Guidelines for the Efficient Extraction and Analysis of Pain Panel from Dried Blood Using the Mitra™ Microsampling Device

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The majority of clinical assays for pain panel are based in serum or plasma obtained from venous sampling in the clinic. Dried matrix microsampling via a fingerstick, using the Mitra Microsampling Device, provides an alternative to this conventional approach and delivers the benefits of convenient remote sampling, improved collection experience, and reduced sample transportation costs. This technical brief highlights a protein precipitation extraction method, requiring no derivatization of target compounds, which is capable of measuring a wide variety of clinically important pain drugs by LC-MS.

Protein Precipitation Extraction Method

A protein precipitation extraction method was evaluated, using a Neoteryx protein precipitation plate (part number 104). This method delivers a very efficient, high throughput extraction that begins with a total reconstitution of the dried blood into an aqueous extract. The extract from this method can be diluted and shot directly into an LC-MS.

1. Collect 20 µL of blood with Mitra microsamplers (see Table 1 for concentration of spiked blood used)
2. Place microsamplers in Mitra Drying AutoRack (part number 108) and allow to dry (at least 3 hours)
3. Place Mitra Drying AutoRack containing dried microsamplers onto a 2 mL, round bottom 8mm 96-well collection plate (part number 103) containing 200 µL of water with 1% formic acid (containing internal standard)
4. Sonicate collection plate and AutoRack assembly for 15 minutes (Qsonica Q700, 1" tip, 100Amp, 2 seconds sonication + 1 second pause)
5. Shake collection plate and AutoRack assembly on Vibramax 100 (Heidolph) for 1 hour @ 1200 rpm
6. Remove AutoRack containing microsamplers from the collec tion plate (microsampling tips should be light pink in color)
7. Add 10uL of 25% zinc sulfate solution in each well. Cover the wells with seal.
8. Shake collection plate on Vibramax 100 (Heidolph) for 5 minutes @ 1200 rpm
9. Centrifuge the extract at 1800G for 5 min
10. Transfer 100 µL of the supernatant to a Neoteryx Protein Precipitation Plate (part number 104)
11. Add 300 µL of acetonitrile into the protein precipitation plate well containing supernatant
12. Vacuum at 5" Hg and collect in collection plate (refer to use instructions neoteryx protein precipitation plate)
13. Evaporate solvent (TurboVap®, 40 °C and flowrate ~23-35)
14. Reconstitute samples in 10% methanol for injection
15. Transfer solvent to autosampler vials and inject into LC-MS

Table 1. Concentration of Analytes in Blood

Analyte Name	Concentration (ng/mL)
alpha-hydroxyalprazolam	50
Benzoylecgnine	2
Clonazepam	90
Codeine	72
Diazepam	16.7
Flunitrazepam	20
Hydrocodone	16.7
Hydromorphone	40
Lorazepam	39
Midazolam	4
Morphine	33.3
Norbuprenorphine	250
Nordiazepam	20
Norhydrocodone	50
Oxazepam	40
Oxycodone	40
Temazapam	43.5
Triazolam	20

Figure 1. **HCT Bias of Protein Precipitation Extraction at 8X LLOQ in Blood**
All analytes are within the +/-20% range at each hematocrit level.

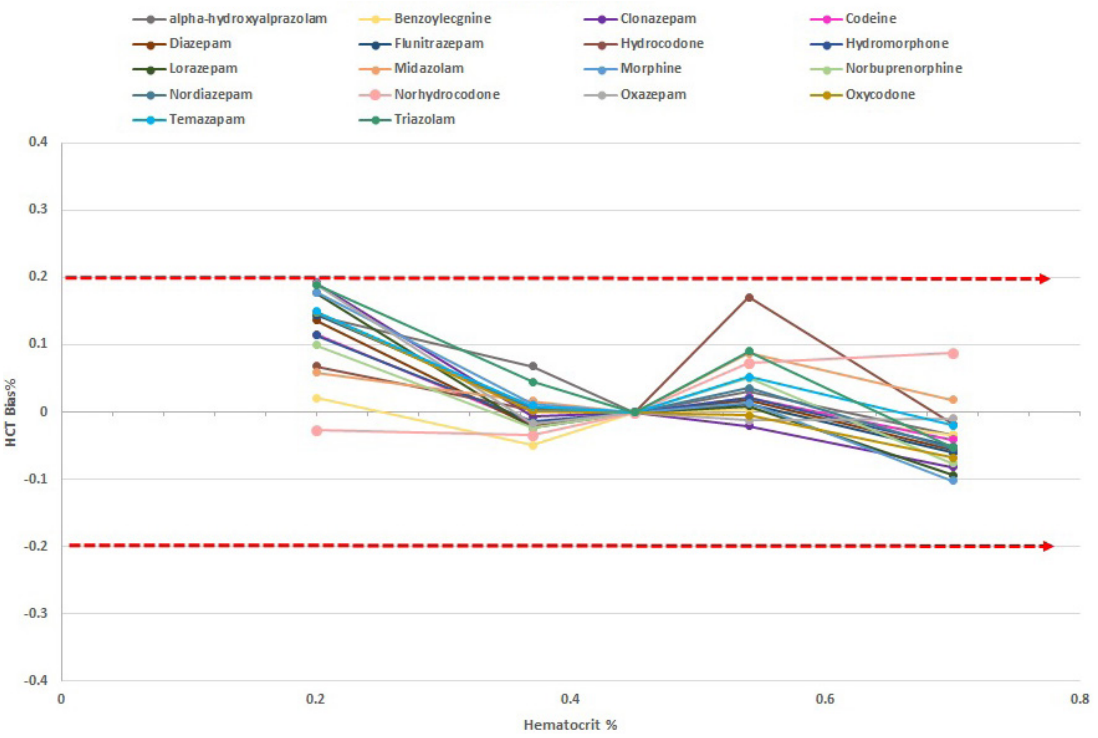


Figure 2. **Extraction Efficiency of Pain Panels at 45% HCT at 8X LLOQ**
All analytes accept Midazolam yield ≤ 120 % recovery. % CV for all analytes is less than 15%.

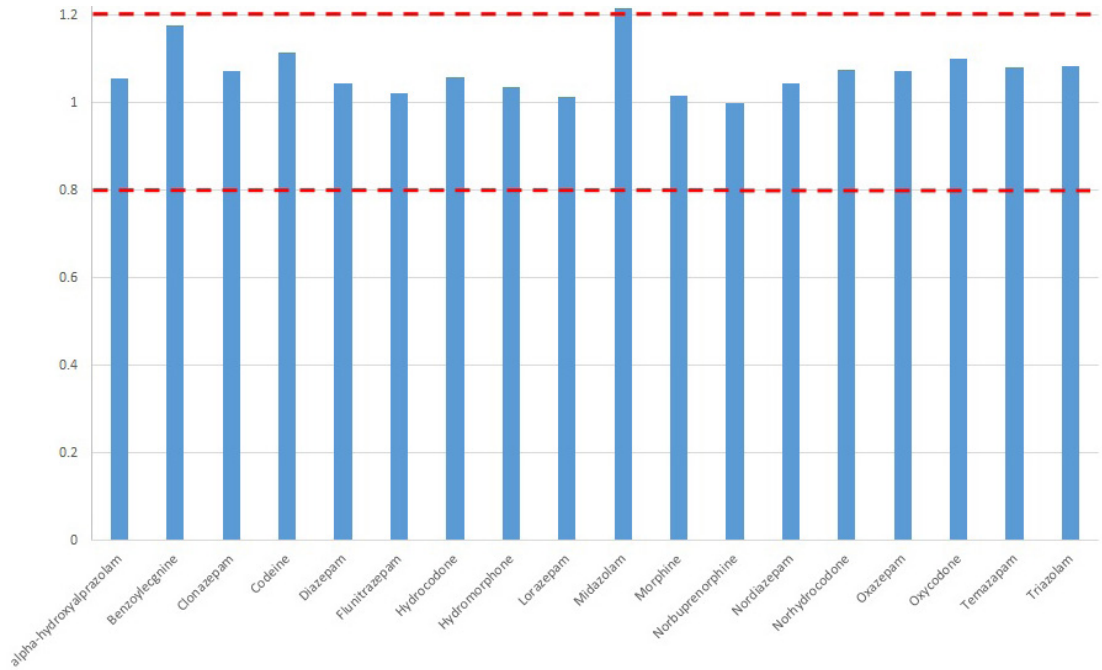


Table 2. **Calibration Curve of Protein Precipitation Method**

A 7-point calibration curves were performed using protein precipitation approach with impact plate. Below is the table at LLOQ level showing accuracy and linearity expected for the assay. The correlation coefficient for all analytes were greater than 0.975 and within standard range.

Analyte Name	Analyte Conc (ng/mL)	Calc Conc (ng/mL)	Accuracy (80-120%)	S/N ≥ 9	r Value Correl Coeff (≥0.975) $1/X^2$
alpha-hydroxyalprazolam	5.00	4.66	93	31	0.997
Benzoylecgnine	0.20	0.18	90	9	0.996
Clonazepam	9.00	8.81	98	31	0.994
Codeine	7.20	7.16	100	21	0.995
Diazepam	1.67	1.75	105	10	0.996
Flunitrazepam	2.00	1.82	91	18	0.995
Hydrocodone	1.67	1.49	89	60	0.997
Hydromorphone	4.00	3.77	94	28	0.996
Lorazepam	3.90	3.79	97	9	0.993
Midazolam	0.40	0.39	97	14	0.996
Morphine	3.33	3.56	107	10	0.996
Norbuprenorphine	25.00	23.70	95	10	0.993
Nordiazepam	2.00	1.96	98	13	0.997
Norhydrocodone	5.00	4.43	89	27	0.993
Oxazepam	4.00	3.98	99	14	0.997
Oxycodone	4.00	3.71	93	16	0.995
Temazapam	4.35	4.39	101	9	0.994
Triazolam	2.00	1.96	98	19	0.997

Table 3. **Accuracy of QC at 3 different concentrations at 45% HCT**

All three QC levels (n=6) for each analyte is within 80-120% range

Pain Panel	Conc. at Low level (ng/mL)	% CV at Low level QC	Accuracy at Low level QC (%)	Conc. at Mid level (ng/mL)	% CV at Mid level QC	Accuracy at Mid level QC (%)	Conc. at High level (ng/mL)	% CV at High level QC	Accuracy at High level QC (%)
alpha-hydroxyalprazolam	25.00	6%	108	100.0	3%	109	400	11%	104
Benzoylecgnine	1.00	3%	107	4.0	4%	104	16	9%	101
Clonazepam	45.00	8%	108	180.0	4%	108	720	11%	99
Codeine	36.00	8%	105	144.0	7%	103	576	9%	96
Diazepam	8.33	3%	108	33.3	3%	106	133	11%	102
Flunitrazepam	10.00	6%	110	40.0	2%	107	160	11%	102
Hydrocodone	8.33	6%	106	33.3	2%	105	133	9%	99
Hydromorphone	20.00	4%	105	80.0	4%	102	320	9%	97
Lorazepam	19.50	5%	114	78.0	2%	110	312	9%	97
Midazolam	2.00	3%	106	8.0	4%	108	32	13%	101
Morphine	16.67	5%	104	66.7	3%	104	267	8%	102
Norbuprenorphine	125.00	4%	115	500.0	3%	111	2000	13%	96
Nordiazepam	10.00	5%	109	40.0	3%	107	160	11%	103
Norhydrocodone	25.00	6%	106	100.0	3%	104	400	10%	97
Oxazepam	20.00	6%	107	80.0	3%	110	320	11%	102
Oxycodone	20.00	2%	106	80.0	3%	105	320	8%	95
Temazapam	21.75	6%	110	87.0	3%	109	348	11%	95
Triazolam	10.00	8%	105	40.0	4%	105	160	9%	103

Figure 3. 7 Point Calibration for Benzoylecgnine Extracted via Protein Precipitation Method.
7-point calibration curve was generated with QCs at 3 different concentrations.
The correlation coefficient for all analytes were within range

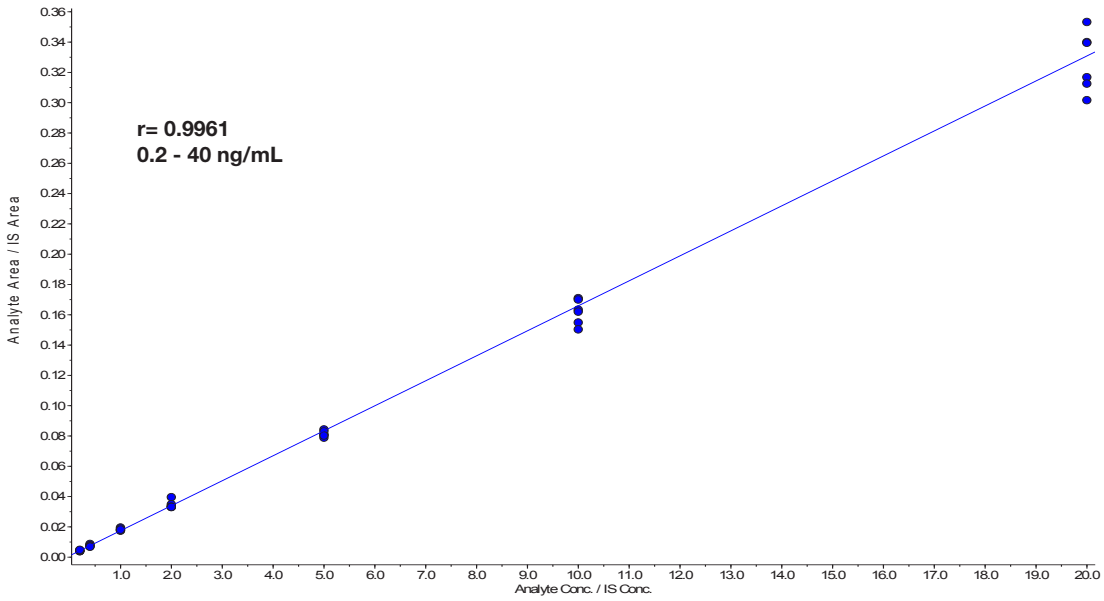
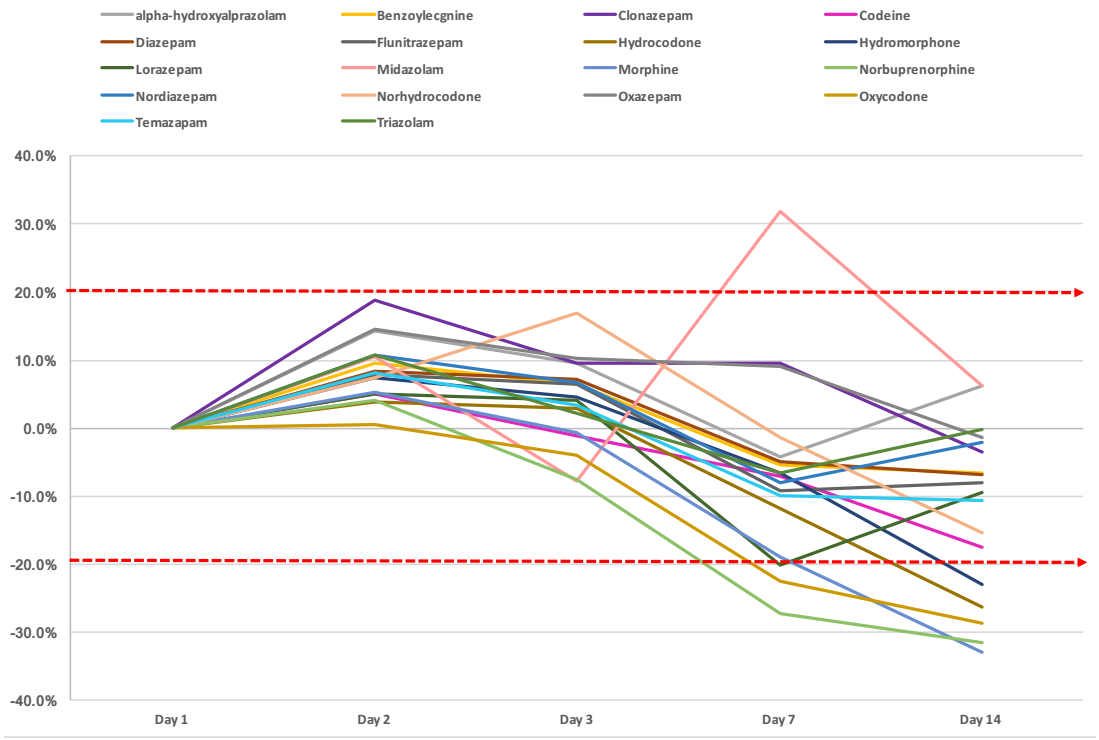
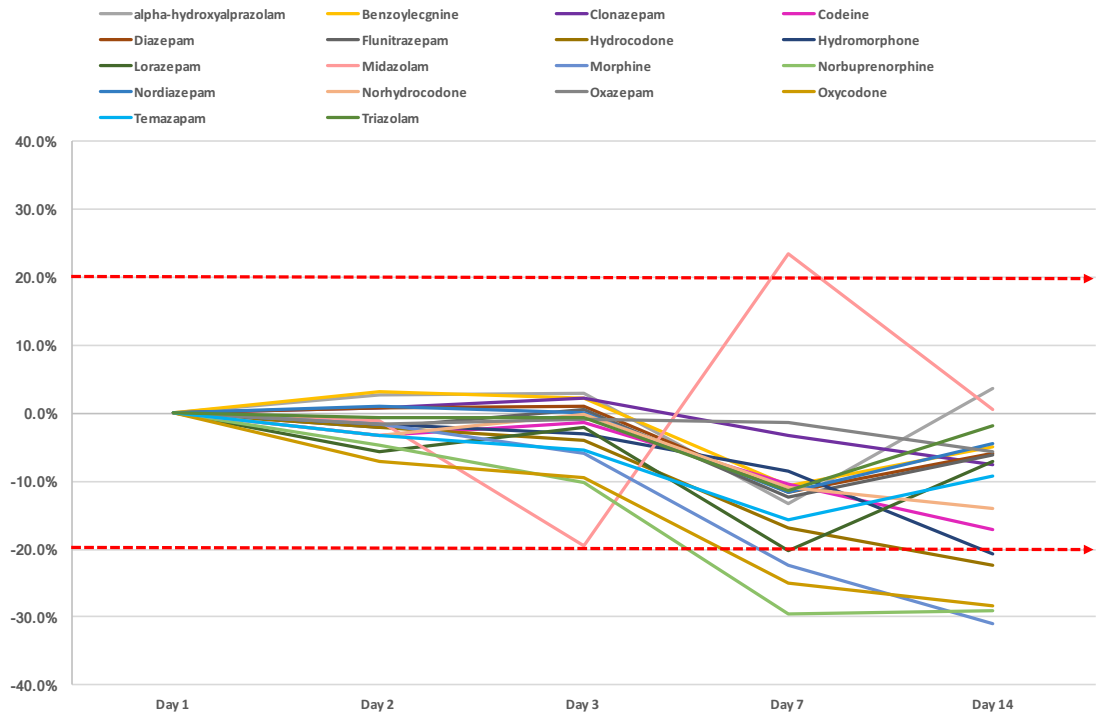


Figure 4. 14 Day Stability Study of Protein Precipitation Method (@ Low QC, Mid QC, and High QC)
Extractions were performed on days 1, 2, 7, and 14 to show the stability over time. The graph shows % bias for each calibration day when compared to day 1. Midazolam went above the 20% range for all QC level on day 7. Hydromorphone for low QC was below the -20% line at 14 days. Morphine, hydrocodone, Oxycodone and Norbuprenorphine was below the -20% line for all QC levels at 14 days.

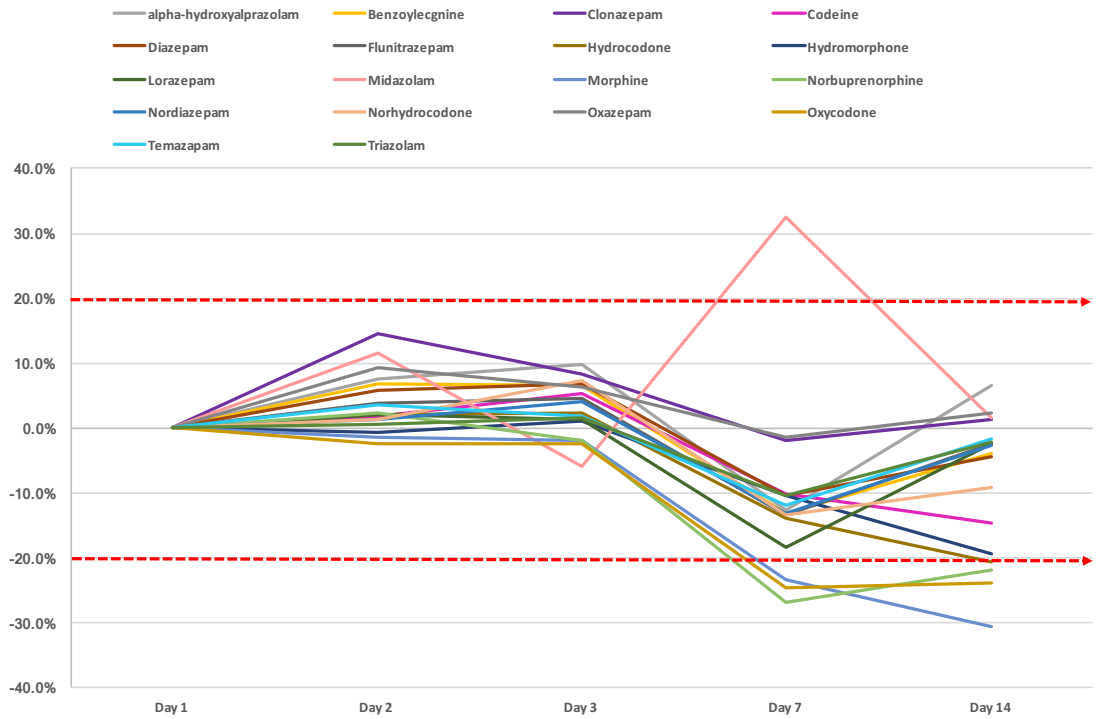
a) Stability Study at Low QC



b) Stability Study at Mid QC



c) Stability Study at High QC



LC-MS Method

This method was used to generate data for all experiments in this technical brief.

System: Agilent 1200
Column: Kinetex® 2.6 µm Biphenyl 50 x 2.1 mm ID (Phenomenex)
Guard Column: SecurityGuard® Ultra Biphenyl Cartridges (Phenomenex)
Temperature: 40 °C
Flow Rate: 0.5 mL/min
Injection Volume: 25 µL
Mobile Phase: A: 0.1% Formic Acid in Water
B: 0.1% Formic Acid in Methanol

Gradient:

Time (min)	Flow Rate	A (%)	B (%)
0.00	500	90	10
0.25	500	90	10
2.75	500	30	70
4.00	500	20	80
4.01	500	10	90
5.00	500	10	90
5.01	500	90	10
6.50	500	90	10

Detection: MS (ESI Positive Mode)
Detector: SCIEX API 4000™ System

MS Conditions

ESI Positive Mode
MS Method: Pain Panel_schmrm_021216.dam
Parameter Table (Period 1 Experiment 1)

CUR:	25.00
GS1:	40.00
GS2:	40.00
IS:	5000.00
TEM:	450.00
ihe:	ON
CAD	7.00
EP:	10.00

MRM transitions and MS paramters

Analyte Name	RT Time	MRM-1	MRM-2	DP	EP	CE	CXP
alpha-hydroxyalprazolam	3.6	325.262	297.1	51	10	37	6
alpha-hydroxyalprazolam-D5	3.6	330.221	302.1	66	10	35	6
Benzoylecgnine	2.4	290.296	168	56	10	27	14
Benzoylecgonine-D8	2.4	298.372	171.1	46	10	29	14
Clonazepam	3.5	316.216	270.1	76	10	35	24
Clonazepam-D4	3.5	320.256	274.1	66	10	35	6
Codeine	1.8	300.316	115.1	76	10	89	10
Codeine-D6	1.8	306.326	152	66	10	77	14
Diazepam	4.2	285.198	193.1	41	10	45	16
Diazepam-D5	4.2	290.291	154.1	71	10	37	14
Flunitrazepam	3.8	314.23	268.2	51	10	35	6
Flunitrazepam-D7	3.8	321.21	275.3	56	10	37	6
Hydrocodone	2	300.308	199.1	91	10	41	16
Hydrocodone-D6	1.9	306.366	202.2	86	10	41	14
Hydromorphone	1.3	286.31	185	86	10	39	16
Hydromorphone-D6	1.3	292.337	185.1	76	10	41	16
Lorazepam	3.5	321.191	274.9	46	10	31	20
Lorazepam-D4	3.4	325.246	279.1	46	10	31	6
Midazolam	3.1	326.301	291.1	71	10	35	6
Morphine	1.1	286.311	152.1	81	10	77	12
Morphine-D3	1.1	289.328	152.1	86	10	75	12
Norbuprenorphine	2.7	414.423	101.1	101	10	53	8
Norbuprenorphine-D3	2.7	417.449	101.1	106	10	51	8
Nordiazepam	3.7	271.235	140	86	10	41	12
Nordiazepam-D5	3.7	276.271	140	81	10	39	12
Norhydrocodone	1.8	286.309	199.1	81	10	37	16
Oxazepam	3.5	287.221	241	61	10	31	22
Oxazepam-D5	3.5	292.228	246.1	46	10	31	22
Oxycodone	1.9	316.317	298.2	56	10	25	6
Oxycodone-D6	1.9	322.346	304.3	71	10	27	8
Temazapam	3.9	301.219	255.1	51	10	29	6
Temazapam-D5	3.9	306.293	260.1	56	10	29	24
Triazolam	3.8	343.161	315.1	71	10	37	8

Corresponding internal standard (IS) used for quantification. Analytes marked with an asterisk are stable labels.

Analyte Name	Corresponding IS
alpha-hydroxyalprazolam	alpha-hydroxyaprazolam-D5*
Benzoylecgnine	Benzoylecgonine-D8*
Clonazepam	Clonazepam-D4*
Codeine	Codeine-D6*
Diazepam	Diazepam-D5*
Flunitrazepam	Flunitrazepam-D7*
Hydrocodone	Hydrocodone-D6*
Hydromorphone	Hydromorphone-D6*
Lorazepam	Lorazepam-D4*
Midazolam	Diazepam-D5*
Morphine	Morphine-D3*
Norbuprenorphine	Norbuprenorphine-D3*
Nordiazepam	Nordiazepam-D5*
Norhydrocodone	Norhydrocodone-D3*
Oxazepam	Oxazepam-D5*
Oxycodone	Oxycodone-D6*
Temazapam	Temazepam-D5*
Triazolam	Temazepam-D5*

20µL 96-autorack (PN 20101) & 20µL Mitra 2-sampler Cartridge (PN 20100)

20µL 96-rack (PN 20006) & 20µL Mitra 4-sampler Clamshell (PN 20004)

Ordering Information

Part No.	Description	Unit
96-rack / 96-autorack format		
20006	20µL Mitra Microsampler 96-rack	6/pk
20101	20µL Mitra Microsampler 96-autorack	1 ea
clamshell format		
20004	20µL Mitra Microsampler 4-sampler Clamshell	52/pk
20002	20µL Mitra Microsampler 3-sampler Clamshell	52/pk
20001	20µL Mitra Microsampler 2-sampler Clamshell	52/pk
cartridge format		
20100	20µL Mitra Microsampler 2-sampler Cartridge / Desiccant Pack	8/pk
reformatting and extraction accessories		
100	Mitra Drying Rack	1 ea
108	Mitra Drying AutoRack	1 ea
104	Neoteryx Protein Precipitation Plate	2/pk
103	96-Well Collection Plate, 2mL Round Well, Round Bottom, 8mm	50/pk
105	Pierceable Sealing Mat, 96-Round Well, 8mm, Silicone	50/pk
106	Pre-Slit Sealing Mat, 96-Round Well, 8mm, Silicone	50/pk
107	Sealing Tape Pad	10/pk

CE IVD

The Mitra Microsampling Device is a FDA listed Class 1 device (D254956). Neoteryx complies with FDA good manufacturing practices, CFR 820 regulations, and ISO 13485

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