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PCT NEWS

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Discovery Starts With Sample Preparation™

Volume 7, Issue 4 April 2013

Pressure Cycling Technology (PCT) Used in Studies of Viral Potency, Lipidomics, Liver Disease, Cancer, and Forensics

Excerpt from Influenza Other Respi Viruses. 2013 Mar 17. doi: 10.1111/irv.12102

Potency under pressure: the impact of hydrostatic pressure on antigenic properties of influenza virus hemagglutinin

Eichelberger SL, Sultana I, Gao J, Getie-Kebtie M, Alterman M, Eichelberger MC. Division of Cellular and Gene Therapies, Office of Cell, Tissue and Gene Therapy, Bethesda, MD, USA

BACKGROUND:
Influenza vaccines are effective in protecting against illness and death caused by this seasonal pathogen. The potency of influenza vaccines is measured by single radial immunodiffusion (SRID) assay that quantifies antigenic forms of hemagglutinin (HA). Hydrostatic pressure results in loss of binding of influenza virus to red blood cells, but it is not known whether this infers loss of potency.

OBJECTIVES:
Our goal was to determine the impact of pressure on HA antigenic structure.

METHODS:
Viruses included in the 2010-2011 trivalent influenza vaccine were subjected to increasing number of cycles at 35 000 psi in a barocycler, and the impact of this treatment measured by determining hemagglutination units (HAU) and potency. Potency was assessed by SRID and immunogenicity in mice.

RESULTS:
After 25 cycles of pressure, the potency measured by SRID assay was below the limit of quantification for the H1N1 and B viruses used in our study, while the H3N2 component retained some potency that was lost after 50 pressure cycles. Pressure treatment also resulted in loss of HAU, but this did not strictly correlate with the potency value. Curiously, loss of potency was abrogated when influenza A, but not B, antigens were exposed to pressure in chicken egg allantoic fluid. Protection against pressure appeared to be mediated by specific interactions because addition of bovine serum albumin did not have the same effect.

CONCLUSIONS:
Our results show that pressure-induced loss of potency is strain dependent and suggests that pressure treatment may be useful for identifying vaccine formulations that improve HA stability.

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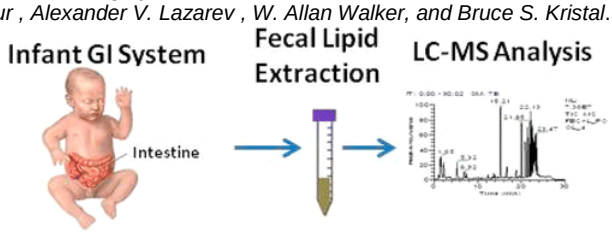
Excerpt from *Anal. Chem.*, 2013, 85 (2), pp 1114–1123 DOI: 10.1021/ac303011k

Method Development for Fecal Lipidomics Profiling

Katherine E Gregory , Susan Schiavo Bird , Vera S. Gross , Vasant R Marur , Alexander V. Lazarev , W. Allan Walker, and Bruce S. Kristal.



Robust methodologies for the analysis of fecal material will facilitate the understanding of gut (patho)physiology and its role in health and disease and will help improve care for individual patients, especially high-risk populations, such as premature infants. Because lipidomics offers a biologically and analytically attractive approach, we developed a simple, sensitive, and quantitatively precise method for profiling intact lipids in fecal material. The method utilizes two separate, complementary extraction chemistries, dichloromethane (DCM) and a methyl *tert*-butyl ether/hexafluoroisopropanol (MTBE) mixture, alone or with high pressure cycling. Extracts were assessed by liquid chromatography–high-resolution mass spectrometry-based profiling with all ion higher energy collisional dissociation fragmentation in both positive and negative ionization modes. This approach provides both class-specific and lipid-specific fragments, enhancing lipid characterization. Solvents preferentially extracted lipids based on hydrophobicity. More polar species preferred MTBE; more hydrophobic compounds preferred DCM. Pressure cycling differentially increased the yield of some lipids. The platform enabled analysis of >500 intact lipophilic species with over 300 lipids spanning 6 LIPID MAPS categories identified in the fecal matter from premature infants. No previous report exists that provides these data; thus, this study represents a new paradigm for assessing nutritional health, inflammation, and infectious disease in vulnerable populations.

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EVENTS

Scientific Meeting: PBI is sponsoring the **15th Annual Symposium on Advances in Separation Science and Mass Spectrometry** (MASSEP and GBMSDG Symposium), May 8, 2013 at Northeastern University, Curry Center, Boston, MA. The Company will showcase its high-pressure systems for proteomic sample preparation, including extraction and digestion.

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Excerpt from *Mol Cell Proteomics. 2013 Feb 6*

Site specific glycoforms of haptoglobin in liver cirrhosis and hepatocellular carcinoma

Pompach P, Brnakova Z, Sanda M, Wu J, Edwards N, and Goldman R.
Georgetown University, United States.

SUMMARY

Haptoglobin is a liver secreted glycoprotein with four N-glycosylation sites. Its glycosylation was reported to change in several cancer diseases which prompted us to examine site specific glycoforms of haptoglobin in liver cirrhosis and hepatocellular carcinoma. To this end, we have used two dimensional separation composed of hydrophilic interaction and nano reverse phase chromatography coupled to QTOF mass spectrometry of the enriched glycopeptides. Our results show increased fucosylation of haptoglobin in liver disease with up to six fucoses associated with specific glycoforms of one glycopeptide. Structural analysis using exoglycosidase treatment and MALDI-MS/MS of detached permethylated glycans led to the identification of Lewis Y type structures observed particularly in the pooled hepatocellular carcinoma sample. To confirm the increase of the Lewis Y structures observed by LC-MS, we have used immunoaffinity detection with Lewis Y specific antibodies. The presence of multiply fucosylated Lewis Y glycoforms of haptoglobin in the disease context could have important functional implications.

Protein digestion and exoglycosidase treatment

Hp was digested as described previously (29). Briefly, 2.5 µg of isolated Hp was resuspended in 20 µL of 50 mM NH4HCO3 at pH 7.8 (Sigma-Aldrich, St. Louis, MO) with 0.05% RapiGest (Waters, Milford, MA), reduced with 5 mM DTT and alkylated with 15 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO). Tryptic digest (2.5 ng/µL) (Promega, Madison, WI) was carried out at 37ºC in Barocycler NEP2320 (Pressure BioSciences, South Easton, MA) or with 8 endoproteinase GluC (60 ng/µL) (Roche, Indianapolis, IN) at 25 ºC overnight. The digests were desalted on a Micro Trap peptide cartridge (Michrom Bioresources, Auburn, CA), and washed 3 times with 250 uL of 0.1% aqueous TFA (Sigma-Aldrich, St. Louis, MO). The peptides were eluted with 100 µL of 60% AcN with 0.1%TFA and the eluate was dried using a SpeedVac concentrator.

Click on the Title for the Complete Publication

Continued on Page 3, Column 1

Excerpt from *Mol Cell Proteomics. 2013 Feb 6*

Quantitative LC-MS-MRM analysis of site-specific glycoforms of haptoglobin in liver disease

Sanda M, Pompach P, Brnakova Z, Wu J, Makambi K, and Goldman R.
Georgetown University, United States

SUMMARY

Development of liver disease is associated with the appearance of multiply fucosylated glycoforms of haptoglobin. To analyze the disease-related haptoglobin glycoforms in liver cirrhosis and hepatocellular carcinoma, we have optimized an LC-MS-MRM workflow for glycopeptide quantification. The final quantitative analysis included twenty four site-specific glycoforms generated by treatment of a tryptic digest of haptoglobin with α(2-3,6,8)neuraminidase and β(1-4)galactosidase. The combination of LC-MS-MRM with exoglycosidase digests allowed resolution of isobaric glycoforms of the Hp-T3 glycopeptide for quantification of the multiply fucosylated Lewis Y-containing glycoforms we have identified in the context of liver disease. Fourteen multiply fucosylated of the twenty examined fucosylated glycoforms increase significantly in the liver disease group compared to healthy controls with an average 5-fold increase in intensity ($p<0.05$). At the same time, two tri-antennary glycoforms without fucoses do not increase in the liver disease group and two tetra-antennary glycoforms without fucoses show a marginal at most 40% increase in intensity. Our analysis of thirty individual patient samples (10 healthy controls (CTL), 10 cirrhosis (CIR) and 10 hepatocellular carcinoma (HCC) patients) shows that these glycoforms are substantially increased in a small subgroup of liver disease patients but do not significantly differ between the groups of HCC and CIR patients. The tri- and tetra-antennary singly fucosylated glycoforms are associated with MELD score and low platelet counts ($p<0.05$). The exoglycosidase-assisted LC-MS-MRM workflow, optimized for the quantification of fucosylated glycoforms of haptoglobin, can be used for quantification of these glycoforms on other glycopeptides with appropriate analytical behavior.

Click on the Title for the Complete Publication

Continued on Page 3, Column 2

SURVEY 1: ACCELERATED PROTEIN DIGESTION

We are conducting a short survey to research the needs of scientists who would benefit from an innovative proteolytic enzyme (e.g. trypsin) digestion process that dramatically reduces the time needed for digestion and that works particularly well for samples that are difficult to digest and/or sequence.

The survey should take less than 5 minutes to complete and participants who complete the survey will be entered for a chance to win a $100 Amazon gift certificate.

Thanks for your participation and good luck!

https://www.surveymonkey.com/s/trypsin-digestion-survey

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Site specific glycoforms of haptoglobin in liver cirrhosis and epatocellular carcinoma: Continued from Page 2, Column 1

Determination of Glyco-site occupancy

Occupancies of all four Hp glycosites were quantified by comparison of XIC precursor ion intensities of unoccupied N-(asparagine) peptides and deglycosylated D-(aspartic acid) peptides following PNGaseF de-glycosylation under 18O water as described previously (30, 31). Briefly, Hp (2 pmoles) was digested after reduction and alkylation by trypsin or endoproteinase GluC as described above. The GluC digest was split in half and one portion was further digested with trypsin while the other was processed as described below; this is done to generate singly glycosylated proteolytic peptides of all four Hp glycosites. All enzymatic digests (tryptic, GluC and combination) were heated for 15 mins at 90℃ to deactivate proteolytic enzymes and 11 evaporated to dryness using speedvac. Separately, 2µl of G7 reaction buffer and 0.5 µl of PNGase F (New England BioLabs, Ipswich, MA) were evaporated and diluted in 20 µl of 18Owater (Cambridge isotope Laboratories, Andover, MA). This solution was used to dissolve the dried peptides for de-glycosylation (1.5 hour at 37℃) using Barocycler NEP2320 (Pressure Bi oSciences, South Easton, MA). De-glycosylated 18O labeled peptides (2pmols) were analyzed directly after the PNGaseF enzymatic treatment on a QTof mass analyzer using an IDA workflow.

DISCUSSION

In summary, we have detected multiply fucosylated glycoforms of Hp with up to six fucoses associated with the presence of Le(y)-type glycoforms of the T3 glycopeptide in the HCC disease group. Analysis of detached permethylated N-glycans and immunostaining with anti-Le(y) antibody confirmed the presence of this glycoform. Function of these unusual minor glycoforms is at present unknown; further studies would be required to establish association of the glycoforms with viral infection or liver disease. The hyper fucosylation likely affects interactions with lectins and other binding partners which could impact the disease progression of viral infection to HCC.

The Barocycler NEP2320

Click Here for PCT Applications

Quantitative LC-MS-MRM analysis of site-specific glycoforms of haptoglobin in liver disease: Continued from Page 2, Column 2

Quantification of Hp glycopeptides

Hp isolated from patient samples (2.5 µg based on peak area of the RP HPLC chromatogram) was spiked with labeled internal standard (VVLHPNYSQVDIGLIK), reduced with DTT, alkylated with IAA, dissolved in 50mM ammonium bicarbonate buffer (Sigma-Aldrich, St. Louis, MO) containing 0.05% RapiGest, and digested with trypsin (Promega, Madison, WI) using Barocycler NEP2320 (Pressure BioSciences, South Easton, MA) at 37℃. Tryptic dig ests were first treated with α(2-3,6,8)neuraminidase (New England BioLabs, Ipswich, MA) at 37℃ in 50mM sodium acetate, 5mm $CaCl_2$, pH 5.5 (New England BioLabs, Ipswich, MA). When appropriate, the digest with α(2-3,6,8)neuraminidase was followed by β(1-4)galactosidase (New England BioLabs, Ipswich, MA) overnight at 37℃ in 50mM sod ium acetate, 5mm $CaCl_2$, pH 5.5 (New England BioLabs, Ipswich, MA).

CONCLUSION

We have developed an exoglycosidase-assisted LC-MS-MRM workflow for quantification of glycoforms of Hp-T3 in liver disease context. The incorporation of exoglycosidase digests into the LC-MS-MRM workflow allows separation of isobaric fucosylated glycoforms associated with cirrhosis and HCC. The fucosylated glycoforms of Hp-T3 are up-regulated on average 5-28 fold in liver disease but the Lewis Y-type glycoforms increase in perhaps 10-20% of the patients. This is not sufficient for a consistent detection of the patients with HCC on the background of liver cirrhosis. Additional appropriately designed studies are needed to determine whether this subset of patients has different prognosis or shares common clinical characteristics. Glycopeptide MRM assays require analyte-specific optimization. To facilitate this task, we suggest a workflow, including fragment-specific CE calculations, for optimization of the LC-MS-MRM analysis of complex N-glycopeptides. Applicability of the workflow is at present limited to isolated N-glycoproteins or their simple mixtures to allow resolution of glycoforms monitored by the common oxonium ions. While general applicability of the assays has limitations, we expect that the exoglycosidase-assisted LC-MS-MRM will provide an excellent tool for verification studies comparing site specific glycoforms of interest in relevant disease conditions.

SURVEY 2: CELL DISRUPTION

We are conducting survey to research the needs of scientists who would benefit from an innovative cell disruption technology platform that provides manual and fully-automated cell disruption from milliliter to liter volumes.

The survey should take less than 5 minutes to complete and participants who complete the survey will be entered for a chance to win a $100 Amazon gift certificate.

Thank you for participating and good luck!

http://www.surveymonkey.com/s/cell-disruption-survey

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Presented at the Mid America Forensic DNA Conference, April, 2013

Pressure Cycling Technology (PCT) Reduces Effects of Inhibitors of the PCR

Pam Marshall, University of North Texas Health Science Center

PCT

- While investigating the use of PCT to attempt to increase DNA yield in challenged samples, we observed that PCT reduced the effects of inhibition on downstream DNA analyses.
- I hypothesized that conditions of extreme pressure alter the conformation of some inhibitors, rendering them less effective at reducing the yield of PCR products in forensic DNA analysis.

UNT HEALTH

Pressure Cycling Equipment and Consumables

Barocycler® NEP3229

- Bench top instrument
- Capable of processing up to three samples simultaneously using PULSE Tubes or up to 48 samples using MicroTubes

Disc-less PULSE Tubes

- **P**ressure **U**sed to **L**yse **S**amples for **E**xtraction hold up to 1.2 mL
- During the PCT process, the Ram compresses the sample and processing buffer against the inside of the Cap, resulting in pressure being transmitted to the sample

PCT MicroTubes with Cartridge Adapter

- Specially designed single use tubes are available in three sizes (50, 100, and 150µl)

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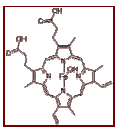
DNA INHIBITORS

Two potent PCR inhibitors, hematin and humic acid, were evaluated

Hematin

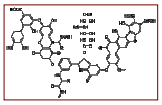
- Metal chelating molecule found in RBCs
- Forms a stable complex with the DNA polymerase
- May also cause a dissociation of the DNA-polymerase complex, thereby inhibiting polymerase activity



www.chemicalbook.com

Humic acid

- Component in soil
- Often encountered in samples that have been buried, such as skeletal remains
- Proposed mechanisms: chelates with magnesium ions needed for DNA polymerase or inhibits the PCR via sequence specific binding to DNA, thus limiting the amount of available template



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Continued on Page 4, Column 2

Pressure Cycling Technology (PCT) Reduces Effects of Inhibitors of the PCR: Continued from Page 4, Column 1

NPC vs PCT: Number of Alleles Detected

	Sample Name	Total RFUs in Sample	Average PH w/n Sample	Standard Deviation PH w/n Sample	# of Alleles Detected
Bone 1	NPC.1.001.1	2518	209.8666667	129.3208369	12
	PCT.1.001.1	2754	130.1904762	91.20211989	21
	NPC.24.001.1	2460	164	144.5425284	15
	PCT.24.001.1	3693	141.0384615	75.80843125	26
Bone 2	NPC.1.002.1	11137	445.48	221.8948925	25
	PCT.1.002.1	16826	653.04	335.1239176	25
	NPC.24.002.1	7005	280.2	147.6211367	25
	PCT.24.002.1	8814	344.56	178.803873	25
Bone 3	NPC.1.003.1	736	92	58.81690716	8
	PCT.1.003.1	1917	91.28571429	58.28987184	21
	NPC.24.003.1				
	PCT.24.003.1	612	102	76.57353516	6

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Discussion

- The results support that pressure cycling technology (PCT) reduces inhibitory effects and thus enhances yield of amplified products of both hematin and humic acid inhibited samples.
- Based on the results obtained in this study, this method can improve the recovery of alleles from challenged or inhibited DNA samples.

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Conclusions

- Results demonstrate enhanced PCR efficiency for inhibited samples following PCT compared with those samples not exposed to pressure technology.
- These results indicate as a proof of concept that PCT is a viable method to overcome the inhibitory effects of hematin and HA on the PCR.
- This research study demonstrates the capabilities and potential of PCT applications for forensic DNA analysis of biological evidentiary samples.

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