National Institute of Standards & Technology

Report of Investigation

Reference Material 8321

Peptide Mixture for Proteomics

This Reference Material (RM) is intended to support investigations used to identify peptides in complex peptide mixtures such as those in mass spectrometry-based proteomics. RM 8321 can be used to help assess the confidence of peptide identification within a laboratory or comparability between laboratories or among different measurement approaches. RM 8321 can also be used in the development and validation of new investigative approaches for identifying peptides in complex peptide mixtures. A unit of RM 8321 consists of three vials, each containing approximately 50 µL of frozen aqueous solution containing 0.1 mL/L formic acid. The peptides in this RM are estimated to be in a concentration range of 0.1 pmol/µL to 10 pmol/µL.

RM 8321 is an aqueous solution of approximately 440 synthetic peptides, present at a range of concentrations that span approximately three orders of magnitude. RM 8321 was designed to provide a complex mixture of peptides for evaluating the performance of proteomics mass spectrometry instruments coupled to liquid chromatography (LC) [1]. Peptides were chosen to cover the chromatographic "space" of a typical reverse phase gradient elution analysis, offering a range of elution profiles. The synthetic peptides in RM 8321 have the same amino acid sequence as tryptic peptides from 50 high abundance human plasma proteins that have been observed as proteotypic peptides through multiple published investigations by the proteomics community. Proteotypic peptides are those peptides which are observed repeatedly by mass spectrometric-based proteomics investigations by different investigators. Therefore, proteotypic peptides are expected to be readily released from enzymatic digestions of the precursor protein, ionize well by electrospray ionization, generate high quality tandem mass spectrometry (MS/MS) spectra, and are stable during the processes of sample preparation and analysis.

Reference Values: A NIST reference value represents the best estimation of the true value based upon the available data [2]. Table 1 lists a set of heuristic rules which describe the confidence of peptide identification in RM 8321. Table 2 lists the peptides present, grouped by confidence level, as determined by two types of LC-MS/MS analyses and comparison to mass spectral libraries [3].

Expiration of Reference Values: RM 8321 is valid, within the specified confidence levels, until **05 June 2020**, provided the RM is handled and stored in accordance with the instructions given in this report (see "Instructions for Storage and Use"). This report is nullified if the RM is damaged, contaminated, or otherwise modified.

Maintenance of RM: NIST will monitor this RM over the period of its validity. If substantive technical changes occur that affect the value assignment before the expiration of this report, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Overall direction and coordination of technical measurements leading to peptide identification were performed by D.M. Bunk of the NIST Biomolecular Measurement Division.

Analyses were performed by A.S. Beasley, M. Lowenthal, and D.M. Bunk of the NIST Biomolecular Measurement Division.

Support aspects involved in the issuance of this RM were coordinated through the NIST Office of Reference Materials.

Michael J. Tarlov, Chief
Biomolecular Measurement Division

Gaithersburg, MD 20899
Report Issue Date: 28 December 2015

Robert L. Watters, Jr., Director
Office of Reference Materials

Reference Values: Table 1 defines the corresponding confidence levels of peptide identification in RM 8321. Table 2 identifies the peptide content, grouped by confidence level, based on both the data dependent acquisition (DDA) and dynamic multiple reaction monitoring (dMRM) analyses.

Table 1. Definitions of Heuristic Rules

Confidence Level	***Heuristic Definition***
High Confidence	Peptides listed were observed in more than 90 % of all laboratory investigations (including technical and process replicate analyses).
Confident	Peptides listed were observed in more than 50 % and less than 90 % of all laboratory investigations.
Low Confidence	Peptides listed were observed in less than 50 % of all laboratory investigations.

Table 2. Reference Peptide Content of RM 8321

High Confidence:

AATVGSLAGQPLQER	HLSLLTTLSNR	SDVVYTDWKKDK
ADLFYDVEALDLESPK	HLVPGAPFLLQALVR	SEETKENEGFTVTAEGK
AEDHFSVIDFNQNIR	HPDYSVVLLLR	SELEEQLTPVAEETR
AEFAEVSK	HQLYIDETVNSNIPTNLR	SELTQQLNALFQDK
AFQPFFVELTMPYSVIR	HQTVPQNTGGKNPDPWAK	SFFSFLGEAFDGAR
AGALNSNDAFVLK	HSIFTPETNPR	SGAQATWTELPWPHEK
AGDFLEANYMNLQR	HSTIFENLANK	SGFPQVSMFFTHTFPK
AGKEPGLQIWR	HTLNQIDEVK	SGKDPNHFRPAGLPEK
AHVSFKPTVAQQR	HTSVQTTSSGSGPFTDVR	SGVQQLIQYYQDQK
AHYGGFTVQNEANK	HVVPNEVVVQR	SHALQLNNR
AIEDYINEFSVR	HYEGSTVPEK	SIEVFGQFNGK
AIGYLNTGYQR	HYQINQQWER	SKEFQLFSSPHGK
AKPALEDLR	IADAHLDR	SKEQLTPLIK
ALDLINKR	IADNKQSSFK	SLAELGGHLDQQVEEFRR
ALLVGEHLNIIVTPK	IAFSATR	SLAPYAQDTQEK
ALMDETMK	IAQWQSFQLEGGLK	SLHTLFGDK
ALTDMPQMR	IDTQDIEASHYR	SPELQAEAK
ALVQQMEQLR	IEGNLIFDPNNYLPK	SPELQAEAKSYFEK
ALYLQYTDETFR	IHWESASLLR	SSALDMENFR
AMAVEDIISR	IIRSSEDPNEDIVER	SSEDPNEDIVER
APNHAVVTR	IIVPLNNR	SSLSVPYVIVPLK
AQLVDMK	IKVLNQELR	SSNLIILEEHLK
AQRQVVAGLNFR	ILGGHLDAK	SVLGQLGITK
ASEAEDASLLSFMQGYMK	IPIEDGSGEVVLSR	SVNDLYIQK
ASSIIDELFQDR	IPLDLVPK	SVSDGIAALDLNAVANK
ASSIIDELFQDRFFTR	ISASAEELR	SVVDENFSWYLEDNIK
ASTPNGYDNGIIWATWK	ISEGLPALEFPNEK	SYFEKSKEQLTPLIK
ATEHLSTLSEK	ITENDIQIALDDAK	SYFPESWLWEVHLVPR
ATFQTPDFIVPLTDLR	ITPNLAEFAFSLYR	SYTITGLQPGTDYK
ATGVLYDYVNK	IVSSAMEPDR	TAAQNLYEK
ATVVYQGER	IYGNQDTSSQLKK	TAAQNLYEKTYLPAVDEK
AVMDDFAAFVEK	IYHSHIDAPK	TAGWNIPMGLLYNK
AVSMPSFSILGSDVR	IYISGMAPRPSLAK	TDAPDLPEENQAR
AYKSELEEQLTPVAEETR	IYLYTLNDNAR	TEDTIFLR
DALSSVQESQVAQQAR	KAMAVEDIISR	TEGDGVYTLNDK
DAQYAPGYDKVK	KATVVYQGER	TEHPFTVEEFVLPK
DDEEFIESNK	KDNEQHVFK	TEHYEEQIEAFK
DDNPNLPR	KELSSFIDK	TELRPGETLNVNFLLR
DFHINLFQVLPWLK	KFPSGTFEQVSQLVK	TEVNVLPGAK
DFVQPPTK	KGEWVALNPLR	TGAQELLR
DGAGDVAFVK	KLSSWVLLMK	TGLQEVEVK
DGNTLTYYR	KLVPFATELHER	THLAPYSDELR
DHAVDLIQK	KLWAYLTINQLLAER	THLPEVFLSK

High Confidence (continued):

DINYVNPVIK
DISEVVTPR
DKVNSFFSTFK
DLATVYVDVLK
DLKVEDIPLAR
DLMEKVKSPELQAEAK
DMYSFLEDMGLK
DRVVEESELAR
DSGRDYVSQFEGSALGK
DSQEEEKTEALTSAK
DSQEEEKTEALTSAKR
DTVIKPLLVEPEGLEK
DTVQIHDITGK
DVVLFEK
DWHGVPGQVDAAMAGR
DYWSTVK
EAQLPVIENK
EDLIWELLNQAQEHFGK
EDTPNSVWEPAK
EELLPAQDIK
EESPLLIGQQSTVSDVPR
EFQLFSSPHGK
ELDESLQVAER
ELSSFIDK
ELSYYSLEDLNNK
EMSGSPASGIPVK
ENADSLQASLRPHADELK
ENISDPTSPLR
EPAHLMSLFGGKPMIIYK
EPTMYVGSTSVQTSR
EQLGPVTQEFWDNLEK
ESDTSYVSLK
ESYSGVTLDPR
ETAVDGELVVLYDVK
EVAFDLEIPK
EVDLKDYEDQQK
EWFWDLATGTMK
EYVLPSFEVIVEPTEK
FAFNLYR
FEDGVLDPDYPR
FFHKNEIWYR
FKDLGEENFK
FLATTPNSLLVSWQPPR
FMETVAEK
FNAVLTNPQGDYDTSTGK
FPEVDVLTK
FPSGTFEQVSQLVK
FPVEMTHNHNFR
FQNSAILTIQPK
FSVPAGIVIPSFQALTAR
FSVVYAK
FSYSKNETYQLFLSYSSK
FSYSSGHVHLSSENK
FTNIGPDTMR
FTVDRPFLFLIYEHR
FVTWIEGVMR
FYNQVSTPLLR
GAYPLSIEPIGVR
GDKVWVYPPEKK
GDSGGAFAVQDPNDK
GDSGGPLIVHK
KSASDLTWDNLK
KTLLSNLEEAKK
KVEQAVETEPEPELR
KVPQVSTPTLVEVSR
KWQEEMELYR
KYFIDFVAR
KYNSQNQSNNQFVLYR
LANLTQGEDQYYLR
LAVYQAGAR
LDAQASFLPK
LDELRDEGK
LDEVKEQVAEVR
LDGKFSVVYAK
LDGSVDFK
LDGSVDFKK
LEEQAQQIR
LFDSDPITVTVPVEVSRK
LGPHAGDVEGHLSFLEK
LGPLVEQGR
LGQYASPTAK
LGVRPSQGGEAPR
LHEAFSPVSYQHDLALLR
LHIMAGR
LKEEIGKELEELR
LKNSLFEYQK
LKSWFEPLVEDMQR
LLDNWDSVTSTFSK
LLIYAVLPTGDVIGDSAK
LNAENNATFYFK
LPPNVVEESAR
LQAEAFQAR
LQGTLPVEAR
LQHLENELTHDIITK
LRDLYSK
LREQLGPVTQEFWDNLEK
LRTEGDGVYTLNDKK
LSINTHPSQKPLSITVR
LSNENHGIAQR
LSPIYNLVPVK
LTIGEGQQHHLGGAK
LVAYYTLIGASGQR
LVDKFLEDVKK
LVNEVTEFAK
LVTDLTK
LWAYLTIQELLAK
MATTMIQSK
MGPTELLIEMEDWK
MGPTELLIEMEDWKGDK
MKGLIDEVNQDFTNR
MKPVPDLVPGNFK
MVETTAYALLTSLNLK
MYLGYEYVTAIR
NANFKFTDHLK
NFPSPVDAAFR
NGNMAGISDQR
NHMQYEIVIK
NKPGVYTDVAYYLAWIR
NNKDSHSLTTNIMEILR
NPANPVQR
NPNLPPETVDSLK
NSLFEYQK
THLPEVFLSKVLEPTLK
TILGTMPAFEVSLQALQK
TLEAQLTPR
TLLPVSKPEIR
TLLSNLEEAK
TMEQFTIHLTVNPQSK
TMTIHNGMFFSTYDR
TNFDNDIALVR
TPSAAYLWVGTGASEAEK
TSNFNAAISLK
TTIEKPVWLGFLGPIIK
TTNIQGINLLFSSR
TVGSDTFYSFK
TVIGPDGHK
TVMVNIENPEGIPVK
TWRNDLISATK
TYETTLEK
TYLPAVDEK
TYLPAVDEKLR
TYLPAVDEKLRDLYSK
TYMLAFDVNDEK
TYNVLDMK
VDKDNEDFQESNR
VDVIPVNLPGEHGQR
VEDPESTLFGSVIR
VFDEFKPLVEEPQNLIK
VFSNGADLSGVTEEAPLK
VGDTLNLNLR
VGFYESDVMGR
VGPEADKYR
VGYVSGWGR
VIGNMGQTMEQLTPELK
VKDISEVVTPR
VKDLATVYVDVLK
VKSPELQAEAK
VKSPELQAEAKSYFEK
VLEPTLK
VLNQELR
VLSLAQEQVGGSPEK
VLVDHFGYTK
VMDKYTFELSR
VNKDDEEFIESNK
VPEARPNSMVVEHPEFLK
VPGLYYFTYHASSR
VPGTSTSATLTGLTR
VPLLLSEPINIIDALEMR
VPQVSTPTLVEVSR
VQHIQLLQK
VQPYLDDFQK
VRGGEGTGYFVDFSVR
VSFLSALEEYTK
VTIMWTPPESAVTGYR
VTWAPPPSIDLTNFLVR
VVGGLVALR
VVLHPNYSQVDIGLIK
VWVYPPEKK
VWVYPPEKK
VYKPSAGNNSLYR
VYSLNDDLKPAK
WFYIASAFR
WKNFPSPVDAAFR

High Confidence (continued):

GDSGGPLIVHKR	NWGLSVYADKPETTK	WLPSSSPVTGYR
GDSPASSKPISINYR	NWIQYK	WQEEMELYR
GDVAFVK	NYNLVESLK	WSRPQAPITGYR
GETHEQVHSILHFK	PALPAGTEDTAKEDAANR	WYEIEKIPTTFENGR
GEVQAMLGQSTEELR	PLVEEPQNLIK	YEASILTHDSSIR
GEVQAMLGQSTEELRVR	PNSMVVEHPEFLK	YEFLNGR
GEWVALNPLR	PPEIAHGYVEHSVR	YEITTIHNLFR
GFEPTLEALFGK	PYTFHSHGITYYK	YFIDFVAR
GFSLDEATNLNGGLLR	QFSFPLSSEPFQGSYK	YGLVTYATYPK
GGEGTGYFVDFSVR	QGHNSVFLIK	YGMVAQVTQTLK
GGETAQSADPQWEQLNNK	RHPDYSVVLLLR	YKEENDDFASFRVDR
GGYTLVSGYPK	RHPYFYAPELLFFAK	YLGEEYVK
GHLFLQTDQPIYNPGQR	RLDGSVDFK	YLQEIYNSNNQK
GHMLENHVER	RLDGSVDFKK	YLQEIYNSNNQKIVNLK
GKWERPFEVK	RLEVDIDIK	YLYEIAR
GSESGIFTNTK	RPYFPVAVGK	YNPVVIDFEMQPIHEVLR
GSFEFPVGDAVSK	RQSEDSTFYLGER	YQISVNK
GSPAINVAVHVFR	RRDGYLFQLLR	YTFELSR
GWVTDGFSSLK	RSFFSFLGEAFDGAR	YVGGQEHFAHLLILR
GWVTDGFSSLKDYWSTVK	RTHLPEVFLSK	YVLPNFEVK
GYSIFSYATK	RVDTVDPPYPR	YVNKEIQNAVNGVK
HGGLYHENMR	RVEPYGENFNK	YYTYLIMNK
HLEVDVWVIEPQGLR	RYIETDPANR	

Confident:

AHVDALR	FNKPFVFLMIEQNTK	NRDVVLTTTFVDDIK
ALFVSEEEKK	GDKVWVYPPEK	RQDNEILIFWSK
ALVEGVDQLFTDYQIK	GETHEQVHSILHFK	RTHLPEVFLSKVLEPTLK
AYYENSPQQVFSTEFEVK	GSPAINVAVHVFRK	SDVVYTDWK
DGYLFQLLR	GSWVNKFPVEMTHNHNFR	SDVVYTDWKK
DIFTGLIGPMK	HGTDDGVVWMNWK	SIEVFGQFNGKR
DLEIEVVLFHPNYNINGK	HTSLGPLEAK	SKEQLTPLIKK
DNDGWLTSDPR	IPKSDVVYTDWKK	SWFEPLVEDMQR
DNENVVNEYSSELEK	IPTTFENGR	SYFEKSKEQLTPLIKK
DNEQHVFK	IQPSGGTNINEALLR	TLLSNLEEAKK
DRLDEVKEQVAEVR	KGEWVALNPLRK	TVIGPDGHKEVTK
DSAHGFLK	KTLLSNLEEAK	VDTVDPPYPR
DSGFQMNQLR	LDDDLEHQGGHVLDHGHK	VQFELHYQEVK
DTEEEDFHVDQVTTVK	LEQGENVFLQATDK	VTFQLTYEEVLK
DYVSQFEGSALGK	LLPHANEVSQK	WDPYKQGFGNVATNTDGK
ERGHMLENHVER	LSPLGEEMR	WFYIASAFRNEEYNK
ESLSSYWESAK	LSSPAVITDK	YVGGQEHFAHLLILRDTK
EYHFGQAVR	MLTPEHVFIHPGWK	

Low Confidence:

DVVLTTTFVDDIK	PVWLGFLGPIIK	TEGDGVYTLNNEK
EDFTSLSLVLYSR	SNLDEDIIAEENIVSR	VELEDWNGR

INSTRUCTIONS FOR STORAGE AND USE

Handling: RM 8321 is a frozen aqueous solution containing approximately 440 synthetic peptides in 0.1 mL/L formic acid. Normal caution and care should be exercised during the material's handling and use.

Storage: The peptide mixture solution is shipped frozen (on dry ice) and, upon receipt, should be stored frozen until ready for use. A freezer temperature of –20 °C is acceptable for storage for up to one week. If a longer storage time is anticipated, the material should be stored at or below –60 °C. The RM should not be exposed to sunlight or ultraviolet radiation. Storage of thawed material at room or refrigerator temperatures may result in degradation or modification of constituent peptides.

Use: Vials of the RM to be analyzed should be removed from the freezer and allowed to stand at room temperature (20 °C to 25 °C) until thawed. After the material is thawed, it should be used immediately. The material should be mixed briefly with a vortex mixer before aliquots are withdrawn.

PREPARATION AND ANALYSIS[(1)]

Material Acquisition and Preparation: The synthetic peptides used in the preparation of the RM were obtained from GenScript USA Inc. (Piscataway, NJ). Aqueous solutions of each synthetic peptide were prepared and characterized by LC-MS using a time-of-flight mass analyzer and by LC-MS/MS using an ion trap mass analyzer. Each synthetic peptide was assessed for identify, purity, and its chromatographic and mass spectrometric behavior. Based on this assessment, solutions of 440 synthetic peptides were blended together to produce the peptide mixture in RM 8321. Two additional LC-MS/MS analyses were performed on the RM to confirm the presence of each expected peptide. Using DDA, the RM was analyzed using reversed-phase LC coupled to a LTQ XL (Thermo Scientific) ion trap mass spectrometer. The data from the DDA was analyzed using theoretical fragmentation libraries of tryptic peptides from all human proteins and a library containing only the peptides used to prepare the RM. The data from the ion trap analysis was also searched using a mass spectral library containing only spectra from the peptides used to prepare the RM. In addition to the ion trap analysis, the presence of peptides in the RM was also confirmed through analysis using reversed-phase LC coupled to a model 6460 (Agilent Technologies) triple quadrupole mass spectrometer operated in dMRM mode. The dMRM method monitored three different MRM transitions for each of the expected peptides in the RM.

Homogeneity Analysis: The homogeneity was assessed at the time the analyses for the reference peptide content of the RM were performed. A stratified random sampling plan was devised to test for homogeneity across the production lot. The results indicated that no appreciable vial-to-vial differences were detected.

REFERENCES

[1] Rudnick, P.A.; Clauser, K.R.; Kilpatrick, L.E.; Tchekhovskoi, D.V.; Neta, P.; Blonder, N.; Billheimer, D.D.; Blackman, R.K.; Bunk, D.M.; Cardasis, H.L.; Ham, A.J.; Jaffe, J.D.; Kinsinger, C.R.; Mesri, M.; Neubert, T.A.; Schilling, B.; Tabb, D.L.; Tegeler, T.J.; Vega-Montoto, L.; Variyath, A.M.; Wang, M.; Wang, P.; Whiteaker, J.R.; Zimmerman, L.J.; Carr, S.A.; Fisher, S.J.; Gibson, B.W.; Paulovich, A.G.; Regnier, F.E.; Rodriguez, H.; Spiegelman, C.; Tempst, P.; Liebler, D.C.; Stein, S.E.; *Performance Metrics for Liquid Chromatography-Tandem Mass Spectrometry Systems in Proteomics Analyses*; Mol. Cell Proteomics, Vol. 9(2), pp. 225–241 (2010).

[2] May, W.; Parris, R.; Beck II, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; *Definition of Terms and Modes Used at NIST for Value Assignment of Reference Materials for Chemical Measurements*; NIST Special Publication 260-136 (2000); available at http://www.nist.gov/srm/upload/SP260-136.PDF (accessed Dec 2015).

[3] Stein, S.; *Mass Spectral Reference Libraries: An Ever-Expanding Resource for Chemical Identificatio*n; Anal. Chem., Vol. 84(17), pp. 7274–7282 (2012).

Users of this RM should ensure that the Report of Investigation in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

[(1)] Certain commercial equipment, instruments, or materials are identified in this report to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.