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technical brief

Guidelines for the Efficient Extraction and Analysis of Hormones/ Steroids from Dried Blood Using the Mitra™ Microsampling Device

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The majority of clinical assays for steroids/hormones 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 patient experience, and reduced sample transportation costs. This technical brief highlights multiple extraction methods, requiring no derivatization of target compounds, which are capable of measuring a wide variety of clinically important steroids/hormones by LC-MS/MS.

Two sample extraction procedures were evaluated using a 96-well plate format. The first is a solid-liquid extraction of the dried blood using 100 % methanol. Advantages of this type of extraction include fast evaporation, fewer steps, and a very clean extraction. However, a methanol extraction may not be the best option if analyzing very polar analytes, if correlation is needed to a protein precipitation reference method, or if a dilute-and-shoot LC/MS approach is preferred.

A protein precipitation, using a Neoteryx protein precipitation plate, was the second extraction method evaluated. This second 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/MS.

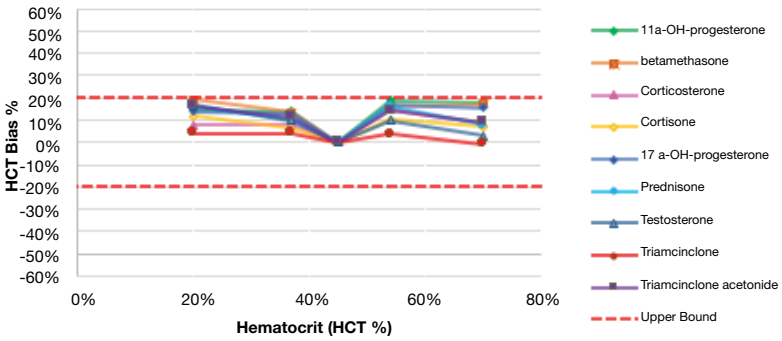
Table 1.
Concentration of Analytes in Blood

Steroids	5X LLOQ-ng/mL	MeOH-500 ng/mL	PPT-1500 ng/mL
11a-OH-progesterone	25	500	1500
betamethasone	12.5	500	1500
Corticosterone	50	500	1500
Cortisone	40	500	1500
17a-OH-progesterone	25	500	1500
Prednisone	50	500	1500
Testosterone	12.5	500	1500
Triamcinolone	12.5	500	1500
Triamcinolone Acetonide	6.25	500	1500

Methanol Extraction Method

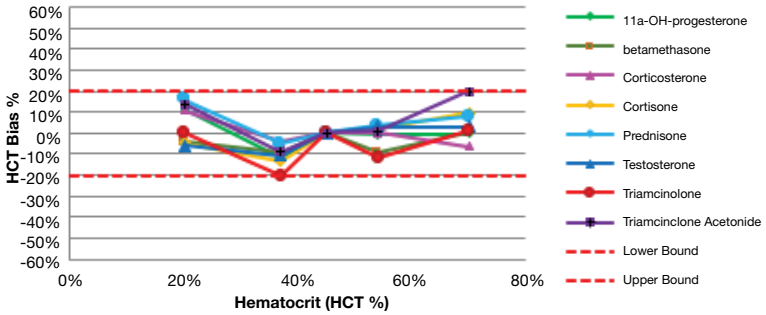
1. Mitra sampling tips collected 10 µL of spiked blood (see Table 1 for concentration)
2. Samples were allowed to completely dry on a rack (at least 3 hours)
3. Dried samplers were removed from the rack and placed into a 96-well collection plate (2 mL) containing 300 µL of methanol (containing internal standard)
4. Collection plate was sonicated for 15 minutes (Qsonica Q700, 1" tip 100Amp, 2 seconds sonication + 1 second pause)
5. Then the plate was shaken on a Vibramax 100 (Heidolph) shaker for 1 hour @ 1200 rpm
6. Remove the samplers from 96-well collection plate (sampling tips should be red in color)
7. Centrifuge the extract 1800G for 5 min
8. Transfer 150 µL of the supernatant to a new 96-well collection plate
9. Evaporate the solvent (TurboVap®, 40 °C and flowrate ~23-35)
10. Reconstitute samples in 20 % acetonitrile for injection
11. Transfer solvent to autosampler vials and inject into LC/MS

Figure 1.
HCT Bias of Methanol Extraction at 5x LLOQ Blood



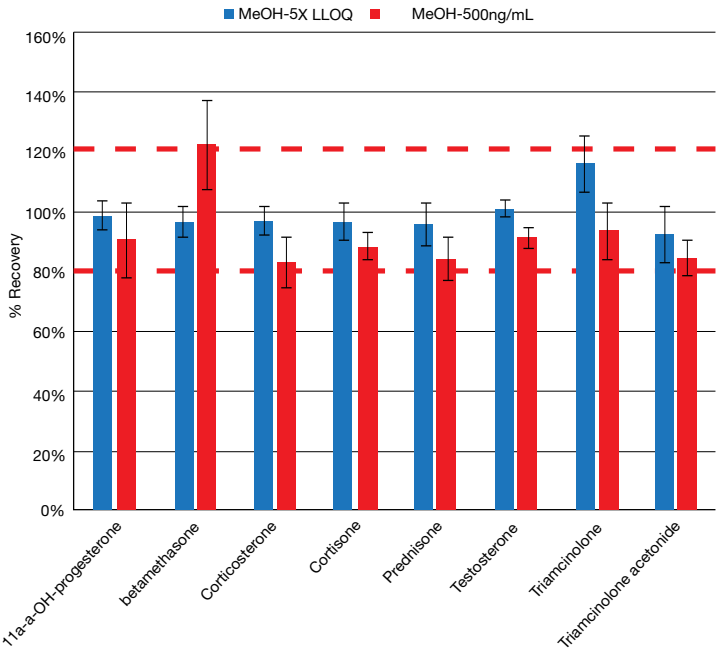
All analytes are with 20 % range for 5 Point HCT bias with a methanol extraction

Figure 2.
HCT Bias of Methanol Extraction at 500 ng/mL in Blood



All analytes are with 20 % range for 5 Point HCT bias with a methanol extraction

Figure 3
Recovery of Steroids with Methanol Extraction -5x LLOQ vs 500 ng/mL in Blood



All analytes yield greater than 80 % recovery, Betamethasone showed high yield at 500 ng/mL conc. % CV for all analytes is less than 15 %

Table 2.
Calibration Curve of Methanol Extraction Method

Analyte Name	Analyte Conc at LLOQ level	Calc Conc.at LLOQ level	Accuracy (80120 %)	S/N	Correl Coeff (≥0.975) 1/X²
	(ng/mL)	(ng/mL)	%		r value
11a-OH-progesterone	5	5.5	110	23	0.995
betamethasone	2.5	2.2	88	12	0.995
Corticosterone	10	9.5	95	12	0.997
Cortisone	8	7.9	99	75	0.998
17a-OH-progesterone	5	4.9	99	44	0.998
Prednisone	10	9.8	98	16	0.998
Testosterone	2.5	2.5	100	100	0.995
Triamcinolone	2.5	2.5	101	25	0.998
Triamcinolone Acetonide	1.25	1.3	104	28	0.998

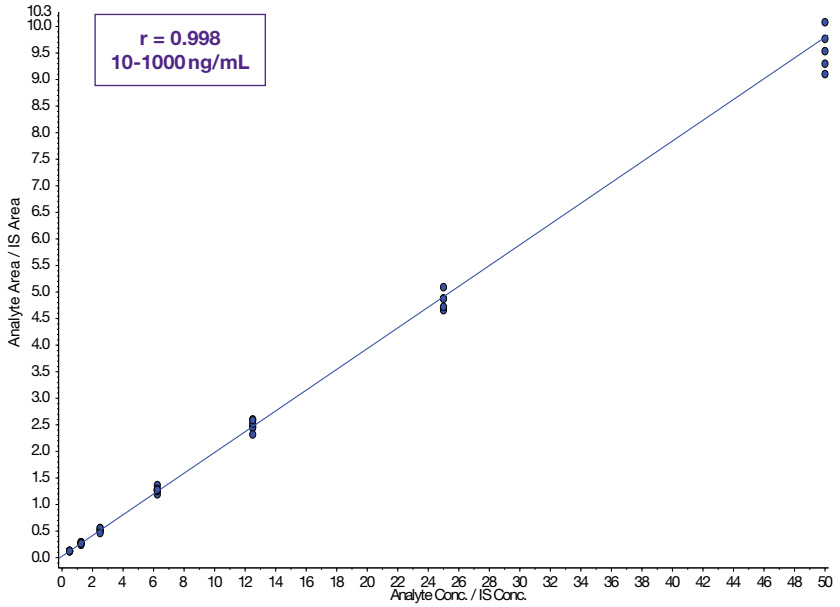
A 7-point calibration curves was performed using methanol. Above is the table at LLOQ level showing accuracy and linearity expected for the assay. The correlation coefficient for all analytes were greater than 0.975 and within standard range.

Table 3.
Accuracy of QC at 3 different concentrations with Methanol Extraction at 45 % HCT

Steroids	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 QC (%)	% CV at High Level QC	Accuracy at High Level QC (%)
11a-OH-progesterone	37.5	6.63	120.3	100	4.74	97.0	200	8.15	101.1
betamethasone	18.75	2.88	102.0	50	1.76	101.5	100	6.08	103.7
Corticosterone	75	4.33	99.7	200	4.41	100.1	400	9.71	104.1
Cortisone	60	9.48	103.6	160	3.14	100.8	320	7.51	102.8
17a-OH-progesterone	37.5	2.94	95.4	100	2.28	99.8	200	9.21	104.7
Prednisone	75	5.23	98.8	200	2.94	98.4	400	6.49	104.0
Testosterone	18.75	3.63	103.8	50	2.53	103.0	100	6.14	101.7
Triamcinolone	18.75	4.30	99.3	50	3.27	98.9	100	8.17	101.3
Triamcinolone Acetonide	9.375	5.96	96.8	25	3.51	100.6	50	5.36	103.7

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

Figure 4
7 Point Calibration for Prednisone Extracted via Methanol Extraction Method

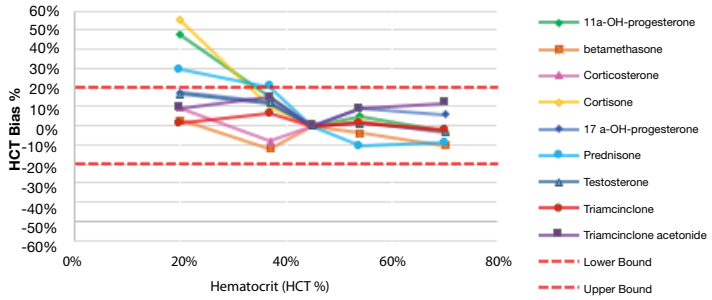


This 7-point calibration curve for prednisone is provided as an example to show the range and linearity expected for the assay.

Protein Precipitation Extraction Method (using a Neoteryx Protein Precipitation Plate)

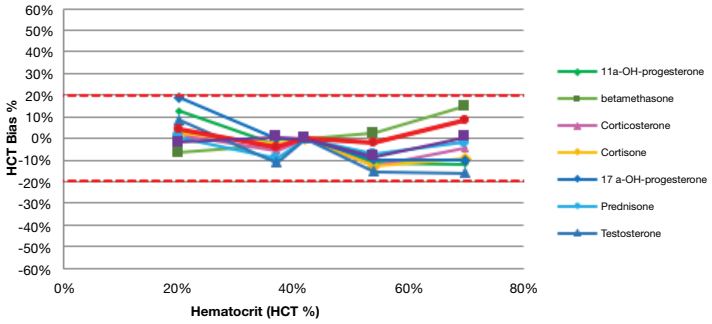
1. Mitra sampling tips collected 10 µL of spiked blood (see Table 1 for blood concentration)
2. Samples were allowed to completely dry on a rack (a least 3 hours)
3. Dried samplers were removed from the rack and placed into a 96-well collection plate (2 mL) containing 200 µL of water with 1 % formic acid (containing internal standard
4. Collection plate was sonicated for 15 minutes (Qsonica Q700, 1" tip 100Amp, 2seconds sonication + 1 second pause)
5. Then the plate was shaken on a Vibramax 100 (Heidolph) shaker for 1 hour @ 1200 rpm
6. Remove the samplers from 96-well collection plate (sampling tips should be light pink in color)
7. Centrifuge the extract 1800G for 5 min
8. Transfer 100 µL of the supernatant to a Neoteryx Protein Precipitation Plate
9. Add 300uL of acetonitrile into the protein precipitation plate well containing supernatant
10. Vacuum at 5" Hg and collect in 96-well collection plate
11. Evaporate solvent (TurboVap®, 40 °C and flowrate ~23-35)
12. Reconstitute samples in 20 % acetonitrile for injection
13. Transfer solvent to autosampler vials and inject into LC/MS

Figure 5
HCT Bias of Protein Precipitation Extraction at 5x LLOQ in Blood



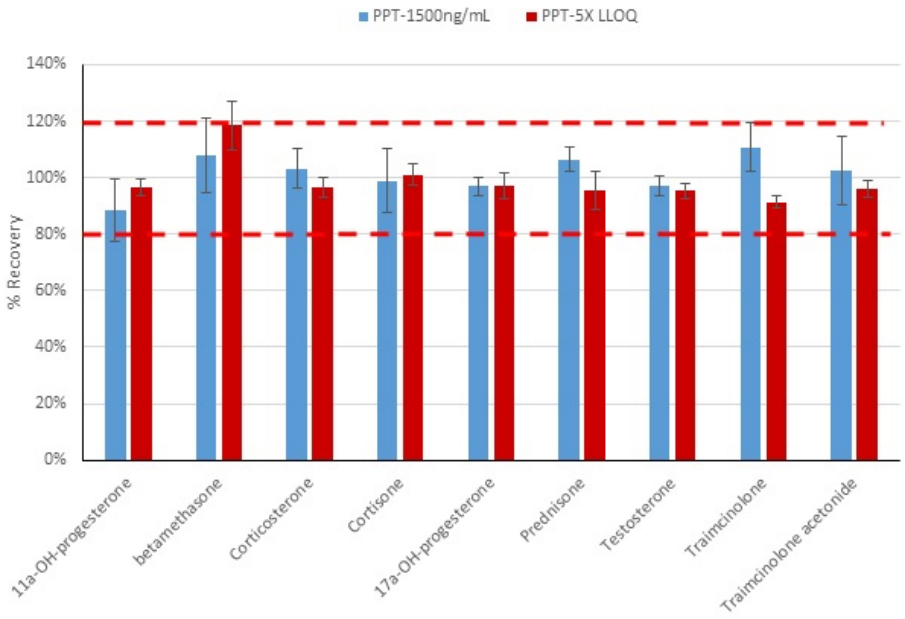
At low hematocrit (20 %) the matrix interference is less and hence the analyte recovery is higher compare to that 45 %. This phenomenon is more enhanced at low concentrations.

Figure 6
HCT Bias of Protein Precipitation Extraction at 1500 ng/mL in Blood



All analytes are with 20 % range for 5 Point HCT bias with a protein precipitation extraction

Figure 7
Recovery of Steroids with Protein Precipitation Extraction at 45 % HCT –5X LLOQ vs 1500 ng/mL



All analytes yield greater than 80 % recovery. % CV for all analytes is less than 15 %

Table 4.
Calibration Curve of Protein Precipitation Method

Analyte Name	Analyte Conc at LLOQ level	Calc Conc.at LLOQ level	Accuracy (80-120 %)	S/N	Correl Coeff (≥0.975) 1/X2
	(ng/mL)	(ng/mL)	%		r value
11a-OH-progesterone	5	5.1	101	13	0.994
betamethasone	2.5	2.8	113	11	0.993
Corticosterone	10	9.1	91	11	0.989
Cortisone	8	8.3	103	11	0.990
17a-OH-progesterone	5	5.1	101	29	0.998
Prednisone	10	10.8	108	10	0.998
Testosterone	2.5	2.5	101	16	0.998
Triamcinolone	2.5	2.4	97	13	0.996
Triamcinolone Acetonide	1.25	1.4	115	28	0.996

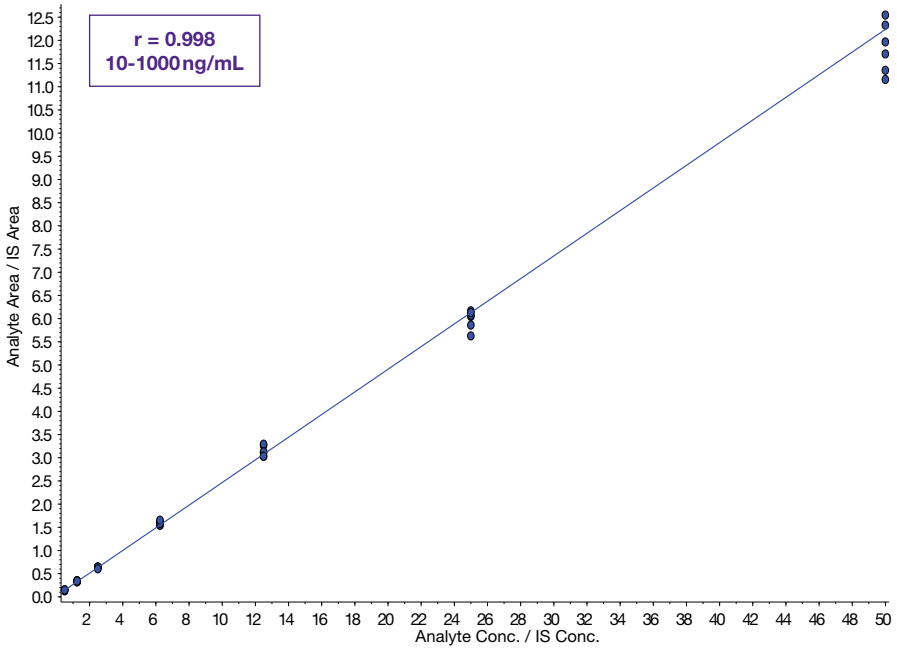
A 7-point calibration curves was performed using protein precipitation approach with impact plate. Below is the table at LLOQ level showing accuracy and linearity expected for the assay. The correlation coefficient for all analytes were greater than 0.975 and within standard range.

Table 5.
Accuracy of QC at 3 different concentrations with Protein Precipitation Extraction at 45 % HCT

Steroids	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 QC (%)	% CV at High Level QC	Accuracy at High Level QC (%)
11a-OH-proges-terone	37.5	4.98	95.8	100	8.70	95.8	200	3.60	104.8
betamethasone	18.75	4.26	96.8	50	2.54	95.2	100	5.17	102.9
Corticosterone	75	6.68	103.9	200	6.47	93.4	400	10.97	101.9
Cortisone	60	10.56	95.7	160	5.67	86.4	320	6.56	97.3
17a-OH-proges-terone	37.5	3.46	96.6	100	3.20	94.8	200	5.50	100.9
Prednisone	75	3.87	106.2	200	2.91	103.3	400	4.92	97.9
Testosterone	18.75	3.85	99.0	50	3.33	96.0	100	2.19	99.6
Triamcinolone	18.75	4.77	104.6	50	7.66	97.9	100	6.61	97.3
Triamcinolone Acetonide	9.375	6.68	100.1	25	6.02	98.0	50	3.16	100.8

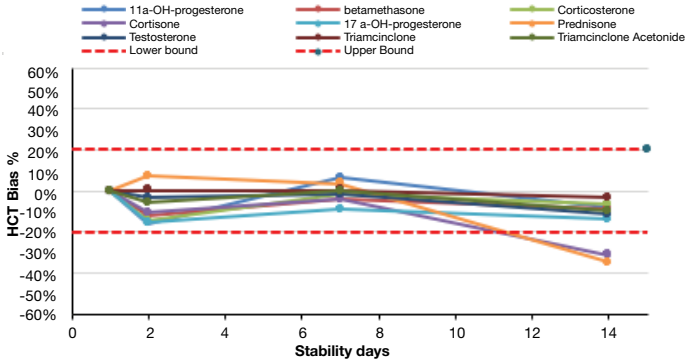
All three QC levels (n=6) for each analyte is within 80-120 % range

Figure 8
7 Point Calibration for Prednisone Extracted via Protein Precipitation Method



This 7-point calibration curve for prednisone is provided as an example to show the range and linearity expected for the protein precipitation assay.

Figure 9
14 Day Stability Study of Protein Precipitation Method (High QC)



Extractions were performed on days 1, 2, 7, and 14 to show the stability over time and shows % bias for each calibration day when compared to day 1. Prednisone and cortisone fell below the -20 % line at 14 days. These were the two lowest LC/MS signal analytes.

MRM transitions and MS parameters:

Analyte	MRM-1	MRM-2	DP	EP	CE	CXP
11a-OH-progesterone	331.4	313.3	50	10	20	10
betamethasone	393.2	373.2	50	10	15	10
Corticosterone	347.4	329.5	50	9	20	10
Cortisone	361.3	163.2	55	14	30	10
17a-OH-progesterone	331.2	109.1	45	12	30	10
Prednisone	359.2	313.2	50	10	15	10
Testosterone	289.1	109	45	10	30	10
Triamcinolone Acetonide	435.3	415.1	45	10	15	12
Triamcinolone	395.2	375.3	40	10	15	10
Triamcinolone-d7*	442.5	404.2	45	10	20	10
Cortisone-d7*	368.3	168.2	55	14	30	10
17a-OH-progesterone-d8*	339.2	113.4	45	12	30	10
Corticosterone-d8*	355.4	337.4	45	10	20	8
Dexamethasone-d4*	397.3	377.2	40	8	15	10
Testosterone-d3*	292.3	109.2	60	9	25	10
Prednisolone*	361.2	147.2	45	10	25	14

Analytes marked with an asterisk are stable labels

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.

LC/MS Method

The method provided below was used to generate data for all experiments within this technical brief

System: Agilent 1200/ ABI 4000
Column: Kinetex® 2.6 µm C18 50 x 2.1 mm ID (Phenomenex)
Guard Column: Security Guard Ultra C18 Cartridges (Phenomenex)
Temperature: 40 °C
Flow Rate: 0.5 mL/min
Injection Volume: 25 µL
Mobile Phase: A: 1 L 100 % water / 1 mL formic acid
B: 1 L 100 % acetonitrile/ 1 mL formic acid
Gradient:

Time (min)	% B
0.00	25
3.00	95
5.0	95
5.10	25
7.00	25

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

Corresponding internal standard (IS) used for quantification:

Analyte	Corresponding IS
11a-OH-progesterone	17a-OH-progesterone-d8
betamethasone	Dexamethasone-d4
Corticosterone	Corticosterone-d8
Cortisone	Cortisone-d7
17a-OH-progesterone	17a-OH-progesterone-d8
Prednisone	Prednisolone
Testosterone	Testosterone-d3
Traimcinolone	Corticosterone-d8
Traimcinolone acetonide	Triamcinolone Acetonide-d7

Ordering Information

Part No.	Description	Unit
10005	Mitra 10 µL Microsampler 96-Rack	1 ea
10006	Mitra 10 µL Microsampler 96-Rack	6/pk
10004	Mitra 10 µL Microsampler 4-Sampler Clamshell	52/pk
10002	Mitra 10 µL Microsampler 3-Sampler Clamshell	52/pk
10001	Mitra 10 µL Microsampler 2-Sampler Clamshell	52/pk
100	Mitra Drying Rack	1 ea
102	Mitra Sampling Tool	1 ea
104	Protein Precipitation Plates	2/pk
103	96-Well Collection Plates, 2 mL Round Well with Round Bottom, 8mm	50/pk
105	Sealing Mats, Pierceable, 96-round well, 8 mm, silicone	50/pk
106	Sealing Mats, Pre-slit, 96-round well, 8 mm, silicone	50/pk
107	Sealing Tape Pad	10/pk

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