Commissioned Papers

Synthetic Genomics: Risks and Benefits for Science and Society

This volume of papers accompanies the report
Synthetic Genomics: Options for Governance

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COMMISSIONED PAPERS

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The following papers were commissioned for the project **Synthetic Genomics: Risks and Benefits for Science and Society.** These papers formed the basis of many discussions at project workshops and at a large invitational meeting. The information elicited from these meetings, and from the commissioned papers themselves, formed the basis of our report *Synthetic Genomics: Options for Governance* (http://dspace.mit.edu/handle/1721.1/39141).

The views and opinions expressed in these commissioned papers are those of the authors of the papers and not necessarily those of the authors of the report, or of the institutions at which the authors work.

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Sanghvi Y. 2005. A Roadmap to the Assembly of Synthetic DNA from Raw Materials. In: *Working Papers for Synthetic Genomics: Risks and Benefits for Science and Society*, pp. 17-33. Garfinkel MS, Endy D, Epstein GL, Friedman RM, editors. 2007.

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Sequence Screening

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Introduction

The use of biological agents in acts of terrorism has received heightened interest since the mailing of anthrax spores in the United States in 2001. Many scenarios have been considered in which bacterial and viral pathogens could be produced and employed as weapons. While some may seem unlikely, the scientific community has a responsibility to assess all threats and to develop ways to monitor, and perhaps counter, any attempts to carry them out. The focus of the current study concerns the use of DNA synthesis and genetic manipulation to create or modify pathogens.

It can be argued that a terrorist group would be much more likely to use a 'conventional' pathogen, such as anthrax, than to design and engineer a modified organism. While this is convincing, there are several strong reasons why someone might wish to employ synthetic DNA. Conventional threats require that the terrorist has access to the pathogen. Some pathogens, such as anthrax, can be isolated in the wild in certain parts of the world. It is clearly possible to culture natural isolates, but the process can be laborious and may yield a strain that is not well suited for use as a biological weapon. In most cases, the easiest sources for pure cultures of pathogens are the laboratories that work on them. In the US such labs are strictly regulated with measures such as background checks on researchers, careful inventory management and high levels of building security. These make it extremely difficult for anyone outside those laboratories to access the pathogens that they contain. Smallpox virus is an example of a pathogen that, having been eradicated in the wild, could only be obtained from a few specific laboratories, all of which operate under tight security.

The alternative approach that concerns us here is that someone could synthesize the entire genome of a dangerous pathogen, such as smallpox, from scratch. This requires no access

to the secure laboratories. Potentially it requires no prior experience in working with the pathogen. Most troubling is the fact that such synthesis could be accomplished in a conventional molecular biology laboratory, without the need for specialized equipment and without attracting attention to the project from others.

The technology required to synthesize the genome of an entire viral pathogen, or genes thereof, is already available. Rapid development in the field of synthetic biology is destined to make this process easier, faster and cheaper.

This evolution in technology brings with it tremendous benefits to biotechnology and medicine but its potential for abuse is a cause for concern. Being able to determine if nefarious activity is underway will become an important requirement for the regulatory authorities.

Here there is some cause for optimism. Currently the vast majority of DNA synthesis is performed by service companies or by in-house central facilities in universities and large companies. The DNA synthesis industry provides researchers with custom DNA at such low cost and with such convenience that almost all synthesis work takes place in a relatively small number of facilities.

A request for DNA synthesis requires that the customer provide the sequence of the molecule. This creates the opportunity to monitor or screen input sequences for matches to a database of pathogen sequences. Finding a positive match at the time the order was received would allow the vendor to alert the relevant authority and to delay shipment of that DNA.

I have written a software package, called BlackWatch, that implements sequence screening. This paper will describe the operation of this system, its current shortcomings and ways that these might be addressed.

1. The Business of Synthetic DNA

At this point it is worth reviewing the state of the synthetic DNA industry as it stands today. Not only does this provide the venue in which to monitor attempts at engineering

pathogens, but its particular constraints and operating procedures have a significant practical impact on the way any sequence screening strategy might be implemented.

The chemical synthesis of oligonucleotides (oligos), short fragments of DNA, became widely available about 20 years ago with the manufacture of desktop DNA synthesizers. Oligos found widespread use in DNA sequencing with an equal, and perhaps greater, application in Polymerase Chain Reaction (PCR) experiments. The high demand for oligos led to the creation of companies that performed contract DNA synthesis on request. The convenience and low cost of using these vendors has driven substantial growth and competition in this industry and today hardly any research laboratories synthesize oligos themselves.

Fierce competition between the synthesis companies has driven prices down to the point where profit margins are minimal. In fact certain companies appear to offer the service as a 'loss leader' in order to attract customers to their other more lucrative products. These companies try to differentiate themselves on the basis of easy ordering via the Web, fast turnaround and value added options, such as chemical modifications of the oligos. The customer can visit the web site of the vendor, create an account, enter in the sequence of the oligo they want synthesized, enter their credit card details and hit submit. A tube containing the DNA will arrive by express delivery the next day or day after. The cost for this service is remarkable. A typical oligo of perhaps 20 to 25 nucleotides in length will cost around $0.30 per nucleotide, a total of less than $10 for a completely custom organic chemical synthesis. As a result, using one of these services is the preferred option for almost all laboratories.

There are probably several hundred companies or university facilities that offer oligo synthesis services around the world. The throughput of the larger companies is impressive. Integrated DNA Technologies (http://www.idtdna.com) of Iowa, states in its press releases that it synthesizes between 15,000 and 25,000 oligos daily and has more than 60,000 customers worldwide.

With improvements in the technologies behind DNA synthesis and gene assembly, it has become feasible to synthesize entire genes from sets of oligos. Several companies provide this service, some of which derive their entire income from gene synthesis. The technology is more involved than oligo synthesis but costs can be kept low, while maintaining accuracy, through the extensive use of laboratory automation. The distinction between companies that synthesize short oligos and those that synthesize entire genes, assembling these from sets of oligos, is important in the context of sequence screening.

The turnaround time for the synthesis of a gene of a few thousand nucleotides is a couple of weeks and the cost can be as low as $1.60 per nucleotide. At this price point it becomes easier to synthesize certain genes than to try to isolate them from their native genomes. There are around 25 companies in the US that offer this service with about the same number in the rest of the world, mostly in Europe. However, it would appear that most of that work is performed in a small subset of these companies.

The next step in the evolution of these technologies is the synthesis of entire genomes. Already the genomes of poliovirus (Wimmer et al. 2002. *Science 297*: 1016-1018) and bacteriophage phiX174 (Smith et al. 2003. *Proc. Natl. Acad. Sci. USA 100*: 15440-15445) have been synthesized from scratch and used to create infectious virus and phage particles, respectively.

Work is underway at the company Synthetic Genomics (http://www.syntheticgenomics.com) and at the J. Craig Venter Institute to identify the minimal set of genes that are necessary to sustain the bacterium *Mycoplasma genitalium*. Once this minimal genome has been defined, the company intends to use it as the foundation for a range of engineered synthetic organisms that possess novel characteristics.

If the history of DNA sequencing, PCR and oligo synthesis serve as a guide, we can expect the synthesis of genes and small genomes to become routine tools for molecular biology over the next decade.

One final aspect of the business of synthetic DNA is of particular importance. Confidentiality and the protection of intellectual property are extremely important to the biotechnology industry. Oligo vendors help ensure confidentiality by not asking customers about the nature of the sequences that they request or the uses to which they will be put. Indeed, most corporate customers would immediately stop using these vendors if they were required to disclose any information about the requested sequences.

This intentional ignorance about the sequences on the part of the vendor could play into the hands of anyone intent on synthesizing or engineering a pathogen. Widespread use of sequence screening software has the potential to remove this vulnerability while still retaining confidentiality for the vast majority of DNA synthesis customers.

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2. Sequence Screening

The basic idea behind sequence screening is straightforward. Sequences of oligos or entire genes that are to be synthesized are compared against a specific curated database of sequences from known pathogens, the 'Select Agents'. Any request that produces a significant match to a pathogen is tagged as being of interest and the site administrator is alerted.

I have implemented this approach in the BlackWatch software system. This consists of a custom sequence database, the BLAST sequence comparison software from NCBI (Altschul et al., Nucleic Acids Res. 1997, v25, pp3389-3402) and a set of Perl wrapper scripts that manage the user interface, run the BLAST searches and process the results.

The system can be accessed from a web interface, the UNIX command line and from custom interfaces to relational databases. A schematic diagram of the system in shown in Figure 1.

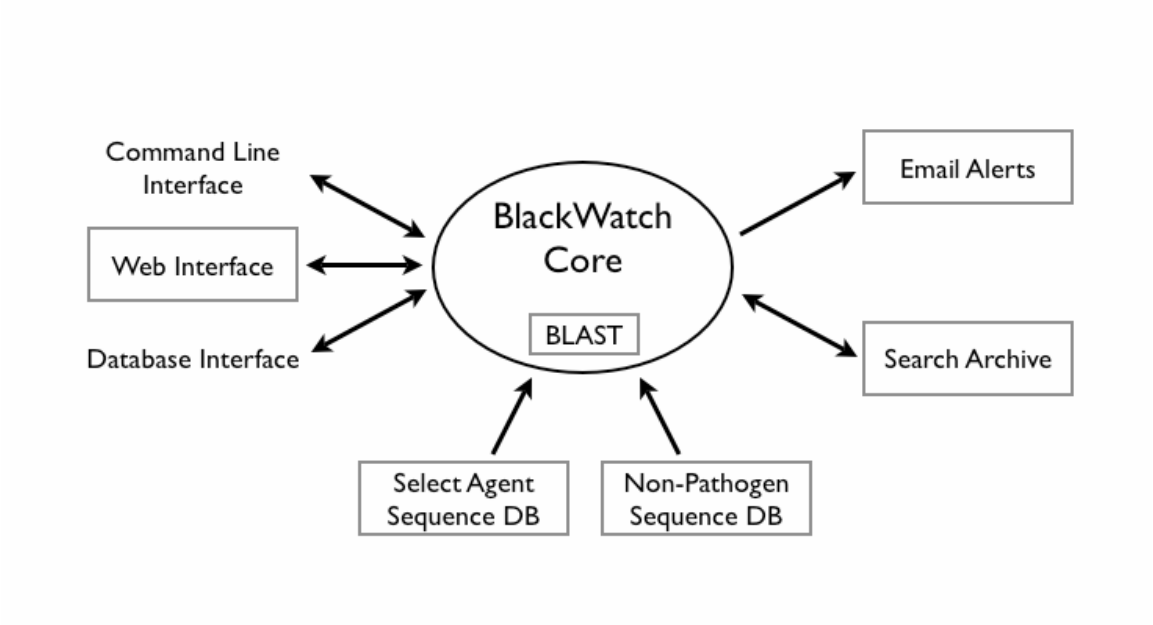

Figure 1: Structure of the BlackWatch Software.

Input sequences are passed to the core scripts from one of the interfaces. An assessment is made on the basis of length as to whether each batch contains short oligos or longer sequences. BLAST searches are initiated against the select agent sequence database. The system currently runs a *blastn* search of input nucleotide sequences against the nucleotide database and a *blastx* search of translated nucleotide sequences against a parallel database of protein sequences from the same pathogens. *tblastx* searches of translated nucleotide sequences against the translated nucleotide database will be introduced in the next version of the software.

BLAST results are processed and matches are assessed based on three criteria – absolute score, statistical significance (E-value), and the coverage of the matching segment. Coverage indicates how much of the query sequence is involved in the match. For an oligonucleotide one would expect the entire query sequence to be included in the alignment, whereas perhaps only part of a larger sequence would be involved. A combination of these criteria is used to select positive matches, with different cutoffs used with oligos relative to long sequences.

Search results for sequences that do not match are discarded, along with the sequences themselves. This is an important component in protecting proprietary information from customers. Positive search results against the select agent database are then searched against the non-pathogen database to see if they also match there. This is to help resolve false positives and is discussed below. Data are archived for each positive result. These include the input sequence, the raw BLAST output and associated information such as the customer identifier, date and time.

The system can be interfaced with relational databases. This will allow it to be driven by production databases at synthesis companies. In the absence of any common architecture for these databases, a custom interface script will have to be written for each company that chooses to set this up. I have successfully integrated the system with an Oracle database during beta testing at a leading oligo synthesis company.

Positive matches can be reported to relevant staff by way of email alerts. These include links to the web interface that will bring up the details of the match.

The search archives can be accessed by customer ID, allowing the history of sequence submissions to be reviewed. This will be important if a customer submits multiple related or overlapping sequences over a period of time. Comments can be added to each match and these are stored in the archive alongside the BLAST output. So one might record why a single match was assessed as a false positive. Later review in the context of other matches might lead you to change that assessment.

Below are some screenshots from the web interface. The first shows the query sequence input screen that will load a FASTA format file or accept sequences that are cut and pasted into the form.

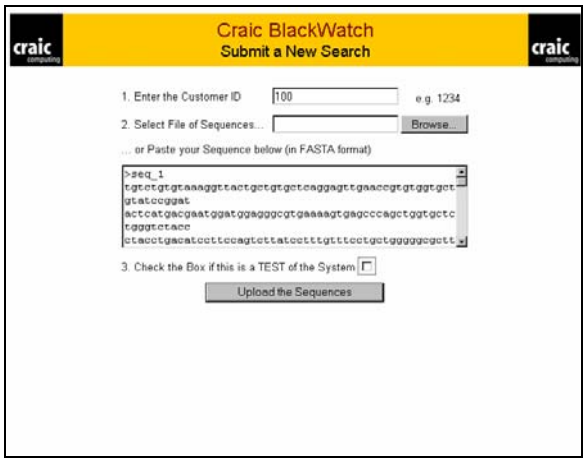

Figure 2: Sequence Input Web Page

Most searches will not produce matches and these are simply acknowledged as having been run. Positive matches are highlighted with links to the GenBank sequence that was hit, the raw BLAST output, the query sequence, etc.

Figure 3: Example of a Positive Match

The BLAST output is available for positive matches, allowing an expert to evaluate the quality of the alignment and thereby assess the likelihood of this being a true or false positive.

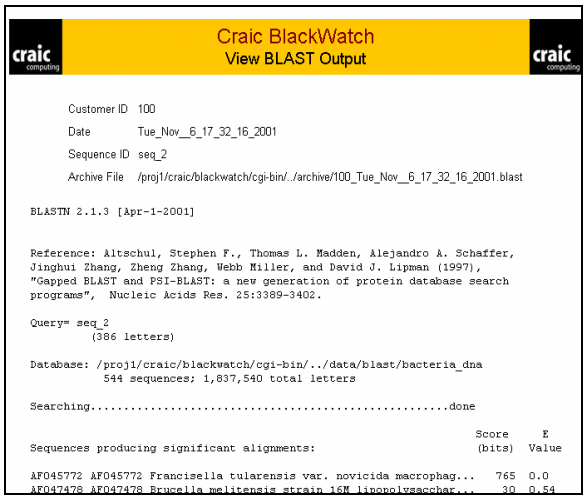

Figure 4: An Example of Detailed BLAST Output for a Positive Match

You can access a demonstration version of BlackWatch at https://biotech.craic.com/blackwatch.

3. The Custom Sequence Database

The database of sequences from select agents is a critical component of the BlackWatch system. Its composition directly influences the numbers of false positive and negative matches, as well as the performance of the search process.

Only sequences from defined select agents are included in the database. A critical issue in sequence screening is the potential disclosure of information about customer sequences. By limiting the database to only select agent sequences, the system minimizes this risk. So oligos related to a human gene would not be expected to match anything in the database. Sequences of bacterial origin, for example, have a much higher risk of matching, especially in light of the approach to false positive control. Understanding the probability of finding such matches will be important in the development and adoption of this system.

There are two approaches to building the sequence database. The first is to limit the sequences to those of genes known to be involved in virulence, toxin synthesis, etc. This highly focused approach would produce a small database with a low probability of false

positive matches. But this approach has several problems. Firstly it requires considerable effort up front in deciding what genes should be included and in extracting only the relevant sequences from GenBank. Secondly it ignores the possibility that genes other than this subset might be employed in the modification of a pathogen.

The alternative approach, which is used in BlackWatch, is to include all sequences that have been assigned to any organism on the select agent list. It is relatively straightforward to extract sequences based on the organism tag in a GenBank record and this selection can be fully automated using simple Perl scripts. Minimal up front effort is required and the data can be made available for searching immediately. It also ensures that all the available data is used in searching, with no preconceptions about how sequences might be used.

The drawbacks of the approach include the potential for redundant data being included in the database, slowing down searches and perhaps creating ambiguity. Some basic checks for redundancy are currently used in the preparation of the database but these could be improved. Perhaps the major problem is that the approach will include sequences of housekeeping genes, such as those for ribosomal proteins, which are highly conserved between diverse species. This raises the probability of false positives significantly.

4. Composition of the Database

All sequences in the database are extracted from the public GenBank database, hosted by the NIH (http://www.ncbi.nlm.nih.gov/Genbank/). This contains sequences for most if not all of the select agents, with complete genomes available for many of the organisms. Anyone attempting to engineer a pathogen using synthetic DNA would be expected to use this same database. No classified or proprietary sequences are included. Not only are these not available to me, but their inclusion would greatly complicate the software and its intended distribution to DNA synthesis companies.

The list of organisms for which all available sequences have been extracted is a composite of those included in the CDC select agent rule (42CFR73), the USDA regulations (7CFR331 and 9CFR121) and the Dept of Commerce Export Administration

"Commerce Control List" (CCL). The composite list specifies a total of 75 organisms and 22 toxins. The breakdown of these is shown in Table 1.

Host	**Human/Animal**	**Animal Only**	**Plant**	**Total**
Pathogen Type				
Viruses	19	12	2	33
Bacteria	15	3	8	26
Fungi	2	0	2	11
Rickettsiae	4	0	9	4
Prions	0	1	0	1
Toxins	22	0	0	22

Table 1: Pathogens in Composite Select Agent List

The list is included as an appendix to this paper and is also available online at: http://biotech.craic.com/blackwatch/regulations/List_of_Select_Agents.pdf

Toxins pose a problem for sequence screening. Protein toxins like abrin, ricin and conotoxin are gene products and so DNA and protein sequences for the toxins themselves are available. In the case of mycotoxins, such as aflatoxin, the molecule is not a protein. In these cases the sequences of genes that encode the biosynthetic pathway may be appropriate targets for sequence screening. This component of the database needs further study.

It might be advisable to include antibiotic resistance genes in the database as an obvious scenario that we need to consider is that of someone introducing antibiotic resistance into an existing bacterial pathogen. Unfortunately the widespread use of these genes in conventional molecular biology would ensure a very large number of false positive matches. This issue should be revisited once progress has been made dealing with the general problem of false positives.

5. Current Implementation of the BlackWatch Software

The software is written in Perl and runs on Linux systems. Porting the scripts to other UNIX variants and Mac OS X would be trivial and a port to the Windows operating system should be straightforward.

The system is in operation on my web server and has been in production use at Blue Heron Biotechnology in Bothell, WA, where it is used to screen requests for entire gene synthesis. It has also been beta tested for a limited period at a leading oligo synthesis company. They chose not to continue using the system for business reasons.

In order for the software to meet the sequence screening needs of the gene and oligo synthesis industry in general, it will require some additional development work. Performance needs to be improved to handle the throughput at large oligo synthesis sites. Integrating the system with existing relational databases that manage orders at these companies needs to be made easier. Most importantly the rate of false positive matches needs to be studied and minimized.

6. False Positives

The primary challenge facing sequence screening is to minimize the number of false positive matches. Every match reported by the system needs to be evaluated at some point by an expert. Those that are deemed to be real may trigger the involvement of the regulatory authorities. Every false positive that passes initial scrutiny will waste considerable time and devalue the importance of the approach in the eyes of those authorities.

Fine tuning the cutoff values for BLAST score, significance and coverage may help reduce false positives in general but will do nothing to address matches to housekeeping genes, etc. The approach that I am experimenting with at the moment is to use a second sequence database of non-pathogens. Any query sequence that hits the pathogen database is then searched against the non-pathogen, or 'reference', database and the corresponding matches, if any are presented to the user alongside the pathogen hits.

Currently the reference database is limited to bacteria and contains the genome sequences for *E.coli* and *B.subtilis*. This screenshot shows the results from a search with a ribosomal protein gene from *S.typhimurium*.

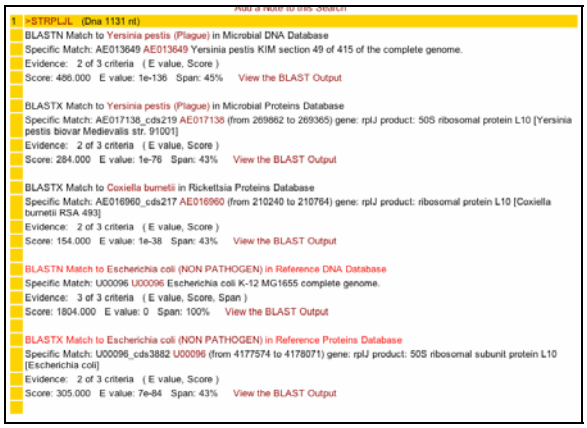

Figure 5: Example of a False Positive Match

This conserved gene has produced a match to the equivalent genes in *Y.pestis* and *Coxiella burnetii* in the pathogen database and also to *E.coli* in the reference database. By comparing the relative scores and significance, a reviewer would judge the query sequence as being more similar to the non-pathogen than to either of the pathogens. Hence this is probably a false positive.

The approach appears quite promising but work needs to be done in creating a comprehensive set of related non-pathogen sequences for viruses, etc., and in automating the process of calling false positives. No approach will catch false positives with 100% accuracy and so an expert reviewer will continue to be required. Perhaps the best that can be achieved is to add weight the scoring of matches according to the biological significance of the matching sequence. A very strong match to a sequence involved in anthrax toxin would be a clear positive match. A match to a less important region of the *B.anthracis* genome would be weighted down. This argues for a sequence database that combines the approach I currently use of capturing all sequences from the pathogens with some degree of expert curation that can define which genes are of particular concern.

False positives are inevitably more likely in the case of oligo sequences because of the sequence length. Here there is the opportunity to do some simulations and real world tests to quantify the problem.

7. Future Developments

There are many scenarios whereby someone who wished to synthesize or modify a pathogen could use the services of synthesis companies and still evade detection by BlackWatch. Minor variation in sequences, such as third position variation in codons, can already be caught by the *blastx* searches against protein sequences. Other scenarios include sending orders for overlapping oligos to different vendors or spreading out orders over a period of time so as to avoid revealing the intent behind a project. One way to address this would be to scan the archived searches across customers, or even across synthesis companies, looking for orders that might be related.

This would require that the results of screening from all vendors be submitted to a central location where these correlations could be made. I return to this idea at the end of the paper. The technical challenges of making these connections are very interesting, but they go hand in hand with a number of important business and confidentiality concerns.

The BLAST sequence comparison software is the obvious choice for comparing relatively large sequences against the database but for oligo comparisons it may be faster to use another approach such as a sequence word lookup table or a suffix tree algorithm. Computational speed could become a problem in high throughput oligo synthesis facilities. The figure of up to 25,000 oligos synthesized per day that Integrated DNA Technologies quotes is sobering. This means that a complete evaluation of each oligo must take place in less than 4 seconds. This can be achieved through a combination of adequate hardware and good software engineering but the system is not currently capable of this throughput.

8. Practical Deployment of Sequence Screening

Beyond the purely technical challenges of the BlackWatch package, its performance and the issue of false positives, there are several broader challenges to its practical deployment in the DNA synthesis industry that need to be overcome.

We need to make it very easy for a synthesis company to obtain, install and operate the software package. The barrier to its procurement can be reduced by making the software available free of charge. Appropriate software engineering can ensure that it is easy to set up and run. External funding from NIH or another agency will be necessary to support the development and deployment of the software. It is unlikely that the synthesis companies would fund the effort themselves.

We need to minimize the cost to the synthesis companies of evaluating the reports that sequence screening will yield. This is the time and effort that staff have to devote to looking at, acting upon, the putative positive matches. Some of these companies, most notably the oligo vendors, operate on very thin profit margins. Any added expense will be most unwelcome, especially if it requires effort on the part of skilled scientists.

But beyond these operational issues there are two major challenges that stand in the way of broad deployment—how to assess the validated, significant matches that do emerge from the screening and what to action to take based on that information. Neither role belongs with the DNA synthesis companies. They require expert knowledge and access to specific staff within the regulatory authorities.

Conclusion

A significant fraction of the synthetic DNA currently being produced today could be monitored by sequence screening at the major oligo and gene synthesis companies. For legitimate customers this process should pose no significant threat to their intellectual property.

For a group wanting to engineer a biological weapon, however, screening could serve as a serious deterrent. They would be faced with the choice of potential discovery by the screening software or having to bring the work in-house and significantly increase the level of effort and expertise needed to accomplish their goal.

Sequence screening has its limitations, as do most technologies that attempt to monitor threats, but I believe it should play an important role in the development of synthetic biology.

A Roadmap to the Assembly of Synthetic DNA from Raw Materials

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Introduction

Until recently, the synthesis of DNA has been a tedious, time consuming, expensive and experimentally challenging task. But advances in automated instrumentation and improved chemistry have now made it possible to make any moderate-length sequence of DNA in any quantity. The ease of automated chemical synthesis of DNA has triggered a whole new industry of low-cost DNA suppliers around the globe. The convenience of ordering DNA sequence by mail has opened new avenues in research both in academia and in the healthcare products developed by pharmaceutical companies. At the same time, these advances have made it theoretically possible to synthesize DNA that could be used to do harm. This article aims to describe the first stages of DNA synthesis, from readily available raw materials to medium-sized segments with a desired sequence (oligonucleotides), and examines whether there are points at which such activities could be, for example, monitored or controlled. Some academic and commercial applications of DNA synthesis require the construction of very small quantities of the desired sequence; others involve synthesis at the gram scale or larger. I provide comments on possible intervention points for both types of application. Terms shown in bold are defined in the glossary.

1. History and key landmarks

Our current methods of DNA synthesis have evolved over almost 150 years. In 1869, Friedrich Miescher first isolated a new substance from human pus cells, which he named nuclein. Two years later, he found that the same material could also be isolated from salmon caught in the river Rhine. Subsequently, Richard Altman in 1889, further purified nuclein as a protein-free product that he called **nucleic acid**. In 1900, Albrecht Kossel

studied the chemical composition of nucleic acids, and found that they contained adenine, cytosine, guanine and thymine bases (Figure 1). Kossel was awarded the Nobel Prize for his work. In his acceptance speech he noted that "nucleic acids possess a great biological significance". In 1902, Emil Fischer received his Nobel Prize for the first chemical synthesis of a **purine** base.

In 1955, the first chemical synthesis of a dimeric block of DNA was accomplished by M. Michelson and Alexander (Lord) Todd. Subsequently, this contribution was recognized by a Nobel Prize to Todd. Next, Har Gobind Khorana and his colleagues showed how a DNA sequence could be assembled via chemical means, now known as the phosphodiester method. In 1976 Khorana with his 19 co-workers reported on the synthesis of a 126-residue long DNA. This project took 8 years; today the same product can be made in one day using an automated DNA synthesizer.

The pioneering work of Robert Letsinger, Kevin Ogilvie and Colin Reese using the phosphotriester method also helped to pave the road to solution-phase synthesis of DNA. In the mid-1970's, the first solid-phase preparation of DNA was performed in the laboratories of Hubert Köster, Michael Gait and K. Itakura. Solid-phase synthesis is the dominant method used today. The specific chemistry we use today came slightly later, in 1981, when Mark Matteucci and Marv Caruthers reported an efficient automated synthesis of DNA employing the P(III) **amidite** chemistry.

2. Transforming raw materials into the building blocks of DNA

DNA is a long chain polymer that is made up of four repeating units called **nucleotides**. Half of the structure is identical for all four nucleotides, and consists of the sugar and phosphate groups (red boxes, Figure 1). The other half of the structure, the **base** (blue boxes, Figure 1) comes in four varieties, divided into two groups. The **pyrimidines** (thymidine and cytosine) each have a six-membered ring containing nitrogen, while the **purines** (adenine and guanine) have a double ring, a fusion of a six-membered ring with a five-membered ring. In the famous double helix of DNA, these nucleotides line up as

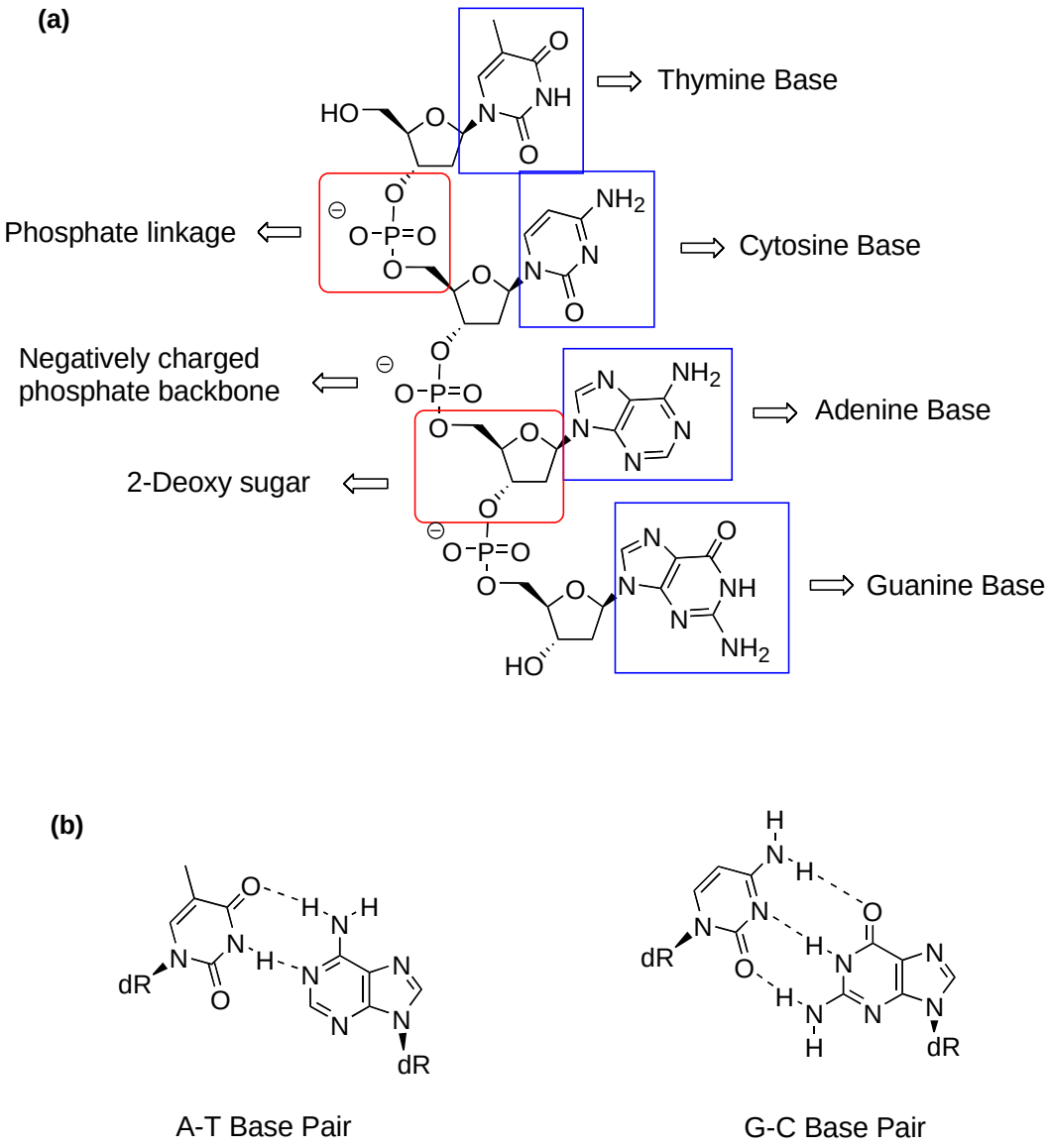

Figure 1: Structural elements of DNA. (a) A single strand of DNA, showing the structure of the bases and the backbone elements. Note that the negative charge on the backbone is balanced by positively charged ions such as Na^+ in a solution of DNA. (b) Structure of the base pairs that form when two matched strands of DNA are allowed to pair to make a double helix.

pairs: as shown in Figure 1, adenine (A) pairs with thymine (T), and cytosine (C) pairs with guanine (G). Because a six-membered-ring base always pairs with a double-ring base, the spacing between the two strands of DNA is maintained, and the overall shape of the molecule is the same no matter what the sequence. The "backbone" of the structure is also always the same, a repeated pattern of sugar-phosphate groups. It is this uniformity of structure that makes it possible to automate the synthesis of DNA. No matter the sequence to be produced, the chemical reaction required is always the same. The problem of making the sequence of DNA needed can thus be reduced to the problem of using the right nucleotide building blocks in the right order.

2.1. Availability of bases

All four bases are available in metric ton quantities from a variety of sources. The cheapest suppliers are in China; they sell their product for under $100/Kg. These products are chemically synthesized and are stable indefinitely when stored appropriately. The chemical synthesis of all four bases is straightforward and it can be carried out almost anywhere with the help of easily accessible reagents in a chemical laboratory. However, easy access to the bases does not lead to easy access to the nucleotides that are essential for the assembly of DNA (see below).

2.2 Availability of nucleotides and nucleosides

Nucleotides are the key reagents used in DNA synthesis. They can readily be made from **nucleosides** by adding phosphate groups. As recently as six years ago, all the nucleotides needed for DNA synthesis were made from nucleosides isolated from natural sources, such as fish milt. A flow chart of this isolation process is shown in Figure 2. Several companies, including Yamasa in Japan, Reliable in the USA and ProBioSint in Italy have used this method to produce nucleosides in metric ton quantities. It is not a rapid process (it can take 1.5 years from beginning to end) and it is very labor intensive. Some years ago, attempts began to develop alternative sources for nucleoside production. Today, at least six Asian companies manufacture the pyrimidine nucleosides at low-cost and in metric ton quantities using a completely chemical process, starting with cane sugar

(Figure 3). Processing of cane sugar furnishes D-glucose, which is transformed into **2-deoxy-D-ribose** in just a few steps. Next, the 2-deoxy-D-ribose is converted into a reactive α-chloro-sugar that is easily converted into the pyrimidine nucleosides (T and C). Mitsui Chemicals has developed a process for producing purine nucleosides at a very large scale, using a phosphate analog of 2-deoxy-D-ribose. The new process is patent protected and currently practiced in Japan for the production of the purine nucleosides (A and G). All four nucleosides are now available in large quantities from chemical synthesis at a significantly lower cost than the nucleosides isolated from fish milt.

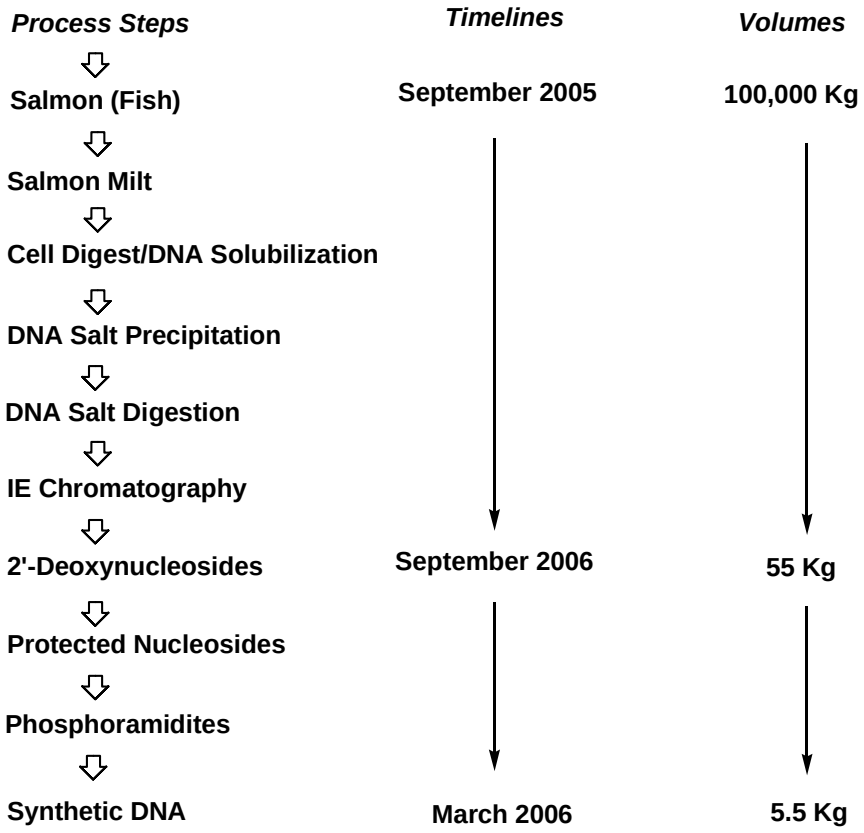

Figure 2: Raw material pipeline from fish to synthetic DNA

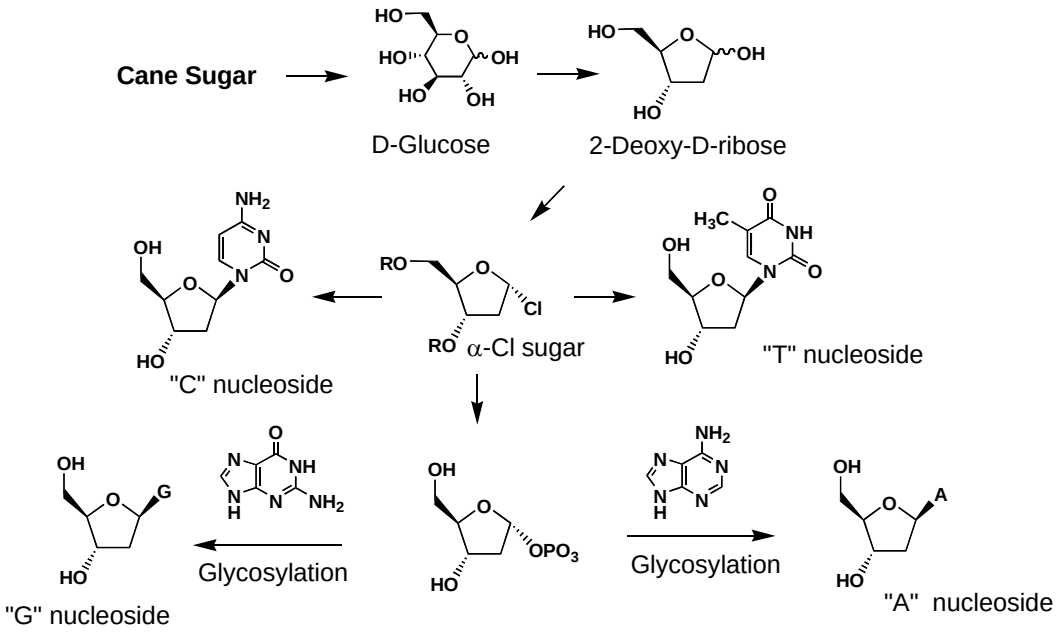

Figure 3: Chemical synthesis pathway for nucleosides from cane sugar

Both of the methods of producing nucleosides require significant skill, especially the chemical synthesis approach. Chemical synthesis of nucleosides requires Ph.D.-level chemistry personnel, specialty chemicals and specialized equipment. The most difficult part in the synthesis of nucleosides is to chemically connect a base to the top face (β-connection) of the sugar. An incorrect linkage from the bottom face will result in the formation of α-nucleosides, which are useless for DNA synthesis. Despite easy access to the bases from China, the synthesis of pure β-nucleosides and high purity amidites is not an easy task for a novice in the field.

In practice, most DNA synthesis today depends on the availability of nucleotide **amidites** (Figure 4), since the amidite chemistry is the dominant chemistry used in automated synthesizers. Good quality amidites are essential for successful synthesis of DNA on an automated machine. The production of good quality amidites is also a skilled task, and large quantities of **anhydrous** solvents and airtight equipment are required.

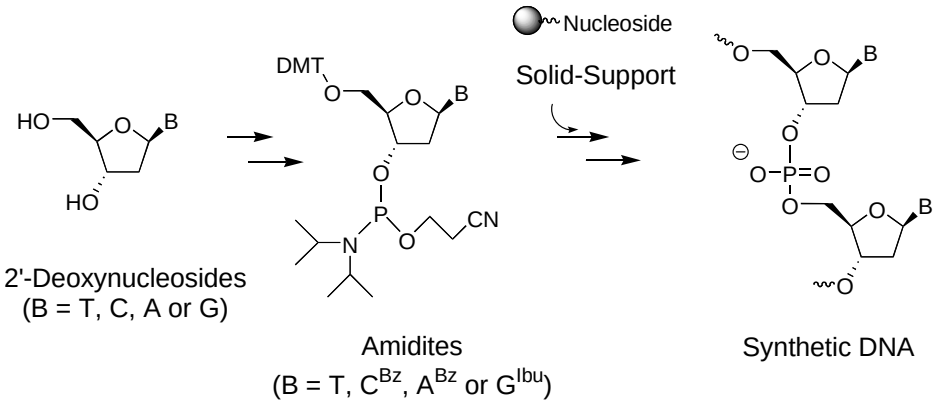

Figure 4: Key raw materials for DNA synthesis. Several steps are required to convert nucleosides to amidites.

2.3 What would it take to make building blocks from scratch?

If a chemist were cut off from all the sources of ready-made nucleotides, nucleosides and bases described above, how would he or she approach the problem of putting together the essential ingredients for DNA synthesis? First, this person would need to be an excellent chemist and have access to a well-equipped chemistry laboratory. The most likely route for such a person to take would be to use the older method of purification from salmon milt. As noted above, isolation of nucleosides from fish is long, tedious and inefficient. To isolate 1Kg of four nucleosides, one would need 1,818 Kg of salmon.

If access to nucleosides is not a problem, the chemist would still need to synthesize nucleotide amidites. This requires the use of special reagents (e.g. phosphitylation reagent), solvents (e.g. anhydrous acetonitrile) and airtight equipment. Isolation, storage and handling of P(III) amidites is an art that is not easily acquired even by an experienced chemist. However, given the tools, training and chemicals, an expert in the field could produce gram quantities of amidites in about six months.

In reality, substantial supplies of nucleosides and all four amidites are already distributed across the globe in large quantities, held by a large number of potential suppliers. It is highly unlikely that even the most concerted international effort would be able to restrict the raw material supplies available to the degree envisioned above.

3. Using the building blocks to make a desired sequence

The assembly of a useful (or harmful) sequence of DNA starts with the assembly of several nucleotides into a medium-length DNA strand, called an **oligonucleotide**. This is done using automated solid-phase synthesis; in other words, the chain of nucleotides is built on a solid bead, one at a time, with washing steps in between. The solid phase is essential to allow multiple steps to be performed with reasonable efficiency. Usually, processes that require a large number of chemical steps give a poor yield; if each step of a six-step synthesis is 95% efficient, the overall yield is only 73%. It would require about 80 individual steps to complete the sequential assembly of a 20-unit long oligonucleotide. The result is that the product is mixed with unreacted starting material and the products of undesired reactions, and can be very hard to purify. The larger the number of steps, the worse the problem gets.

Solid phase synthesis makes multi-step synthesis easier in two ways. First, it is very easy to separate the product (which is attached to the bead) from the unreacted starting material (which is in solution) by simply washing the beads extensively. Second, this ease of separation makes it possible to use large excesses of starting material to drive the reaction very close to completion. In the case of the amidite chemistry that is typically used, each reaction occurs with >99% efficiency. The only impurity left is the product of partial reactions.

Partial reactions are inevitable in any chemical process. For example, if one is trying to construct the sequence ATGCCAA, one would start with an A attached to a bead, then react it with a T. In most cases, the sequence AT will be made, but in a few cases the T will not be added. If the unreacted A is allowed to continue in the elongation reaction, the end result would be the wrong sequence, AGCCAA, which might have a completely different biological effect from the desired sequence. The same problem can occur at any step of the elongation process. Current DNA synthesis technology uses a trick to minimize the problems caused by incomplete reaction, "capping" unreacted sequences with special blocks that prevent their further elongation.

Each cycle of elongation takes place in four main steps, with wash steps in between: **(1)** deprotection, in which a group that prevents premature reaction is removed from the end of a growing nucleotide chain; **(2)** coupling, in which a new nucleotide is added; **(3)** oxidation to stabilize the newly formed linkage; and **(4)** capping of partial products. Finally, the completed chain must be cleaved from the bead. The reagents needed for each step are discussed below. I will focus primarily on the amidite method, because of its widespread use on automated machines that produce thousands of DNA sequences every day around the world. Several other reaction schemes are possible, although they are less efficient.

3.1. Building blocks and reagents required for solid-phase synthesis

The most important reagent required for amidite chemistry is a protected stable amidite derivative (see Figure 4), which provides extremely high (>99%) reaction efficiencies. These amidites are easily synthesized from nucleosides in just a few chemical steps, none of which would be challenging for a reasonably well-trained chemist with access to a sophisticated laboratory. Until recently the manufacturing and sales of these amidites was restricted due to the Köster patent. With the expiration of the patent this year, a number of low-cost Asian suppliers are now producing amidites in commercial quantities. This is one of the key reasons for the recent reduction in the cost of synthetic DNA. The four amidites of interest are wax-like, hygroscopic and easily decomposed upon heating. They must be carefully protected from air, water and heat. For most DNA synthesis applications, the amidites are sold in convenient pre-packed bottles that are simply plugged into a synthesizer without exposing them to air.

The solid support is the second most important raw material needed for DNA synthesis (Figure 5). In essence, the solid support is a small mechanically sturdy polymeric porous bead that is chemically inert during DNA synthesis. The bead must have a reasonable surface area so that each bead can accommodate many growing chains. The most popular solid-support for small-scale synthesis is controlled pore glass (CPG) made from glass or silica (Figure 5). CPG is a special bead custom-made for the synthesis of DNA by a

handful of companies. Synthesis of DNA on ordinary glass is possible but less efficient and would lead to decreased production of the desired DNA strand. Beads made of cross-linked polymers (reminiscent of nylon, but more rigid) can also be used as an alternative support.

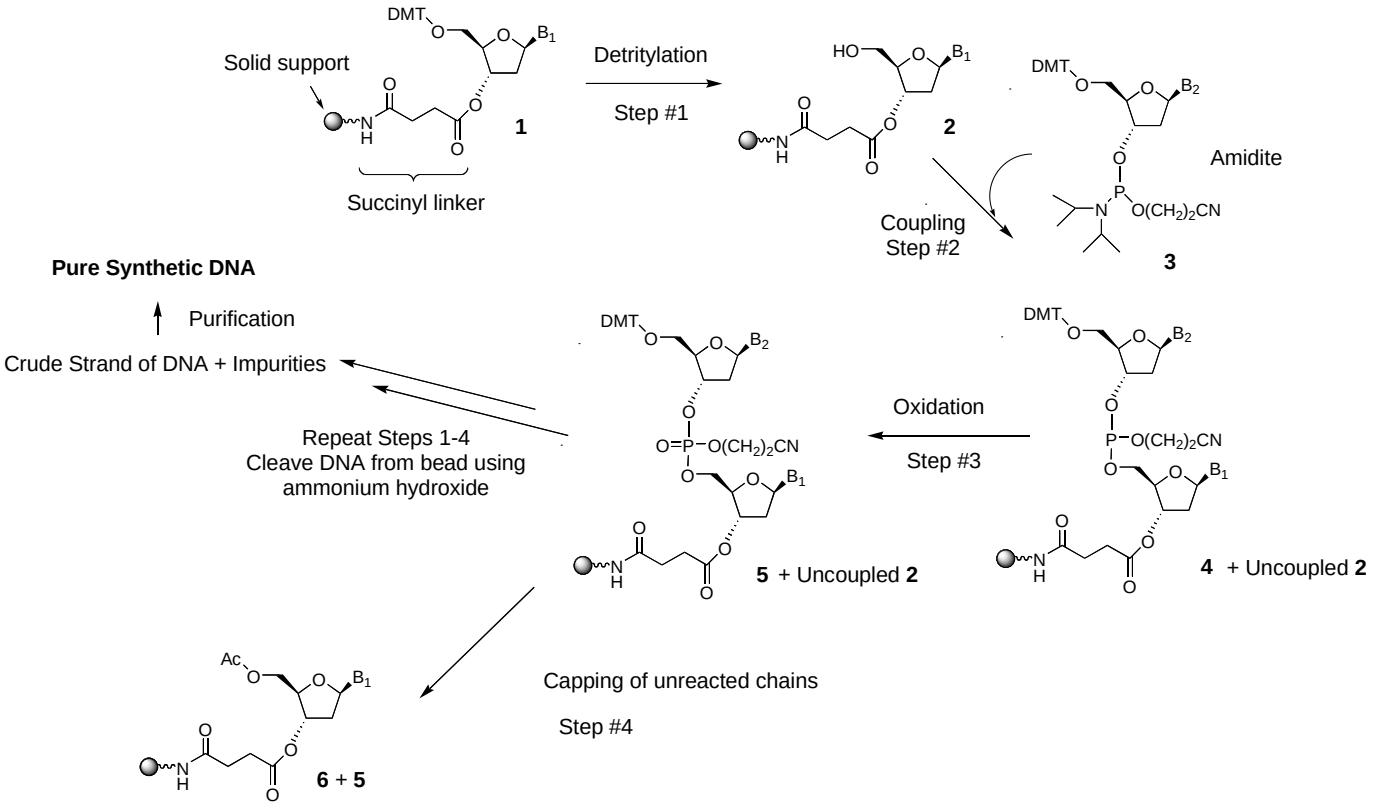

Figure 5: General scheme for automated synthesis of DNA using amidites

Generally solid-supports are sold with the first nucleoside unit already anchored to the surface of the bead via a short cleavable succinyl linker (Figure 4c). The support is placed in a reactor (column) and connected to the automated synthesizer for the chain extension. The first step in oligonucleotide synthesis is the coupling of the first nucleotide to the

nucleoside already attached to the surface of the bead. The addition of each nucleotide unit requires four individual chemical steps and a number of reagents.

These reagents include: (a) a deblocking solution that contains an acid such as dichloroacetic acid (DCA) in dichloromethane (DCM) or toluene; (b) an activator solution such as 1H-tetrazole or 4,5-dicyanoimidazole in acetonitrile; (c) an oxidation solution such as iodine in pyridine, THF and water; (d) two capping solutions, one containing N-methyl imidazole in pyridine and acetonitrile, the other containing acetic anhydride in acetonitrile. These are the only special reagents needed for the four-step repetitive synthesis cycle used in DNA construction (Figure 4). All of them are easily produced from common materials that would be next to impossible to control; alternative reagents have also been described in a variety of publications. In the final step, ammonium hydroxide solution is used to cleave the succinyl linker arm that holds the DNA chain attached to the surface of the solid support and removes the protecting groups that avoid side reactions during synthesis. Ammonium hydroxide is also a very common reagent.

One of the important reagents used for DNA synthesis is the anhydrous acetonitrile required for the washing steps. Because the amidites are very sensitive to water, the grade of acetonitrile needed is higher than for most other applications.

3.2. Chemical steps during assembly

The specifics of the chemical reactions that take place in an average DNA synthesizer are shown in Figure 4. The beads that make up the solid support, with the first nucleoside residue **1** attached**,** are packed into a column to allow solvents to be flowed through them efficiently. The synthesizer is programmed to pump reagents and solvent through the column, and the order of amidite addition is determined by the sequence of DNA needed. The chemical steps shown are: (1) detritylation, consisting of the removal of an acid-labile protecting group from the 5'-hydroxyl group of the nucleoside residue at the end of the growing oligonucleotide chain; (2) coupling of an activated amidite with the 5'-hydroxyl group generated in step 1. (3) oxidation of the labile P(III) intermediate **4** to

stable P(V) product **5** (Figure 4); and (4) capping using a mixture of two solutions, cap A (N-methyl imidazole in pyridine and acetonitrile) and cap B (acetic anhydride in acetonitrile), pumped through the column at the same time. A washing cycle between each step is essential. The four-step protocol is short and very efficient with each cycle completed in just a few minutes.

Note that the "cap" added to the chain that failed to complete the desired reaction is an acetyl (Ac) residue, which is chemically different from the dimethoxytrityl (DMT) blocking group. The DMT group can be removed by gentle acid treatment, freeing it to react in the next cycle. The Ac group withstands this treatment, preventing the chain from elongating. DMT serves two purposes in the cycle; it prevents the amidites from reacting with themselves, and it prevents a chain that has been successfully elongated from being capped. After the capping step is complete the DMT can be removed to allow a new coupling reaction.

3.3. Automated synthesizers

In the early 1980's, the first commercial DNA synthesizers were built and sold by Applied Biosystems. These were single-column 380A and 380B instruments with capabilities to make one DNA sequence at a time on a very small scale (0.2 – 10 μmol). Today, there are a number of instruments on the market with the ability to produce hundreds or thousands of DNA sequences in parallel using both commercial and proprietary instrumentations. For example, Applied Biosystems 3900 DNA synthesizers use 96- or 384-well plates, making a different sequence in each well; specialized companies such as Illumina have adapted this strategy to use synthesizers with large platforms that carry many 384-well plates, again making an individual sequence in each well of each plate. Similarly, high throughput DNA synthesis is available on the MerMade Bioautomation or the Oligator Farm. Although synthesis of DNA without an automated synthesizer is in principle possible, in practice it would be highly inconvenient.

4. Purification

Some uses for oligonucleotides require a purification step to remove the products of incomplete reactions. The most widely used purification technologies are: (1) anion exchange, in which the oligonucleotide (after being cleaved from the solid support used in synthesis) is passed over positively-charged beads that retard the progress of individual oligonucleotides depending on how many negative charges are present on the molecule; and (2) reverse-phase chromatography, which separates molecules based on their degree of hydrophobicity. Both of these purification methods are very widely used for a variety of biochemical applications in academia and industry. New purification methods currently being explored include membrane-based chromatography and simulated moving bed (SMB) chromatography. SMB in particular looks promising, with the potential for >98% purity at the kilogram scale.

5. Points of intervention

The reagents required for oligonucleotide synthesis are almost all so common, or so readily produced, as to defy restriction. The possibilities for restriction differ depending on whether the application to be controlled requires small amounts of material (as is the case for most genetic engineering applications) or large amounts (as for many medical applications). In both cases I focus on the amidite chemistry, since this is by far the most efficient chemistry currently available. Other chemistries can be used, but no sensible chemist would use them unless there was no other option.

5.1 Small-scale synthesis

- *Nucleosides, nucleotides and amidites*. These are the key building blocks of oligonucleotide synthesis. They are used in a range of peaceful industries, including the production of important medicines such as AZT for the treatment of HIV. They are made and sold in very large scale; for example, Proligo (recently acquired by Sigma Aldrich) produces tons of amidites per year. Denial of all ready nucleoside supplies to an oligonucleotide chemist might slow the progress of DNA synthesis for months or years. At the same time, such restrictions would destroy or severely hamper the biotechnology

industry, and the progress of biomedical research. Given the ready availability of amidites from many suppliers across the world, it is hard to imagine an effective program to restrict access to these chemicals.

- *Solid support*. A handful of companies produce the beads that permit efficient DNA synthesis on automated machines. Because of the highly specialized equipment and training required for the preparation of these beads, a skilled individual cannot make these products alone. Restricting access to beads may be worth exploring as a method of controlling DNA synthesis. Note, however, that unless one is willing to destroy the entire DNA synthesis industry, there will be a large number of companies that have legitimate uses for these beads. It would be a significant challenge to track every shipment of beads to every DNA synthesis company and ensure that all the beads are used for legitimate purposes. It is also increasingly possible to make oligonucleotides on derivatized glass slides, which are relatively easy to make by hand.

- *DNA synthesizer*. A number of automated DNA synthesizers are available in the market place. It is possible that tracking the sales of new instruments might allow identification of potential terrorists. However, a very large number of existing instruments have already been sold and would be hard to track in this way; furthermore, an experienced engineer could construct one from spare parts with little difficulty.

5.2 Large-scale synthesis

- *Raw materials*. The amount of raw materials needed for large-scale synthesis provides significant logistical challenges. Only a handful of companies are able to mass-produce these building blocks in the purity required for DNA synthesis. It should therefore be possible to identify and track bulk users of these chemicals. Furthermore, the capital investment in building and running a facility to produce DNA on large-scale is significant. For example, a kilo-scale plant used for the production of DNA would cost $2-5 million in capital investment alone. This does not include the cost of running the facilities. It would be relatively easy to keep track of the construction of such plants world-wide.

- *Solvents and reagents*. Although there are a number of choices for solvents and reagents for DNA synthesis, anhydrous acetonitrile is one solvent that is absolutely essential for the coupling step. A limited number of companies are producing DNA synthesis grade acetonitrile. Monitoring the sales of high purity acetonitrile might allow suspicious organizations to be identified if they are performing large-scale reactions. For small-scale oligonucleotide synthesis, however, anhydrous acetonitrile can be readily produced in the laboratory using a still.

- *Plant permit*. Because a majority of large-scale (e.g. >10kg/year) plants are manufacturing medicines based on DNA they are regulated by the FDA for their GMP compliance. Therefore, it should be possible to monitor any suspicious or non-therapeutic activities and to require careful reagent tracking to minimize the risk that beads or solvents are diverted to other purposes. One possible hurdle could be put in place for such activity is to require a permit of some kind from an official entity before an organization is allowed to produce DNA in kilo quantities.

- *Product registration*. It is possible to envision a system where someone requesting kilo-scale custom synthesis of DNA is required to register with an organization describing its potential use. This system may create a barrier for the synthesis of DNA for harmful applications.

Conclusion

It would not be an easy matter to restrict the supply of the reagents needed for DNA synthesis to such an extent as to prevent a motivated individual from making oligonucleotides at a small scale. As noted above, the least implausible option for tracking and restriction would seem to be solid support beads. However, since these are widely used by the legitimate DNA synthesis industry, the restrictions must also include protocols for monitoring reagent use within a company and reporting their disposition. Several of the companies making and using these reagents reside outside the USA, complicating the task of imposing effective tracking policies.

Glossary

AMIDITE (also known as phosphoramidite): This is a protected version of a **nucleoside** that is easily activated for the coupling reaction. The P atom, which will eventually form part of the phosphate backbone, is protected with β-cyanoethyl and diisopropylamine groups. In the first stage of the coupling reaction, a weak acid protonates the nitrogen atom of the diisopropylamine protecting group, causing it to become positively charged and making it into a good leaving group. This allows nucleophilic attack by the free 5' hydroxyl group of the bead-attached monomer on the phosphorous atom, forming the molecule referred to as **4** in Figure 4.

Different protecting groups are also attached to the amines ($-NH_2$) that are not part of a ring in the bases A, G and C, to prevent them from becoming protonated and causing unwanted reactions. These protecting groups, and the cyanoethyl protecting group on the phosphate, remain on the growing chain until it is finally released from the bead.

ANHYDROUS: Water-free. Because the **amidite** chemistry depends on the hydroxyl group of the bead-attached monomer performing a nucleophilic attack on the positively charged diisopropylamine group, any other nucleophiles in the solution will reduce the efficiency of the coupling reaction. Water can act as a nucleophile, and must be rigorously excluded from the reaction.

BASE: The structures of the bases are shown in Figure 1. The information content of a DNA molecule consists of the linear arrangement of the bases A, T, G and C along a phosphate/sugar backbone (also shown in Figure 1). It is the pairing of the bases, A with T and G with C, that allows DNA to be copied.

2-DEOXY-D-RIBOSE: The particular form of sugar that is used in DNA synthesis. The D in DNA stands for "deoxy"; this refers to the fact that carbon number 2 in the ribose ring does not carry an oxygen in the DNA structure. In RNA, the sugar used is ribose, not deoxyribose.

NUCLEIC ACID: A polymer of nucleotide subunits.

NUCLEOTIDES: A base connected to a sugar (ribose) ring and one or more phosphate groups. In the DNA structure, the nucleotides have one phosphate group each, which forms part of the backbone of the DNA. Each phosphate group is linked to two sugar groups, through two different oxygen atoms (see Figure 1).

NUCLEOSIDE: The structures of the four nucleosides relevant to DNA synthesis are shown in Figure 3. A phosphate group must be added to nucleosides before they can be linked together to form DNA. In amidite chemistry, the phosphate group is formed by oxidation after the coupling step (see Figure 5).

OLIGONUCLEOTIDE: A short stretch of DNA (for example, 20 nucleotide subunits linked together).

PURINE: A base in which the **pyrimidine** ring is fused to a second ring, the imidazole ring. Imidazole is a five-membered aromatic ring with two nitrogens.

PYRIMIDINE: A base consisting of a six-membered aromatic ring with two nitrogen atoms at positions 1 and 3.

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Synthetic Viral Genomics: Risks and Benefits for Science and Society

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I. Introduction

A. Viruses and Biological Warfare

Viral disease outbreaks have long inspired fear in human populations. Highly pathogenic infectious disease has shaped world history, primarily by impacting the outcome of wars and other global conflicts and precipitating human movement. Historic accounts have documented the catastrophic consequences and human suffering associated with widespread viral outbreaks like smallpox virus, yellow fever virus, measles virus, human immunodeficiency virus (HIV), the severe acute respiratory syndrome coronavirus (SARS-CoV), the 1918 influenza virus and others (51). News accounts and film have reinforced the serious threat posed by the emergence of new viral diseases as well as the catastrophic consequences of intentional release of highly pathogenic viruses in human populations. As illustrated by the SARS epidemic and the continuing evolution of the H5N1 avian influenza, global and national infectious disease outbreaks can overwhelm disaster medical response networks and medical facilities, disrupt global economies, and paralyze health and medical services by targeting health care workers and medical staff (21). This review focuses on viruses of humans, animals and plants that are viewed as potential weapons of mass disruption to human populations, critical plant and animal food sources, and national economies; and will consider whether and how the availability of synthetic genomics technologies will change this landscape.

Biological warfare (BW) agents are microorganisms or toxins that are intended to kill, injure or incapacitate the enemy, elicit fear and devastate national economies. Because small amounts of microorganisms might cause high numbers of casualties, they are classified as weapons of mass destruction. A number of naturally occurring viruses have

potential uses as BW agents, although the availability of these agents is oftentimes limited. This report discusses the potential use of recombinant and synthetic DNAs to resurrect recombinant BW viruses de novo and the potential for altering the pathogenic properties of viruses for nefarious purposes. Examples of weaponized viruses include Variola major (Smallpox), Venezuelan equine encephalitis virus (VEE), and the filoviruses Marburg and Ebola viruses, with the classic example being the use of smallpox virus-contaminated blankets against indigenous North American Indian populations (76). It is now clear that many viruses possess properties consistent with applications in biological warfare and bioterrorism.

B. Properties of Select BW Agents

Traditionally, biological warfare concerns have focused on a relatively limited, select group of naturally occurring pathogens viewed as having a set of desirable characteristics: 1) highly pathogenic, 2) readily available, 3) easily produced, 4) weaponizable, 5) stable, 6) infectious at a low dose, 7) easily transmissible, and 8) inspiring of fear (32). Viruses of concern include pathogens that replicate and produce serious morbidity and mortality in humans to pathogens that target farm animals and plants of economic importance. Historically, weaponization of agents has been constrained by availability, the biological characteristics specified within the genome of these organisms, the ability to replicate and produce large quantities of the material, and by the lack of appropriate associated technologies. Culture (growth) and containment conditions for most of the virus agents of concern have been solved and are readily available in the literature. Natural hosts and reservoirs of many viral agents have been identified, providing a means of readily acquiring these pathogens in nature, although this is not always the case. Most recently, full length genome sequences have been solved for many important human, animal and plant pathogens, providing a genetic template for understanding the molecular mechanisms of pathogenesis and replication. Structural studies have identified contact points between the virus and the host receptors needed for docking and entry, providing the means to humanize animal pathogens (42). With the advent of synthetic biology, recombinant DNA technology, reverse genetic approaches (i.e. the development of molecular clones of infectious genomes) and the identification of

virulence alleles, not only are new avenues available for obtaining these pathogens, but more ominously, tools exist for simultaneously modifying the genomes for increased virulence, immunogenicity, transmissibility, host range and pathogenesis (22, 59). Moreover, these approaches can be used to molecularly resurrect extinct human and animal pathogens, like the 1918 human influenza virus (81).

National biodefense strategies are focused on threats posed by this small group of plant, animal and human pathogens that occur in nature. However, counterterrorism think-tanks anticipate that these particular threats will ameliorate over the next decade because of medical countermeasures (e.g., drugs, vaccines, diagnostics), coupled with a limited set of pathogens that include all of the biological warfare characteristics. More important, the anticipated long-term threat in biological warfare is in recognizing and designing countermeasures to protect against genetically modified and designer pathogens, made possible by newly emerging technologies in recombinant DNA, synthetic biology, reverse genetics and directed evolution (59). How will synthetic genomics effect future biological weapons development? What are the risks and benefits of these new technologies and how serious a threat do they pose for human health and the global economy? This paper builds upon earlier work and seeks to review the methodologies in isolating recombinant viruses in vitro and the application of these methods globally to biological warfare and biodefense (27).

II. Virus Classification and Reverse Genetic Approaches

A. Overview of Virus Classification and Reverse Genetics

From the genome, all viruses must generate a positive strand mRNA that is translated into proteins essential for genome replication and the assembly and formation of progeny virions. Depending upon the nature of the genome, all viruses can be clustered into seven fundamentally different groups, which utilize different strategies to synthesize mRNA

from the input genome, a scheme called the Baltimore Classification (Figure 1).[1] Because virus infectivity is dependent upon the ability to transcribe mRNAs, reverse genetic strategies are designed to insure expression of critical viral mRNAs that encode essential replicase proteins needed to "boot" (initiate) genome infectivity and initiate genome replication.

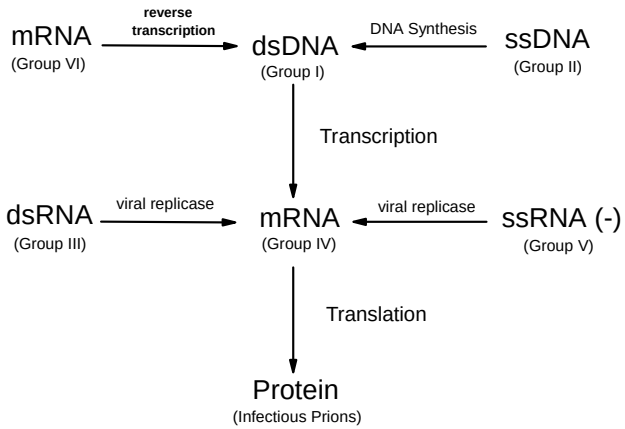


Figure 1. Baltimore Classification Scheme.

Group I viruses include the double-stranded DNA (dsDNA) viruses, like the Herpes viruses and Poxviruses which replicate in the nucleus or cytoplasm, respectively. The dsDNA viruses use cellular and/or virally-encoded transcriptase components to mediate expression of viral mRNAs. Poxviruses for instance require one or more viral proteins to initiate mRNA transcription and boot infectivity of the viral genome. Hence, smallpox virus genomes are not infectious unless the appropriate suite of viral proteins is provided in *trans* (in addition to the genome itself). In contrast, the Herpes virus genome is infectious in the absence of any viral proteins as cellular transcriptase machinery induces expression of early mRNAs and proteins that regulate expression of other viral genes and replication. Using vaccinia (poxvirus) as a model, an approach to successfully initiate/jump start and boot the infectivity of poxviruses has been developed, providing a template strategy for the family (11, 24). Herpes virus genomes are infectious in the absence of additional viral factors. Group II viruses encode single stranded DNA genomes which must be used as templates for the synthesis of a dsDNA before

[1] Named for the virologist David Baltimore, who proposed the system.

transcription and translation of mRNAs can occur within cells. At this time, group II BW agents have not been identified.

The Group III viruses contain double stranded RNA viruses, like reoviruses. Reovirus genomes consist of complementary positive and negative strands of RNA that are bound by hydrogen bonding, wrapped within a multistructured icosahedral core that is essential for virus transcription. The virion structure contains the necessary proteins required for initiating mRNA synthesis. Unlike many of the single-stranded RNA viruses, the dsRNA virus genomes are not infectious in isolation and the components necessary for booting genome infectivity remain unresolved.

Group IV viruses contain a single-stranded positive polarity RNA genome and include the flaviviruses, alphaviruses, picornaviruses (including poliovirus), coronaviruses (including the SARS virus), caliciviruses and others. Upon entry into cells, positive strand RNA genomes are immediately recognized by host translational machinery and the genome is translated into a suite of viral proteins, including the replicase proteins and RNA-dependent RNA polymerase which is necessary for initiating the viral replication cycle. Consequently, genome infectivity usually does require viral proteins or transcripts provided in trans to boot genome infectivity, although some exceptions have been reported (13). Group V viruses contain a single-stranded negative polarity RNA genome and include filoviruses (Ebola/Marburg), myxoviruses (influenza), and paramyxoviruses (Hendra). Group V genomes come in two different flavors, segmented (e.g., myxoviruses) or nonsegmented (e.g., paramyxoviruses and filoviruses). In either case, the genome is not infectious because it is complementary in sequence (anti-sense); it is the opposite of the positive strand that specifies amino acids and thus cannot be translated directly into any of the critical viral structural or replicase proteins needed for producing infectious virions. Negative strand RNA genomes are encapsidated into a complex ribonucleoprotein structure (RNP) usually composed of several virally encoded replicase proteins (e.g., polymerase complex proteins, support proteins, trans-acting proteins) that are incorporated into the virion during assembly. Together, these compose a functional replication complex. Upon entry, these RNP complexes immediately transcribe the genome negative strand RNA into mRNA that can be translated into the viral proteins.

Consequently, genome infectivity requires the presence of full length RNA and a set of virally encoded replicase proteins that function as a transcriptional complex to express mRNAs. If mRNAs encoding the transcripton complex are provided in trans, group V genomes become infectious and virus will be successfully recovered.

Group VI viruses, retroviruses (including HIV) and lentiviruses, encode single stranded positive polarity RNA genomes, but virions encode a reverse transcriptase enzyme to convert the mRNA genome into a complementary DNA (cDNA) which serves as template for dsDNA synthesis. Following the synthesis of dsDNA, group VI viruses use cellular transcriptional and translational machinery to express viral transcripts encoding structural and nonstructural proteins. At this time, the group VI viruses do not include any BW agents.

B. Infectious Genomes, Molecular Clones and Reverse Genetics

The basic concepts central to understanding virus reverse genetics and molecular clones are summarized in Figures 1 and 2. The central idea is that the virion is an extracellular vehicle that transfers the viral genome (e.g., RNA or DNA genomes) between susceptible cells and protects the nucleic acid genome from degradation in the environment (Figure 2, Part A). Following entry, the viral genome is programmed to initiate a series of events that result in the production of a replicase complex that transcribes mRNA and replicates the genome. As discussed in the previous section, nucleic acid structure and organization determines the pathway of events needed to express mRNA and initiate virus gene expression and infection. Not all viruses, however, require virion attachment and entry to mediate a productive infection. In these cases, viral genomes can be isolated from virions and transfected directly into susceptible host's cells. If the genome is infectious, viral RNAs and proteins will be expressed allowing for the production and release of progeny virions (Figure 2, Part B). Classic examples of viruses with "infectious genomes" include the herpes viruses, polioviruses, alphaviruses, polyomaviruses, and flaviviruses which are classified among the Group I, II or IV viruses. However, not all viral genomes are infectious upon delivery into cells. Viruses with Group III or V genomes have never

been demonstrated to be infectious upon genome delivery into susceptible cells. Some Group I (poxviruses) and group IV virus genomes (e.g., norovirus, a causative agent of non-bacterial gastroenteritis, or "cruise ship disease" and the coronavirus infectious bronchitis virus) are not infectious upon delivery into susceptible cells (13). In these instances, genome infectivity requires the presence of specific cofactors to initiate viral replication. These cofactors typically represent one or more proteins that encode essential replicase proteins or encapsidate the genome into an RNP structure necessary for initiating transcription of mRNA from the genome. In this example, infectious bronchitis virus genome infectivity requires the nucleocapsid protein in trans while the components needed to boot norovirus genome infectivity remain unknown (13).

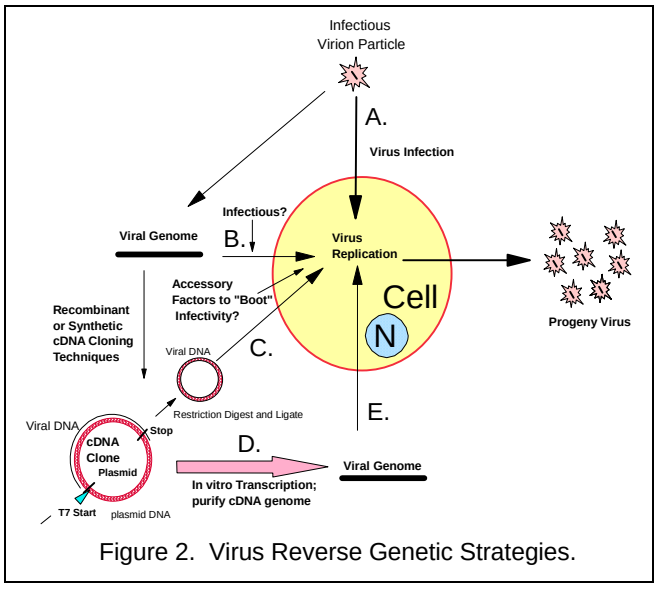


Figure 2. Virus Reverse Genetic Strategies.

In the late 1970's, a simple observation altered the course of virology research globally. Using a small dsDNA virus genome as a model (the Group I polyomavirus SV40) researchers cloned the viral genome into a bacterial plasmid and propagated the viral genome in bacteria. Upon isolation of the plasmid DNA from bacteria, restriction enzymes were used to excise the dsDNA viral genome, re-ligate the genome in vitro into a circular dsDNA and rescue virus following transfection of the genome into susceptible cells (Figure 2, Part C)(28). (Many advances in biotechnology have been, and continue to be, dependent upon this restrict-isolate-ligate technique, or variations of it.) Shortly

thereafter, full length cDNAs of positive strand RNA genomes were isolated following reverse transcription, the cDNAs cloned and propagated in bacterial plasmids, and following introduction of full length DNA into eukaryotic cells, recombinant viruses were rescued from the transfected cultures, although very inefficiently. The major problems with this approach were the difficulty in generating the appropriate termini, accurate genome sequence, problems in nuclear transport of the full length RNA genome, and splicing of the viral genomic RNA. To rectify the efficiency problems, bacteriophage promoters (T7, SP6, T3) were introduced upstream of the cloned viral cDNAs, allowing in vitro transcription of full length RNA copies of the viral genome using the appropriate phage RNA polymerase, nucleotide triphosphates, and other constituents (Figure 2, Part D). The full length RNAs, near exact replicas of the viral genome, were highly infectious upon transfection of susceptible host cells (Figure 2, Part E)(2, 65, 66). The ability to clone full length copies of viral genomes allowed for ease of manipulation of the genome and the introduction of specific mutations. Recovered viruses contained the introduced mutations that were encoded within the full length cDNA clones, providing a ready means of performing detailed genetic analyses of virus replication and pathogenesis.

As noted earlier not all viral genomes are infectious, complicating the development of full length cDNAs and the recovery of recombinant viruses. Isolated dsRNA genomes from Group V negative sense RNA viruses are not infectious because the genome sequence cannot be translated directly into a functional replicase complex needed to transcribe the incoming genomic RNA. As Group V virions contain a replicase protein complex essential for transcription, genome infectivity requires that cells be co-transfected with plasmids that express the genomic RNA and plasmids expressing transcripts that encode the replicase protein complex are needed for genome infectivity (Figure 3a). For most group V viruses, both genome negative and positive sense RNA infectivity can be booted using this approach with most investigators expressing full length plus (coding) strands from the initial transcript. The plus strands are transcribed to full length negative strands, which are used to express the appropriate set of mRNA encoding the full component of positive and negative strand RNAs. Using this approach

Schnell et al. successfully recovered the first recombinant negative stranded RNA virus, rabies virus, from a cloned cDNA, ushering in an era of Group V virus reverse genetics (68, 82). These findings were rapidly extended to other linear negative stranded RNAs like paramyxoviruses and then to segmented negative strand RNA viruses like influenza and other myxoviruses, and then select bunyaviruses and arenaviruses (20). Reverse genetic strategies for group V viruses with segmented genomes are most complex as multiple plasmids expressing copies of each genome segment must be simultaneously delivered to a cell along with the support plasmids encoding the transcriptase complex.

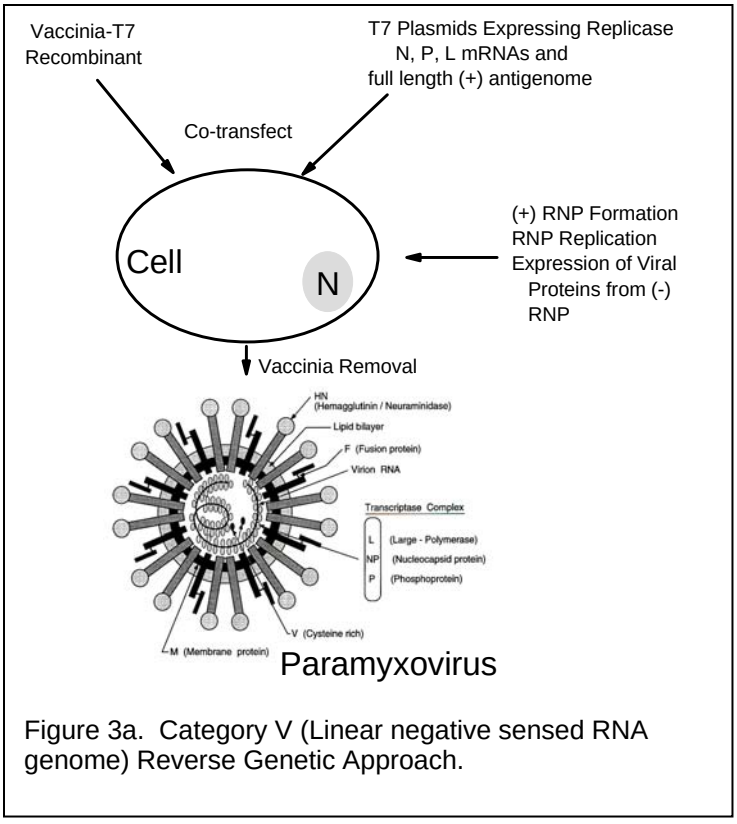


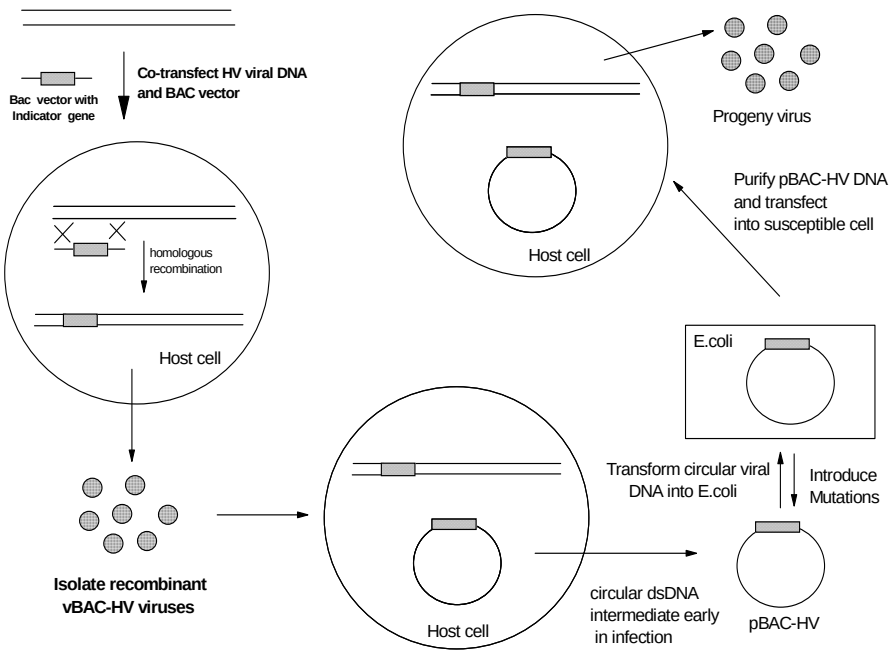
Figure 3a. Category V (Linear negative sensed RNA genome) Reverse Genetic Approach.

Most of the RNA viruses have relatively small genomes (under approximately 20,000 bases or base-pairs). Viruses with extremely large genomes (over 100,000 base-pairs, e.g., herpes viruses, poxviruses, or ~20,000-30,000 base pairs, e.g., coronaviruses, filoviruses) have presented additional obstacles in the development of stable molecular clones. Generation of infectious clones for viruses encoding large RNA or DNA genomes is complicated by the need for sequence accuracy (e.g., incorrect sequences usually contain lethal mutations), the lack of suitable cloning vectors that stably maintain large DNA inserts, large genome size, and that the genomes oftentimes encode regions that are toxic or unstable in bacteria. In poxviruses for example, the ~200 kilobase pair (kbp) genome has covalently closed hairpin ends (structures formed by the DNA itself) that are required for genome replication and virion encoded products are also essential for booting genome infectivity (24).

Herpes virus genomes are ~150 kbp in size. One solution was to stably clone large viral genomes as bacterial artificial chromosome (BAC) vectors. BAC vectors are based on the replication of F factor in E.coli, which is tightly controlled and allows stable maintenance of large, complex DNA fragments up to 600 kbp and both herpesvirus and poxvirus genomes can be stably maintained in BAC vectors (17, 24). For Herpes viruses, BAC shuttle vector sequences encoding a marker are inserted by homologous recombination into the genome. Circular viral DNA, which is generated during the Herpes virus replication cycle, is purified from infected cells (so-called Hirt prep) and introduced in bacterial cells, which essentially generates a large plasmid containing the Herpes virus genome (49). As herpesvirus genomes are infectious, the BAC DNA sequences are rapidly lost after delivery to a suitable host cell, along with some surrounding viral sequences, because they are dispensable for viral DNA replication (71). Using the Cre/lox system (another basic tool of molecular biology), a self-recombining full length

pseudorabies virus BAC was developed where the full length genome is automatically removed from the BAC sequences by the expression of Cre recombinase after transfection, reducing the potential for random deletions of viral sequences (72) (Figure 3b). Recombinant Herpes virus genomes that have been successfully cloned include mouse cytomegalovirus, herpes simplex virus 1, human cytomegalovirus, pseudorabies virus, and Kaposi's Sarcoma virus (11, 24, 49).

Figure 3b. Bac Vector Based Recombinant Clones for Herpesviruses (HV).



Poxvirus genome structure and replication modes make the development of an infectious poxvirus molecular clone an order of magnitude more difficult than generation of the Herpes virus molecular clone. Poxvirus genomes replicate in the cytoplasm and require several viral proteins to mediate mRNA transcription and a unique DNA-dependent RNA polymerase that are normally contained within the virion to initiate virus infection.

Consequently, purified poxvirus DNA is not infectious. In addition, the linear dsDNA genome has closed hairpins at each end of the genome that are essential for DNA replication. How were these problems solved? As described with Herpes viruses, a mini BAC encoding a marker called green fluorescent protein (GFP) was recombined into the thymidine kinase gene encoded in the vaccinia genome (a model for smallpox). Recombinant viruses harboring the BAC cassette were identified by GFP expression. However, transformation of Vaccinia BAC vectors into E.coli required conversion of the linear genome with covalently closed ends into a closed circular DNA. To accomplish this, Domi and Moss blocked late viral gene expression knowing that this favored additional recombination events that allowed head to tail concatamers of full length genome from which monomeric recombinant genome in a covalently closed circle would result, a favored genome orientation for insertion into E.coli. Transfection of VAC-BAC DNA into mammalian cells, previously infected with a helper fowl pox virus whose replication is defective in mammalian cells, allowed recovery of recombinant vaccinia virus (23, 24).

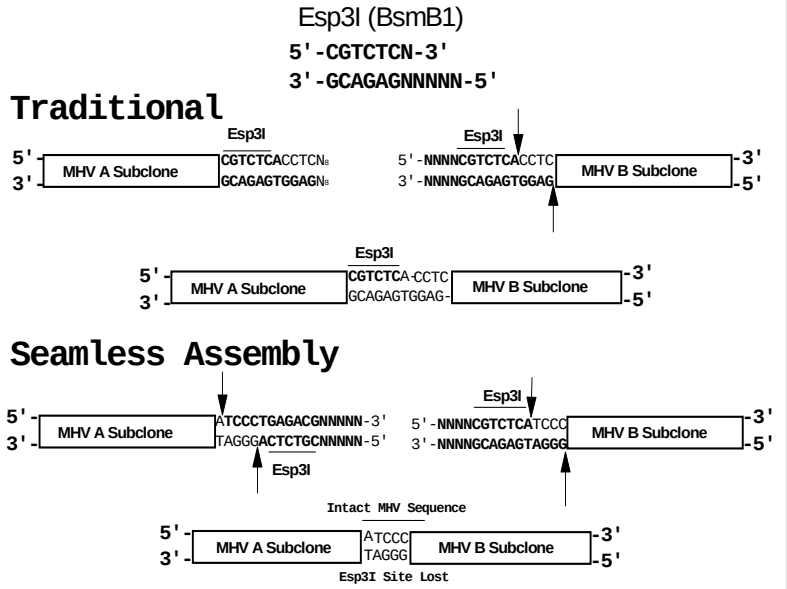
Although BACs are remarkably stable, both poxviruses and herpesvirus genomes contain repetitive sequence elements and other sequences that might be unstable with passage as no biological selective pressure exists to maintain virus genome sequence fidelity in E. coli. Because the large genome size makes it impractical to sequence the entire genome, in vivo pathogenesis studies have been used to demonstrate equivalent levels of pathogenicity and virulence between wildtype and recombinant herpes viruses, further supporting the hypothesis that BAC recombinant genomes are highly stable in E.coli (12). The availability of large dsDNA genomes in BACs provides two major opportunities for future research, the construction of expression vectors for treatment of human diseases and the mutagenesis of the viral genome for understanding gene function, virus replication and pathogenesis.

A second solution to large genome instability was developed using coronaviruses as models. Seven contiguous cDNA clones that spanned the 31.5 kilobase (kb) coronavirus genome (e.g., mouse hepatitis virus [MHV] or SARS-CoV) were amplified, isolated and ligated into standard polymerase chain reaction (PCR) cloning vectors (PCR is one

technique used to amplify sequences that are rare and/or not available in large quantities, to provide enough material for subsequent experiments). The ends of the cDNAs were engineered with unique junctions, generated by class IIS restriction endonucleases like BglI or Esp3I. These enzymes leave asymmetric ends, which are designed to seamlessly reproduce the exact virus sequence, allow directional assembly of adjacent cDNA subclones, and direct the production of an intact full length cDNA construct of ~31.5 Kb in length. With enzymes like Esp3I, interconnecting restriction site junctions can be located at the ends of each cDNA and systematically removed during the assembly of the complete full-length cDNA product (Figure 4a). The availability of a contiguous set of DNAs containing unique interconnecting junctions provides for the systematic assembly of large DNA molecules greater than 1,000,000 base pairs by in vitro ligation (85). In the case of coronaviruses (Figure 4b), full length cDNAs are assembled that contain a T7 transcription site at the 5' end of the genome. RNA transcripts driven from the full length cDNA were infectious upon delivery into susceptible cells (85, 87). Alternatively, coronavirus genomes can be stably cloned into BAC vectors. T7 or eukaryotic promoters encoded upstream of the viral sequences allow for the synthesis of full length RNA genome sequences, which are infectious upon introduction into cells (1).

Seamless assembly (also called No See'm Sites (85)) cascades have been used to assemble full length cDNAs of the coronaviruses mouse hepatitis virus, transmissible gastroenteritis virus, infectious bronchitis virus and the SARS-CoV (85,86,87). Because certain type IIS restriction endonucleases (e.g., Esp3I, AarI, Sap1) recognize asymmetric binding sites and leave asymmetric ends, these enzymes can be used to create the unique interconnecting junctions, which can be subsequently removed from the final assembly product allowing for the seamless reconstruction of an exact sequence (Figure 4b). This approach avoids the introduction of nucleotide changes that are normally associated with building a full-length cDNA product of a viral genome. These non-palindrome restriction sites will also provide other novel recombinant DNA applications. For example, by PCR

Figure 4a. Systematic Whole Genome Assembly Techniques.



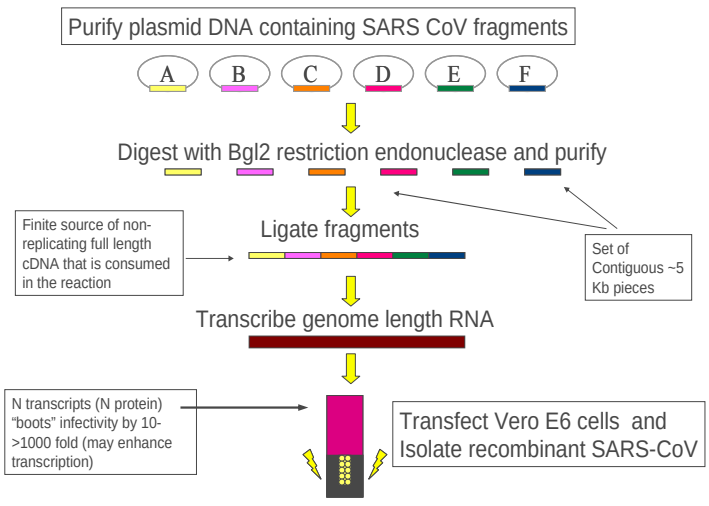
it will be possible to insert Esp3I or a related non-palindromic restriction site at any given nucleotide in a viral genome and use the variable domain for simple and rapid site-specific mutagenesis. By orientating the restriction sites as "No See'm", the sites are removed during reassembly, leaving only the desired mutation in the final DNA product. The dual properties of strand specificity and a variable end overhang that can be tailored to match any sequence allow for Esp3I sites to be engineered as "universal connectors" that can be joined with any other four nucleotide restriction site overhangs (e.g. EcoRI, PstX1, BamH1). Alternatively, "No See'm" sites can be used to insert foreign genes into viral, eukaryotic, or microbial genome or vector, simultaneously removing all evidence of the restriction sites that were used in the recombinant DNA manipulation.

Finally, these restriction sites allow for the rapid assembly of small synthetically produced cDNAs into progressively larger cDNAs. For example, enzymes like AarI recognize a 7 nucleotide recognition sequence and leave a four nucleotide asymmetric end (usually). In a random DNA sequence, this site occurs every 8,000 base pairs or so.

Using a recursive assembly cascade $2^{\sim 256}$ different 8Kb cDNAs can be assembled into extremely large >1,000,000 bp DNAs designed in BACs for stable maintenance in bacteria (85-87).

At this time, well developed molecular clones have been constructed with representative viruses in most of the known virus families; specifically, the Groups I-IV genomes, thus providing a systematic approach for generating molecular clones of many Categories I, III, and IV BW agents. In addition, recent advances in synthetic biology provides promise for reconstructing microbial genomes de novo (15), as has been elegantly demonstrated with the recovery of recombinant poliovirus and ΦX174 viruses (14, 73) from synthetically derived genomes. In these instances, accurate sequences were available for

Figure 4b. Systematic Assembly of Coronavirus Genomes.



de novo synthesis, as functional molecular clones had existed for both viruses for many years. Consequently, the combination of proof of principle, available templates for genome construction and sequence information make it likely that any virus genome

could be synthetically reconstructed from sequence databases, assuming that the sequence is correct (18, 36).

C. Review of Controlled Viruses

The United States Department of Health and Human Services (HHS), the Centers for Disease Control and Prevention (CDC), and the United States Department of Agriculture (USDA) have identified bacteria, viruses, toxins, rickettsia, and fungi that pose a potential threat to public health or welfare. Some of these organisms are considered Select Agents and High Consequence Livestock Pathogens and all research laboratories with access to these agents must submits names and fingerprints of all individuals listed as working with Select Agents to the Department of Justice. Every person who enters a laboratory containing registered Select Agents must have FBI security clearance or be accompanied and monitored by such a cleared person. This includes visitors and employees performing routine cleaning, maintenance, and repairs. The CDC oversees and regulates all laboratories that possess or use select agents and the transfer of select agents and toxins that may be used to threaten the overall public health and safety as published in the Federal Register on March 18, 2005 (42 C.F.R. Part 73, 7 C.F.R. Part 331, and 9 C.F.R. Part 121) (Appendix 1). In addition, the Department of Commerce regulates the transport of many pathogenic agents deemed important for maintaining the public health or that could impact the economic vitality of the US. Many, but not all, overlap with the Select Agent List and the USDA High Consequence Livestock Pathogens. Finally, the National Institutes of Health has assembled a list of high priority agents for biodefense research, and provides special funding for basic science, vaccines and therapeutics. Select agents are typically grouped among category A agents that pose the most serious perceived risk to national security while category B agents include many important food and waterborne agents that are easy to disseminate. The category C agents are emerging pathogens of special concern or pathogens that could be engineered for mass dissemination.

All work with microbes that might be harmful to workers or to the environment is conducted according to a variety of regulations directed to the general area of "biosafety

and containment". What is important here is that biosafety and containment are accomplished through a suite of institutional and worker actions and these activities are referred to by the level of containment achieved. "Biosafety Level 1" (BSL-1) is the least stringent containment; BSL-4 the most stringent (used for the deadliest pathogens for which there are no treatments).

Priority viruses will be discussed according to the Baltimore Classification Scheme. The key columns in these tables are the last three, *Nature*, *Laboratory*, and *Synthetic*. A "yes" in *Nature* indicates that the virus can be found in nature (thus, all viruses on the list except smallpox, 1918 H1N1 and 1957 H2N2 influenza, and the 2002-2003 strain of SARS CoV). A "yes" under *Laboratory* means that the virus can be found in some kind of lab, be it a research laboratory, a reference laboratory (e.g., the American Type Culture Collection), a commercial laboratory, etc. This is virtually all viruses on the list (smallpox is closely guarded, and the recently resurrected 1918 influenza virus, at least for now, is in a limited number of known laboratories). *Synthetic* captures two characteristics. First, is it possible to synthetically construct a virus of a specific family? These are indicated in bold, and takes into account both whether a synthetic DNA construct can supply the appropriate nucleic acid, and if enough is known about the other aspects of booting the system that it is imaginable that a synthetic approach would be taken. Second, for the individual viruses on the list, the range of possibility takes into account both whether it is possible to construct, and whether this would be an attractive possibility compared to finding it in nature, or trying to steal it from a laboratory (in the case of a bioterrorist). So for example, even though foot-and-mouth disease virus is easy to find in nature and highly contagious, it is also easy enough to synthesize that bioterrorists hoping to hide their tracks may prefer the synthetic route.

The Group I agents include the dsDNA viruses contained among the Herpes viruses, Poxviruses and Asfarviruses (Figure 5). Herpes viruses contain linear dsDNA genomes of about 150,000 base pairs and include Herpes B virus (primate) and Malignant catarrhal fever viruses (swine), both of which are readily available in nature and for which culture conditions have been detailed in the literature. Herpes virus genomes are infectious; full length molecular clones and recombinant viruses have been described for several human

and animal herpes viruses (72). Although molecular clones for Herpes B virus and Malignant catarrhal fever virus have not been described, a significant body of literature provides a theoretical template and guide for the development of similar constructs with a high probability of success.

Poxvirus genomes range in size from 150,000 to 196,000 base pairs in length and the genomes are not infectious upon introduction into susceptible cells. However, poxvirus genome infectivity can be booted by coinfection with an avian poxvirus that has an abortive infection in mammalian cell lines, but provides essential proteins for transcribing the poxvirus genome. A molecular clone has been described for vaccinia virus, providing a theoretical template for guiding similar technology with other members in the family (23, 24). Poxviruses like Variola major and Variola minor (smallpox) and monkey pox

Figure 5. Category I Restricted Agents.

Family	Virus	Genome Size	Infectious/ Boot Infectivity	HMS-CDC	NIH A-C	Commerce	USDA	Nature	Laboratory	Synthetic
Category I	dsDNA Genome	Linear	Mixed/yes							Yes but Difficult
Herpesviruses			Yes/Yes							
	Herpes B Virus	156,789		Y				Yes	Yes	Unlikely
	Malignant catarrhal fever virus	156,789					Y	Yes	Yes	Unlikely
Poxviruses			No/Yes*							Yes, but Difficult
	Variola Major	186,103-185,578	No/No	Y	A	Y		No	No* (Limited)	Plausible but difficult
	Variola Minor	186,986	No/No	Y	A	Y		No	No* (Limited)	Plausible, but difficult
	Monkey pox	196,858	No/No	Y	A	Y		Yes	Yes	Unlikely
	White pox				A	Y		Yes	Yes	Unlikely
	Goat pox	149,999	No/No		A		Y	Yes	Yes	Unlikely
	Sheep pox virus	149,955	No/No		A		Y	Yes	Yes	Unlikely
	Camel pox		No/No		A		Y	Yes	Yes	Unlikely
	Lumpy skin disease virus	150,773	No/No				Y	Yes	Yes	Unlikely
Asfarvirus	African swine fever virus	170,101	No/No				Y	Yes	Yes	Possible

*Variola samples are maintained in two laboratories worldwide.

viruses are select agents. Although most poxviruses can be readily found in nature and/or are maintained in laboratory settings, Variola major and minor are notable exceptions that are thought extinct in the wild. These two viruses are maintained in high security facilities in the US and Russia and it is very unlikely that these agents can be recovered from natural settings.

Group III priority agents include the reoviruses African horse sickness and exotic bluetongue strains, which primarily infect domesticated animals (Figure 6). Reovirus genomes contain ten segments of double stranded RNA and these genomes are not infectious in isolation. Reproducible schemes to boot reovirus genome infectivity have recently been developed by the Dermody laboratory. Although these viruses are available in nature and in laboratory settings, the inability to initiate genome infectivity had hampered the successful development of reverse genetic approaches and molecular clones. Consequently, the use of natural or laboratory acquired strains represented the

Family Category III	Virus	Genome	Infectious/ Boot Infectivity	HMS/CDC	NIH A-C	Commerce	USDA	Nature	Laboratory	Synthetic
REOVIRUS	**dsRNA Segmented Genome (10)**	**Linear, dsRNA**	**No, Yes*,**							**Not Possible**
Reovirus	African horse sickness virus	1-3965; 6-1566 2-3203; 7-1179 3-2792; 8-1166 4-1978; 9-1169 5-1566; 10-798	**No, No**				**Y**	**Yes**	**Yes**	Unlikely
	Bluetongue virus (exotic)	1-3944; 6-1658 2-2953; 7-1156 3-2772; 8-1125 4-1981; 9-1049 5-1769; 10-822	**No, No**				**Y**	**Yes**	**Yes**	Unlikely

Figure 6. Category III Priority Viruses.

most likely approach to acquiring these agents for bioterrorism purposes, although the reovirus reverse genetic system should be an appropriate template for developing molecular clones to other reoviruses..

Group IV viruses contain single stranded positive polarity RNA genomes and include agents in the calicivirus, potyvirus, picornavirus, alphavirus, flavivirus and coronavirus families (Figure 7). These viruses have dramatically different virion structures, genome organizations, and transmission modes between hosts; they target different tissues, display different virulence and pathogenic determinants and use different replication strategies upon entry into susceptible cells. Common features, however, include an infectious positive sense RNA genome and relatively straightforward and well developed approaches for obtaining full length cDNA clones from which recombinant viruses can be easily isolated in culture. In most cases these viruses replicate efficiently in culture, and animal models of disease exist, allowing for easy cultivation, maintenance, and testing in a laboratory setting. A general rule of thumb is that the BSL2 positive single stranded RNA (e.g., human noroviruses) pathogens are more readily accessible than the BSL3 pathogens (e.g., SARS-CoV, VEE, etc.) in laboratory settings. BSL4 pathogens are the least accessible. Poliovirus, which is targeted for eradication, is not included among any of the high priority pathogen lists but has been synthetically reconstructed by the Wimmer laboratory. Wild poliovirus is eradicated from the North and South American continents and Europe, but is still prevalent in Africa and parts of Asia. The virus has been present in many laboratories throughout the world, although current efforts are aimed at limiting the availability of wildtype stocks to a few locations in the US. Should eradication efforts prove successful, poliovirus should almost certainly be listed as a high priority agent. In the future, poliovirus might represent a likely candidate for synthetic reconstruction efforts because whole genome sequence is available, genome size is small and could be purchased for about $10,000 US dollars, and synthetic polioviruses have been reconstructed in the laboratory. This possibility, however, may be several decades away and is also dependent upon an end to global vaccination efforts.

The Group IV viruses are also very abundant in nature and many are present in laboratories. The main exception is the human 2002-3 SARS-CoV epidemic strain that is likely extinct in the wild, but is present in many laboratories throughout the world. Globally, most SARS-CoV isolates were late phase epidemic strains because many early and zoonotic (animal) isolates were never successfully cultured and not distributed outside of China (19, 41). Molecular clones have been described for prototype animal caliciviruses, picoronaviruses, potyviruses, alphaviruses, flaviviruses and coronaviruses, including many, but not all of the agents of interest in Figure 7. At this time, molecular clones for human noroviruses have not been successfully developed.

Group V viruses contain a single stranded negative polarity RNA genome and include members of the bunyavirus, arenavirus, filovirus, paramyxovirus, rhabdovirus, and influenza virus families (Figure 8, below). As with the group IV viruses, these viruses differ dramatically in virion structure, genome organization, transmission modes, human disease severity, virulence and pathogenesis. In general, negative stranded RNA genomes are either nonsegmented and linear (e.g., paramyxovirus, filoviruses, rhabdovirus) or segmented and linear (e.g., bunyavirus, arenavirus, myxoviruses). These viruses are readily found in nature either in human and animal hosts or vectors; all of which have been well described in the literature. Most are easily cultured in laboratory settings. Again, laboratory availability diminishes with increased BSL ratings, so that BSL3 (e.g., 1918 influenza, Rift Valley Fever) and BSL4 (e.g., Ebola, Marburg, Lassa Fever, etc.) are the least available. The exceptions include the 1918 Spanish influenza virus and H2N2 (1957 pandemic) Asian influenza viruses which are likely extinct in the wild. The 1918 Spanish influenza was resurrected from a molecular clone and is only available in a few laboratories worldwide, but the H2N2 strain is more prevalent in laboratory settings (81). Both viruses are likely capable of producing pandemic disease,

Figure 7. Category IV Priority Viruses.

Family	Virus	Genome	Infectivity/Boot Infection	HMS/CDC	NIH A-C	Commerce	USDA	Nature	Laboratory	Synthetic
Category IV	**Positive Polarity RNA Genomes**	**Linear**	**Yes/Yes**							
Calicivirus		**Linear**	**Yes/Yes**							**Possible**
	Human Norovirus	7,654	No/No		B			Yes	Yes	Not yet
	Vesicular exanthema virus	8284	?/No				Y	Yes	Yes	Plausible
	Rabbit Hemorrhagic virus	7467	?/No				Y	Yes	Yes	Unlikely
Picornavirus			**Yes/Yes**							**Yes**
	HAV	7,478	Yes/Yes		B			Yes	Yes	Unlikely
	Foot&Mouth Virus	8,161	Yes/Yes				Y	Yes	Yes	Plausible
	Poliovirus*	7,440	Yes/Yes					Yes	Yes	Done
	Swine vesicular disease virus	7,401	Yes/Yes				Y	Yes	Yes	Plausible
Potyvirus	**ssRNA + polarity**									**Yes**
	Plum Pox Virus	9741	Yes/Yes	Yes		Yes		Yes	Yes	Unlikely
Alphavirus			**Yes/Yes**							**Yes**
	VEE	11,444	Yes,Yes	Y	B	Y	Y	Yes	Yes	Plausible
	EEE	11,675	Yes,Yes	Y	B	Y	Y	Yes	Yes	Unlikely
	WEE	11,484	Yes,Yes		B	Y		Yes	Yes	Unlikely
	Chikungunya virus	11,826	Yes			Y		Yes	Yes	Unlikely
Flavivirus			**Yes/Yes**							**Yes**
	Dengue	10,735	Yes/Yes		A	Y		Yes	Yes	Unlikely
	West Nile	10,962	Yes/Yes		B			Yes	Yes	Unlikely
	Yellow Fever	100,862	Yes/Yes		C	Y		Yes	Yes	Unlikely
	Wesselsbron disease virus	NA	Yes/No				Y	Yes	Yes	Unlikely
	Japanese Encephalitis Virus	10,976	Yes/Yes		B		Y	Yes	Yes	Unlikely
	Central European TB-encephalitis	10,978-10,871	Yes/Yes	Y	C	?		Yes	Yes	Unlikely
	Far Eastern TB encephalitis virus	NA	Yes/Yes	Y	C	?		Yes	Yes	Unlikely
	Louping ill virus	10,871	No/No				Y	Yes	Yes	**Unlikely**
	Kyasanur Forest virus	Incomplete	Yes/No	Y	B	?		Yes	Yes	Unlikely
	Omsk HF Virus	10,787	Yes/No	Y	C	?		Yes	Yes	Unlikely
	Russian Spring/Summer Encephalitis virus		Yes/No	Y	C	Y		Yes	Yes	Unlikely
	Classical swine fever virus	12,301	Yes/				Y	Yes	Yes	Unlikely
Coronavirus	SARS-CoV	29,751	**Yes/Yes**		C			No[1]	Yes	**Yes**

[1]The 2002-2003 epidemic strain is likely extinct in the wild; many zoonotic forms exist; *poliovirus is not included in any priority pathogen lists.

as the Spanish Flu H1N1 and Asian H2N2 strains have not circulated in human populations for over 90 and 50 years, respectively. Reverse genetics systems for prototypic members of each virus family have been reported in the literature although success is more rare with arenaviruses and bunyaviruses. In contrast, well documented reverse genetic systems have been described for paramyxoviruses, rhabdoviruses, myxoviruses, and filoviruses providing clear templates for reconstruction of synthetic viruses.

Although many Category I-V agents are available in laboratory settings, serial passage of virus in cell culture oftentimes selects for "culture adapted" variants that display altered or reduced pathogenicity in the original host. In fact, serial passage in cell culture or alternative animal model has been used to attenuate virus pathogenesis and was used as a method to develop live attenuated poliovirus and measles virus vaccines. Consequently, laboratory strains may not reproduce wildtype virus pathogenicity and virulence when reintroduced into the natural host and may not represent the preferred source of starting material for bioterrorism applications.

III. Barriers to Synthesizing and Resurrecting Viruses by Synthetic Biology and Reverse Genetics

Genetic engineering of viruses requires the development of infectious clones from which recombinant viruses can be isolated. Two basic strategies exist to develop and molecularly clone a viral genome: classic recombinant DNA approaches or synthetic biology. Although the basic methodology is different, the outcome is the same, a full length DNA copy of the viral genome is constructed which is infectious upon delivery to a permissive host cell. Classic recombinant DNA approaches require the availability of viral nucleic acid, which is normally isolated from infected tissues or cells and used as template for cloning and sequence analysis. For RNA viruses, the approach includes using reverse transcriptase and polymerase chain reaction to clone overlapping pieces of the viral genome and then whole genome assembly and sequence validation before successful recovery of recombinant viruses (10). Virus genome availability is an important issue and until recently, a major bottleneck in constructing a molecular clone to

any BW virus. Most, though not all, viral BW agents are not readily available except in high containment BSL3 and BSL4 laboratories throughout the world. The few sites and lack of funding support historically limited access to a small number of researchers, although increased support for BW research has greatly increased the distribution and availability of these agents throughout the world (31). Most viruses are also available in zoonotic reservoirs although successful isolation may require an outbreak or knowledgeable individuals carrying out systematic sampling of hosts in endemic areas. Then, containment facilities for replicating virus are necessary. Some exceptions to this general availability of controlled viruses include early 20th century influenza viruses like the 1918 H1N1 (Spanish flu), the 1957 H2N2 (Asian Flu), smallpox viruses (extinct 1977) and perhaps the 2002-2003 epidemic SARS-CoV strains, all of which are likely extinct in the wild given the lack of recent human disease. With the molecular resurrection of the 1918 H1N1 strain using recombinant DNA techniques (81), these viruses only exist in select laboratories distributed throughout the world.

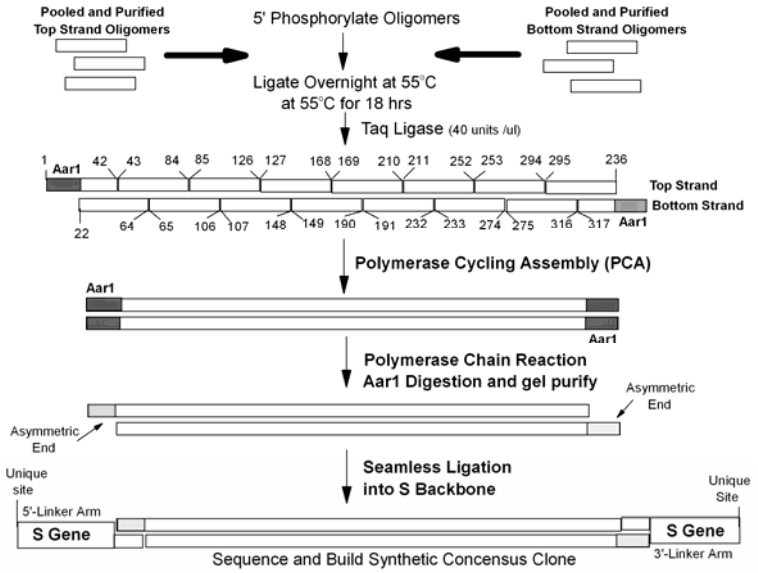
Two general approaches exist for synthetic reconstruction of microbial genomes from published sequence databases: de novo DNA synthesis and polymerase cycling assembly (PCA). Roughly 50 commercial suppliers worldwide provide synthetic DNAs using either approach, mostly in the range of <5.0Kb, although at this time only a few companies can assemble DNAs >30Kb. For example, Blue Heron's GeneMaker™ is a proprietary, high-throughput gene synthesis platform with a ~3-4 week turnaround time and is reported to be able to synthesize any gene, DNA sequence, mutation or variant-including SNPs, insertions, deletions and domain-swaps with perfect accuracy regardless of sequence or size (http://www.blueheronbio.com/). Most commercial suppliers, however, use polymerase cycling assembly (PCA), a variation on PCR. Using published sequence, sequential ~42 nucleotide oligomers are synthesized and oriented in both the top and bottom strand, as pioneered for ΦX174 (73) (Figure 9). Top and bottom strand oligomers overlap by ~22 bp. The PCA approach involves: 1) phosphorylation of high purity 42-mers (oligonucleotide strands of DNA) in the top and bottom strand, respectively, 2) annealing of the primers under high stringency conditions and ligation with the Taq ligase at 55^{o}C, 3) assembly by polymerase cycling assembly (PCA) using

the HF polymerase mixture from Clontech (N-terminal deletion mutant of Taq DNA polymerase lacking 5'-exonuclease activity and Deep $Vent_R$ polymerase [NEB] with 3' exonuclease proofreading activity), 4) PCR amplification and cloning of full length amplicons (Figure 9). The key issue is to use HPLC to maximize oligomer purity and to minimize the numbers of prematurely truncated oligmers used in assemblages. As PCR is an error prone process, the PCA approach is also error prone and it requires sequence verification to ensure accurate sequence. PCA is also limited to DNAs of 5-10 Kb in length which is well within the genome sizes of many viral genomes, although improvements in PCR technologies could extend this limitation. Both approaches, coupled with systematic genome assembly techniques shown in Figure 4, will allow assembly of extremely large viral genomes, including poxviruses and herpes viruses.

Consequently, knowledgeable experts can theoretically reconstruct full length synthetic genomes for any of the high priority virus pathogens, although technical concerns may limit the robustness of these approaches. It is conceivable that a bioterrorist could order

Figure 9. PCA Technique. Synthetic Reconstruction of Exotic SARS-CoV Spike Glycoproteins.

Synthetic S glycoproteins are synthesized and inserted into the SARS-CoV molecular clone; allowing for recovery of recombinant viruses encoding zoonotic S glycoproteins.



genome portions from various synthesis facilities distributed in different countries throughout the world and then assemble an infectious genome without ever having access to the virus. To our knowledge, no international regulatory group reviews the body of synthetic DNAs ordered globally to determine if a highly pathogenic recombinant virus genome is being constructed.

What, then, are the technical barriers to the reconstruction of viral genomes? Three major issues are generally recognized: sequence accuracy, genome size and stability, and expertise. They are discussed in this order below.

Sequence databases record submissions from research facilities throughout the world. However, they have limited ability to review the accuracy of the sequence submission. Consequently, these databases are littered with mistakes ranging from 1 in 500 to 1 in 10,000 base pairs. In general, large sequencing centers are more accurate than independent research laboratories (18, 36). Accurate sequence is absolutely essential for rescuing recombinant viruses that are fully pathogenic (7, 10, 30, 85, 86) as even a single nucleotide change can result in viable virus that are completely attenuated in vivo (74). Sequence accuracy represents a significant barrier to the synthetic reconstruction of these highly pathogenic viruses. RNA viruses exist in heterogeneous "swarms" of "microspecies," thus requiring the identification of a "master sequence;" i.e., the predominant sequence identified after sequencing the genome numerous times. Consequently, full length sequence information may have been reported, but the published sequence may actually not be infectious. Problems with sequence accuracy are proportional to genome size, as reported sequence for large viral genomes will more likely include a higher number of mutations than small genomes. In many instances, sequence errors will reside at the ends of viral genomes because the ends are oftentimes more difficult to clone and sequence.

Using state of the art facilities, the smallpox genome from a Bangladesh 1975 strain was sequenced (47). However, an error rate of 1:10,000 would result in about 19-20 mistakes and 10-14 amino acid changes in the recombinant genome. Should these mistakes occur within essential viral proteins or occur in virulence alleles, recovery of highly pathogenic

recombinant viruses might be impossible. More recently, another genome sequence of Variola major (India 1967) has been reported in the literature (Bangladesh 75, and India 67; Accession # X69198 and L22579). These full length genomes differ in size by 525 base pairs, contain ~1500 other allelic changes scattered throughout the genomes, and also differ in size and sequence with the Variola minor genome (Figure 5). Although roughly 99.1% identical, which of these reported sequences are correct? Will pathogenic virus be recovered from a putative molecular clone of either, both or neither? If neither is infectious, which changes are responsible for the lethal phenotype? In the absence of documentation of the infectivity of a reported sequence, it becomes difficult to accurately predict the correct sequence that will allow for the recovery of infectious virus. At best, a combination of bioinformatics, evolutionary genetic and phylogenetic comparisons among family members may identify likely codon and nucleotide inconsistencies, simultaneously suggesting the appropriate nucleotide/codon at a given position. In the case of poxviruses, only two full length sequences of Variola major have been reported, hampering such sequence comparisons. Ultimately this approach only allows informed guesses that may not result in the production of recombinant virus. Obviously, reported full length genomic sequences that have been demonstrated to generate infectious viral progeny provide an exact sequence design for synthetic resurrection of a recombinant virus, greatly increasing the probability of success. In the absence of this data, multiple full length submissions are needed to enhance the probability of success.

Another problem hampering the development of synthetic DNA genomes for genetic manipulation are genome size and sequence stability in microbial vectors. Many viral full-length cDNAs, including coronavirus genomes and certain flavivirus genomes like yellow fever virus are unstable in microbial vectors (10). Low copy BAC vectors and stable cloning plasmids oftentimes reduce the scope of this problem although instability has been reported with large inserts following passage (1, 85). Plasmid instability might be caused by sequence toxicity associated with the expression of viral gene products in microbial cells or the primary sequence might simply be unstable in microbial vectors, especially sequences that are A:T rich. To circumvent this problem, plasmid vectors have been developed that contain poly-cloning regions flanked by several transcriptional

and translational stops to attenuate potential expression of toxic products (86). The development of wide host range, low copy vectors that can be used in Gram positive or lactic acid bacteria may also allow amplification of sequences that are unstable in E. coli hosts. Alternatively, theta-replicating plasmids that are structurally more stable and that accommodate larger inserts than plasmids that replicate by rolling circle models may alleviate these concerns in the future (3, 35, 58). Poxvirus vectors also provide an alternative approach for stably incorporating large viral genome inserts, although long-term stability of these vectors is unknown (1, 77).

The technical skill needed to develop full length infectious cDNAs of viruses is not simple and requires a great deal of expertise and support: technically trained staff, the availability of state of the art research facilities, and funding. Theoretically, the ability to purchase a full length DNA of many viral biodefense pathogens is now possible, especially for those virus genomes that are less than 10 kb in length. In addition, defined infectious sequences are documented and methods have been reported in the literature. Infectious genomes of many Class IV viruses could be purchased and the need for trained staff becomes minimized. Today, a picornavirus or flavivirus genome could be purchased for as little as $15,000, a coronavirus genome for less than $40,000. It is much more difficult to reconstruct large viral genomes, meaning that trained staff and state of the art facilities become very essential to the process.

However, it is conceivable that technical advances over the next decade may even render large viral genomes commercially available for use by legitimate researchers, but perhaps also by bioterrorists.

IV. Risk and Benefits of Synthetic Organisms

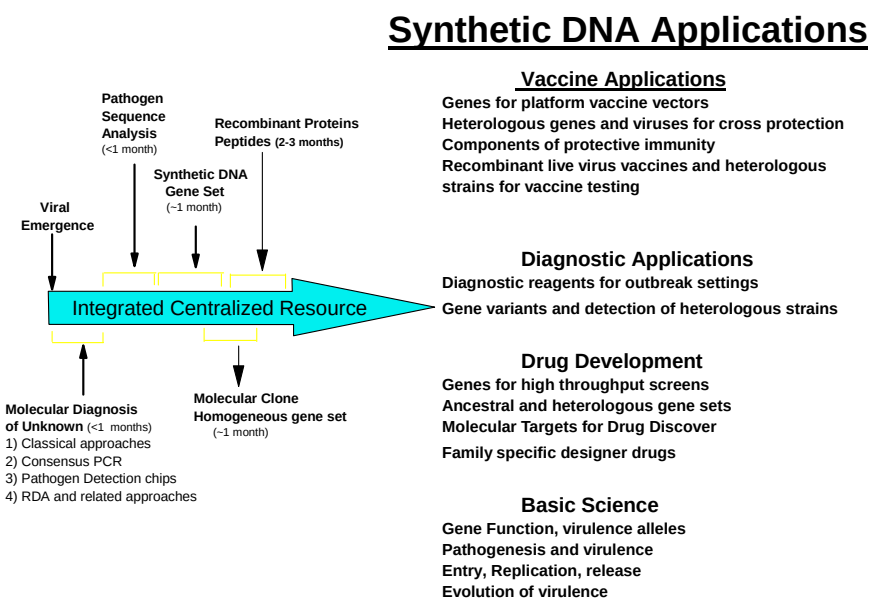
A. Benefits to Society

The benefits of recombinant DNA have been heavily reviewed in the literature and include the development of safe and effective virus platform technologies for vaccine design and gene therapy, the production of large quantities of drugs and other human and animal medicines, and agricultural and other products key to robust national economies. Genetic engineering of bacteria and plants may allow for the production of large quantities of clean burning fuels, produce complex drugs, design highly stable biomolecules with new functions, and develop organisms that rapidly degrade complex pollutants (52, 56, 64, 78). Comparative genomics also provides numerous insights into the biology of disease-causing agents and is allowing for the development of new diagnostic approaches, new drugs and vaccines (27). Synthetic biology enhances all of the opportunities provided by recombinant DNA research. The main advantages of synthetic genomics over classic recombinant DNA approaches are speed and a mutagenesis capacity that allow for whole genome design in a cost effective manner (6). How will synthetic biology protect the overall public health?

A major advantage is in the development of rapid response networks to prevent the spread of new emerging diseases. Platform technologies allow for rapid detection and sequencing of new emerging pathogens. The SARS-CoV was rapidly identified as a new coronavirus by gene discovery arrays and whole genome sequencing techniques within a month after spread outside of China (37, 46, 83, 84). Similar advances were also made in the identification of highly pathogenic avian H5N1 influenza strains, hendra virus and in other outbreaks. Sequence information allowed for immediate synthesis of SARS and H5N1 structural genes for vaccines and diagnosis and the rapid development of candidate vaccines and diagnostic tools within a few months of discovery. Classic recombinant DNA approaches requires template nucleic acid from infected cells and tissues (limited supply), followed by more tedious cloning and sequence analysis in independent labs throughout the world. As access to viral nucleic acids historically limited response

efforts to only a few groups globally, research productivity was stifled. Synthetic biology results in a true paradigm shift in virus vaccine, therapeutic and diagnostic discovery, resulting in the near simultaneously engagement of multiple laboratories as genome sequence becomes available (Figure 10).

Figure 10. Synthetic DNA Rapid Response Applications.



Genome sequence provides for rapid incorporation of synthetic genes into platform technologies that allow for rapid diagnosis and epidemiologic characterization of the incidence, prevalence and distribution of new pathogens in human and animal hosts. Synthetic genes can be immediately incorporated into recombinant virus or bacterial vaccine platforms and tested in animal models and/or humans. Synthetic genes and proteins become essentially immediately available for structural studies, for high throughput identification of small molecule inhibitors and for the rational design of

drugs. Synthetic full length molecular clones become available for genetic analysis of virus pathogenicity and replication, construction of heterotypic strains for vaccine and drug testing, rapid development of recombinant viruses containing indicator genes for high-throughput screens and for the development of live attenuated viruses as vaccines or seed stocks for killed vaccines.

Thus, the availability of synthetic genes and genomes provides for rapid development of candidate drugs and vaccines, although significant bureaucratic hurdles must be overcome to allow for rapid use in vulnerable human populations. We note that highly pathogenic respiratory viruses can be rapidly distributed worldwide, providing only limited opportunities and time for the prevention of global pandemics and the preservation of the overall public health.

B. Risks to Society

1. Bioterrorism

The historical record clearly shows that many nations have had biological weapons programs (of varying degrees of development) throughout the 20th century including many European nations, the USSR and the United States, Japan and Iraq. From relatively unscientific programs early in the 20th century, progressively more sophisticated scientific programs developed during WWI and the Cold War. There is little doubt that the genomics revolution could stimulate a new generation of potential program development (27, 76). It is also well established that the biological revolution, coupled with advances in biotechnology could be used to enhance the offensive biological properties of viruses simply by altering resistance to antiviral agents (e.g., herpes viruses, poxviruses, influenza), modifying antigenic properties (e.g, T cell epitopes or neutralizing epitopes), modifying tissue tropism, pathogenesis and transmissibility, "humanizing" zoonotic viruses, and creating designer super pathogens (27, 59). These bioweapons could be targeted to humans, domesticated animals or crops, causing a devastating impact on human civilization. Moreover, applications of these approaches are certainly not limited to the list of pathogens recorded throughout this report—well developed

engineering tools have been developed for only a few BW agents, making them relatively poor substrates for biodesign. A clever bioterrorist might start with a relatively benign, easily obtainable virus (BSL2) and obtain an existing molecular clone by simply requesting it from the scientists who work with these agents. Then, using the expanding database of genomic sequences and identified virulence genes, the benign viral genome could be modified into more lethal combinations for nefarious use.

As recombinant DNA approaches, infectious DNA clones and the general methods needed to bioengineer RNA and DNA viruses have been available since the 1980-1990's, what new capabilities does synthetic biology bring to a biowarrior's arsenal? Clearly, recombinant viral genomes and bioweapon design can be accomplished using either or a combination of both approaches, suggesting that synthetic biology will have little impact on the overall capabilities of bioweapons research. However, synthetic biology provides several attractive advantages as compared with standard recombinant DNA approaches; specifically 1) speed, 2) mutagenic superiority, 3) ease of genome construction and 4) low cost. The main paradigm shift may be that the approach is less technically demanding and more design-based, requiring only limited technical expertise because the genome can be synthesized and purchased from commercial vendors, government sponsored facilities, or from rogue basement operations (e.g., bioterrorist sponsored or private entrepreneur). Main technical support might include a competent research technician and minimal equipment to isolate recombinant pathogens from the recombinant DNAs.

Standard recombinant DNA techniques are hands-on, laborious and slow, requiring multiple rounds of mutagenesis and sequence validation of the final product. At the end of this effort, there is no guarantee that the designer or synthetic genome will function as intended (see other sections), dictating the need for high throughput strategies. Synthetic genomes can be devised fairly rapidly using a variety of bioinformatics tools and purchased fairly cheaply ($1.10/base at current rates), allowing for rapid production of numerous candidate bioweapons that can be simultaneously released (e.g., survival of the fittest approach) or lab tested and then the best candidate used for nefarious purposes. The latter approach assumes that an organization has funded the development of a secure

facility, has provided trained personnel and is willing to test the agents and/or passage them in humans, as animal models may be unreliable predictors of human pathogenesis. Assuming the technology continues to advance and spread globally, synthetic biology will allow for rapid synthesis of large designer genomes (e.g., ~30 Kb genome in less than a couple of weeks); larger genomes become technically more demanding. It seems likely that a standard approach could be designed for recovering each synthetic virus, further minimizing the need for highly trained personnel.

Will synthetic or recombinant bioweapons be developed for BW use? If the main purpose is to kill and inspire fear in human populations, natural source pathogens likely provide a more reliable source of starting material. Stealing the BW agent from a laboratory or obtaining the pathogen from natural outbreak conditions is still easier than the synthetic reconstruction of a pathogenic virus. These conditions, however, change as 1st and 2nd generation candidate vaccines and drugs are developed against this select list of pathogens, limiting future attempts to newly emerged viruses. If notoriety, fear and directing foreign government policies are principle objectives, then the release and subsequent discovery of a synthetically derived virus bioweapon will certainly garner tremendous media coverage, inspire fear and terrorize human populations and direct severe pressure on government officials to respond in predicted ways.

2. Prospects for Designer Super Pathogens

Advances in genomics may provide new approaches for mixing and matching genetic traits encoded from different viral pathogens, as over 1532 genome length sequences are available in Genbank. A large number of recombinant viruses have been assembled using reverse genetic approaches including chimeric flaviviruses, chimeric enteroviruses and coronaviruses, HIV, lentiviruses and others usually for the purposes of generating vaccines or dissecting basic questions about, e.g., viral metabolism (29, 34, 39, 40, 50). Importantly, recombinant viruses are actively being designed with programmed pathogenic traits as a means of controlling certain insect and animal pests, providing both theoretical and practical strategies for conducting effective biowarfare (53, 69). More importantly, the identification of numerous virus virulence genes that target the innate

immune response (e.g., interferons, tumor necrosis factors, interleukins, complement, chemokines, etc.), apoptosis (programmed cell death) and other host signaling pathways provides a gene repository that can be used to potentially manage virus virulence (5, 8, 9, 26, 70). Poxviruses and Herpes viruses, for example, encode a suite of immune evasion genes and pro-apoptotic genes (48, 54). More recently, virus encoded microRNAs were identified in Epstein Barr Virus (EBV) and other herpes viruses, which function to silence specific cellular mRNAs or repress translation of host genes that function in cell proliferation, apoptosis, transcription regulators and components of signal transduction pathways (62). Although the function of many viral micro-RNAs are unknown, it is likely that they regulate protein coding gene expression in animals and influence pathogenesis (61). Moreover, microRNAs could also be designed and targeted to downregulate specific human signaling pathways.

The identification of virulence alleles is traditionally a first step to attenuating virus virulence. However, highly virulent murine pox virus (ectromelia) were recovered after the host IL-4 gene was incorporated into the genome. IL-4 expression altered the host Th1/Th2 immune response leading to severe immunosuppression of cellular immune responses, high viremias, and increased pathogenesis following infection. The recombinant virus was lethal in both control and in immunized or therapeutically treated mice (33, 67). More troubling was the belated recognition that this outcome could have been predicted based on our understanding of pox molecular virology and pathogenesis, suggesting that increased virulence can be rationally modeled into existing pathogens (55) and subsequent extension of these findings to other, but not all animal poxviruses (75). Many key questions remain unanswered regarding the ability to translate results with inbred mouse strains and murine poxviruses to outcome responses in outbred human populations infected with recombinant human poxviruses. Today, these outcomes cannot be predicted. Is it possible to enhance virulence by recombinant DNA approaches in other virus families and animal models? The influenza NS1 gene (an interferon antagonist gene) also enhances the replication efficiency of avian Newcastle disease virus in human cells (57), although the in vivo pathogenesis of these isolates has not been evaluated.

More recently the SARS-CoV ORF6, but not the ORF3a group specific antigens (specific proteins of the virus) were shown to enhance mouse hepatitis virus virulence in inbred mice strains. The mechanism by which the SARS-CoV ORF6 product enhances MHV virulence is not known at this time (60). Finally, viral gene discovery and sequence recovery using DNA microarrays will greatly increase the electronic availability of sequences from many novel human, animal and insect viruses (83, 84). This revolution in pathogen detection, coupled with rapid genome sequencing, provides a rich parts list for designing novel features into the genome of viruses.

Another approach might be to "humanize" zoonotic viruses by inserting mutations into virus attachment proteins or constructing chimeric proteins that regulate virus species specificity (viral attachment proteins bind receptors, mediating virus docking and entry into cells). For example, the mouse hepatitis virus (MHV) attachment protein, the S glycoprotein, typically targets murine cells and is highly species specific. Recombinant viruses contain chimeric S glycoproteins that are composed of the ecto-domain of a feline coronaviruses fused with the c-terminal domain of MHV S glycoproteins targets feline, not murine cells for infection. The pathogenicity of these chimeric coronaviruses is unknown (39). As information regarding the structure and interactions between virus attachment proteins and their receptors accumulate, data will provide detailed predictions regarding easy approaches to humanize zoonotic strains by retargeting the attachment proteins to recognize human, not the animal receptors (43-45). Conversely, it is not clear whether species retargeting mutations will result in viruses that produce clinical disease in the human host.

Synthetic DNAs and systematic assembly approaches also provide unparalleled power for building genomes of any given sequence, simultaneously providing novel capabilities for nefarious use. For example, genome sequences represent fingerprints that allow geographic mapping of the likely origin of a given virus. Recombinant viruses generated from classic recombinant DNA techniques will carry the signature of the parental virus used in the process as well as novel restriction sites that were engineered into the genome during the cloning process. In contrast, synthetic viral genomes can be designed to be

identical with exact virus strains circulating in any given location from any year. This powerful technique provides the bioterrorist with a "scapegoat" option; leaving a sequence signature that misdirects efforts at tracking the true originators of the crime. Even better, the approach could be used to build mistrust and/or precipitate open warfare between nations. A simple example might involve the use of the picornavirus foot and mouth disease virus, which is not present on the North American continent, yet is endemic in Africa, Asia, the Middle East and South America. North American herds are not vaccinated against this pathogen, the virus is highly contagious, and the disease is subject to international quarantine. Geographically distinct FMDV strains contain unique sequence signatures allowing ready determination of origin. A North American outbreak of an infectious "synthetic" FMDV virus containing signature sequences reminiscent of strains found in select Middle East or Asian nations that are viewed as terrorist states by the US government would inflame worsening tensions and could provide a ready excuse for military retaliation. Project costs would likely be less than $50K, including synthesis, recovery and distribution. Another possibility may be to optimize replication efficiency by optimizing for human codon use, especially useful in "humanizing" zoonotic viruses although to our knowledge codon optimization has never been linked to increased replication or pathogenesis. In both examples, standard recombinant DNA approaches would be difficult and tedious, while synthetically derived genomes could be readily manufactured within weeks.

Virus pathogenesis is a complex phenotype governed by multiple genes and is heavily influenced by the host genetic background. Virus genes influence virus-receptor interaction, tissue tropism, virus-host interactions within cells, spread throughout the host, virion stability and transmission between hosts. Colonization of hosts is influenced by ecologic factors including herd immunity, cross immunity and host susceptibility alleles. In general, the rules governing virulence shifts are hard to predict because of the lack of research and ethical concerns that have historically limited this type of research. In fact, the research itself promotes an emerging conundrum as to the limits of biodefense research: the need to know to protect the overall public health versus the development of models to elucidate the fundamental principles of pathogen design (4). Synthetic biology

and recombinant DNA approaches provide numerous opportunities to construct designer pathogens encoding a repertoire of virulence genes from other pathogens, while simultaneously providing a rapid response network for preventing the emergence and spread of new human and animal diseases. The state of knowledge prevents accurate predictions regarding the pathogenic potential of designer viruses; most likely, replication and pathogenesis would be attenuated. As a principle goal of bioterrorism is to inspire fear, highly pathogenic outcomes may not be necessary as large scale panic would likely result after the release of designer pathogens in US cities. Given the reported findings and the large repertoire of host, viral and microbial virulence genes identified in the literature, the most robust defense against the development of designer viral pathogens for malicious use is basic research into the mechanisms by which viral pathogenesis might be manipulated and applied counter measures that ameliorate these pathogenic mechanisms. This justification, however, blurs the distinction between fundamental academic research and bio-weapon development.

3. Ancient Pathogen Resurrection

Paleomicrobiology is an emerging field dedicated to identifying and characterizing ancient microorganisms in fossilized remains (25). Mega-genomic high throughput large scale sequencing of DNA isolated from mammoths preserved in the permafrost not only identified over 13 million base pairs of mammoth DNA sequence, but also identified novel bacterial and 278 viral sequences that could be assigned to dsDNA viruses, retroviruses and ssRNA viruses (63). Although DNA genomes can survive for almost 20,000 years (25), RNA virus fossil records do not exist beyond a ~90-100 year window, making it difficult to understand the evolution of virulence, molecular evolution, and the function of modern day viral genes. Among RNA viruses, the current record is the molecular resurrection of the highly pathogenic 1918 influenza virus, which required almost 10 years of intensive effort using standard recombinant DNA approaches from many laboratories (81). Obviously, synthetic reconstruction of ancient viral genomes may provide a rapid alternative as sequence database grow more robust over the next few decades. How pathogenic are these ancient pathogens? Will vaccines and anti-virals protect humans from ancient virus diseases?

Moreover, alternative approaches also exist to regenerate ancient viral sequences. Ancestral gene resurrection using bioinformatics approaches offers a powerful approach to experimentally test hypotheses about the function of genes from the deep evolutionary past (79). Using phylogenetic methods (38), ancestral sequences can be inferred but the approach suffers from the lack of empirical data to refute or corroborate the robustness of the method. More recently, the sequence of ancestral genes was accurately predicted as evidenced by the synthetic reconstruction of a functional ancestral steroid receptor, Archosaur visual pigment and other genes (15, 16, 79, 80). To our knowledge, phylogenic reconstruction of ancient virus sequences has not been tested empirically but it may be possible to construct replacement viruses encoding ancient structural genes from inferred sequence. Such viruses would have unpredictable pathogenicity, but would likely be highly resistant to vaccines and therapeutics targeted to modern day strains.

4. Summary

Chemical synthesis of viral genomes will become less tedious over the coming years. Costs will likely decrease as synthesis capabilities increase. Moreover, the technology to synthesize DNA and reconstruct whole viral genomes is spreading across the globe with dozens of commercial outfits providing synthetic DNAs for research purposes. DNA synthesizers can be purchased through on-line sites such as eBay. It is likely that engineering design improvements will allow for simple construction of larger genomes. The technology to synthetically reconstruct genomes is fairly straightforward and will be used, if not by the United States, then by other Nations throughout the world. It is also likely that synthetic genes and synthetic life forms will be constructed for improving the human condition and they will be released into the environment. As with most technology, synthetic biology contains risks and benefits ranging from a network to protect the public health from new emerging diseases to the development of designer pathogens. Synthetic genome technology will certainly allow for greater access to rare viral pathogens and allow for the opportunity to attempt rationale design of super pathogens. It is likely that the threat grows over time, as technology and information provide for more rational genome design. The most robust defense against the development of designer viral pathogens for malicious use may be basic research into the

mechanisms by which viral pathogenesis might be manipulated so that applied counter-measures can be developed.

Addendum (November 2007): Since the writing of this initial report, recent studies have demonstrated the availability of a reverse genetic systems for reovirus, a group III dsRNA virus (Kobayashi T, Antar AA, Boehme KW, Danthi P, Eby EA, Guglielmi KM, Holm GH, Johnson EM, Maginnis MS, Naik S, Skelton WB, Wetzel JD, Wilson GJ, Chappell JD, Dermody TS. A plasmid-based reverse genetics system for animal double-stranded RNA viruses. Cell Host Microbe. 2007 Apr 19;1(2):147-57) and for additional group V single stranded negative polarity RNA viruses like Rift Valley Fever Virus (Ikegami T, Won S, Peters CJ, Makino S. Rescue of infectious rift valley fever virus entirely from cDNA, analysis of virus lacking the NS gene, and expression of a foreign gene. J Virol. 2006 Mar;80(6):2933-40.)

Appendix 1. EHS/CDC Select Agent List (Viruses)

1. African horse sickness virus [1]
2. African swine fever virus 1
3. Akabane virus 1
4. Avian influenza virus (highly pathogenic) 1
5. Blue tongue virus (exotic) 1
6. Camel pox virus 1
7. Cercopithecine herpes virus (Herpes B virus) 3
8. Classical swine fever virus 1
9. Crimean-Congo haemorrhagic fever virus 3
10. Eastern equine encephalitis virus 2
11. Ebola viruses 3
12. Foot and mouth disease virus 1
13. Goat pox virus1
14. Japanese encephalitis virus 1
15. Lassa fever virus 3
16. Lumpy skin disease virus 1
17. Malignant catarrhal fever 1
18. Marburg virus 3
19. Menangle virus 1
20. Monkey pox virus 1
21. Newcastle disease virus (exotic) 1
22. Nipah and Hendra complex viruses 2
23. Peste des petits ruminants 1
24. Plum pox potyvirus4
25. Rift Valley fever virus 2
26. Rinderpest virus 1
27. Sheep pox 1
28. South American haemorrhagic fever viruses (Junin, Machupo, Sabia, Flexal, Guanarito) 3
29. Swine vesicular disease virus 1
30. Tick-borne encephalitis complex (flavi) viruses (Central European Tick-borne encephalitis, Far Eastern Tick-borne encephalitis, Russian Spring and Summer encephalitis, Kyasanur Forest disease, Omsk Hemorrhagic Fever) 3
31. Variola major virus (Smallpox virus) and Variola minor (Alastrim) 3
32. Venezuelan equine encephalitis virus 2
33. Vesicular stomatitis virus (exotic) 1
34. Reconstructed replication competent forms of the 1918 pandemic influenza virus containing any portion of the coding regions of all eight gene segments (Reconstructed 1918 Influenza virus)

[1]USDA High Consequence Livestock Pathogens or Toxin

[2]USDA/HSS Overlap Agent

[3]HHS Select Infectious Agent

[4]APHIS Plant Pathogens (Animal and Plant Health Inspection Service, a division of USDA)

Appendix I, cont.

Prions

1.Bovine spongiform encephalopathy agent (USDA High Consequence Livestock Pathogens or Toxin)

Appendix II. US Department of Commerce-Pathogen and Zoonotic virus list
a. Viruses:

a.1. Chikungunya virus;
a.2. Congo-Crimean haemorrhagic fever virus;
a.3. Dengue fever virus;
a.4. Eastern equine encephalitis virus;
a.5. Ebola virus;
a.6. Hantaan virus;
a.7. Japanese encephalitis virus;
a.8. Junin virus;
a.9. Lassa fever virus
a.10. Lymphocytic choriomeningitis virus;
a.11. Machupo virus;
a.12. Marburg virus;
a.13. Monkey pox virus;
a.14. Rift Valley fever virus;
a.15. Tick-borne encephalitis virus (Russian Spring-Summer encephalitis virus);
a.16. Variola virus;
a.17. Venezuelan equine encephalitis virus;
a.18. Western equine encephalitis virus;
a.19. White pox; or
a.20. Yellow fever virus.

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Impact of Synthetic Genomics on the Threat of Bioterrorism with Viral Agents

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Introduction

In 2002, a team of researchers at the State University of New York led by Eckard Wimmer assembled a DNA template for the RNA poliovirus using an internet-available nucleotide sequence and mail order synthetic oligonucleotides. Using a routine laboratory procedure, they then converted the DNA into RNA and produced an infectious, neurovirulent poliovirus capable of paralyzing and killing mice.[1]

This work demonstrated clearly for the first time the feasibility of chemically synthesizing a pathogen knowing only its nucleotide sequence. Some called the work "irresponsible," and there was widespread speculation in the press that bioterrorists might use the technology to create more virulent viruses, such as smallpox, from published gene sequences or create novel, more lethal viruses. Wimmer countered that "an evildoer would not use that very tedious method to synthesize a virus. That terrorist would rather use already existing viruses in nature." [2]

Indeed, all viruses, from the common cold to the deadliest, originate in nature, being identified and isolated from infected humans or animals or the virus's animal or insect vector. However, the rapidly advancing technology of whole genome assembly ("synthetic genomics") is making the chemical synthesis of viral genomes a much less tedious endeavor.[3]

This paper will explore the potential impact of synthetic genomics technology on the risks of a bioterrorist attack using viral pathogens. More succinctly: Does the ability to chemically synthesize and assemble a DNA copy of a pathogenic virus genome in the lab increase the risk of a bioterrorist attack using that pathogen? For the purposes of this

paper, it will be assumed that the synthetic technology is capable of preparing a DNA copy of any virus for which its nucleotide sequence is known.

While only "acquisition" of dangerous viruses will be considered here, it is important to maintain the present discussion in the context of the overall challenges of assessing the risks of a bioterrorism attack. The nature and sophistication of an attack can vary tremendously depending on the intent and capabilities of the would-be bioterrorist. If the desired impact of an attack is extensive human morbidity and mortality (a "high consequence event") such as with the widespread dissemination of smallpox virus several aspects of implementation, in addition to threat agent acquisition, must be executed successfully. These include the biological propagation and scale production of the threat agent, its packaging, storage and transport, and finally, its delivery or dissemination. There are numerous significant technical and logistical challenges at each step. In this case, the overall risk of an effective bioterrorism attack is the product of the probabilities of success of each component step. On the other hand, if the bioterrorist's objective is public fear or panic (societal or economic disruption), a simple isolated detection of an exotic pathogen may serve that end, and would require little technical or logistical expertise. For example, the detection of a broken blood sample tube in a New York City subway station containing infectious Marburg virus smuggled in from the recent outbreak in Angola, or a case of foot-and-mouth-disease in a Texas cattle feedlot caused by virus released from a vial stolen from a research lab in South America, would to the trick.

Synthetic genomics technologies could affect the availability of bioterrorism threat viruses, potentially providing a new or alternative means to obtain a pathogen. It might also facilitate the engineering of new or novel pathogens. This paper will review the viruses considered bioterrorism threat agents, their current sources and availability, and the potential impact of synthetic genomics on threat agent acquisition. The potential impact of synthetic technologies on the generation of new or novel viral pathogens will also be discussed briefly.

1. Viruses Considered Bioterrorism Threat Agents

The viral agents considered to pose severe threats to public health and safety are represented on various lists prepared by several government and international organizations. The three most widely-referenced lists are as follows:

1.1. The Centers for Disease Control and Prevention (CDC) established a list of high priority, exclusively human, "bioterrorism agents" ranked according to three categories.[4] Category A agents pose the most serious risk to national security because they can be easily disseminated or transmitted from person to person, are associated with high mortality rates, might cause public panic and social disruption, and require special action for public health preparedness. Category B agents include those that are relatively easy to disseminate; result in moderate morbidity rates and low mortality rates; and require specific diagnostic and surveillance enhancements. Finally, Category C agents comprise newly emerging pathogens, or pathogens that could be engineered for mass dissemination in the future because of their availability; ease of production and dissemination; and potential for high morbidity and mortality rates.

1.2. The National Institute of Allergy and Infectious Disease of the National Institutes of Health (NIAID, NIH) has developed a Category agents list of human pathogens quite similar, but not identical, to the CDC list.[5]

1.3. The Select Agents list, developed by CDC, the Department of Human and Health Services (HHS) and the United States Department of Agriculture (USDA), is a broader treatment and encompasses most Category A, B and C agents, additional human pathogens, and disease agents of livestock and plants.[6]

While there has been spirited debate on the constitution of the various pathogen lists, taken together, these lists provide the spectrum of virus pathogens that, if released in non-endemic areas or used in a bioterrorism attack, could cause a level of physical, economic and societal harm and disruption. Whether particular entries on the lists represent credible bioterrorism risks should be an area of continued review and assessment.

Table 1 is a compilation of the viruses from these lists, grouped alphabetically according to virus type (taxonomic family). Table 1 also indicates the government agency responsible for agent selection (HHS, CDC, NIAID, USDA), showing agency overlaps, non-overlaps, and for "Category" viruses, priority. The Table further provides the specific virus genome type, size, and infectivity.

There are three DNA virus families represented in Table 1, comprising the threat agents African swine fever virus (Asfarviridae), herpes B virus (Herpersviridae) and a number of poxviruses including smallpox virus (Poxviridae). These viruses have large double-stranded DNA genomes (150-205 kilobase pairs, kbp) and encode on the order of 100 gene products that are essential for virus replication, as well as another 100 "nonessential" gene products, many of which are involved in the modulation of host responses to virus infection and viral pathogenesis.

All remaining threat viruses, derived from 13 distinct virus families, are RNA viruses with genomes of positive (messenger) RNA sense (6 families), negative RNA sense (6 families) or double-stranded RNA (1 family). RNA virus genomes range in length from about 8 kb (Picornaviridae) to about 30 kb (Coronaviridae). Generally, most or all of the gene products encoded by RNA viruses are essential for virus replication. Some are also involved in the modulation of host responses to virus infection and viral pathogenesis.

2. Sources of Viral Threat Agents

All viruses listed in Table 1 were originally identified in and isolated from diseased humans, animals or, in one case, plants; or from animal or insect vectors of the respective pathogens. With the exception of smallpox (variola) virus, which was eradicated globally in 1977, and arguably the 1918 influenza virus, all viruses listed in Table 1 continue to circulate in nature. Therefore, nature represents a current and ongoing source of these viral pathogens.

All viruses listed in Table 1 also exist in numerous laboratories throughout the World, including academic research labs, diagnostic, hospital and nation state health labs, as well

as in biologics repositories (collectively, "laboratories"). For smallpox virus, the only known stocks remain in two high security laboratories. For all the other viruses, many research laboratories around the world have studied, and continue to study their structure, biology, molecular biology, genetics, immunology, pathogenesis and epidemiology.

Thus, nature and laboratories represent two current sources from which would-be bioterrorists could acquire viral threat agents. Since the complete genomic sequence for almost all viruses in Table 1 is known and publicly available, application of synthetic genomics represents a potential third source for any of these pathogens.

3. Does the Source of the Virus Matter?

Is the virus isolated from nature the same virus as the one found in laboratories, and would a synthetic replica of either be the same? Maybe, maybe not.

From a bioterrorism perspective, viruses isolated from nature are a sure bet. Their virulence and transmissibility are known. Their effect and impact can be predicted or calculated. Viruses isolated directly from diseased hosts (called "primary isolates") demonstrate clearly the consequences of their infection. Additionally, the virus's ability to survive, persist and spread in the environment and among susceptible hosts is generally known. Finally, pathogenic viruses isolated from diseased hosts are typically "hot" viruses; that is, primary isolates of pathogenic viruses tend to cause severe disease in their host.

When passaged in the laboratory (in either cell culture or lab animals), primary isolates often become attenuated. The attenuation is the result of adaptive genetic changes that the virus acquires in order to survive in its new environment. These genetic changes can be subtle (single nucleotide changes) or dramatic (genome deletions or rearrangements). Generally, the longer a virus is propagated in cell culture, or through non-natural animal hosts, the greater the attenuation. In fact, this is the basic methodology for the development of many live attenuated virus vaccines.

In order to retain as closely as possible the characteristics of the natural virus, many labs maintain low passage virus stocks. However, not all labs are so fastidious. Laboratory-adapted relatives of primary pathogenic virus isolates, while often well characterized for their *in vitro* attributes, may or may not have been characterized in a living host for infectivity, fitness, virulence and transmissibility, or compared to their primary isolate for these biological features. As a result, the degree of attenuation of laboratory-passaged viruses may or may not be known.

Most viral genome sequences deposited in databases are derived from laboratory-passaged viruses. While many sequences may be derived from low-passage viruses, and are therefore more likely to be close to their primary isolates, in some cases the passage history of the virus from which the sequence was derived is unclear, as are the biological attributes associated with that virus. Thus, there can in some cases be uncertainty regarding the biological attributes of a synthetic replica of a gene bank virus sequence.

That the biological attributes of a virus can be dramatically altered with very subtle genetic changes is exemplified by the first demonstration of viral synthetic genomics. For poliovirus, the introduction (for technical reasons) of several silent nucleotide changes into the virus genome resulted in a synthetic virus that was four orders of magnitude less virulent in mice than the natural virus.[1] Our understanding of the contribution of nucleotide sequence on genome structure, and in turn on biological attributes like virus replicative capacity, fitness, stability, and living host virulence and transmissibility are rudimentary at best.

The source of the virus may matter from a bioterrorism perspective.

4. From DNA Copy to Infectious Virus

There are descriptions of methodologies in the literature for the recovery of infectious virus from molecularly cloned DNA for member viruses from almost all the virus families listed in Table 1. These techniques are outlined generically below. Thus, in principle, it should be technically feasible to go from synthetic or recombinant DNA to infectious virus for any of the viral threat agents. However, in reality, for many of the

actual pathogens on the list, reverse genetics systems have not been demonstrated directly, and there may be unanticipated technical challenges for particular viruses. For example, a reverse genetics system has been demonstrated for Ebola virus, but has yet to be successful for the closely related Marburg virus. Therefore, while some of these methods are relatively straightforward, others require significant technical expertise and finesse.

Of the large dsDNA viruses, herpes B virus genomic DNA is itself infectious. African swine virus and poxvirus genomic DNAs are not infectious because of the requirement for activities of viral enzymes packaged within the virion. This requirement can be fulfilled for poxviruses, for example, by transfecting the viral genomic DNA into cells previously infected with another poxvirus. The resident "helper" virus provides the *trans*-acting systems needed to activate the transfected DNA and yield fully competent infectious virus.[7]

For the (+)ssRNA viruses, simply transfecting a DNA copy of the mRNA-sense genome into cells generally yields infectious virus.[8]

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For (–)ssRNA viruses, infectious virus can be recovered from cDNA designed with transcriptional promoters to yield full-length anti-genomic RNA upon transfection, either alone or together with plasmids encoding the expression of various viral proteins, into cells that provide the appropriate RNA polymerase. For segmented genomes, simultaneous transfection of multiple anti-genome plasmids is involved.[9, 10]

For the one dsRNA virus (Reoviridae), the system for the recovery of infectious virus directly from DNA has not been described. However, a reverse genetics system that involves the lipofection of cells with plus strand RNA transcripts or dsRNAs representing the 10 genomic segment of reovirus, together with a rabbit reticulocyte lysate in which ssRNA or melted dsRNA has been translated, can yield infectious virus after provision of a helper virus.[11]

5. Three Examples

Below, examples of three viral threat agents are considered to illustrate the opportunities for acquiring the pathogen from each of the sources mentioned above. The potential impact that synthetic genomics might have on the acquisition of each agent is then discussed. The three examples are:

1. Smallpox (variola) virus, a large double-stranded DNA virus,
2. Ebola and Marburg hemorrhagic fever filoviruses, negative-strand RNA viruses, and
3. Foot-and-mouth disease virus, a small positive-strand RNA virus.

5.1 Example 1 / Smallpox virus. Smallpox is caused by variola virus. Variola virus was declared eliminated from the world in 1979 by an aggressive global vaccination program. Once eradicated, immunization against smallpox ended, so individuals born after this time are immunologically naïve to the virus. Moreover, the level of immunity among persons who were vaccinated before eradication is uncertain but is likely low. Because of this high level of population susceptibility, smallpox (variola) virus is often considered the number one bioterrorism threat virus. Transmission of variola virus generally requires close contact with an infected individual. While this makes it possible to effectively interrupt chains of transmission by quarantine and restrictive movement methods, the average number of cases infected by a primary case is estimated at 3.5 to 6, indicating that an outbreak would produce a rapid rise in cases before control measures could be put in place. In addition to the significant morbidity associated with infection, death occurs in up to 30% of cases.

a) Agent Source and Availability (Variola Virus)

i) Nature. Humans were the only host for variola virus. Once eradicated by global immunization, smallpox virus ceased to exist in nature.

ii) Research laboratories or repositories. The only known stocks of variola virus have been retained in two World Health Organization

(WHO)-approved, high security laboratories: at the CDC in Atlanta and at the Russian State Center for Research on Virology and Biotechnology in Koltsovo, Novosibirsk Region, Russian Federation. However, some believe that secret caches of variola virus still remain undeclared or undetected and could be used by foreign governments or terrorist groups.[12]

iii) **Potential impact of synthetic genomics.** Since variola virus is not available from nature, and assuming there are no secret stores of the virus in covert laboratories, reconstruction of the dsDNA genome from the known variola virus nucleotide sequence may be the only path to the infectious agent available to bioterrorists. Consequently, while it would represent a considerable technical challenge for even a State-sponsored program, synthetic genomics technology could provide the means for the re-creation of variola virus, and therefore could affect the availability of this agent for malevolent use. Due to the large size of the poxvirus genome, however, it would be anticipated that well-established poxvirus recombination techniques would play a significant supportive or alternative role in producing an entire poxvirus genome. For example, smaller genome segments of a sequence derived from variola virus may be readily incorporated into a "base" monkeypox virus, resulting in chimeric orthopoxviruses with unknown and unpredictable biological characteristics.

5.2 Example 2 / Ebola and Marburg Hemorrhagic Fever Filoviruses. A number of distinct viral pathogens fall into the hemorrhagic fever virus group, including the arenaviruses (Lassa and South American hemorrhagic fever viruses), bunyaviruses (Rift Valley fever virus), flaviviruses (yellow fever virus) and filoviruses (Ebola and Marburg). All are "Category A" bioweapons-bioterrorism agents. All are negative-strand RNA viruses. All are endemic in various parts of the world. All cause severe disease, characterized by fever,

multiple organ involvement with extensive vascular damage and bleeding diathesis, which in many cases is fatal.

For this example, attention will be limited to perhaps the best recognized of the hemorrhagic fever viruses, the filoviruses Ebola and Marburg. These viruses cause sporadic and recurrent disease in central Africa with case fatality rates ranging from 25% to 90%. Filoviruses are readily transmitted and disseminated by aerosol, droplets, and contact of oral mucosa or conjunctivae with any body fluids of the diseased. The world population is susceptible to infection by these viruses, and there are presently no vaccines and no specific treatments for Ebola or Marburg.

a) Agent Source and Availability (Ebola and Marburg Viruses)

i) Nature. While both diseases remain relatively rare, outbreaks have become more common since the mid-1990s. The 2005 outbreaks of Ebola in the Republic of the Congo and Marburg in Angola are recent examples. However, in the absence of an outbreak, filoviruses are hidden. Their reservoir in nature remains unknown. Therefore, to obtain these viruses from nature requires that it be done during an outbreak of human disease. Blood and other body fluids of infected individuals are rich sources of virus.

ii) Research laboratories or repositories. Because of their virulence, filoviruses are handled in high containment laboratory facilities to prevent virus release into the environment, and also to protect those working with these highly pathogenic viruses. Consequently, the number of labs in possession of these viruses is limited, as is access to these labs. However, during outbreaks, unsecured local hospitals and medical field teams collect, hold and transport numerous infectious patient specimens. Additionally, there may be covert stores of virus outside known containment laboratories. Hemorrhagic fever viruses were the subject of biowarfare research in the former Soviet Union,

where weaponized Marburg virus was produced and research on Ebola was conducted. Upon the dissolution of the Soviet Union and these programs, the disposition of laboratory biological materials was not tracked.

iii) Potential impact of synthetic genomics. While Ebola and Marburg viruses may be readily obtained from diseased individuals, synthetic genomics technologies could provide an alternative source for these pathogens. Recovery of infectious virus from DNA has been demonstrated for Ebola virus, but not for Marburg virus.

5.3 Example 3 / Foot-and-Mouth Disease Virus. The potential for a terrorist attack against agricultural targets (agroterrorism) represents a daunting national security threat.[13-15] This reality is acknowledged by the inclusion of numerous livestock pathogens in the Select Agents list (Table 1). Deliberate introduction of an exotic animal or plant pathogen would elicit widespread public fear and would cause substantial economic loss and instability.

Foot-and-mouth-disease-virus (FMDV) is the most frequently mentioned disease agent of agroterrorism, and also the most likely terrorist threat. FMDV is extremely contagious and causes severe disease in cattle, swine, sheep, goats, and other cloven-hoofed animals. FMDV is not present in North America, and FMDV vaccination is not allowed. Consequently, the entire host animal population of North America is susceptible to infection and disease. The disease is "reportable" (i.e., subject to international quarantine) under rules of the Office International des Epizooties (OIE). So in addition to crippling national animal industries through lost production and mortality, an outbreak of FMD in the U.S. would suspend all exportation of meat and milk products until such time that disease (virus) eradication could be assured. For this example, the probability for catastrophic economic damage and social disruption is exceptionally high.

Unlike the previously discussed examples (smallpox and hemorrhagic fever viruses), where irrespective of how the pathogenic virus is sourced, there are considerable logistic challenges regarding the propagating and handling such hazardous human pathogens, none exist for FMDV. Humans are not susceptible to infection by FMDV; the virus is not a threat to human health or food safety.

a) Agent Source and Availability (Foot-and-Mouth Disease Virus)

i) Nature. FMDV is endemic in large regions of Asia, Africa, the Middle East and South America, and consequent readily available for animal sources. In a recent, and typical, 18-month period, the OIE recorded FMD outbreaks in 15 countries. Sporadic outbreaks also occasionally occur in "disease-free" areas (e.g., Japan, 2000; United Kingdom, 2001).

ii) Research laboratories or repositories. FMDV was first identified in 1898 and has been researched widely ever since. Suffice it to say, the number of academic and veterinary research facilities globally in possession of stocks of FMDV is quite large. The virus is readily available from these institutions. In endemic areas, there are typically no security measures employed in handling FMDV.

iii) Potential impact of synthetic genomics. FMDV is a small (+)ssRNA virus within the same virus family as poliovirus. The total synthesis and recovery of infectious virus is without technological challenge. However, based on the ready availability of the virus in nature and from innumerable research labs, it is unlikely synthetic genomic technology would have any impact on the availability of FMDV for use in a bioterrorism attack.

6. The Dark Side – Making "Super-pathogens"

While nature has provided would-be bioterrorists an ample supply and selection of quite virulent viruses (Table 1), there is concern that genetic technologies will be used to modify these already pathogenic agents and create "super-pathogens", viruses that are

more lethal and disruptive than naturally occurring pathogens, and that are designed to evade vaccines or to be resistant to drugs.

The potential of this dark side was brightly illuminated in 2001 when Australian workers inadvertently created an unusually virulent mousepox (ectromelia) virus.[16] While trying to improve their experimental mouse contraceptive vaccine, they engineered the expression of cytokine IL-4 from ectromelia virus, hoping that infection with this recombinant poxvirus would enhance antibody production by their vaccine. It instead resulted in severe suppression of cellular immune responses in the mice, uncontrolled virus replication, and animal death. Even mice previously immunized against normal ectromelia virus [16] or treated with the antiviral drug cidofovir[17] were unable to survive ectromelia-IL-4 virus challenge. Although humans are not susceptible to ectromelia virus, there is clear concern that smallpox could be similarly modified to make it more deadly.

Indeed, there may be a number of ways to augment a viral bioweapon. Virus infectivity, virulence or transmissibility might be enhanced by, for example:

- Increasing the replicative capacity of the virus by modifying the viral polymerase or gene expression by optimizing for human codon usage,
- Changing the tropism of the virus by incorporating genes encoding particular cellular receptor binding proteins,
- Engineering drug-resistance determinants into the virus (should there exist antiviral drugs for the virus), or
- Compromising or overwhelming the host immune response to infection or vaccine-induced immunity by incorporating into the virus genes encoding human immune system antagonists (as with mouse genes in mousepox as mentioned above).

Additionally, random approaches, such as DNA shuffling (accelerated or directed molecular evolution) or combining genetic elements of distinct pathogenic viruses to create chimeric viruses, could be applied to bioweapons enhancement.

However, while all of these "pathogen enhancements" are theoretically possible, they require significant technical sophistication, and, importantly, the outcomes are not predictable. After their creation, putative super-pathogens would require characterization

of their infectivity, fitness and stability, and verification of their virulence and transmissibility in a living host to establish or confirm their super pathogenic powers. This would likely require some degree of physical containment during the agent's development (construction, propagation and production) and for animal studies, so as to prevent harm to the creators, as well as to maintain the covert nature of the operation. Finally, animal test systems may not be predictive of human pathogenicity, particularly if the enhancing modifications are designed to be human-specific (e.g., human codon optimization, human receptor binding proteins, human immune response antagonists).

Thus, for the high-tech bioterrorist embarking on a virus bioweapons enhancement strategy, there is a considerable level of complexity and risk, as well as uncertainty of the outcome and impact of an attack with such modified agents.

6.1 Potential impact of synthetic genomics. Does the availability of synthetic genomics technology influence the likelihood of super-pathogen construction? Certain of the approaches to bioweapons enhancement could be facilitated by the technology; for example human codon optimization. However, numerous other approaches involving gene insertions (as with the mousepox example above), genetic shuffling, mutagenesis, and recombination require only standard recombinant DNA procedures using readily available genetic materials and reagents. Synthetic genomics technology will likely have little impact on increasing the risk of the creation, or use, of a novel viral pathogen in a bioterrorism attack.

7. Summary Comments

All new technologies and technological advances have the potential to be used in malevolent ways. So when posed with the question: "Does the ability to chemically synthesize and assemble a pathogenic virus genome in the lab increase the risk of a bioterrorist attack using that virus?" The answer is: "Probably." The question then

becomes: "How much is the risk increased?" Here, the answer is: "Probably not substantially."

To expand on this opinion, and to provide a platform of further discussion, several component questions may be considered.

1. **Does the increased availability of a pathogenic virus increase the risk of its use in a bioterrorism attack?**

   Probably. If the pathogen is readily available, its use in an attack can be considered by the bioterrorist. Conversely, if you cannot obtain the virus, you cannot use it.

2. **Can synthetic genomics technology increase the availability of virus pathogens?**

   Clearly for one pathogen (variola virus), yes; for others, perhaps to some degree (e.g., Ebola/Marburg viruses), and for most, not at all (e.g., FMDV). Nature and laboratories already provide logistically easier, technologically less demanding, and sufficiently rich sources of all agents listed in Table 1 (except variola virus).

3. **If it were assumed that synthetic genomics technology does increase the availability of a particular pathogen, does that portend its greater likelihood of being used in a bioterrorism attack?**

   Probably not. Mere acquisition (by whatever means) of a viable infectious viral pathogen does not necessarily increase the threat of its use in an attack. Estimating the risk that a pathogen (once obtained) might be used in a bioterrorism attack involves a number of factors. Among these are: (1) the intent or desired outcome of the bioterrorist, which may vary from local panic to widespread dissemination with mass casualties, and (2) the level of technical sophistication and skill of the would-be terrorist to carry out an attack, which may range from little or no basic microbiology ability to biocontainment and weaponization capabilities. In view of these two factors, the risk (likelihood) of a bioterrorism attack must to be considered in the context of the overall risk at the "point of delivery." For example, if the intent of an attack is broad pathogen dissemination with high human mortality, there are many

steps that must be executed successfully after the acquisition of the threat agent. These include the propagation and scale production of a hazardous human pathogen, its packaging, storage, transport, and finally, its delivery or dissemination. In such a case, the contribution to the risk of an attack attributable to the pathogen acquisition step is quite low. If, on the other hand, the desired outcome of an attack is more modest, such as local panic and disruption, acquiring the suitable threat agent becomes more pivotal. In this situation, it would seem that simplicity and opportunism would dictate methods. It would be surprising if a technologically challenging, sophisticated approach such as whole genome synthesis was employed to acquire a pathogen for this purpose, particularly given the other available sources of the pathogen.

4. Does synthetic genomics technology increase the likelihood of engineering a new or more virulent viral pathogen?

Not substantially. Other currently available technologies are sufficient to undertake such a pathogen enhancement effort. Moreover, creating a new human pathogen, or a more lethal variant of a known pathogen, is associated with significant technical and logistic challenges as mentioned earlier. The availability of synthetic genomics technology does not remove or lessen these challenges.

8. Closing Remarks

- Bioterrorism using viral agents can readily proceed in the absence of synthetic genomics technology. Synthetic genomics is not an enabling technology in this context.
- A bioterrorism organism need not be extremely virulent, or virulent at all to humans (e.g., agroterrorism agents). But, it must be deliverable in order to be effective for its intended purpose.
- A bioterrorism attack employing either "low technology" (for example, natural FMDV) or "high technology" (for example, synthetic variola virus) has the capacity to be high consequence event.

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Table 1. Compilation of Viral Threat Agents[1]

Virus Name	Virus Genus / Family	Select Agent[2]	CDC (NIAID) Category[3]	Genome Type[4]	Genome Size (kb)[5]	Genome Infectivity[6]
Junín	Arenavirus / Arenaviridae	HHS	A(A)	2(–) ssRNA	10.5	No
Machupo	Arenavirus / Arenaviridae	HHS	A(A)		10.6	
Sabiá	Arenavirus / Arenaviridae	HHS	A(A)		10.5	
Guanarito	Arenavirus / Arenaviridae	HHS	A(A)		10.4	
Lassa	Arenavirus / Arenaviridae	HHS	A(A)		10.7	
Lymphocytic choriomeningitis	Arenavirus / Arenaviridae		(A)		10.1	
Flexal	Arenavirus / Arenaviridae	HHS			NR	
African swine fever	Asfivirus / Asfarviridae	USDA		dsDNA	170.1	No
Rift Valley fever	Phlebovirus / Bunyaviridae	HHS/USDA	A(A)	3(–) ssRNA	12.2	No
Hanta	Hantavirus / Bunyaviridae		C(A)	3(–) ssRNA	12.3	
Crimean-Congo	Nairovirus / Bunyaviridae	HHS	A(C)	3(–) ssRNA	18.9	
Akabane	Orthobunyavirus/ Bunyaviridae	USDA		3(–) ssRNA	NR	
La Crosse	Orthobunyavirus/ Bunyaviridae		(C)		12.5	
Swine vesicular disease	Vesivirus / Caliciviridae	USDA		(+) ssRNA	8.3	Yes

Virus Name	**Virus Genus / Family**	**Select Agent[2]**	**CDC (NIAID) Category[3]**	**Genome Type[4]**	**Genome Size (kb)[5]**	**Genome Infectivity[6]**
Japanese encephalitis	Flavivirus / Flaviviridae	USDA	(B)	(+) ssRNA	11.0	Yes
West Nile	Flavivirus / Flaviviridae		(B)		11.0	
Dengue	Flavivirus / Flaviviridae		(A)		10.7	
Yellow fever	Flavivirus / Flaviviridae		(C)		10.9	
Tick-borne encephalitis complex	Flavivirus / Flaviviridae	HHS	B(C)			
Omsk hemorrhagic fever					10.8	
Central European TBE					NR	
Far Eastern TBE					NR	
Russian spring summer					NR	
Kyasamur Forest					NR	
Classic swine fever	Pestivirus / Flaviviridae	USDA		(+) ssRNA	12.3	Yes
Cerceopithecine herpes (B virus)	Simplexvirus / Herpesviridae	HHS		dsDNA	156.8	Yes
SARS	Coronavirus / Coronaviridae		(C)	(+) ssRNA	29.8	Yes
Ebola	Ebolavirus / Filoviridae	HHS	A(A)	(–) ssRNA	19.0	No
Marburg	Marburgvirus / Filoviridae	HHS	A(A)	(–) ssRNA	19.1	

Virus Name	**Virus Genus / Family**	**Select Agent[2]**	**CDC (NIAID) Category[3]**	**Genome Type[4]**	**Genome Size (kb)[5]**	**Genome Infectivity[6]**
Avian influenza (HPAI) Reconstructed 1918 influenza	Influenzavirus A / Orthomyxoviridae Influenzavirus A / Orthomyxoviridae	USDA HHS	(C)	8(–) ssRNA	13.5	No
Hendra Nipah	Henipavirus / Paramyxoviridae Henipavirus / Paramyxoviridae	HHS/USDA HHS/USDA	C(C) C(C)	(–) ssRNA	18.2 18.2	No
Newcastle disease Rinderpest	Avulavirus / Paramyxoviridae Avulavirus / Paramyxoviridae	USDA USDA		(–) ssRNA	15.2 15.9	
Peste Des Petits Ruminants Menangle	Rubilavirus / Paramyxoviridae Rubilavirus / Paramyxoviridae	USDA USDA		(–) ssRNA	16.0 NR	
Foot and mouth disease	Aphthovirus / Picornaviridae	USDA		(+) ssRNA	8.2	Yes
Variola major (Smallpox) Variola minor (Alastrim) Monkeypox Camel pox	Orthopoxvirus / Poxviridae Orthopoxvirus / Poxviridae Orthopoxvirus / Poxviridae Orthopoxvirus / Poxviridae	HHS HHS HHS USDA	A(A)	dsDNA	185.6 NR 196.9 205.7	No
Lumpy skin disease Goat pox Sheep pox	Capripoxvirus / Poxviridae Capripoxvirus / Poxviridae Capripoxvirus / Poxviridae	USDA USDA USDA		dsDNA	150.8 149.6 150.0	
African horse sickness Bluetongue / catarrhal fever	Orbivirus / Reoviridae Orbivirus / Reoviridae	USDA USDA		10 dsRNA	19.5 19.2	No
Rabies	Lyssavirus / Rhabdoviridae		(C)	(–) ssRNA	11.9	No
Vesicular stomatitis	Vesiculovirus / Rhabdoviridae	USDA		(–) ssRNA	11.2	
Eastern equine encephalitis Western equine encephalitis Venezuelan equine encephalitis Chikungunya	Alphavirus / Togaviridae Alphavirus / Togaviridae Alphavirus / Togaviridae Alphavirus / Togaviridae	HHS/USDA HHS/USDA 	B(B) B(B) B(B) (C)	(+) ssRNA	11.7 11.5 11.4 11.8	Yes

[1]According to documents accessed October 2007; http://www.cdc.gov/od/sap/docs/salist.pdf; http://www.bt.cdc.gov/agent/agentlist-category.asp; http://www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/PDF/Cat.pdf .
[2] Indicated is the agency responsible for entry on Select Agent List (HHS, USDA)
[3] Indicated are those agents listed on the CDC or NIAID (in parenthesis) Category A, B, and C lists
[4] All genomes are non-segmented (single molecules) except for those proceeded with a number, which indicates the number of genome segments. This is followed in parenthesis by the genome polarity (–, negative; +, positive), genome type (ss, single strand, ds, double strand; RNA or DNA).
[5] kb-kilobases, NR-not reported
[6] Infectivity of purified genomic nucleic acid

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Risk Assessment of Synthetic Genomics: A Biosafety and Biosecurity Perspective

Diane O. Fleming

Introduction

The ability to synthesize molecules found in living organisms is not new for scientists in the fields of biochemistry and molecular biology. However, the "synthetic biology" made possible by the genetic mapping of microorganisms, plants and animals, including the human genome, has taken this area of science into new and relatively uncharted territory. The focus here will be on "synthetic genomics" in which genetic information is synthesized using chemical components and the genomic DNA sequence of an organism. This is how investigators at the State University of New York in Stony Brook, using a published genetic sequence, synthesized a DNA version of poliovirus in 2002. Using an enzyme, reverse transcriptase, they converted the DNA to RNA and were able to grow the virus in a cell-free extract. Their synthesized poliovirus caused paralysis in animals (Cello et al., 2002). One of the authors, Eckard Wimmer, warned: "*The world had better be prepared. This shows you can re-create a virus from written information*."

From a biosafety and biosecurity perspective the synthesis of etiologic agents is of concern because of the potential to create completely new combinations or chimeric genomes with enhanced virulence, extended host range, and resistance to antimicrobials, antivirals or vaccines. A major concern is that an agent which has been eradicated as a source of infectious disease, such as smallpox, and one which is in the process of being eradicated, such as poliovirus, will never be truly eliminated because the information for their synthesis is readily available in sequence databases.

The potential benefits of synthetic genomics include but are not limited to new sources of fuel, food, therapeutics and environmental remediation. Plans to utilize synthetic

genomics to obtain specific products or outcomes are well underway. A bacteriophage genome was synthesized by the Institute of Biological Energy Alternatives (Smith et al, 2003). They now plan to synthesize larger microorganisms, including a mycoplasma with a "minimal genome" (Hutchinson et al, 1999). Such an approach could be useful in beginning to address alternative biologically-based fuel sources, and other applications. Additionally, through rearranging genes, called "gene shuffling", and repeatedly selecting for specific traits, an organism can be made to make more of a desired product that it already produces. As explained by Maxygen: "The parents are a series of related genes. These are cut into pieces, shuffled together and then assembled to form a new genetic generation. Some of these daughter genes can manufacture proteins that are much better at certain tasks than nature's originals. The best ones can be screened out and shuffled to produce whole lineages of superior descendants, in a process mimicking evolution by natural selection" (Crameri et al., 1998).

The risks associated with the synthesis of these genomes could have biological, chemical and physical components. Unknown and thus unquantifiable risks associated with new organisms or products from this technology could include various levels of harm to humans, animals, plants, other microorganisms and the environment in the event of an unplanned release. This is not unlike the risks perceived to lurk in recombinant DNA research in the early '70s or the risks potentially associated with the return of the first lunar astronauts and the recent samples from Mars. For example, in an attempt to protect against the introduction of unknown organisms or materials from space, the National Aeronautics and Space Administration (NASA) developed the Lunar Receiving Laboratory where astronauts were to be quarantined. Built from plans developed at Fort Detrick in Maryland, it included ethylene oxide chambers for sterilization. Such situations, when approached with a rational, scientific risk assessment of the known and unknown factors, can result in appropriate recommendations for biosafety as well as biosecurity. The guidelines and regulations currently in use in these areas can and do encompass synthetic genomics.

It may be useful to start with definitions for relevant terms to assist in understanding precisely the scope of possible safety concerns that could be triggered by a widespread introduction of synthetic genomics technologies (or by any new technology):

Laboratory biosafety "is used to describe the containment principles, technologies and practices that are implemented to prevent unintentional exposure to pathogens and toxins, or their accidental release" (Chapter 9, page 47, WHO, 2004). Biosafety for larger scale and industrial work also focuses on providing a safe environment for work with biohazardous agents and materials (Cipriano, 2000:2002; NIH, 2002, Appendix K).

The definition given in Biosafety in Microbiological and Biomedical Laboratories is similar: **Biosafety:** Development and implementation of administrative policies, work practices, facility design, and safety equipment to prevent transmission of biologic agents to workers, other persons, and the environment (CDC/NIH, 1999). (It should be noted here that the term "biosafety" may not be appropriate for use in protection of the integrity of the "species", as in the 2003 Cartagena protocol of the Conference of the Parties to the Convention on Biological Diversity. "Species integrity" "species purity" or even "species safety" would better define the actual use of the term and prevent the confusion which currently exists.)

Laboratory biosecurity "refers to institutional and personal security measures designed to prevent the loss, theft, misuse, diversion or intentional release of pathogens or toxins. Effective biosafety practices are the very foundation of laboratory biosecurity activities" (Chapter 9, page 47, WHO, 2004.)

The definition given in BMBL, although similar, is focused on select agents: **Biosecurity:** Protection of high-consequence microbial agents and toxins, or critical relevant information, against theft or diversion by those who intend to pursue intentional misuse. (See also Appendix 1, this document.)

1. Biosafety in research laboratories in the US

An overview of control of biohazards shows that the information for personal and community protection has been available for over forty years, but it has not necessarily been used for training at sites of use nor has it been incorporated into many training curricula.

1.1 EARLY HISTORY OF LABORATORY BIOSAFETY IN THE USA. In classified work done during the 1940s to 1969 at Fort Detrick, MD, and other U.S. Army Chemical Corps installations, there was an urgent need to focus on protection of the worker as well as the community from agents of biological warfare.[2] The work was so secret that the advances in protection were not well known outside the field of experts who worked there and who met in closed conferences. Information was shared in the first Biological Safety Conference held at Ft. Detrick in 1955. By 1966, the conference had grown to include universities, private laboratories, hospitals, industrial complexes and 17 government installations with presentations no longer bound by security. At that 11th conference, Dr. Arnold G. Wedum, now known as the "Father of Biosafety" discussed the revised edition of *Assessment of Risk of Human Infection in the Microbiological Laboratory* in which he provided four indicators of risk to serve as guidelines for the safe handling of microorganisms:

- number of laboratory infections observed
- infectious human dose
- infection of uninoculated control animals caged with or near inoculated cagemates
- presence of microorganisms in urine and/or feces of inoculated animal.

[2] Much of the early history of Ft. Detrick can be found at http://www.detrick.army.mil/cutting_edge/index.cfm?chapter=titlepage. The historical content of the early meetings and resultant papers published has been compiled by Manny Barbeito and Dick Kruse and published in the Journal of the American Biological Safety Association, now called "Applied Biosafety." This history of these conferences is also available on the ABSA website www.ABSA.org.

The published edition included 530 references and assumptions for 130 organisms or diseases. Charles Baldwin of Dow Chemical described the inception of the red-on-yellow biohazard symbol, which was first displayed at this conference and which a professional opinion group selected as the symbol they deemed unique, with easily recognized distinctive colors (Barbeito and Kruse, 1997).[3]

After President Nixon ended the biological warfare research program in 1969 and with the advent of the recombinant DNA guidelines in 1976 and the related NIH-sponsored training of biological safety officers in the early 1980s, these pioneers welcomed many newcomers to the field of biosafety. The 49th Biological Safety Conference is scheduled to be held in Boston in October of this year (2006). The American Biological Safety Association was formed in 1984.

1.2 CLASSIFICATION OF ETIOLOGIC AGENTS ON THE BASIS OF HAZARD: THE FIRST WRITTEN GUIDELINES. In 1969, the Public Health Service and the USDA, as part of their "regulatory responsibility for quarantine and interstate shipment of etiologic agents", worked together to produce the first edition of the "Classification of Etiologic Agents on the Basis of Hazard", the first documented guidelines for work with infectious agents. They noted in their introduction: "This document provides a standard for evaluating the hazards associated with various etiological agents and defines minimal safety conditions for their management without restricting or hampering bona fide microbiological investigations." This small booklet included lists of agents in four categories of increasing risk to the healthy adult worker and gave the basis for the agent classifications, as well as descriptions of the level of competency and the containment requirements. (See Appendix 2, this document.) A fifth category, known as Class 5, consisted of animal agents excluded from the USA by law (Foot-and-Mouth-Disease virus) and a list of agents excluded by USDA administrative policy

[3] For a firsthand account of the development of the symbol, and a graphic of it, see http://www.hms.harvard.edu/orsp/coms/BiosafetyResources/History-of-Biohazard-Symbol.htm

It should be noted that the number of academic departments of microbiology is shrinking every year, and very little if any hands-on training in good microbiological practices, including aseptic techniques, occurs outside these departments; sometimes, it does not even occur within these departments. In newer documents physical containment, given in terms of four Biosafety Levels, has become the focus of the recommendations and is given in agent summary statements in the guidelines from the Department of Health and Human Services (CDC/NIH, 1999).

The 4th edition of Classification of Etiologic Agents on the Basis of Hazard, published in 1974 and reprinted through 1976, continued to offer very concise guidelines for the general safety in handling of microorganisms: "The best way to maintain laboratory safety is to practice correct and careful laboratory techniques, including effective decontamination and sterilization procedures, at all times. The laboratory's isolation and containment requirements are to supplement, not to supplant, good laboratory practices and sound scientific judgment. However, in an adequately isolated and properly equipped laboratory with correctly directed airflow, a scientifically and technically competent investigator can confidently work even with the most hazardous agents, provided the safety cabinets are selected to meet the requirements of the work. Of the several available cabinet types, the investigator should select the one which meets requirements for the maximum risk he expects to encounter." The CDC's Office of Biosafety (now called Health and Safety) was available for consultation on the handling of etiologic agents.

The CDC list of the four classes of human pathogens and the USDA restricted agents was widely disseminated and continued to be used, for example, in an Appendix of the NIH guidelines, well into the 90's, long after it had been replaced in 1984 by the first edition of the CDC/NIH "Biosafety in Microbiological and Biomedical Laboratories." The World Health Organization, the European Union, Canada, Australia and New Zealand built on the older

agent classification model to provide definitions for four Risk Groups (RG) of agents. (Appendix 3, this document)

1.3 BIOSAFETY IN MICROBIOLOGICAL AND BIOMEDICAL LABORATORIES (BMBL). The information given in the BMBL included a new format of agent summary statements to assist in the selection of the appropriate containment for diagnostic clinical work, research and animal studies. More detailed recommendations for personal practices, safety equipment and facility design were given for each of the four biosafety levels of containment along with a separate set of four animal biosafety levels, due to the unique hazards associated with work in animals. The BMBL did not retain the list of etiologic agents based on hazard assessment, due to an unfavorable response from microbiologists who were concerned about costs and restrictions. The new format put the responsibility for risk assessment on the principal investigator or laboratory director and provided a limited number of agent summary statements for pathogens which have caused laboratory acquired infections (LAI) or could be of significant risk to the laboratory worker. Every known microorganism, and especially new or re-emerging pathogens, could not be addressed in BMBL. The mechanism for publication of timely information not covered by the current edition is to publish on the CDC website and in Morbidity and Mortality Weekly Report (MMWR) as well as professional journals. The current 4th edition is available online at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm Revised every 5 years, the 5th edition of BMBL is expected in 2006. The BMBL guidelines are considered the state of the art for the handling of infectious and toxic etiological agents of human disease in the USA. Section V on Risk Assessment can be found in Appendix 4 of this document.

1.4 NIH GUIDELINES FOR RESEARCH WITH RECOMBINANT DNA MOLECULES. With the advent of recombinant DNA research, and the Asilomar conference of potentially self-governing researchers in the mid 70s, guidelines were written for the biological and physical containment of such work. The first recombinant

DNA guidelines were published in 1976, and a series of technical advisory bulletins followed. The Office of the Director, NIH, whose signature was required for approval of potentially problematic experiments, also appointed a committee of experts, known as the Recombinant DNA Advisory Committee (RAC), to review and recommend revision, rejection or containment precautions for the work.

Institutional Biosafety Committees, formed to provide local oversight and approval, were provided with some training by the NIH for IBC representatives. An NIH sponsored train-the-trainer course was prepared by Dr. Donald Vesley with outlines and slides made available though the National Audiovisual Center as "Introduction to Biohazard Control". This material was to be used by local biological safety officers or other experienced professionals for training research workers (Appendix F of NRC, 1989). The World Health Organization Special Programme on Safety Measures in Microbiology sponsored the first WHO Global biosafety train-the-trainer course "Laboratory Biosafety Principles and Practices: An Instructor's Guide for Biosafety Training" in 1983 which included much of this material. There has been a recent outreach from NIH's Office of Biotechnology Activities (OBA) to provide updated training for IBC members throughout the country. The slides are available on the OBA website.[4] The current focus of the training is on the prescriptive requirements for compliance with the NIH guidelines, and not on the procedure-related training of those who do the hands-on work.

Over time, with an apparent lack of true hazards associated with the process of recombinant DNA, the guidelines were revised and relaxed. Most approvals were done locally by IBCs, although certain experiments were still to be approved by the RAC and NIH Director. Laboratory infections that have been reported were not related to the recombinant procedures and could have been prevented by using the biosafety guidelines and practices recommended for work with the infectious agent involved. For example, a vaccinia eye infection

[4] http://www4.od.nih.gov/oba/IBC/IBCindexpg.htm

and a skin infection occurred in workers who refused the recommended vaccine. Eye protection in one case and gloves in the second could have provided an appropriate barrier (Lewis et al., 2006; Mempel et al., 2003). Risk assessment from Section II of the NIH guidelines and the requirements for research scale (Appendix G) and large scale (Appendix K) work are provided in Appendix 5 of this document.

That the NIH guidelines can apply to synthetic genomics is seen from *Section I-B. Definition of Recombinant DNA Molecules.* In the context of the *NIH Guidelines*, recombinant DNA molecules are defined as either: (i) molecules that are constructed outside living cells by joining natural or synthetic DNA segments to DNA molecules that can replicate in a living cell, or (ii) molecules that result from the replication of those described in (i) above. Synthetic DNA segments which are likely to yield a potentially harmful polynucleotide or polypeptide (e.g., a toxin or a pharmacologically active agent) are considered as equivalent to their natural DNA counterpart. If the synthetic DNA segment is not expressed *in vivo* as a biologically active polynucleotide or polypeptide product, it is exempt from the *NIH Guidelines*. Genomic DNA of plants and bacteria that have acquired a transposable element, even if the latter was donated from a recombinant vector no longer present, are not subject to the *NIH Guidelines* unless the transposon itself contains recombinant DNA.[5]

Although the scope of the NIH guidelines clearly includes the biosafety of synthetic genomic work at laboratory and at large scale, the biosecurity issue has not been addressed in these guidelines. The NRC report *Biotechnology Research in an Age of Bioterrorism* (the Fink Committee report) begins to address some of these issues through its Recommendation #2 (establishment of a review system for "experiments of concern."). If the RAC or a similar body is to review experiments of concern in synthetic genomics, they and the local IBCs

[5] (http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html)

can utilize the information provided by the WHO and in the BMBL. Training programs available on the web can be modified for specific local use.

1.5 BIOSECURITY. Following the anthrax dissemination and deaths in the USA in 2001, regulations were promulgated to restrict the use of certain select agents. The CDC added a Select Agent Program with biosecurity as a major component. The Public Health Security and Bioterrorism Preparedness and Response Act of 2002 (the Act) required institutions to notify the US Department of Health and Human Services (DHHS) or the US Department of Agriculture (USDA) of the possession of specific pathogens or toxins (i.e., select agents), as defined by DHHS, or certain animal and plant pathogens or toxins (i.e., high-consequence pathogens), as defined by USDA. Details can be found in the regulations:

- CDC and OIG, 2005; http://www.cdc.gov/od/sap/;
- APHIS,USDA,2005; http://www.aphis.usda.gov/programs/ag_selectagent/index.html
- Appendix F of BMBL, the 5th edition of which is to include further guidance on biosecurity policies and procedures, such as:

  - risk and threat assessment;
  - facility security plans;
  - physical security;
  - data and electronic technology systems;
  - security policies for personnel;
  - policies regarding accessing the laboratory and animal areas;
  - specimen accountability;
  - receipt of agents into the laboratory;
  - transfer or shipping of select agents from the laboratory;
  - emergency response plans; and
  - reporting of incidents, unintentional injuries, and security breaches

The National Science Advisory Board on Biosecurity (NSABB) is expected to offer more insight on requirements in this area: "The NSABB has been established to provide advice to federal departments and agencies on ways to minimize the possibility that knowledge and technologies emanating from vitally important biological research will be misused to threaten public health or national security. The NSABB is a critical component of a set of federal initiatives to promote biosecurity in life science research.

The NSABB is charged specifically with guiding the development of:

- A system of institutional and federal research review that allows for fulfillment of important research objectives while addressing national security concerns;
- Guidelines for the identification and conduct of research that may require special attention and security surveillance;
- Professional codes of conduct for scientists and laboratory workers that can be adopted by professional organizations and institutions engaged in life science research; and
- Materials and resources to educate the research community about effective biosecurity.

The NSABB is chartered to have up to 25 voting members with a broad range of expertise in molecular biology, microbiology, infectious diseases, biosafety, public health, veterinary medicine, plant health, national security, biodefense, law enforcement, scientific publishing, and related field. The NSABB also includes nonvoting ex officio members from 15 federal agencies and departments." (http://www.biosecurityboard.gov/)

A sample biosecurity training program developed by the Veteran's Administration can be found at
http://www1.va.gov/resdev/programs/biosafety/default.cfm#

1.6 OSHA'S BLOODBORNE PATHOGEN STANDARD. Finalized in 1992, this was the first regulation in which the Occupational Safety and Health Administration became involved with etiologic agents and infectious diseases to ensure a safe workplace for those potentially exposed to human blood and certain body materials as well as the viruses of hepatitis B and AIDS. The regulation was the result of petitions by the Service Employees International Union, a union of many frontline healthcare workers. The regulation has had a major impact on clinical laboratories and hospitals as well as on research with the agents covered. Concern about unsafe equipment led to further regulations requiring review of needle-stick and sharps injuries and selection of appropriate safety devices (OHSA, 2001). OSHA also monitors other infectious agents and materials under the general duty clause, the requirement for employers to provide a workplace free of recognized hazards. The OSHA website now contains fact sheets about infectious agents of concern in the workplace.

1.7 USDA'S SELECT AGENTS AND RESTRICTED (CLASS 5) AGENTS. 5) USDA'S SELECT AGENTS AND RESTRICTED (CLASS 5) AGENTS. The USDA has a list of agents that require permits for use some of which are handled under biosafety level 3 (BSL-3) or ABSL 3 (for animal containment) and even BSL 3 enhanced (BSL-3 with additional modifications, but clearly not a BSL-4 facility). Some specific containment conditions, BSL-3-Ag are applied when large animals cannot be caged and the room becomes the containment barrier. Use of such animals with eleven infectious agents requires BSL-3 Ag (Heckert and Kozlovac, 2006). (See below for detailed description of various containment facilities, and see Appendix 6, this document.)

1.8 OVERVIEW OF PREVIOUS FAILURES OF BIOLOGICAL CONTAINMENT. Workers assume the most risk in research enterprises including pathogenic agents. Most of the laboratory-acquired infections reported involved the person working with the agent, many of whom did not recall an accident (Harding and Byers, 2000).

A few accidents did involve some spread to co-workers (see Appendices 7 and 8, this document). Animals naturally infected with *Coxiella burnetii* in a San Francisco research facility were the source of infections in children and nurses who visited an area which should have been off-limits. According to the CDC web site: "Q fever outbreaks have resulted mainly from occupational exposure involving veterinarians, meat processing plant workers, sheep and dairy workers, livestock farmers, and researchers at facilities housing sheep. Prevention and control efforts should be directed primarily toward these groups and environments."

Community and other external populations are rarely involved. One exception was the release of anthrax in a 1979 industrial accident in Sverdlovsk, Russia. Finding the actual cause took many years of investigation (Miselson et al, 1994) and it was determined finally as the failure by maintenance personnel to replace a critical filter in a vent serving the anthrax production facility. The accidental smallpox release in a laboratory in Birmingham, England, resulted in two deaths but did not cause a community epidemic.

Release into community or environment has not been reported from US labs at Ft. Detrick, CDC or USDA (Cutting Edge. The History of Ft. Detrick, 4th ed. Oct, 2000 http://www.detrick.army.mil/cutting_edge/index.cfm?chapter=titlepage). There are sporadic cases of internal laboratory releases which infect workers and less frequently co-workers (see LAI, Collins, 1983, Harding and Byers, 2000, 2006 and Appendix 7, this document).

2. Concept of risk assessment

"*If reasonable precautions are to be taken against laboratory-acquired infections it is necessary to assess realistically the hazards that might be imposed on the laboratory worker and on the community during and as a result of work with any particular micro-organisms. It is a waste of time and resources to take elaborate precautions*

when the risks are negligible but foolish to take none if they are considerable. The precautions should be appropriate to the organism being investigated and the techniques used." (Collins, 1983, pg 53)

2.1 *Comprehensive risk assessment involves evaluating the agent-host-activity triad*

- Agent factors: Information on synthetic genomic constructs must come from the genetic sequence to be used and/or the sources of the genetic material to be combined. There is information on "wild type" or "type strains" of the pathogens in resources such as Bergey's Manual; ATCC catalogue; medical microbiology texts, BMBL agent summary statements, etc.)
- "Host factors" of lack of training in microbiology, recombinant DNA techniques, and specific techniques for synthetic genomics, and lack of competency in these techniques need to be addressed. This failure to train will not be resolved by promulgating regulations restricting the use of synthetic genomic processes. There are also other factors such as impaired immunity to be addressed.

2.2 Concept of chain of infection, which if broken reduces the risk and prevents disease

- Agent must be able to cause illness (pathogenic virus, toxigenic bacteria, etc.). For example, if the agent is inactivated or attenuated to a lesser degree of virulence it will not usually cause disease, even if it can still infect, but verification is needed.
- Reservoir: Agent must have a place to survive or replicate (intermediate host or reservoir). Example: Drain standing pools of water to prevent the breeding of an insect vector; kill the snail intermediate host of a parasitic disease.
- Exit point: Agent must be able to exit from the reservoir or host. Example: use algacide in cooling towers to kill algae and thus prevent amplification

and aerosol release of *Legionella;* send high temperature or flush with bleach to remove from water supply in hospital, thus preventing release from reservoir.

- Means of spread: mode of transmission. Direct (ingestion via mouth pipetting, injection with a contaminated needle or inoculation via animal bite) or indirect contact with agent such as contaminants on surfaces and particles in aerosols.
- Washing hands to remove transient contaminants and prevents many infections.
- Entry site: Agent must have a way to enter the host (route of entry): percutaneous (injection), ingestion, inhalation or contact with mucous membranes
- Susceptible host: the unimmunized or the immunologically impaired by disease or extremes of age (very young and very old). Note: immunization can be overwhelmed by a large dose of infectious agent; it changes the level of susceptibility.

The spread of infectious diseases at work can be stopped by breaking the chain:

- Killing the agent or replacing it with a non-pathogen
- Changing the environment so that the agent cannot survive
- Removing the agent's means of spread (mode of transmission)
- Making sure workers are immune to the agent and/or have protective equipment.
- Properly training workers on work practices to prevent illness.

2.3 Risk groups (WHO, NIH, EU, Canada, Australia, New Zealand)

The Risk Groups (RG) are based on:

- Severity of disease
- Individual and community risk (low to high)
- Host range (restricted or broad)

- Availability of treatment or prophylaxis (antibiotics, vaccines, etc)
- Endemicity (already present in the environment?)

2.4 Biosafety levels (CDC/NIH, 1999) used for risk management are based on

- The agent factors (see agent summary statements)
- The work to be done (clinical, research, large scale)
- The worker (host) factors (training, health, immunity)

Risk Assessment resources include:

- Chapter 5, BMBL: http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s5.htm
- Section II and appendix B, NIH guidelines http://www4.od.nih.gov/oba/rac/guidelines_02/NIH_Guidelines_Apr_02.htm#_Toc72615z http://www4.od.nih.gov/oba/rac/guidelines_02/APPENDIX_B.htm (See Appendix 4, this document).

Also see Appendix 8, this document, for a risk assessment matrix for agent hazards, and Appendix 9 for protocol hazards (Appendix 10 documents concentration and particle size of aerosols created during laboratory techniques). Finally, Appendix 11 is a risk assessment matrix for susceptibility to disease.

3. Selection of appropriate level of containment

(See NIH guidelines, Section II and CDC/NIH guidelines, BMBL, 4th ed)

It is the responsibility of the principal investigator or laboratory director to select the appropriate containment based upon their risk assessment. The institutional biosafety officer can provide assistance. Final local approval would come from the IBC or local biosafety committee, including the IRB (human subjects) and the IACUC (animal work) if appropriate. Higher level approvals from the RAC under NIH/OBA (or perhaps, at some point, the new NSABB) may be required. Select or restricted agents

are regulated by CDC or USDA, in which case that agency sets compliance and containment requirements. A Responsible Official (RO) and Assistant Responsible Official (ARO) represent the facility to ensure compliance with containment requirements.

3.1 Technologies for containment (See BSLs and ABSLs in BMBL and NIH guidelines Appendices G and K)

3.1.1 Administrative controls include standard operating procedures for:

- Housekeeping, spill clean up, decontamination, disinfection, sterilization and waste handling, packaging and disposal
- Hand washing, personal protective equipment, cleaning of uniforms etc.
- Reporting incidents, illnesses, accidents and injuries
- Medical program; vaccinations
- Training requirements and documentation
- Effective and safe use of equipment (biological safety cabinets, centrifuge, autoclave); equipment certification and validation requirements and records
- Limiting the number of workers exposed (access control)
- Monitoring and auditing checklists and procedures
- Avoiding exposure to infectious agents using the following practices at all times:
    - Do not mouth pipette
    - Manipulate infectious fluids carefully to avoid spills and the production of aerosols and droplets
    - Restrict the use of needles and syringes to those procedures for which there are no alternatives
    - Use needles, syringes and other "sharps" carefully to avoid self-inoculation
    - Dispose of "sharps" in leak and puncture resistant container

- Use protective laboratory coats and gloves
- Wash hands following all laboratory activities, following the removal of gloves, and immediately following contact with infectious materials
- Decontaminate work surfaces before and after use, and immediately after spills
- Do not eat, drink, store foods or smoke in the laboratory (NRC, 1989)

3.1.2 Engineering Controls

- General ventilation: maintaining a building at the proper temperature and humidity
- Local ventilation: such as isolation rooms, laboratory hoods, biological safety cabinets, and other means to control infectious agents
- Using safe needles and sharp devices designed to reduce the risk of needle sticks or other skin punctures and using puncture-proof sharps disposal containers
- Autoclaving or other sterilization methods

3.1.3 Primary barriers (personal protective equipment, safety cabinets,, other safety equipment, etc)

Personal protective equipment (PPE) includes:

- Respirators
- Gloves
- Face shields and eye protection
- Gowns, scrubs, head covers, booties, boots and other protective clothing

3.1.4 Secondary barriers: the physical facility

- If the facility is inappropriate for the proposed work, either the facility, the work or the method proposed for doing the work should be modified (NRC, 1989). BSL 1 and 2 do not require containment facilities
- BSL 3 and 4 are containment and high/maximum containment facilities respectively with specific features as described in BMBL
- BSL 3 Ag requires a containment facility that holds pressure and is used for loose, usually large animals (cattle, ostriches, etc) for work with 11 agents of concern (Heckert and Kozlovac, 2006)

4. Oversight mechanisms (See also Section 3)

A number of actors and agencies are responsible for ensuring that appropriate rules and guidelines are followed. These include, but are not necessarily limited to, the following:

Institutional: the principal investigator, the institutional biosafety officer, the institute's biosafety committee, IACUC auditing, certifications and commissionings.

Agency: CDC (import and select agents), EPA (TSCA and FIFRA rules), FDA (drugs, vaccines, and devices), USDA (APHIS, import and interstate movement, and select agents), DOT (transportation), Department of Commerce (export rules).

These and other cognizant bodies are reviewed at http://www.absa.org/resrules.html.

5. International considerations of interest

Because research is an international endeavor, it is critical to also understand rules and guidelines as asserted in other countries, not just in the United States, and not only by overarching bodies such as the World Health Organization. It seems very unlikely that there could ever be true harmonization of biosafety regulations, both for scientific reasons (the endemicity of microbes varies from locale to locale) and for cultural reasons. However, it will be worth considering if there could be any useful

global guidelines for biosafety with respect to synthetic genomics particularly (see Section VII).

See resource list for the WHO manual, regulations in Canada, EU, AU/NZ and some stringent country regulations, e.g., by competent authorities in UK. See also the commissioned paper on the oversight of biosafety in other countries by Franco Furger.

6. Special considerations for synthetic genomics

6.1 Any new safety issues?

It is difficult to foresee problems over and above what was expected with the early recombinant DNA experiments. As in the past, we will not know until they occur. However, it is worth reconsidering generally the types of problems or failures that have or could occur; in some cases these are directly relevant to synthetic genomics.

The problems could include:

- *Problem of unforeseen results*, particularly if the result is an unexpected increase in pathogenicity or virulence. A recent example of mice immunized against mousepox or naturally resistant mice that were nonetheless susceptible to a mousepox virus that had been modified by the addition of the interleukin IL-4. The purpose of the experiment was to create a mouse contraceptive; the outcome was clearly not what was expected. While the problem of unforeseen results is not unique to synthetic genomics, the combining of multiple sources of DNA sequence (not just, say, a bacterial vector and a specific gene as is exemplified by standard recombinant DNA techniques), particularly when this can occur very rapidly, may be of some concern.

- Broader host range than wild type. Synthetic genomics techniques might make quite simple, for example, the humanizing of zoonotic pathogens.
- Sheer volume of work that can be done. Using chip-based technologies, thousands or tens of thousand of "experiments" can potentially be done at one time. Although most of the synthetic genomics work occurring now is still at the "art" or "craft" level, it might be worth anticipating biosafety concerns now.

6.2 Lack of training in microbiology and/or recombinant DNA is problematic

Whether or not synthetic genomics is unique as a biotechnological tool, it is within the realm of technologies that rely heavily on good basic microbiological techniques. Although, as discussed above, the teaching of good microbiological techniques has faded somewhat over time in all departments, there is at least some tacit knowledge that is passed on and a good bit of structured training that still does take place in most biologically-oriented departments. Where synthetic genomics (and synthetic biology more generally) might be unique is the possibility that if the field does expand rapidly, as many are predicting, there could be an infusion of workers to the field who have literally no background in biology, let alone in microbiology. These could be people coming from engineering or physics background, and may never have stepped into a biology lab before they go about conducting their first experiment. If this is the case, the concern would then be about the general sorts of failures that can occur as a result of the use of poor technique:

- Failure to use aseptic technique and good microbiological practices can contaminate work or infect workers (SARS infections).
- Failure to understand routes of disease transmission can result in laboratory-acquired infections.
- Workers exposing co-workers, family or community in addition to themselves.

Further, not just for these new entrants to the field, but for many biologists, training has not included biosecurity aspects. For both biosafety and biosecurity, it is worth considering structured programs to train and mentor new investigators in synthetic genomics in good microbiological principles and practices, including specific procedures. NIH in fact developed in the 1980s an audiovisual program for non-microbiologists working with DNA (see Appendix F, NRC, 1989). The use of these structured programs, and a recommitment on the part of the community to training, may need to be considered as part of any research done in synthetic genomics.

6.3 Authority responsible for selection of containment (See also Section IV)

The same authorities that are responsible for oversight of biosafety are likely to be involved in the oversight of safety in synthetic genomics experiments and applications. The first point of contact in the chain is the principal investigator; for now, this is the person initially responsible for risk assessment. At the institutional level, the biosafety officer, the institutional biosafety committee, and the institutional animal care and use committee, if relevant, would all have some say.

At the national level, it remains to be seen to some degree how synthetic genomics is considered. Certainly, the NSABB (and thus NIH's Office of Biotechnology Activities) has taken an interest in synthetic genomics specifically. Both CDC and USDA will have an interest in synthetic genomics, particularly on the applications end as synthetic constructs begin to be used outside the laboratory.

Internationally, there has so far been little specific notice of synthetic genomics by the relevant offices that oversee biotechnology (including GMOs) in most of Europe. This is slowly changing, and whether the respective countries treat synthetic genomes as GMOs, as a generic biotechnology application, or in some

other way is not yet determined, and will be the focus of some discussion at the workshop.

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Biosafety Resource List

Guidelines, Manuals, Data Sheets

- Canadian Laboratory Biosafety Guidelines, 3rd Edition, 2004. Public Health Agency of Canada http://www.phac-aspc.gc.ca/publicat/lbg-ldmbl-04/index.html
- Canadian Material Safety Data Sheets (MSDS) for microorganisms. Health Canada, Office of Laboratory Security. http://www.phac-aspc.gc.ca/msds-ftss/index.html#menu
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Organization and Other Government Websites

- American Society for Microbiology (ASM) – http://www.asm.org
- American Biological Safety Association (ABSA) – http://www.absa.org
- American Type Culture Collection (ATCC) – http://www.atcc.org *(Check catalogs for detailed information and ATCC containment recommendations for cells and microorganisms.)*
- Department of Agriculture, Animal and Plant Health Inspection Service Plant virus lists and noxious weeds – www.aphis.usda.gov/ppq Veterinary Services – http://www.aphis.usda.gov/vs/ncie/ *(Information to import or to domestically transfer etiologic agents of livestock, poultry & other animals or materials that might contain these etiologic agents.)*
- Information Systems for Biotechnology – http://www.isb.vt.edu (*A National Resource in Agbiotech)*

Additional Websites for Risk Assessment of Organisms

- Belgium - Risk classification of organisms (human, animal and plant pathogens)

(Moniteur Belge 26.02.2002) http://www.biosafety.be/

- International - ABSA compilation of Risk Group classification for infectious substances http://www.absa.org/resriskgroup.html
- UK - Health Directorate, Health and Safety Executive (HSE). The Approved list of Biological Agents. 2004. http://www.hse.gov.uk/pubns/misc208.pdf

Risk Group Resources

- Australian/New Zealand Standard AS/NZS 2243.3:2002. Safety in laboratories Part 3: Microbiological aspects and containment facilities.(www.standards.com.au and www.standards.com.nz)
- Canada. Minister of Health. Population and Public Health Branch. Center for Emergency Preparedness and Response. 2004. "Laboratory Biosafety Guidelines". 3rd Edition http://www.phac-aspc.gc.ca/publicat/lbg-ldmbl-04/index.html2.CDC/NIH. 1999. Biosafety in Microbiological and Biomedical Laboratories" 4th Edition. Government Printing Office http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm (5th edition in press).
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Select Agent Information

- Department of Agriculture. Animal and Plant Health Inspection (APHIS). 7 CFR Part 331 (Plant diseases & Pests); 9 CFR Part 121 (Animal diseases). Possession, Use, and Transfer of Biological Agents and Toxins – http://www.aphis.usda.gov/vs/ncie/pdf/btarule.pdf
- Department of Health and Human Services. Centers for Disease Control (CDC). 42 CFR 73 Possession, Use, and Transfer of Select Agents and Toxins; Interim Final Rule – http://www.cdc.gov/od/sap/docs/42cfr73.pdf

- Department of Health and Human Services, Centers for Disease Control, Select Agent Program website – http://www.cdc.gov/od/sap/

Miscellaneous

- Belgian Biosafety Server – http://biosafety.ihe.be/ (This site also has multi-links to additional European information)
- Brooks, G. F., J. S. Butel, &S. A. Morse,. (eds.) 2004. Jawetz, Melnick, & Adelberg's *Medical Microbiology*. 23rd edition. The McGraw Hill Co.
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- Emerging Infectious Diseases Journal (On-line), Centers for Disease Control http://www.cdc.gov/ncidod/EID/index.htm *(Subscription information available.)*
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APPENDIX 1. DEFINITIONS

From BMBL 4th edition, Appendix F: Definitions (CDC/NIH,1999)

BIOSAFETY: Development and implementation of administrative policies, work practices, facility design, and safety equipment to prevent transmission of biologic agents to workers, other persons, and the environment.

BIOSECURITY: Protection of high-consequence microbial agents and toxins, or critical relevant information, against theft or diversion by those who intend to pursue intentional misuse.

BIOLOGIC TERRORISM: Use of biologic agents or toxins (e.g., pathogenic organisms that affect humans, animals, or plants) for terrorist purposes.

RESPONSIBLE OFFICIAL: A facility official who has been designated the responsibility and authority to ensure that the requirements of Title 42, CFR, Part 73, are met.

RISK: A measure of the potential loss of a specific biologic agent of concern, on the basis of the probability of occurrence of an adversary event, effectiveness of protection, and consequence of loss.

SELECT AGENT: Specifically regulated pathogens and toxins as defined in Title 42, CFR, Part 73, including pathogens and toxins regulated by both DHHS and USDA (i.e., overlapping agents or toxins).

THREAT: The capability of an adversary, coupled with intentions, to undertake malevolent actions.

THREAT ASSESSMENT: A judgment, based on available information, of the actual or potential threat of malevolent action.

VULNERABILITY: An exploitable capability, security weakness, or deficiency at a facility. Exploitable capabilities or weaknesses are those inherent in the design or layout of the biologic laboratory and its protection, or those existing because of the failure to meet or maintain prescribed security standards when evaluated against defined threats.

VULNERABILITY ASSESSMENT: A systematic evaluation process in which qualitative and quantitative techniques are applied to arrive at an effectiveness level for a security system to protect biologic laboratories and operations from specifically defined acts that can oppose or harm a person's interest.

APPENDIX 2. Classification of Etiologic Agents on the Basis of Hazard (CDC,1974)

The basis for the agent classifications:

Class 1. Agents of no or minimal hazard under ordinary conditions of handling.

Class 2. Agents of ordinary potential hazard. This class includes agents which may produce disease of varying degrees of severity from accidental inoculation or injection or other means of cutaneous penetration but which are contained by ordinary laboratory techniques.

Class 3. Agents involving special hazard or agents derived from outside the United States which require a federal permit for importation unless they are specified for higher classification. This class includes pathogens which require special conditions for containment.

Class 4. Agents that require the most stringent conditions for their containment because they are extremely hazardous to laboratory personnel or may cause serious epidemic disease. This class includes Class 3 agents from outside the US when they are employed in entomological experiments or when other entomological experiments are conducted in the same laboratory area.

Containment and training requirements. Recommendations describing the level of competence and physical containment for working with agents of each Class:

Class 1 .Distribution to all users; no special competence or containment required.
(This recommendation still applies for healthy human adults, but host factors must be taken into account when working with opportunistic pathogens, thus good microbiological practices must be learned and used)

Class 2.Distribution to laboratories whose staffs have levels of competency equal to or greater than one would expect in a college department of microbiology. Requests for agents in Class 2 are placed on institutional letterhead. (This requirement assumes that the institution has determined worker competency and facility acceptability; an erroneous assumption).

Class 3. Distribution to laboratories whose staffs have levels of competency equal to or greater than one would expect in a college department of microbiology and who have had special training in handling dangerous agents and are supervised by competent scientists. For aerosol studies, passage in animals, and infection of arthropod vectors, the laboratory should be located in a geographical area in which the chance of accidental establishment of the agent in a susceptible ecologic focus is minimal. Requests for agents in Class 3 are signed by the chairman of the department or the head of the laboratory or research institute where the work will be carried out. Conditions for containment include:

1. A controlled access facility: suite or room separated from the activities of individuals not engaged in handling Class 3 agents and form the general traffic pattern of the rest of the building or laboratory.
2. Negative air pressure is maintained at the site of work n a preparation cubicle or under a hood. Air is recirculated only after it has been adequately decontaminated through high efficiency filters.
3. Animal experiments, including cage sterilization, refuse handling, disposal of animals, etc., are conducted with a level of precaution equivalent to conditions required for laboratory experiments.
4. Personnel at risk are immunized against agents for which immune prophylaxis is available.

Class 4. Distribution to laboratories whose staffs have levels of competency equal to or greater than one would expect in a college department of microbiology and who have had special training in handling dangerous pathogens and are supervised by competent scientists. For aerosol studies, passage in animals, and infection of arthropod vectors, the laboratory should be located in a geographical area in which the chance of accidental establishment of the agent in a susceptible ecologic focus is minimal. Requests for agents in Class 4 are signed by the director of the institute or laboratory where the work is to be carried out. Conditions for containment include all those required for Class 3 agents and the following:

1. Work areas are in a facility which is in effect a separate building, or they are separated from other work areas by effective airlocks.
2. If the work area is not in a separate building, the entire area used for Class 4 agents has a separate air exhaust and negative pressure with respect to other areas of the building. Exhaust air is decontaminated by filtration through high efficiency filters or by some other suitable process. Class 4 agents are manipulated only in safety cabinets equipped with absolute filters.
3. Access to work areas is restricted to individuals immunized or otherwise under specific control.
4. Protective clothing is worn, and it is decontaminated before being removed from the laboratory area.
5. When an agent is used in entomological experiments, the windows, walls, floor, ceiling, and airlock of the work area are insect-proof, and pure pyrethrum insecticide or a suitable insect killing device is available in the airlock.

APPENDIX 3. Risk Classification Criteria for World Health Organization (WHO), Australia, Canada, European Union (EU), and for the USA, the NIH for RDNA and the CDC/NIH.

1. **WHO Classification of Infective Microorganisms by Risk Group (2004)**. WHO Basis for Risk Grouping: Each country classifies the agents in that country by risk group based on pathogenicity of the organism, modes of transmission and host range of the organism. These may be influenced by existing levels of immunity, density and movement of host population presence of appropriate vectors and standards of environmental hygiene.

    - Availability of effective preventive measures. Such measures may include: prophylaxis by vaccination or antisera; sanitary measures, e.g. food and water hygiene; the control of animal reservoirs or arthropod vectors; the movement of people or animals; and the importation of infected animals or animal products.
    - Availability of effective treatment. This includes passive immunization and post-exposure vaccination, antibiotics, and chemotherapeutic agents, taking into consideration the possibility of the emergence of resistant strains. It is important to take prevailing conditions in the geographical area in which the microorganisms are handled into account. Note: Individual governments may decide to prohibit the handling or importation of certain pathogens except for diagnostic purposes.

    - **WHO Risk Group 1** (no or low individual and community risk). A microorganism that is unlikely to cause human disease or animal disease
    - **WHO Risk Group 2** (moderate individual risk, low community risk). A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventative measures are available and the risk of spread of infection is limited.
    - **WHO Risk Group 3** (high individual risk, low community risk). A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.
    - **WHO Risk Group 4** (high individual and community risk). A pathogen that usually causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

2. **Australian/New Zealand Standard (2002).** Standard AS/NZS 2243.3:2002. Safety in laboratories Part 3: Microbiological aspects and containment facilities.. The following classification is based on the pathogenicity of the agent, the mode of transmission and host range of the agent, the availability of effective preventive measures and the availability of effective treatment.

- **Group 1** (low individual and community risk). A microorganism that is unlikely to cause human, plant or animal disease.
- **Group 2** (moderate individual risk, limited community risk). A pathogen that can cause human, animal or plant disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause infection, but effective treatment and preventive measures are available and the risk of spread is limited.
- **Group 3** (high individual risk, limited community risk). A pathogen that usually causes serious human or animal disease and may present a serious hazard to laboratory workers. It could present a risk if spread in the community or the environment, but there are usually effective preventive measures or treatment available.
- **Group 4** (high individual and community risk). A pathogen that usually produces life-threatening human or animal disease represents a serious hazard to laboratory workers and is readily transmissible from one individual to another. Effective treatment and preventive measures are not usually available.

3. **Canadian Laboratory Safety Guidelines (2004; list not available)** Inherent risks of a pathogen made on basis of factors such as severity of disease caused, routes of infection, virulence and infectivity takes into account existence of effective therapies, possibilities for immunization, presence of vectors, quantity of agent and whether agent is indigenous to Canada, possible effects on other species, including plants, or possible economic environmental effects.
   - **Risk Group 1** (low individual and community risk). Any biological agent that is unlikely to cause disease in healthy workers or animals.
   - **Risk Group 2** (moderate individual risk, limited community risk). Any pathogen that can cause human disease, but under normal circumstances is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures rarely cause infection leading to serious disease, effective treatment and preventive measures are available and the risk of spread is limited.
   - **Risk Group 3** (high individual risk, low community risk). Any pathogen that usually causes serious human disease, or can result in serious economic consequences but does not ordinarily spread by casual contact from one individual to another, or that causes disease treatable by antimicrobial or antiparasitic agents.
   - **Risk Group 4** (high individual and community risk). Any pathogen that usually produces very serious human disease, often untreatable, and may be readily transmitted from one individual to another, or from animal to human or vice-versa, directly or indirectly, or by casual contact.

4. **European Economic Community (2000)**. Directive 2000/54/EC and Directive 90/679/EEC (adopted 20 November, 1990; revised 18 September 2000) on the protection of workers from risks related to exposure to biological agents at work provides for the Classification of biological agents into four infection risk groups on the basis of the following criteria:

   - **Group 1** biological agent means one that is unlikely to cause human disease.
   - **Group 2** biological agent means one that can cause human disease and might be a hazard to workers; it is unlikely to spread to the community; there is usually effective prophylaxis or treatment available.
   - **Group 3** biological agent means one that can cause severe human disease and present a serious hazard to workers; it may present a risk of spreading to the community, but there is usually effective prophylaxis or treatment available.
   - **Group 4** biological agent means one that causes severe human disease and is a serious hazard to workers; it may present a high risk of spreading to the community; there is usually no effective prophylaxis or treatment available. (See also Official Journal of the European Communities No L262/21 dated September 18, 2000.) Article 2. Definitions; Article 18. Classification of biological agents; Annex III. Community Classification. Introductory Notes)

5. **NIH Recombinant DNA Guidelines** (**USA, 2002).** April 2002. Appendix B. http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html

   - **Risk Group 1** (RG1) Agents that are not associated with disease in healthy adult humans. Includes a list of animal viral etiologic agents in common use.
   - **Risk Group 2** (RG2) Agents that are associated with human disease which is rarely serious and for which preventive or therapeutic interventions are often available.
   - **Risk Group 3** (RG3) Agents that are associated with serious or lethal human disease for which preventive or therapeutic interventions may be available (high individual risk but low community risk).
   - **Risk Group 4** (RG4) Agents that are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are not usually available (high individual risk and high community risk).

6. **CDC/NIH Guidelines (1999).** "Biosafety in Microbiological and Biomedical Laboratories" 4th Edition, 1999. Section III gives criteria for placing work into a biosafety containment level; not yet translated into Risk groups.

   - **Biosafety Level 1 (BSL 1)**: well characterized agents not consistently known to cause disease in healthy adult humans, of minimal potential hazard to laboratory personnel and the environment
   - **Biosafety Level 2 (BSL 2)**: agents of moderate potential hazard to personnel and the environment

- **Biosafety Level 3 (BSL 3)**: indigenous or exotic agents which may cause serious or potentially lethal disease as a result of exposure by the inhalation route (applicable to clinical, diagnostic, teaching, research or production facilities)
- **Biosafety Level 4 (BSL 4)**: dangerous and exotic agents which pose a high individual risk of aerosol-transmitted laboratory infections and life-threatening disease

References

1. Australian/New Zealand Standard AS/NZS 2243.3:2002. Safety in laboratories Part 3: Microbiological aspects and containment facilities.(www.standards.com.au and www.standards.com.nz)
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3. National Institutes of Health. 2002. *NIH Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines)* 59 FR 34496 (July 5, 1994), as amended. The current amended version of the *NIH Guidelines* can be accessed at: http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html
4. European Union. 2000. Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work.(seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EC Official Journal of the European Communities L262/21. October 17,2000
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APPENDIX 4. Biosafety in Biomedical and Microbiological Laboratories

BMBL Section V Risk Assessment

"Risk" implies the probability that harm, injury, or disease will occur. In the context of the microbiological and biomedical laboratories, the assessment of risk focuses primarily on the prevention of laboratory-associated infections. When addressing laboratory activities involving infectious or potentially infectious material, risk assessment is a critical and productive exercise. It helps to assign the biosafety levels (facilities, equipment, and practices) that reduce the worker's and the environment's risk of exposure to an agent to an absolute minimum. The intent of this section is to provide guidance and to establish a framework for selecting the appropriate biosafety level.

Risk assessment can be qualitative or quantitative. In the presence of known hazards (e.g., residual levels of formaldehyde gas after a laboratory decontamination), quantitative assessments can be done. But in many cases, quantitative data will be incomplete or even absent (e.g., investigation of an unknown agent or receipt of an unlabeled sample). Types, subtypes, and variants of infectious agents involving different or unusual vectors, the difficulty of assays to measure an agent's amplification potential, and the unique considerations of genetic recombinants are but a few of the challenges to the safe conduct of laboratory work. In the face of such complexity, meaningful quantitative sampling methods are frequently unavailable. Therefore, the process of doing a risk assessment for work with biohazardous materials cannot depend on a prescribed algorithm.

The laboratory director or principal investigator is responsible for assessing risks in order to set the biosafety level for the work. This should be done in close collaboration with the Institutional Biosafety Committee (and/or other biosafety professionals as needed) to ensure compliance with established guidelines and regulations.

In performing a qualitative risk assessment, all the risk factors are first identified and explored. There may be related information available, such as this manual, the *NIH Recombinant DNA Guidelines*, the *Canadian Laboratory Biosafety Guidelines*, or the *WHO Biosafety Guidelines*. In some cases, one must rely on other sources of information such as field data from subject matter experts. This information is interpreted for its tendency to raise or lower the risk of laboratory-acquired infection.(1)

The challenge of risk assessment lies in those cases where complete information on these factors is unavailable. A conservative approach is generally advisable when insufficient information forces subjective judgment. Universal precautions are always advisable.

The factors of interest in a risk assessment include:

- The *pathogenicity* of the infectious or suspected infectious agent, including disease incidence and severity (i.e., mild morbidity versus high mortality, acute versus chronic disease). The more severe the potentially acquired disease, the

higher the risk. For example, *staphylococcus aureus* only rarely causes a severe or life threatening disease in a laboratory situation and is relegated to BSL-2. Viruses such as Ebola, Marburg, and Lassa fever, which cause diseases with high mortality rates and for which there are no vaccines or treatment, are worked with at BSL-4. However, disease severity needs to be tempered by other factors. Work with human immunodeficiency virus (HIV) and hepatitis B virus is also done at BSL-2, although they can cause potentially lethal disease. But they are not transmitted by the aerosol route, the incidence of laboratory-acquired infection is extremely low for HIV, and an effective vaccine is available for hepatitis B.

- The *route of transmission* (e.g., parenteral, airborne, or by ingestion) of newly isolated agents may not be definitively established. Agents that can be transmitted by the aerosol route have caused most laboratory infections. It is wise, when planning work with a relatively uncharacterized agent with an uncertain mode of transmission, to consider the *potential for aerosol transmission.* The greater the aerosol potential, the higher the risk.
- *Agent stability* is a consideration that involves not only aerosol infectivity (e.g., from spore-forming bacteria), but also the agent's ability to survive over time in the environment. Factors such as desiccation, exposure to sunlight or ultraviolet light, or exposure to chemical disinfectants must be considered.
- The *infectious dose* of the agent is another factor to consider. Infectious dose can vary from one to hundreds of thousands of units. The complex nature of the interaction of microorganisms and the host presents a significant challenge even to the healthiest immunized laboratory worker, and may pose a serious risk to those with lesser resistance. The laboratory worker's *immune* status is directly related to his/her susceptibility to disease when working with an infectious agent.
- The *concentration* (number of infectious organisms per unit volume) will be important in determining the risk. Such a determination will include consideration of the milieu containing the organism (e.g., solid tissue, viscous blood or sputum, or liquid medium) and the laboratory activity planned (e.g., agent amplification, sonication, or centrifugation). The volume of concentrated material being handled is also important. In most instances, the risk factors increase as the working volume of high-titered microorganisms increases, since additional handling of the materials is often required.
- The *origin* of the potentially infectious material is also critical in doing a risk assessment. "Origin" may refer to geographic location (e.g., domestic or foreign); host (e.g., infected or uninfected human or animal); or nature of source (potential zoonotic or associated with a disease outbreak). From another perspective, this factor can also consider the potential of agents to endanger American livestock and poultry.
- The *availability of data from animal studies*, in the absence of human data, may provide useful information in a risk assessment. Information about pathogenicity, infectivity, and route of transmission in animals may provide valuable clues. Caution must always be exercised, however, in translating infectivity data from one species of animal to another species.
- The established *availability of an effective prophylaxis* or therapeutic intervention is another essential factor to be considered. The most common form of

prophylaxis is immunization with an effective vaccine. Risk assessment includes determining the availability of effective immunizations. In some instances, immunization may affect the biosafety level (e.g., the BSL-4 Junin virus can be worked on at BSL-3 by an immunized worker). Immunization may also be passive (e.g., the use of serum immunoglobulin in HBV exposures). However important, immunization only serves as an additional layer of protection beyond engineering controls, proper practices and procedures, and the use of personal protective equipment. Occasionally, immunization or therapeutic intervention (antibiotic or antiviral therapy) may be particularly important in field conditions. The offer of immunizations is part of risk management.

- *Medical surveillance* ensures that the safeguards decided upon in fact produce the expected health outcomes. Medical surveillance is part of risk management. It may include serum banking, monitoring employee health status, and participating in post-exposure management.
- Risk assessment must also include an evaluation of the *experience and skill level of at-risk personnel* such as laboratorians and maintenance, housekeeping, and animal care personnel (see Section III). Additional education may be necessary to ensure the safety of persons working at each biosafety level.

The infectious agents whose risk is evaluated often will fall into the following discrete categories:

- **Materials containing known infectious agents**. The characteristics of most known infectious agents have been well identified. Information useful to risk assessment can be obtained from laboratory investigations, disease surveillance, and epidemiological studies. Infectious agents known to have caused laboratory-associated infections are included in this volume's agent summary statements (see Section VII). Other sources include the American Public Health Association's manual, *Control of Communicable Diseases*.(2) Literature reviews on laboratory acquired infections also may be helpful.(3)(4)(5)(6)(7)(8)

- **Materials containing unknown infectious agents.** The challenge here is to establish the most appropriate biosafety level with the limited information available. Often these are clinical specimens. Some questions that may help in this risk assessment include:

  1. Why is an infectious agent suspected?
  2. What epidemiological data are available? What route of transmission is indicated? What is the morbidity or mortality rate associated with the agent?
  3. What medical data are available?

  The responses to these questions may identify the agent or a surrogate agent whose existing agent summary statement can be used to determine a biosafety level. In the absence of hard data, a conservative approach is advisable.

- **Materials containing recombinant DNA molecules.** This category of agents includes microorganisms that have been genetically modified through recombinant DNA technologies. These technologies continue to evolve rapidly. Experimental procedures designed to derive novel recombinant viruses, bacteria, yeast, and other microorganisms have become commonplace in recent years. It is highly likely that future applications of recombinant DNA technology will produce new hybrid viruses. The National Institutes of Health publication, *Guidelines for Research Involving Recombinant DNA Molecules*,(9) is a key reference in establishing an appropriate biosafety level for work involving recombinant microorganisms.

In selecting an appropriate biosafety level for such work, perhaps the greatest challenge is to evaluate the potential increased biohazard associated with a particular genetic modification. In most such cases, the selection of an appropriate biosafety level begins by establishing the classification of the non-modified virus. Among the recombinant viruses now routinely developed are adenoviruses, alphaviruses, retroviruses, vaccinia viruses, herpesviruses, and others designed to express heterologous gene products. However, the nature of the genetic modification and the quantity of virus must be carefully considered when selecting the appropriate biosafety level for work with a recombinant virus.

Among the points to consider in work with recombinant microorganisms are:

1. Does the inserted gene encode a known toxin or a relatively uncharacterized toxin?
2. Does the modification have the potential to alter the host range or cell tropism of the virus?
3. Does the modification have the potential to increase the replication capacity of the virus?
4. Does the inserted gene encode a known oncogene?
5. Does the inserted gene have the potential for altering the cell cycle?
6. Does the viral DNA integrate into the host genome?
7. What is the probability of generating replication-competent viruses?

This list of questions is not meant to be inclusive. Rather, it serves as an example of the information needed to judge whether a higher biosafety level is needed in work with genetically modified microorganisms. Since in many cases the answers to the above questions will not be definitive, it is important that the organization have a properly constituted and informed Institutional Biosafety Committee, as outlined in the NIH guidelines, to evaluate the risk assessment.

- **Materials that may or may not contain unknown infectious agents.** In the absence of information that suggests an infectious agent, universal precautions are indicated.

- **Animal studies.** Laboratory studies involving animals may present many different kinds of physical, environmental, and biological hazards. The specific

hazards present in any particular animal facility are unique, varying according to the species involved and the nature of the research activity. The risk assessment for biological hazard should particularly focus on the animal facility's potential for increased exposure, both to human pathogens and to zoonotic agents.

The animals themselves can introduce new biological hazards to the facility. Latent infections are most common in field-captured animals or in animals coming from unscreened herds. For example, monkey b-virus presents a latent risk to individuals who handle macaques. The animal routes of transmission must also be considered in the risk assessment. Animals that shed virus through respiratory dissemination or dissemination in urine or feces are far more hazardous than those that do not. Animal handlers in research facilities working on infectious agents have a greater risk of exposure from the animals' aerosols, bites, and scratches. Section IV describes the practices and facilities applicable to work on animals infected with agents assigned to corresponding Biosafety Levels 1-4.(1)

- **Other applications** The described risk assessment process is also applicable to laboratory operations other than those involving the use of primary agents of human disease. It is true that microbiological studies of animal host-specific pathogens, soil, water, food, feeds, and other natural or manufactured materials, pose comparatively lower risks for the laboratory worker. Nonetheless, microbiologists and other scientists working with such materials may find the practices, containment equipment, and facility recommendations described in this publication of value in developing operational standards to meet their own assessed needs.

- **Other Resources** *NIH Guidelines for Recombinant DNA Molecules*: http://www.nih.gov/od/orda/toc.htm NIH Office of Recombinant DNA Activities: http://www.nih.gov/od/orda

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APPENDIX 5. NIH Guidelines for Research Involving Recombinant DNA Molecules (April 2002) Section II Risk Assessment; Appendix G (Lab scale) and K (Large Scale) NIH, 2002

Section II-B. Containment

Effective biological safety programs have been operative in a variety of laboratories for many years. Considerable information already exists about the design of physical containment facilities and selection of laboratory procedures applicable to organisms carrying recombinant DNA (see Section V-B, Footnotes and References of Sections I-IV). The existing programs rely upon mechanisms that can be divided into two categories: (i) a set of standard practices that are generally used in microbiological laboratories; and (ii) special procedures, equipment, and laboratory installations that provide physical barriers that are applied in varying degrees according to the estimated biohazard. Four biosafety levels are described in Appendix G, Physical Containment. These biosafety levels consist of combinations of laboratory practices and techniques, safety equipment, and laboratory facilities appropriate for the operations performed and are based on the potential hazards imposed by the agents used and for the laboratory function and activity. Biosafety Level 4 provides the most stringent containment conditions, Biosafety Level 1 the least stringent. Experiments involving recombinant DNA lend themselves to a third containment mechanism, namely, the application of highly specific biological barriers. Natural barriers exist that limit either: (i) the infectivity of a vector or vehicle (plasmid or virus) for specific hosts, or (ii) its dissemination and survival in the environment. Vectors, which provide the means for recombinant DNA and/or host cell replication, can be genetically designed to decrease, by many orders of magnitude, the probability of dissemination of recombinant DNA outside the laboratory (see Appendix I, Biological Containment).

NIH APPENDIX G: Physical Containment

Appendix G specifies physical containment for standard laboratory experiments and defines Biosafety Level 1through Biosafety Level 4. For large-scale (over 10 liters) research or production, Appendix K (*Physical Containment for Large Scale Uses of*

Organisms Containing Recombinant DNA Molecules) supersedes Appendix G. Appendix K defines Good Large Scale Practice through Biosafety Level 3 - Large Scale. For certain work with plants, Appendix P (*Physical and Biological Containment for Recombinant DNA Research Involving Plants*) supersedes Appendix G. Appendix P defines Biosafety Levels 1 through 4 - Plants. For certain work with animals, Appendix Q (*Physical and Biological Containment for Recombinant DNA Research Involving Animals*) supersedes Appendix G. Appendix Q defines Biosafety Levels 1 through 4 - Animals.

APPENDIX G-I. Standard Practices and Training

The first principle of containment is strict adherence to good microbiological practices (see Appendices G-III-A through G-III-J, *Footnotes and References of Appendix G*). Consequently, all personnel directly or indirectly involved in experiments using recombinant DNA shall receive adequate instruction (see Sections IV-B-1-h, *Responsibilities of the Institution--General Information*, and IV-B-7-d, *Responsibilities of the Principal Investigator Prior to Initiating Research*). At a minimum, these instructions include training in aseptic techniques and in the biology of the organisms used in the experiments so that the potential biohazards can be understood and appreciated.

Any research group working with agents that are known or potential biohazards shall have an emergency plan that describes the procedures to be followed if an accident contaminates personnel or the environment. The Principal Investigator shall ensure that everyone in the laboratory is familiar with both the potential hazards of the work and the emergency plan (see Sections IV -B-7-d, *Responsibilities of the Principal Investigator Prior to Initiating Research* and IV-B-7-e, *Responsibilities of the Principal Investigator During the Conduct of the Research*). If a research group is working with a known pathogen for which there is an effective vaccine, the vaccine should be made available to all workers. Serological monitoring, when clearly appropriate, will be provided (see Section IV -B-1-f, *Responsibilities of the Institution--General Information*).

The *Laboratory Safety Monograph* (see Appendix G-III-O, *Footnotes and References of Appendix G*) and *Biosafety in Microbiological and Biomedical Laboratories* (see Appendix G-III-B, *Footnotes and References of Appendix G*) describe practices, equipment, and facilities in detail.

APPENDIX G-II. Physical Containment Levels

The objective of physical containment is to confine organisms containing recombinant DNA molecules and to reduce the potential for exposure of the laboratory worker, persons outside of the laboratory, and the environment to organisms containing recombinant DNA molecules. Physical containment is achieved through the use of laboratory practices, containment equipment, and special laboratory design. Emphasis is placed on primary means of physical containment which are provided by laboratory practices and containment equipment.

Special laboratory design provides a secondary means of protection against the accidental release of organisms outside the laboratory or to the environment. Special laboratory design is used primarily in facilities in which experiments of moderate to high potential hazard are performed.

Combinations of laboratory practices, containment equipment, and special laboratory design can be made to achieve different levels of physical containment. Four levels of physical containment, which are designated as BL1, BL2, BL3, and BL4 are described. It should be emphasized that the descriptions and assignments of physical containment detailed below are based on existing approaches to containment of pathogenic organisms (see Appendix G-III-B, *Footnotes and References of Appendix G*).

The National Cancer Institute describes three levels for research on oncogenic viruses which roughly correspond to our BL2, BL3, and BL4 levels (see Appendix G-III-C, *Footnotes and References of Appendix G*). It is recognized that several different combinations of laboratory practices, containment equipment, and special laboratory design may be appropriate for containment of specific research activities. The *NIH Guidelines*, therefore, allow alternative selections of primary containment equipment within facilities that have been designed to provide BL3 and BL4 levels of physical containment.

NIH APPENDIX K. Physical Containment for Large Scale Uses of Organisms Containing Recombinant DNA Molecules

Appendix K specifies physical containment guidelines for large-scale (greater than 10 liters of culture) research or production involving viable organisms containing recombinant DNA molecules. It shall apply to large-scale research or production activities as specified in Section III-D-6, *Experiments Involving More than 10 Liters of Culture*. It is important to note that this appendix addresses only the biological hazard associated with organisms containing recombinant DNA. Other hazards accompanying the large-scale cultivation of such organisms (e.g., toxic properties of products; physical, mechanical, and chemical aspects of downstream processing) are not addressed and shall be considered separately, albeit in conjunction with this appendix.

All provisions shall apply to large-scale research or production activities with the following modifications: (i) Appendix K shall supersede Appendix G, *Physical Containment*, when quantities in excess of 10 liters of culture are involved in research or production. Appendix K-II applies to Good Large Scale Practice; (ii) the institution shall appoint a Biological Safety Officer if it engages in large-scale research or production activities involving viable organisms containing recombinant DNA molecules. The duties of the Biological Safety Officer shall include those specified in Section IV-B-3, *Biological Safety Officer*; (iii) the institution shall establish and maintain a health surveillance program for personnel engaged in large-scale research or production activities involving viable organisms containing recombinant DNA molecules which require Biosafety Level (BL) 3 containment at the laboratory scale. The program shall include: preassignment and periodic physical and medical examinations; collection,

maintenance, and analysis of serum specimens for monitoring serologic changes that may result from the employee's work experience; and provisions for the investigation of any serious, unusual, or extended illnesses of employees to determine possible occupational origin.

APPENDIX K-I. Selection of Physical Containment Levels

The selection of the physical containment level required for recombinant DNA research or production involving more than 10 liters of culture is based on the containment guidelines established in Section III, *Experiments Covered by the NIH Guidelines*. For purposes of large-scale research or production, four physical containment levels are established. The four levels set containment conditions at those appropriate for the degree of hazard to health or the environment posed by the organism, judged by experience with similar organisms unmodified by recombinant DNA techniques and consistent with Good Large Scale Practice.

The four biosafety levels of large-scale physical containment are referred to as Good Large Scale Practice, BL1-Large Scale, BL2-Large Scale, and BL3-Large Scale. Good Large Scale Practice is recommended for large-scale research or production involving viable, non-pathogenic, and non-toxigenic recombinant strains derived from host organisms that have an extended history of safe large-scale use. Good Large Scale Practice is recommended for organisms such as those included in Appendix C, *Exemptions under Section III-F-6*, which have built-in environmental limitations that permit optimum growth in the large-scale setting but limited survival without adverse consequences in the environment. BL1-Large Scale is recommended for large-scale research or production of viable organisms containing recombinant DNA molecules that require BL1 containment at the laboratory scale and that do not qualify for Good Large Scale Practice. BL2-Large Scale is recommended for large-scale research or production of viable organisms containing recombinant DNA molecules that require BL2 containment at the laboratory scale. BL3-Large Scale is recommended for large-scale research or production of viable organisms containing recombinant DNA molecules that require BL3 containment at the laboratory scale. No provisions are made for large-scale research or production of viable organisms containing recombinant DNA molecules that require BL4 containment at the laboratory scale. If necessary, these requirements will be established by NIH on an individual basis.

Page 13 - NIH Guidelines for Research Involving Recombinant DNA Molecules (April 2002) Since these three means of containment are complementary, different levels of containment can be established that apply various combinations of the physical and biological barriers along with a constant use of standard practices. Categories of containment are considered separately in order that such combinations can be conveniently expressed in the NIH Guidelines.

Physical containment conditions within laboratories, described in Appendix G, Physical Containment, may not always be appropriate for all organisms because of their physical size, the number of organisms needed for an experiment, or the particular growth

requirements of the organism. Likewise, biological containment for microorganisms described in Appendix I, Biological Containment, may not be appropriate for all organisms, particularly higher eukaryotic organisms. However, significant information exists about the design of research facilities and experimental procedures that are applicable to organisms containing recombinant DNA that is either integrated into the genome or into microorganisms associated with the higher organism as a symbiont, pathogen, or other relationship. This information describes facilities for physical containment of organisms used in non-traditional laboratory settings and special practices for limiting or excluding the unwanted establishment, transfer of genetic information, and dissemination of organisms beyond the intended location, based on both physical and biological containment principles. Research conducted in accordance with these conditions effectively confines the organism.

For research involving plants, four biosafety levels (BL1-P through BL4-P) are described in Appendix P, Physical and Biological Containment for Recombinant DNA Research Involving Plants. BL1-P is designed to provide a moderate level of containment for experiments for which there is convincing biological evidence that precludes the possibility of survival, transfer, or dissemination of recombinant DNA into the environment, or in which there is no recognizable and predictable risk to the environment in the event of accidental release. BL2-P is designed to provide a greater level of containment for experiments involving plants and certain associated organisms in which there is a recognized possibility of survival, transmission, or dissemination of recombinant DNA containing organisms, but the consequence of such an inadvertent release has a predictably minimal biological impact. BL3-P and BL4-P describe additional containment conditions for research with plants and certain pathogens and other organisms that require special containment because of their recognized potential for significant detrimental impact on managed or natural ecosystems. BL1-P relies upon accepted scientific practices for conducting research in most ordinary greenhouse or growth chamber facilities and incorporates accepted procedures for good pest control and cultural practices. BL1-P facilities and procedures provide a modified and protected environment for the propagation of plants and microorganisms associated with the plants and a degree of containment that adequately controls the potential for release of biologically viable plants, plant parts, and microorganisms associated with them. BL2-P and BL3-P rely upon accepted scientific practices for conducting research in greenhouses with organisms infecting or infesting plants in a manner that minimizes or prevents inadvertent contamination of plants within or surrounding the greenhouse. BL4-P describes facilities and practices known to provide containment of certain exotic plant pathogens.

For research involving animals, which are of a size or have growth requirements that preclude the use of conventional primary containment systems used for small laboratory animals, four biosafety levels (BL1-N through BL4-N) are described in Appendix Q, Physical and Biological Containment for Recombinant DNA Research Involving Animals. BL1-N describes containment for animals that have been modified by stable introduction of recombinant DNA, or DNA derived therefrom, into the germ-line (transgenic animals) and experiments involving viable recombinant DNA-modified

microorganisms and is designed to eliminate the possibility of sexual transmission of the modified genome or transmission of recombinant DNA-derived viruses known to be transmitted from animal parent to offspring only by sexual reproduction. Procedures, practices, and facilities follow classical methods of avoiding genetic exchange between animals. BL2-N describes containment which is used for transgenic animals associated with recombinant DNA-derived organisms and is designed to eliminate the possibility of vertical or horizontal transmission.

Page 14 - NIH Guidelines for Research Involving Recombinant DNA Molecules (April 2002). In constructing the NIH Guidelines, it was necessary to define boundary conditions for the different levels of physical and biological containment and for the classes of experiments to which they apply. These definitions do not take into account all existing and anticipated information on special procedures that will allow particular experiments to be conducted under different conditions than indicated here without affecting risk. Individual investigators and Institutional Biosafety Committees are urged to devise simple and more effective containment procedures and to submit recommended changes in the NIH Guidelines to permit the use of these procedures.

APPENDIX K. *Physical Containment for Large Scale Uses of Organisms Containing Recombinant DNA Molecules 1*

Appendix K-II. Good Large Scale Practice (GLSP)

Appendix K-II-A. Institutional codes of practice shall be formulated and implemented to assure adequate control of health and safety matters.

Appendix K-II-B. Written instructions and training of personnel shall be provided to assure that cultures of viable organisms containing recombinant DNA molecules are handled prudently and that the work place is kept clean and orderly.

Appendix K-II-C. In the interest of good personal hygiene, facilities (e.g., hand washing sink, shower, and changing room) and protective clothing (e.g., uniforms, laboratory

coats) shall be provided that are appropriate for the risk of exposure to viable organisms containing recombinant DNA molecules. Eating, drinking, smoking, applying cosmetics, and mouth pipetting shall be prohibited in the work area.

Appendix K-II-D. Cultures of viable organisms containing recombinant DNA molecules shall be handled in facilities intended to safeguard health during work with microorganisms that do not require containment.

Appendix K-II-E. Discharges containing viable recombinant organisms shall be handled in accordance with applicable governmental environmental regulations.

Appendix K-II-F. Addition of materials to a system, sample collection, transfer of culture fluids within/between systems, and processing of culture fluids shall be conducted in a manner that maintains employee's exposure to viable organisms containing recombinant DNA molecules at a level that does not adversely affect the health and safety of employees.

Appendix K-II-G. The facility's emergency response plan shall include provisions for handling spills.

Appendix K-III. Biosafety Level 1 (BL1) - Large Scale

Appendix K-III-A. Spills and accidents which result in overt exposures to organisms containing recombinant DNA molecules are immediately reported to the Laboratory Director. Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

Appendix K-III-B. Cultures of viable organisms containing recombinant DNA molecules shall be handled in a closed system (e.g., closed vessel used for the propagation and growth of cultures) or other primary containment equipment (e.g., biological safety cabinet containing a centrifuge used to process culture fluids) which is designed to reduce the potential for escape of viable organisms. Volumes less than 10 liters may be handled outside of a closed system or other primary containment equipment provided all physical containment requirements specified in Appendix G-II-A, *Physical Containment Levels--Biosafety Level 1*, are met.

Appendix K-III-C. Culture fluids (except as allowed in Appendix K-III-D) shall not be removed from a closed system or other primary containment equipment unless the viable organisms containing recombinant DNA molecules have been inactivated by a validated inactivation procedure. A validated inactivation procedure is one which has been demonstrated to be effective using the organism that will serve as the host for propagating the recombinant DNA molecules. Culture fluids that contain viable organisms or viral vectors intended as final product may be removed from the primary containment equipment by way of closed systems for sample analysis, further processing or final fill.

Appendix K-III-D. Sample collection from a closed system, the addition of materials to a closed system, and the transfer of culture fluids from one closed system to another shall be conducted in a manner which minimizes the release of aerosols or contamination of exposed surfaces.

Appendix K-III-E. Exhaust gases removed from a closed system or other primary containment equipment shall be treated by filters which have efficiencies equivalent to high efficiency particulate air/HEPA filters or by other equivalent procedures (e.g., incineration) to minimize the release of viable organisms containing recombinant DNA molecules to the environment.

Appendix K-III-F. A closed system or other primary containment equipment that has contained viable organisms containing recombinant DNA molecules shall not be opened for maintenance or other purposes unless it has been sterilized by a validated sterilization procedure except when the culture fluids contain viable organisms or vectors intended as final product as described in Appendix K-III-C above. A validated sterilization procedure is one which has been demonstrated to be effective using the organism that will serve as the host for propagating the recombinant DNA molecules.

Appendix K-III-G. Emergency plans required by Sections IV-B-2-b-(6), *Institutional Biosafety Committee*, and IV-B-3-c-(3), *Biological Safety Officer*, shall include methods and procedures for handling large losses of culture on an emergency basis.

Appendix K-IV. Biosafety Level 2 (BL2) - Large Scale

Appendix K-IV-A. Spills and accidents which result in overt exposures to organisms containing recombinant DNA molecules are immediately reported to the Biological Safety Officer, Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities (if applicable). Reports to NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

Appendix K-IV-B. Cultures of viable organisms containing recombinant DNA molecules shall be handled in a closed system (e.g., closed vessel used for the propagation and growth of cultures) or other primary containment equipment (e.g., Class III biological safety cabinet containing a centrifuge used to process culture fluids) which is designed to prevent the escape of viable organisms. Volumes less than 10 liters may be handled outside of a closed system or other primary containment equipment provided all physical containment requirements specified in Appendix G-II-B, *Physical Containment Levels--Biosafety Level 2*, are met.

Appendix K-IV-C. Culture fluids (except as allowed in Appendix K-IV-D) shall not be removed from a closed system or other primary containment equipment unless the viable organisms containing recombinant DNA molecules have been inactivated by a validated

inactivation procedure. A validated inactivation procedure is one which has been demonstrated to be effective using the organism that will serve as the host for propagating the recombinant DNA molecules. Culture fluids that contain viable organisms or viral vectors intended as final product may be removed from the primary containment equipment by way of closed systems for sample analysis, further processing or final fill.

Appendix K-IV-D. Sample collection from a closed system, the addition of materials to a closed system, and the transfer of cultures fluids from one closed system to another shall be conducted in a manner which prevents the release of aerosols or contamination of exposed surfaces.

Appendix K-IV-E. Exhaust gases removed from a closed system or other primary containment equipment shall be treated by filters which have efficiencies equivalent to high efficiency particulate air/HEPA filters or by other equivalent procedures (e.g., incineration) to prevent the release of viable organisms containing recombinant DNA molecules to the environment.

Appendix K-IV-F. A closed system or other primary containment equipment that has contained viable organisms containing recombinant DNA molecules shall not be opened for maintenance or other purposes unless it has been sterilized by a validated sterilization procedure except when the culture fluids contain viable organisms or vectors intended as final product as described in Appendix K-IV-C above. A validated sterilization procedure is one which has been demonstrated to be effective using the organisms that will serve as the host for propagating the recombinant DNA molecules.

Appendix K-IV-G. Rotating seals and other mechanical devices directly associated with a closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules shall be designed to prevent leakage or shall be fully enclosed in ventilated housings that are exhausted through filters which have efficiencies equivalent to high efficiency particulate air/HEPA filters or through other equivalent treatment devices.

Appendix K-IV-H. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules and other primary containment equipment used to contain operations involving viable organisms containing sensing devices that monitor the integrity of containment during operations.

Appendix K-IV-I. A closed system used for the propagation and growth of viable organisms containing the recombinant DNA molecules shall be tested for integrity of the containment features using the organism that will serve as the host for propagating recombinant DNA molecules. Testing shall be accomplished prior to the introduction of viable organisms containing recombinant DNA molecules and following modification or replacement of essential containment features. Procedures and methods used in the testing shall be appropriate for the equipment design and for recovery and demonstration of the test organism. Records of tests and results shall be maintained on file.

Appendix K-IV-J. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules shall be permanently identified. This identification shall be used in all records reflecting testing, operation, and maintenance and in all documentation relating to use of this equipment for research or production activities involving viable organisms containing recombinant DNA molecules.

Appendix K-IV-K. The universal biosafety sign shall be posted on each closed system and primary containment equipment when used to contain viable organisms containing recombinant DNA molecules.

Appendix K-IV-L. Emergency plans required by Sections IV-B-2-b-(6), *Institutional Biosafety Committee*, and IV-B-3-c-(3), *Biological Safety Officer*, shall include methods and procedures for handling large losses of culture on an emergency basis.

Appendix K-V. Biosafety Level 3 (BL3) - Large Scale

Appendix K-V-A. Spills and accidents which result in overt exposures to organisms containing recombinant DNA molecules are immediately reported to the Biological Safety Officer, Institutional Biosafety Committee, NIH/OBA, and other appropriate authorities (if applicable). Reports to NIH/OBA shall be sent to the Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Drive, Suite 750, MSC 7985, Bethesda, MD 20892-7985 (20817 for non-USPS mail), 301-496-9838, 301-496-9839 (fax). Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

Appendix K-V-B. Cultures of viable organisms containing recombinant DNA molecules shall be handled in a closed system (e.g., closed vessels used for the propagation and growth of cultures) or other primary containment equipment (e.g., Class III biological safety cabinet containing a centrifuge used to process culture fluids) which is designed to prevent the escape of viable organisms. Volumes less than 10 liters may be handled outside of a closed system provided all physical containment requirements specified in Appendix G-II-C, *Physical Containment Levels--Biosafety Level 3*, are met.

Appendix K-V-C. Culture fluids (except as allowed in Appendix K-V-D) shall not be removed from a closed system or other primary containment equipment unless the viable organisms containing recombinant DNA molecules have been inactivated by a validated inactivation procedure. A validated inactivation procedure is one which has been demonstrated to be effective using the organisms that will serve as the host for propagating the recombinant DNA molecules. Culture fluids that contain viable organisms or viral vectors intended as final product may be removed from the primary containment equipment by way of closed systems for sample analysis, further processing or final fill.

Appendix K-V-D. Sample collection from a closed system, the addition of materials to a closed system, and the transfer of culture fluids from one closed system to another shall

be conducted in a manner which prevents the release of aerosols or contamination of exposed surfaces.

Appendix K-V-E. Exhaust gases removed from a closed system or other primary containment equipment shall be treated by filters which have efficiencies equivalent to high efficiency particulate air/HEPA filters or by other equivalent procedures (e.g., incineration) to prevent the release of viable organisms containing recombinant DNA molecules to the environment.

Appendix K-V-F. A closed system or other primary containment equipment that has contained viable organisms containing recombinant DNA molecules shall not be opened for maintenance or other purposes unless it has been sterilized by a validated sterilization procedure except when the culture fluids contain viable organisms or vectors intended as final product as described in Appendix K-V-C above. A validated sterilization procedure is one which has been demonstrated to be effective using the organisms that will serve as the host for propagating the recombinant DNA molecules.

Appendix K-V-G. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules shall be operated so that the space above the culture level will be maintained at a pressure as low as possible, consistent with equipment design, in order to maintain the integrity of containment features.

Appendix K-V-H. Rotating seals and other mechanical devices directly associated with a closed system used to contain viable organisms containing recombinant DNA molecules shall be designed to prevent leakage or shall be fully enclosed in ventilated housings that are exhausted through filters which have efficiencies equivalent to high efficiency particulate air/HEPA filters or through other equivalent treatment devices.

Appendix K-V-I. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules and other primary containment equipment used to contain operations involving viable organisms containing recombinant DNA molecules shall include monitoring or sensing devices that monitor the integrity of containment during operations.

Appendix K-V-J. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules shall be tested for integrity of the containment features using the organisms that will serve as the host for propagating the recombinant DNA molecules. Testing shall be accomplished prior to the introduction of viable organisms containing recombinant DNA molecules and following modification or replacement of essential containment features. Procedures and methods used in the testing shall be appropriate for the equipment design and for recovery and demonstration of the test organism. Records of tests and results shall be maintained on file.

Appendix K-V-K. A closed system used for the propagation and growth of viable organisms containing recombinant DNA molecules shall be permanently identified. This identification shall be used in all records reflecting testing, operation, maintenance, and

use of this equipment for research production activities involving viable organisms containing recombinant DNA molecules.

Appendix K-V-L. The universal biosafety sign shall be posted on each closed system and primary containment equipment when used to contain viable organisms containing recombinant DNA molecules.

Appendix K-V-M. Emergency plans required by Sections IV-B-2-b-(6), *Institutional Biosafety Committee*, and IV-B-3-c-(3), *Biological Safety Officer*, shall include methods and procedures for handling large losses of culture on an emergency basis.

Appendix K-V-N. Closed systems and other primary containment equipment used in handling cultures of viable organisms containing recombinant DNA molecules shall be located within a controlled area which meets the following requirements:

Appendix K-V-N-1. The controlled area shall have a separate entry area. The entry area shall be a double-doored space such as an air lock, anteroom, or change room that separates the controlled area from the balance of the facility.

Appendix K-V-N-2. The surfaces of walls, ceilings, and floors in the controlled area shall be such as to permit ready cleaning and decontamination.

Appendix K-V-N-3. Penetrations into the controlled area shall be sealed to permit liquid or vapor phase space decontamination.

Appendix K-V-N-4. All utilities and service or process piping and wiring entering the controlled area shall be protected against contamination.

Appendix K-V-N-5. Hand washing facilities equipped with foot, elbow, or automatically operated valves shall be located at each major work area and near each primary exit.

Appendix K-V-N-6. A shower facility shall be provided. This facility shall be located in close proximity to the controlled area.

Appendix K-V-N-7. The controlled area shall be designed to preclude release of culture fluids outside the controlled area in the event of an accidental spill or release from the closed systems or other primary containment equipment.

Appendix K-V-N-8. The controlled area shall have a ventilation system that is capable of controlling air movement. The movement of air shall be from areas of lower contamination potential to areas of higher contamination potential. If the ventilation system provides positive pressure supply air, the system shall operate in a manner that prevents the reversal of the direction of air movement or shall be equipped with an alarm that would be actuated in the event that reversal in the direction of air movement were to occur. The exhaust air from the controlled area shall not be recirculated to other areas of

the facility. The exhaust air from the controlled area may not be discharged to the outdoors without being high efficiency particulate air/HEPA filtered, subjected to thermal oxidation, or otherwise treated to prevent the release of viable organisms.

Appendix K-V-O. The following personnel and operational practices shall be required:

Appendix K-V-O-1. Personnel entry into the controlled area shall be through the entry area specified in Appendix K-V-N-1.

Appendix K-V-O-2. Persons entering the controlled area shall exchange or cover their personal clothing with work garments such as jump suits, laboratory coats, pants and shirts, head cover, and shoes or shoe covers. On exit from the controlled area the work clothing may be stored in a locker separate from that used for personal clothing or discarded for laundering. Clothing shall be decontaminated before laundering.

Appendix K-V-O-3. Entry into the controlled area during periods when work is in progress shall be restricted to those persons required to meet program or support needs. Prior to entry, all persons shall be informed of the operating practices, emergency procedures, and the nature of the work conducted.

Appendix K-V-O-4. Persons under 18 years of age shall not be permitted to enter the controlled area.

Appendix K-V-O-5. The universal biosafety sign shall be posted on entry doors to the controlled area and all internal doors when any work involving the organism is in progress. This includes periods when decontamination procedures are in progress. The sign posted on the entry doors to the controlled area shall include a statement of agents in use and personnel authorized to enter the controlled area.

Appendix K-V-O-6. The controlled area shall be kept neat and clean.

Appendix K-V-O-7. Eating, drinking, smoking, and storage of food are prohibited in the controlled area.

Appendix K-V-O-8. Animals and plants shall be excluded from the controlled area.

Appendix K-V-O-9. An effective insect and rodent control program shall be maintained.

Appendix K-V-O-10. Access doors to the controlled area shall be kept closed, except as necessary for access, while work is in progress. Serve doors leading directly outdoors shall be sealed and locked while work is in progress.

Appendix K-V-O-11. Persons shall wash their hands when exiting the controlled area.

Appendix K-V-O-12. Persons working in the controlled area shall be trained in emergency procedures.

Appendix K-V-O-13. Equipment and materials required for the management of accidents involving viable organisms containing recombinant DNA molecules shall be available in the controlled area.

Appendix K-V-O-14. The controlled area shall be decontaminated in accordance with established procedures following spills or other accidental release of viable organisms containing recombinant DNA molecules.

Appendix K-VI. Footnotes of Appendix K

Appendix K-VII. Definitions

Appendix K-VII-A. Accidental Release. An accidental release is the unintentional discharge of a microbiological agent (i.e., microorganism or virus) or eukaryotic cell due to a failure in the containment system.

Appendix K-VII-B. Biological Barrier. A biological barrier is an impediment (naturally occurring or introduced) to the infectivity and/or survival of a microbiological agent or eukaryotic cell once it has been released into the environment.

Appendix K-VII-C. Closed System. A closed system is one in which by its design and proper operation, prevents release of a microbiological agent or eukaryotic cell contained therein.

Appendix K-VII-D. Containment. Containment is the confinement of a microbiological agent or eukaryotic cell that is being cultured, stored, manipulated, transported, or destroyed in order to prevent or limit its contact with people and/or the environment. Methods used to achieve this include: physical and biological barriers and inactivation using physical or chemical means.

Appendix K-VII-E. *De minimis* Release. *De minimis* release is the release of: (i) viable microbiological agents or eukaryotic cells that does not result in the establishment of disease in healthy people, plants, or animals; or (ii) in uncontrolled proliferation of any microbiological agents or eukaryotic cells.

Appendix K-VII-F. Disinfection. Disinfection is a process by which viable microbiological agents or eukaryotic cells are reduced to a level unlikely to produce disease in healthy people, plants, or animals.

Appendix K-VII-G. Good Large Scale Practice Organism. For an organism to qualify for Good Large Scale Practice consideration, it must meet the following criteria [Reference: Organization for Economic Cooperation and Development, *Recombinant DNA Safety Considerations*, 1987, p. 34-35]: (i) the host organism should be non-pathogenic, should not contain adventitious agents and should have an extended history of safe large-scale use or have built-in environmental limitations that permit optimum

growth in the large-scale setting but limited survival without adverse consequences in the environment; (ii) the recombinant DNA-engineered organism should be non-pathogenic, should be as safe in the large-scale setting as the host organism, and without adverse consequences in the environment; and (iii) the vector/insert should be well characterized and free from known harmful sequences; should be limited in size as much as possible to the DNA required to perform the intended function; should not increase the stability of the construct in the environment unless that is a requirement of the intended function; should be poorly mobilizable; and should not transfer any resistance markers to microorganisms unknown to acquire them naturally if such acquisition could compromise the use of a drug to control disease agents in human or veterinary medicine or agriculture.

Appendix K-VII-H. Inactivation. Inactivation is any process that destroys the ability of a specific microbiological agent or eukaryotic cell to self-replicate.

Appendix K-VII-I. Incidental Release. An incidental release is the discharge of a microbiological agent or eukaryotic cell from a containment system that is expected when the system is appropriately designed and properly operated and maintained.

Appendix K-VII-J. Minimization. Minimization is the design and operation of containment systems in order that any incidental release is a *de minimis* release.

Appendix K-VII-K. Pathogen. A pathogen is any microbiological agent or eukaryotic cell containing sufficient genetic information, which upon expression of such information, is capable of producing disease in healthy people, plants, or animals.

Appendix K-VII-L. Physical Barrier. A physical barrier is considered any equipment, facilities, or devices (e.g., fermentors, factories, filters, thermal oxidizers) which are designed to achieve containment.

Appendix K-VII-M. Release. Release is the discharge of a microbiological agent or eukaryotic cell from a containment system. Discharges can be incidental or accidental. Incidental releases are *de minimis* in nature; accidental releases may be *de minimis* in nature.

APPENDIX 6. Agents requiring BSL 3 Ag for work with loose animals

- Avian influenza virus (highly pathogenic)
- African swine fever virus
- Classical swine fever
- Foot and mouth disease virus
- Lumpy skin disease virus
- Mycoplasma mycoides subsp. mycoides, (small colony type)
- Mycoplasma capricolum
- Newcastle disease virus (velogenic strains)
- Peste des petits ruminants (plague of small ruminants)

- Rift Valley fever virus
- Rinderpest virus

APPENDIX 7. Laboratory acquired infections between 1979-2004 (From Harding and Byers, 2006.in press)

Category of Agent	**Symptomatic LAIs[a]**	**Asymptomatic LAIs[a]**	**Total primary LAIs**	**# Deaths**	**2nd Infections**	**# Publications**
Bacteria	598	60	658	17[b]	7	125
Rickettsia	187	214	401	1	0	13
Viruses	608	430	1,038	18[c]	10	97
Parasites	49	4	53	0	0	30
Fungi	6	0	6	0	0	5
Total	1,448	708	2,156	36	17	270

[a]LAIs resulting from primary infections; secondary infections not included in totals.

[b]Four deaths were attributed to aborted fetuses resulting from *Brucella melitensis* exposures and 1 to a secondary contact exposed to a multi-drug resistant *Salmonella agoni*.

[c]One death was attributed to an aborted fetus associated with Parvovirus infection and 1 to a secondary contact exposed to SARS.

*APPENDIX 8. Risk assessment matrix for agent hazards**

RISK FACTORS / AGENT HAZARDS	DEGREE OF LABORATORY RISK		
	LOW TO MODERATE	**MODERATE TO HIGH**	**HIGH**
Pathogenicity	Mild to moderate disease (*Salmonella typhimurium*)	Moderate to serious disease (*Mycobacterium tuberculosis*)	Severe disease (Cercopithecine herpes virus)
Virulence	Mild to moderate disease or low infectivity	Severe disease or moderate infectivity	Lethal disease or high infectivity
Infective dose	$>10^6$ IU (*Vibrio cholerae*)	10^6 – 100 IU (Influenza A virus)	<100 IU (*Francisella tularensis*}
Transmission	Indirect contact (contact with contaminated surfaces, animal bedding)	Direct contact (droplet, tissue, fluid, secretion contact with mucous membranes; ingestion)	Inhalation or percutaneous inoculation (needle stick)
Stability	Survive minutes to hours on surfaces (Measles virus)	Survive days to weeks on surfaces (Hepatitis B virus)	Survive weeks to months in hostile environment (*Coxiella burnetii*)
Animal host range	Not likely to cross species barrier	Broad host range but not known to cause disease in humans	Zoonoses (Hanta virus)
Occurrence of natural disease	Endemic	Not endemic	Importation controlled by CDC or USDA
Probable causes of laboratory-associated infections	Absence of LAI reports	Accidents; percutaneous; ingestion; unknown	Evidence of inhalation transmission
WHO Risk Group**	Risk Group 2 (moderate individual risk, low community risk)	Risk Group 3 (high individual risk, low community risk)	Risk Group 4 (high individual and community risk)

*adapted from W. E. Barkley, personal communication

** See WHO RG definitions in Appendix 3.

APPENDIX 9. RISK ASSESSMENT MATRIX FOR PROTOCOL HAZARDS*

Protocol Hazards	Low Risk	Moderate Risk	High Risk
Agent Concentration[a]	$<10^3$ IU/ml	$10^3 - 10^6$ IU/ml	$>10^9$ IU/ml
Suspension volume	<1 ml	1 ml – 1 L	>1L
Equipment/procedures that generate droplets and 2-10 μm particle aerosols	Streaking "smooth" agar on a Petri dish	Opening blender lid after 1 min; pipetting with minimal bubbles; Streaking "rough" agar on a Petri dish	Opening blender lid after stop; Flaming an inoculating loop; pipetting with bubbles
Protocol Complexity	Standard repetitive procedures	Periodic change in procedures	Frequent change and complex procedures
Use of Animals	Use of safe animal care practices	Necropsies; large animals handling	Aerosol challenge protocols
Use of Sharps		With protective devices; safety sharps	Without protective devices

*adapted from W. E. Barkley, personal communication

[a]The risk related to the agent concentration depends upon the infectious dose, which can be very small. (for Coxiella burnetti high risk is 1-10 IU).

APPENDIX 10. Concentration and particle size of aerosols created during representative laboratory techniques[a]

Operation	No. of viable colonies[b]	Particle size[c] (μm)
Mixing culture with:		
Pipet	6.6	2.3 ± 1.0
Vortex mixer (15 sec.)	0.0	0.0
Mixer overflow	9.4	4.8 ± 1.9
Use of blender:		
Top on	119.6	1.9 ± 0.7
Top off	1,500.0	1.7 ± 0.5
Use of a sonicator	6.3	4.8 ± 1.6
Lyophilized cultures:		
Opened carefully	134.0	10.0 ± 4.3
Dropped and broken	4,838.0	10.0 ± 4.8

[a]Adapted from Kenny and Sabel,1968.

[b]Mean number of viable colonies per cubic foot of air sampled.

[c]Count median diameter of particle.

(Table 3 from Harding and Byers, 2006)

APPENDIX 11. Risk assessment matrix for Susceptibility to Disease*

RISK FACTORS	DEGREE OF LABORATORY RISK		
	LOW TO MODERATE	MODERATE TO HIGH	HIGH
Susceptibility to disease			
Potential for exposure	Non-lab person associated with the lab; Intermittent visitor to lab	Lab worker in room where agent is handled	Lab worker who handles agent
Individual susceptibility	Effective immunization	Competent immune status	Compromised immune status
Availability of effective vaccine or other protective prophylaxis	Yes	Less effective prophylaxis	No
Availability of effective treatments and therapeutic agents	Yes	Treatments and therapeutic agents offer some value	No

*adapted from W. E. Barkley, personal communication

From Genetically Modified Organisms To Synthetic Biology: Legislation in the European Union, in Six Member Countries and in Switzerland

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Introduction

This report is based on the assumption that in Europe and in its member countries—with the exception perhaps of Switzerland—"synthetic genomics" as a distinct policy domain does not yet exist. This conclusion is based on considerable empirical evidence. Since I was approached last October (2005) by the project leaders I have systematically been monitoring the use of this term in the news media around the (English speaking) world. I have done so in two ways: with the help of several Google search robots and by scanning various Lexis-Nexis news databases using the term "synthetic genomics" and its translation in German, Italian French, Spanish and Dutch.

The search has produced very modest results. To date, there has been very few instances of news reporting focused on synthetic genomics. One of them was the founding by Dr. Craig Venter of Synthetic Genomics. Another one has been the launch of the project this review paper has been prepared for. And the third one was the appointment of Dr. Ari Patrinos to President of Synthetic Genomics. In addition, there has been sporadic report on the discipline itself, but rarely in connection with possible novel risks. With regard to Europe, no news stories have been found focusing on synthetic genomics per se or on possible new dangers stemming from its development.

The results emerging from the media analysis are entirely consistent with the EU priorities in the area of public health and consumer protection. The EU Directorate General on Consumer Protection and Safety over the years has established numerous scientific committtess and advisory boards. Each of these committees is focused on a specific policy domain and is responsible for advising the European Commission on

matters pertaining to its area of expertise. Based on the available information, it appears that none of the currently chartered scientific and regulatory committees has included synthetic genomics in its policy agenda. In 2003 the Scientific Committee on Emerging and Newly Identified Health Risks prepared a report for the Commission titled "Priorities in the EU for Risk Assessment in the Non-Food Area". The report examines a wide variety of possible future threats to the health of European citizens and to the natural environment. The document runs 76 pages but contains no reference to either synthetic genomics or synthetic biology.

It would have been highly interesting to discuss with the Committee members whether the subject of synthetic genomics has been identified as a possible future area of concern, and if so the reasons for not including it in their report. Unfortunately, several attempts to get in touch with this committee and its members have produced no tangible results. In 2004 the Commission enacted very stringent procedural rules designed to prevent or mitigate attempts at influencing the Committee members, to ensure transparency and more generally good governance. These rules have prevented me from obtaining any feedback, formal or informal, for attribution or on background, from the Committee secretariat or the Committee members. Among other things, these rules do not allow the Committee to provide any information to individual citizens, i.e. individuals with no recognizable institutional affiliation. These limitations notwithstanding it is reasonable to assume that "synthetic genomics" currently is not on the agenda of European policy-makers. For this reason this report does not attempt to identify legislative or regulatory measures taken by the EU or its member countries and targeted at synthetic genomics proper, as these measures simply have not been taken.

A more interesting question is whether "synthetic biology," as opposed to synthetic genomics, has attracted the attention of European policy makers. Unlike synthetic genomics, synthetic biology is being regarded by the Research Directorate-General as a scientific discipline in its own right, albeit a very young one. Synthetic biology was not included in the 6th Framework Program (FP), and it is unclear whether it be will included it in the 7th FP. However, the EU has begun funding synthetic biology as part of its NEST (New and Emerging Science and Technology) activities. Synthetic biology, whose

research agenda and priorities have been described in a recently published expert report, appears to share many of the goals synthetic genomics purports to pursue. For example, both disciplines envision adopting a rigorous engineering approach to the design of organisms as it is common in mechanical and electrical engineering. That these two fields are quite similar is not entirely surprising, considering that two of the authors of the EU experts report are based in the United States, with strong ties to the scientific leaders in synthetic genomics. For this reason, in this report I will be focusing on the synthetic biology rather than on synthetic genomics.

Whether EU policymakers regard synthetic biology as a scientific development distinct from genetic engineering, and whether this scientific discipline is likely to create genuine new challenges is a question that at this time cannot be answered with any degree of confidence. As mentioned earlier, EU officials have not been willing to provide any insight. On the other end, anecdotal evidence suggests that the scientists—when it comes to assessing possible risks and dangers associated with synthetic biology—do not believe that this scientific development is likely to create any fundamental new challenge. In this view when it comes to the risks to human health, safety and the natural environment synthetic biology is no different than genetic engineering as practiced over the last 25 years. Accordingly, these scientists have expressed the view that the rules and regulations currently in place and governing research activities in contained environments, as well as the regulations designed to protect the health and safety of the personnel working in these environments and the measure taken to minimize the risks associated with the release in the natural environment of genetically modified organisms, with few exceptions, are more than adequate for the task at hand.

Whether this conclusion is shared by a majority of the scientists operating in this field remains to be seen. It appears that regulators may differ somewhat in their assessment of the risks posed by this new scientific development. Also largely unclear at this point is whether malicious intent is being considered by either policymakers or scientists as a serious threat.

Based on the working hypothesis that synthetic biology, for a while at least, is not likely to pose many new threats to health, safety and the natural environment in the remainder of this paper I have compiled the laws and regulations pertaining to the contained use of genetically manipulated (or modified) organisms (GMOs), to the safety of the personnel involved in contained facilities and their surroundings and concerning the release of GMOs in the natural environment. The review includes EU norms as well as laws and regulations adopted in Germany, Britain, France, Italy, Austria, Belgium and Switzerland. The selection of these countries was motivated by both conceptual and practical considerations. I have included European leaders in science and engineering such as Germany and the United Kingdom, but also smaller countries such as Belgium, Austria and Switzerland. Switzerland, while formally not a member of the European Union, is actively involved in synthetic biology and entertains close ties with the European Union in all policy areas, including science policy.

Austria

Statutory Framework

- **BUNDESGESETZ, mit dem Arbeiten mit gentechnisch veränderten Organismen, das Freisetzen und Inverkehrbringen von gentechnisch veränderten Organismen** und die Anwendung von Genanalyse und Gentherapie am Menschen geregelt und das Produkthaftungsgesetz (Gentechnikgesetz (GTG) – BGBl. I Nr. 510, July 7, 1994)
- **BUNDESGESETZ über Sicherheit und Gesundheitsschutz bei der Arbeit** (ArbeitnehmerInnenschutzgesetz (ASchG) – BGBl. Nr. 450, June 17, 1994)

Deliberate Release of GMOs in the Environment

- **VERORDNUNG** der Bundesministerin für Gesundheit und Frauen über Inhalt, Umfang und Form von Anträgen auf Genehmigung einer Freisetzung oder des Inverkehrbringens von gentechnisch veränderten Organismen, die Sicherheitsbewertung und den Überwachungsplan –(Freisetzungsverordnung – BGBl. II Nr. 260, August 24, 1997)
-

Contained Use of Pathogenic and/or Genetically Modified Organisms

- **VERORDNUNG** des Bundesministers für soziale Sicherheit und Generationen über die Sicherheit bei Arbeiten mit gentechnisch veränderten Organismen in

geschlossenen Systemen (Systemverordnung – BGBl. II Nr. 431, November 29, 2002)

- **VERORDNUNG** der Bundesministerin für Gesundheit und Konsumentenschutz über das Anhörungsverfahren gemäß dem Gentechnikgesetz (Anhörungsverordnung – BGBl. II Nr. 61, February 28, 1997)

Protection of Workers Exposed to Biological Agents at Work

- **VERORDNUNG** der Bundesministerin für Arbeit, Gesundheit und Soziales über den Schutz der Arbeitnehmer/innen gegen Gefährdung durch biologische Arbeitsstoffe (Verordnung biologische Arbeitsstoffe (VbA) – BGBl. II Nr. 237, July 23, 1998).

Regulatory Authority

- Bundesministerium für Arbeit Gesundheit und Frauen: Health related rules and regulations
- Bundesministerium für Bildung Wissenschaft und Kultur, Bereich Gentechnik und Tierversuchwsen: Contained use of GMOs
- Bundesministerium für sozial Sicherheit, Generationen und Konsumentenschutz: Contained use of GMOs
- Bundesministerium für Gesundheit und Frauen: deliberate release of GMOs
- Bundesminister für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft: commercial distribution of GMOs

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Belgium

Legal Framework

Belgium, unlike other European countries, has not enacted comprehensive legislation pertaining to the contained use and intentional release of GMOs in the environment. Rather, it has adopted and implemented most relevant European directives.

While in the case of the deliberate release of GMOs Belgium has passed regulations at the federal level, it has delegated to the regions the task of implementing European legislation regarding contained use of GMOs. However, the evaluation of research proposals involving the genetically modified organisms is conducted by a national scientific committee. This common advisory system is founded by a Cooperation Agreement concerning Biosafety.

Deliberate Release of GMOs in the Environment

- **ARRÊTÉ ROYAL** du 21 février 2005 réglementant la dissémination volontaire dans l'environnement ainsi que la mise sur le marché d'organismes génétiquement modifiés ou de produits en contenant. (Moniteur Belge, February 24, 2005, p. 7129) : This Decree implements and enforces Directive 2001/18/EC.
- **LAW of February 22, 1998 (amending the law of July 20, 1991), especially articles 222 and 226.**
- **LAW of July 20, 1991, especially article 132**: provisions concerning the deliberate release in the environment of genetically modified organisms.

Contained Use of Pathogenic and/or Genetically Modified Organisms

- The European legislation has been transposed at the regional level as a part of the Regional Environmental laws for classified installations.
- These regulations implement European Directive 98/81/EC revising Directive 90/219/EEC, and related Decisions 2000/608/EC and 2001/204/EC.

Protection of Workers Exposed to Biological Agents at Work

- **ARRÊTÉ ROYAL** modifiant l'arrêté royal du 4 août 1996 concernant la protection des travailleurs contre les risques liés à l'exposition à des agents biologiques au travail (Moniteur Belge, p. 37917, October 7, 1999)
  - This royal decree implements European Directives 90/679/EEC and Amendments 93/88/EEC, 95/30/CE, 97/59/CE and 97/65/CE.

The Cooperation Agreement

- The "Cooperation Agreement between the Federal State and the Regions on the Administrative and Scientific Co-ordination Concerning Biosafety" establishes a single scientific advisory system common to the Federal and Regional authorities scientific aspects related to the uses of genetically modified organisms (GMOs) and pathogens are assessed in a coordinated way.
- The common scientific evaluation system is composed of the Biosafety Advisory Council (BAC) and the Service of Biosafety and Biotechnology (SBB).

Advisory Boards

- **Biosafety Advisory Council**
  - The BAC advises the competent authorities on the safety of any activities using GMOs and pathogens, including genetic and ecological aspects related to biodiversity.
  - The Council can be consulted by the Regions or the SBB for the contained use activities (laboratories, greenhouses, animal husbandries, production plants). It must be consulted for the deliberate release of GMOs in the environment and the placing on the market of all GMOs and GMO-based products.
  - The Council consists of representatives of the Regional and Federal authorities. It is assisted by experts in its scientific work. The secretariat of the Council is ensured by the SBB.

- **Service of Biosafety and Biotechnology**
  - The Service of Biosafety and Biotechnology (SBB) is composed of an administrative secretariat, a multidisciplinary group of scientists and a laboratory for biosafety research and expertise.

- **Regulatory authority**
  - Service public fédéral (SPF) Santé publique, Sécurité de la Chaîne alimentaire et Environnement (Federal Public Service (FPS) Health, Food Chain Security and Environment): Deliberate release into the environment of GMOs.
  - The regulatory authority for the contained use of GMOs is delegated to the three federal regions (the Flemish, Walloon and Brussels-Capital region).
  - Ministère fédéral de l'Emploi et du Travail, Administration de l'hygiène et de la médecine du travail (Federal Ministry for Employment, Work, Hygiene and employment medicine Administration): responsible for Worker safety.

Entry Points

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European Union

Deliberate Release into the Environment of Genetically Modified Organisms

- **COMMISSION DECISION 2004/204/EC** of 23 February 2004 laying down detailed arrangements for the operation of the registers for recording information on genetic modifications in GMOs, provided for in Directive 2001/18/EC of the European Parliament and of the Council (Official Journal L 65, 3.3.2004, p. 20)
- **COMMISSION REGULATION (EC) No 65/2004** of 14 January 2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms (OJ L 10, 16.1.2004, p. 5)
- **COUNCIL DECISION 2002/813/EC** of 3 October 2002 establishing, pursuant to Directive 2001/18/EC of the European Parliament and of the Council, the summary notification information format for notifications concerning the deliberate release into the environment of genetically modified organisms for purposes other than for placing on the market (OJ L 280, 18.10.2002, p. 62)

- **COUNCIL DECISION 2002/812/EC** of 3 October 2002 establishing pursuant to Directive 2001/18/EC of the European Parliament and of the Council the summary information format relating to the placing on the market of genetically modified organisms as or in products (OJ L 280, 18.10.2002, p. 37)
- **COUNCIL DECISION 2002/811/EC** of 3 October 2002 establishing guidance notes supplementing Annex VII to Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC (OJ L 280, 18.10.2002, p. 27)
- **COMMISSION DECISION 2002/623/EC** of 24 July 2002 establishing guidance notes supplementing Annex II to Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC (OJ L 200, 30.7.2002, p. 22)
- **DIRECTIVE 2001/18/EC** of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC (OJ L 106, 17.4.2001, p. 1)
  - Directive 90/220 CEE du 23 avril 1990 relative à la dissémination volontaire dans l'environnement d'Organismes Génétiquement Modifiés (OGM).

Contained Use of Pathogenic and/or Genetically Modified Organisms

- **COMMISSION DECISION 005/174/EC** of 28 February 2005 establishing guidance notes supplementing part B of Annex II to Council Directive 90/219/EEC on the contained use of genetically modified microorganisms (notified under document number C(2005) 413) (Text with EEA relevance). Official Journal L 059, 05/03/2005 p. 20 – 25.
- **COUNCIL DECISION 2001/204/EC** of 8 March 2001 supplementing Directive 90/219/EEC as regards the criteria for establishing the safety, for human health and the environment, of types of genetically modified microorganisms (Text with EEA relevance) Official Journal L 073, 15/03/2001 p. 32 – 34.
- **COMMISSION DECISION 2000/608/EC** of 27 September 2000 concerning the guidance notes for risk assessment outlined in Annex III of Directive 90/219/EEC on the contained use of genetically modified microorganisms (notified under document number C(2000) 2736) (Text with EEA relevance) Official Journal L 258 , 12/10/2000 p. 43 – 48.
- **COUNCIL DIRECTIVE 98/81/EC** of 26 October 1998 amending Directive 90/219/EEC on the contained use of genetically modified microorganisms. Official Journal L 330 , 05/12/1998 p. 13 – 31.
- **COUNCIL DIRECTIVE 90/219/EEC** of 23 April 1990 on the contained use of genetically modified microorganisms. Official Journal L 117, 08/05/1990 p. 1 – 14.

Protection of Workers Exposed to Biological Agents at Work

- **DIRECTIVE 2000/54/EC** of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work. Seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EEC (OJ Journal L 262, 17/10/2000 p. 21 – 45).
  - Directive 2000/54/EC codifies Directive 90/679/EEC as well as its successive amendments. It therefore repeals these different Directives and replaces them.
- **Council Directive 89/391/EEC** of 12 June 1989 on the introduction of measures to encourage improvements in the safety and health of workers at work (OJ L 183, 29/06/1989 p. 1 – 8).
  - Council Directive 90/679/EEC of 26 November 1990 on the protection of workers from risks related to exposure to biological agents at work (seventh individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC) (Official Journal L 374 – 31.12.1990).

  Amended by the following acts:
  - Council Directive 93/88/EEC of 12 October 1993 (Official Journal L 268 of 29.10.1993)
  - Commission Directive 95/30/EC of 30 June 1995 (Official Journal L 155 of 06.07.1995)
  - Commission Directive 97/59/EC of 7 October 1997 (Official Journal L 282 of 15.10.1997)
  - Commission Directive 97/65/EC of 26 November 1997 (Official Journal L 335 of 06.12.1997)

Advisory Boards

Scientific Committee on Health and Environmental Risks

Examines questions relating to examinations of the toxicity and ecotoxicity of chemicals, biochemicals and biological compounds whose use may have harmful consequences for human health and the environment.

The Committee will address questions in relation to new and existing chemicals, the restriction and marketing of dangerous substances, biocides, waste, environmental contaminants, plastic and other materials used for water pipe work (e.g. new organics substances), drinking water, indoor and ambient air quality. It will address questions relating to human exposure to mixtures of chemicals, sensitisation and identification of endocrine disrupters.

Scientific Committee on emerging and Newly-Identified Health Risks

Examines questions concerning emerging or newly-identified risks and on broad, complex or multi-disciplinary issues requiring a comprehensive assessment of risks to consumer safety or public health and related issues not covered by other Community risk-assessment bodies.

As of this writing, it has been impossible to discuss with representatives of this Committee, formally or informally, for personal attribution or on background, whether this Committee has included synthetic biology in its work and what is its assessment of the potential risks and dangers associated with this new scientific developments.

Policy-Making/Regulatory Bodies

Directorate General – Health and Consumer Protection, Directorate Public Health – Risk Assessment

France

Statutory Framework

- **CODE DE L'ENVIRONNEMENT (Partie Législative)**

  Livre V: Prévention des pollutions, des risques et des nuisances
  Titre III: Organismes génétiquement modifiés
  Chapitre Ier: Dispositions générales
  Chapitre II: Utilisation confinée des organismes génétiquement modifiés
  Chapitre III: Dissémination volontaire et mise sur le marché d'organismes génétiquement modifiés
- **CODE DU TRAVAIL (Partie Législative)**

  Livre II: Réglementation du travail
  Titre III: Hygiène, sécurité et conditions de travail

Deliberate Release into the Environment of Genetically Modified Organisms

- **DIRECTIVE 2001/18/EC** of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC (OJ L 106, 17.4.2001, p. 1)

Contained Use of Pathogenic and/or Genetically Modified Organisms

- **COUNCIL DIRECTIVE 98/81/EC** of 26 October 1998 amending Directive 90/219/EEC on the contained use of genetically modified micro-organisms. Official Journal L 330, 05/12/1998 p. 13 – 31.
- **COUNCIL DIRECTIVE 90/219/EEC** of 23 April 1990 on the contained use of genetically modified micro-organisms. Official Journal L 117, 08/05/1990 p. 1 – 14.

Protection of Workers Exposed to Biological Agents at Work

- **ARRÊTÉ DU 30 JUILLET 2004** relatif à la mise en oeuvre, l'importation, l'exportation, la détention, la cession à titre gratuit ou onéreux, l'acquisition et le transport de certains agents responsables de maladies infectieuses, micro-organismes pathogènes et toxines. (NOR: SANP0422322A - JO n° 182 du 7 août 2004 page 14114).
  - Decree concerning the use, importation, exportation, distribution (free or commercial), receiving and the transport of certain biological agents responsible for infectious diseases, pathogens and toxins.
- **ARRÊTÉ DU 18 JUILLET 1994** fixant la liste des agents biologiques pathogènes (NOR: TEFT9400844A - JO n° 175 du 30 juillet 1994 page 11078): list of pathogens.
- **DÉCRET NO 94-352 DU 4 MAI 1994** relatif à la protection des travailleurs contre les risques résultant de leur exposition à des agents biologiques et modifiant le code du travail (deuxième partie: Décrets en Conseil d'Etat - Section VI "Prevention du risque biologique"). (NOR: TEFT9400313D - JO n° 105 du 6 mai 1994 page 6620)
  - These and other regulatory measures are integrated in the "code du travail – partie réglementaire" as follows:
- **CODE DU TRAVAIL (Partie Réglementaire - Décrets en Conseil d'Etat):** Livre II: Réglementation du travail, Titre III: Hygiène et sécurité, Chapitre Ier , Section 6: Prévention du risque biologique:
  - Sous-section 1: Définitions
  - Sous-section 2: Règles générales d'évaluation et de prévention du risque biologique
  - Sous-section 3: Formation et information
  - Sous-section 4: Dispositions particulières à certaines activités
  - Sous-section 5: Surveillance médicale spéciale

Advisory Boards

- **Commission de Génie Génétique**
  - The Law 92-654 of July 13, 1992 article 3-1 defines the Genetic Engineering Commission's mission (Commission de Génie Génétique, CGG) as follows (summary):
  - The CGG is charged of evaluating the risks and dankers created by genetically modified organisms and the procedures used for their creation, as well as the potential risks and dangers associated with the use of genetic engineering technologies.
  - Concerning the contained use, the CGG proposes containment measures to prevent the risks assocaited with the use of GMOs, of procedures and techniques.

Regulatory Authority

- Ministère de la santé et de la protection sociale
- Centre national de la recherche scientifique / Ministère chargé de la recherche
- Ministère de l'agriculture, de l'alimentation, de la pêche et des affaires rurales

Germany

Statutory Framework

- **GENTECHNIKGESETZ (GenTG) 1993:** govern all uses of genetically modified organisms except for human applications.
- **GESETZ zur Anpassung von Zuständigkeiten im Gentechnikrecht 2004**: decribes changes in regulatory authority as required by the "Gentechnikrecht 2004".
- **EG-GENTECHNIK-DURCHFÜHRUNGSGESETZ (EGGenTDurchfG) 2004**: statute governing the implementation of EU regulations pertaining to biotechnology.

Deliberate Release into the Environment of Genetically Modified Organisms

- **VERORDNUNG über Aufzeichnungen bei gentechnischen Arbeiten und bei Freisetzungen (Gentechnik-Aufzeichnungsverordnung – GenTAufzV) 1996**: Regulation establishing mandatory protocols for conducting genetic manipulations and for the release of genetically modified organisms (BGBl. I S. 1645 – 4. November 1996)

Contained Use of Pathogenic and/or Genetically Modified Organisms

- **VERORDNUNG über die Erstellung von außerbetrieblichen Notfallplänen und über Informations-, Melde- und Unterrichtungspflichten (GenTNotfV) 1997**: regulation for the implementation of community emergency plans. Also estabishes information and reporting requirements (BGBl. (Bundesgesetzblatt) I S. 2882 – 10. Oktober 1997)
- **VERORDNUNG über Anhörungsverfahren nach dem Gentechnikgesetz (Gentechnik-Anhörungsverordnung – GenTAnhV) 1996**: regulation mandating public hearings for the etablishment or modification of commercial facilities conducting safety level 3 or level 4 activities (BGBl. I S. 1649 – 4. November 1996)
- **VERORDNUNG über die Sicherheitsstufen und Sicherheitsmaßnahmen bei gentechnischen Arbeiten in gentechnischen Anlagen (Gentechnik Sicherheitsverordnung – GenTSV) 1995**: defines safety levels and describes safety measures (BGBl. I S. 297 – 14. März 1995)

Advisory Boards

Zentrale Kommission für die Biologische Sicherheit

The "central commission for biosafety" is an expert body charged with examining possible risks for humans, animals and the natural environment. associated with genetically manipulated organisms. Established by the "Gentechnikgesetz" §§ 4 und 5 regulation ZKBSV.

- **VERORDNUNG über die Zentrale Kommission für die Biologische Sicherheit (ZKBS-Verordnung – ZKBSV) 1996**: creates a "biosafety commission", describes its duties and responsibilities, and establishes procedural norms (BGBl. I S. 1232 – 5. August 1996).

Protection of Workers Exposed to Biological Agents at Work

- **VERORDNUNG zum Schutz vor Gefahrstoffen (Gefahrenstoffverordnung – GefStoffV) 2005**: Article 8 of this regulation affects BioStoffV with regard to the definition of affected individuals, procedures of risk assessment, employee information and medical record-keeping requirements.
- **VERORDNUNG über Sicherheit und Gesundheitsschutz bei Tätigkeiten mit biologischen Arbeitsstoffen (Biostoffverordnung BioStoffV) 1999**: implements EU directive 90/679/EEC of November 1990 (seventh individual directive within the meaning of article 16(1) of directive 89/391/EEC) (BGBl. I S. 50 – 27. Januar 1999)

Regulatory Authority

- Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL)

Italy

Deliberate Release into the Environment of Genetically Modified Organisms

- **DECRETO LEGISLATIVO 8 luglio 2003, n.224**: Attuazione della direttiva 2001/18/CE concernente l'emissione deliberata nell'ambiente di organismi geneticamente modificati: implements EU Directive 2001/18/EC regulating the intentional release of genetically modified organisms.

Contained Use of Pathogenic and/or Genetically Modified Organisms

- **DECRETO LEGISLATIVO 12 aprile 2001, n. 206**: attuazione della direttiva 98/81/CE del Consiglio che modifica la direttiva 90/219/CEE concernente l'impiego confinato di microorganismi geneticamente modificati: implements EU Directive 98/81/EC governing the contained use of genetically modified organisms.

Protection of Workers Exposed to Biological Agents at Work

- **DIRECTIVE 2000/54/EC** of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work. Seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EEC (OJ Journal L 262, 17/10/2000 p. 21 – 45): this directive replaces and therefore repeals directive 90/679/EEC.
- **DECRETO LEGISLATIVO 19 settembre 1994 n. 626**: Attuazione delle direttive 89/391/CEE, 89/654/CEE, 89/655/CEE, 89/656/CEE, 90/269/CEE, 90/270/CEE, 90/394/CEE e 90/679/CEE riguardanti il miglioramento della sicurezza e della salute dei lavoratori sul luogo di lavoro.
  - As the title implies, this legislative decree implements several major individual EU directives designed to improve worker health and safety. As discussed in section xx above, directive 89/391/EEC establishes a general framework for maintaining health and worker safety, while directive 90/679/EEC deals specifically with biological risks.

Advisory Boards

- **Commissione interministeriale di valutazione**
  - The "Comitato Nazionale per la Biosicurezza e le Biotecnologie (CNBB)", i.e. the National Committee for Biosafety and Biotechnologies has been established by decree in 2001 (decreto istitutivo del Comitato per la Biosicurezza a le Biotecnologie – DPCM 14.XI.2001).
  - This committee does more than just discharging the functions described in EU directive 98/81/EC Art 14. It performs numerous coordination functions and is also responsible for evaluating possible health and safety risks from the release of genetically manipulated organisms in the environment. Enabling legislation:

Regulatory Authority

- Ministero della Sanità (Health Ministry): Contained use of GMOs
- Ministero dell'Ambiente e della Tutela del Territorio (Ministry of the Environment): Intentional release of GMOs.

Switzerland

While Switzerland technically is not a member of the European Union, it actively collaborates with the Union in numerous policy areas, including science and technology policy. Switzerland has a long-standing policy of crafting "euro-compatible" legislation. This means that laws and regulations are routinely checked for their consistency with European norms. Furthermore, in the area of synthetic biology Swiss scientists (or scientists operating at Swiss Universities) play a key role. In addition, Switzerland is represented with one expert on the Committee on emerging and Newly Identified Health Risks. Finally, there is some evidence that of all European countries Switzerland is the only one that has begun informally exploring posible societial consequences of synthetic biology.

Statutory Framework

- **BUNDESGESETZ vom 21. März 2003 über die Gentechnik im Ausserhumanbereich (Gentechnikgesetz, GTG):** legal framework governing the creation and use of genetically modified organisms (medical applications excluded)
- **BUNDESGESETZ über die Bekämpfung übertragbarer Krankheiten des Menschen (Epidemiengesetz) vom 18. Dezember 1970:** prevention and mitigation of communicable diseases.
- **BUNDESGESETZ über den Umweltschutz (Umweltschutzgesetz, USG) vom 7. Oktober 1983:** protection of the natural environment.

- **BUNDESGESETZ über die Unfallversicherung (UVG) vom 20. März 1981:** statute governing the personal injury insurance.
- **VERORDNUNG vom 20. November 1996 über die Eidgenössische Fachkommission für Biologische Sicherheit**

Deliberate Release into the Environment of Genetically Modified Organisms

- **VERORDNUNG über den Umgang mit Organismen in der Umwelt (Freisetzungsverordnung, FrSV) Entwurf vom 21. November 2005**: proposed revision of the FrSV
- **VERORDNUNG vom 25. August 1999 über den Umgang mit Organismen in der Umwelt (Freisetzungsverordnung, FrSV)**: regulates the release of genetically modified organisms in the natural environment.

Contained Use of Pathogenic and/or Genetically Modified Organisms

- **VERORDNUNG vom 25. August 1999 über den Umgang mit Organismen in geschlossenen Systemen (Einschliessungsverordnung, ESV)**

Protection of Workers Exposed to Biological Agents at Work

- **VERORDNUNG vom 25. August 1999 über den Schutz der Arbeitnehmerinnen und Arbeitnehmer vor Gefährdung durch Mikroorganismen (SAMV)**

Advisory Boards

- **Eidgenössische Fachkommission für biologische Sicherheit (EFBS)**
  - Federal Expert Commission for Biological Safety. This commission advices the government and the administration on health and safety issues. It also delivers technical opinions on proposal for conducting experiments on GMOs and pathogens. Statutory authority for the establishment of this commission are the Umweltschutzgesetz and the Epidemiengesetz and the Gentechnikgesetz. The commission work is governed by the following regulation:

- **Eidgenössische Ethikkommission für die Biotechnologie im Ausserhumanbereich (EKAH)**
  - Federal Ethics Commission for Biotechnology (medical applications excluded). EKAH is an independent commission created by the Swiss government in 1998 to advice the government and the administration on the ethical aspects of "green" biotechnology. Established by government decree, its involvement in the

authorization of contained uses and releases of GMOs is governend by the Einschliessungsverordnung and the Freisetzungsverordnung (see below).

Regulatory Authority

- Bundesamt für Umwelt (BAFU) (Federal Agency for the Environment)
- Bundesamt für Gesundheit (BAG) (Federal Health Agency)

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United Kingdom

Statutory Framework

- **Environmental Protection Act 1990 (c. 43) 1990 c. 43**: Part VI – Genetically Modified Organisms
- **Health and Safety at Work Act 1974**

Deliberate Release into the Environment of Genetically Modified Organisms

- **Genetically Modified Organisms (Deliberate Release) Regulations 2002** – Statutory Instrument 2002 No. 2443
- **The Genetically Modified Organisms (Deliberate Release and Risk Assessment-Amendment) Regulations 1997** – Statutory Instrument 1997 No. 1900
- **Environmental Protection Act 1990 (c. 43) 1990 c. 43**: Section 108(1)

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- **The Genetically Modified Organisms (Contained Use) (Amendment) Regulations 2005** – Statutory Instrument 2005 No. 2466
- **The Genetically Modified Organisms (Contained Use) (Amendment) Regulations 2002** – Statutory Instrument 2002 No. 63
- **The Genetically Modified Organisms (Contained Use) Regulations 2000** – Statutory Instrument 2000 No. 2831
- **The Genetically Modified Organisms (Risk Assessment) (Records and Exemptions) Regulations 1996** – Statutory Instrument 1996 No. 1106

Protection of Workers Exposed to Biological Agents at Work

- The Control of Substances Hazardous to Health (Amendment) Regulations 2004 - Statutory Instrument 2004 No. 3386
- The Carriage of Dangerous Goods and Use of Transportable Pressure Equipment Regulations 2004 – Statutory Instrument 2004 No. 568
- The Control of Substances Hazardous to Health Regulations 2002 – Statutory Instrument 2002 No. 2677
- The Management of Health and Safety at Work Regulations 1999 – Statutory Instrument 1999 No. 3242

Competent Authorities

The Health and Safety Executive (HSE) and the Secretary of State for the Department for Environment, Food and Rural Affairs (Defra) form the Competent Authority in England and Wales for GMO(CU).

Advisory Boards

Advisory Committee on Releases to the Environment (ACRE)

- ACRE is an independent Advisory Committee composed of leading scientists. Its main function is to give statutory advice to Ministers in the UK on the risks to human health and the environment from the release and marketing of genetically modified organisms (GMOs).
- ACRE is a statutory advisory committee appointed under section 124 of the Environmental Protection Act 1990.
- The Committee works within the legislative framework set out by Part VI of the EPA and the GMO Deliberate Release Regulations 2002 which together implement Directive 2001/18/EC.

Scientific Advisory Committee on Genetic Modification (Contained Use) (SACGM(CU))

- The Scientific Advisory Committee on Genetically Modified Organisms (Contained Use) - SACGM (CU) - provides technical and scientific advice to the UK Competent Authorities (UK CAs) on all aspects of the human and environmental risks of the contained use of genetically modified organisms (GMOs). In particular:
  - To advise on the technical issues on individual activities notified under the Genetically Modified Organisms (Contained Use) Regulations 2000;
  - To provide advice on risk assessments for contained use activities involving GMOs; and
  - To develop and update guidance on all aspects of contained use of GMOs including the Compendium of Guidance.
  - The SACGM(CU) was set up as a Government scientific advisory committee in accordance with the Office of Science and Technology's Code of Practice for scientific advisory committees and operates in accordance with the Nolan principles.