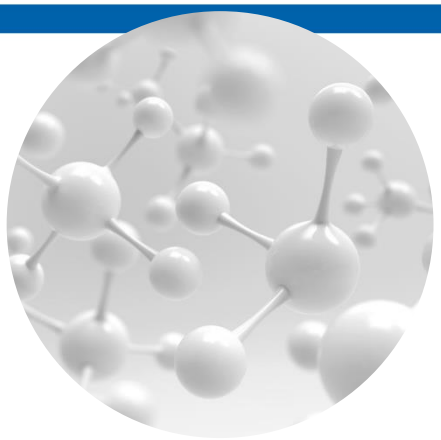

TN-0153

Simplifying Urine Drug Testing by Combining IMCSzyme® RT and β-Gone™ Plus β-Glucuronidase Removal

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Introduction

β-Glucuronidase is used to hydrolyze Glucuronic Acid from phase II metabolites present in biological fluids to simplify parent analyte detection using mass spectrometry. After enzymatic hydrolysis, the sample may be further processed with sample clean-up techniques to remove residual enzyme to improve LC column life, minimize matrix effects, or enhance signal-to-noise levels. In this technical note, we combine enzymatic hydrolysis and protein removal into a single cohesive workflow that is both fast and simple. First, we examine the compatibility of IMCSzyme RT (genetically modified β-Glucuronidase) with β-Gone Plus (96-well plate that removes β-Glucuronidase). Second, we assess β-Gone Plus performance by determining three factors: extent of protein removal from samples, impact on matrix effects, and various analyte recoveries.

Compared to other protein crash and SPE clean-up workflows, β-Gone Plus reduces plastic consumables, organic waste, and preparation time by performing sample hydrolysis within the filter plate. In-well hydrolysis bypasses the need for an extra 96-well plate to mix the urine sample, enzyme, buffer, internal standards, and an additional time-consuming transfer step. Compared to the traditional protein precipitation process using organic solvents, the ratio of organic solvent to sample is < 50 % lower with β-Gone Plus, as most protein precipitation processes will use 3- to 4-fold more organic solvent to achieve similar results. Similarly, solid-phase extraction (SPE) requires sorbent conditioning and equilibration steps that increase organic solvent use and time.

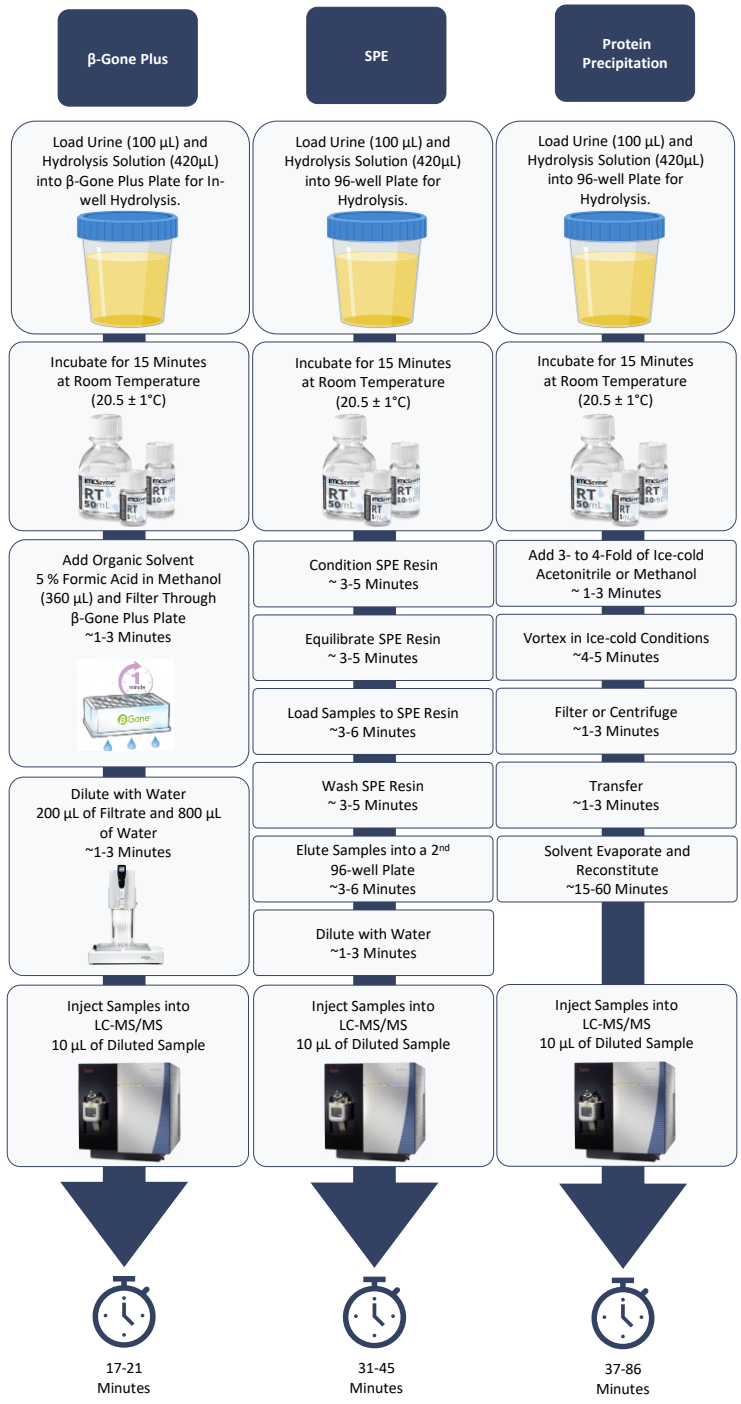
Sample Preparation

Urine and hydrolysis solution (**Table 1**) were loaded into a β-Gone Plus plate (Part No.: 8E-S323-TGA) for in-well hydrolysis. Due to the modified top filter, the aqueous solution remains above the filter plate until methanol is mixed into the sample solution. After a 15-minute incubation at room temperature, 360 µL of 5 % Formic Acid in Methanol was added, and then samples were filtered through the plate using a centrifuge at 500 × g for 1 minute. Filtered samples (200 µL) were diluted with 800 µL of water, and 10 µL of diluted sample was injected for analysis. Urine controls were spiked with 15 Glucuronide standards at an equivalent to 500 ng/mL.

Table 1. Hydrolysis Using Different Amounts of IMCSzyme RT

Sample (100 µL)	IMCSzyme RT (µL)	Enzyme Buffer (µL)	Room Temperature Hydrolysis Buffer (µL)	Internal Standard (µL)
Surine™ (synthetic urine control)	0	100	300	20
	10	90		
or	20	80		
Certified Drug-free Urine (DFU) (urine control)	40	60		
	60	40		
or	80	20		
Urine	100	0		

Sample Preparation Workflow



Have questions or want more details on implementing this method? We would love to help! Visit www.phenomenex.com/Chat to get in touch with one of our Technical Specialists

Revision: 2
PHEN-RUO-00172

LC Conditions

Column: Kinetex™ 2.6 µm Biphenyl
Dimensions: 50 x 4.6 mm
Part No.: 00B-4622-E0
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile

Gradient:

Time (min)	%B
0	5
0.5	5
8.5	95
9	95
9.2	5

Flow Rate: 0.6 mL/min
Injection Volume: 10 µL
Temperature: 40 °C
Instrument: Thermo Scientific™ Vanquish™ HPLC
Detector: Thermo Scientific TSQ Endura™ MS
Detection: MS/MS

MS/MS Conditions

Electrospray: 1000 V
Sheath Gas: 55 arb
Auxiliary Gas: 11 arb
Sweep Gas: 1 arb
Ion Transfer Tube Temperature: 300 °C
Vaporizer Temperature: 300 °C

Table 2. MRM Transitions

Analyte	Retention Time (min)	Precursor (m/z)	Product (m/z)
Oxymorphone-Glucuronide (OMOR gluc)	1.10	478.183	284.04, 460.097
Morphine-Glucuronide (MOR gluc)	1.12	462.183	211.012, 286.04
Hydromorphone-Glucuronide (HMOR gluc)	1.23	462.183	184.986, 286.054
Morphine (MOR)	2.09	286.4	157.1, 165.1, 183.1
Morphine-D3 (MOR-D3)	2.09	289.1	165.054
Oxymorphone (OMOR)	2.29	302.152	227.058, 284.058
Oxymorphone-D3 (OMOR-D3)	2.29	305.152	287.04
Naloxone-Glucunoride (NXONE gluc)	2.34	504.17	310.169, 328.222, 486.222
Hydromorphone (HMOR)	2.54	286.122	157, 184.986
Hydromorphone-D3 (HMOR-D3)	2.54	289.183	184.929
Naltrexol-Glucuronide (NXOL gluc)	2.77	520.27	326.468, 501.994
Dihydrocodeine-Glucuronide (DCOD gluc)	2.83	478.183	199.04, 302.111
Codeine-Glucuronide (COD gluc)	2.85	476.213	282.169, 300.111
Naloxone (NXONE)	2.91	328.22	267.879, 310.022
Naloxone-D5 (NXONE-D5)	2.91	333.2	315.151
Dihydrocodeine (DCOD)	2.93	302.183	199.058, 201.058
Dihydrocodeine-D6 (DCOD-D6)	2.93	308.239	202
Codeine (COD)	2.99	300.19	165.111, 215.111
Codeine-D6 (COD-D6)	2.99	306.183	218.111
Naltrexol (NXOL)	3.17	343.865	254.24, 326.169
Naltrexol-D3 (NXOL-D3)	3.17	347.24	329.24
Tapentadol-Glucuronide (TAP gluc)	3.24	398.183	107.169, 222.183
Norbuprenorphine-Glucuronide (NBUP gluc)	3.34	590.304	396.208, 414.222
Tapentadol (TAP)	3.79	222.183	107.111, 121.111
Tapentadol-D3 (TAP-D3)	3.79	225.183	107.111
Buprenorphine-Glucuronide (BUP gluc)	4.07	644.243	369.222, 414.222, 468.262
Norbuprenorphine (NBUP)	4.15	414.27	340.11, 396.208
Norbupenorphine-D3 (NBUP-D3)	4.15	417.274	343.165
Oxazepam-Glucuronide (OXZ gluc)	4.54	463	241, 287
Lorazepam-Glucuronide (LOR gluc)	4.63	498.7	276.875, 322.889
Amitriptyline-Glucuronide (AMT gluc)	4.81	454.183	233.04, 278.111
Temazepam-Glucuronide (TEM gluc)	4.83	477.152	255, 301.058
Buprenorphine (BUP)	4.9	468.365	396.151, 414.222
Buprenorphine-D4 (BUP-D4)	4.9	472.274	400.222
Oxazepam (OXZ)	5.34	287	241, 269
Oxazepam-D5 (OXZ-D5)	5.34	292	245.986
Amitriptyline (AMT)	5.39	278.243	117.111, 233.111
Amitriptyline-D3 (AMT-D3)	5.39	281.183	233.04
Lorazepam (LOR)	5.42	322.091	275.96, 303.875
Lorazepam-D4 (LOR-D4)	5.42	326.7	280.946
Temazepam (TEM)	5.9	301.091	255.058, 282.986
Temazepam-D5 (TEM-D5)	5.9	306.091	260.04
11-Nor-9-carboxy-Δ^9-tetahydrocannabinol-Glucuronide (cTHC gluc)	6.29	521.335	327.111, 345.111
11-Nor-9-carboxy-Δ^9-tetahydrocannabinol (cTHC)	7.14	345.274	299.111, 327.04
11-Nor-9-carboxy-Δ^9-tetahydrocannabinol-D3 (cTHC-D3)	7.14	348.312	330.111

Results and Discussion

Synthetic urine (Surine™) and drug-free urine (DFU) were hydrolyzed using different amounts of IMCSzyme® RT, ranging from 0 to 100 µL. Analytes were considered hydrolyzed when ≥ 400 ng/mL was quantified, or > 80 % recovery of target concentration of 500 ng/mL was reached, and Glucuronide remaining was ≤ 20 % compared to unhydrolyzed samples. Codeine recoveries were above 400 ng/mL when both Surine and DFU used ≥ 10 µL of IMCSzyme RT (**Figure 1**). Oxymorphone recoveries were also like Codeine recoveries for both urine sources when using ≥ 10 µL of IMCSzyme RT (**Figure 1**). Hydrolysis efficiency was further confirmed by quantifying ≤ 20 % Glucuronide remaining (**Figure 1**). Morphine, Hydromorphone, Naloxone, Dihydrocodeine, Naltrexol, Tapentadol, Norbuprenorphine, Buprenorphine, Amitriptyline, Oxazepam, Temazepam, Lorazepam, and cTHC were also hydrolyzed using ≥ 10 µL of IMCSzyme RT (data not shown).

In **Figure 2**, Urine samples (P1 – P7) were positive for Oxymorphone Glucuronide at different concentrations. Samples were processed with 10 µL of IMCSzyme RT for 15 minutes at room temperature and then filtered with β-Gone™ Plus. Recovered Oxymorphone ranged from 500 to 1,700 ng/mL. Hydrolysis was confirmed by quantifying Oxymorphone Glucuronide remaining when compared to unhydrolyzed samples which ranged from 6 % to 11 %. Sample hydrolysis and clean-up were finished in less than 20 minutes by performing in-well hydrolysis with IMCSzyme RT and β-Gone Plus, where samples are processed on top of the filter plate.

Drug-free urine (100 µL) and hydrolysis solution (420 µL) were loaded into a β-Gone Plus plate with organic solvent. Organic solvent was modified because Formic Acid or > 10 % Methanol is note compatible with the colorimetric protein assay. Protein removal was determined by performing a colorimetric protein assay on samples before and after β-Gone Plus filtration. The amount of protein in samples prior to filtration was 0.35 mg/mL, and the amount of protein in samples after filtration was -0.04 mg/mL, which is below the detection limit (**Figure 3**). β-Gone Plus effectively removed the enzyme from samples.

Analyte recovery was evaluated by comparing area counts of samples where the standard solution was added either before or after filtration. All analyte recoveries were > 80 % except for cTHC and its corresponding Glucuronide, which were 70 % and 40 % respectively (**Figure 4**). cTHC has a high logP value, resulting in poor solubility and low recovery in aqueous solutions. Increasing the organic solvent could result in higher recoveries. Overall, analyte recoveries using β-Gone Plus were robust towards a broad range of parent analytes and glucuronidated analytes.

Matrix samples included blank matrix (Surine), DFU, and three urine samples (P8-P10). Hydrolysis solution and organic solvent were added to matrix samples and filtered. Internal standards were added to 200 µL of filtrate and diluted with water. Matrix effects were evaluated by comparing internal standard area counts in blank matrix to area counts in DFU and urine samples. Morphine, Codeine, and Naltrexol exhibited suppression > -20 %, while Naloxone exhibited enhancement > 20 % (**Figure 5**). The remaining analytes were within ± 20 % of enhancement or suppression (**Figure 4**). β-Gone Plus minimized matrix effects for most analytes.

Figure 1. Urine Controls (100 µL), Fortified with Each Glucuronide, were Hydrolyzed with Different Volumes of IMCSzyme RT.

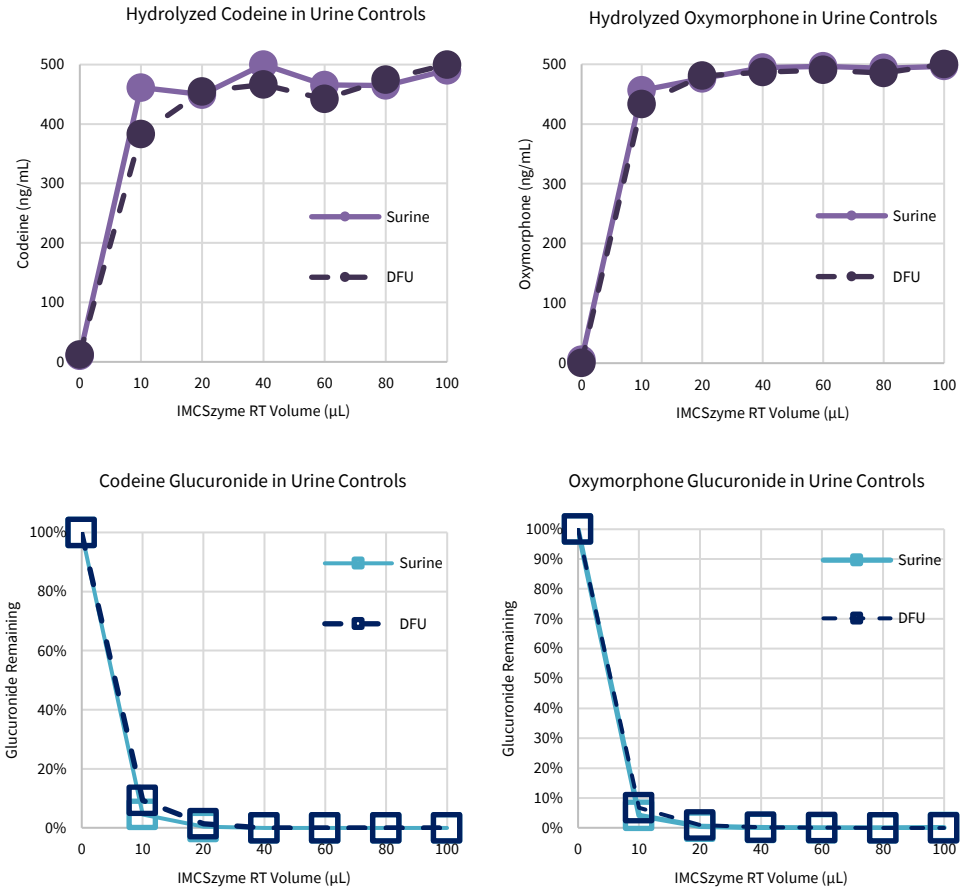


Figure 2. Urine Samples Processed with 10 µL of IMCSzyme® RT. (**a**) Recovered Oxymorphone Ranged from 500 to 1700 ng/mL. (**b**) All Samples had < 20 % of Oxymorphone Glucuronide Remaining.

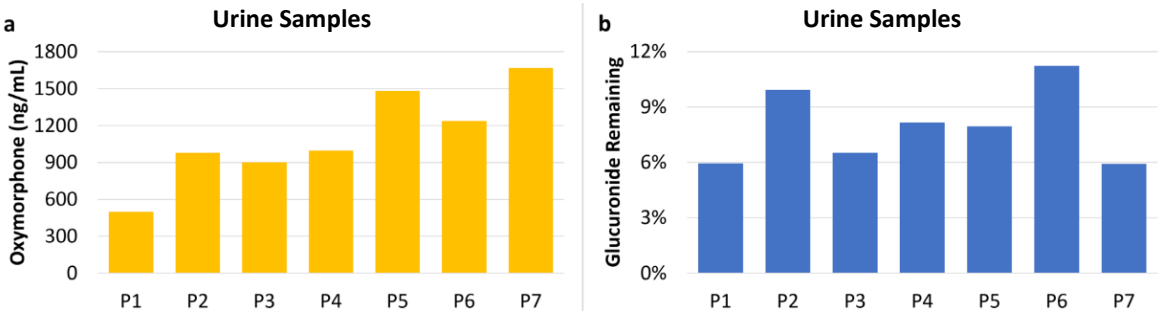


Figure 3. The Amount of Protein in Samples was Measured Before and After β-Gone™ Plus Filtration.

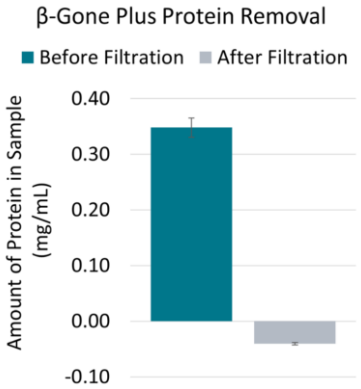


Figure 4. Analyte Recoveries after Samples were Filtered Through β-Gone Plus.

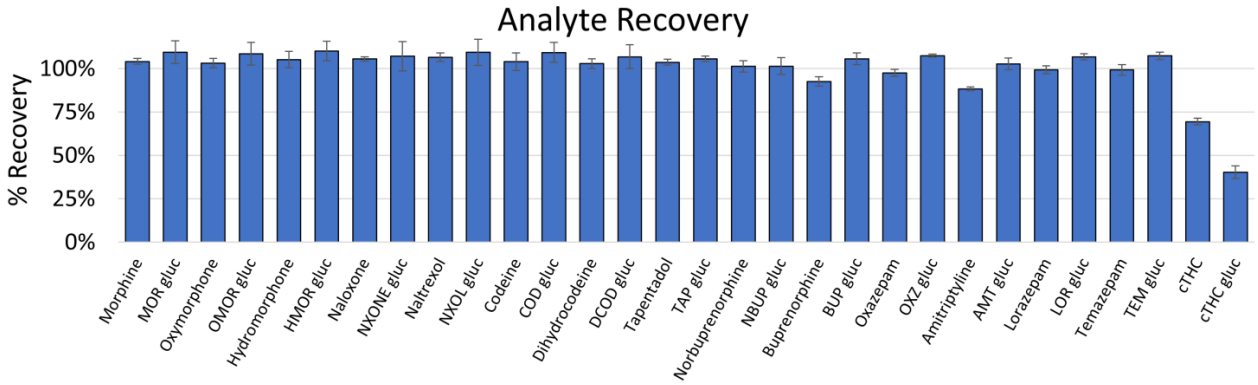
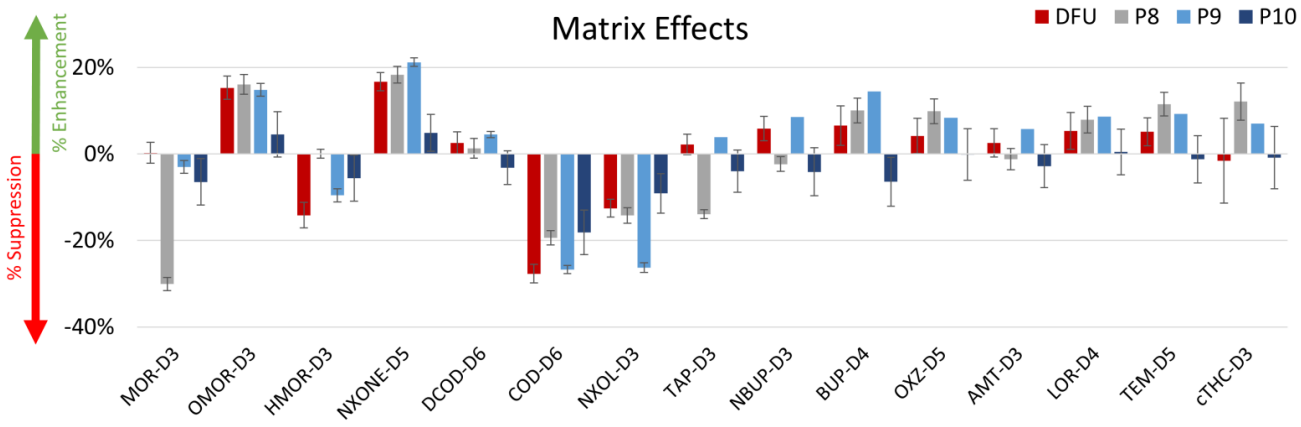


Figure 5 . Analyte Matrix Effects of DFU and Three Samples Compared to Surine™.



Conclusions

The presented method shows several examples where β-Gone™ Plus can produce more efficient drug analysis workflows. We have shown that in-well hydrolysis with IMCSzyme® RT hydrolyzed Glucuronides in 15 minutes at room temperature. Secondly, post hydrolysis clean-up of IMCSzyme RT with β-Gone Plus can be completed in less than 5 minutes. Thirdly, β-Gone Plus removes enzymes, reduces matrix effects, and recovered > 80 % of most analytes. Additionally, room temperature hydrolysis means this workflow can be easily adapted for full automation using a liquid handling system.

Ordering Information

Kinetex™ Analytical Columns

2.6 µm Analytical Columns (mm)							SecurityGuard™ ULTRA Cartridges‡
Phases	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
EVO C18	00A-4725-E0	00B-4725-E0	—	00D-4725-E0	00F-4725-E0	00G-4725-E0	AJ0-9296
PS C18	00A-4780-E0	00B-4780-E0	—	00D-4780-E0	00F-4780-E0	00G-4780-E0	AJ0-8949
Polar C18	00A-4759-E0	00B-4759-E0	—	00D-4759-E0	00F-4759-E0	—	AJ0-9530
Biphenyl	—	00B-4622-E0	—	00D-4622-E0	00F-4622-E0	—	AJ0-9207
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	—	AJ0-8768
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	—	AJ0-8768
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	—	AJ0-8770
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	—	AJ0-8772
Phenyl-Hexyl	—	00B-4495-E0	00C-4495-E0	00D-4495-E0	00F-4495-E0	—	AJ0-8774
F5	00A-4723-E0	00B-4723-E0	—	00D-4723-E0	00F-4723-E0	—	AJ0-9320
							for 4.6 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

β-Gone β-Glucuronidase Removal Products

Part No.	Description	Unit
8B-S139-TAK	1 mL Tubes, Recombinant Enzyme	100/Box
8B-S322-DAK	1 mL Tubes, Non-Recombinant Enzyme	100/Box
8E-S139-TGA	96-Well Plate, Recombinant Enzyme	1/Box
8E-S322-DGA	96-Well Plate, Non-Recombinant Enzyme	1/Box
8E-S323-TGA	96-Well Plate Plus 30 mg/well, Recombinant/Non-Recombinant Enzyme	1/Box
8E-S323-UGA	96-Well Plate Plus 60 mg/well, Recombinant/Non-Recombinant Enzyme	1/Box
8N-S323-TUK	2 mL Centrifuge Tubes, Recombinant and Non-Recombinant Enzyme	100/Box

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