Study Director(s)	Date
Periodic Status Report	July 10, 2007

This study will be included in the next periodic status report as completed.

Based on a review of this study, it has been concluded that this report accurately describes the methods and standard operating procedures, and that the reported results accurately reflect the raw data of the study. This study has been reviewed in accordance with the provisions of the FDA Good Laboratory Practice Regulations (21 CFR, Part 58).

QA Representative: Julie A McClure 7-17-07

Julie A. McClure, B.S.
Quality Assurance Auditor

Date

STORE IN REFRIGERATOR (+4°C)
CALIBRATION #: 7420
TECH/DATE: DMW 6-7-07

GLP SAI FORM

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25447_001 25447

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*Annotates a required field

SPONSOR FINAL REPORT WILL BE ADDRESSED AND MAILED TO

ViRexx Medical Corp — ~~Don Pohl~~ Paul Tiege MEL 6-11-07
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CITY* — **STATE*** — **ZIP***
Canada
COUNTRY*
780-433-4411
PHONE*
780-436-0068
FAX*
ptiege@virexx .com
E-MAIL*

INVOICE INFORMATION
Same, Attn: Erin Horowitz
BILLING ADDRESS (include Company Name if different from mailed to)*

V0725-186PT
PURCHASE ORDER NUMBER*
T07 2708
COST ESTIMATE AND PROPOSAL NUMBER
☐ VISA ☐ MasterCard ☐ American Exp.
CARD HOLDER NAME
CREDIT CARD NUMBER — **EXPIRATION DATE**
ACCOUNTS PAYABLE PHONE* — **ACCOUNTS PAYABLE FAX***

Occlusin™ 505 Artificial Embolization Device
TEST ARTICLE NAME *USE EXACT WORDING DESIRED ON FINAL REPORT* * +*

Embolotherapy
INTENDED CLINICAL USE OF TEST ARTICLE:*

X BATCH ☐ CODE ☐ LOT — FL288
CHECK ONE — **IDENTIFICATION NUMBER***

CONTROL ARTICLE NAME*

☐ BATCH ☐ CODE ☐ LOT
CHECK ONE — **IDENTIFICATION NUMBER***
NAMSA recommends only one lot, batch, or code per test article submission.

QUANTITY SUBMITTED:* 26 Vials Occlusin™ 505 Artificial Embolization Device Batch FL288
(please specify quantities for each lot/batch/code provided)
Glass vials containing white beads
PHYSICAL DESCRIPTION OF TEST ARTICLE (Chemical/Material type/Color)*

TEST ARTICLE IS CATEGORIZED AS BEING A (check all that apply):* +
X MEDICAL DEVICE ☐ BIOLOGIC ☐ TISSUE
☐ PHARMACEUTICAL ☐ CHEMICAL ☐ OTHER

+ A detailed composition list and current MSDS sheet must accompany any chemical or biologic test article. A certificate of testing or reprocessing must be submitted for any human tissue derived sample or clinically used medical device

TEST ARTICLE BEING SUBMITTED IS:*
X STERILIZED ☐ NOT STERILIZED
☐ **NAMSA TO STERILIZE BY:** ☐ EO (additional charge) ☐ STEAM

Mixtures of test or control articles with carriers require analysis to demonstrate proper concentration, homogeneity, and stability.*
☐ Sponsor will provide analytical methods; or
☐ Sponsor will perform analysis on representative aliquots provided by NAMSA.

STORAGE CONDITIONS*
☐ ROOM TEMPERATURE X REFRIGERATION ☐ FREEZER
☐ OTHER:

TEST AND CONTROL ARTICLE CHARACTERIZATION: The sponsor assures the above test article has been characterized for identity, strength, purity, and composition as required by FDA Good Laboratory Practice Regulations of 21 CFR Part 58.105. Stability testing is the responsibility of the sponsor and is subject to FDA audit. Characterization and stability information are also required for control articles. Please check the statement(s) applicable to the test and control articles for both Stability and Strength, Purity and Composition sections below.

Test Article	Control Article	Stability (Choose One)
X	☐	Stability testing is in progress; article is stable for duration of intended testing.
☐	☐	Stability testing is complete and on file with sponsor. Expiration date (test): Expiration date (control):
☐	☐	Marketed product stability characterized by its labeling.

Test Article	Control Article	Strength, Purity, and Composition (Choose One)
☐	☐	Sponsor provided data in a Certificate of Analysis or other appropriate documentation and results will be reflected in the final report.
X	☐	Sponsor elects not to provide this information to NAMSA and takes full responsibility for this data and can supply this information if requested to do so.

If requesting to return sample, please check the courier and include your:
☐ UPS ☐ Federal Express ☐ Other: Account Number:

T060707_002
FEDEX VIREXX
DMW 6-7-07

AUTHORIZED BY SPONSOR: Michelle C. Longstaff — DATE: 06 JUN 07 6-11-07

GLP PROTOCOL

TEST FACILITY:

NAMSA
6750 Wales Road
Northwood, OH 43619

SPONSOR:

Paul Tiege
ViRexx Medical Corporation
8223 Roper Road NW
Edmonton, Alberta,
Canada

STUDY TITLE:

ASTM Hemolysis

TABLE OF CONTENTS

		Page
Approvals		3
1.	Introduction	4
2.	Materials	4
3.	Test System	5
4.	Animal Management	5
5.	Preparation of Standards and Controls	6
6.	Method	7
7.	Evaluation and Statistical Analysis	7
8.	Report	8
9.	Quality Assurance	8
10.	Proposed Dates	8
11.	Records	8
12.	References	8
13.	Protocol Changes	8

Approvals

Sponsor Representative (Sponsor): _____

Date Approved: 30 MAY 07

Study Director (NAMSA): Michelle E. Longstreet

Michelle E. Longstreet, B.S.
Study Director

Date Initiated: 6-11-07

1. Introduction

Purpose
This procedure describes the method used to determine whether an extract of a biomaterial or a medical device causes hemolysis (lysis of red blood cells) per ASTM F756-00, Standard Practice for Assessment of Hemolytic Properties of Materials. This study will be based on the requirements of the International Organization for Standardization: Biological evaluation of medical devices, Part 4: Selection of Tests for Interactions with Blood.

GLP Compliance
Good Laboratory Practice – This nonclinical laboratory study will be conducted in accordance with the United States Food and Drug Administration Good Laboratory Practice Regulations, 21 CFR Part 58.

2. Materials

Test Article
The sponsor will submit the test article to be evaluated. Detailed information about the test article will be provided by the sponsor on the NAMSA Sample Submission Form or on a similar attachment to the protocol.

Preparation
The following is to be completed by the sponsor or study director. Further instructions may be attached to the protocol. The sample will be prepared as follows:

① **Ratio of test article to extraction vehicle (select one):**

- ✓ Material thickness less than 0.5 mm - ratio of 60 cm^2:10 ml (based on the USP ratio 120 cm^2:20 ml)
- ____ Material thickness greater than or equal to 0.5 mm - ratio of 30 cm^2:10 ml (based on the USP ratio 60 cm^2:20 ml)
- ____ Irregularly shaped objects and/or sponsor option - ratio of 2 g:10 ml (based on the USP ratio 4 g:20 ml)
- ____ Other (explain): _____

NOTE: The test article will be prepared in triplicate for both extraction and direct contact; therefore, the amount of test article required is six times that indicated above.

① **Test Article Preparation Instructions:**
occlusin 505 has a total SA per vial of ~44 cm^2
please extract 3 times two vials (two vials = 88 cm^2) in appropriate volumes for the extraction study
for direct contact please use (60 cm^2 / 44 cm^2 / 400 mg = 545 mg, approx. 1.5 vials)
extraction procedure should be done under constant agitation, ie end over end, to prevent particles from clumping.

Vehicle: Calcium and magnesium free phosphate buffered saline (CMF-PBS)

The extraction conditions should not in any instance cause physical changes such as fusion or melting, with results in a change in the available surface area. A slight adherence of the pieces can be tolerated.

① **Extraction Conditions (select one):**

- ✓ 37°C, 72 hours
- ____ 50°C, 72 hours
- ____ 70°C, 24 hours
- ____ 121°C, 1 hour
- ____ Other (specify): _____

① **Disposition of Test/Control Article (select one):**

✓ Discard ____ Return unused article ____ Return unused and used article

①completed by sponsor MEL 6-11-07

Special Laboratory Instructions:

Control Article

Negative Control: High density polyethylene will be prepared in triplicate using a ratio of 60 cm^2:20 ml for direct contact and extraction in CMF-PBS using the same extraction conditions as described for the test article.

Positive Control: A 10 ml aliquot of Sterile Water for Injection (SWFI) prepared in triplicate using the extraction conditions as described for the test article will be used as the extraction control. Triplicate 7.0 ml aliquots of SWFI will be used as the direct contact control.

3. Test System

Test System

Species:	Rabbit (*Oryctolagus cuniculus*)
Breed:	New Zealand White
Source:	USDA licensed supplier
Sex:	No particular gender is prescribed for this test
Body Weight Range:	No particular weight range is prescribed for this test
Age:	No particular age is prescribed for this test
Acclimation Period:	Minimum 5 days
Number of Animals:	Three
Identification Method:	Ear tag

Justification of Test System

Hemolysis testing of medical device materials has historically been used to measure blood compatibility *in vitro*. Whole blood samples for use in this test will be collected from the rabbits in EDTA vacuum tubes. A sample of clot-free blood will be collected from each rabbit and the tubes will be pooled in a borosilicate screw cap tube and mixed gently to prevent hemolysis.

4. Animal Management

Husbandry:	Conditions will conform to Standard Operating Procedures that are based on the "Guide for the Care and Use of Laboratory Animals."
Food:	A commercially available rabbit feed will be provided daily.
Water:	Potable water will be provided *ad libitum* through species appropriate water containers or delivered through an automatic watering system.
Contaminants:	Reasonably expected contaminants in feed or water supplies should not have the potential to influence the outcome of this test.
Housing:	Animals will be individually housed in stainless steel suspended cages identified by a card indicating the animal number and sex.
Environment:	The room temperature will be monitored daily. The recommended temperature range for the room is 61-72°F. The room humidity will be monitored daily. The humidity range for the room is 30-70%. The light cycle will be controlled using an automatic timer (12 hours light, 12 hours dark).
Accreditation:	NAMSA is an AAALAC International accredited facility and is registered with the United States Department of Agriculture. Additionally, NAMSA maintains an approved Animal Welfare Assurance on file with the National Institutes of Health, Office for Laboratory Animal Welfare.
Personnel:	Associates involved will be appropriately qualified and trained.

Selection:	Only healthy animals will be selected. To reduce the number of animals used for testing, and to comply with the directives of the NAMSA Institutional Animal Care and Use Committees (IACUC), rabbits on this study may have been used previously in an unrelated test system. Complete history of animal usage is traceable in laboratory records. Animals used for previous evaluations will be identified in the report.
Sedation, Analgesia or Anesthesia:	It has been determined that the use of sedation, analgesia or anesthesia will be necessary during the routine course of this procedure.
Veterinary Care:	In the unlikely event that an animal should become injured, ill, or moribund, care will be conducted in accordance with current veterinary medical practice.
IACUC:	This protocol has been approved by NAMSA IACUC, and is reviewed at least annually by the same committees. Any significant changes to this protocol must be approved by the IACUC prior to conduct.

5. Preparation of Standards and Controls

Dilution Factors For Calculations

Throughout the course of this study, several dilutions of the whole blood or the blood plasma will be conducted. To account for these dilutions in the calculations that will be performed, the following dilution factors (DF) will be used:

Plasma Hemoglobin Determination:

750 µl of plasma added to 750 µl of Drabkin's or Cyanmethemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume plasma}} = \frac{1500\,\mu\text{l solution}}{750\,\mu\text{l plasma}} = 2$$

Whole Blood Hemoglobin Determination:

20 µl of whole blood added to 5 ml of Drabkin's or Cyanmethemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume blood}} = \frac{5.02\text{ ml solution}}{0.02\text{ ml blood}} = 251$$

Diluted Blood Hemoglobin Determination:

400 µl diluted blood added to 5 ml of Drabkin's or Cyanmethemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume diluted blood}} = \frac{5.4\text{ ml solution}}{0.4\text{ ml diluted blood}} = 13.5$$

Sample Hemoglobin Determination:

1.0 ml of Supernatant added to 1.0 ml of Drabkin's or Cyanmethemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume supernatant}} = \frac{2.0\text{ ml solution}}{1.0\text{ ml supernatant}} = 2$$

To Determine Total Hemoglobin present in each tube:

1.0 ml of diluted blood added to 7.0 ml CMF-PBS

$$DF = \frac{\text{Final volume blood/PBS - CMF}}{\text{Volume diluted blood}} = \frac{8.0\text{ ml}}{1.0\text{ ml}} = 8$$

Standards Preparation

The Human Hemoglobin Standard will be reconstituted in Drabkin's or Cyanmethemoglobin reagent at a concentration of 1.44 mg/ml. The reconstituted standard may be stored at 5 ±3°C for up to 2 months from the date of preparation. The reconstituted standard will be diluted to 0.800, 0.600, 0.300, 0.150, 0.0750, 0.0375, and 0.00188 mg/ml. The absorbances of the dilutions and the starting standard solution will be read against a Drabkin's or Cyanmethemoglobin reagent blank at a wavelength of 540 nm. Using the information obtained from the absorbance readings and concentrations, a standard curve will be generated by linear regression.

Plasma Hemoglobin Determination

A 3 ml aliquot of the anticoagulated pooled rabbit blood will be centrifuged at 700-800 Xg for 15 minutes. A 750 µl portion of the plasma (supernatant) will be added to 750 µl Drabkin's or Cyanmethemoglobin reagent. The solution will be allowed to stand at room temperature for 15 minutes for Drabkin's reagent or 5 minutes for Cyanmethemoglobin reagent and then the absorbance (A) of the solution will be read at a wavelength of 540 nm. The plasma hemoglobin concentration in the blood sample will be calculated as follows using the slope of the standard curve.

Plasma Free Hemoglobin Concentration (PFH) = A (Plasma) * Slope * 2 (DF)

If the plasma hemoglobin is greater than 2 mg/ml, the blood will not be used for this test.

Blood Hemoglobin Determination

A 20 µl portion of well-mixed, pooled whole blood (plasma hemoglobin ≤2 mg/ml) will be added to 5 ml of Drabkin's or Cyanmethemoglobin Reagent. This solution will be allowed to stand at room temperature for 15 minutes for Drabkin's Reagent or 5 minutes for Cyanmethemoglobin reagent and then the absorbance of the solution will be read at 540 nm. The hemoglobin concentration of the pooled blood sample will be calculated as follows using the slope of the standard curve.

Whole Blood Hemoglobin Concentration (WB) = A * Slope * 251(DF)

The total hemoglobin content of the pooled blood sample will be adjusted to 10 ± 1 mg/ml by diluting with an appropriate amount of CMF-PBS. The hemoglobin concentration will be confirmed by taking 400 µl of the well-mixed, diluted blood and adding it to 5 ml of Drabkin's or Cyanmethemoglobin Reagent. This solution will be allowed to stand at room temperature for 15 minutes for Drabkin's reagent or 5 minutes for Cyanmethemoglobin reagent and then the absorbance of the solution will be read at 540 nm. The concentration of hemoglobin in the diluted blood will be calculated as follows using the slope of the standard curve.

Diluted Blood Hemoglobin Concentration (DB) = A * Slope * 13.5(DF)

This number will be divided by 8 (DF) to obtain the hemoglobin concentration present in each tube.

6. Method

Following the extraction procedure, the appropriate volume of diluted blood (based on a ratio of 1 ml diluted blood/7 ml vehicle) will be added to the following: triplicate tubes of the test, negative control, positive control, and blank extracts as well as triplicate tubes of the test, negative control, positive control, and blanks in direct contact. The tubes will be maintained for 3 hours at 37 ± 2°C with gentle periodic inversions. Following incubation, all tubes will be centrifuged at 700-800 Xg for 15 minutes.

NOTE: At this point, samples may be decanted, capped and stored in a freezer for up to 96 hours before subsequent hemoglobin analysis.

A 1 ml portion of each supernatant will be added to separate tubes containing 1 ml of Drabkin's or Cyanmethemoglobin reagent. The test and control solutions will stand at room temperature for 15 minutes for Drabkin's reagent or 5 minutes for Cyanmethemoglobin reagent and the absorbances will be read at 540 nm.

7. Evaluation and Statistical Analysis

Using the slope of the standard curve, the hemoglobin concentration in each sample supernatant (hemoglobin released) will be determined as follows:

Supernatant Hemoglobin Concentrations = A * Slope * 2 (DF). The blank corrected percent hemolysis will be calculated for each test article solution and the negative and positive controls as follows:

$$\text{Blank Corrected \% Hemolysis} = \frac{\text{ABS(Sample)} - \text{ABS(Blank)}}{(0.844)\text{ABS(Diluted Blood)} - \text{ABS(Blank)}} \times 100$$

The mean blank corrected % hemolysis will be calculated by averaging the blank corrected % hemolysis values determined for each of the triplicate test samples. This value will be reported to the nearest 1%. The standard deviation for the replicates will also be determined.

An average hemolytic index of the triplicate test samples will also be calculated compared to the negative control. A hemolytic index of 2% or less will be considered to be nonhemolytic. A hemolytic grade will be assigned based on the following scoring scheme:

Hemolytic Index	Hemolytic Grade
0 - 2%	Nonhemolytic
2 - 5%	Slightly Hemolytic
> 5%	Hemolytic

For the suitability of the system to be confirmed, the negative control must have a blank corrected % hemolysis value < 2% and the positive control must have a blank corrected % hemolysis value of ≥ 8%. If either of these values is not within the acceptable range, the test will be repeated with fresh rabbit blood.

8. Report

The final report will include a description of the methods employed, absorbance values/results for the test and controls in direct contact and as extracts, the hemolytic index and grade of the test in direct contact and as an extract, and any additional pertinent observations.

9. Quality Assurance

Inspections will be conducted at intervals adequate to assure the integrity of the study in conformance with 21 CFR 58.35(b) (3). The final report will also be reviewed for conformance to Section 58.185, Subpart J, of the GLP Regulations. A Statement of Quality Assurance Activities will be provided with the final report.

10. Proposed Dates

The study dates will be finalized by the study director following receipt of the sponsor-approved protocol and appropriate material for the study. Initiation of the study will be the date on which the study director signs the GLP protocol. Projected dates for starting the study (first treatment) and for the completion of the study (final report release) will be provided to the sponsor (or representative of the sponsor).

11. Records

Test article and control preparation, the source of the blood used in the test, dates of relevant activities (such as the test initiation and completion), absorbance values, and individual hemoglobin concentration values will be recorded.

All raw data pertaining to this study and a copy of the final report will be retained in designated NAMSA archive files.

12. References

21 CFR 58 (GLP Regulations).

ASTM F756-00, Standard Practice for Assessment of Hemolytic Properties of Materials.

ISO 10993-4 (2002) Biological evaluation of medical devices - Part 4: Selection of tests for interactions with blood.

Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, National Academy of Sciences (Washington: National Academy Press, 1996).

OLAW, Public Health Service Policy on Humane Care and use of Laboratory Animals (NIH Publication).

United States Code of Federal Regulation (CFR) 9: The Animal Welfare Act.

13. Protocol Changes

Any necessary changes to the protocol after sponsor approval or study initiation will be documented and approved by the study director as protocol amendments. Copies will be distributed to the sponsor, the raw data file, and the NAMSA Quality Assurance department.

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June 12, 2007

Paul Tiege
ViRexx Medical Corporation
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PROTOCOL AMENDMENT I

Test Article: Occlusin™ 505 Artificial Embolization Device

Identification: Batch: FL288

NAMSA Submission ID.: 07T_38228

We have received appropriate test article and approved protocol(s) for the program to be conducted in accordance with the Good Laboratory Practice (GLP) Regulations on the material described above. Below is a projected schedule for the work to be performed.

NAMSA Code	NAMSA Lab Number	Study	Estimated Start Date:	Estimated Report Release Date:
V0607_100	07T_38228_02	ASTM Hemolysis - CMF-PBS Extract	June 18, 2007	July 13, 2007

Michelle E. Longstreet

Michelle E. Longstreet, B.S.
Study Director

6-12-07
Date

cc: QA (NAMSA)
GLP study file

NAMSA

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June 26, 2007

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PROTOCOL AMENDMENT II

Test Article: Occlusin™ 505 Artificial Embolization Device

Identification: Batch: FL288

Protocol: V0607_100 ASTM Hemolysis – CMF-PBS Extract

NAMSA Lab No.: 07T_38228_02

This amendment has been written to correct the Preparation section of the study protocol:

- Both the extraction and direct contact methods will be prepared by the sponsor supplied surface area. 3 vials will be used for the direct contact preparation.

This amendment was written prior to testing.

Michelle E. Longstreet

Michelle E. Longstreet, B.S.
Study Director

6-27-07
Date

cc: QA (NAMSA)
GLP study file

NAMSA

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PROTOCOL AMENDMENT III

Test Article:	Occlusin™ 505 Artificial Embolization Device
Identification:	Batch: FL288
Protocol:	V0607_100 ASTM Hemolysis – CMF-PBS Extract
NAMSA Lab No.:	07T_38228_02

This amendment has been written to correct the **Standards Preparation** section of the study protocol:

- The reconstituted standard will be diluted to 0.800, 0.600, 0.300, 0.150, 0.0750, 0.0375, and **0.0188** mg/ml.

This amendment was written prior to testing.

Michelle E. Longstreet

Michelle E. Longstreet, B.S.
Study Director

6-28-07
Date

cc: QA (NAMSA)
GLP study file

NAMSA

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June 29, 2007

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PROTOCOL AMENDMENT IV

Test Article:	Occlusin™ 505 Artificial Embolization Device
Identification:	Batch: FL288
Protocol:	V0607_100 ASTM Hemolysis – CMF-PBS Extract
NAMSA Lab No.:	07T_38228_02

This amendment has been written to clarify the storage conditions of the test article:

- The test article was not stored with refrigeration (2-8 degrees C). The test article was stored at room temperature for one day. Per sponsor, it is acceptable to use the test article.

This amendment was written prior to testing.

Michelle E. Longstreet

Michelle E. Longstreet, B.S.
Study Director

6-29-07
Date

cc: QA (NAMSA)
GLP study file