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# REPORT DOCUMENTATION PAGE

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## 1-Introduction

Most tumors appear to contain heterogeneous populations of cancer cells and cancer stem cells (CSC). These CSCs are more resistant to apoptosis, survive chemotherapy and eventually give rise to secondary tumors, which are harder to eliminate after the first-line of treatment. The reasons for resilience of CSCs to therapy are not well known at present. Understanding the biology of tumors and their CSCs will aid in developing novel anti-cancer therapies. We set course to investigate if cells positive for CD133 (an important CSC marker) exist in the culture of primary human malignant peripheral nerve sheath tumors (MPNST) cells and if their elimination by an oncolytic virus can result in significant tumor regression.

We developed the first mutated version of herpes simplex virus-1 (HSV-1) that is transcriptionally targeted against CD133+ cells (SS2). CD133 is identified as one of the most important biomarkers for cancer stem cells involved in the biology of a multitude of human cancers, including those of the liver, brain, colon, skin and pancreas. By programming an oncolytic virus to infect and efficiently destroy the fraction of tumor cells that act as the stem cell backbone of the tumor, will result in a robust regression in tumor growth.

In this proposal, we introduce our data in detection of CSCs MPNSTs and explain our plans for targeting these cells as a novel therapeutic strategy.

## 2-Keywords

MPNST, CD133, HSV-1, cancer stem cells, NF1

## 3. Accomplishments

### **Specific Aim 1: Specific Aim 1: To study the effects of SS2 virus on primary MPNST cells along with their non-malignant counterpart (normal primary human Schwann cells) (months 1-12):**

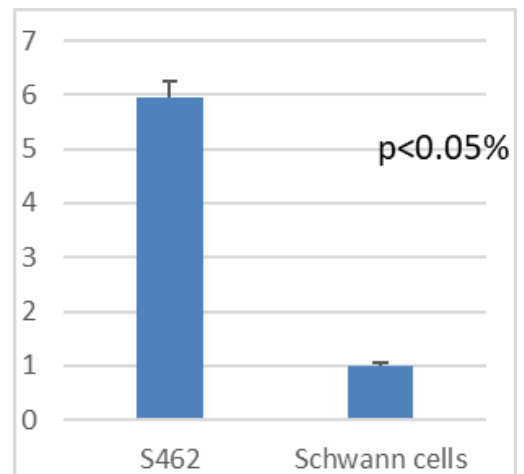
Subtask 1: Evaluation of promoter activity of CD133 expression in primary MPNST cells and normal HSCs.

*Methods: Dual luciferase reporter assay.*

Percentage accomplished: 100%

#### Luciferase Assay

Dual-Luciferase® Reporter Assay System (Promega, WI) was used for the promoter assay. The CD133 promoter (0.9 kb) was cloned into reporter vector pGL4.10 directly upstream of the firefly luciferase gene. The pGL4.75 plasmid, expressing the Renilla luciferase using cytomegalovirus promoter (CMV) promoter, was used as internal normalization control. The assay was performed following the manufacturer's instruction. (Promega). Briefly, 1 µg experimental plasmid and 1 ng control plasmid were co-transfected into 1-2x10<sup>5</sup> cells, which were cultured at 24 well plate, using lipofectamine® 2000 reagent (Lifetechnologies, CA). Cell lysates were prepared 24 hours post transfection using Passive Lysis Buffer (Promega). Cell debris was removed via centrifugation and Luciferase Assay Reagent was added to the supernatant. Bioluminescence was measured and compared using a GloMax illuminometer (Promega). The results shown here portrays 6 folds more activity in S462 compared to Schwann cells.



*Subtask 2: Evaluation of generation of SS2 viral proteins in primary MPNST cells and normal HSCs. Methods: Titration assay, immune-histochemistry and western blotting.*

*Percentage accomplished: 75%*

The expression profile of CD133 in cultured cells were analyzed by flow cytometry. About  $1-10^6$  cells were incubated with 100 L of 1% BSA in PBS containing 1  $\mu$ g of CD16/CD32 for 30 mins on ice to block unspecific Fc intersection, then labeled with PE-conjugated anti-CD133 for 1 h. labeled cells were resuspended in PBS with 1% FBS, and analyzed by flow cytometer.

Unstained cells served as negative controls. The relative percentages of cell expressing CD133 shows that 25.9 % of S462 cells are CD133+. Two series of NF1 and Schwann cells were tested and different results are obtained by flow cytometry analysis. The first NF1 and Schwann cell lines analysis shows that 20.5 % of NF1 Cells and 9.2 % of Schwann cells are CD133+.

The second NF1 and Schwann cell lines flow cytometry results indicates that a very low percentage of CD133 are present in Schwann cells (0.69%), while NF1 cells present a relatively higher percentage of CD133 (9.5 %). An interesting observation was from both series of NF1 and Schwann cell lines which were a pair meaning that schwann cell was derived from a patient with NF1 tumor, as NF1 cell lines were significantly higher in CD133 percentage.

**Protocol for CD133 expression analysis**

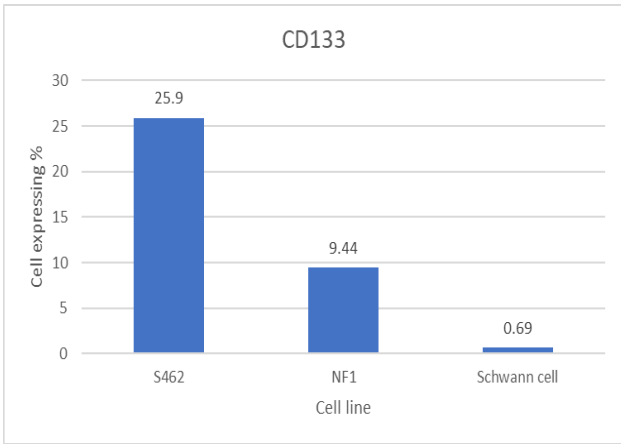
Specimen	Attune Live Cell Count cell/ul	Attune Live Cell Count cell/ml	To Be Stained	Volume in ml for $10^6$	Note
NF1 S2	2.29E+03	2.29E+06	1.00E+06	0.437	Stained $10^6$ cells per tube.
Schwann Cell S2	4.19E+03	4.19E+06	1.00E+06	0.239	Stained $10^6$ cells per tube.

Antibody/Stain	Concentration in $\mu$ g/ml	Best Signal to Noise Amount	$\mu$ l Needed for Best S/N
Invitrogen CD133 APC	25	0.125	5

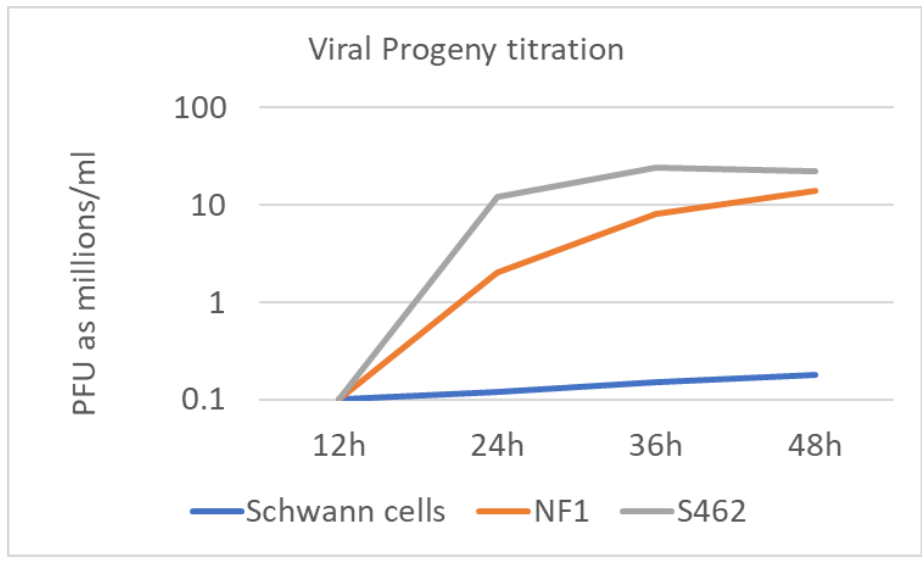
**Method:**

1. Received 2 conical tube of cells.
2. Counted cells on the Attune, used PI to discriminate live cells from dead cells. Cells were found to be >96% live.
3. Aliquoted out the antibody as in the table above. Prepared an unstained control, as ell.
4. Add  $10^6$  cells in 100 ul of wash buffer (PBS + 2% FCS).
5. Incubate on ice for 30 minutes, wash with 3 ml of wash buffer, spin down and resuspend in 350  $\mu$ l/tube.
6. Run samples, collect 10k cells/tube.

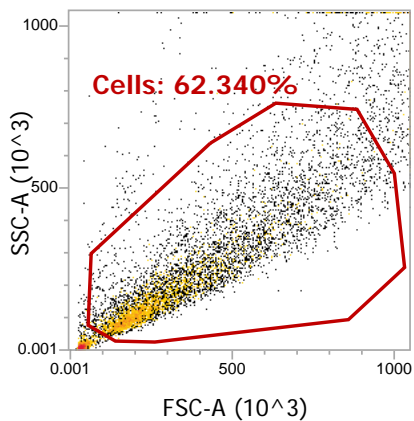
Cell line	Cell Expressing CD133%
S462	25.9
NF1 (Series 1)	20.8
Schwann cell (Series 1)	9.2
NF1 (Series 2)	9.5
Schwann cell (Series 2)	0.6



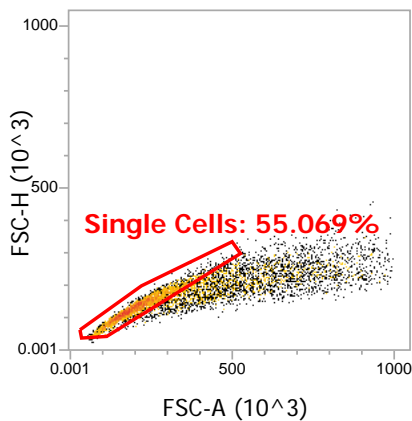
In order to do the titration assay cells were grown in eight-well slide chambers (Falcon) and infected with HSV-1 at a multiplicity of infection (MOI) of 0.5 plaque-forming units (p.f.u.) per cell, or mock-infected. Twenty hours after infection, the cells were fixed in acetone (100%) for 10 min and then left at room temperature to dry. The fixed and dried cells were incubated with a fluorescein-labelled mouse monoclonal antibody to the HSV-1 gC antigen (SyvaMicrotrak from Behring) for 30 min at 37 °C. The slides were then washed with distilled water, dried and mounted in 90% glycerol containing 0.1% phenylenediamine, and viewed with a Zeiss Axiophot microscope on which a Carl Zeiss camera was mounted. The viral progeny production seems to be extremely higher in 462 cells.



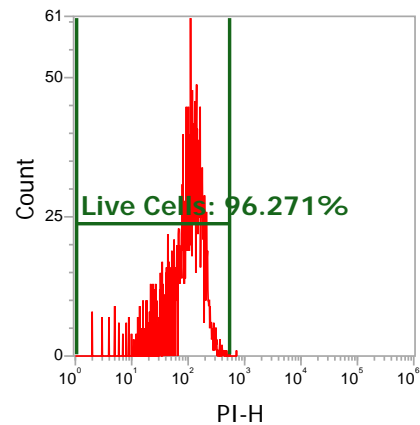
All Events - NF1 S2



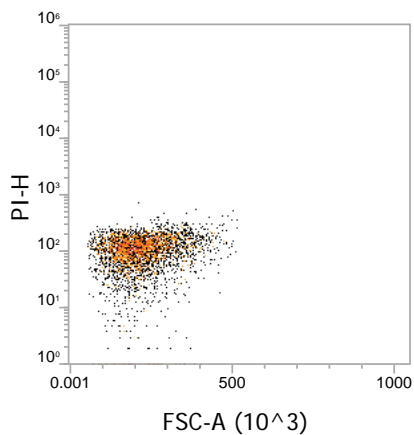
Cells - NF1 S2



Single Cells - NF1 S2



Single Cells - NF1 S2



Experiment: **20 Feb 25 Count**

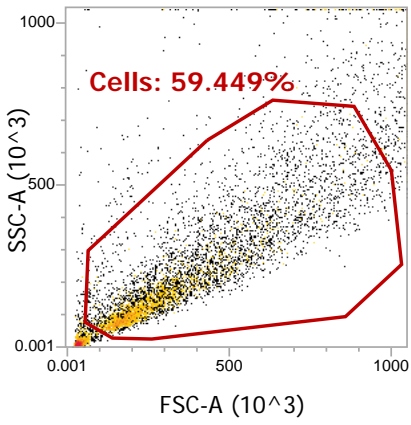
Group: **Group**

Sample: **NF1 S2**

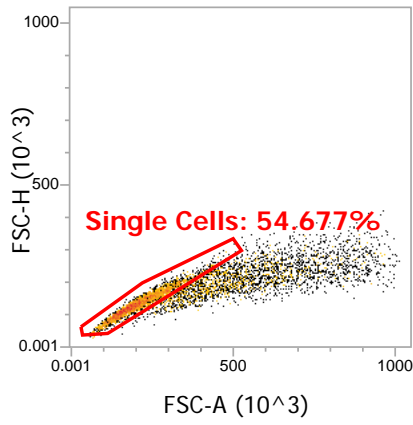
Time Recorded: **11:41:04**

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
▢ All Events	All Events	N/A	N/A	10,000	100.000	100.000
▢ Cells	Cells	FSC-A	SSC-A	6,234	62.340	62.340
▢ Single Cells	Single Cells	FSC-A	FSC-H	3,433	34.330	55.069
▢ Live Cells	Live Cells	BL2-H		3,305	33.050	96.271

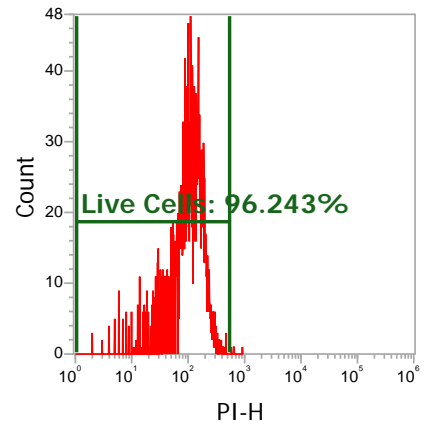
All Events - NF1 S2 + PI



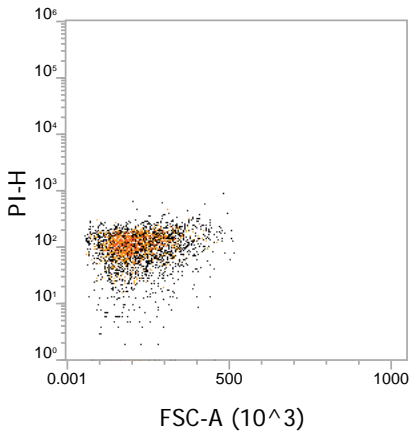
Cells - NF1 S2 + PI



Single Cells - NF1 S2 + PI



Single Cells - NF1 S2 + PI



Experiment: **20 Feb 25 Count**

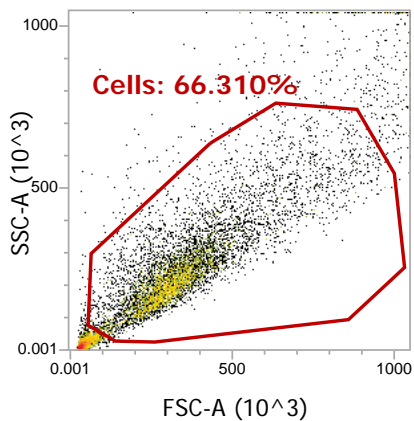
Group: **Group**

Sample: **NF1 S2 + PI**

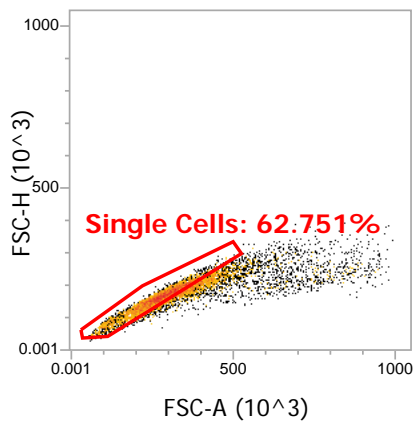
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Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
▢ All Events	All Events	N/A	N/A	9,334	100.000	100.000
▢ Cells	Cells	FSC-A	SSC-A	5,549	59.449	59.449
▢ Single Cells	Single Cells	FSC-A	FSC-H	3,034	32.505	54.677
▢ Live Cells	Live Cells	BL2-H		2,920	31.283	96.243

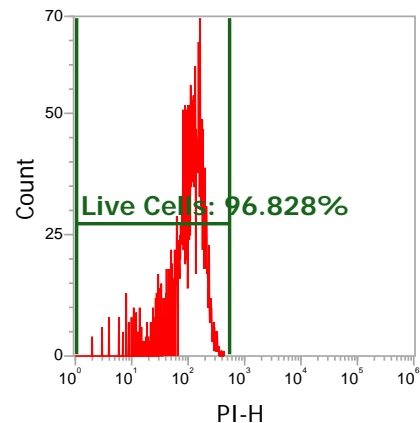
All Events - Schwann Cell S2



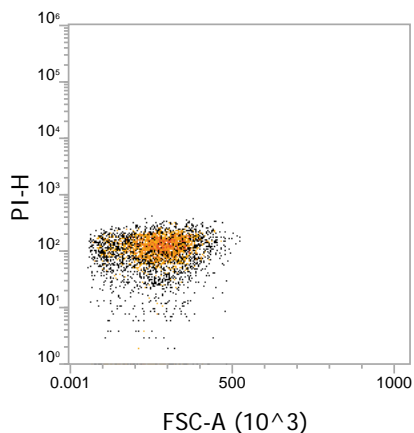
Cells - Schwann Cell S2



Single Cells - Schwann Cell S2



Single Cells - Schwann Cell S2



Experiment: **20 Feb 25 Count**

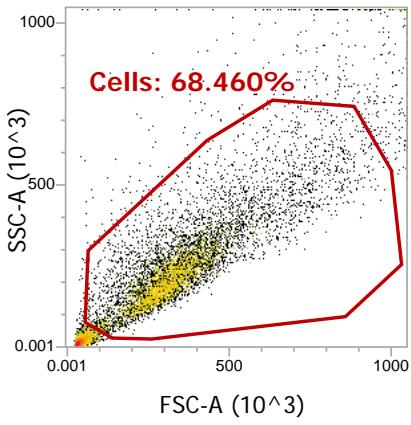
Group: **Group**

Sample: **Schwann Cell S2**

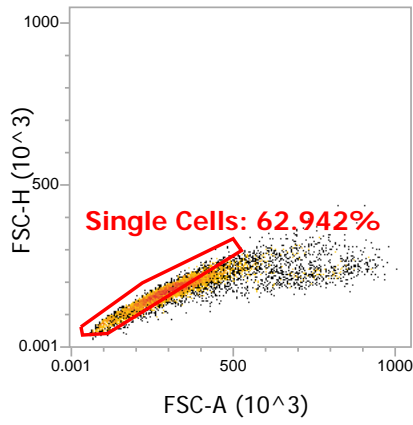
Time Recorded: **11:48:48**

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
▢ All Events	All Events	N/A	N/A	10,000	100.000	100.000
▢ Cells	Cells	FSC-A	SSC-A	6,631	66.310	66.310
▢ Single Cells	Single Cells	FSC-A	FSC-H	4,161	41.610	62.751
▢ Live Cells	Live Cells	BL2-H		4,029	40.290	96.828

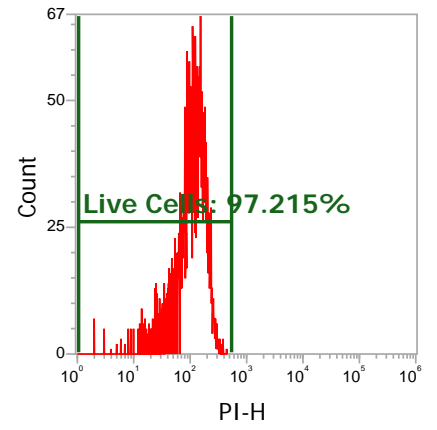
All Events - Schwann Cell S2 + PI



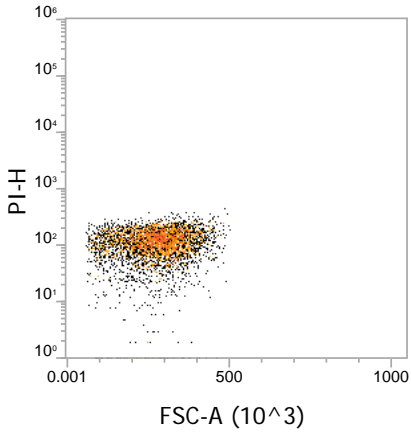
Cells - Schwann Cell S2 + PI



Single Cells - Schwann Cell S2 + PI



Single Cells - Schwann Cell S2 + PI



Experiment: **20 Feb 25 Count**

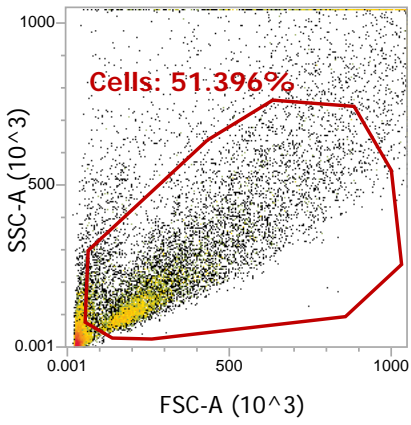
Group: **Group**

Sample: **Schwann Cell S2 + PI**

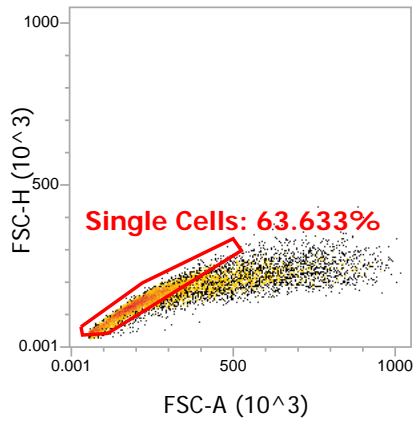
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Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
▢ All Events	All Events	N/A	N/A	10,000	100.000	100.000
▢ Cells	Cells	FSC-A	SSC-A	6,846	68.460	68.460
▢ Single Cells	Single Cells	FSC-A	FSC-H	4,309	43.090	62.942
▢ Live Cells	Live Cells	BL2-H		4,189	41.890	97.215

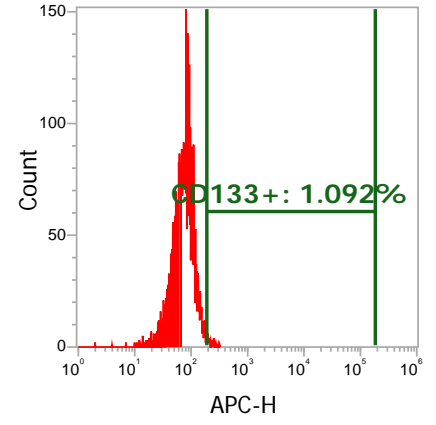
All Events - NF1 S2 Unstained



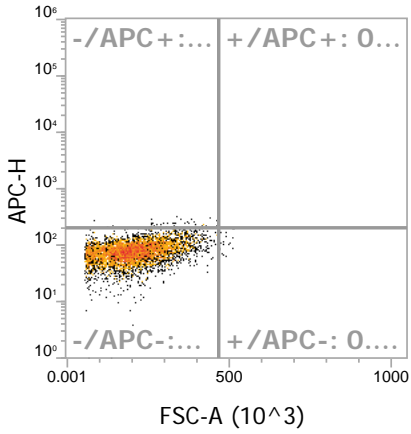
Cells - NF1 S2 Unstained



Single Cells - NF1 S2 Unstained



Single Cells - NF1 S2 Unstained



Experiment: 20 Feb 25 NF1 S2 CD133

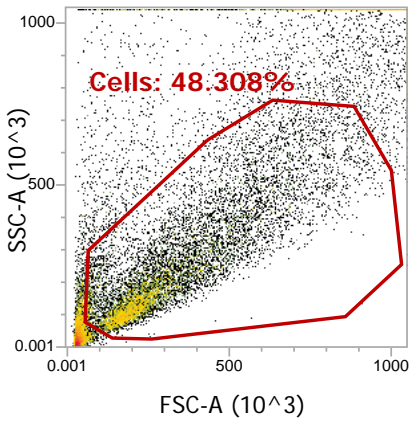
Group: Group

Sample: NF1 S2 Unstained

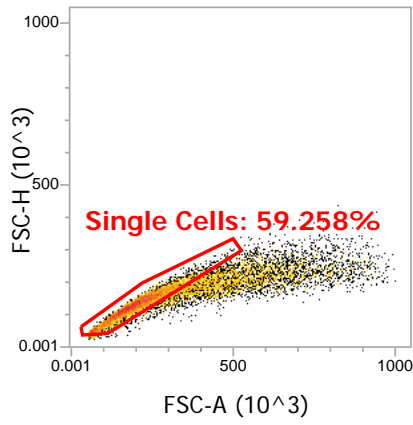
Time Recorded: 13:33:40

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
▢ All Events	All Events	N/A	N/A	17,083	100.000	100.000
▢ Cells	Cells	FSC-A	SSC-A	8,780	51.396	51.396
▢ Single Cells	Single Cells	FSC-A	FSC-H	5,587	32.705	63.633
▢ CD133+	CD133+	RL1-H	RL1-H	61	0.357	1.092
▢ -/APC+	-/APC+	FSC-A	RL1-H	59	0.345	1.056
▢ +/APC+	+/APC+	FSC-A	RL1-H	2	0.012	0.036
▢ -/APC-	-/APC-	FSC-A	RL1-H	5,513	32.272	98.675
▢ +/APC-	+/APC-	FSC-A	RL1-H	13	0.076	0.233

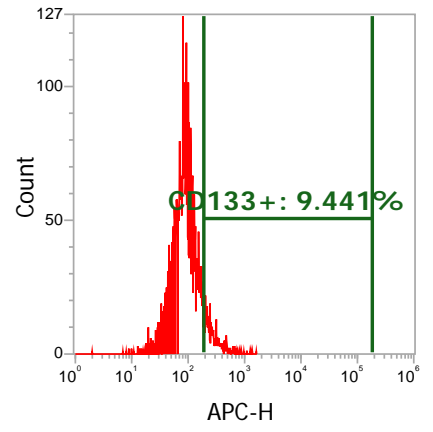
All Events - NF1 S2 + CD133



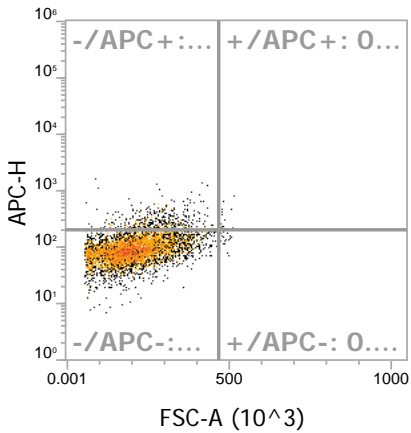
Cells - NF1 S2 + CD133



Single Cells - NF1 S2 + CD133



Single Cells - NF1 S2 + CD133



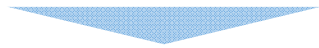
Experiment: 20 Feb 25 NF1 S2 CD133

Group: Group

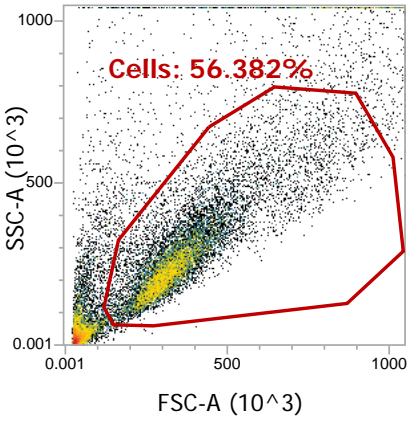
Sample: NF1 S2 + CD133

Time Recorded: 13:36:53

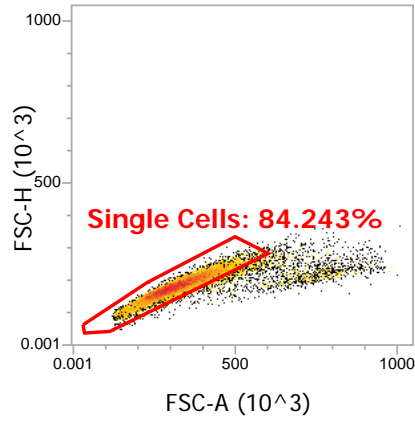
Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
▢ All Events	All Events	N/A	N/A	20,425	100.000	100.000
▢ Cells	Cells	FSC-A	SSC-A	9,867	48.308	48.308
▢ Single Cells	Single Cells	FSC-A	FSC-H	5,847	28.627	59.258
▢ CD133+	CD133+	RL1-H	RL1-H	552	2.703	9.441
▢ -/APC+	-/APC+	FSC-A	RL1-H	537	2.629	9.184
▢ +/APC+	+/APC+	FSC-A	RL1-H	15	0.073	0.257
▢ -/APC-	-/APC-	FSC-A	RL1-H	5,280	25.851	90.303
▢ +/APC-	+/APC-	FSC-A	RL1-H	15	0.073	0.257



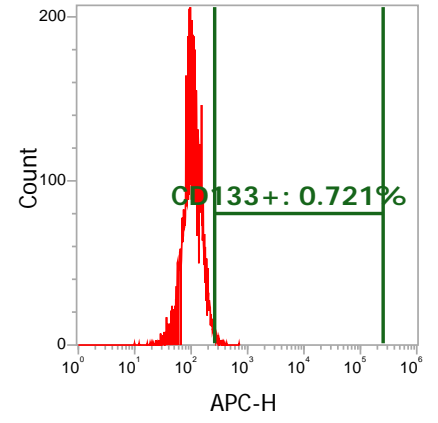
All Events - Schwann Cell S2 Unstained



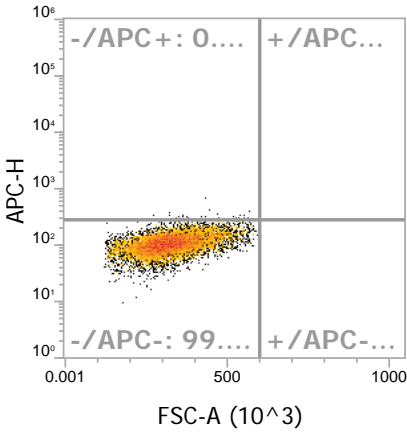
Cells - Schwann Cell S2 Unstained



Single Cells - Schwann Cell S2 Unstained



Single Cells - Schwann Cell S2 Unstained



Experiment: 20 Feb 25 Schwann Cells S2 CD133(1)

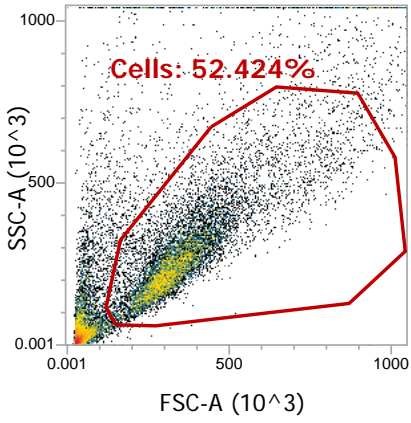
Group: Group

Sample: Schwann Cell S2 Unstained

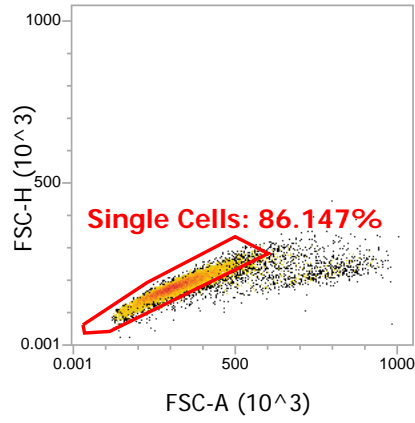
Time Recorded: 13:45:59

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▢ All Events	All Events	N/A	N/A	19,563	100.000	100.000
▢ Cells	Cells	FSC-A	SSC-A	11,030	56.382	56.382
▢ Single Cells	Single Cells	FSC-A	FSC-H	9,292	47.498	84.243
▢ CD133+	CD133+	RL1-H	RL1-H	67	0.342	0.721
▢ -/APC+	-/APC+	FSC-A	RL1-H	67	0.342	0.721
▢ +/APC+	+/APC+	FSC-A	RL1-H	0	0.000	0.000
▢ -/APC-	-/APC-	FSC-A	RL1-H	9,225	47.155	99.279
▢ +/APC-	+/APC-	FSC-A	RL1-H	0	0.000	0.000

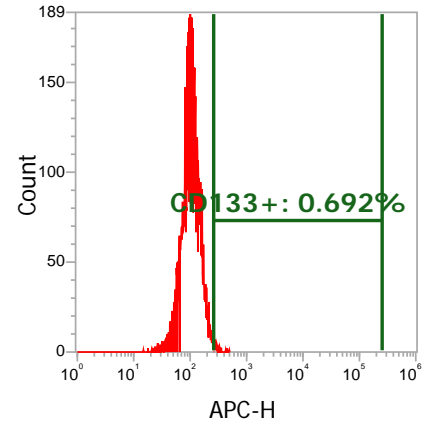
All Events - Schwann Cell S2 + CD133



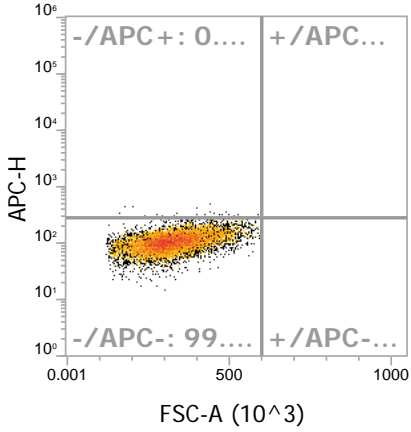
Cells - Schwann Cell S2 + CD133



Single Cells - Schwann Cell S2 + CD133



Single Cells - Schwann Cell S2 + CD133



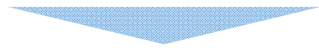
Experiment: 20 Feb 25 Schwann Cells S2 CD133(1)

Group: Group

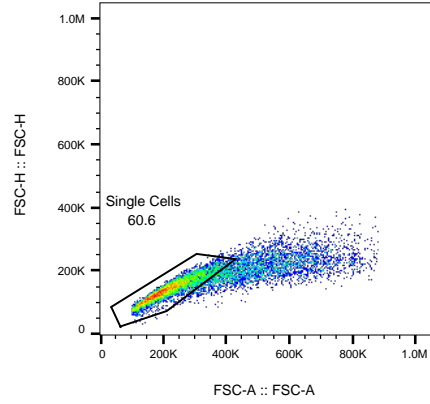
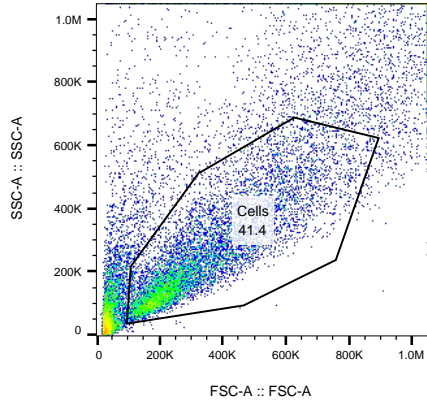
Sample: Schwann Cell S2 + CD133

Time Recorded: 13:51:21

Name	Gate	X Parameter	Y Parameter	Count	%Total	%Gated
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▾ Cells	Cells	FSC-A	SSC-A	9,731	52.424	52.424
▾ Single Cells	Single Cells	FSC-A	FSC-H	8,383	45.162	86.147
▾ CD133+	CD133+	RL1-H		58	0.312	0.692
▾ -/APC+	-/APC+	FSC-A	RL1-H	58	0.312	0.692
▾ +/APC+	+/APC+	FSC-A	RL1-H	0	0.000	0.000
▾ -/APC-	-/APC-	FSC-A	RL1-H	8,325	44.850	99.308
▾ +/APC-	+/APC-	FSC-A	RL1-H	0	0.000	0.000

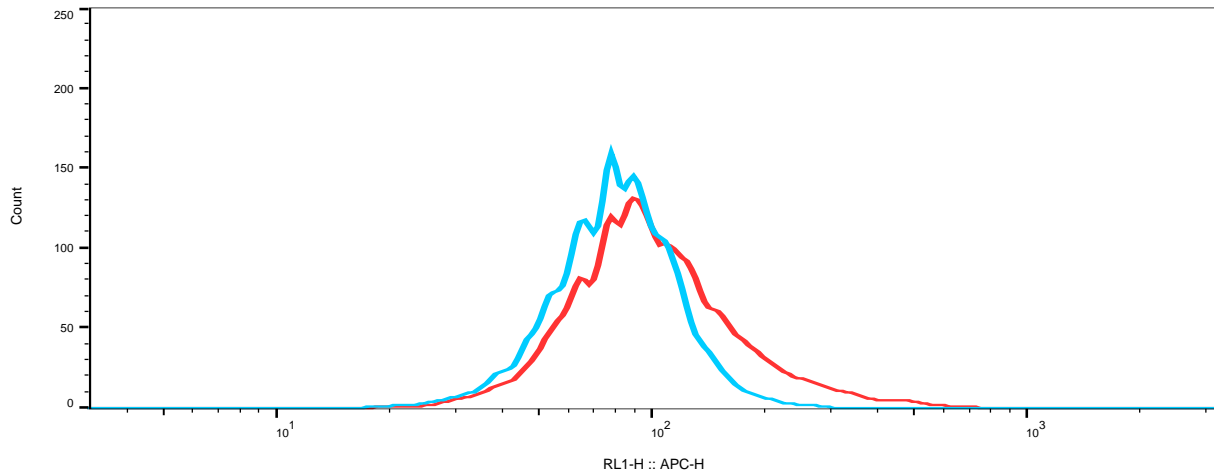


	Sample Name	Count	\$DATE
█	NF1 S2 + CD133.fcs	20425	25-Feb-2020



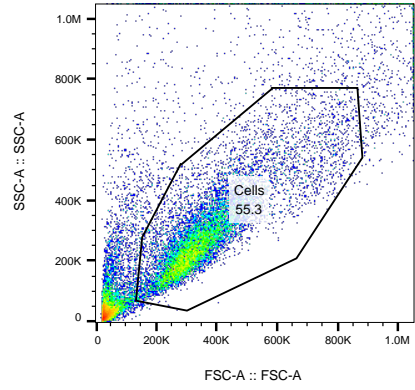
NF1 S2 + CD133.fcs  
Ungated  
20425

NF1 S2 + CD133.fcs  
Cells  
8454

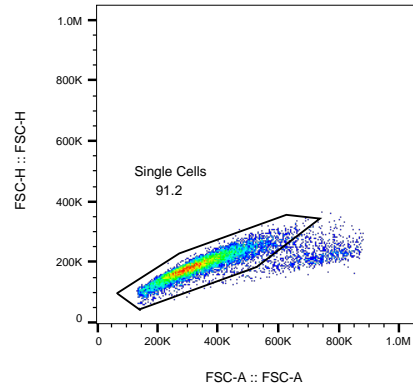


	Sample Name	Subset Name	Count	\$DATE	Median : RL1-H	Mean : RL1-H
█	NF1 S2 + PI.fcs	Single Cells	4850	25-Feb-2020	80.1	85.4
█	NF1 S2 + CD133.fcs	Single Cells	5125	25-Feb-2020	96.0	117

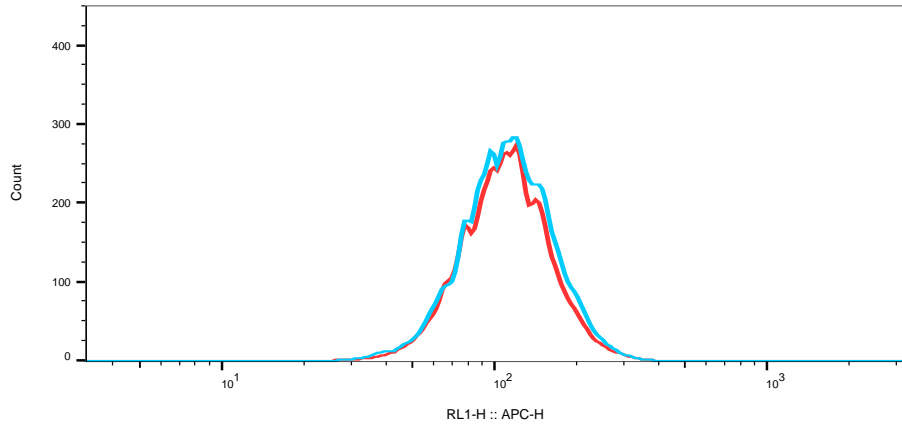
Sample Name	Count	\$DATE
Schwann Cell S2.fcs	19563	25-Feb-2020



Schwann Cell S2.fcs  
Ungated  
19563



Schwann Cell S2.fcs  
Cells  
10820



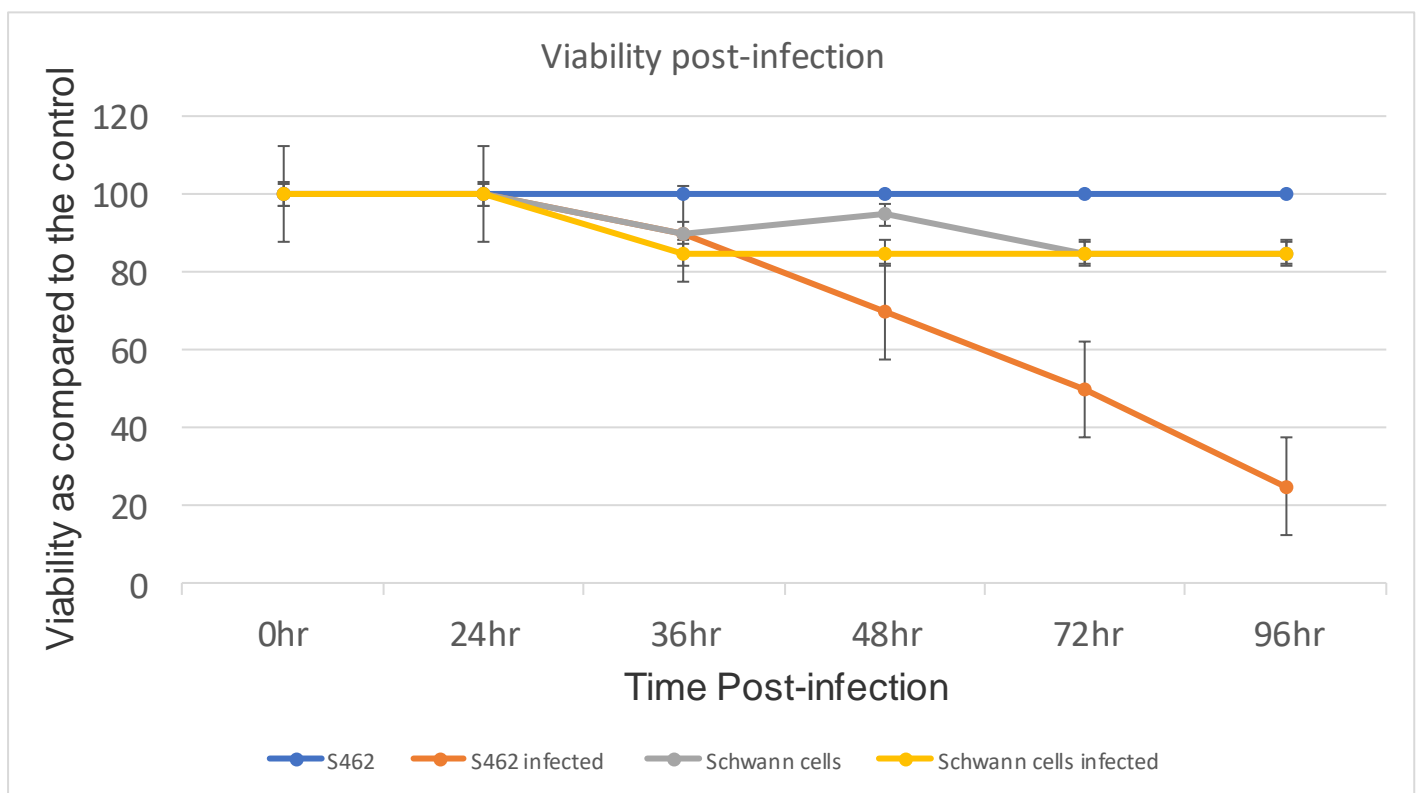
Sample Name	Subset Name	Count	\$DATE	Median : RL1-H	Mean : RL1-H
Schwann Cell S2.fcs	Single Cells	9866	25-Feb-2020	112	120
Schwann Cell S2 + CD133.fcs	Single Cells	8875	25-Feb-2020	110	117

Subtask 3: Evaluation of the effects of SS2 on proliferation, invasiveness and colony formation in primary MPNST cells. *Methods: WST-1, Matrigel-invasion and colony formation assays.*

*Percentage completed: 50%*

#### WST-1 Proliferation assay

One thousand cells were plated onto 96 well plates (BD Biosciences) and incubated overnight in 100  $\mu$ l of DMEM. The next day, designated wells were trypsinized and cells counted. Wells of interest were infected starting at multiplicity of infection of 3 (MOI~3). Each 24 hours up to 144 hours, 49  $\mu$ l XTT reagent (Cell Signaling) and 1  $\mu$ l electron coupling solution were added to three control and three infected wells and incubated for one hour. Colorimetric viability was assessed by spectrophotometer at 450nm. The results show a significant decrease in the viability of S462 cells while Schwann are much less affected. Viability of S462 cells is significantly decreased at 48-96 hours post-infection.



Subtask 4: Evaluation of the effects of SS2 virus on sphere formation capability of CD133+ MPNST cells. Method: Colony formation assay.

At this time our efforts to have colonies of CD133+ cells have not been successful. We will continue our efforts once the stay-home-order has been removed from our state and staff return.

**Specific Aim 2: We will investigate the effects of SS2 virus in treatment of MPNST in orthotopic xenograft model for MPNST (months 12-24):**

Being pursued after re-opening of the KUMC animal research center.

### **3.1-Future directions:**

Upon return to the research activities and removal of the stay-home order next week, we will resume following experiments:

- 1- Colony formation assay
- 2- Invasion assay

In parallel to continuing the in-vitro experiments, we will pursue establishing the xenograft model for MPNST and treating the tumors with the anti-CD133 virus. In order to get a more comprehensive set of data, we are planning to use two MPNST cell lines for this specific aim.

### **4-Impact**

To the best of our knowledge, this is the first time that a virus is being developed to transcriptionally target CD133+ CSCs in a cell-specific manner. If proven to be successful, such strategy can significantly diminish the growth, regeneration and maintenance capabilities of MPNST. Therefore, the virus proposed here could be used as a gene therapy tool against MPNST. Also, once the overall strategy in targeting CD133 cells is proven to be efficient in causing tumor regression, it would open a new horizon towards developing other strategies such as drug therapy against CD133.

From clinical standpoint, different members of oncolytic viruses have entered clinical trials and therefore such therapeutic strategy is highly relevant to the clinic.

### **5-Changes/Problems**

We faced some issues with hiring the correct personnel due to the government shutdown in 2019. Later, we had problems with viability of cells sent to us by ATCC which was reported back and replaced with new batches. At this point, we face a level of inconsistency in the CD133 expression in tested cells. We are now ordering our 4<sup>th</sup> badge of cells and attempting to repeat the experiments along with performing other in-vitro assays necessary. We have been also facing with the outcome of the Coronavirus pandemic in terms of shut down of the research facilities that we are all aware of.

### **6-Products**

**1-Encapsulated Oncolytic Viruses Can Cure Cancer?** M. Hosseini, R. Hejazi and F. Farassati. *Oncolytic Virotherapy*, In Press, 2020.

**2-MPNST stem cells and the possibility of targeting them:** M. Hosseini, F. Farassati. *OncoTarget and Therapy*, In Press, 2020.

### **7-Participants & Other Collaborating Organizations**

Mahsa Hosseini, PhD

### **8-Special Reporting Requirements: N/A**

### **9-Appendices; N/A**