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MEGBI Research in development of a synthetic peptide vaccine against H5N1 based on MHC-I epitopes

February-2011
First version

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Last update: 23-Feb-11

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1 Introduction

Influenza virus particles are highly pleiomorphic (variable), mostly spherical/ovoid, 80-120nm diameter, but many forms occur, including long filamentous particles (up to 2000nm long x 80-120nm diameter). Different strains of virus vary in their tendency to form filaments - this property maps to the matrix protein

www.microbiologybytes.com/introductio.

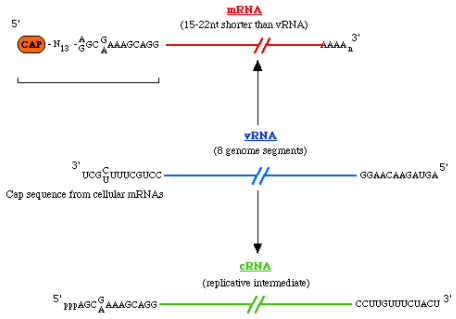
2 Theoretical basis

2.1 Influenza-A-virus H5N1

The avian influenza is causes by the influenza–A-virus H5N1 that belongs to the family of Orthomyxoviruses

2.1.1 Structure of virus particles and the genome of influenza A virus

- The influenza virus consists of 8 negative-strand RNA molecules surrounded by an envelope. The envelope contains the HA and NA proteins.
- Influenza enters cells by receptor-mediated endocytosis.
- Once inside the host, the viral RNAs are transcribed in the nucleus, stealing 5' caps from host mRNAs. These are then translated at ribosomes.



www.microbiologybytes.com/virology/Or

- The replicase then makes negative-strand RNA copies and the 8 RNAs of the viral genome assemble at the membrane and bud from the cell.

On the outside surface of the influenza virus is a lipid bilayer with HA, NA and M2 proteins inserted into it. Inside the bilayer are eight separate, linear RNA segments that make up the viral genome[1].

2.1.1.1 Genome structure of influenza virus

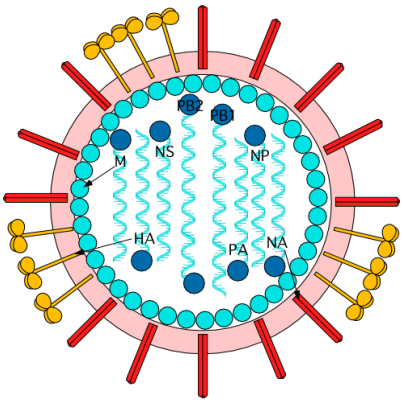
Segment:	Size(nt)	Polypeptide(s)	Function
1	2341	PB2	Transcriptase: cap binding

[1] www.microbiologytext.com/index

2	2341	PB1	Transcriptase: elongation
3	2233	PA	Transcriptase: protease activity (?)
4	1778	HA	Haemagglutinin
5	1565	NP	Nucleoprotein: RNA binding; part of transcriptase complex; nuclear/cytoplasmic transport of vRNA
6	1413	NA	Neuraminidase: release of virus
7	1027	M1	Matrix protein: major component of virion
		M2	Integral membrane protein - ion channel
8	890	NS1	Non-structural: nucleus; effects on cellular RNA transport, splicing, translation. Anti-interferon protein.
		NS2	Non-structural: nucleus+cytoplasm, function unknown

Genetics of influenza viruses. Ann Rev Genet. 2002 36: 305-332.

A representation of the flu virus that shows the outer shell and a cutaway revealing the 8 RNA pieces that comprise the genome of the virus. The outer shell is composed of lipids obtained from the last host cell. This is decorated with hemagglutinin (HA, yellow) and neuraminidase (NA, pink). HA is necessary for entrance into cells, while NA is needed for release from cells. NP is the viral polymerase. This copies the RNA genome into mRNA for protein synthesis and later in the life cycle makes copies of each RNA for new viral particles. NP is very error prone and creates many mutations in the viral genome. HA and NA therefore change rapidly, and escape recognition by our immune systems.



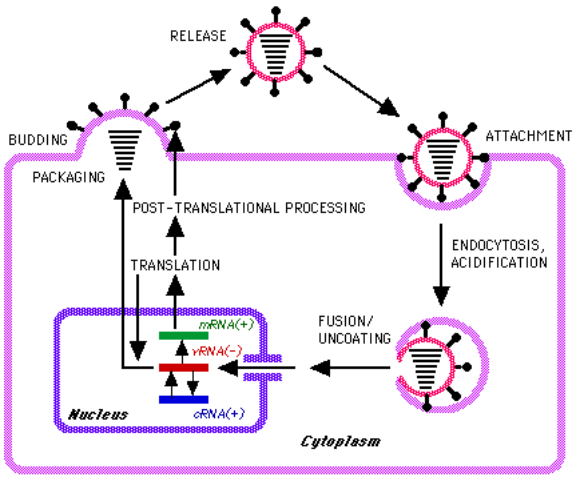
The genome of the virus is composed of eight (-) single-stranded RNAs (these (-) strands cannot be translated into protein) with each segment being complementary to one mRNA. Six of the eight mRNAs code for single proteins, while the remaining two code for two proteins by differential splicing of the RNA. Each mRNA segment is associated with multiple copies of the nucleocapsid protein (NP) and an RNA polymerase (made from the viral proteins PB1, PB2 and PA)[2].

Replication:

The following steps take place during viral replication;-

1. Adsorption
2. Penetration
3. Uncoating
4. Viral genome replication
5. Maturation
6. Release

[2] www.microbiologytext.com/index



Source: www.microbiologytext.com/index

1. Adsorption

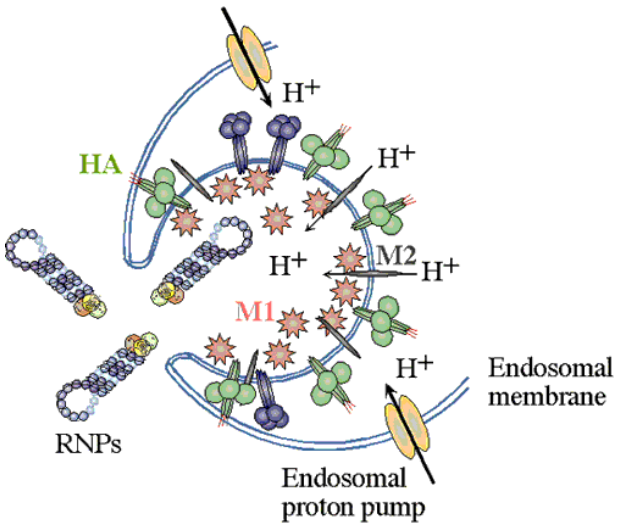
The virus becomes attached to the cells, and at this stage, it can be recovered in the infectious form without cell lysis by procedures that either destroy the receptors or weaken their bonds to the virions. Animal viruses have specialized attachment sites distributed over the surface of the virion e.g. orthomyxoviruses and paramyxoviruses attach through glycoprotein spikes, and adenoviruses attach through the penton fibers. Adsorption occurs to specific cellular receptors. Some receptors are glycoproteins, others are phospholipids or glycolipids. These are usually macromolecules with specific physiological functions, such as complement receptors for EBV. Whether or not receptors for a certain virus are present on a cell depends on the species, the tissue and its physiological state. Cells lacking specific receptors are resistant. Attachment is blocked by antibodies that bind to the viral or cellular sites involved.

2. Penetration

Penetration rapidly follows adsorption, and the virus can no longer be recovered from the intact cell. The most common mechanism is receptor mediated endocytosis, the process by which many hormones and toxins enter cells. The virion is endocytosed and contained within a cytoplasmic vacuole.

3. Uncoating

A key step in uncoating is the acidification of the content of the endosome to a pH of about 5, owing to the activity of a proton pump present in the membrane. The low pH causes rearrangement of coat components, which then expose normally hidden hydrophobic sites. They bind to the lipid bilayer of the membrane, causing the extrusion of the viral core into the cytosol. For influenza virus, the acid-sensitive component is the core HA_2 unit of the haemagglutinin, for adenoviruses, it is the penton base.



© Paul Digard, Dept Pathology, University of Cambridge.

4. Viral Nucleic Acid Replication

Virulent viruses, either DNA and RNA, shut off cellular protein synthesis and disaggregate cellular polyribosomes, favouring a shift to viral synthesis. The mechanism of protein synthesis shut-off varies even within the same viral family. Poliovirus, using a viral protease, causes cleavage of a 200 Kd cap-binding protein, which is required for initiation of translation of capped cellular messengers. In contrast to virulent viruses, moderate viruses e.g. polyomaviruses may stimulate the synthesis of host DNA, mRNA, and protein. This phenomenon is of considerable interest for viral carcinogenesis.

Maturation and Release

Maturation proceeds differently for naked, enveloped, and complex viruses[3].

2.2 MHC-I Molecule

- MHC molecules are membrane-bound proteins: MHC I molecules are found on almost all tissues of the body, while MHC II molecules are found only on antigen-presenting cells.
- MHC molecules possess a deep groove that is capable of holding a short peptide. MHC I molecules process proteins present inside the cell and present them on their surface.

[3] http://virology-online.com/general/Replication.htm

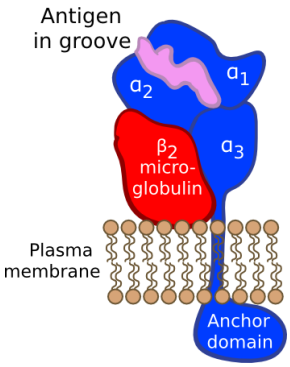
MHC II molecules present antigens taken from the phagosome digestion, most often foreign cells, and present them to the immune system.

- The immune system monitors the proteins present on MHC I molecules and activates when a foreign protein, from an intracellular parasite, is detected. This normally results in the destruction of the cell.

Almost all human tissues contain MHC molecules, which serve as signal beacons for the immune system. There are two types of MHC molecules. MHC I molecules are found on almost all types of body cells, whereas MHC II molecules are only found on macrophages, dendritic cells and B cells, which can serve as professional antigen-presenting cells. MHC I molecules are status monitors for all cells of the body, while MHC II molecules are important in presenting foreign antigens to other cells of the immune system.

The MHC I molecule consists of a α chain and a β_2-microglobulin. One segment of the β_2-microglobulin is anchored to the membrane, with the rest of the molecule protruding outside the cell. Class II MHC molecules are also transmembrane proteins consisting of α and β chains. The two chains fold to give a structure similar to that of the MHC I molecule. Both MHC I and MHC II molecules contain a deep groove in the domain of the molecule farthest away from the membrane and therefore exposed to the environment. This groove is capable of binding short peptides or other antigens that are not part of the MHC molecule. The peptides in the groove are from various sources, as you will see below. The MHC I groove is closed off and can contain only short peptide sequences, while the MHC II molecule is open and can bind to peptides of longer lengths. The following figure depicts the structure of MHC proteins[4].

Structure of an MHC molecule



[4] http://www.microbiologytext.com/index

2.3 β2m

This gene encodes a serum protein found in association with the major histocompatibility complex (MHC) class I heavy chain on the surface of nearly all nucleated cells. The protein has a predominantly beta-pleated sheet structure that can form amyloid fibrils in some pathological conditions. A mutation in this gene has been shown to result in hypercatabolic hypoproteinemia[5]

[5] http://www.ncbi.nlm.nih.gov/

3 MATERIAL AND METHOD

The sequence of HLA-A*0201 was extracted from ncbi: http://www.ncbi.nlm.nih.gov/

```
ORIGIN
        1 atggccgtca tggcgccccg aaccctcgtc ctgctactct cgggggctct ggccctgacc
       61 cagacctggg cgggctctca ctccatgagg tatttcttca catccgtgtc ccggcccggc
      121 cgcggggagc cccgcttcat cgcagtgggc tacgtggacg acacgcagtt cgtgcggttc
      181 gacagcgacg ccgcgagcca gaggatggag ccgcgggcgc cgtggataga gcaggagggt
      241 ccggagtatt gggacgggga gacacggaaa gtgaaggccc actcacagac tcaccgagtg
      301 gacctgggga ccctgcgcgg ctactacaac cagagcgagg ccggttctca caccgtccag
      361 aggatgtatg gctgcgacgt ggggtcggac tggcgcttcc tccgcgggta ccaccagtac
      421 gcctacgacg gcaaggatta catcgccctg aaagaggacc tgcgctcttg gaccgcggcg
      481 gacatggcag ctcagaccac caagcacaag tgggaggcgg cccatgtggc ggagcagttg
      541 agagcctacc tggagggcac gtgcgtggag tggctccgca gatacctgga gaacgggaag
      601 gagacgctgc agcgcacgga cgcccccaaa acgcatatga ctcaccacgc tgtctctgac
      661 catgaagcca ccctgaggtg ctgggccctg agcttctacc ctgcggagat cacactgacc
      721 tggcagcggg atggggagga ccagacccag gacacggagc tcgtggagac caggcctgca
      781 ggggatggaa ccttccagaa gtgggcggct gtggtggtgc cttctggaca ggagcagaga
      841 tacacctgcc atgtgcagca tgagggtttg cccaagcccc tcaccctgag atgggagccg
      901 tcttcccagc ccaccatccc catcgtgggc atcattgctg gcctggttct ctttggagct
      961 gtgatcactg gagctgtggt cgctgctgtg atgtggagga ggaagagctc agatagaaaa
     1021 ggagggagct actctcaggc tgcaagcagt gacagtgccc agggctctga tgtgtctctc
     1081 acagcttgta aagtgtga
```

The sequence of β2-m was extracted from ncbi: http://www.ncbi.nlm.nih.gov/

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ORIGIN
        1 aatataagtg gaggcgtcgc gctggcgggc attcctgaag ctgacagcat tcgggccgag
       61 atgtctcgct ccgtggcctt agctgtgctc gcgctactct ctctttctgg cctggaggct
      121 atccagcgta ctccaaagat tcaggtttac tcacgtcatc cagcagagaa tggaaagtca
      181 aatttcctga attgctatgt gtctgggttt catccatccg acattgaagt tgacttactg
      241 aagaatggag agagaattga aaaagtggag cattcagact tgtctttcag caaggactgg
      301 tctttctatc tcttgtacta cactgaattc acccccactg aaaaagatga gtatgcctgc
      361 cgtgtgaacc atgtgacttt gtcacagccc aagatagtta agtgggatcg agacatgtaa
      421 gcagcatcat ggaggtttga agatgccgca tttggattgg atgaattcca aattctgctt
      481 gcttgctttt taatattgat atgcttatac acttacactt tatgcacaaa atgtagggtt
      541 ataataatgt taacatggac atgatcttct ttataattct actttgagtg ctgtctccat
      601 gtttgatgta tctgagcagg ttgctccaca ggtagctcta ggagggctgg caacttagag
      661 gtggggagca gagaattctc ttatccaaca tcaacatctt ggtcagattt gaactcttca
      721 atctcttgca ctcaaagctt gttaagatag ttaagcgtgc ataagttaac ttccaattta
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781 catactctgc ttagaatttg ggggaaaatt tagaaatata attgacagga ttattggaaa
841 tttgttataa tgaatgaaac attttgtcat ataagattca tatttacttc ttatacattt
901 gataaagtaa ggcatggttg tggttaatct ggtttatttt tgttccacaa gttaaataaa
961 tcataaaact tgatgtgtta tctctta
```

3.1 Cloning of the HLA-A*0201 and b-2m gene from PBMC[6]

Protocol:

1. Isolate[7] the total RNA that was extracted from the peripheric blood monocytes (PBMC) from 10 ml human_s anticoagulative venous blood was dissolved in 50μL ddH2O.

   RESULT: total RNA only

2. Quantify by ultraviolet or spectrophotometer. *Goal: for incertitude that find RNA if the number < 1.3.*
3. Amplify The extracellular fragment of HLA-A*0201 (including the fragment of transmembrane) using total RNA as a template with the forward primer: 5′-CCCTGACCCAGACCTGGGCGG-3′ and the reverse primer 3′-AGGGTCGGGTGGTAGGGGTAG-5′.by PCR as follows:

*RESULT: Amplification the DNA segment of HLA-A*0201 only.*

4. Then using the PCR-product as a template amplified the just extracellular fragment of HLA-A*0201 with the forward primer 5′-GGCTCCCACTCCATGAGGTAT-3′ and the reverse primer 3′-GGGAGTGGGACTCTACCCTCG -5′.

*RESULT: by nested PCR can surely the amplification of DNA segment of HLA-A*0201.*

5. Analogously, we constructed b-2m expression vector; however, there were some differences between them. The fragment encoding b-2m was amplified using total RNA as a template with the forward primer 5′-GCATCCAGCGTACTCCAAAGA-3′ and the reverse primer 3′-

[6] This step was extracted from Protein Expression and Purification 35 (2004) 210-21, form *science direct* www.sciencedirect.com.

[7] The procedures of this step as follows in peqGOLD kit and for more details please see the part 4.6.

ATTCACCCTAGCTCTGTACCG-5′. The PCR protocol was the same as that used in amplification of HLA-A*0201.

RESULT: Amplification the DNA segment of β2m only.

6. After purification from an agarose gel[8]. The DNA sequences were verified by sequencing.

3.2 Expression of HLA-A*0201 and b-2m

We referred on this step to expression by pETvector with BL21 (DE3) (host strain for expression).

3.2.1 Prepare pET Vector:

To digest and gel-purify the vector[9]:

Reagent:

-pET vector

-10X restriction enzyme buffer

-EcoR I restriction enzyme

-calf intestinal alkaline phosphatase

1. Assemble the following components in a microcentrifuge tube:
   - 3 µg pET vector
   - 3 µl 10X restriction enzyme buffer
   - 10–20 U EcoR I restriction enzyme (assuming compatible buffer; the total volume of enzyme added should not exceed 10% of the reaction volume to avoid high glycerol concentrations)
   - x µl Nuclease-free water brought to volume
     30 µl Total volume
2. Incubate at the appropriate temperature (usually 37°C) for 2–4 h.

[8] Refer to MEGBI training courses book part I for purification on agarose gel.

1. [9] This step from Novagen."
   lifeserv.bgu.ac.il/wb/zarivach/.../Novagen%20**pET**%20**system**%20**manual**.pdf"

3. Run a 3 μl sample together with Perfect DNA™ Markers on an agarose gel to check the extent of digestion.
4. When digestion is complete, add calf intestinal alkaline phosphatase (Calbiochem Cat. No.524576) directly to the remainder of the digestion. The enzyme functions in most restriction buffers under the conditions described here. It is important to use the correct amount of enzyme; too much can cause unwanted deletions and can be difficult to remove for future steps. Three μg of a typical pET vector (5 kbp) corresponds to about 2 pmol DNA ends when linearized, or about 4 pmol ends if two enzymes were used for digestion. We recommend using 0.05 units of alkaline phosphatase per pmol ends. Dilute the enzyme in water or 50 mM Tris-HCl, pH 9.0 just before use.
5. Incubate at 37°C for 30 min.
6. Add gel sample buffer to the reaction and load the entire sample into a large well (0.5–1.0 cm wide) on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Run the gel far enough to separate the linear plasmid from nicked and supercoiled species. It is useful to run uncut vector DNA in an adjacent lane to help distinguish undigested from linearized plasmid DNA.
7. Visualize the DNA band with a long wave UV light source and cut the band from the gel using a clean razor blade. Avoid over exposure to the light source, which can cause nicks and double strand breaks in the DNA.
8. Recover the DNA from the gel slice. The SpinPrep™ Gel DNA Kit (Cat. No. 70852-3) is ideal for this application. Resuspend the final product in a total volume of 30 μl (usually about 50 ng/μl DNA). The DNA can be quantified spectophotometrically or using the PicoGreen kit from Molecular Probes. Assume recoveries in the range of 50% for the ligation step.
9. Store the treated vector at –20°C until use.

3.2.2 Prepare the insert DNA with the adapter

Suggested Conditions for Adapter Addition to DNA[10].
Reagent:
1. 5X adapter buffer
2. *Eco*R I(*Not* I) adapter (1 mg/ml)
3. 0.1 M DTT
4. T4 DNA ligase (1 U/μl)
5. T4 polynucleotide kinase.

1. Add the following reagents to a microcentrifuge tube on ice.
Up to 5 μg blunt-ended DNA
-10 μl 5X adapter buffer (330 mM Tris-HCl (pH 7.6),50 mM MgCl2, 5 mM ATP)

[10] This step was extracted from invitrogen life technologies.

-10 µl *Eco*R I(*Not* I) adapter (1 mg/ml)[11]
-7 µl 0.1 M DTT
-distilled water sufficient to bring the volume to 45 µl.
2. Then add 5 µl of T4 DNA ligase (1 U/µl) and mix gently.
3. Incubate the reaction for a minimum of 16 h at 16°C.
4. Heat the reaction at 70°C for 10 min to inactivate the ligase.
5. Place the reaction on ice. The adapted DNA, after adapter removal (step 9) may be ligated directly to any phosphorylated, *Eco*R I-digested vector.
However, if a dephosphorylated *Eco*R I-digested vector is used, the adapted cDNA must be phosphorylated first before adapter removal (step 6).
6. Add 3 µl (30 units) of T4 polynucleotide kinase to the reaction from step 5.
7. Mix gently, and incubate the reaction for 30 min at 37°C.
8. Heat the reaction at 70°C for 10 min to inactivate the kinase and place the reaction on ice.
9. Remove the excess *Eco*R I(*Not* I) adapters by column chromatography (ie. cDNA Size Fractionation Columns, Cat. No. 18092-015) or by some other method (ie. gel electrophoresis) and then ligate into the appropriate vector.

3.2.3 LIGATION

Reagent:

-10X Ligase Buffer (200 mM Tris-HCl pH 7.6,

-100 mM MgCl2

-100 mM DTT

-10 mM ATP

- 50 ng/µl prepared pET vector

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[11] *Eco*R I(*Not* I) adapter may be used to add *Eco*R I cohesive ends directly to any blunt-ended DNA fragment. The adapter is formed by annealing two oligonucleotides together forming a phosphorylated, blunt end and a nonphosphorylated *Eco*R I half-site. Ligation to blunt ended DNA results in *Eco*R I half-sites on both ends of the DNA. The adapted DNA may be ligated into any *Eco*R I containing vector. The adapter also contains the recognition sequences for *Not* I and *Sal* I restriction enzymes. Thus, DNA inserts may be cleaved from the vector using *Eco*R I or either of the rare cutting enzymes, *Not* I or *Sal* I.
Adapter sequence: 5'-pGTCGACGCGGCCGCG
CAGCTGCGCCGGCGCTTAA-OH-5'
Storage Buffer
10 mM Tris-HCl (pH 7.5)
100 mM NaCl
1 mM EDTA
Quality Control Assays:
This product has passed a self-ligation quality control assay.
Doc. Rev.: 100901

- T4 DNA ligase, diluted (with ligase dilution buffer) 0.2–0.4 Weiss units/µl
- Prepared target gene insert (0.2 pmol)
-Nuclease-free water
-Ligase

Procedures:

One consistently successful protocol for ligation is presented here.

1. For a standard reaction using DNA fragments with 2–4 base sticky ends, use 50–100 ng (0.015–0.03 pmol) of pET vector with 0.2 pmol insert (50 ng of a 500 bp fragment) in a volume of 20 µl. Assemble the following components in a 1.5 ml tube (these components are available separately in the DNA Ligation Kit, Cat. No. 69838-3) or use the Clonables™ 2X Ligation Premix (Cat. No. 70573-3). Add the ligase last.
   - 2 µl 10X Ligase Buffer (200 mM Tris-HCl pH 7.6,
   - 100 mM MgCl2
   - 2 µl 100 mM DTT
   - 1 µl 10 mM ATP
   - 2 µl 50 ng/µl prepared pET vector
   - 1 µl T4 DNA ligase, diluted (with ligase dilution buffer) 0.2–0.4 Weiss units/µl
   - x µl Prepared target gene insert (0.2 pmol)
   - y µl Nuclease-free water to volume

     20 µl Total volume

2. Add the ligase last, and gently mix by stirring with a pipet tip. Incubate at 16°C for 2 h to overnight. Also set up a control reaction in which the insert is omitted to check for nonrecombinant background.

Note: For blunt ends, use 10X more ligase (i.e., undiluted enzyme), reduce the ATP concentration to 0.1 mM and incubate for 6–16 h at 16°C or 2 h at room temperature.

3.2.4 Transformation INTO EXPRESSION HOST

Reagent:

- competent cell.
- SOC Medium.
- LB agar plates

Procedures:

1. Remove the appropriate number of competent cell tubes from the freezer (include one extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is surrounded by ice. If the standard cells are to be used, place the required number of empty 1.5 ml polypropylene microcentrifuge tubes on ice to prechill. Allow the cells to thaw on ice for ~2–5 min.

2. Visually check the cells to see that they have thawed and gently flick the cells 1–2 times to evenly resuspend the cells.
3. *Standard Competent Cells*:
Pipet 20 µl aliquots of cells into the pre-chilled tubes.
Singles Competent Cells:
Proceed to Step 4 or 5, depending on whether a Test Plasmid sample is included as a positive control.
4. (Optional) to determine transformation efficiency, add 0.2 ng (1 µl) Test Plasmid provided with Competent Cells to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.
5. Add 1 µl of a ligation reaction or purified plasmid DNA directly to the cells. Stir gently to mix and return the tube to the ice, making sure that the tube is surrounded by ice except for the cap. Repeat for additional samples.
6. Incubate the tubes on ice for 5 min.
7. Place the tubes in a 42°C water bath for exactly 30 sec; do not shake.
8. Place the tubes on ice for 2 min.
9. *Standard Competent Cells:*
Add 80 µl of room temperature SOC Medium to each tube. Keep the tubes on ice until all have received SOC.
Singles Competent Cells
Add 250 µl of room temperature SOC Medium to each tube. Keep the tubes on ice until all have received SOC.
10. Selection for transformation is accomplished by plating on medium containing antibiotic for the plasmid encoded drug resistance. Additional host-specific antibiotics may also be appropriate to ensure maintenance of the host feature(s).
When using NovaBlue: if selecting for β-lactamase (carbR/ampR), no outgrowth (shaking incubation) step is required, although slightly higher cloning efficiencies may be obtained with 30–60 min outgrowth. Plate 5–50 µl cells directly on selective media. If selecting for kanamycin resistance, shake at 37°C (250 rpm) for 30 min prior to plating.
When using strains other than NovaBlue: shake at 37°C (250 rpm) for 60 min prior to plating.
Prepare LB agar plates with appropriate antibiotic ahead of time
Notes: The outgrowth incubation is conveniently performed in a shaking incubator using a test tube rack anchored to the shaking platform. Place each transformation tube in an empty 13 mm x 100 mm glass test tube in the rack. The snap-caps on the transformation tubes prevent them from falling to the bottom of the test tubes, and all transformation tubes remain vertical.
During the outgrowth (or earlier if omitting outgrowth), place the *plates at 37°C. If the plates contain a lot of moisture, place them cover-side up and ope*n the cover ~1/3 of the way to allow the plates to dry for 30–45 min. If the plates do not need drying, keep them closed and place them cover-side down in the 37°C incubator for ~20 min prior to plating.

11. Spread 5–50 µl of each transformation on LB agar plates containing the appropriate antibiotic for the plasmid and host strain. When plating less than 25 µl, first pipet a "pool" of SOC onto the plate and then pipet the cells into the SOC. Please see the part 4.6 for additional details on plating technique.

Important: The appropriate amount of transformation mixture to plate varies with the efficiency of both the ligation and the competent cells. As little as 2 µl will yield several hundred transformants under highly efficient conditions (e.g., with NovaBlue cells giving > 4 × 10 8 cfu/µg). For recombinants in NovaBlue, expect 10 5–10 7 transformants/µg plasmid, depending on the particular insert and the ligation efficiency.

When using the Test Plasmid, plate no more than 5 µl (e.g., 5 µl of NovaBlue cells at 1 × 10 8 efficiency) or 10 µl (e.g., 10 µl of cells at 1 × 10 6 efficiency) of the final transformation mix in a pool of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the ampR gene).

12. Let the plates sit on the bench for several min to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

3.3 Extraction and solubilization of inclusion bodies

- **Reagent:**
- 10mM tris HCl , pH8
- 10 mM tris HCl, pH7
- 8M Urea, 100 mM Tris HCl, pH8
- 8M urea 20 mM Tris ,PH 8
- LYSOSYM (100 µg /ml)
- Phenyl methyl sulfonyl fluorid (50µg/ml)
- DNase (20µg/ml)
- RNase (20µg/ml)
- 1mM EDTA
- 0-100 mM NaCl

Purification of recombinant proteins:

1- The cell were harvested by centrifugation at an OD650 OF 1.8-2.0
2- The cell pellets were resuspended in 10 mM Tris HCl.pH 8 (20 ml) resuspension, containing lysozyme (100 µg / ml), phenyl methyl sulfonyl fluoride (50 µg / ml), DNase(20 µg/ml),RNase (20 µg / ml),and 1mM EDTA and incubated at 22◦c for 20 min.
3- The cells were lysed by sonication[12] and then centrifuged (10000xg).for 20 min.

[12] **Sonication** is the act of applying sound (usually ultrasound) energy to agitate particles in a sample.

4- The pellet containing recombinant protein was washed with 10 mM Tris HCl, pH 8(20 ml).
5- It is then dissolved in 100 mM Tris HCl, pH8/8 M urea (10 ml), and centrifuged at 4°C for 1 hour.(150000xg)
6- The recombinant protein purified on Q Sepharose fast flow[13].

Using a HiTrap 1-ml column with a syringe. **(A)** Prepare buffers and sample. Remove the column's top cap and snap off the end. Wash and equilibrate. **(B)** Load the sample and begin collecting fractions. **(C)** Wash, elute, and continue collecting fractions[14].

7- fractions, the purified HLA heavy chains contained stored at-20°C for use in the ELISA experiment

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In biological applications, sonication may be sufficient to disrupt or deactivate a biological material. This process is called sonoporation. Sonication is also use to fragment molecules of DNA. This is an alternative to the freeze-pump-thaw(**by Schlenk flask**) and sparging methods. It is especially useful when it is not possible to stir the sample, as with NMR tubes

[13] The ion exchange chromatography Q sepharose FF was purchased from GE Healthcare. see www.gelifesciences.com/hitrap for more details

[14] Took from GE Healthcare.

3.4 ELISA assay of peptide-MHC complex formation:

Material:

- Pan specific mouse anti HLA class I antibody,W6/32
- Polyclonal rabbit antihuman β2m-HRP
- dextran polymer conjugated with goat antirabbit IgG and HRP
- 96 well Maxisorp ELISA Plates
- Pluronic lutrol F-68
- peptide
- 100mM carbonate buffer (PH 9.6)
- 10٪ w/v skimmed milk powder in PBS (SMP-PBS)
- 0.05٪ Tween-20 in PBS
- 0.3 mM tris-maleat buffer(PH 6.6)

Day 1

-96 well Maxisorp ELISA Plates were coated and let overnight at 4 °C with W6/32.

Using 50 µl/well at 5 µl/ml, in 100 mM carbonate buffer, PH 9.6.

Day 2

- Add 320 µl/ well 10٪ w/v skimmed milk powder in PBS (SMP-PBS) to block the residual bind.
- Wash twice with 600 µl/ well of 0.05 ٪ Tween-20 in PBS at room temperature using an automated plate washer to remove unbound W6/32 and blocking reagent
- On ice, purified recombinant HLA molecule in 8M Urea and 20mM tris PH8 were diluted 100-fold into a 0.3 mM tris maleat buffer, PH 6.6, contaning human β2m, peptide and lutrol F- 68 at the concentrations indicated.

3nM MHC-I HC is optimal

1g/L lutrol F-68

100nM β2m is optimal

10,000nM petid (optimal 1nM to 1µM)

- To allow complex formation the reaction mixtures were incubated at 18 °C for 48h.

Day 3

Incubation

Day 4

- Just prior to the ELISA analysis, the reaction volume was diluted 10 times into 2% SMP/PBS at 4°C.
- 50 µl/well with PBS/0.05% Tween 20 were transferred in triplicate to a W6/32 coated plate.
- The plate was incubated for 2h at 4°C.
- Washed 6 times with 600 µl/well with PBS/0.05% Tween 20 at room temperature.
- detect the binding complex,the plate was incubated for 1h at 4°c , with 50 µl/ well of a polyclonal rabbit antihuman β2m-HRP diluted 1:2500 into 2% SMP-PBS and the washed 6*600 µl/well with PBS 0.05% tween 20 at room temperature
- To enhance the detection, the plate was subsequently incubated for 30 min at room temperature with a dextran polymer conjugated with goat antirabbit IgG and HRP diluted in 1:15 in 2% SMP-PBS containing 1% normal mouse serum.
- Washed 6*600 µl/well with PBS/0.05% Tween 20 at room temperature.
- ELISA was developed with 3,3′ 5,5′-tetramethylbenzidine hydrogenperoxide for 30 min at room temperature.
- Colorimetric reaction was read at 450nm using a Victor Multilabel ELISA counter

3.5 More detials:

I- The peqGOLD total RNA Kit

INTRODUCTION

The peqGOLD Total RNA Kit provides a rapid and easy method for the isolation of up to 100 µg of total RNA from eukaryotic cells and tissues. This kit allows processing of a single or multiple samples in less than 30 min. Normally, up to 1 x 107 cells or 40 mg tissue can be used in a single experiment. There is no need for phenol/chloroform extractions and time-consuming steps such as CsCl gradient ultracentrifugation or precipitation with isopropanol. While this kit may be used for isolation of RNA from whole blood, we recommend to use the

peqGOLD Blood RNA Kit (product # 12-6814) as it is specifically designed for effective hemolysis and hemoglobin removal and gives therefore higher RNA yields from blood.
RNA purified using the peqGOLD Total RNA Kit is ready for applications such as RT-PCR, Northern blotting, poly(A)+-RNA (mRNA) purification, nuclease protection assays and in vitro translation.
peqGOLD Total RNA Kits are available with Safety-Line (Order No. 12-6834-xx) or Classic Line (Order No. 12-6634-xx) columns. Safety-Line columns can be closed tightly by lids to avoid cross-contamination more effectively. Classic-Line colums do not have lids for a more comfortable handling.

PRINCIPLE

The peqGOLD Total RNA Kit uses the reversible binding properties of the PerfectBind RNA Column, a new silica-based material. This is combined with the speed of minicolumn spin technology. A specifically formulated high salt buffer system allows more than 100 µg of RNA molecules greater than 200 bases to bind to the matrix. Cells or tissues are first lysed under denaturing conditions that practically inactivate RNases. Samples are then applied to the PerfectBind RNA Columns to which total RNA binds, while cellular debris and other contaminants are effectively washed out. High quality RNA is finally eluted in RNase-free sterile water.

KIT COMPONENTS

peqGOLD Total RNA Kit
Order No. Safety-Line
Order No. Classic-Line
Components
PerfectBind RNA Columns
DNA Removing Columns
2.0 ml Collection Tubes
RNA Lysis Buffer T
RNA Wash Buffer I
RNA Wash Buffer II (conc.)
RNase-free Water
Instruction manual

STORAGE AND STABILITY

The peqGOLD Total RNA Kit components should be stored at room temperature. If stored under these conditions, all components are stable for at least 12 months from the date of purchase. During shipment crystals may form in the RNA Lysis Buffer T. Warm up to 37°C to dissolve.

BEFORE STARTING

Briefly examine this booklet and become familiar with each step. Prepare all components and have the necessary materials ready before starting.

! Whenever working with RNA, always wear one-way gloves to minimize RNase contamination. Use only fresh RNase-free disposable plastic pipette tips when using the supplied reagents.
! Work carefully but as quickly as possible during the procedure.
! Under cool ambient conditions, crystals may form in RNA Lysis Buffer T. This is normal and the bottle should be warmed (37 °C) to dissolve the salt before use.
! RNA Wash Buffer II is concentrated and has to be diluted with absolute ethanol as follows:
12-6834-00 Add 8 ml 100% EtOH to 2 ml Wash Buffer II.
12-6834-01 Add 80 ml 100% EtOH to 20 ml Wash Buffer II.
12-6834-02 Add 3 x 80 ml 100% EtOH to 3 x 20 ml Wash Buffer II.
! Store diluted RNA Wash Buffer II at room temperature.
! All steps must be carried out at room temperature (22 – 25 °C).

PEQGOLD TOTAL RNA ISOLATION PROTOCOL

Eucaryotic cells and tissue

Materials to be supplied by the user:
! 100 % Ethanol
! 70 % Ethanol in sterile RNase-free dH2O
! Sterile RNase-free pipet tips and centrifuge tubes

1. Homogenization and lysis

a. Tissue

Excise tissue (~ 40 mg, 3 mm3) and promptly freeze in a small volume of liquid nitrogen.
Grind tissue with a ceramic mortar and pestle under approximately 10 ml of liquid nitrogen.
Wear gloves and take great care when working with liquid nitrogen.
Transfer the suspension into a pre-cooled 15 ml polypropylene tube. If the tube is not precooled (in liquid nitrogen), the suspension will boil vigorously possibly causing loss of tissue. When the liquid nitrogen has completely evaporated, add 400 µl RNA Lysis Buffer T. Transfer the lysate directly into a DNA Removing Column placed in a 2.0 ml Collection Tube. Centrifuge at 12.000 x g for 1 min at room temperature. Transfer the flow-through lysate into a new 1.5 ml tube.
For RNase rich tissues or more than 40 mg tissue, use 600 µl of RNA Lysis Buffer T. However, do not use more than 50 mg tissue.
For homogenization, you may also use glass-, teflon- or electric homogenisators.

b. Monolayer Cells

For tissue culture cells grown in monolayer (adherent fibroblasts, endothelial cells etc.), lyse the cells directly in the culture vessel as follows. Aspirate culture medium completely and add RNA Lysis Buffer T directly to the cells. Use 800 µl for T35 flasks or 10 cm dishes, and 400 µl for smaller vessels. Pipet buffer over entire surface of vessel to ensure complete lysis. Transfer the lysate directly into a DNA Removing Column placed in a 2.0 ml Collection Tube. Centrifuge at 12.000 x g for 1 min at room temperature. Transfer the flow-through lysate into a new 1.5 ml tube.

This method is preferable to trypsinization followed by washing because it minimizes RNA degradation by nuclease contamination.

c. Suspension culture

For cells grown in suspension cultures, pellet cells at 1.500 rpm (400 x g) for 5 min. Pour off supernatant and add 400 µl RNA Lysis Buffer T per 1 x 107 cells. Transfer the lysate directly into a DNA Removing Column placed in 2.0 ml Collection Tube. Centrifuge at 12.000 x g for 1 min at room temperature. Transfer the flow-through lysate into a new 1.5 ml tube.

2. Load and Bind

Add an equal volume (400 µl, 600 µl or 800 µl) 70 % Ethanol to the lysate and mix thoroughly by vortexing. Place a PerfectBind RNA Column in a new 2.0 ml Collection Tube (supplied) and add the lysate directly to the membrane. Centrifuge the PerfectBind RNA Column / Collection Tube assembly at 10.000 x g for 1 min. Discard the flowthrough liquid and Collection Tube.

A precipitate may form on addition of 70 % ethanol. Vortex and add the entire mixture to the column. The maximum capacity of the PerfectBind RNA Column is 750 µl, larger volumes can be loaded successively. However, the total binding capacity of a PerfectBind RNA Column is approx.
100 µg RNA.

3. Wash I

Place the PerfectBind RNA Column in a fresh 2.0 ml Collection Tube, add 500 µl RNA Wash Buffer I to the PerfectBind RNA Column and centrifuge for 15 sec at 10.000 x g. (supplied). Discard the flow-throw liquid and reuse the collection tube in the next step.

4. DNase Digestion (optional)

Since PerfectBind RNA Column technology actually removes most of DNA without a DNase treatment, it is not necessary to do DNase digestion for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Following steps provide on-membrane DNase I digestion (Order No. 12-1091).

a. For each PerfectBind RNA Column, prepare this DNase I digestion reaction mix:
DNase I Digestion Buffer 73.5 µl, RNase-free DNase I (20 Kunitz units/µl) 1.5 µl, Total volume 75.0 µl.

Note:
1. DNase I is very sensitive to physical denaturation, so do not vortex this DNase I mixture!
Mix gently by inverting the tube.
Prepare fresh DNase I digestion mixture directly before RNA isolation.
2. DNase I digestion buffer is supplied with RNase-free DNase set.
Standard DNase buffers are not compatible with on-membrane DNase digestion!

b. Pipet 75 µl of the DNase I digestion reaction mix directly onto the surface of PerfectBind RNA resin in each column. Make sure to pipet the DNase I digestion mixture directly onto the membrane.

DNase I digestion will not be complete if some of the mix stick to the wall or the O-ring of the PerfectBind RNA Column.

c. Incubate at room temperature (25 – 30 °C) for 15 minutes.

d. Place the PerfectBind RNA Column into a 2.0 ml Collection Tube and add 400 µl RNA Wash Buffer I. Incubate the PerfectBind RNA Column at benchtop for 5 minutes.
Centrifuge at 10.000 x g for 5 minutes and discard flow-through. Re-use collection tube in the next step. Continue with step 5.

5. Wash II

Add 600 µl completed RNA Wash Buffer II to the PerfectBind RNA Column and centrifuge for 15 sec at 10.000 x g. Discard the flow-through liquid. Repeat this wash step and discard the flow-through liquid.

6. Dry (Important! Do not skip this step!)

Place the PerfectBind RNA Column containing your RNA in the collection tube used in step 5 and centrifuge for 1 min at 10.000 x g to completely dry the column matrix. This step is essential to remove ethanol from the column.

7. Elution

Place the PerfectBind RNA Column (step 6) into a fresh 1.5 ml microcentrifuge tube. Add 50 - 100 µl (depending on the desired final concentration of RNA) sterile RNAse-free dH2O directly to the binding matrix in the PerfectBind RNA Column and centrifuge for 1min at 5.000 x g to elute RNA.

A second elution may be necessary if the expected yield of RNA is > 50 µg. Alternatively, RNA may be eluted with a higher volume of water. While an additional elution increase total RNA yield, the concentration will be lowered since more than 80 % of RNA is recovered with the first elution. Pre-heating RNase-free dH2O to 70 °C before adding to the PerfectBind RNA Column and incubating the PerfectBind RNA Column for 5 min at room temperature before centrifugation may increase yield.

DNA CONTAMINATION

No RNA extraction procedure can completely remove genomic DNA. For sensitive work (such as RT-PCR or differential display) we suggest that you treat the eluted RNA with RNase-free DNase. On-membrane DNase I Digestion is a simple and fast method and can be integrated into the standard protocol between the washing steps (see page 14/15).

Also for RT-PCR, use intron-spanning primers that allow easy identification of DNAcontamination.

A PCR reaction, which uses the RNA as template, will also allow the detection of DNA contamination.

QUANTITATION AND STORAGE OF RNA

Determine the absorption of an appropriate dilution (10- to 50-fold) of the sample at 260 nm and 280 nm.

RNase-free water is slightly acidic and can dramatically lower absorption values. We suggest that you dilute the sample in a buffered solution (TE) for spectrophotometric analysis.

One A260-unit is about 40 µg RNA/ml. The RNA concentration is calculated as follows:

RNA conc. (µg /ml) = Absorption260 × 40 × Dilution Factor

The ratio of A260/280 is an indication of nucleic acid purity. A value higher than 1.8 indicates > 90 % nucleic acid.

Store RNA samples at – 70 oC in sterile RNase-free dH2O. Under such conditions RNA prepared with the peqGOLD system is stable for at least one year.

RNA QUALITY

It is highly recommended to determine the RNA quality prior to further applications.

Denaturing agarose gel electrophoresis and ethidium bromide staining can best assess the quality of RNA. Two sharp bands should appear on the gel. These represent the 28S and 18S ribosomal RNA bands. If these bands smear towards lower molecular weight RNA, then the RNA has undergone major degradation during preparation, handling or storage.

Although RNA molecules less than 200 bases in length do not efficiently bind to the PerfectBind RNA Column, a third RNA band, the tRNA band, may be visible when a large number of cells are used.

II- Transformation[15]

Initial cloning should be done in a *recA*– cloning strain, such as NovaBlue, or other similar host that lacks the gene for T7 RNA polymerase. This enables high percentage monomer plasmid yields for examination of the construct sequence, as well as separation of cloning from expression. This separation can be valuable in troubleshooting any difficulties that might arise during later procedures.

The strains described above for cloning and expression with pET vectors can be prepared for transformation by standard procedures. Expect BL21 (an expression strain) and its derivatives to be transformed at about 1/10 the efficiency of the other strains. For convenience and consistent performance, Novagen offers the relevant host strains as prepared competent cells, ready for high-efficiency transformation.

DNA in ligation reactions containing high-quality reagents is suitable for direct addition to Novagen's Competent Cells (no more than 1 µl ligation should be used per 20 µl cells).

Inactivation of the ligase is not required prior to transformation. Plasmid DNA isolated using standard preparation procedures is also usually satisfactory; however, for maximum efficiency, the sample DNA should be free of phenol, ethanol, salts, protein and detergents, and dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) or in water.

Novagen's Competent Cells are provided in 0.2 ml aliquots. The standard transformation reaction requires 20 µl cells, so each tube contains enough cells for 10 transformations. Singles™ Competent Cells are provided in 50 µl aliquots, which are used "as is" for single 50 µl transformations. Note that there are a few steps in the protocol that vary for the Singles™ vs. standard cells. Novagen's NovaBlue and BL21(DE3) Competent Cells are also offered in a highthroughput 96-well plate format known as HT96™ Competent Cells (see Technical Bulletin 313).

[15] Extracted from Novagen

Handling Tips

1. Upon receipt from Novagen, verify that the competent cells are still frozen and that dry ice is still present in the shipping container. Immediately place the competent cells at –70°C or below. For optimal results, do not allow the cells to thaw at any time prior to use.
2. Handle only the very top of the tube and the tube cap to prevent the cells from warming. Keep the cells on ice whenever possible.
3. To mix cells, flick the tube 1–3 times. *NEVER* vortex the competent cells.
4. To avoid multiple freeze-thaw cycles of the standard 0.2 ml cells, dispense the cells into aliquots after the initial thaw and store them at –70°C or below (note that Singles™ Competent Cells are provided as 50 µl aliquots, which are used "as is" and do not require dispensing. To dispense aliquots of cells from the 0.2 ml stock, remove the stock tube quickly from the ice and flick 1–2 times to mix prior to opening the tube. Remove a 20 µl aliquot from the middle of the cells, and replace the tube immediately on ice. Place the aliquot immediately into the bottom of a pre-chilled 1.5 ml tube, mix by pipetting once up and down, and then immediately close the tube and replace on ice. After all of the aliquots are taken, return any unused tubes to the freezer before proceeding with the transformation.

Plating techniques

1. Remove the plates from the incubator. If plating less than 25 µl of the transformation, we recommend plating onto a pool of SOC, which facilitates even colony distribution on the plate surface. Using a sterile pipet tip, place 40–60 µl of SOC in the center of a plate for a plating cushion.
2. To remove the transformation sample, flick the transformation tube 5–8 times, open the cap and immediately remove the sample volume from the middle of the transformation reaction.
3. Transfer the sample to the plate by dispensing the sample volume into the SOC cushion. After the sample is out of the pipet tip, use the same tip to pipet up the sample volume's worth of SOC from the cushion edge and dispense that SOC back into the cushion. (This effectively rinses out your pipet tip.)

ColiRollers™ Plating Beads

To use ColiRollers, simply dispense 10–20 beads per plate. The beads can be dispensed before or after pipetting the transformation mix on the plate. Cover the plate with its lid and move the plate back and forth several times. The rolling action of the beads distributes the cells. Several plates can be stacked up and shaken at one time. After all plates have been spread, discard the ColiRollers by inverting the plate over a collection container. Cover and incubate (step 12 above).

ColiRollers™ Plating Beads are treated glass beads that eliminate the use of the spreader and alcohol flame while evenly and consistently distributing cells without damage.

Standard spreader

Completely immerse the plating spreader (bent glass rod or equivalent) into ethanol and flame to sterilize. After the flame is extinguished, allow the spreader to cool ~10 sec prior to placing the spreader on the plate. Place the spreader on the LB agar at the outside of the plate (not touching the pool of cells). This further cools the spreader on the LB agar before spreading the cells. Slowly turn the plate while supporting the weight of the spreader.

Important: Do not press down on the spreader – use just enough pressure to spread the cells.

Spread until the sample is evenly distributed on the plate. If the plates are fairly dry, the sample and cushion will quickly absorb into the plate. After the moisture is absorbed, do not continue spreading. If the plates are wet, spread until the sample is evenly distributed. Do not spread until the sample and cushion have absorbed completely into the plate, because overspreading can decrease transformation efficiency. Instead, after spreading briefly, allow the plates to sit upright at room temperature for ~15 min prior to placing them in the 37ºC incubator. This will allow excess moisture to absorb into the plates before the plates are inverted and placed in the incubator.

Incubate all plates, cover-side down, in the 37ºC incubator for 15–18 h. To obtain larger colonies, extend the incubation time slightly (1–2 h), but beware of the potential for development of satellite colonies with extended incubations (usually > 36 h at 37°C; satellites are not commonly observed when using carbenicillin or kanamycin). Once the colonies are at the desired size, the plates can be placed at 4ºC.