<table>
<thead>
<tr>
<th>Task: <strong>NMR Sample Preparation</strong></th>
<th><strong>Location</strong>: Rooms G14, G16, G23 and G24 in the Henry Wellcome Building for Biomolecular NMR Spectroscopy, University of Birmingham</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Equipment Used</strong>: Centrifuge, pH meter, NMR spectrometers, fumehood, fridges, freezers, tube cleaner</td>
<td><strong>Hazard</strong> (e.g. physical, ergonomic, biological, chemical, radiation):</td>
</tr>
<tr>
<td><strong>Hazard</strong></td>
<td><strong>Risk Assessment</strong> ('high', 'medium' or 'low'):</td>
</tr>
<tr>
<td>1. <strong>Radiation</strong>: danger to people and equipment due to inappropriate submission of radioactive samples.</td>
<td>1. <strong>Low</strong>: All Users must understand and agree that no samples are allowed that require any form of radiological monitoring</td>
</tr>
<tr>
<td>2. <strong>Chemical</strong>: danger to people and equipment due to submission of carcinogenic, corrosive, flammable or toxic samples.</td>
<td>2. <strong>Medium</strong>: Use of hazardous chemicals is discouraged. Users must follow all Control of Substances Hazardous to Health (COSHH) regulations. Hazardous chemicals brought into the building must be reported and approved by HWB-NMR staff, and labelled and stored in a safe manner at all times. Excluded Chemical Weapons Convention Scheduled Chemicals are listed in Appendix 2. COSHH Assessment NMR002 ‘Protein NMR Sample Preparation’ on the Cancer Studies COSHH database covers a number of chemicals commonly used in biomolecular NMR. Copies are available from S. Rhodes.</td>
</tr>
<tr>
<td>3. <strong>Biological</strong>: danger to people due to submission of biological hazards such as toxins, infectious agents, viruses, pathogenic bacteria.</td>
<td>3. <strong>Low</strong>: Use of any biological hazard is discouraged and must follow all COSHH regulations. Some of the disallowed biological agents are listed in Appendix 1. All samples must be labelled and stored in a safe manner at all times.</td>
</tr>
<tr>
<td>4. <strong>Ethical</strong>: samples submitted may be of human or animal origins, requiring consent.</td>
<td>4. <strong>Low</strong>: No testing of human or animal subjects shall be conducted on the premises. Ethical approvals for human or animal tissues or biofluids must be obtained from the appropriate ethical review body and submitted to HWB-NMR staff prior to initiation of NMR analysis.</td>
</tr>
<tr>
<td>5. <strong>Physical</strong>: Inappropriate use of manual centrifuge can cause sample breakage and debris, and involves spinning an exposed rotor. The insertion of samples into NMR magnets requires climbing access platform near the magnets.</td>
<td>5. <strong>Low</strong>: Use of equipment in the laboratory and NMR chambers requires appropriate user induction and appropriate access systems.</td>
</tr>
<tr>
<td>6. <strong>Financial</strong> (e.g. damage to NMR systems): NMR sample tubes can break and release their contents onto and damage probes. Probes can be damaged by insertion of hazardous samples, stripping the probe tuning rods by forced overturning, or use of excessive RF pulse power or probe temperatures. These probes can cost over £100000, and full insurance cover is not affordable.</td>
<td>6. <strong>Medium</strong>: NMR samples can only be inserted and experiments initiated by fully trained Users and Staff who understand the risks and accept their responsibilities. Users are not permitted to modify the NMR hardware or control software or attempt new pulse sequences unless these actions have been authorized by the NMR staff.</td>
</tr>
<tr>
<td><strong>Safety Pre-Requisites</strong> (e.g. secure access, training, work order, supervision, warning signs, protective equipment):</td>
<td><strong>Prepared by</strong></td>
</tr>
<tr>
<td>1. Ensure compliance with COSHH regulations (see <a href="http://www.coshh-essentials.org.uk/">http://www.coshh-essentials.org.uk/</a>)</td>
<td>1. Prof Michael Overduin, phone 4143802, <a href="mailto:M.Overduin@bham.ac.uk">M.Overduin@bham.ac.uk</a></td>
</tr>
<tr>
<td>2. Submit completed form for NMR time or sample analysis indicating all hazards. Provide MSDS datasheets for any hazardous chemicals.</td>
<td>2. Dr Ulrich Günther, phone 4148361, <a href="mailto:U.L.Gunther@bham.ac.uk">U.L.Gunther@bham.ac.uk</a></td>
</tr>
<tr>
<td>3. All Users must obtain approval from the Operations Manager before initiating NMR experiments, including the completion of an orientation for the safe use of the NMR systems.</td>
<td></td>
</tr>
</tbody>
</table>
Key Points (warning, check points, emergency/first aid information):
1. In case of any accident, concern or question regarding these protocols please notify the Preparers (see above)
2. It is the user’s responsibility to be familiar with and comply with these procedures.
3. Negligence or non-compliance can result in barring from future use of HWB-NMR resources.
4. Disputes will be handled by the Health and Safety Committee.
5. Approved chemicals (e.g. $^2$H$_2$O) and glassware (e.g. NMR tubes) may be stocked in the HWB-NMR sample preparation laboratory, and are available upon request from Sue Rhodes.

Considerations for Protein NMR Sample Preparation

1. Molecular weight: Larger molecules have longer correlation times, leading to faster relaxation and increased line-widths. NMR is best suited to characterizing folded proteins in the 5-40 kDa range, larger proteins and complexes require exponentially more time and expense. Tags used for affinity purification (e.g. glutathione S transferase or thioredoxin) should be cleaved and removed unless they are small (under 10 residues, e.g. His-tags).

2. Concentration: Protein concentrations should ideally be between 0.5 and 1.5 mM for structural analysis (1mM = 10mg/ml for a 10kDa protein), noting that doubling the concentration requires approximately a quarter of the acquisition time to obtain a given signal to noise ratio, but increases sample viscosity. Proteins should be purified to >90\% homogeneity, i.e. a single clean band on a SDS-PAGE gel or, even better, a single peak on a size exclusion chromatography column. The sequence and exact size of the construct should be verified by DNA or protein sequencing.

3. Stability: The sample may be in the NMR spectrometer for many hours or days, with temperatures for data collection ranging from 4 - 40ºC. The standard operating temperature is 25ºC. Highly concentrated proteins tend to aggregate and precipitate, yielding poor NMR spectra.

4. Buffer: Ideally 20 mM, although 0-50 mM is common. Phosphate buffer is an economical non-protonated buffer, and there also are a variety of perdeuterated buffers (d-Tris, d-HEPES) available that do not obscure protein NMR signals.

5. pH: Usually acidic pH is required (typically 5-7) because many NMR experiments require observation of exchangeable amide protons which are difficult to observe at higher pH. Protein structure and interactions can be pH sensitive, so the final sample pH should be verified.

6. Ionic Strength: Salt (e.g. KCl) can increase protein solubility. However, high ionic strength (>150mM) demands longer 90º pulses, increases sample heating, reduces signal to noise (especially using cryogenic probes) and makes it more difficult to tune the probe.

7. Paramagnetics: Paramagnetic metals such as Cu(II), Mn(II), Cr(III), Fe(III) and Co(II) lead to NMR line broadening, and should typically be avoided.

8. Volume: Each NMR sample should consist of a final volume of 550 \mu L, including all additives, for a standard tube (e.g. Wilmad 535).

9. Additives: $^2$H$_2$O is added to 10\% (or 5\% for high value samples) for locking on the NMR signal frequencies, unless a 100\% $^2$H$_2$O solution is being used to clearly observe nonexchangeable protons. Sodium azide (usually 1-3mM) is added to prevent microbial contamination. A reducing agent such as DTT (<1mM or higher concentration - 30mM - if perdeuterated DTT) can be added to prevent protein oxidation. The internal standard 2,2-dimethylsilapentane-5-sulfonic acid (DSS) can be added (typically 50 \mu M) to reference the chemical shifts. If sample gets proteolysed, protease inhibitors can be added at low concentrations (<50\mu M).

10. Sample Tubes: Wilmad 535 NMR tubes (7 inch) are standard for protein NMR. New NMR tubes are not ‘analytically clean’ when delivered, but usually have organic or inorganic residues. Ensure tubes are clean (a rinse with water or buffer is advisable) and not chipped or warped by excessive heat. Shigemi tubes can be used for low volume samples, but require special care to eliminate bubbles. Tubes should be capped and the cap parafilmed to avoid evaporative loss.

11. Isotope labelling: Although proteins can initially be assessed for suitability for structural analysis in unlabelled forms, detailed studies require labelling with $^15$N for small (~50-100 residues), $^15$N and $^13$C for medium (~100-150 residues), and $^{15}$N, $^{13}$C and $^2$H for large (>~150 residues) proteins.

Procedure – Operational Notes

1. Use of concentrators: Proteins are often exchanged into their final solution conditions using concentrators with molecular weight cut off filters. The filters are stored with glycerol, which must be removed by at least three washes or spins of the concentrator with deionized water.

2. Washing NMR tubes: NMR tube washers or Solvent Jet Cleaners can be purchased from GPE Limited, Sigma Aldrich and Wilmad, an economical 9 tube washer unit can be built by the School of Chemistry glassblowing shop, and will be provided by the HWB-NMR wet-lab and Overduin’s laboratory (Institute for Cancer Studies S313 ). Strong acids such as Nochromix (Godax Laboratories) are available to remove adhered materials and deposits by overnight soaking, followed by washes with water or buffer.

3. Transport of NMR tubes: Individual NMR tubes can be safely transported in NMR tube racks made by, for example, Kimble-Kontes or Wilmad, graduated cylinders, or inverted and taped 15 mL Falcon tubes.

4. Short term storage of NMR tubes: use specialized NMR tube racks at the consoles and refrigerator to minimize
risks of breakage of the delicate NMR tubes.

5. **Long term storage of NMR tubes**: We recommend flash freezing proteins in liquid nitrogen and storage at -80°C to minimize risks of oxidation or degradation. A small scale freeze and thaw trial experiment is advised to assess the risk of protein precipitation during sample warm up.

**Sources and references**

The following companies sell NMR reagents and consumables:

2. Cambridge Isotope Laboratories - [http://www.isotope.com](http://www.isotope.com) sells isotope labelled reagents for NMR
3. Isotec - (a division of Sigma/Aldrich) sells isotope labelled reagents for NMR
4. Silantes GmbH - [http://www.silantes.com](http://www.silantes.com) sells isotope labelled reagents for NMR
5. C/D/N ISOTOPES - [http://www.cdniiso.com](http://www.cdniiso.com) sells isotope labelled reagents for NMR
6. Medical Isotopes, Inc. - [http://www.medicalisotopes.com](http://www.medicalisotopes.com) sells isotope labelled reagents for NMR
7. Wilmad Glass Company: The standard NMR tube is Wilmad product 5mm 535-PP 7, although a less expensive tube (528-PP 7) can also be used for routine use.
8. Shigemi, Inc: For low volume samples use 5 mm tubes from Shigemi, Inc (412-444-3011). Ensure that tubes are matched to the appropriate solvent.
9. GPE Limited [http://www.gpelimited.co.uk/](http://www.gpelimited.co.uk/) sells glassware for NMR
10. Godax Laboratories sells Nochromix

The following articles may provide useful information:

**Chemical shift standards**

DS Wishart et al. (1995) 1H, 13C and 15N chemical shift referencing in biomolecular NMR, J Biomol NMR 6, 135-140.

**NMR Buffers**

CH Schein (1990) Solubility as a function of protein structure and solvent components, Biotechnology 8, 308-316


**General NMR**

Three Methods in Enzymology volumes (176, 177 and 239) are dedicated to biomolecular NMR. See Norman Oppenheimer's article "Sample Preparation" pp 78-89, Vol 176 for useful hints on preparing a NMR sample.

**Protein solubility**


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**Appendix 1. Excluded Infectious Agents, Bacteria, Viruses and Toxins:**

Note: The following list of excluded substances is not necessarily exhaustive. Please contact NMR staff if you are unsure about a particular substance.

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**ANTI-TERRORISM, CRIME AND SECURITY ACT 2001**

Part 7 of the Anti-Terrorism, Crime and Security Act 2001 is concerned with the security of dangerous substances that may be targeted or used by terrorists. These substances are listed in Schedule 5 of the Act. The Schedule was amended in 2007. The current list is shown below and includes viruses, rickettsiae, fungi, bacteria and toxins. The toxins are also included in HAZDAT.

The provisions set out in Part 7 (and Schedules 5 and 6) place an obligation on managers of laboratories and other premises holding stocks of specified disease-causing micro-organisms and toxins to notify their holdings, and to comply with any reasonable security requirements which the police may impose.

It also requires managers of laboratories and other premises, on request, to furnish the police with details of people with access to the dangerous substances held there. The Secretary of State is given power to direct that a named individual must not be allowed access to such disease strains or the premises in which they are held.

Reporting etc, is co-ordinated through the University Health and Safety Unit.

**VIRUSES**

- Chikungunya virus
- Mobala virus
- Congo-crimean haemorrhagic fever virus
- Monkey pox virus
<table>
<thead>
<tr>
<th>Virus Name</th>
<th>Virus Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dengue fever virus</td>
<td>Mucambo virus</td>
</tr>
<tr>
<td>Dobrava/Belgrade virus</td>
<td>Murray Valley encephalitis virus</td>
</tr>
<tr>
<td>Eastern equine encephalitis virus</td>
<td>Ndumu virus</td>
</tr>
<tr>
<td>Ebola virus</td>
<td>Nipah virus</td>
</tr>
<tr>
<td>Everglades virus</td>
<td>Omsk haemorrhagic fever virus</td>
</tr>
<tr>
<td>Getah virus</td>
<td>Polio virus</td>
</tr>
<tr>
<td>Guanarito virus</td>
<td>Powassan virus</td>
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<tr>
<td>Hantaan virus</td>
<td>Rabies virus</td>
</tr>
<tr>
<td>Hendra virus (Equine morbillivirus)</td>
<td>Rift Valley fever virus</td>
</tr>
<tr>
<td>Herpes simiae (B virus)</td>
<td>Rocio virus</td>
</tr>
<tr>
<td>Influenza viruses (pandemic strains)</td>
<td>Sabia virus</td>
</tr>
<tr>
<td>Japanese encephalitis virus</td>
<td>Sagiyama virus</td>
</tr>
<tr>
<td>Junin virus</td>
<td>Sin Nombre virus</td>
</tr>
<tr>
<td>Kyasanur Forest virus</td>
<td>St Louis encephalitis virus</td>
</tr>
<tr>
<td>Lassa fever virus</td>
<td>Tick-borne encephalitis virus (Russian Spring-Summer encephalitis virus)</td>
</tr>
<tr>
<td>Louping ill virus</td>
<td>Variola virus</td>
</tr>
<tr>
<td>Lymphocytic choriomeningitis virus</td>
<td>Venezuelan equine encephalitis virus</td>
</tr>
<tr>
<td>Machupo virus</td>
<td>West Nile fever virus</td>
</tr>
<tr>
<td>Marburg virus</td>
<td>Western equine encephalitis virus</td>
</tr>
<tr>
<td>Mayaro virus</td>
<td>Yellow fever virus</td>
</tr>
<tr>
<td>Middleburg virus</td>
<td></td>
</tr>
</tbody>
</table>

**RICKETTSSIAE**

- Coxiella burnetii
- Rickettsia prowazeki
- Rickettsia rickettsii
- Rickettsia typhi (mooseri)

**BACTERIA**
Bacillus anthracis  Francisella tularensis
Brucella abortus  Multiple-drug resistant Salmonella paratyphi
Brucella canis  Mycobacterium tuberculosis
Brucella melitensis  Salmonella paratyphi A, B, C
Brucella suis  Salmonella typhi
Burkholderia mallei (Pseudomonas mallei)  Shigella boydii
Burkholderia pseudomallei (Pseudomonas pseudomallei)  Shigella dysenteriae
Chlamydia psittaci  Shigella flexneri.
Clostridium botulinum  Vibrio cholerae
Clostridium perfringens  Yersinia pestis
Enterohemorrhagic Escherichia coli, serotype 0157 and verotoxin producing strains

FUNGI
Cladophialophora bantiana
Cryptococcus neoformans.

TOXINS

<table>
<thead>
<tr>
<th>TOXIN</th>
<th>CAS NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abrin Modeccin toxin</td>
<td>1393-62-0</td>
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<tr>
<td>Abrin</td>
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<tr>
<td>Botulinum toxins</td>
<td>93384-46-4</td>
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<td>Botulin D</td>
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<tr>
<td>Botulin toxin A</td>
<td>93384-43-1</td>
</tr>
<tr>
<td>Botulinum toxin B</td>
<td></td>
</tr>
<tr>
<td>Botulinum toxin F</td>
<td></td>
</tr>
<tr>
<td>Clostridium botulinum toxin</td>
<td></td>
</tr>
<tr>
<td>Clostridium perfringens toxins</td>
<td></td>
</tr>
<tr>
<td>Clostridium botulinum neurotoxin</td>
<td></td>
</tr>
<tr>
<td>Clostridium perfringens, epsilon toxin</td>
<td></td>
</tr>
<tr>
<td>Clostridium perfringens, type A enterotoxin</td>
<td></td>
</tr>
<tr>
<td>Conotoxin</td>
<td>123210-68-4</td>
</tr>
<tr>
<td>Conotoxin</td>
<td></td>
</tr>
<tr>
<td>Modeccin</td>
<td>65988-88-7</td>
</tr>
</tbody>
</table>
Modeccin

Ricin
Ricin 9009-86-3

Saxitoxin
Saxitoxin 35523-89-8

Shiga and Shiga-like toxins
Shiga toxin

DNA (Escherichia coli strain KY-019 clone pKTN1054 Shiga-like toxin SLT-II subunit A gene plus Shiga-like toxin SLT-II subunit B gene) 153834-56-1

DNA (Escherichia coli strain KY-019 clone pKTN1054 Shiga-like toxin SLT-II subunit A gene) 153834-58-3

DNA (Escherichia coli strain KY-019 clone pKTN1054 Shiga-like toxin SLT-II subunit B gene) 153834-60-7

DNA (Escherichia coli strain TK-051 clone pKTN1050 Shiga-like toxin SLT-II subunit A gene plus Shiga-like toxin SLT-II subunit B gene) 153834-57-2

DNA (Escherichia coli strain TK-051 clone pKTN1050 Shiga-like toxin SLT-II subunit A gene) 153834-59-4

Verotoxin 1 (Shiga shigella B subunit) 620190-09-2

Staphylococcal enterotoxins
Staphylococcal enterotoxin A (Staphylococcus aureus gene SEA) 915245-87-3

Staphylococcal enterotoxin B (Staphylococcus aureus aureus strain COL gene seb) 811333-16-1

Staphylococcal enterotoxin C-bovine (Staphylococcus aureus host cattle gene sec-bov) 349587-80-0

Staphylococcal enterotoxin E (Staphylococcus aureus) 197981-85-4

Tetrodotoxin
Tetrodotoxin 4368-28-9

Viscum Album Lectin 1 (Viscumin)
Viscum Album Lectin 1 83590-17-4

Volkensin toxin
Volkensin toxin 91933-11-8

Any reference to a micro-organism or toxin includes:

(a) any genetic material containing any nucleic acid sequence
   - associated with the pathogenicity of the micro-organism or
   - for the coding of the toxin; and

(b) any genetically modified organism containing any such sequence.

Any reference to a toxin includes subunits of the toxin.

<table>
<thead>
<tr>
<th>Abrin</th>
<th>Marburg virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxins</td>
<td>Menangle virus</td>
</tr>
<tr>
<td>African horsesickness virus</td>
<td>Mycoplasma capricolum /M. F38/M. mycoides capri (contagious caprine)</td>
</tr>
<tr>
<td>African swine fever</td>
<td></td>
</tr>
</tbody>
</table>
Akabane virus | Mycoplasma mycoides mycoides (contagious bovine pleuropneumonia)  
Avian influenza (highly pathogenic) | Newcastle disease virus (exotic)  
Bacillus anthracis | Nipah virus  
Bluetongue virus (exotic) | Peronosclerospora philippinensis  
Botulinum toxins | Peste des petits ruminants  
Bovine spongiform encephalopathy agent | Phakopsora pachyrhizi pleuropneumonia)  
Brucella abortus, Brucella melitensis, Brucella suis | Plum pox potyvirus  
Burkholderia (Pseudomonas) mallei, Burkholderia (Pseudomonas) pseudomallei | Ralstonia solanacearum Race 3  
Camel pox virus | Ricin  
Classical swine fever | Rickettsia prowazekii  
Clostridium botulinum | Rickettsia rickettsii  
Clostridium perfringens epsilon toxin | Rift Valley fever virus  
Coccidioides immitis | Rinderpest virus  
Conotoxins | Saxitoxin  
Cowdria ruminantium (heartwater) | Sheeppox  
Coxiella burnetii | Shigatoxin  
Crimean-Congo haemorrhagic fever virus | South American haemorrhagic fever viruses  
Diacetoxyscerpinol | Staphylococcal enterotoxins  
Eastern equine encephalitis virus | Swine vesicular disease virus  
Ebola viruses | Sycnutriyum endobioticum  
Equine morbillivirus (Hendra virus) | T-2 toxin  
Foot-and-mouth disease virus | Tetrodotoxin  
Francisella tularensis | Tick-borne encephalitis complex viruses  
Goat pox virus | Variola major virus (smallpox)  
Japanese encephalitis virus | Venezuelan equine encephalitis virus  
Lassa fever virus | Vesicular stomatitis (exotic)  
Liberobacter africanus | Viruses causing hantavirus pulmonary syndrome  
Liberobacter asiaticus | Xanthomonas oryzae pv. oryzicola  
Lumpy skin disease virus | Xylella fastidiosa (citrus variegated chlorosis strain)  
Malignant catarrhal fever | Yellow fever virus  

Appendix 2: Excluded chemicals:

**Chemical Weapons Convention Scheduled Chemicals.**

The following Schedules list toxic chemicals and their precursors.

(Whenever reference is made to groups of dialkylated chemicals, followed by a list of alkyl groups in parentheses, all chemicals possible by all possible combinations of alkyl groups listed in the parentheses are considered as listed in the respective Schedule as long as they are not explicitly exempted.)

**Schedule 1**

**A. Toxic chemicals:**

<table>
<thead>
<tr>
<th>Description</th>
<th>CAS registry number</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) O-Alkyl (&lt;C10, incl. cycloalkyl) alkyl(Me, Et, n-Pr or i-Pr)-phosphonofluoridates</td>
<td></td>
</tr>
</tbody>
</table>
  e.g. Sarin: O-Isopropyl methylphosphonofluoridate 107-44-8  
  Soman: O-Pinacolyl methylphosphonofluoridate 96-64-0  |
| (2) O-Alkyl (<C10, incl. cycloalkyl) N,N-dialkyl (Me, Et, n-Pr or i-Pr) phosphoramidocyanidates |  
  e.g. Tabun: O-Ethyl N,N-dimethylphosphoramidocyanidate 77-81-6  |
| (3) O-Alkyl (H or <C10, incl. cycloalkyl) S-2-dialkyl(Me, Et, n-Pr or i-Pr)-aminoethyl alkyl (Me, Et, n-Pr or i-Pr) phosphonothiolates and corresponding alkylated or protonated salts |  
  e.g. VX: O-Ethyl S-2-diisopropylaminoethyl methyl phosphonothiolate 50782-69-9  |
| (4) Sulfur mustards: |  
  2-Chloroethylchloromethylsulfide 2625-76-5  
  Mustard gas: Bis(2-chloroethyl)sulfide 505-60-2  
  Bis(2-chloroethylthio)methane 63869-13-6  
  Sesquimustard: 1,2-Bis(2-chloroethylthio)ethane 3563-36-8  
  1,3-Bis(2-chloroethylthio)-n-propane 63905-10-2  
  1,4-Bis(2-chloroethylthio)-n-butane 142868-93-7  
  1,5-Bis(2-chloroethylthio)-n-pentane 142868-94-8  
  Bis(2-chloroethylthiomethyl)ether 63918-90-1  
  O-Mustard: Bis(2-chloroethylthioethyl)ether 63918-89-8  |
| (5) Lewisites: |  
  Lewisite 1: 2-Chlorovinylchloroarsine 541-25-3  
  Lewisite 2: Bis(2-chlorovinyl)chloroarsine 40334-69-8  |
Lewisite 3: Tris(2-chlorovinyl)arsine 40334-70-1
(6) Nitrogen mustards:
HN1: Bis(2-chloroethyl)ethylamine 538-07-8
HN2: Bis(2-chloroethyl)methylamine 51-75-2
HN3: Tris(2-chloroethyl)amine 555-77-1
(7) Saxitoxin 35523-89-8
(8) Ricin 9009-86-3

B. Precursors:
(9) Alkyl (Me, Et, n-Pr or i-Pr) phosphonyldifluorides
e.g. DF: Methylphosphonyldifluoride 676-99-3
(10) O-Alkyl (H or <C10, incl. cycloalkyl) O-2-dialkyl (Me, Et, n-Pr or i-Pr)-aminoethyl
alkyl (Me, Et, n-Pr or i-Pr) phosphonites and corresponding alkylated or protonated salts
e.g. QL: O-Ethyl O-2-diisopropylaminoethylmethylphosphonite 57856-11-8
(11) Chlorosarin: O-Isopropyl methylphosphonochloridate 1445-76-7
(12) Chlorosoman: O-Pinacolyl methylphosphonochloridate 7040-57-5

Schedule 2

A. Toxic chemicals:
(1) Amiton: O,O-Diethyl S-[2-(diethylamino)ethyl]phosphorothiolate and corresponding alkylated or protonated salts 78-53-5
(2) PFIB: 1,1,3,3,3-Pentafluoro-2-(trifluoromethyl)-1-propene 382-21-8
(3) BZ: 3-Quinuclidinyl benzilate (*) 6581-06-2

B. Precursors:
(4) Chemicals, except for those listed in Schedule 1, containing a phosphorus atom to which is bonded one methyl, ethyl or propyl (normal or iso) group but not further carbon atoms,
e.g. Methylphosphonyl dichloride 676-97-1
Dimethyl methylphosphonate 756-79-6
Exemption: Fonofos: O-Ethyl S-phenylethylphosphonothiolothionate 944-22-9
(5) N,N-Dialkyl (Me, Et, n-Pr or i-Pr) phosphoramidic dihalides
(6) Dialkyl (Me, Et, n-Pr or i-Pr) N,N-dialkyl (Me, Et, n-Pr or i-Pr)-phosphoramidates
(7) Arsenic trichloride 7784-34-1
(8) 2,2-Diphenyl-2-hydroxyacetic acid 76-93-7
(9) Quinuclidin-3-ol 1619-34-7
(10) N,N-Dialkyl (Me, Et, n-Pr or i-Pr) aminoethyl-2-chlorides and corresponding protonated salts
(11) N,N-Dialkyl (Me, Et, n-Pr or i-Pr) aminoethane-2-ols and corresponding protonated salts
Exemptions: N,N-Dimethylaminethanol and corresponding protonated salts 108-01-0
N,N-Diethylaminethanol and corresponding protonated salts 100-37-8
(12) N,N-Dialkyl (Me, Et, n-Pr or i-Pr) aminoethane-2-thiols and corresponding protonated salts
(13) Thiodiglycol: Bis(2-hydroxyethyl)sulfide 111-48-8
(14) Pinacolyl alcohol: 3,3-Dimethylbutan-2-ol 464-07-3

Schedule 3

A. Toxic chemicals:
(1) Phosgene: Carbonyl dichloride 75-44-5
(2) Cyanogen chloride 506-77-4
(3) Hydrogen cyanide 74-90-8
(4) Chloropicrin: Trichloronitromethane 76-06-2

B. Precursors:
(5) Phosphorus oxychloride 10025-87-3
(6) Phosphorus trichloride 7719-12-2
(7) Phosphorus pentachloride 10026-13-8
(8) Trimethyl phosphite 121-45-9
(9) Triethyl phosphite 122-52-1
(10) Dimethyl phosphite 868-85-9
(11) Diethyl phosphite 762-04-9
<table>
<thead>
<tr>
<th>Number</th>
<th>Chemical Name</th>
<th>CAS Number</th>
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<tbody>
<tr>
<td>12</td>
<td>Sulfur monochloride</td>
<td>10025-67-9</td>
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<td>13</td>
<td>Sulfur dichloride</td>
<td>10545-99-0</td>
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<td>14</td>
<td>Thionyl chloride</td>
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<td>15</td>
<td>Ethyldiethanolamine</td>
<td>139-87-7</td>
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<td>16</td>
<td>Methyldiethanolamine</td>
<td>105-59-9</td>
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<td>17</td>
<td>Triethanolamine * Please just inform us if you intend to use TEA</td>
<td>102-71-6</td>
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