NEUROSYNTEK
NEW MEDICINE - ROBOTICS - MEDIA

PRECLINICAL TRIALS REPORT

TUMORIGENICITY TEST IN VIVO: ADIPOSE-DERIVED MESENCHYMAL STEM CELLS PROCESSED WITH STEMOPTIMIZER NS 6.X

NeuroSyntek:
Pavel G. Molchanov
Elena N. Golubeva
Pavel M. Bulai
Taras N. Pitlik
Victor V. Boksha

Supported by:
Sylvia Jeewon Kim
Jieying Yang
Hong Zeng
Transgenic, Knockout and Tumor model Center, Stanford Cancer Institute, School of Medicine, Stanford University, USA

November 2015 - April 2016

CONTENTS

FACILITIES

Stem cells culture and expansion was conducted in San Jose BioCube, 5941 Optical Court, San Jose, CA 95138.

All animal tests were conducted by Jeewon Kim, PhD, Jieying Yang, MS in Transgenic, Knockout and Tumor model Center, Stanford Cancer Institute, School of Medicine, Stanford University, Stanford CA 94305, USA.

All animal experimentation protocols were approved by the Stanford University Animal Care and Use Committee.

PROTOCOLS

I Stem cell cultivation procedure

Cell Sample

Adipose-Derived Mesenchymal Stem Cells; Normal, Human (Product Code: PT-5006; Lot Number: 0000421627; Manufacture Date: 23-Jun-2014) were obtained from Lonza Walkersville Inc. (USA) (Appendix A, B).

General terms

Culture medium: DMEM-A (Dulbecco's Modified Eagle Medium with Streptomycin/Penicillin, containing 10% fetal bovine serum (FBS)

Additional reagents: phosphate-buffer solution pH = 7.2 (PBS), trypsin:EDTA (1:4)), saline.

Thaw the cells as described in [1] (if cells were previously cryopreserved). Expand for one week.

Cell culture procedure

1. Remove DMEM-A from Petri dish with cells gently. Wash cells with 4 ml of PBS. Remove the PBS.

2. Add 2 mL of trypsin:EDTA, place the dish in the incubator at 37^{o}C for 3 minutes. Then add 4mL of DMEM-A (containing 20% of FBS) into the Petri. Pipette cells, put into a centrifuge tube.

3. Centrifuge at 1500rpm for 10 minutes at room temperature. Remove the supernatant, add 3mL of DMEM-A.

4. Determine cells viability using trypan blue. Take 10μL of cell suspension; mix with 10μL of trypan blue. Put 10μL of suspension on the counting chamber. Count cells.

5. Seed cells on the chip of the NS 6.x system (hAdMSCs-NS) or in Petri dishes (hAdMSCs control) in DMEM-A containing 20% of FBS. Place into the CO_2-incubator at 37^oC.

6. If cells are not used within 24h, change the medium for DMEM-A (10% of FBS).

Important: Avoid high cells confluences (should be less than 30% - 40%), expand to 5x10^6 of cells, use only for 1 - 4 passages. Freeze if needed.

7. For the pre-clinical and clinical studies: trypsinize cells, wash with DMEM-A, centrifuge, replace DMEM-A medium, resuspend in the pharma-grade sterile saline (or saline with Sodium hyaluronate), obtain necessary cells concentration (see below).

Stem cells cryopreservation

1. Wash cells with 4mL PBS per 100mm Petri dish. Remove PBS gently.

2. Add 2mL trypsin:EDTA (1:1) to each Petri dish. Place in the CO_2 incubator at 37^oC for 3 minutes. Add 4mL of DMEM-A (20% FBS) per each P. dish. Resuspend, put the suspension into a centrifuge tube.

3. Centrifuge at 1500rpm, 20C for 10 minutes. Remove supernatant gently, add 6mL DMEM-A (20% FBS), resuspend. Count cells (as described above). Centrifuge.

4. Prepare cells freezing medium: 950μL DMEM-A (20% FBS)+50 μL DMSO per 1x10^6 of cells.

5. Remove supernatant from the tube, add freezing medium (1x10^6 of cells per 1mL of freezing medium). Resuspend the cells. Put 1mL of freezing medium with cells in a vial for freezing.

6. Keep at room temperature for 15 minutes. Keep at + 4^oC for 15 minutes. Keep at -20^oC for 2 hours. Afterwards, keep cells at -80^oC until cells need to be used.

II Electroporation procedure (NeuroSyntek 6.x)

Sample preparation:

1. Remove medium from each well of the chip with cells.

2. Wash cells with 4mL of HEPES buffered saline.

3. Add 2.5mL of HEPES buffered saline into each well. Work with cells not more than 3h.

Prepare HEPES buffered saline with the following concentrations, filter before use:

	n [mol/L]	M [g/mol]	V [L]	M [g]
NaCl	0.140	58.44	0.05	0.400
KCl	0.004	74.55	0.05	0.015

$CaCl_2 \times 2H_2O$	0.002	147.02	0.05	0.015
$MgCl_2 \times 6H_2O$	0.001	203.31	0.05	0.010
HEPES (Na salt)	0.010	260.30	0.05	0.130
$C_6H_{12}O_6$	0.005	180.16	0.05	0.045

Adjust pH to 7.2 - 7.3

For cells staining media use the following buffer solution:

HEPES buffered saline + BSA (BSA-MACS) [9.5 ml + 0.5 ml respectively]

Light-induced electroporation procedure

1. Wash cells with HEPES-BSA. Remove buffer.
2. In a separate tube mix 1mL HEPES-BSA and 50µL FcR Blocker, add to a well on the chip.
3. Incubate for 5 minutes at RT.
4. In a separate tube mix 1mL of HEPES-BSA and 50µL of CD105 PE (or any other marker of interest, labeled with PE), add to a well on the chip.
5. Incubate for 15 minutes at RT in the dark. Remove medium.
6. Wash with HEPES-BSA for 3 minutes. Remove medium.
7. Add 2mL - 3mL of HEPES (without BSA). Avoid light exposure. Install the chip in the NS 6.x system. Switch to the luminescence mode. Choose the area of the electroporation. Distinguish the target cells using NS6.x Software. Start the automated light-induced electroporation procedure.
8. After finishing the electroporation procedure, close the program, turn of the device.
9. Remove the module with cells from the slot.
10. Remove the media from each well, wash the cells with HEPES, add 4mL of DMEM (10% of FBS), place the module in the CO_2-incubator for further cells expansion.

III Animal model tumorigenicity test

Cell sample

1. Cells provided for all tests were generated in a lab-grade cell processing facility.
2. Cell lines used in the trial included:

Mesenchymal Stem Cells

Lipoaspirate derived from white adipose tissue

Homo sapiens, human

Product Code: PT-5006; Lot Number: 0000421627; Manufacture Date: 23-Jun-2014, Lonza Walkersville Inc. (USA) (Appendix A, B).

Quality control records of Mesenchymal Stem Cells included:

Input control:

Sterility Test

Bacteria and Yeast: Negative

Mycoplasma: Negative

Viral Test

Hepatitis B: Negative

Hepatitis C: Negative

HIV: Negative

Specific Staining

Positive expression for CD13, CD29, CD44, CD73, CD90, CD105, and CD166.

Negative expression for CD14, CD19, and CD45.

Output control:

IDEXX IMPACT II: All negative

HeLa cells

Cervix epithelial adenocarcinoma

Homo sapiens, human negroid

Lot Number 14G005, ECACC, purchased from Sigma, USA (Appendix A,B)

Quality control records of HeLa cells included:

Input control:

Sterility Test

Bacteria and Yeast: Negative

Mycoplasma: Negative

Viral Test

Hepatitis B: Negative

Hepatitis C: Negative

HIV: Negative

Output control:

IDEXX IMPACT II: All negative

General Terms

1. Six- to eight-week-old female BALB/c nude mice should be used as the animal model (obtained from Charles River Laboratories Inc.) All mice should be kept under standard temperature, humidity, and timed lighting conditions and were provided with mouse chow and water *ad libitum.*

Note: All animal experimentation protocols should be approved by the animal ethics committee (Stanford University Animal Care and Use Committee).

2. Mice should be divided into the following groups:

I. 6 mice - control-dose group ($5x10^6$ hAdMSCs-Control/kg B.W) (Approx. $1x10^5$ cells per animal)

II. 5 mice - study-dose group ($5x10^6$ hAdMSCs-NS/kg B.W) (Approx. 10^5 cells per animal)

III. 4 mice - positive control group (HeLa cells; human cervix adenocarcinoma; $5x10^6$ cells/kg B.W.), (Approx. 10^5 cells per animal)

Cells injection procedure

1. Adjust the concentration of the cell suspension to 5×10^6/mL viable cells in PBS.

2. Set cell injection volume to 0.2mL in a low dead-volume syringe with 26G needle.

3. Deliver cells to 36.6 C prior to the injection.

4. Inject 0.2mL of the cell suspension subcutaneously unilaterally once with a slow rate 10uL/s for the injection volume.

Animals observation

1. Animals to be observed once a week for their clinical symptoms (vital signs, appearance, presence and extent of any abnormal response, etc.) for up to 4 first weeks.

2. After 4 weeks the observation rate should be set to once per two weeks.

3. Assess the presence of tumors, if any, once in a two weeks interval between the injection and study completion. Measure the tumor dimensions (if there are any tumors) using a caliper in two perpendicular dimensions, and calculate the tumor size using the equation 0.52×Width×Width×Height (mm).

4. After 12 weeks of observation sacrifice animals.

Reporting results and findings

1. Reports of macroscopic findings should be given for stem cells. Macroscopic findings include the conditions of mice during the test period and macroscopic tumor growth, if any. Visible metastases, if any, their location, and frequency should be reported.

RESULTS

Adipose-derived mesenchymal stem cells were purchased from Lonza and seeded on the 18th Jan 2016, HeLa cells (for positive control group of mice) were obtained from Sigma and seeded on the 21st Jan 2016 (Appendix A). Cells were cultured according to protocols provided by suppliers, in order to minimize adverse effects of unexpected cells transformation, proliferation activity modification, etc.

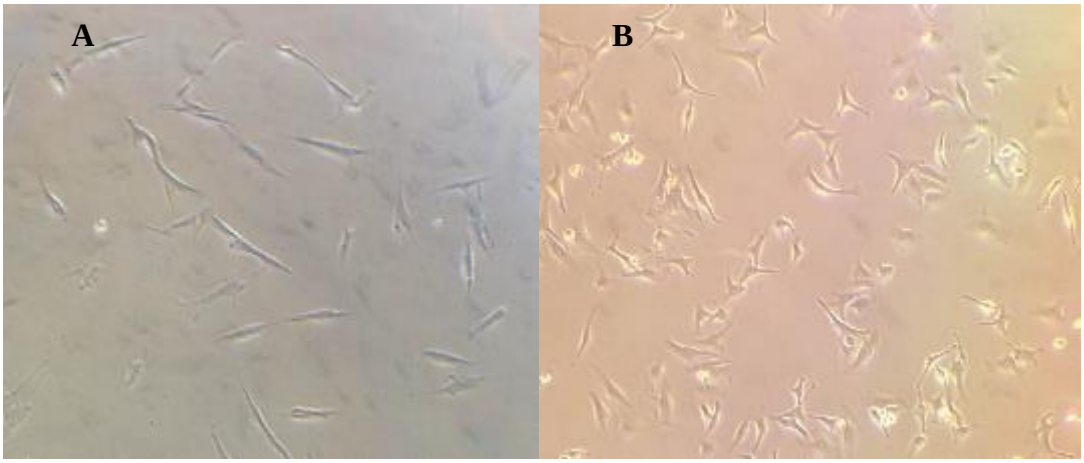

Fig. 1 Reflected light microscopy of cells for the injections. A – ADSCs, B – HeLa.

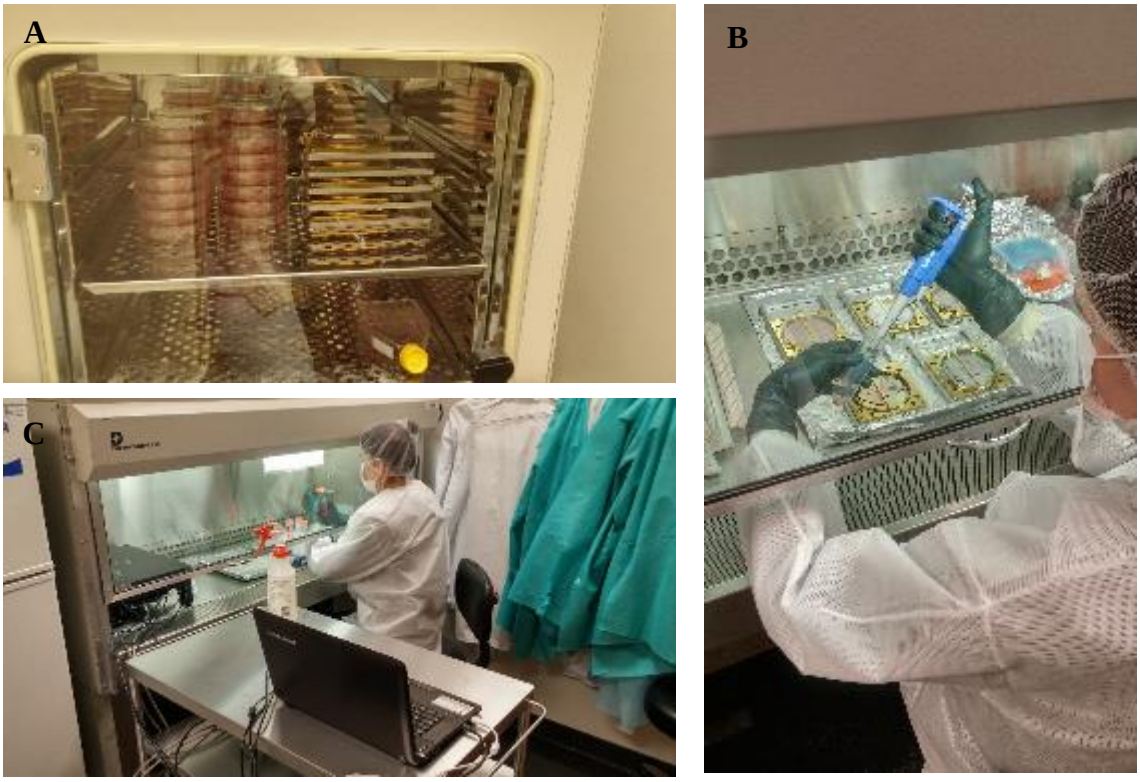

Fig. 2 Cells preparation with NS StemOptimizer. A – Control cells (Petri dishes) and processed stem cells (in NS-chips); B – on-chip cells sorting procedure; C – NS StemOptimizer setup

On the 24th of Jan cells (both cell lines) were partially frozen for further rodent pathogen, bacteria and mycoplasma testing and shipped to IDEXX BioResearch on the next day for the independent evaluation.

IDEXX report was received in 5 days with all positions tested being negative (Appendix C). One half of ADMSCs cells were processed with NS StemOptimizer in accordance with the previously developed protocol. Another half was expanded in polystyrene cell-culture treated Petri dishes (negative control). Cells viability was determined by trypan blue test. Both cell groups and HeLa cells were used for the injection in immunocompromised mice.

Cells injections were provided in two batches:

Table 1. Cells injections schedule

03 Feb 2016 (1st Part)	Number of animals	Cells number injected per an. (viability)
MSCs control	2 mice	4.0×10^5 (96 %) 4.6×10^5 (98 %)
MSCs-operated with NeuroSyntek study	1 mouse	6.0×10^5 (93 %)
HeLa w/FBS (10%) positive control	2 mice	1.0×10^6 (80 %) 1.0×10^6 (80 %)
HeLa w/o FBS positive control	2 mice	1.8×10^6 (82 %) 1.8×10^6 (80 %)
10 Feb 2016 (2nd Part)	Number of animals	Cells number injected per an. (viability)
MSCs control	4 mice	8.0×10^5 (95 %)
MSCs-operated with NeuroSyntek study	4 mice	4.8×10^5 (98 %)

Three groups of mice were controlled as described above.

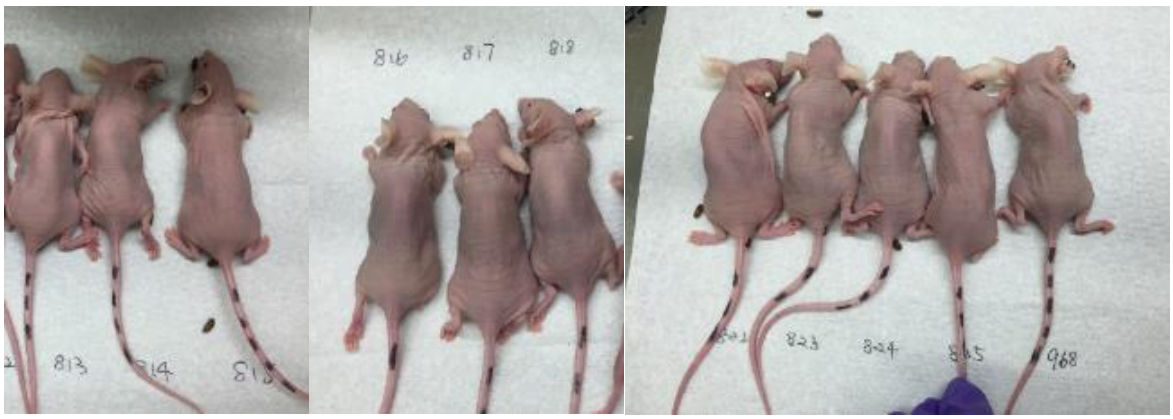

Fig. 3 Animals after the injections. Description: *Control group ADSCs - ## 817, 818, 825; ADSCs processed with NS StemOptimizer - ## 813-816, 822-824, 968*

Fig. 4. Standard mice observation procedure

CONCLUSION

No tumors in the control group of mice and mice, injected with ADSCs, processed with NS StemOptimizer, were detected. ADSCs, processed with NS StemOptimizer, showed no the tumor-forming potential after transplantation, as they did not form tumors at the site of transplantation or at distal sites.

REFERENCES

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APPENDIX A

Lonza Walkersville Inc.
8830 Biggs Ford Road
Walkersville, MD 21793 8415
Tel (301) 898 7025
Fax (301) 845 4024

Lonza

Printed on, 20-Jan-2016 07:52 Page 1 / 1

CERTIFICATE OF ANALYSIS

Product Code: PT-5006
Product: Adipose-Derived Stem Cells (Amp)

Lot Number: 0000421627
Manufacture Date: 23-Jun-2014

TEST (Method)	SPECIFICATIONS Min.	Max.	Results
Tissue Acquisition Number	***	***	27739
DONOR CHARACTERISTICS			
Age	***	***	38 Y
Sex	***	***	FEMALE
Race	***	***	H
BMI	***	***	28
VIRUS TESTING			
HIV Test	***	***	Not Detected
HBV Test	***	***	Not Detected
HCV Test	***	***	Not Detected
MICROBIAL TESTING			
Sterility Test	***	***	Negative
Mycoplasma	***	***	Negative
CELL PERFORMANCE TESTING			
Cell Passage Frozen			1
Viability	>=70%	*****	95 %
Cell Count (Cells/ml)	>=1,000,000	*****	2260000
Seeding Efficiency	>=20%	*****	70 %
Doubling Time (hours)	15	70	15 hrs
CD13,CD29,CD44,CD73,CD90,CD105,CD166	>=90% Pos.	*****	Pass
CD14, CD31, CD45	<=5% Pos.	*****	Pass
CD34	% Pos. FIO	*****	

These cells were isolated from donated human tissue after obtaining permission for their use in research applications by informed consent or legal authorization. This product is for research use only. Details concerning the use of our cell and media products can be downloaded from our website at www.lonza.com/cell-protocols.

Public Health England

Certificate of Analysis

ECACC
European Collection of Cell Cultures
Operated by Public Health England

Product Description: HeLa

Lot Number: 14G005

Test Description:
Sterility Testing of Cell Banks. SOP ECC92

Acceptance Criterion/Specification:
All positive controls (Bacillus subtilis and Candida albicans) show evidence of microbial growth (turbidity) and the negative controls show no evidence of microbial growth (clear).
The criterion for a positive test is turbidity in any of the test broths. All broths should be clear for a negative test result.

Test Number: 52053

Test Date: 05/08/2014

Result: Pass

Authorised by M Brooks **ECACC, Head of Quality** **Date** 09 APR 15.

Culture Collections, Public Health England, Porton Down, Salisbury, SP4 0JG, UK
Tel: +44 (0)1980 612512 Email: culturecollections@phe.gov.uk

Public Health England

Certificate of Analysis

ECACC European Collection of Cell Cultures
Operated by Public Health England

Product Description: HeLa

Lot Number: 14G005

Test Description:
Cell count, viability and confluency of cells on resuscitation from frozen. SOP ECC108

Acceptance Criterion/Specification:
were judged acceptable if they meet two of the following criteria:
- >70% viable cells
- >2 x 10^6 total cells/amp
- Cell growth as expected within 2 days.

Test Number: 52087

Test Date: 01/08/2014

Result: 2.4x10E6 95.6%

Test Description:
Detection of Mycoplasma by PCR using Mycoplasma-specific PCR Primers Validated by ECACC. SOP ECC73

Acceptance Criterion/Specification:
Positive controls yield a single 280 bp amplification product. Negative Control yields no amplified product. The criteria for a positive test result is the yield of a single 280bp PCR product.

Test Number: 52087

Test Date: 01/08/2014

Result: Pass

Authorised by _____ **ECACC, Head of Quality** **Date** 09 APR 15.

Culture Collections, Public Health England, Porton Down, Salisbury, SP4 0JG, UK
Tel: +44 (0)1980 612512 Email: culturecollections@phe.gov.uk

Public Health England

Certificate of Analysis

ECACC European Collection of Cell Cultures
Operated by Public Health England

Product Description: HeLa

Lot Number: 14G005

Test Description:
Detection of Mycoplasma using a Vero indicator cell line and Hoechst 33258 fluorescent detection system. SOP ECC123

Acceptance Criterion/Specification:
The Vero cells in the negative control are clearly seen as fluorescing nuclei with no cytoplasmic fluorescence. Positive control (M.hyorhiris) must show evidence of mycoplasma as fluorescing nuclei plus extra nuclear fluorescence of mycoplasma DNA. Positive test results appear as extra nuclear fluorescence of mycoplasma DNA. Negative results show no cytoplasmic fluorescence.

Test Number: 52087

Test Date: 01/08/2014

Result: Pass

Authorised by _____ **ECACC, Head of Quality** **Date** 09 APR 15

Culture Collections, Public Health England, Porton Down, Salisbury, SP4 0JG, UK
Tel: +44 (0)1980 612512 Email: culturecollections@phe.gov.uk

Public Health England

Certificate of Analysis

ECACC
European Collection of Cell Cultures
Operated by Public Health England

Product Description: HeLa

Lot Number: 14G005

Test Description:
The authentication of human cell lines by STR Profiling. SOP ECC86.
Human cell lines are authenticated using the PowerPlex® 16 HS PCR amplification kit. The Powerplex kit amplifies 15 STR loci plus the gender marker.

Acceptance Criterion/Specification:
Three controls are used for each STR Profiling, two positives; PowerPlex® 16 HS control and K562, and a negative.
For the test to be deemed valid the control samples must give the correct algorithms.
For a result to be accepted as a pass, the algorithm for the test sample must exhibit a minimum of 80% homology of the nine core alleles with an agreed source.
This criterion does not apply where the sample itself constitutes the source.

Test Number: 52087

Test Date: 26/08/2014

Result: Pass

Authorised by _____ **ECACC, Head of Quality** **Date** 09 APR 15.

Culture Collections, Public Health England, Porton Down, Salisbury, SP4 0JG, UK
Tel: +44 (0)1980 612512 Email: culturecollections@phe.gov.uk

Public Health England

Certificate of Analysis Supplementary data sheet

ECACC European Collection of Cell Cultures
Operated by Public Health England

Product Description: HeLa
Lot Number: 14G005
Accession Number: 93021013

Human Pathogenic Virus Testing

Results:

Test Type	Assay Subtype	Referral Lab ID	Referral Lab Ref No.	Report Date	Result
HIV-1 (PCR)	HIV-1 env	TDL(920)	14T644147	17/12/2014	NOT Detected
	HIV-1 LTR	TDL(920)	14T644147	17/12/2014	NOT Detected
	HIV-1 gag	TDL(920)	14T644147	17/12/2014	NOT Detected
	HIV-1 pol	TDL(920)	14T644147	17/12/2014	NOT Detected
Hep C (PCR)	n/a	TDL(920)	15T452364	24/03/2015	NOT Detected
Hep B (PCR)	n/a	TDL(920)	14T644147	17/12/2014	NOT Detected

Comments:

This virus testing report is supplementary to the original certificate of analysis.
Virus testing was carried out by an external referral laboratory.

Authorised by [signature] ECACC, Head of Quality Date 09 APR 15.

Culture Collections, Public Health England, Porton Down, Salisbury, SP4 0JG, United Kingdom
T: +44 (0) 1980 612512 F: +44 (0) 1980 611315 E: culturecollections@phe.gov.uk
W: www.phe-culturecollections.org.uk

APPENDIX B

Lonza

Lonza Walkersville Inc.
Walkersville Warehouse
8830 Biggs Ford Road
Walkersville 21793-0127
Phone:
Fax:

1 / 1

0007417300

Packing List

0007417300

Shipping Point:	Walkersville 21793-0127		
Shipping Point:	21793-0127Walkersville		
Delivery Date:	15-Jan-2016	**Shipping Terms:**	FCA - Free carrier Incoterms2010
Freight Carrier:	FED EX		
Freight Mode:	LBS Overnight Air		
Route:	ZLAL1-LBS Overnight Air		

Customer No.: 6438533

Ship to:
Company
Neurosyntek
Pavel Molchanov
5941 Optical Court
SAN JOSE CA 95138
USA

Sold to:
Neurosyntek
5941 Optical Court
SAN JOSE CA 95138
USA

Bill to:

Neurosyntek Ophthalmic LLC
Elizabeth Garibay
4704 Montrose Boulevard
HOUSTON TX 77006
USA

Order No.	Order Date	Customer Order No.	Customer Contact
40067677	12-Jan-2016	LZ160112-1	Pavel Molchanov - 832 643 7648

Line Item	Product Code/ Description	Order Qty	Ship Qty	UOM	Lot No./Ser.Nr.	Expiration date
010	PT-5006 Adipose-Derived Stem Cells (Amp)	1	1	AMP	0000421627	

Delivery Instructions:

Footer Notes:

In the event of an emergency with the products contained in this package, please contact 800-424-9300 or 703-527-3887 (collect calls).
Returns: Products may not be returned for credit without first obtaining authorization from Lonza. Proof of proper storage may be required. A restocking fee ($50 or 25% of product price) may be applied without return authorization or when purchaser is at fault. Damaged, discrepancies or missing product: must be reported to Customer Service within 5 business days of receipt.
Customer Service: 800-638-8174 Scientific Support: 800-521-0390
Federal ID: 95-3917176

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PAVEL MOLCHANOV
5941 OPTICAL CT
SAN JOSE CA 95138

Customer Service 800-325-3010 • sigma-aldrich.com/custserv

PAGE 1 of 1 DELIVERY# 852652605

DATE	SOLD TO ACCT.	SOLD TO NAME	PURCHASE ORDER NUMBER	REFERENCE #
01/19/2016	49981906	NEUROSYNTEK OPHTHALMIC	SA160118-1	SEE BELOW

ROUTE	PERSON TO CONTACT	PHONE NUMBER
FEDEX OVERNIGHT CA SHIPPING POINT	PAVEL MOLCHANOV Ref: 3015608683	6502292983

STOCK NO.	LOT NO.	ORDERED	SHIPPED	BACK ORD.	DESCRIPTION	
93021013-1VL	14G005	1	1	0	HELA CELLS, HUMAN NEGROID CERVIX EPITHEL	
					CNTRY OF OR: GB	
*** Shipped in Dry Ice ***					526.00/1 EA	526.00
					Sub Total	526.00
					Trans / Handling	42.91
					Total Tax	46.03
					TOTAL	614.94

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APPENDIX C

IDEXX BioResearch

FINAL REPORT OF LABORATORY EXAMINATION

4011 Discovery Drive, Columbia, MO 65201
1-800-669-0825 1-573-499-5700
idexxbioresearch@idexx.com www.idexxbioresearch.com

IDEXX BioResearch Case # 3849-2016

Received: 1/26/2016
Completed: 1/31/2016

Submitted By

Pavel Molchanov
Neurosyntek
932 Lundy Ln
Los Altos, CA 94024

Phone: 650-229-2983
Email: pgm@neurosyntek.com

Specimen Description

Species: mouse
Description: Cell culture lines
Number of Specimens/Animals: 2

Purchase Order #: IDX160122-1

Client ID	Addl ID	Investigator	Date Placed		
ADSCLZ9	421627	MOLCHANOV	1/24/2016	PT-5006	MFG 08/23/2014
HELASA3	14G005	MOLCHANOV	1/24/2016	93021013-1VL	MFG 07/18/2014

Services/Tests Performed: IMPACT II

PCR evaluation for: Ectromelia, EDIM, LCMV, LDEV, MAV1, MAV2, mCMV, MHV, MNV, MPV, MVM, *Mycoplasma pulmonis*, *Mycoplasma* sp., Polyoma, PVM, REO3, Sendai, TMEV

General Comments: Cell culture lines for rodent pathogen test, frozen, freeze media, min 1M per vial

Summary: All test results were negative.

PCR EVALUATION

cells	ADSCL29	HELASA3
Ectromelia	-	-
EDIM	-	-
LCMV	-	-
LDEV	-	-
MAV1	-	-
MAV2	-	-
mCMV	-	-
MHV	-	-
MNV	-	-
Mycoplasma pulmonis	-	-
Mycoplasma sp.	-	-
MVM	-	-
MPV	-	-
Polyoma	-	-
PVM	-	-
REO3	-	-
Sendai	-	-
TMEV	-	-

Legend: + = positive - = negative id:id = pooled sample range id+id+id = non-range pooled sample NT or blank = no test performed wps = weak positive