



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.

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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

<i>Confidence Level</i>	<i>Heuristic Definition</i>
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:

AATVGS LAGQPLQER	HLSLLT T LSNR	SDVVYTDWKKDK
ADLFYDVEALDLESPK	HLVPGAPFLQALVR	SEETKENEGFTVTAEGK
AEDHFSVIDFNQNR	HPDYSV V LLLR	SELEEQLTPVAEETR
AEFAEVSK	HQLYIDETVNSNIPTNLR	SELTQQLNALFQDK
AFQPFFVELTMPYSVIR	HQTVPQNTGGKNPDPWAK	SFFSFLGEAFD GAR
AGALNSND AFVLK	HSIFTPETNPR	SGAQATWTELPWPHEK
AGDFLEANYMNLQR	HSTIFENLANK	SGFPQVSMFFTHTFPK
AGKEPGLQIWR	HTLNQIDEVK	SGKDPNHFRPAGLPEK
AHVSFKPTVAQQR	HTSVQTTSSGSGPFTDVR	SGVQQLIQYYQDQK
AHYGGFTVQNEANK	HVVPNEV V VQR	SHALQLNNR
AIEDYINEFSVR	HYEGSTVPEK	SIEVFGQFNGK
AIGYLNTGYQR	HYQINQQWER	SKEFQLFSSPHGK
AKPALEDLR	IADAHLDR	SKEQLTPLIK
ALDLINKR	IADNKQSSFK	SLAELGGHLDQQVEEFR
ALLVGEHLNIIVTPK	IAFSATR	SLAPYAQDTQEK
ALMDETMK	IAQWQSFQLEGGLK	SLHTLFGDK
ALTDMPQMR	IDTQDIEASHYR	SPELQAEAK
ALVQQMEQLR	IEGNLIFDPNNYLPK	SPELQAEAKSYFEK
ALYLQYTD ETR	IHWESASLLR	SSALDMENFR
AMAVEDIISR	IIRSSDPNEDIVER	SSEDPNEDIVER
APNHAVVTR	IIVPLNNR	SSLSVPYVIVPLK
AQLVDMK	IKVLNQELR	SSNLIILEEHLK
AQRQVVAGLNFR	ILGGHLD AK	SVLGQLGITK
ASEAEDASLLSFMQGYMK	IPIEDGSGEVVLSR	SVNDLYIQK
ASSIIDELFQDR	IPLDLVPK	SVSDGIAALDLNAVANK
ASSIIDELFQDRFFTR	ISASAEELR	SVVDENFSWYLEDNIK
ASTPNGYDNGI I WATWK	ISEGLPALEFPNEK	SYFEKSKEQLTPLIK
ATEHLSTLSEK	ITENDIQIALDDAK	SYFPESWLWEVHLVPR
ATFQTPDFIVPLTDLR	ITPNLAEF AFSLYR	SYTITGLQPGTDYK
ATGVLYDYVNK	IVSSAMEPDR	TAAQNLYEK
ATVVYQGER	IYGNQDTSSQLKK	TAAQNLYEKTYLPAVDEK
AVMDDFAAFVEK	IYHSHIDAPK	TAGWNIPMGLLYNK
AVSMPSFSILGSDVR	IYISGMAPRPSLAK	TDAPDLPEENQAR
AYKSELEEQLTPVAEETR	IYLYTLNDNAR	TEDTIFLR
DALSSVQESQVAQQR	KAMAVEDIISR	TEGDGVYTLNDK
DAQYAPGYDKVK	KATVVYQGER	TEHPFTVEEFVLPK
DDEEFIESNK	KDNEQHVFVK	TEHYEEQIEAFK
DDNPNLPR	KELSSFIDK	TELRPGETLNVN FLLR
DFHINLFQVLPWLK	KFPSGTFEQVSQLVK	TEVNVLP GAK
DFVQPPTK	KGEWVALNPLR	TGAQELLR
DGAGDVAFVK	KLSSWVLLMK	TGLQEVEVK
DGNTLTYYR	KLVPFATELHER	THLAPYSDEL R
DHAVDLIQK	KLWAYLTINQLLAER	THLPEVFLSK

High Confidence (continued):

DINYVNPVIK	KSASDLTWDNLK	THLPEVFLSKVLEPTLK
DISEVVTTPR	KTLLSNLEEAKK	TILGTMPAFEVSLQALQK
DKVNSFFSTFK	KVEQAVETEPEPELR	TLEAQLTPR
DLATVYVDVLK	KVPQVSTPTLVEVSR	TLLPVSKPEIR
DLKVEDIPLAR	KWQEEMELYR	TLLSNLEEAK
DLMEKVKSPELQAEAK	KYFIDFVAR	TMEQFTIHLTVNPQSK
DMYSFLEDMGLK	KYNSQNQSNNQFVLYR	TMTIHNGMFFSTYDR
DRVVEESELAR	LANLTQGEDQYYLR	TNFDNDIALVR
DSGRDYVSQFEGSALGK	LAVYQAGAR	TPSAAYLWVG TGASEAEK
DSQEEKTEALTSK	LDAQASFLPK	TSNFNAAISLK
DSQEEKTEALTSKR	LDEL RDEGK	TTIEKPVWLGLGPIIK
DTVIKPLLVEPEGLEK	LDEVKEQVAEVR	TTNIQGINLLFSSR
DTVQIHDITGK	LDGKFSVVYAK	TVGSDTFYSFK
DVVLFEK	LDGSVDFK	TVIGPDGHK
DWHGVPGQVDAAMAGR	LDGSVDFKK	TVMVNINPEGIPVK
DYWSTVK	LEEQAQIR	TWRNDLISATK
EAQLPVIENK	LFDSDPITVTPVEVSRK	TYETTLEK
EDLIWELLNQAQEHFGK	LGPHAGDVEGHL SFLEK	TYLPAVDEK
EDTPNSVWEPK	LGPLVEQGR	TYLPAVDEKLR
EELLPAQDIK	LGQYASPTAK	TYLPAVDEKLRDLYSK
EESPLLIGQQSTVSDVPR	LGVRPSQGGEAPR	TYMLAFDVNDEK
EFQLFSSPHGK	LHEAFSPVSQHD LALLR	TYNV LDMK
ELDESLQVAER	LHIMAGR	VDKDNEDFQESNR
ELSSFIDK	LKEEIGKELEELR	VDVIPVNLPGEHGQR
ELSYYSLEDLNNK	LKNSLF EYQK	VEDPESTLFGSVIR
EMSGSPASGIPVK	LKSWFEPLVEDMQR	VFDEFKPLVEEPQNLK
ENADSLQASLRPHADELK	LLDNWDSVTSTFSK	VFSNGADLSGVTEEAPLK
ENISDPTSPLR	LLIYAVLPTGDVIGDSAK	VGDTLNLNLR
EPAHLSLFGGKPMIYK	LNAENNATFYFK	VG FYESDVMGR
EPTMYVGSTSVQTSR	LPPNVVEESAR	VGPEADKYR
EQLGPVTQEFWDNLEK	LQAEAFQAR	VG YVSGWGR
ESDTSYVSLK	LQGTLPVEAR	VIGNMGQTMEQLTPELK
ESYSGVTLDPR	LQHLENELTHDIITK	VKDISEVVTTPR
ETAVDGELVVLYDVK	LRDLYSK	VKDLATVYVDVLK
EVAFDLEIPK	LREQLGPVTQEFWDNLEK	VKSPELQAEAK
EVDLKD YEDQQK	LRTEGDGVYTLNDKK	VKSPELQAEAKSYFEK
EWFWDLATGTMK	LSINTHPSQKPLSITVR	VLEPTLK
EYVLPSFEVIVEPTEK	LSNENHGIAQR	VLNQELR
FAFNLYR	LSPIYNLVPVK	VLSLAQEYVGG SPEK
FEDGVLPDPYPR	LTIGEGQQHHLGGAK	VLVDHFGYTK
FFHKNEIWYR	LVAYYTLIGASGQR	VMDKYTFELSR
FKDLGEENFK	LVDKFLEDVKK	VNKDDEEFIESNK
FLATTPNSLLVSWQPPR	LVNEVTEFAK	VPEARPNM VVEHPEFLK
FMETVAEK	LVTDLTK	VPGLYYFTYHASSR
FNAVLTNPQGDYDTSTGK	LWAYLTIQELLAK	VPGTSTSATLTGLTR
FPEVDVLTK	MAT TMIQSK	VPLLLSEPINIIDALEMR
FPSGTFEQVSQLVK	MGPTELLIEMEDWK	VPQVSTPTLVEVSR
FPVEMTHNHNFR	MGPTELLIEMEDWKGDK	VQHIQLLQK
FQNSAILTIQPK	MKGLIDEVNQDFTNR	VQPYLDDFQK
FSVPAGIVIPSFQALTAR	MKPVPDLVPGNFK	VRGGEGTGYFVDFSVR
FSVVYAK	MVETTAYALLTSLNLK	VSFLSALEEYTK
FSYSKNETYQLFLSYSSK	MYLGYEYVTAIR	VTIMWTPPESAVTGYR
FSYSSGHVHLSENK	NANFKFTDHLK	VTWAPPPSIDLTNFLVR
FTNIGPDTMR	NFPSPVDAAFR	VVGGLVALR
FTVDRPFLFLIYEHR	NGNMAGISDQR	VVLHPNYSQVDIGLIK
FVTWIEGVMR	NHMQYEIVIK	VWVYPPEKK
FYNQVSTPLLR	NKPGVYTDVAYYLAWIR	VWVYPPEKK
GAYPLSIEPIGVR	NNKDSHSLTTNIMEILR	VYKPSAGNNSLYR
GDKVWVYPPEKK	NPANPVQR	VYSLNDDLKPAK
GDSGGAFAVQDPNDK	NPNLPPETVDSLK	WFYIASAFR
GDSGGPLIVHK	NSLFEYQK	WKNFPSPVDAAFR

High Confidence (continued):

GDSGGPLIVHKK	NWGLSVYADKPETTK	WLPSSSPVTGYR
GDSPASSKPISINYR	NWIQYK	WQEEMELYR
GDVAFVK	NYNLVESLK	WSRPQAPITGYR
GETHEQVHSILHFK	PALPAGTEDTAKEDAANR	WYEIEKIPTTFENGR
GEVQAMLGQSTEELR	PLVEEPQNLIK	YEASILTHDSSIR
GEVQAMLGQSTEELRVR	PNSMVVEHPEFLK	YEFLNGR
GEWVALNPLR	PPEIAHGYPEHSVR	YEITTIHNLFR
GFEPTLEALFGK	PYTFHSHGITYYK	YFIDFVAR
GFSLDEATNLNGLLR	QFSFPLSSEPFQGSYK	YGLVTYATYPK
GGETGYFVDFSVR	QGHNSVFLIK	YGMVAQVTQTLK
GGETAQSADPQWEQLNNK	RHPDYSVVLRLR	YKEENDDFASFRVDR
GGYTLVSGYPK	RHPYFYAPELLFFAK	YLGEEYVK
GHLFLQTDQPIYNPGQR	RLDGSVDFK	YLQEIYNSNNQK
GHMLNHVER	RLDGSVDFKK	YLQEIYNSNNQKIVNLK
GKWERPFEVK	RLEVDIDIK	YLYEIR
GSESGIFTNTK	RPYFPVAVGK	YNPVVIDFEMQPIHEVLR
GSFEFPVGDAVSK	RQSEDSTFYLGER	YQISVNK
GSPAINVAVHVFR	RRDGYLFQLLR	YTFELSR
GWVTDGFSSLK	RSFFSFLGEAFDGAR	YVGGQEHFAHLLILR
GWVTDGFSSSLKDYWSTVK	RTHLPEVFLSK	YVLPNFEVK
GYSISYATK	RVDTVDPYPYR	YVNKEIQNAVNGVK
HGGLYHENMR	RVEPYGENFNK	YYTYLIMNK
HLEV DVWVIEPQGLR	RYIETDPANR	

Confident:

AHVDALR	FNKPFVFLMIEQNTK	NRDVVLTTTFVDDIK
ALFVSEEEKK	GDKVWVYPPEK	RQDNEILIFWSK
ALVEGVDQLFTDYQIK	GETHEQVHSILHFK	RTHLPEVFLSKVLEPTLK
AYYENSPQQVFSTEFVK	GSPAINVAVHVFRK	SDVVYTDWK
DGYLFQLLR	GSWVNKFPVEMTHNHNFR	SDVVYTDWKK
DIFTGLIGPMK	HGTDDGVVWMNWK	SIEVFGQFNGKR
DLEIEVVLFPNYNINGK	HTSLGPLEAK	SKEQLTPLIKK
DNDGWLTSDPR	IPKSDVVYTDWKK	SWFEPLVEDMQR
DNENVVNEYSSELEK	IPTTFENGR	SYFEKSKEQLTPLIKK
DNEQHVK	IQPSGGTNINEALLR	TLLSNLEEAKK
DRLDEVKEQVAEVR	KGEWVALNPLRK	TVIGPDGHKEVTK
DSAHGFLK	KTLLSNLEEAK	VDTVDPYPYR
DSGFQMNQLR	LDDDLEHQGGHVLDHGHK	VQFELHYQEVK
DTEEDFHVDQVTTVK	LEQGENVFLQATDK	VTFQLTYEEVLK
DYVSQFEGSALGK	LLPHANEVSQK	WDPYKQGFGNVATNTDGK
ERGHMLNHVER	LSPLGEEMR	WFYIASAFRNEEYNK
ESLSSYWESAK	LSSPAVITDK	YVGGQEHFAHLLILRDTK
EYHFGQAVR	MLTPEHVFIHPGWK	

Low Confidence:

DVVLTTTFVDDIK	PVWLGFGLPIIK	TEGDGVYTLNNEK
EDFTSLSLVLYSR	SNLDEDIIAENIVSR	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 identity, 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

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

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⁽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.