InterVivo Solutions Final Study Report

Effect of Lofexidine against Somatic Signs of Morphine Withdrawal

Study Numbers: VRI33-18070-RE

Test Articles:	Lofexidine
Sponsor name and address:	
Testing facility name and address:	Vivocore Inc. 8224 Sideroad 15, RR#3 Fergus, Ontario N1M 2W4
Scientific Director name and address:	Guy A. Higgins, InterVivo Solutions Inc. 120 Carlton Street, Suite 203 Toronto, Ontario M5A 4K2
Study initiation date: *Study completion date:*	2018-05-29 2018-06-18
Experimental initiation date: *Experimental completion date:*	2018-05-29 2018-06-18

Scientific Director's signature and date:

_____	_____
Guy A. Higgins, PhD	Date

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A. STUDY TITLE

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B. STUDY NUMBER

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C. SPONSOR REPRESENTATIVE

D. SCIENTIFIC DIRECTOR

Guy A. Higgins, InterVivo Solutions Inc.
120 Carlton Street, Suite 203, Toronto, Ontario M5A 4K2
Email: guyh@intervivo.com

E. STUDY COORDINATOR

Leo Silenieks, InterVivo Solutions.
8224 Sideroad 15, RR#3, Fergus, Ontario N1M 2W4
Email: leos@intervivo.com

F. LOCATION OF FACILITIES

Animal location:
8224 Sideroad 15, RR#3
Fergus, Ontario N1M 2W4

Bioanalytical facility:
InterVivo Solutions
Mercury Biosciences Centre
2820 Argentia Road, Unit 8
Mississauga, Ontario L5N 8G4

G. OBJECTIVE

Opioids represent a class of drugs used to treat pain conditions, and include oxycodone (OxyContin and Percocet), hydrocodone (Vicodin), and fentanyl, morphine and heroin. The rapid increase in the use and misuse of prescription and non-prescription opioid drugs in the United States and Canada has resulted in an opioid epidemic. The identification of new treatment strategies to relieve dependency to opioids is considered an urgent medical need (Volkow and Collins, 2017).

Opioids such as morphine, elicit a physical dependence upon repeated usage which may be manifest as an aversive withdrawal state on cessation of use. Avoidance of this withdrawal state is regarded as a significant driver of continued usage. In animals the withdrawal state can be modelled by repeated treatment of an opioid such as morphine over a period of days and precipitating a withdrawal state by the acute injection of an opioid antagonist such as naloxone. The resulting withdrawal state can be studied by measuring somatic signs of withdrawal (e.g. elevated startle, chewing, tremors, weight loss) (see Higgins and Sellers, 1994). The purpose of this study was to investigate the effect of Lofexidine against the somatic signs of naloxone precipitated withdrawal in morphine dependent rats. Lofexidine (Lucemyra®) was recently FDA approved for the treatment of opioid dependency and so forms a useful benchmark (https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm607884.htm).

H. STUDY DESIGN

The study was run in two phases, the first tested Lofexidine. In Phase 1, thirty-two (32) male Wistar rats were assigned to 4 groups of 8. Group A was dosed twice daily with saline vehicle (*s.c.*), while Groups B, C and D were dosed twice daily with morphine solution (10 mg/kg; *s.c.*). Dosing was at approximately 7:00 and 18:00 (±1 hour) for 12 consecutive days. This treatment regimen was based on published work (Erami *et al.,* 2012) and was designed to induce opioid dependence.

On Day 9 and 12, animals were tested for a naloxone precipitated withdrawal. On Day 9, all rats received either saline (*s.c.*) or naloxone hydrochloride (1 mg/kg; *s.c.*). On Day 12, treatment types were crossed over. Following each treatment (10 minutes), rats received either saline (*i.p.*) (Groups A and B) or Lofexidine (Lucemyra®) (*i.p.*) 0.1 mg/kg (Group C) or 0.3 mg/kg[1] (Group D). AM doses of morphine were administered 2 hours prior to naloxone administration. Using this design, Lofexidine, could be examined against an opioid precipitated withdrawal. Lofexidine doses were based on previous literature (Shearman *et al.,* 1979).

Rats were transferred to activity test chambers after 10 minutes from vehicle or Lofexidine administration. Locomotor activity was measured in the test chambers for 30 minutes, where they were also visually assessed for somatic signs associated with opioid withdrawal (i.e.

[1] Initially a 0.6 mg/kg lofexidine dose was scheduled, however reduced to 0.3 mg/kg due to toxicity noted in 2 rats treated with morphine/naloxone/lofexidine 0.6 mg/kg combination.

writhes, chews, ptosis, WDS, tremors, body weight change; see Higgins and Sellers, 1994). Simultaneously, the activity pattern (distance travelled, rearing) of each rat was automatically recorded. The tracking arena (17"W x 17"L x 12"H) was equipped with sensor bars secured 1" and ~5" above the floor to measure distance travelled and rearing activity respectively.

On Days 3, 6, 9 and 12, at 1-hour post morphine injection, approximately 100 µL of blood was collected by saphenous vein from Group B animals (n=5 randomly selected rats/day) for measurement of morphine plasma levels over the 12 days dosing period. Blood was collected into 1.5 mL microcentrifuge tubes containing potassium EDTA. Tubes were inverted gently to ensure mixing of the anticoagulant and were placed on wet ice. Within 2 hours of collection, blood was centrifuged at 2500 g for 10 minutes at 4°C and 50 pL aliquots of plasma transferred into pre-labelled tubes containing 150 pL of Hepes 0.1 N buffer (pH 7.0-7.5). Samples were stored at approximately -80°C until shipment on dry ice to the bioanalytical laboratory.

I. TREATMENT GROUPS

Table 1. Treatment Group Summary

Group	A	B	C	D
Treatment A	Vehicle	Morphine	Morphine	Morphine
Treatment B	Veh/Nal	Veh/Nal	Veh/Nal	Veh/Nal
Treatment C	Vehicle	Vehicle	Lofexidine (0.1mg/kg)	Lofexidine (0.3 mg/kg)

J. SCHEDULE

Table 2. Schedule of Operations

Date	Study Day	Procedures
2018-05-29	-8	Animal arrival
2018-06-07 to 2018-06-08	1 to 2	Twice Daily Morphine/Vehicle Administration
2018-06-09	3	Twice Daily Morphine/Vehicle Administration Blood Collection
2018-06-10 to 2018-06-11	4 to 5	Twice Daily Morphine/Vehicle Administration
2018-06-12	6	Twice Daily Morphine/Vehicle Administration Blood Collection
2018-06-13 to 2018-06-14	7 to 8	Twice Daily Morphine/Vehicle Administration
2018-06-15	9	Twice Daily Morphine/Vehicle Administration Treatment with Naloxone/Lofexidine Assessment of Somatic Signs and Activity
2018-06-16 to 2018-06-17	10 to 11	Twice Daily Morphine/Vehicle Administration
2018-06-18	12	Twice Daily Morphine/Vehicle Administration Treatment with Naloxone/Lofexidine Assessment of Somatic Signs and Activity

K. EXPERIMENTAL MATERIALS

Table 3. Test Compound Summary

Compound	Lofexidine Hydrochloride	Morphine Sulfate[2]	Naloxone Hydrochloride Dihydrate
Doses Tested	0.1, 0.3 mg/kg	10 mg/kg	1 mg/kg
Route of Administration	IP	SC	SC
Dose Volume	1 ml/kg	1 ml/kg	2 ml/kg
Batch Number	044M4758V	HG4285	SLBV7396
Catalog Number	SML1019-50MG	5060401	N7758
Molecular Weight	295.6 g/mol	285.3 g/mol	399.9 g/mol
Pre-Treatment Time	10 minutes	120 minutes	15 minutes
Source	Sigma	Sandoz	Sigma Aldrich
Vehicle	Saline	Saline	Saline

L. MATERIALS AND METHODS

1 Study Subjects

Thirty-two male Wistar rats, weighing between 275-300g, were purchased from Charles River Laboratories for the purpose of the experiment. All animal use procedures were performed in accordance with the principles of the Canadian Council of Animal Care and were reviewed by an internal Animal Care Committee.

2 Selection and Allocation of Animals

Animals in good health and of similar weight were included in the study. Group allocation was performed prior to dosing using a random number generator.

3 Acclimation and Pre-Treatment of Test System

All animals were given a minimum of 7 days of acclimation to the testing facility prior to initiation of study related procedures.

4 Preparation and Administration of Test Articles

Preparation of naloxone hydrochloride dihydrate and Lofexidine hydrochloride included mixing with saline followed by sonication. Morphine sulfate was provided in pre-prepared vials and therefore did not require further preparation.

5 Housing and Management of Test System

Rats were pair housed in polycarbonate cages with standard corn cob bedding. Cages were

[2] Morphine sulfate was provided under Health Canada Exemption document – ref # 45083.03.18

changed and enrichment was provided according to standard operating procedures. Following surgical procedures in study Phase 2, animals were individually housed.

Rats were housed under an automated 12-hour light/12-hour dark cycle with all experimental activity occurring during the animals' light cycle. Environmental controls were set to maintain the animal room in a temperature range from 17°C - 23°C, with a relative humidity range of 30 - 70%. The test room ventilation provided a minimum of 15 filtered air changes per hour.

Subjects had access to food and water *ad libitum* for the duration of the study. There was no difference in the feeding program between subjects, as food and water were replenished following identical regimen. Certified Rodent Diet (LabDiet® 5001) was provided on stainless steel feeders. Tap water was provided in glass bottles with stainless steel sippers.

6 Procedures and Data Recorded

(1) Health Observations

The health of the animals was observed daily over the course of the study for mortality, abnormalities, and signs of pain or distress.

(2) Body Weights

Animals were weighed daily over the course of the study in order to determine accurate dose volumes. Refer to Appendix Appendix B for animal body weight data.

(3) Withdrawal Data

Refer to Appendix A for a summary of somatic signs.

(4) Activity Data

Refer to Appendix A for a summary activity data.

(5) Sample Analysis

Refer to Appendix F for plasma concentrations of morphine.

M. STATISTICAL ANALYSIS

Body weight data are presented as mean±SEM for pellet group and analyzed using two-way ANOVA with treatment group as a between subjects factor, and day as a within subjects factor. On test days 9 and 12, withdrawal was assessed by comparing naloxone pretreatment with saline in test subjects subchronically treated with either vehicle or morphine using a crossover design. Data collected included checked signs which were recorded manually (see Appendix A for description) during the test observation period, and which were summed to give a total withdrawal score. The withdrawal score was further subcategorised into signs of "Affect" and "Malaise" (see Appendix A for description). Locomotor measures of distance travelled and rearing counts were measured concurrently by automated tracking apparatus. All data collected on days 9 and 12 were analyzed using two-way ANOVA with morphine/saline treatment as a between subjects factor, and naloxone

(i.e. naloxone or vehicle) as a within subjects factor. In the event of a significant main effect or interaction, between group comparisons between treatment and control groups were made using Dunnett's test.

All statistical analysis was conducted using Statistica software (http://www.statsoft.com/Products/STATISTICA-Features). Sigma restricted model for the parameterization of effects is default used by Statistica for coding categorical predictors (see http://documentation.statsoft.com/STATISTICAHelp.aspx?path=glossary/GlossaryTwo/S/SigmaRestrictedModel).

N. RESULTS AND DISCUSSION

Rats treated with morphine (10 mg/kg SC b.i.d) had lower body weight gain compared to saline treated rats (see Appendix B). A main effect of group ($F_{3,27}=9.1$; $P<0.01$), day ($F_{12,324}=114.9$; $P<0.01$) and group x day interaction ($F_{36,324}=10.9$; $P<0.01$) reflected that from Days 3-12 all morphine treated groups had lower body weight compared to vehicle treated group. Body weights between all morphine-treated groups were similar at Days 9 and 12 (see Appendix B). Plasma levels of morphine measured 1h after dosage on Days 3, 6, 9 and 12 were found to be consistent over days, being in the range 400-600 ng/ml (see Appendix F).

On days 9 and 12 post implantation, subjects were tested for opioid precipitated withdrawal in a counterbalanced cross-over design. All rats in each group received either an acute dose of saline or naloxone hydrochloride (1 mg/kg SC). Five minutes preceding the vehicle or naloxone treatment, rats received either saline (Groups A and B) or Lofexidine (Group C: 0.1 mg/kg IP; Group D: 0.3 mg/kg IP)[3].

Opioid withdrawal was determined by the resultant incidence of specific checked behavioural signs. A significant main effect of Treatment (i.e. Vehicle versus Morphine)/Pretreatment (i.e. Vehicle or Lofexidine) ($F_{3,27}=27.1$, $P<0.01$), naloxone (i.e. Vehicle versus Naloxone) ($F_{1,27}=57.9$, $P<0.01$) and Treatment/Pretreatment x naloxone dose interaction ($F_{3,27}=23.8$, $P<0.01$) was recorded. The Vehicle pretreated group treated with morphine/naloxone combination had significantly higher checked somatic scores compared to groups treated with vehicle/naloxone, or morphine/vehicle, consistent with a naloxone precipitated withdrawal in morphine dependent rats (see Appendices C-E).

The overall withdrawal score was subcategorised into behaviours potentially associated with affect (i.e startle, reactivity to touch) and malaise (i.e chewing, paw shakes, chin rubbing, salivation) (see Appendix A). On both sub-categories main effects of Treatment/Pretreatment ($F_{3,27}\geq3.9$, $P\leq0.01$), naloxone dose ($F_{1,27}\geq62.8$, $P<0.01$) and Treatment/Pretreatment x naloxone dose interaction ($F_{3,27}\geq3.2$, $P<0.05$) was recorded, reflecting that the withdrawal state was associated with

[3] Two animals were excluded from the study due to dosing with lofexidine 0.6 mg/kg which was poorly tolerated.

behavioural signs of affect and malaise.

Comparison between Morphine/naloxone+vehicle and Morphine/naloxone + lofexidine groups identified a significant difference consistent with lofexidine (0.1 – 0.3 mg/kg IP) pretreatment significantly attenuating the expression of checked somatic withdrawal signs (see Appendix C, D and E). Indeed at the 0.3 mg/kg dose, lofexidine reduced the incidence of checked withdrawal signs to a level equivalent to non-dependent (i.e Group A, vehicle treated) rats. Lofexidine significantly reduced both signs classified as “affect” and “malaise” related.

Motor effects were measured by distance travelled and number of rears. For both measures a main effect of Treatment/Pretreatment (distance: $F_{3,27}=4.2$, $P=0.01$; rearing: $F_{3,27}=3.3$, $P<0.05$), a main effect of Naloxone dose (distance: $F_{1,27}=27.6$, $P<0.01$; rearing: $F_{1,27}=26.3$, $P<0.01$), and a Treatment/Pretreatment x Naloxone interaction (distance: $F_{3,27}=6.6$, $P<0.01$; rearing: $F_{3,27}=6.1$, $P<0.01$). These main effects reflected an increase in motor activity in morphine/vehicle treated rats, and a decrease in these measures in morphine/naloxone treated rats relative to Vehicle (Group A) controls. Lofexidine pretreatment reduced the morphine-induced hyperactivity but did not reverse the withdrawal-induced hypoactivity (see Appendices C-E).

O. SCIENTIFIC DIRECTOR’S ADDITIONAL COMMENTS

Pretreatment with naloxone to rats sub-chronically treated with morphine (10 mg/kg b.i.d.) induced a withdrawal syndrome characterised by the expression of somatic signs including signs characterised as “malaise” and “affect”. Measures of locomotor activity were also affected, with a hyperactivity evident in morphine rats treated with vehicle (i.e. no naloxone, non-withdrawal), and a hypoactivity evident in the withdrawal group, possibly reflective of a dysphoric state.

Lofexidine (0.1-0.3 mg/kg IP) attenuated the expression of all somatic withdrawal signs relative to controls, reducing the withdrawal score to a level equivalent to non-dependent animals at the 0.3 mg/kg dose. A partial reduction of withdrawal signs was noted at the 0.1 mg/kg dose. Lofexidine also reduced the hyperactivity in morphine rats treated with vehicle. However, Lofexidine did not reverse the opioid withdrawal-induced hypoactivity.

P. ANIMAL DISPOSITION

Animals were euthanized a study conclusion. Carcasses and tissues from euthanized or deceased animals were disposed of according to standard operating procedures.

Q. DATA INTEGRITY STATEMENT

There were no unforeseen circumstances that affected the quality or integrity of the data.

R. REFERENCES

Erami, E., Azhdari-Zarmehri, H., Rahmani, A., Ghasemi-Dashkhasan, E., Semnanian, S., & Haghparast, A. (2012). Blockade of orexin receptor 1 attenuates the development of morphine tolerance and physical dependence in rats. Pharmacol. Biochem. Behav., 103(2), 212-219.

Higgins GA & Sellers EM (1994) Antagonist-precipitated opioid withdrawal in rats: evidence for dissociations between physical and motivational signs. Pharmacol. Biochem. Behav. 48(1): 1-8.

Shearman, G. T., Lal, H., & Ursillo, R. C. (1980). Effectiveness of lofexidine in blocking morphine-withdrawal signs in the rat. Pharmacol. Biochem. Behav. 12(4), 573-575.

Volkow ND, Collins FS (2017) The Role of Science in Addressing the Opioid Crisis. NEJM 377: 391-394.

S. ACCURACY OF REPORT STATEMENT

I certify that this report is a complete and accurate representation of all study observations.

Guy A. Higgins, Scientific Director Date

Appendix A: SUMMARY OF SOMATIC SIGNS OF OPIOID WITHDRAWAL

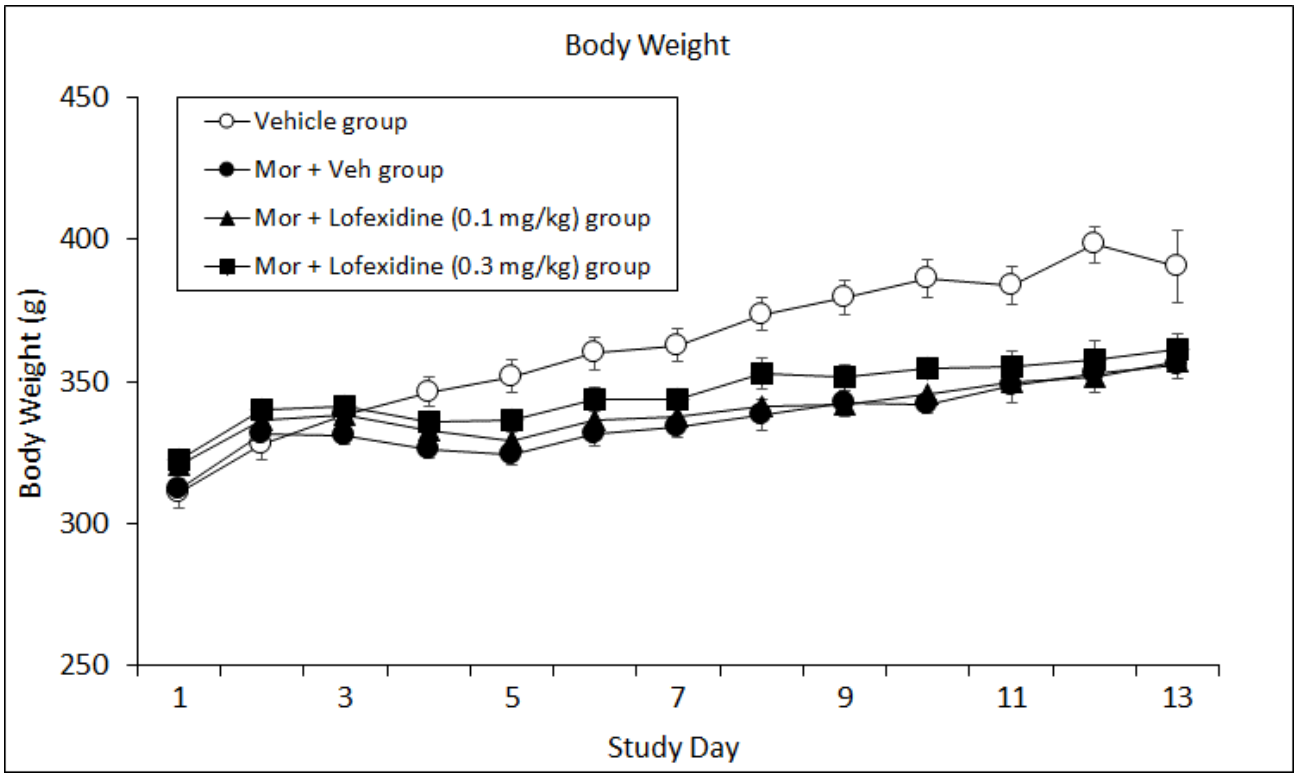
Sign	Description of scoring
Wet dog shakes	Counts (N)
Chin rubs	Counts (N)
Paw shakes	Distinct bouts of paw shakes (N)
Chewing	Distinct bouts of vacuous chewing (N)
Teeth chatter	Distinct bouts of teeth chattering (N)
Ptosis* (eyelid closure)	Distinct periods of eyelid closure. Typically this behaviour is continuous. During phase 1, the occurrence of at least one sustained episode (1 minute) of ptosis every 10 minutes would be scored once (N), resulting in a maximum total score of 6 for each animal. During phase 2, the occurrence of at least one sustained episode of ptosis every 5 minutes would be scored once, resulting in a maximum total score of 6 for each animal.
Startle*	Using a hand-held clicker (Clik-R Trainer), animals were startled once during every time-bin and given a score between 0-2 based on the magnitude of their response
Salivation*	Scored once at the end of every time-bin with a value between 0-2 based on intensity of the response.
Flat body posture*	Distinct bouts of the animal adopting a flattened body posture with face resting on the cage floor (N).
Lacrimation*	Presence of lacrimation was counted (N) once at the end of every time-bin.
Reactivity to touch*	Scored once at the end of every time-bin with a value between 0-2 based on intensity of the response.

* During phase 1 time-bins were 10 minutes long, during phase 2 time-bins were 5 minutes long. Somatic signs were measured once per time-bin.

Total withdrawal score was sub-categorised into “Affect” and “Malaise” related behaviours.

“Affect” related	“Malaise” related
Startle, Reactivity to touch	Chewing, Chin rubbing, Salivation, Paw shakes
Supporting references: http://www.ratbehavior.org/WhatIsMyRatDoingFAQ.htm Davis (1990) Pharmacol. Ther. 47: 147-165 Cryan & Sweeney (2011) BJP 164: 1129-1161	Supporting references: Higgins et al (1992) JPET 264:1440-1449. Parker & MacLeod (1991) PBB 40: 983-986. Grill et al (1992) Brain Res. 573: 95-104.

Appendix B: ANIMAL BODY WEIGHTS



Effect	SS	Degr. of Freedom	MS	F	p
Repeated Measures Analysis of Variance (Horus Body weight) Sigma-restricted parameterization Effective hypothesis decomposition; Std. Error of Estimate: 39.03251					
Intercept	4807409	1	4807409	31554.26	0.000000
Group	41385	3	13795	9.05	0.000258
Error	41136	27	1524		
DAY	76402	12	6367	114.92	0.000000
DAY*Group	21631	36	601	10.85	0.000000
Error	17951	324	55		

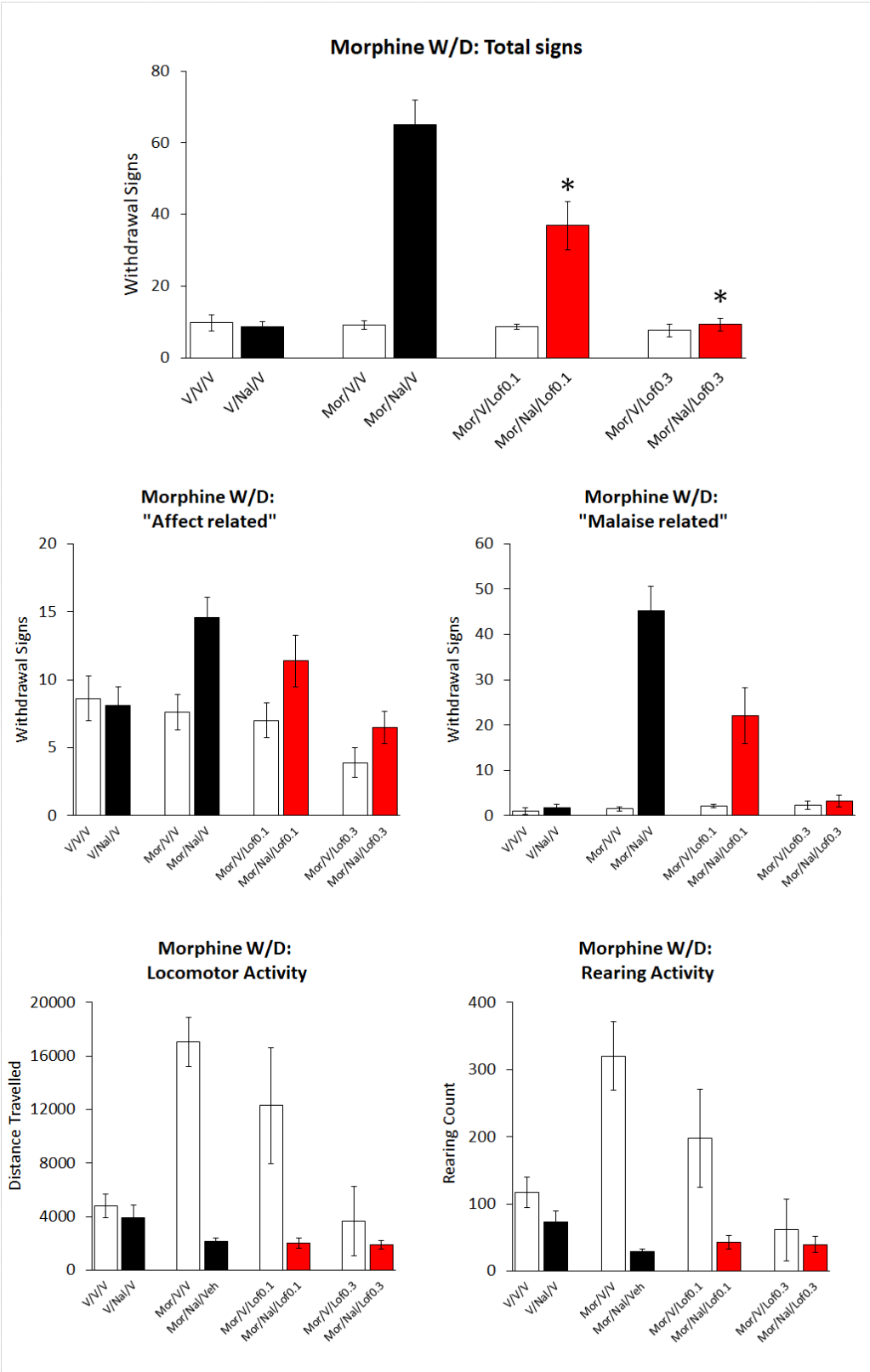
Group

Cell No.	Group	{1} 362.18
Dunnett test; variable DV_1 (Horus Body weight) Probabilities for Post Hoc Tests (2-sided) Error: Between MSE = 1523.5, df = 27.000		
1	A	
2	B	0.000153
3	C	0.000890
4	D	0.018386

Appendix C: LOFEXIDINE VS. NALOXONE PRECIPITATED OPIOID WITHDRAWAL: Raw Data

			Somatic Signs												Activity		
	Treatment	Animal ID	WDS	Chewing	Paw Shakes + chin rub	Teeth Chatter	Startle	Ptosis	Salivation	Lacrimation	Flat body posture	Reactivity	Total	"Anxiety related" Startle + Reactivity	"Malaise related" chewing+paw shakes+salivation	Distance Travelled (cm)	Ambulatory Episodes	Rears
Saline	Vehicle + Vehicle	A1	0	1	0	0	7	0	0	0	0	0	8	7	1	7141.6	234.0	190.0
		A2	0	6	0	0	6	0	0	0	0	11	23	17	6	3332.6	105.0	84.0
		A3	0	0	0	0	4	0	0	0	0	1	5	5	0	3182.2	106.0	76.0
		A4	0	1	0	0	4	0	0	0	0	1	6	5	1	3487.5	116.0	122.0
		A5	0	0	0	0	6	0	0	0	0	2	8	8	0	3505.9	112.0	67.0
		A6	1	0	0	0	6	0	0	0	0	0	7	6	0	7168.5	217.0	186.0
		A7	0	0	0	0	3	0	0	0	0	3	6	6	0	1791.1	43.0	23.0
		A8	0	0	0	0	6	0	0	0	0	9	15	15	0	8588.5	252.0	188.0
		AVG	0.1	1.0	0.0	0.0	5.3	0.0	0.0	0.0	0.0	3.4	9.8	8.6	1.0	4774.7	148.1	117.0
		SEM	0.1	0.7	0.0	0.0	0.5	0.0	0.0	0.0	0.0	1.5	2.2	1.7	0.7	872.8	26.7	22.9
Saline	Naloxone + Vehicle	A1	0	3	0	0	6	0	0	0	0	2	11	8	3	3984.0	134.0	96.0
		A2	0	7	0	0	5	0	0	0	0	6	12	11	7	2237.4	68.0	48.0
		A3	0	1	0	0	3	0	0	0	0	0	4	3	1	1996.0	56.0	57.0
		A4	0	1	0	0	3	0	0	0	0	0	4	3	1	3439.4	105.0	44.0
		A5	0	1	0	0	6	0	0	0	0	4	11	10	1	1587.3	52.0	22.0
		A6	0	0	0	0	6	0	0	0	1	0	7	6	0	6513.3	216.0	149.0
		A7	0	0	0	0	6	0	0	0	0	8	14	14	0	2202.5	63.0	28.0
		A8	0	0	1	0	5	0	0	0	0	5	6	10	1	9202.1	263.0	135.0
		AVG	0.0	1.6	0.1	0.0	5.0	0.0	0.0	0.0	0.1	3.1	8.6	8.1	1.8	3895.2	119.6	72.4
		SEM	0.0	0.8	0.1	0.0	0.5	0.0	0.0	0.0	0.1	1.1	1.4	1.4	0.8	942.8	28.3	17.2
Morphine 10mg/kg/day	Vehicle + Vehicle	B1	0	0	0	0	3	0	0	0	0	3	6	6	0	24278.0	775.0	404.0
		B2	0	1	0	0	6	0	0	0	0	6	13	12	1	21587.2	663.0	424.0
		B3	0	3	0	0	6	0	0	0	0	0	9	6	3	17194.9	559.0	256.0
		B4	0	1	0	0	6	0	0	0	0	7	14	13	1	18754.1	627.0	430.0
		B5	0	4	0	0	2	0	0	0	0	0	6	2	4	7958.4	270.0	267.0
		B6	0	0	0	0	6	0	0	0	0	4	10	10	0	16652.8	542.0	125.0
		B7	0	1	0	0	6	0	0	0	0	0	7	6	1	11940.4	433.0	144.0
		B8	0	2	0	0	6	0	0	0	0	0	8	6	2	17983.9	640.0	514.0
		AVG	0.0	1.5	0.0	0.0	5.1	0.0	0.0	0.0	0.0	2.5	9.1	7.6	1.5	17043.7	563.6	320.5
		SEM	0.0	0.5	0.0	0.0	0.6	0.0	0.0	0.0	0.0	1.0	1.1	1.3	0.5	1820.3	54.8	50.6
Morphine 10mg/kg/day	Naloxone + Vehicle	B1	0	33	6	0	7	0	0	0	0	7	53	14	39	2094.3	60.0	32.0
		B2	6	14	5	0	7	4	0	0	1	8	45	15	19	1387.4	38.0	18.0
		B3	2	63	0	0	7	0	0	0	3	12	87	19	63	1744.9	53.0	32.0
		B4	0	29	10	2	4	1	2	0	0	10	48	14	41	3490.2	93.0	27.0
		B5	3	46	5	0	10	4	0	0	3	11	82	21	51	1735.3	49.0	25.0
		B6	1	18	14	0	6	5	4	0	0	10	48	16	36	1187.4	33.0	25.0
		B7	0	41	1	7	6	4	0	0	0	1	60	7	42	2803.0	87.0	53.0
		B8	0	40	20	10	6	6	5	0	0	5	92	11	65	2521.7	61.0	24.0
		AVG	1.5	35.5	7.6	2.4	6.6	3.0	1.4	0.0	0.9	8.0	64.4	14.6	44.5	2120.5	59.3	29.5
		SEM	0.8	5.6	2.4	1.4	0.6	0.8	0.7	0.0	0.5	1.3	6.9	1.5	5.3	273.9	7.6	3.7
Morphine 10mg/kg/day	Vehicle + Lofexidine (0.1 mg/kg)	C1	0	3	0	0	5	0	0	0	1	1	10	6	3	2043.2	64.0	51.0
		C2	0	4	0	0	5	0	0	0	3	0	12	5	4	838.6	22.0	16.0
		C3	0	1	0	0	7	0	0	0	0	8	8	15	1	28858.4	798.0	526.0
		C4	0	2	0	0	6	0	0	0	0	0	8	6	2	28648.1	802.0	419.0
		C5	0	3	0	0	4	0	0	0	3	0	7	4	3	298.0	12.0	13.0
		C6	0	0	0	0	6	0	0	0	0	1	7	7	0	20207.4	603.0	348.0
		C7	0	2	0	0	6	0	0	0	0	3	11	9	2	13156.0	395.0	190.0
		C8	0	2	0	0	4	0	0	0	0	0	6	4	2	4317.4	98.0	17.0
		AVG	0.0	2.1	0.0	0.0	5.4	0.0	0.0	0.0	0.9	1.6	8.6	7.0	2.1	12295.9	349.3	197.5
		SEM	0.0	0.4	0.0	0.0	0.4	0.0	0.0	0.0	0.5	1.0	0.8	1.3	0.4	4328.1	122.3	73.3
Morphine 10mg/kg/day	Naloxone + Lofexidine (0.1 mg/kg)	C1	0	26	0	0	6	0	0	0	0	8	32	14	26	1562.8	40.0	37.0
		C2	0	6	0	2	2	0	0	0	3	0	13	2	6	1486.6	48.0	19.0
		C3	0	4	0	9	6	0	0	0	0	11	30	17	4	3595.9	117.0	94.0
		C4	0	43	2	0	6	0	0	0	4	10	65	16	45	1791.1	43.0	23.0
		C5	0	41	0	1	6	2	0	0	5	1	56	7	41	1606.2	54.0	36.0
		C6	0	8	0	7	6	0	0	0	2	5	21	11	8	3780.5	98.0	83.0
		C7	0	31	2	0	6	1	0	0	0	10	50	16	33	1293.4	38.0	28.0
		C8	0	6	4	0	3	5	0	0	1	5	24	8	10	1122.9	30.0	22.0
		AVG	0.0	20.6	1.0	2.4	5.1	1.0	0.0	0.0	1.9	6.3	36.4	11.4	21.6	2029.9	58.5	42.8
		SEM	0.0	5.8	0.5	1.3	0.6	0.6	0.0	0.0	0.7	1.5	6.5	1.9	5.9	369.2	11.1	10.3
Morphine 10mg/kg/day	Vehicle + Lofexidine (0.3 mg/kg)	D1	0	0	0	0	0	0	0	0	4	0	0	0	0	148.7	1.0	7.0
		D2	0	0	0	0	0	0	0	0	6	0	6	0	0	115.8	3.0	5.0
		D3	0	8	0	2	3	0	0	0	3	0	13	3	8	986.7	25.0	27.0
		D4	0	2	0	0	5	0	0	0	4	0	11	5	2	737.6	21.0	17.0
		D5	0	3	0	0	6	0	0	0	0	1	10	7	3	18996.5	680.0	337.0
		D6	0	2	0	0	6	0	0	0	1	0	9	6	2	1536.6	40.0	16.0
		D7	0	3	0	0	6	0	0	0	3	0	12	6	3	3111.8	92.0	22.0
		D8																
		AVG	0.0	2.6	0.0	0.3	3.7	0.0	0.0	0.0	3.0	0.1	8.7	3.9	2.6	3661.9	123.1	61.6
		SEM	0.0	1.0	0.0	0.3	1.0	0.0	0.0	0.0	0.8	0.1	1.7	1.1	1.0	2584.7	93.5	46.0
Morphine 10mg/kg/day	Naloxone + Lofexidine (0.3 mg/kg)	D1	0	0	0	0	2	0	0	0	0	5	7	7	0	1976.5	71.0	55.0
		D2	0	9	0	0	5	0	0	0	0	5	14	10	9	1889.1	59.0	28.0
		D3	0	4	0	0	4	0	0	0	3	1	12	5	4	3711.8	134.0	100.0
		D4	0	5	1	0	2	0	0	0	2	8	8	10	6	1847.3	55.0	51.0
		D5	0	0	0	0	5	0	0	0	1	1	7	6	0	913.4	28.0	14.0
		D6	0	6	1	0	5	0	0	0	1	5	18	10	7	1182.5	37.0	16.0
		D7	0	0	0	0	2	0	0	0	1	0	3	2	0	1660.1	46.0	11.0
		D8	0	0	0	1	2	0	0	0	2	0	5	2	0			
		AVG	0.0	3.0	0.3	0.1	3.4	0.0	0.0	0.0	1.3	3.1	9.3	6.5	3.3	1883.0	61.4	39.3
		SEM	0.0	1.2	0.2	0.1	0.5	0.0	0.0	0.0	0.4	1.1	1.8	1.2	1.3	339.3	13.2	12.1
		QC																
			Day 9															
			Day 12															

Appendix D: LOFEXIDINE VS. NALOXONE PRECIPITATED OPIOID WITHDRAWAL: Graphs



Appendix E: LOFEXIDINE VS. NALOXONE PRECIPITATED OPIOID WITHDRAWAL: Statistical Analysis

Total Withdrawal Score

Repeated Measures Analysis of Variance (horus)
Sigma-restricted parameterization
Effective hypothesis decomposition; Std. Error of Estimate: 9.951353

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	23330.36	1	23330.36	235.5902	0.000000
Treatment	8063.08	3	2687.69	27.1403	0.000000
Error	2673.79	27	99.03		
NAL	6654.31	1	6654.31	57.9501	0.000000
NAL*Treatment	8210.31	3	2736.77	23.8336	0.000000
Error	3100.37	27	114.83		

Dunnett test; variable DV_1 (horus anx revised)
Probabilities for Post Hoc Tests (2-sided)
Error: Between; Within; Pooled MSE = 106.93, df = 53.707

Cell No.	Treatment	NAL	{4} 64.375
1	Saline	TotalVeh	0.000007
2	Saline	TotalNal	0.000007
3	Morphine	TotalVeh	0.000007
4	Morphine	TotalNal	
5	Lof0.1	TotalVeh	0.000007
6	Lof0.1	TotalNal	0.000017
7	Lof0.3	TotalVeh	0.000007
8	Lof0.3	TotalNal	0.000007

"Affect" related Withdrawal

Repeated Measures Analysis of Variance (horus anx revised)
Sigma-restricted parameterization
Effective hypothesis decomposition; Std. Error of Estimate: 4.555979

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	4513.929	1	4513.929	217.4660	0.000000
Treatment	242.756	3	80.919	3.8984	0.019538
Error	560.438	27	20.757		
NAL	193.611	1	193.611	15.8576	0.000464
NAL*Treatment	116.187	3	38.729	3.1721	0.040261
Error	329.652	27	12.209		

Dunnett test; variable DV_1 (horus anx revised)
Probabilities for Post Hoc Tests (2-sided)
Error: Between; Within; Pooled MSE = 16.483, df = 50.598

Cell No.	Treatment	NAL	{4} 14.625
1	Saline	AnxVeh	0.027023
2	Saline	AnxNal	0.014022
3	Morphine	AnxVeh	0.002659
4	Morphine	AnxNal	
5	Lof0.1	AnxVeh	0.002841
6	Lof0.1	AnxNal	0.448973
7	Lof0.3	AnxVeh	0.000038
8	Lof0.3	AnxNal	0.005072

"Malaise" related withdrawal

Repeated Measures Analysis of Variance (horus)
Sigma-restricted parameterization
Effective hypothesis decomposition; Std. Error of Estimate: 8.656434

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	5993.148	1	5993.148	79.97917	0.000000
Treatment	4608.528	3	1536.176	20.50042	0.000000
Error	2023.214	27	74.934		
NAL	4003.459	1	4003.459	62.83848	0.000000
NAL*Treatment	4662.596	3	1554.199	24.39477	0.000000
Error	1720.179	27	63.710		

Dunnett test; variable DV_1 (horus anx revised)
Probabilities for Post Hoc Tests (2-sided)
Error: Between; Within; Pooled MSE = 69.322, df = 53.648

Cell No.	Treatment	NAL	{4} 44.500
1	Saline	MalVeh	0.000007
2	Saline	MalNal	0.000007
3	Morphine	MalVeh	0.000007
4	Morphine	MalNal	
5	Lof0.1	MalVeh	0.000007
6	Lof0.1	MalNal	0.000015
7	Lof0.3	MalVeh	0.000007
8	Lof0.3	MalNal	0.000007

Total Distance Travelled

Repeated Measures Analysis of Variance (horus)
Sigma-restricted parameterization
Effective hypothesis decomposition; Std. Error of Estimate: 5749.568

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	2.197283E+09	1	2.197283E+09	66.46845	0.000000
Treatment	4.168672E+08	3	1.389557E+08	4.20345	0.014556
Error	8.925536E+08	27	3.305754E+07		
NAL	7.487455E+08	1	7.487455E+08	27.62742	0.000015
NAL*Treatment	5.387635E+08	3	1.795878E+08	6.62648	0.001688
Error	7.317415E+08	27	2.710154E+07		

Dunnett test; variable DV_1 (horus anx revised)
Probabilities for Post Hoc Tests (2-sided)
Error: Between; Within; Pooled MSE = 3008E4, df = 53.476

Cell No.	Treatment	NAL	{4} 2120.5
1	Saline	DistVeh	0.876096
2	Saline	DistNal	0.981737
3	Morphine	DistVeh	0.000035
4	Morphine	DistNal	
5	Lof0.1	DistVeh	0.003127
6	Lof0.1	DistNal	1.000000
7	Lof0.3	DistVeh	0.993181
8	Lof0.3	DistNal	1.000000

Rearing counts

Repeated Measures Analysis of Variance (horus)
Sigma-restricted parameterization
Effective hypothesis decomposition; Std. Error of Estimate: 110.7116

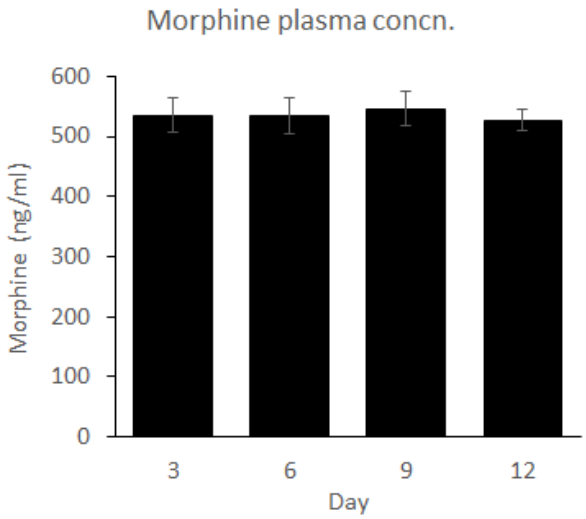
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	748516.1	1	748516.1	61.06807	0.000000
Treatment	122430.4	3	40810.1	3.32951	0.034330
Error	330941.1	27	12257.1		
NAL	253758.2	1	253758.2	26.27326	0.000022
NAL*Treatment	175859.4	3	58619.8	6.06929	0.002696
Error	260777.4	27	9658.4		

Dunnett test; variable DV_1 (horus anx revised)
Probabilities for Post Hoc Tests (2-sided)
Error: Between; Within; Pooled MSE = 10958., df = 53.251

Cell No.	Treatment	NAL	{4} 29.500
1	Saline	RearVeh	0.402934
2	Saline	RearNal	0.940056
3	Morphine	RearVeh	0.000024
4	Morphine	RearNal	
5	Lof0.1	RearVeh	0.013389
6	Lof0.1	RearNal	0.999955
7	Lof0.3	RearVeh	0.988827
8	Lof0.3	RearNal	0.999996

Appendix F: PHASE 1 PLASMA CONCENTRATION DATA

Animal ID	Study Day	Plasma Concentration of Morphine (mg/mL)	Animal ID	Study Day	Plasma Concentration of Morphine (mg/mL)
B1	Day 3	926	B3	Day 9	529
B2	Day 3	803	B4	Day 9	452
B3	Day 3	456	B5	Day 9	732
B4	Day 3	412	B6	Day 9	621
B5	Day 3	463	B7	Day 9	381
C1	Day 3	375	C3	Day 9	543
C2	Day 3	609	C4	Day 9	546
C3	Day 3	586	C5	Day 9	566
C4	Day 3	452	C6	Day 9	514
C5	Day 3	684	C7	Day 9	525
D1	Day 3	487	D3	Day 9	801
D2	Day 3	520	D4	Day 9	418
D3	Day 3	637	D5	Day 9	511
D4	Day 3	665	D6	Day 9	460
D5	Day 3	615	D7	Day 9	590
B1	Day 6	523	B2	Day 12	505
B2	Day 6	374	B4	Day 12	507
B6	Day 6	580	B6	Day 12	450
B7	Day 6	491	B7	Day 12	551
B8	Day 6	622	B8	Day 12	544
C1	Day 6	579	C1	Day 12	552
C2	Day 6	416	C3	Day 12	607
C6	Day 6	826	C5	Day 12	490
C7	Day 6	454	C7	Day 12	644
C8	Day 6	673	C8	Day 12	570
D1	Day 6	493	D1	Day 12	418
D2	Day 6	399	D2	Day 12	474
D6	Day 6	543	D4	Day 12	483
D7	Day 6	461	D6	Day 12	576
D8	Day 6	592			



For samples highlighted in RED, only 100 uL of sample was in original tube (according to protocol should be 200 uL [50 uL of plasma and 150 uL of HEPES]).
It was assumed that these samples were diluted 2x with HEPES instead of 4x. 100 uL of HEPES was added to these samples to make up to total volume of 200 uL.