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بسم الله الرحمن الرحيم

DEVELOPMENT OF A SYNTHETIC PEPTIDE VACCINE AGAINST H5N1 BASED ON MHC-I EPITOPES

بحث في تطوير لقاح الببتيد المصنع ضد فيروس إنفلونزا الطيور المستند على حواتم أليلات المركب الرئيسي للتلاؤم النسيجي.(MHC-I)

Step 2: Verification of the peptid candidate FLKDVMESM through ELISA and IFN-γ ELISPOT analysis in a laboratory setting

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Overview / نظرة عامة

إن بحث تطوير لقاح الببتيد المصنع ضد فيروس إنفلونزا الطيور المستند على حواتم أليلات المركب الرئيسي للتلاؤم النسيجي.(MHC-I)، هو بحث يستوجب أربع مراحل لإتمامه منها المرحلة الثانية المشروحة بالتفصيل في هذا الكتاب والتي هي إختبار إيليزا الكمي للتحقق من نسبة الربط الفعلي بين الببتيدات المصنعة وأليلات المركب الرئيسي للتلاؤم النسيجي (MHC-I) من مستضدات كريات البيض البشرية (HLA) للببتيدات التي تم تنبؤها في المرحلة الأولى بواسطة NetCTL 1.2.

يتضمن هذا الكتاب في القسم الأول منه المفاهيم والمصطلحات العلمية المستخدمة في البحث، القسم الثاني يتضمن البروتوكولات المستخدمة لتطبيق هذه المرحلة وهي مأخوذة من مواقع عن الإنترنت أما القسم الثالث يتضمن نفس بروتوكولات القسم الثاني مع إجراء بعض التعديلات عليها بما يتناسب مع الآلات والمواد المؤسس عليها المختبر أما القسم الرابع يعرض النتائج الأخيرة مع الصور من التجارب التي تم العمل عليها في مختبر MEGBI وأخيراً مضاف إلى هذا الكتاب الجدول الزمني المستغرق لإتمام كل عملية من هذه المرحلة.

والله ولي التوفيق.

1 Introduction / المقدمة

Avian influenza is caused by the influenza-A-A virus H5N1 which is found in birds. The influenza-A-type virus is characterized by the HA (hemagluttinin) and NA (neuraminidase) proteins. H5N1 has one HA type 5 proteins (H5) as well as one NA type 1 protein (N1).

The majority of humans do not possess any kind of immunity against the H5N1 virus. The virus is able to replicate efficiently inside the human body, for this reason should be opposite this pandemic. However, a mutation in the H5N1 virus, only two amino acid exchanges at the HA receptor binding site of H5N1 are required in order to optimize binding of the virus to N-acetyl-neuraminicacid, which is found on epithelial cells of the human lung. It causes a fast and effective spreading of H5N1 in the human population.

And the **goal of the research** is the identification of peptides of the potential H5N1 mutants which might elicit an immune response in humans. Humans could be immunized with these peptides before the outbreak of a pandemic.

Approach

- Computer-based analysis of candidate peptides of the mutated H5N1 which bind to MHC-I with high affinity and hence are immunogenic.
- Verification of these candidates through ELISA and IFN-γ ELISPOT analysis in a laboratory setting.
- Animal testing of the candidate peptides which have found to be promising in the above mentioned tests.

إن فيروسات إنفلونزا آي (H5N1) تسبب إنفلونزا الطيور. ويميز هذه الفيروسات النوع الخامس من البروتينات السكرية الراصة الدموية (H5 hemaglutinin) والنوع الأول من بروتين النوروراميديناز (Neuraminidase N1) المغلفة للمادة النووية للفيروس.

وبما إن غالبية البشر لا تملك أي نوع من الحصانة ضد فيروس H5N1 فإن هذا الفيروس قادر على التكرار بكفاءة داخل جسم الإنسان لذا يجب مواجهة هذا الوباء. إلا أن التغيير الجيني المفاجئ في الفيروس من خلال تبادل الأحماض الأمينية في مستقبل موقع ملزم لفيروس h5n1 من أجل تحسين ربط الفيروس بحمض أن–أسيتيل نورامينيك الذي يوجد على الخلايا الظاهرية لرئة الإنسان، يسبب الإنتشار السريع والفعال له بين البشر.

لتطوير هذا اللقاح يجب معرفة ببتيدات طفرات H5N1 المحتملة التي قد تثير رد فعل مناعة لدى البشر.

المنهج:

- يستند الحاسوب على تحليل الببتيد المرشح للتغير الجيني المفاجئ في فيروس H5N1 الذي يرتبط ب MHC-I في نسبة عالية لذا فهي المناعة.
- التحقق من هذه المرشاحات من خلال فحص إيليزا و IFN-γ ELISPOT في المختبر.
- إختبار الببتيدات المرشحة، التي وجدت في الفحوصات المذكورة أعلاه لتكون محفزة للمناعة، على الحيوان.

الطرق

المرحلة الأولى: هي إستناد الحاسوب على تنبؤ MHC-I تم إنجازها في ألمانيا.

المرحلة الثانية: هي إختبار إيليزا الكمي للتحقق من نسبة الربط الفعلي

Methods

Step 1 Computer-based MHC-I prediction (worked in Germany).
Step 2 quantitative ELISA Experiment for investigated from the actual binding affinity between the synthetically peptides and the MHC-I alleles from the respective HLA types for which peptides have been predicted by NetCTL1.2.
Step 3 Animal studies and IFN-γ ELISPOT analysis.
Step 4 Clinical Studies

The second step is the step described in this book and it is now under discussion

بين الببتيدات المصنعة وأليلات المركب الرئيسي للتلاؤم النسيجي (MHC-I) من مستضدات كريات البيض البشرية (HLA) للببتيدات التي تم تنبؤها بواسطة NetCTL 1.2.

المرحلة الثالثة: هي الدراسات الحيوانية وتحليل IFN-Y ELISPOT للتحقق من فعالية لقاح الببتيد.

المرحلة الرابعة: هي الدراسات السريرية

المرحلة الثانية هي المرحلة الوارد ذكرها في هذا الكتاب وهي الآن قيد البحث

2 Theoretical basis / النظرية الاساسية

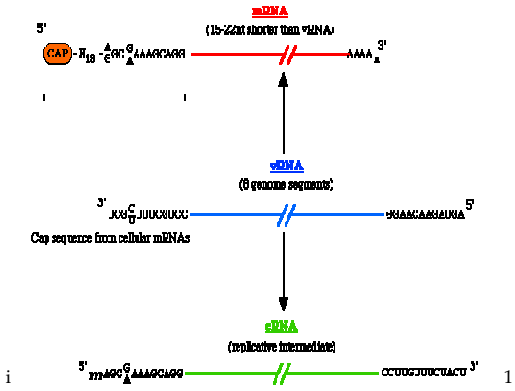
2.1 Influenza-A-virus H5N1 / انفلونزا "آي" للفيروس H5N1

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

الانفلونزا "آي" H5N1 هي التي تسبب انفلونزا الطيور الذي ينتمي إلى عائلة Orthomyxoviruses

.1 Structure of virus particles and the genome of influenza A virus / هيكل جزيئات الفيروس وجينوم فيروس الانفلونزا آي

- 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.



- فيروس الانفلونزا يتكون من سلالات الحمض النووي الريبي 8 السلبية محاطة بمغلف. المغلف يحتوي على البروتينات ها HA و نا NA.
- الأنفلونزا يدخل الخلايا بوساطة.مستقبلات الالتقام.
- ينسخ الحمض النووي الريبي الفيروسي في النواة، مرة واحدة داخل الخلية المضيفة ويأخذ قبعة 5' من الحمض النووي الريبي المضيف. ثم يتم تحويلها الى ريبوسومات

[1] www.microbiologybytes.com/virology/Or

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[ii2].

السطح الخارجي لفيروس الانفلونزا هو طبقة ثنائية من الدهون مع HA و NA و البروتينات M2 المدرجة فيه. داخل هذه الطبقة الثنائية شرائح الحمض النووي الريبي الثمانية التي يتشكل الجينوم الفيروسي.

2.1.1.1 Genome structure of influenza virus / هيكل الجينوم لفيروس الانفلونزا

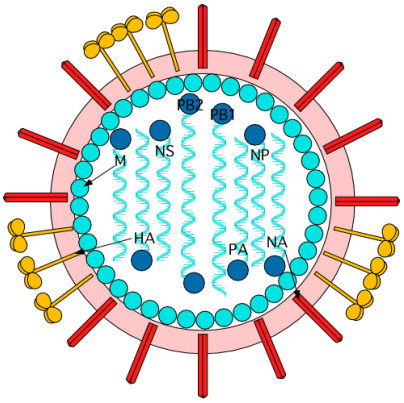
Segment:	Size(nt)	Polypeptide(s)	Function
1	2341	PB2	Transcriptase: cap binding
2	2341	PB1	Transcriptase: elongation
3	2233	PA	Transcriptase: protease activity (?)
4	1778	HA	Haemagglutinin
5	1565	NP	Nucleoprotein: RNA binding; part of transcriptase complex; nuclear/cytoplasmic transport of vRNA
6	1413	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.

[2] www.microbiologytext.com/index

		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.



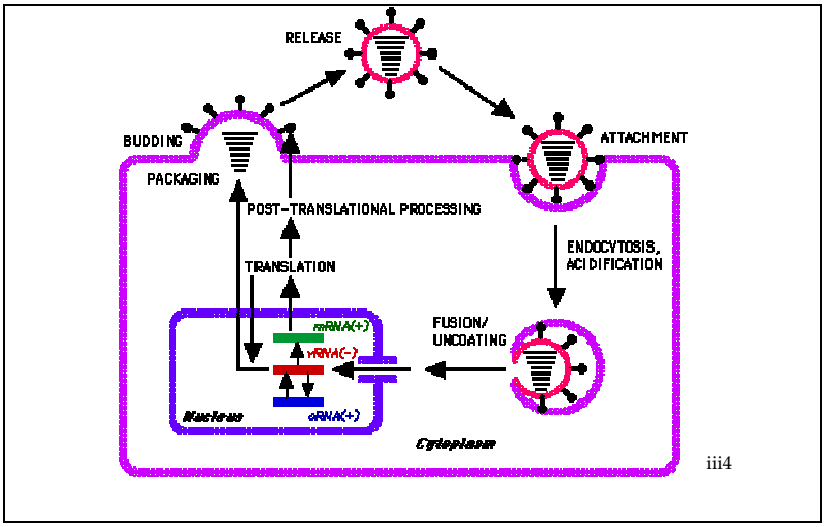
هذا رسم لفيروس الانفلونزا الذي يظهر الغلاف الخارجي ويكشف عن 8 قطع من الحمض النووي الريبي التي يشكل الجينوم للفيروس. وتتألف القشرة الخارجية من الدهون التي تم الحصول عليها من الخلية المضيفة الماضية. ويزين هذا مع هيماغلوتينين (ها، أصفر) والنورامينيداز (نا، وردي). ها هو ضروري للدخول إلى الخلايا ، في حين أن NA يساعد على الخروج منها. النيكليوبروتين (NP) هو بوليميراز الفيروس.

These 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.

هذه النسخ من الرنا الجينومي داخل الرنا "م" لتصنيع البروتين وبعد ذلك في دورة الحياة يجعل نسخ لكل حمض نووي ريبي لجزيئات فيروسية جديدة. نيكليوبروتين هو معرض جدا للخطأ ويخلق العديد من الطفرات في الجينوم الفيروسي وبالتالي الطفرات. "ها" و "نا" تتغير بسرعة، لتهرب قبل أن تتعرف عليها أنظمة المناعة لدينا.

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)[3].

ويتكون الجينوم للفيروس من ثمانية سلالات سلبية للرنا المفرد (هذه السلالات السلبية لا يمكن أن تترجم إلى فروع البروتين) مع كل قطعة يتم اكتمال mRNA واحد. ستة من ثمانية mRNA ترمز لنوع واحد من البروتينات، في حين أن الإثنان المتبقيان يرمزان إلى إثنين من البروتينات بواسطة الربط التفضلي من الحمض النووي الريبي. ويرتبط كل جزء من mRNA مع نسخ متعددة من النيكلويوبروتين وبوليميراز الحمض النووي الريبي (مصنوعة من بروتينات فيروسية PB1، PB2 وPA).



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1. Adsorption

1.الالتصاق

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

في هذه المرحلة تلتصق الفيروس على الخلية ويمكنها إستعادة عافيتها على الشكل المعدي دون تحلل الخلية بواسطة الاجراءات التي تدمر

[3] www.microbiologytext.com/index

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

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.

المستقبلات أو تضعف روابطهم الى الاجسام الفيروسية. الفيروسات الحيوانية تمتلك مواقع متخصصة للالتصاق موزعة على سطح الجسم الفيروسي.مثل paramyxoviruses و orthomyxoviruses يلتصقان من خلال الجليكوبروتين، و adenoviruses بلتصق من خلال الالياف الخماسية. الالتصاق يحدث لمستقبلات خلايا معينة، بعض المستقبلات هي الجليكوبروتين و الاخرين هم الفوسفوليبيد او الجليكوليبيد. وعادة تكون جزيئات ضخمة ضمن وظائف فيزيولوجية محددة مثل تكملة لمستقبلات EBV. سواء كانت أم لم تكن مستقبلات لفيروسات معينة موجودة على خلية تعتمد على الأنواع ، والأنسجة وحالته الفيزيولوجية. تفتقر الخلايا الى مستقبلات خاصة مقاومة. تم منع التعلق عن طريق المضادات الحيوية التي تربط المواقع المشتركة بين الفيروس او الخلايا .

2. Penetration

2.الدخول

Penetration rapidly follows adsorption, and the virus can no longer be recovered from the intact cell.

الدخول السريع يلي عملية الامتصاص، وهنا لا يستطيع الفيروس من استرداد عافيته من خلايا سليمة.

The most common mechanism is receptor mediated endocytosis, the process by which many hormones and toxins enter cells

الالتقام بواسطة المستقبلات هو الالية الاكثر شيوعياً. حيث من خلالها يدخل العديد من الهرمونات و السموم إلى الخلايا .

The virion is endocytosed and contained within a cytoplasmic vacuole

تُلتقم أجزاء الفيروس داخل تجويف سيتوبلاسمي في الخلية

3. Uncoating

3.نزع الغلاف

A key step in uncoating is the acidification of the content of the endosome to a pH of about 5,

الخطوة الاساسية في نزع الغلاف هي في نسبة الأسيد

owing to the activity of a proton pump present in the membrane

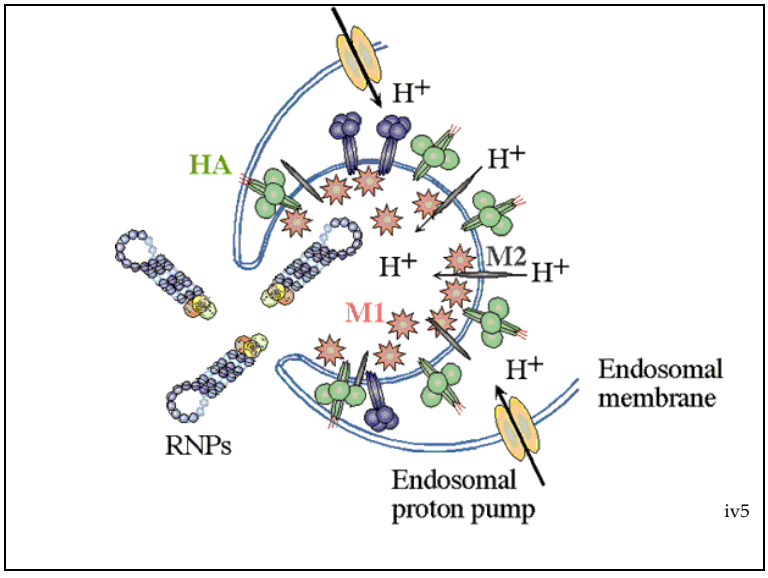
The low pH causes rearrangement of coat components, which then expose normally hidden hydrophobic sites.

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.

الموجود(PH 5) في الدخلول (هو حيز محاط بالغشاء الخلوي endosome) وذلك بسبب مضخة البروتين الموجودة في الغلاف.

يسبب انخفاض الرقم الهيدروجيني إلى إعادة مكونات الطبقة,الذي يكشف عادة المواقع الغير المائية التي تربط المادة الدهنية في الطبقة الثنائية للغشاء مما تسبب في قدف النواة الفيروسي داخل السيتوسول.

، عنصر الأحماض الحساسة هي وحدة الHA_2 الأساسية من الهيماغلوتينين لفيروس الانفلونزا ، وهي القاعدة الخماسية لل adenoviruses



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4. Viral Nucleic Acid Replication

النسخ المتماثل للحمض النووي الفيروسي

Virulent viruses, either DNA and RNA, shut off cellular protein synthesis and disaggregate

الفيروسات الفتاكة سواء الرنا أو الدنا توقف تصنيع بروتينات الخلية و

[5] © Paul Digard, Dept Pathology, University of Cambridge

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.

تفصل البوليريبوسوم لصالح حدوث تحول في تصنيع الفيروس.

إن إيقاف آلية تصنيع البروتين تختلف حتى داخل الأسرة الفيروسية نفسها

فيروس شلل الأطفال يستخدم انزيم لقطع البروتين الفيروسي ويسبب الانقسام ل200 Kd لقبعة-الملزمة للبروتينات وهو مطلوب للبدء في ترجمة رسائل الخلايا المغطاة.

وعلى العكس من الفيروسات الفتاكة هناك فيروسات معدلة مثل polyomaviruse يمكنها أن تحفز تصنيع الرنا"م" والدنا و البروتينات للخلية المضافة

هذه الظاهرة أخذت قدرا كبيرا من الإهتمام في السرطان الفيروسي.

Maturation and Release

النضوج و التحرر

Maturation proceeds differently for naked, enveloped, and complex viruses[v6]

نضوج الحصيلة بطريقة مختلفة عن الفيروسات المجردة، المغلفة، و المعقدة

2.2 MHC-I Molecule / *مقعد التوافق النسيجي الكبير الجزئي*

- 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.

- جزيئات معقد التوافق النسيجي الكبير هي بروتينات غشاء محددة: تم العثور على جزيئات معقد التوافق النسيجي الكبير الأول في أنسجة كلها تقريبا من الجسم، في حين تم العثور على جزيئات معقد التوافق

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

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

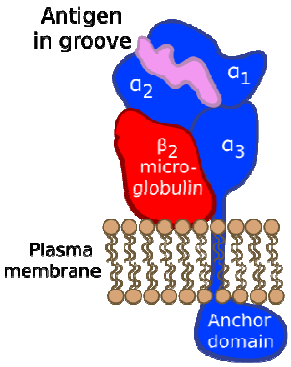
النسيجي الكبير الثاني فقط على مستضد تقديم الخلايا.

- جزيئات معقد التوافق النسيجي الكبير تمتلك تجويف عميق قادر على عقد الببتيد القصير. عملية جزيئات معقد التوافق النسيجي الكبير الأول معالجة البروتينات الموجودة داخل الخلية و عرضها على سطحها. أما جزيئات معقد التوافق النسيجي الكبير الثاني يظهر المستضدات المأخوذة من خلايا الهضم، وفي أغلب الأحيان الخلايا الأجنبية ، وعرضها على الجهاز المناعي.

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.

- الجهاز المناعي يراقب البروتينات الموجودة على جزيئات معقد التوافق النسيجي الكبير الأول وينشط عندما يتم الكشف عن البروتينات الأجنبية، من الطفيليات داخل الخلايا. هذه النتائج تظهر عادة في تدمير الخلية

Structure of an MHC molecule



2.3 β2m./ بيتا 2 ميكروغلوبيلين

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

يرمز هذا الجين على بروتين المصل الموجود بالتعاون مع معقد التوافق النسيجي الكبير الصنف الأول للسلسلة الثقيلة على سطح ما يقارب جميع الخلايا التي تمتلك نواة. البروتين له هيكل ورقة مطوية-بيتا في الغالب التي يمكن أن تشكل ألياف نسيجية أميلودية في بعض الحالات

pathological conditions. A mutation in this gene has been shown to result in hypercatabolic hypoproteinemia[vi7]

المرضية. وقد تبين طفرة في هذا الجين إلى نقص بروتين الدم المفرط الأيض (hypercatabolism)

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

3 MATERIAL AND METHOD

A- Part I

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/

```
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
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[8vii]

Protocol:

1. Isolate[9] 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

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

[9] The procedures of this step as follows in peqGOLD kit and for more details please see the part 3.5.

primer 3′-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. 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[10][viii]*:*

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.

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

1. [10] This step from Novagen." lifeserv.bgu.ac.il/wb/zarivach/.../Novagen%20**pET**%20**system**%20**manual**.pdf"

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[11ix].

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 μl *Eco*R I(*Not* I) adapter (1 mg/ml)[12]

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

[12] *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

- 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
- 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

---

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

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[13] and then centrifuged (10000xg).for 20 min.

4- The pellet containing recombinant protein was washed with 10 mM Tris HCl, pH 8(20 ml).

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

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

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[14x].

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[15].

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

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.

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

[15] Took from GE Healthcare.

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.

B- Part II

The protocol that will be practiced in MEGBI lab contains many modifications for the protocol of part I to fit with the materials and the devices presented in the lab.

3.6 Amplification of the HLA-A*0201 and β-2m gene from PBMC

Reagent:

peqGOLD kit.
Forward primer for HLA-A*0201: 5′-CCCTGACCCAGACCTGGGCGG-3′
Reverse primer for HLA-A*0201: 3′-AGGGTCGGGTGGTAGGGGTAG-5′
Forward primer 3 for HLA-A*0201: 5′-GGCTCCCACTCCATGAGGTAT-3′
Reverse primer 4 for HLA-A*0201: 3′-GGGAGTGGGACTCTACCCTCG -5′.
Forward primer for β2-m: 5′-GCATCCAGCGTACTCCAAAGA-3′
Reverse primer for β2-m: 3′-ATTCACCCTAGCTCTGTACCG-5′
dNTP Mix
Sterile, distilled water
M DTT
(200 units) of M-MLV (RT)
RNase A
10XPCR Buffer [200mM Tris-HCl (PH 8.4), 500 mM KCl]
50 mM $MgCl_2$
10 mM dNTP Mix
Amplification primer 1(10μM)
Amplification primer 2 (10μM)
Taq DNA/polymerase

Protocol:

1. Isolate[16] 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: the total RNA only

[16] The procedures of this step as follows in peqGOLD kit and for more details see the last page.

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:

a. Add the following components to a nuclease-free microcentrifuge tube:

- Take 0.6µl of the Primer.

- Take 5µl of the total RNA.

- 1µl (10mM) dNTP Mix (10mM each dATP,dGTP,dCTP and dTTP at neutral PH).

- Add sterile, distilled water to 12 µl.

b. Heat mixture to 65°C for 5 min and quick chill on ice.

C. collect the content of the tube by brief centrifugation (e.g. 20s , 10 000 rpm)

And add:

- 4µl 5X First-Strand Buffer

- 2µl 0.1 M DTT[17]

c. Mix contents of the tube gently and incubate at 37 °C for 2 min

d. Add 1µl (200 units) of M-MLV (RT)[18] , and mix by pipetting gently up and down.

e. Incubate 50 min at 37°C.

f. Inactivate the reaction by heating at 70°C for 15 min.

g. Incubate 40s at 90 °C (with PCR machine)

[17] DTT, Molecular Grade, is an antioxidant used to stabilize enzymes and other proteins containing sulfhydryl groups. The liquid form of the product is a 100mM solution of DTT in water(Promega)

[18] take the tube of M-MLV out and put in ice until thawed

h. Immediately put the tube into ice for 1 min

i. Add 3µl RNase A

j. Incubate 20 min at 37°C (with PCR machine)

Now we have cDNA.

4-Add the above to a PCR reaction tube for a final reaction volume of 50 µl:

- 2.5µl 10×Reaction buffer S
- 2.5µl 10×Reaction buffer Y
- 10µl enhancer solution
- 1µl 40 mM dNTP Mix
- 1µl amplification primer 1(10µM)
- 1µl amplification primer 2 (10µM)
- 0.5 µl Taq DNA/polymerase (5U/µl)
- 2µl cDNA (from first-strand reaction)
- Autoclaved, distilled water to 50 µl
- Mix gently and layer 1-2 drops (50µl) of silicone oil over the reaction
- (Note: the addition of silicone oil is unnecessary in thermal cyclers equipped with a heated lid.)
- Heat reaction to 94°C for 2 min to denature.
- PCR program: 35 cycles:
- 10s 95 °C
- 10s 60 °C
- 30-60s 72 °C
- Take 5µl from the result and put in the second round PCR and repeat the same step.

(Note: we use inner primers 3 and 4 in the second round of PCR)

5-Put the PCR product on the gel agarose.

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

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

Purify from an agarose gel by QIA quick gel extraction kit[19xi] . The DNA sequences were verified by sequencing.

3.7 Prepare the insert DNA (HLA-A*0201 and *β2m*) with the adapter

Suggested Conditions for Adapter Addition to DNA[20].
Reagent:
5X adapter buffer
*Eco*R I(*Not* I) adapter (1 mg/ml)
0.1 M DTT
T4 DNA ligase (1 U/μl)
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 μl *Eco*R I(*Not* I) adapter (1 mg/ml)[21]

[19] See MEGBI training courses book part I page 54-58

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

[21] *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

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[22] 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*RI (*Not*I) adapters by gel electrophoresis and then ligate into the appropriate vector.

Prepare the T7 RNA polymerase with the adapter
The protocol was the same as that used in preparation of insert DNA with the adapter.

3.8 Expression of HLA-A*0201 and β-2m

3.8.1 Vector preparation:

To digest and gel-purify the vector:
Reagent:
pET vector
10X restriction enzyme buffer
EcoR I restriction enzyme

---

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

[22] T4 Polynucleotide Kinase (T4 PNK) catalyzes the transfer of the γ-phosphate from ATP to the 5´-terminus of polynucleotides or to mononucleotides bearing a 3´-phosphate group (1). T4 PNK is widely used to end-label short oligonucleotide probes (2), DNA (3) and RNA (4) molecules (Promega)

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.
3. Run a 3 μl sample 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 by purification with QIA quick gel extraction kit[23xii] Resuspend the final product in a total volume of 30 μl (usually about 50 ng/μl DNA). Assume recoveries in the range of 50% for the ligation step.
9. Store the treated vector at –20°C until use.

[23] Refer to MEGBI training courses book part I page 54-58.

3.8.2 Ligation

N.B: this step should be done in 3 eppendorfs tubes for HLA-A*0201, β2-m and T7 RNA polymerase.

Reagent:

10X Ligase Buffer (200 mM Tris-HCl pH 7.6,
100 mM MgCl2
100 mM DTT
10 mM ATP, (or 2× rapid ligation buffer contains ATP)
50 ng/µl prepared pET vector
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

Procedures:

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:

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. 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.

3.8.3 Transformation

Preparation of E.coli competent cells[24].

Reagent:

Competent cells JM109.

[24] Refer to MEGBI training courses book part I page 62.

LB 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 each ligation reaction (ligation of HLA-A*0201 and ligation of T7 RNA polymerase) 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 LB medium to each tube. Keep the tubes on ice until all have received LB.
Singles Competent Cells
Add 250 µl of room temperature LB Medium to each tube. Keep the tubes on ice until all have received LB.
10. Selection for transformation is accomplished by plating on medium containing antibiotic (ampicillin) for the plasmid encoded drug resistance.

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. Please see the last page 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).
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 LB 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.

3.9 Extraction and solubilization of inclusion bodies

Materiel:
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 fluoride (50µg/ml)
DNase (20µg/ml)

RNase (20μg/ml)
1mM EDTA
0-100 mM NaCl

Purification of recombinant proteins:

Purification of recombinant proteins:

Incubation of some colonies overnight with lb medium with ampicillin.
Centrifugation at 4000 rpm for 5min.
The cell pellets were resuspended in 10 mM Tris HCl.pH 8 (250μl) 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.
The cells were lysed by heat shock for 40s in boiling water and then centrifuged (10000xg) for 20 min.
The pellet containing recombinant protein was washed with 10 mM Tris HCl, pH 8(250μl).
It is then dissolved in 100 mM Tris HCl, pH8/8 M urea (125μl), and centrifuged at 4°C for 1 hour (150000xg)
The recombinant protein purified by an ion exchange chromatography Q Sepharose fast flow[25].
Fractions, the purified HLA heavy chains contained stored at-20°C for use in the ELISA experiment

3.10 ELISA assay of peptide-MHC complex formation:

Material:

- Pan specific mouse anti HLA class I antibody, W6/32
- Polyclonal rabbit antihuman β2m-HRP or post primary block (from Novolink compact polymer Detection kit)
- dextran polymer conjugated with goat antirabbit IgG and HRP or Novolink polymer **(**from Novolink compact polymer Detection kit**)**
- 96 well Maxisorp ELISA Plates
- Pluronic lutrol F-68
- Peptide

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

- 100mM carbonate buffer (PH 9.6)
- 10٪ w/v skimmed milk powder in PBS (SMP-PBS) or Protein block (from Novolink compact polymer Detection kit).
- 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 50µl/well at 5µl/ml, in 100 mM carbonate buffer, PH 9.6.

Day 2

Add 320 µl/ well 10٪ w/v protein block 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, containing 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 peptide (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٪ protein block 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 post primary bock with 2% protein block 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. Novolink polymer.

Washed 6*600 µl/well with PBS/0.05% Tween 20 at room temperature.

ELISA was developed with 3, 3′ 5, 5′-tetramethylbenzidine hydrogenperoxide[26] for 30 min at room temperature.

Colorimetric reaction was read at 450nm using a Victor Multilabel ELISA counter.

3.11 For more details:

The peqGOLD viral RNA Kit

INTRODUCTION

The peqGOLD viral RNA Kit is designed for isolation of viral RNA from cell free fluids such as plasma, serum, urine and cell culture supernatants.

The kit is also suitable for isolation of total RNA from cultured cells, tissues and bacteria.

RNA purified using the peqGOLD viral RNA Kit method is ready for applications such as RT-PCR

Protocol: Purification of Viral RNA (Spin Protocol)

This protocol is for purification of viral RNA from 140 µl plasma, serum, urine, cell culture.

Important points before starting

■ Read "Important Notes" (pages–30)

■ All centrifugation steps are carried out at room temperature (15–25°C).

Things to do before starting

■ Equilibrate samples to room temperature (15–25°C).

■ Equilibrate Buffer AVE to room temperature for elution in step 11.

■ Check that Buffer AW1 and Buffer AW2 have been prepared according to the instructions on page 32.

■ Add carrier RNA reconstituted in Buffer AVE to Buffer AVL

[26] Ready-to-use sensitive substrate for the detection of horseradish peroxidase activity. Absorbs at 450 nm (yellow end-product). Ideal for ELISA and solution assays.

Important; if not have QIAamp Viral RNA mini , we can use peqGOLD Viral RNA Kit protocoles visit www.peqlab.de

procedure

1. Pipet 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.

If the sample volume is larger than 140 µl, increase the amount of Buffer AVL–carrier RNA proportionally (e.g., a 280 µl sample will require 1120 µl Buffer AVL–carrier RNA) and use a larger tube.

* Fully automatable on the QIAcube. See www.qiagen.com/MyQIAcube for protocols.

2. Add 140 µl plasma, serum, urine, cell-culture supernatant, or cell-free body fluid to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.

To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used.

3. Incubate at room temperature (15–25°C) for 10 min.

Viral particle lysis is complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA.

Potentially infectious agents and RNases are inactivated in Buffer AVL.

4. Briefly centrifuge the tube to remove drops from the inside of the lid.

5. Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.

Only ethanol should be used since other alcohols may result in reduced RNA yield and purity. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280 µl sample will require 1120 µl of ethanol). In order to ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

6. Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.

Close each spin column in order to avoid cross-contamination during centrifugation.

Centrifugation is performed at 6000 x g (8000 rpm) in order to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

7. Carefully open the QIAamp Mini column, and repeat step 6.

If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.

8. Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl.

9. Carefully open the QIAamp Mini column, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Continue directly with step 11, or to eliminate any chance of possible Buffer AW2 carryover, perform step 10, and then continue with step 11.

Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flow-through, containing Buffer AW2, contacting the QIAamp Mini column.

Removing the QIAamp Mini column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp Mini column. In these cases, the optional step 10 should be performed.

10. Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.

Centrifuge at 6000 x g (8000 rpm) for 1 min.

A single elution with 60 µl of Buffer AVE is sufficient to elute at least 90% of the viral RNA from the QIAamp Mini column. Performing a double elution using 2 x 40 µl of Buffer AVE will

increase yield by up to 10%. Elution with volumes of less than 30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate.
Viral RNA is stable for up to one year when stored at –20°C or –70°C.

Transformation[27]

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).

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.

[27] Extracted from Novagen

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.

The Host Strains

Host Strains[28xiii]

After plasmids are established in a non-expression host, they are most often transformed into a host bearing the T7 RNA polymerase gene (λDE3 lysogen) for expression of target proteins. Figure 1 illustrates in schematic form the host and vector elements available for control of T7 RNA polymerase levels and the subsequent transcription of a target gene in a pET vector. In λDE3 lysogens, the T7 RNA polymerase gene is under the control of the *lacUV5* promoter. This allows some degree of transcription in the uninduced state and in the absence of further controls is suitable for expression of many genes whose products have innocuous effects on

[28] **United States & Canada 800-207-0144**
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United Kingdom 0800 622935
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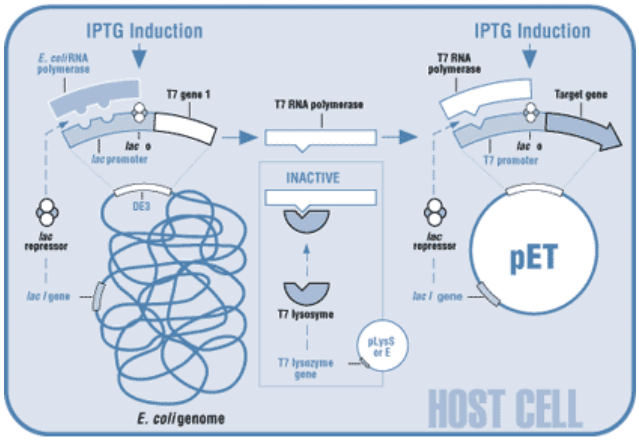
host cell growth. For more stringent control, hosts carrying either pLysS or pLysE are available. The pLys plasmids encode T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase, and thus reduces its ability to transcribe target genes in uninduced cells. pLysS hosts produce low amounts of T7 lysozyme, while pLysE hosts produce much more enzyme and, therefore, represent the most stringent control available in λDE3 lysogens (4).

Figure 1. Control elements of the pET system



Several different host strains are available as λDE3 lysogens. The most widely used host is BL21, which has the advantage of being deficient in both *lon* (5) and *ompT* proteases. Novagen has introduced two derivatives of BL21 designed for special purposes. The B834 series is methionine deficient and, therefore, enables high specific activity labeling of target proteins with ^{35}S-methionine or selenomethionine (6). The BLR strain is a *recA*- derivative that improves plasmid monomer yields and may help stabilize target plasmids containing repetitive sequences. The AD494 strains are thioredoxin reductase (*trxB*) mutants that enable disulfide bond formation in the *E.coli* cytoplasm. This allows for the potential production of properly folded, active proteins (7). Other available strain backgrounds include the K-12 strains HMS174 and NovaBlue, which are *recA*-, like BLR. These strains may stabilize certain target genes whose products may cause the loss of the DE3 prophage. NovaBlue is potentially useful as a stringent host due to the presence of the high affinity *lacI*^{q} repressor encoded by the F episome. In addition, Novagen offers the λDE3 Lysogenization Kit for making new expression hosts with other genetic backgrounds.

An alternative for expressing extremely toxic genes or preparing a new λDE3 lysogen is to provide T7 RNA polymerase by infection with λCE6. Although not as convenient as inducing a λDE3 lysogen with IPTG, this strategy may be preferred for certain applications.

High Stringency T7lac Promoter

In addition to the choice of three basic expression stringencies at the host level, the pET system provides two different stringency options at the level of the T7 promoter itself: the "plain" T7 promoter and the T7*lac* promoter (8; also shown in Fig. 1). The T7*lac* promoter contains a 25 bp *lac* operator sequence immediately downstream from the 17 bp promoter region. Binding of the *lac* repressor at this site effectively reduces transcription by T7 RNA polymerase, thus providing a second *lacI*-based mechanism (besides the repression at *lacUV5)* to suppress basal expression in λDE3 lysogens. Plasmids with the T7*lac* promoter also carry their own copy of *lacI* to ensure that enough repressor is made to titrate all available operator sites.

In practice, it is usually worthwhile to test several different vector/host combinations to obtain the best possible yield of protein in its desired form (9). Figure 2 illustrates dramatic differences in the expression of two target proteins with various combinations.

4 Results / النتائج

4.1 Amplification of the HLA-A*0201 and β-2m gene from PBMC / مضاعفة جين هلا*0201 وبيتا-2م من المحيط الخارجي لخلايا الدم البيضاء

First: we isolated the total RNA form PBMC as follows:

We drew 10ml of blood from a person, we putted it in 4ml of EDTA (fig.1) to prevent the agglutination (EDTA is an anticoagulant) and we centrifugated it into a centrifugation at 4000rpm for 8 minutes (fig.2), we obtained the result designed in fig.3 then we aspirated the phase 2 that contains the white blood cells with a micropipette 1000µl, we putted it in a 1.5ml microcentrifuge tube finally we isolated the total RNA by the peqGold viral RNA kit (can be stored the result at 4°C).

أولاً: قمنا بعزل الرنا الكامل من المحيط الخارجي لخلايا الدم البيضاء كما يلي:

قمنا بسحب 10مل من دم إنسان، ووضعناه في 4مل من إيثلين ثنائي الأمين رباعي حمض الخل EDTA) (صورة 1) لمنع تخثر الدم (منع التجلط) ومن ثم وضعناه في آلة الطاردة لفصل الدم على سرعة 4000 آر.بي.آم لمدة 8 دقائق (صورة 2)، وبعدها حصلنا على ثلاث طبقات من الدم كما في الصورة 3 وبواسطة الماصة الأوتوماتيكية الخاصة ب 1000µl. سحبنا الطبقة الثانية من الدم المحتوية على كريات الدم البيضاء ووضعناها في أنبوب 1.5مل وبدأنا بعملية عزل الرنا الكامل بواسطة مجموعة الرنا الفيروسي بك غولد.

(تحفظ النتيجة على 4م°)

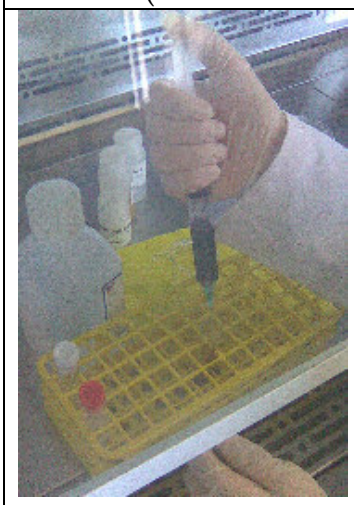


Figure 1: putting the blood in 4ml EDTA / الصورة 1: وضع 10 مل من الدم في 4مل من مضاد التخثر

Figure 2: centrifugation at 4000rpm for 8min / الصورة 2: عملية الفصل على 4000آر.بي.آم لمدة 8دقائق

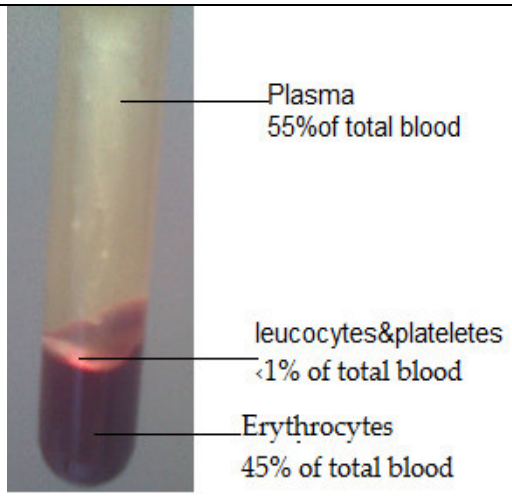


Figure 3: Blood components after centrifugation / الصورة 3: مكونات الدم بعد الفصل.

Second: we retrotranscripted the tRNA to cDNA by M-MLV reverse transcriptase as described in part 3.6

ثانياً: قمنا بعملية إعادة نسخ الرنا الناقل إلى الدنا الناسخ بواسطة أنزيم M-MLV reverse transcriptase كما هو مذكور في الجزء 3.6 المرحلة 3.

step 3. **Third:** we amplified the cDNA by PCR as described in part 3.6 step 4, and we obtained a quantity amplified of HLA-A*0201 and β2-m.	**ثالثاً:** بعدما حصلنا على الدنا الناسخ قمنا بمضاعفة كميته بواسطة آلة تفاعل البلمرة المتسلسل كما هو مذكور في الجزء 3.6 المرحلة 4، وهكذا نكون قد حصلنا على كمية مضاعفة من ال هلا*0201 وبيتا2-آم.

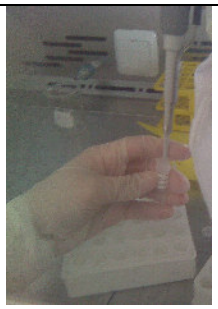


Figure 4: Pipetting of 1µl of M-MLV(RT) / الصورة 4: إمتصاص 1 ميكروليتر من M-MLV

Figure 5: PCR machine / الصورة 5: آلة تفاعل البلمرة المتسلسل

Fourth: we purified the PCR product on an agarose gel**;** 1. we prepared the agarose gel as follows**:** The preparation of an agarose gel: - Put 1g of agarose in 100ml of TAE buffer 1×PH and dissolve it at 90°C. - Let the gel cool down to about 60°C at room temperature. - Put the gel on the gel rack then insert the comb and let it to solidify.	**رابعاً:** قمنا بتنقية منتج آلة تفاعل البلمرة المتسلسل بواسطة هلام الأغاروز: 1. قمنا بتحضير الهلام الأعاروزي كما يلي: طريقة التحضير: - وضعنا 1غ من الأغاروز في 100مل من منظم TAE 1×درجة الحموضة ثم قمنا بتذويبه على 90°م. - تركناه ليبرد إلى 60°م على درجة حرارة الغرفة. - أضفناه على طبق الهلام وأضفنا عليه المشط ثم تركناه حتى يجمد.

Figure 6: Dissolve the gel at 90°C with agitation / الصورة 6: تذويب الهلام على 90°م مع التحريك.

Figure 7: putting of the gel on the gel rack / الصورة 7: وضع الهلام على الطبق

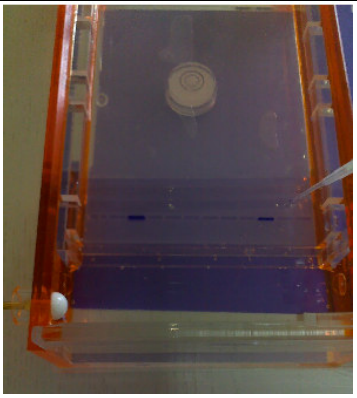


Figure 8: injetion of 10µl of sample into the wells of gel / الصورة 8: وضع 10ميكروليتر من العينة في فتحة الهلام

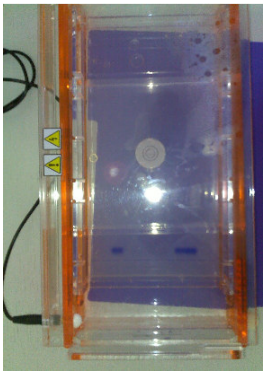


Figure 9: migration of the three bands (2 bands for HLA-A*0201& 1 band for β2-m) الصورة 9: هجرة البقع الثلاثة (بقعتين للهلا وبقعة لبيتا2–آم)

2. After the solidification of gel, we drew the comb and the border of the gel rack, we added 1liter of TAE buffer to cover all the gel, then we injected 10µl of each sample (we putted in each new eppendorf tube 6 µl of the DNA (HLA-A*0201 or β-2m) + 3µl of glycerol + 3µl of bormophenol blue) into the wells of the gel prepared, we closed the lid of electrophoresis chamber and applied the voltage at 120 volts for 30min, (400mA, 400 Watts).

3. After the migration of bands we turned off the power supply we removed the gel and putted it into a deep vessel we covered it by 250ml of dH_2O and we added 30µl of ethidium bromide and late it for 30min. then we washed the gel 3 times from ethidium bromide with dist.water.(250ml in each times).

WARNING: be curful with the ethidium bromide!!! Use specific gloves, put the waste liquid of ethidium bromide in a specific place, and do not throw it in the nature.

2. بعد جماد الهلام جيداً، قمنا بسحب المشط وأطراف طبق الهلام، وأضفنا 1 ليتر من منظم TAE 1×درجة الحموضة لتغطية الهلام كاملاً ووضعنا في الفتحات الصغيرة للهلام 10ميكروليتر من كل عينة (تحتوي العينة في كل أنبوب على 6ميكروليتر من الدنا إما الهلا 0201وإما بيتا2–آم + 3 ميكروليتر من الغليسيرول + 3 ميكروليتر من البروموفينول الأزرق) ثم أغلقنا غطاء الرحلان الكهربائي وقمنا بتشغيل التيار الكهربائي على 120فولت لمدة 30 دقيقة.

3. بعد هجرة بقع الدنا قمنا بإطفاء التيار الكهربائي عن الآلة، أخذنا الهلام ووضعناه في وعاء عميق أضفنا عليه 250مل من الماء المعقم مع 30 ميكروليتر من بروميد الإثيديوم وتركناه لمدة 30 دقيقة، بعدها قمنا بغسله من بروميد الإثيديوم 3 مرات بواسطة الماء المعقم (وضعنا في كل مرة 250مل من الماء المعقم).

تحذير: يجب الحذر في التعامل مع الإتيديوم !!! إستخدم القفازات الخاصة , ضع الماء المحتوية على بروميد الإثيديوم في مكان خاص و لا ترميه في الطبيعة . لأنه قد يسبب السرطان ويحدث الطفرات (التغيير المفاجئ للدنا).

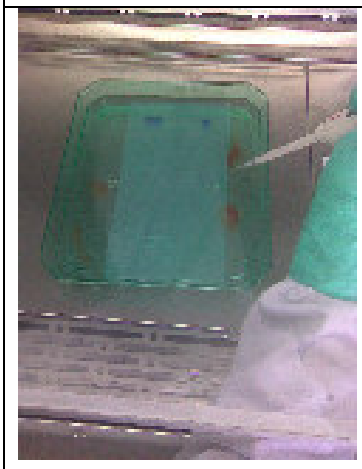
4. بعد غسله جيداً من بروميد الإثيديوم وضعناه على آلة الأشعة ما فوق البنفسجية لنرى إذا كان هناك بقع من الدنا، إلا إن هذه المرحلة ستبين لنا نتيجة ما تم فعله حتى الآن.

النتيجة: الحمدلله تمكنا من رؤية البقع الثلاثة التي تم وضعهم (بقعتين للهلا وبقعة لبيتا2–آم) وتمكنا من إكمال العمل (الصورة–12).

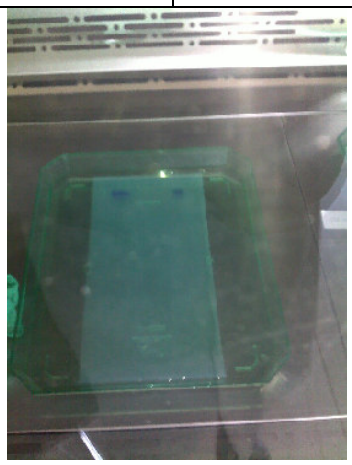
Ethidium bromide could cause a cancer and it is mutagen.

4. We putted the gel on UV-machine to see if we have bands or no, if yes, so the steps are correct and we can continue the protocol.

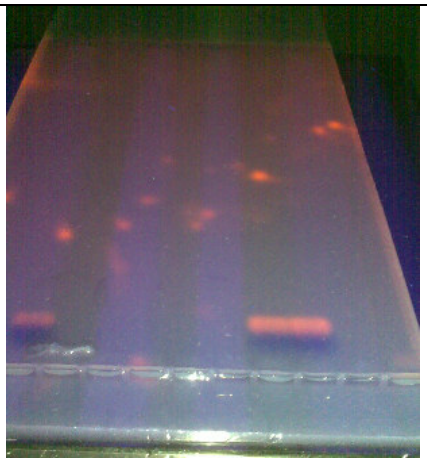
Result: AlHamdullillah we observed the 3 bands and we could continue the protocol (fig.12).



:injection of 30µl of ethidium 10Figure bromde in the gel recovered with 250ml dH_2O. الصورة 10: إضافة 30 ميكروليتر من بروميد الإثيديوم في الهلام المغطى ب 250مل من الماء المعقم



: soaking the gel with 11Figure ethidium bromide for 30min/ الصورة 11: نقع الهلام ببروميد الإثيديوم لمدة 30 دقيقة



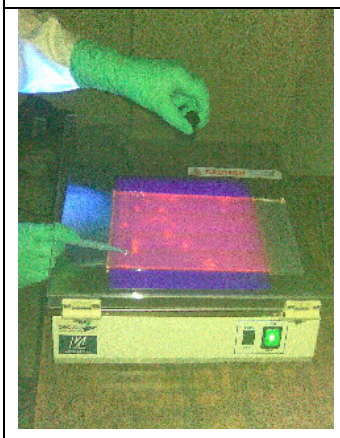
: observation of 2 bands of HLA-A*0201 12Figure at the right an 1 band of β2-m at the left on UV-machine / الصورة 12: ملاحظة بقعتي الدنا من الهلا 0201 من الجهة اليمنى للهلام وبقعة للبيتا2–آم على الجهة اليسرى منه على آلة الأشعة ما فوق البنفسجية.

5. We marked off the border of each band on the UV-machine (fig.14), then we putted the gel in the deep vessel to cut the bands and should be remove all the excess of gel (fig.15) then we putted each band in a new eppendorf tube (fig.16), we balanced each tube before and after the addition of band in it for obtain the weight of bands to begin the purification of those bands with the Qiaquick gel extraction kit[29] (the weight of HLA-A*0201 was

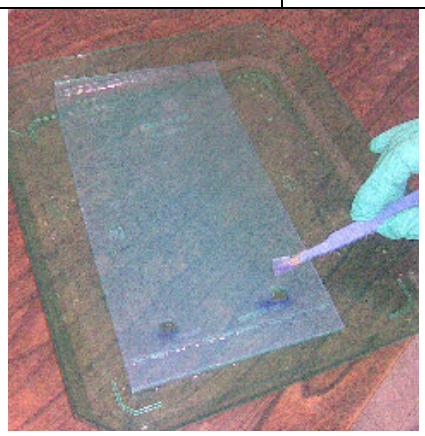
5. بينما كان الهلام على آلة الأشعة مافوق البنفسجية قمنا بتعيين حدود البقع (الصورة 14)، ثم نقلناه إلى وعاء عميق ثم قطعنا البقع جيداً مع إزالة الهلام الزائد (الصورة 15) وضعنا كل بقعة في أنبوب خاص (الصورة 16)، ثم قمنا بأخذ وزن كل أنبوب بداخله البقعة من الهلام وأنبوب فارغ لنحصل على الوزن الصافي للهلام لنبدأ بتصفيته عبر مجموعة إستخلاص هلام كياكويك (الوزن الصافي للهلا 0201 كان 1.32غ وبيتا كان 1.31غ) وبعدها قمنا بحفظ الدنا المنقى على – 20°م حتى إستعماله في المراحل القادة.

[29] For more detail see MEGBI training courses book part I page 54-58.

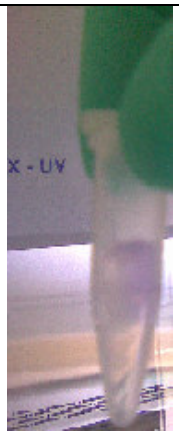
1.32g, and for β2-m was 1.31g) then we stored the purified DNA at -20°C until use.



: marking off the border of bands/ 13Figure
الصورة 13: تعيين أطراف البقع



: cutting the DNA band from the 14Figure gel/الصورة 14: إزالة الهلام عن بقع الدنا ووضعهم



: DNA band in eppendorf tube 15Figure
الصورة 15: وضع بقع الدنا في الأنبوب/

4.2 Preparation of vector / تحضير الناقل

To digest the vector we assembled the following components in a microcentrifuge tube:
3 µl pET vector
3 µl 10X restriction enzyme buffer
1 µl (10–20 U) EcoR I restriction enzyme
23 µl Nuclease-free water brought to volume
30 µl Total volume

We incubated at the appropriate temperature (usually 37°C) for 2–4 h in the water bath. We ran a 3 µl sample on an agarose gel to check the extent of digestion (fig.16), when digestion is complete, we added 1µl of calf intestinal alkaline phosphatase (we diluted 0.2µl of calf intestinal alkaline phosphatase in 0.8 µl of water for obtain

لقطع الناقل قمنا بجمع المكونات التالية في أنبوب النابذة:

3 ميكروليتر من الناقل pET.

3 ميكروليتر من منظم أنزيم القطع 10×.

1 ميكروليتر (10–20وحدة) من أنزيم القطع إيكو.آر.وان

23 ميكروليتر من ماء خال من النيوكياز.

الحجم النهائي في الأنبوب 30 ميكروليتر.

قمنا بحضن الأنبوب لمدة ساعتين على 37°م في حوض ماء. ومن ثم وضعنا 3 ميكروليتر من العينة على هلام الأغاروز لنتحقق من قطعه (صورة 16)، وعندما تأكدنا من تقطيعه أضفنا 1 ميكروليتر من أنزيم الفوسفاتيز القلوي المعوي للعجل (ولكن لنحصل على تركيز 0.05وحدة من الأنزيم أضفنا 0.8ميكروليتر من الماء على 0.2ميكروليتر من الأنزيم) مباشرة لإبقاء عملية القطع ووضعناه في حوض ماء على 37°م لمدة 30دقيقة ثم قمنا بوضعه في 4 فتحات من الهلام الأغاروزي لتنقيته (الصورة 17)، بعد هجرة البقع على الهلام كما في المرحلة السابقة قمنا بتقطيعهم من الهلام الزائد بواسطة مشرط حاد ونظيف (الصورة 18).

[30] Refer to MEGBI training courses book part I page 54-58.

0.05U) directly to the remainder of the digestion. We incubated at 37°C for 30 min in the water bath then we injected the sample in 4 wells in the gel (fig.17), we ran the gel to separate the linear plasmid from nicked and supercoiled species. We visualized the DNA band with a long wave UV light source (fig.18) and we cut the band from the gel using a clean razor blade.

WARNING: Avoid over exposure to the light source, which can cause nicks and double strand breaks in the DNA.

We balanced the bands to begin that purification with QIA quick gel extraction kit[30] Resuspended the final product in a total volume of 30 µl (usually about 50 ng/µl DNA). Assume recoveries in the range of 50% for the ligation step. Then we stored the treated vector at –20°C until use.

تحذير: يجب تجنب تعريض الزائد للهلام على الأشعة ما فوق البنفسجية لأن ذلك قد يسبب شقوق وكسر للحبلين المزدوجين للدنا.

ومن ثم قمنا بقياس وزن البقع لنبدأ بعملية تنقيتهم من الهلام بواسطة مجموعة إستخلاص هلام كياكويك. الحجم النهائي من المنتج هو 30ميكروليتر ويمكن حفظه على -20م° إلى حين إستعماله مرة أخرى.

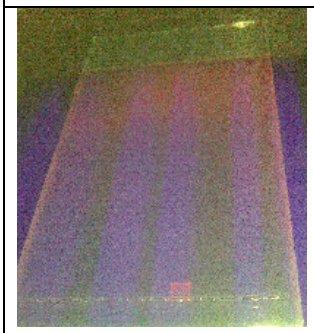


Figure 16: visualization the band of the digestion vector in the gel / الصورة 16: رؤية بقع دنا الناقل المقطع على الهلام

Figure 17: injection the samples in 4 wells in gel / الصورة 17: وضع العينات في 4 فتحات في الهلام

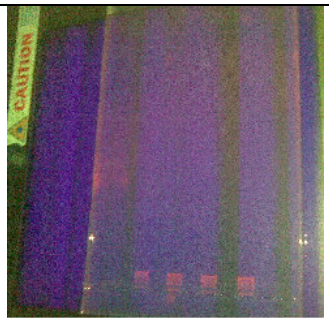


Figure 18: observation the bands on UV-machine / الصورة 18: ملاحظة البقع على ألة الأشعة ما فوق البنفسجية

4.3 Ligation / عملية الربط

We prepared 3 eppendorfs tubes and we marked each eppendorf tube (the first for the ligation of vector with HLA-A*0201, the second for the vector with β2-m, the third for the vector with T7 RNA Polymerase).

In this step we would tried if the insert can be legated with the vector without adapter!

Figure 19: Marking of each tube / الصورة 19: وضع إشارة على كل أنبوب

في هذه العملية قمنا بتجهيز 3 أنابيب من النابذة مع الكتابة على كل أنبوب ما الدنا الذي سيحتويه (مثلاً: الأنبوب الأول كتبنا عليه الناقل مع الهلا0201، الثاني الناقل مع بينا-2آم أما الثالث كتبنا عليه الناقل مع تي7 بوليميراز الرنا).

في هذه المرحلة كنا نريد أن نجرب هل من الممكن ربط الدنا الزائد بالناقل من دون الوصيلة (adapter).

We added in each eppendorf tube (1.5ml):

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

8 µl 25 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

2 µl Prepared target gene insert (0.2 pmol)

2 µl Nuclease-free water to volume

20 µl Total volume

Then gently we mixed by stirring with a pipet tip,

ثم أضفنا في كل أنبوب (1.5مل):

2 ميكروليتر من منظم الليغاز 10×.

8 ميكروليتر من كلور الماغنيزيوم 25mM.

2 ميكروليتر من 100mM DTT.

1 ميكروليتر من 10Mm ATP.

2 ميكروليتر من الناقل pET المحضر 50ng/µl.

1 ميكروليتر من ليغاز الدنا تي4، المخفف (مع المنظم المخفف ليغاز) 0.2-0.4 وحدة/ ميكروليتر.

2 ميكروليتر من الدنا المحضر (0.2 pmol).

2 ميكروليتر من ماء خالٍ من النيوكلياز.

20 ميكروليتر الحجم النهائي.

we incubated at 16°C to overnight.	ومن ثم مزجناهم بلطف بواسطة الممصة صعوداً ونزولاً وتركناهم على 16°م طيلة الليل.

4.4 Transformation / *عملية النقل*

Transformation into host strain for cloning In this step we would to prove if the ligation of the insert with the vector without adapter was possible or no, for this reason we transformed them in the host strain for cloning then we platted them and if in the next day the colonies was amplified on the plate this mean that ligation is possible and vice versa. For this step we brought a plate contains e.coli from the hospital because when we worked in the e.coli that presented in our lab we found it death and not lost the time we worked this step as follows: 1- We took some colonies from the plate then we putted it in 5ml LB medium. 2- We incubated over night at 37°C with shaking 3- In the second day we observed a big quantity of e.coli in the medium we distributed the medium in 4 eppendorf tubes and we chilled them on ice for 10 min, centrifuged them at 4000 rpm for 10 min at 4°C, discarded the supernatant and resuspended the pellets each in 600	**عملية النقل إلى داخل خلايا الإستنساخ** في هذه المرحلة أردنا أن نقوم بتجربة ربط الدنا الزائد بالناقل من غير الموصل (adapter)، وللتأكد من ذلك يجب نقل المنتج من عملية الربط إلى داخل خلايا البكتريا وزرعها على وسط غذائي ملائم ثم مراقبتهم في اليوم التالي فإذا تبين أن خلايا البكتيريا تكاثرت فهذا يعني أن عملية الربط قد نجحت والعكس صحيح. ولهذه التجربة جلبنا بكتريا مزروعة من المستشفى وذلك لأن البكتريا التي كانت موجودة عندنا في المختبر وجدناه ميتة بينما كنا نجهزها لنعمل بها ولكي لا نخسر الكثير من الوقت بإنتظار إحضار بكتيريا أخرى من الشركة، قمنا بالعمل ببكيريا المستشفى كما يلي: 1- أخذنا بعض من البكتريا المزروعة في صحن بتري ووضعناهم في 5 مل من وسط آل بي. 2- حضناهم طيلة الليل على 37م° مع الإهتزاز. 3- في اليوم التالي لاحظنا أن خلايا تكاثرت بشكل ملحوظ في الوسط آل بي عندئذ وزعنا كمية الوسط التي كانت في الأنبوب إلى 4 أنابيب نابذة ووضعناهم في الثلج لمدة 10 دقائق، ومن ثم نبذناهم على 4000 آر.بي.آم لمدة 10 دقائق على 4م°،

µl of cold 0.1M calcium chloride, leaved them on ice for 25 min, centrifuged them at 4000 rpm for 10 min at 4°C, resuspended each pellet in 60 µl of cold 0.1M calcium chloride (this step for competent cells) then we let some microliter for the next step and we stored the remaining at -20°C

4- We did the transformation as follows: We pipetted 20 µl aliquots of cells e.coli from the hospital into pre-chilled tube we added 1 µl of each ligation reaction (we added the pET that without cutting as control positive), stirred gently to mix and we returned to the ice we incubated 5 min on ice , we placed the tubes in a 42°C water for exactly 30 sec. (do not shake), we placed the tubes on ice for 2 min, we added 80 µl LB medium to each tube, kept the tube on ice until all have received LB, incubated for 1 hour at 37°C with shaking.

5- We Platted them onto plate contain LB medium with ampicillin then incubated overnight at 37°C

6- We observed in the second day colonies on the pate and that mean the ligation is possible and we can continue with the expression.

7- For the plate that contains the pET (the control positive) we took some colonies from it and we putted it the 2.5ml LB medium with ampicillin then

ثم إرمي السائل وأضف 600ميكروليتر من كلورايد الكالسيوم 0.1M البارد على المترقد في الأنبوب وإتركه في الثلج لمدة25 دقيقة ومن بعدها إنبذهم على4000 آر.بي.آم لمدة 10 دقائق على 4م° ثم أضف 60ميكروليتر من كلورايد الكالسيوم 0.1M على المترقد في الأنبوب (هذه المرحلة هي لفتح الخلايا) وأخيراً تركنا بعض الميكروليتر لنعمل بهم في المرحلة التالية وقمنا بتخزين الباقي في الثلاجة على -20م°.

4- قمنا بعملية النقل كالتالي: أخذنا 20 ميكروليتر من خلايا البكتريا ووضعناهم في أنبوب بارد وأضفنا 1 ميكروليتر من كل من تفاعل الربط (وأضفنا أيضاً إحدى الأنابيب الناقل pET لوحده كتحكم إيجابي)، حركناهم بنعومة لخلطهم ووضعناهم في الثلج لمدة 5 دقائق ثم في الماء الساخن على 42م° لمدة 30 ثانية (من دون تحريك) ثم في الثلج مرة ثانية لمدة 2 دقيقتين وأضفنا 80 ميكروليتر من وسط آل بي لكل أنبوب، وتركناهم في الثلج إلى أن يصل الوسط إلى الجميع في الأنبوب ثم وضعناهم في الحاضنة لمدة ساعة على 37 م° مع التحريك.

5- ثم زرعناهم على صحن بتري المحتوي على وسط آل بي مع الأمبيسيلين ومن بعدها وتركناهم طيلة الليل على 37 م°.

6- وفي اليوم الثاني لاحظنا مجموعات متكاثرة من البكتريا على الصحن وهذا يعني أن عملية الربط ممكنة ويمكننا تكملة العمل في التعبير عن البروتين.

7- أما بالنسبة لصحن الناقل الذي أخذناه كتحكم إيجابي أخذنا منه بعض مجموعات البكتريا ووضعناهم في 2.5مل من

we incubated it overnight at 37°C for 12-16 hours. 8- In the next day we centrifuged it and we purified it with Qiaprep spin Miniprep kit then we stored the result at -20°C.	وسط آل بي مع الأمبيسيلين وتركناهم طيلة الليل على 37م° لمدة 12-16ساعة. 8- وفي اليوم التالي نبذناهم ثم قمنا بتصفيتهم بواسطة مجموعة "كيابرب سبين مينيبرب" والناتج الذي حصلنا عليه خزناه على -20م°.

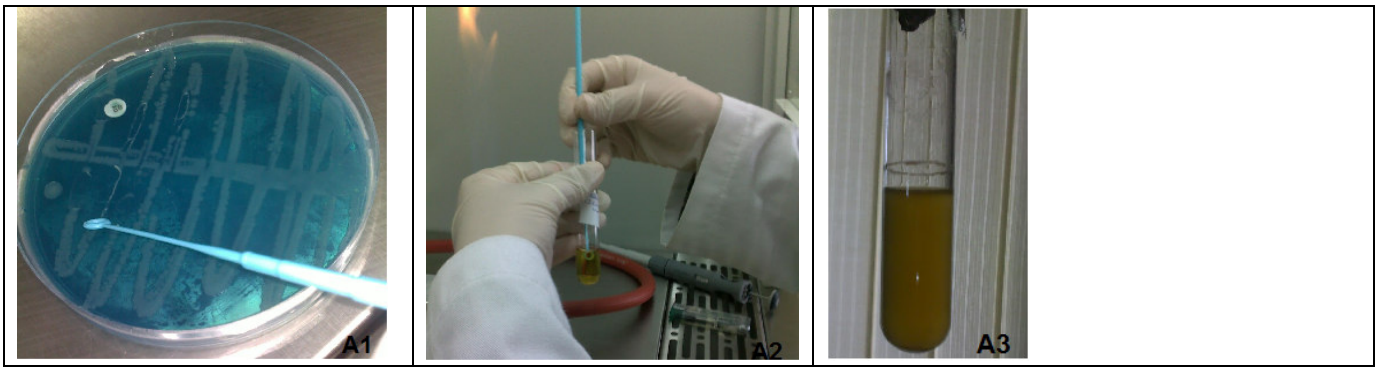


Figure 20: A1) take some colonies from the plate. A2) put the colonies in LB medium and let overnight at 37°C with shaking, next day observe the result as in A3, continue with the competent cells, and the transformation.

الصورة 20: أ) خذ بعض مجموعات البكتريا من الصحن، أ**2**) وضعهم في وسط آل بي وتركهم طيلة الليل على **37**م° مع التحريك، في اليوم التالي لاحظنا أن الوسط تغير لونه كما في أ**3**، أكمل في فتح خلايا البكتريا، ومن ثم في عملية النقل

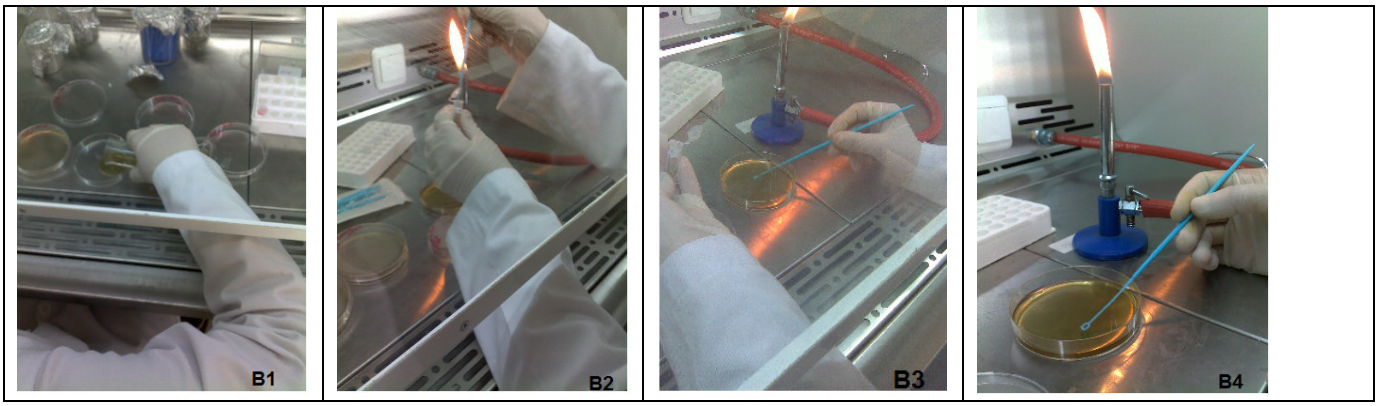


Figure 21: after preparing the LB medium with the ampicillin, B1) put it in the plate with attention from introducing air bubbles, B2) take some microliter from the transformation and plate it on the medium as fig. B3, B4)

الصورة 21: بعد تحضير وسط آل بي مع الآمبيسلين، ب**2**) ضعه في الصحن مع الإنتباه من تكوين فقاقيع من الهواء، ب**2**) خذ بعض الميكروليتر من عينة الناقل وازرعهم على الوسط كما في ب**3**) وب**4**).

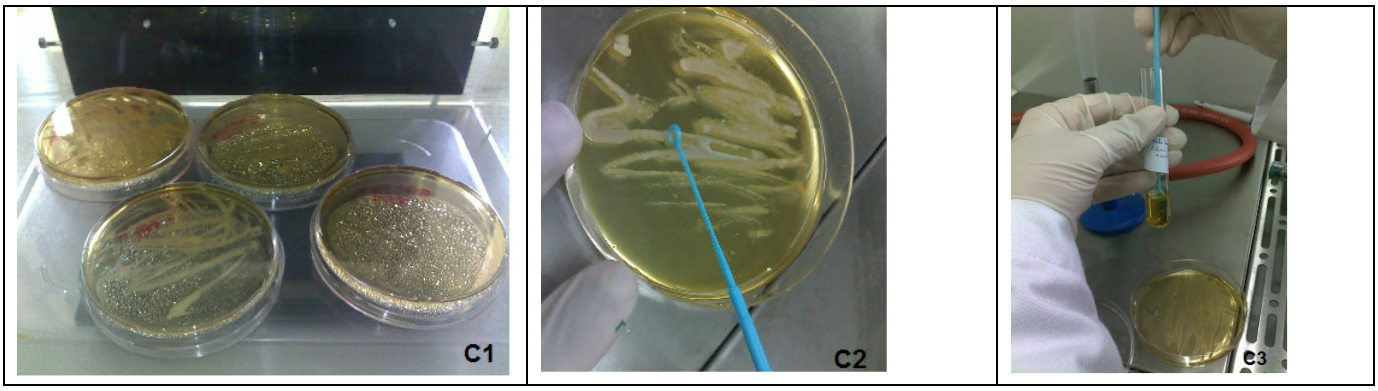


Figure 22: C1) after plating, incubate the plates overnight at 37°C, C2) in the next day take some colonies from the plate and put it in LB medium with ampicillin as C3) and let it between 12-16h at 37°C.

الصورة 22: ج **1**) بعد عملية الزرع، أترك الصحون طيلة الليل على **37**م°، ج**2**) في اليوم التالي خذ بعض المجموعات من الصحن وضعها في وسط آل بي مع أمبيسيلين كما في ج**3**) وأتركهم ما بين **12**– **16** ساعة على **37**م°.

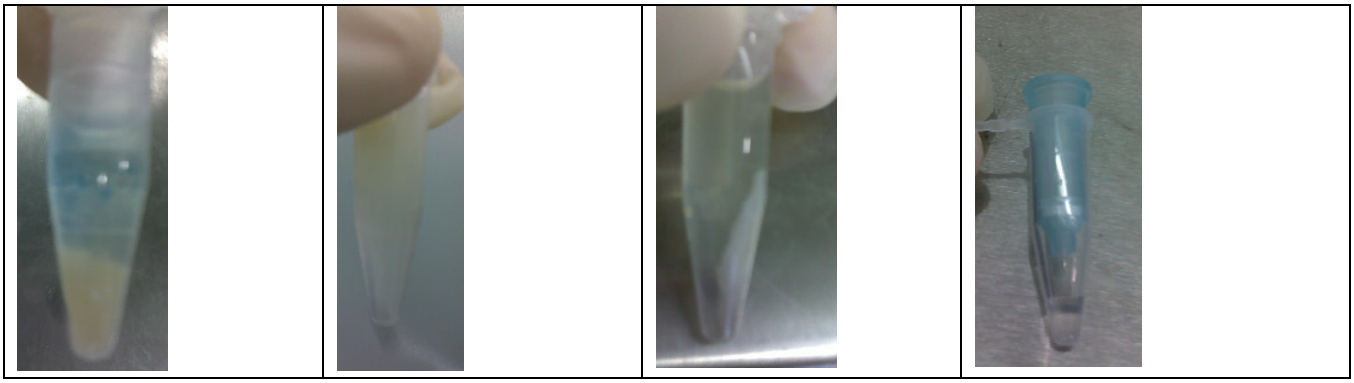


Figure 23: this the result in each step when worked in the purification of plasmid not cutting for restored at -20°C./

الصورة 23: هذه الأنابيب هي نتيج كل مرحلة من مرحلة تنقية الناقل الغير مقطع والذي سيحفظ على –**20**م°.

Transformation into expression host	**عملية النقل إلى داخل الخلايا المعبرة**

في هذه المرحلة قمنا بتجربة العمل على الخلايا البكتيرية المخصصة للإستنساخ مع إضافة جين بوليمراز الرنا ت7 المؤتلف مع الناقل pET (الذي جهز في مرحلة الربط) وأضفناه مع كل عملية نقل سواء كانت نقل هلا 0201 المؤتلف أو بيتا2-آم المؤتلف ثم زرعناهم على صحون بتري وأكملنا بهم العمل حتى فحص إيليزا لنرى إن كان هناك مركب فهذا يعني أن هذه المرحلة صحيحة والعكس صحيح.

ولفعل ذلك يجب أولاً تحضير سلسلة الخلايا، ثانياً يجب نقل المنتج من الربط إلى الخلايا المحضرة ومن ثم زرعهم ومن بعدها إستخراج التعبير البروتييني من سلسلة الخلايا وتصفيته، وأخيراً يجب التأكد منه بواسطة إيليزا.

في البداية أخذنا بكتريا إشيريكي القلونية top 10 من درجة -70مº حيث يجب أن تكون مفتوحة إلا أن أثناء العمل وزرعها على صحن بتري لم يتبين لنا في اليوم التالي أنها تكاثرت لهذا السبب ظننا بأنها ميتة وللتاكد من ذلك قمنا بوضع بعض الميكروليترات في 5 من وسط آل بي وتركناهم طيلة الليل على 37مº مع التحريك وفي اليوم الثاني لاحظنا أن البكتريا تكاثرت وهذا يوكد بأن البكتريا ليست ميتة ولكن كانت غير مفتوحة لذا قمنا بعملية فتحها على الشكل التالي:

وزعنا ال5مل من وسط آل بي المتكاثر فيها البكتريا على 4 أنابيب نابذة (1.5مل) وتركناهم في الثلج لمدة 10 دقائق، ثم نبذناهم على 4000آر.بي.آم لمدة 10 دقائق على 4مº، ومن بعدها رمينا السائل الطائف في الأنبوب ووضعنا 600 ميكروليتر من كلورايد الكالسيوم 0.1M البارد، ثم تركناهم في الثلج لمدة 25 دقيقة، ثم نبذناهم على 4000آر.بي.آم لمدة 10 دقائق على 4مº، وأخيراً رمينا السائل الطائف في الأنبوب ووضعنا 60 ميكروليتر من كلورايد الكالسيوم 0.1M

We tried in this step to work in host for cloning and we added in it the T7 RNA Polymerase recombinant with the pET vector (step of ligation) and we added it with the transformation of HLA-A*0201 recombinant and β2-m recombinant then we platted them onto petri dishes and we continued the protocol until ELISA test for see if there was a complex that meaning there is a protein and this step is correct and vice versa.

To do this first should be prepared the host strain, second should be done the transformation, the plating then the extraction the protein from the strain and purified it, finally should be done the ELISA test.

We took the e.coli top 10 from -70°C and that should be competent and we worked in it but when we plated onto petri dish we didn't saw in the second day any colonies, for this reason we think that the e.coli was died but for prove that we putted some micro liter of this e.coli into 5ml LB medium and we incubated them at 37°C with shaking for overnight and in the second day we saw the amplification of e.coli and that meaning the e.coli wasn't died but it wasn't competent, we did the competent for the e.coli as follows:

We dispensed the 5ml of sample that in the tube in 4 eppendorf tube 1.5ml and we let them in glace for 10 min, then we centrifuged them at 4000 rpm at 4°C for 10

البارد، ثم قمنا بتخزين 3 أنابيب على -20م° وتركنا أنبوب لنكمل العمل به في عملية النقل.

ثانياً قمنا بعملية النقل كالتالي:

أخذنا 20 ميكروليتر من خلايا top 10 ووضعناهم في أنبوب مبرد وأضفنا 1 ميكروليتر من تفاعل الربط (الأنبوب الأول يحتوي على رابط هلا0201 مع رابط بوليمراز الرنا ت7، الثاني يحتوي على رابط بيتا2-آم مع رابط بوليمراز الرنا ت7)، حركناهم بنعومة لخلطهم وأعدناهم إلى الثلج لمدة 5 دقائق ثم وضعناهم في الماء الساخن على 42م° لمدة 30 ثانية (من دون تحريك) ثم في الثلج مرة ثانية لمدة 2 دقيقتين وأضفنا 80 ميكروليتر من وسط آل بي لكل أنبوب، وتركناهم في الثلج إلى أن يصل الوسط إلى الجميع في الأنبوب ثم وضعناهم في الحاضنة لمدة ساعة على 37 م° مع التحريك.

ثم زرعناهم على صحنين بتري المحتوي على وسط آل بي مع الأمبيسيلين ومن بعدها تركناهم طيلة الليل على 37 م°، وفي اليوم التالي لاحظنا تكاثر البكتريا على الوسط، فأخذنا بعض من المجموعات المتكاثرة ووضعناهم في 2.5 مل من وسط آل بي مع الأمبيسيلين وتركناهم على 37 م° لمدة 12-16ساعة ومن ثم نبذناهم على 4000 آر.بي.آم لمدة 5 دقائق، ومن أضفنا على المترقد في أسفل الأنبوب 229.84 ميكروليتر من تريس حمض الهيدروكلوريك (0.1M) وأضفنا عليه 25 ميكروغرام من اللايزوزيم، 10 ميكروليتر من فنيل ميثيل سولفونيل الفلورايد (50mg/ml)، 0.08 ميكروليتر من أنزيم الدنا (1000U) و 0.08 ميكروليتر من أنزيم الرنا (10mg/ml)، 1 ميكروليتر من إي.دي.تي.آي (1mM)، ثم تركناهم على 22م° لمدة 20 دقيقة، ومن بعدها قمنا بصدمة حرارية للأنبوب لدة 40 ثانية في ماء مغلية، ثم نبذناهم

min, we discarded the supernatant and we added 600µl of $CaCl_2$ 0.1M cold, we let them in glace for 25 min then we centrifuged at 4000 rpm for 10 min at 4°C finally we resuspended each pellet in 60µl of $CaCl_2$ 0.1M cold, then we stored 3 eppendorf tubes at -20°C and we let one eppendorf for use it in the transformation.

Secondly, we did the transformation as follows:

We pipetted 20 µl aliquots of cells top 10 into pre-chilled tube we added 1 µl of each ligation reaction (first tube contains ligation of HLA-A0201 and T7RNA polymerase, second it contains the ligation of β2-m and T7RNA polymerase), stirred gently to mix and we returned to the ice, incubated 5 min on ice, placed the tubes in a 42°C water for exactly 30 sec. (do not shake), turned the tubes on ice for 2 min, added 80 µl LB medium to each tube kept the tube on ice until all have received LB, incubated for 1 hour at 37°C with shaking.

then we platted them onto 2 plates contain LB medium with ampicillin then incubated overnight at 37°C, we observed in the second day colonies on the plate, we took some colonies from each tube and putted in 2.5ml LB medium contains ampicillin we incubated them for 12-16 hours at 37°C then we centrifuged them at 4000rpm for 5 min, resupended the pellets in 229.84 µl of tris HCl (10mM) and we added in it 25 µg lyososyme, 10 µl

phenylmethylsulfonylfluoride (50mg/ml), 0.08 µl DNase (1000U) 0.08 µl RNase (10mg/ml) 1 µl EDTA (1mM), incubated at 22°C for20 min, heat shock for 40s in a boiling water, centrifuged at 10000×g for 20min, washed the pellet with 250 µl 10 mM tris HCl, PH 8, dissolved it in 125 µl of 100mM tris HCl, PH8, 8M urea, then should be centrifuged at 4°C for 1 hour at 150000×g but because we didn't have a centrifuge it speed more than 13000×g we centrifuge them at 13000×g for 2 hours and saw a pellet for this reason we continued in this way,	على 10000×g لمدة 20 دقيقة، ومن ثم قمنا بغسل المترقد في أسفل الأنبوب ب250 ميكروليتر من تريس حمض الهيدروكلوريك (10mM)، درجة الحموضة 8، ومن ثم ذوبه في 125 ميكروليتر من تريس حمض الهيدروكلوريك (100mM)، درجة الحموضة 8، مع اليوريا (8M)، ثم كان يجب نبذهم على 4م° لمدة ساعة على 150000×g ولكن بما أن النابذة الموجودة في المختبر السرعة القصوى لديها 13000×g قمنا بنذ الأنبوبين على 13000×g لمدة 2ساعتين وعندما لاحظنا أن هناك كمية من المترقد في أسفل الأنبوب أكملنا العمل على هذا النحو،

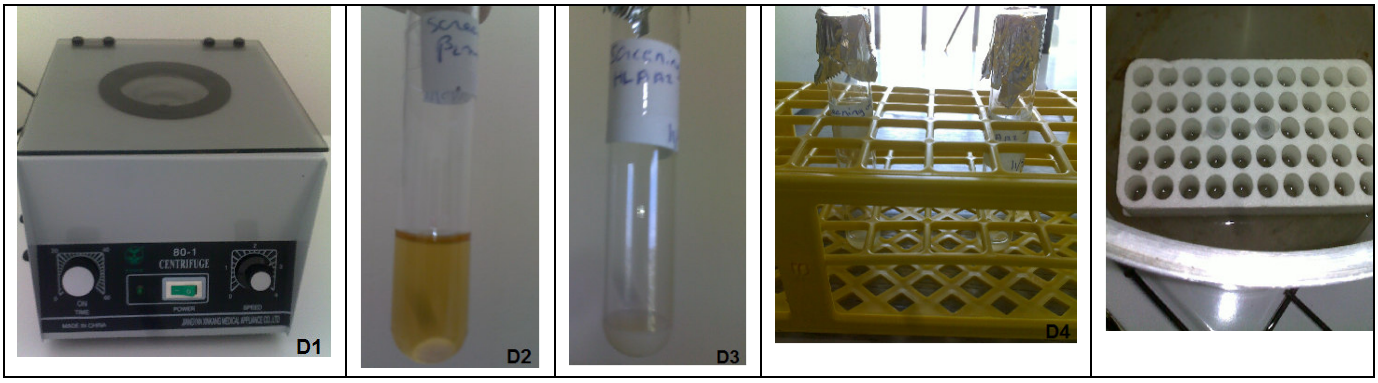


Figure 24: after incubation the colonies that contain the insert recombinant,D1) centrifuge them at 4000rpm for 5 min and obtain the result as D2), then resuspend the pellet with the buffer as D3) and incubated at 22°C for 20 min as D4) then transfer them to microcentrifuge tube, heat shock 40s as D5).

الصورة 24: بعد حضن المجموعات التي تحتوي على الدنا في الوسط، د1) انبذ الوسط على 4000أر.بي.آم لمدة 5 دقائق وبعدها ستحصل على النتيجة كما في الصورة د2)، ومن بعدها ذوب المترقد بالمنظم كما في د3) واحضنه على 22م° لمدة 20 دقيقة كما في د4) وانقلهم إلى أنابيب نابذة وقم بصدمة حرارية لهم على 40 ثانية كما في د5).

We purified the protein of HLA-A0201 and β2-m by anion exchange chromatography Q sepharose fast flow (QFF) as follows: 1. We filled the syringe with the start	قمنا بتصفية بروتيين الهلا0201 وبيتا2-آم بواسطة غروماتوغرافيا تبادل الإيون السيفاروزي للتدفق السريع كما يلي: 1. أملأنا الإبرة بمنظم البدء، ثم أزلنا السدادة التي على العامود

buffer, removed the stopper and connected the column to the syringe with the provided connector, drop to drop to avoid introducing air into the column. 2. Removed the snap-off end at the column outlet. 3. Washed out the preservatives with 5 column volumes of start buffer, at 1ml/min for the HiTrap 1ml. 4. Washed with 5 column volumes of elution buffer. 5. Finally equilibrated with 5 column volumes of start buffer. 6. applied the sample (the protein of HLA-A0201) at 1ml/min for HiTrap 1ml using a syringe fitted to the luer connector 7. Washed with at least 5 column volumes of start buffer or until no material appears in the effluent. 8. Eluted with 5-10 column volumes of elution buffer. 9. After completed elution, regenerated the column by washing with 5 column volumes of regeneration buffer (elution buffer) followed by 5-10 column volumes of start buffer. The column is now ready for a new sample. 10. Applied the sample (the protein of β2-m) at 1ml/min for HiTrap 1ml using a syringe fitted to the luer connector. 11. Washed with at least 5 column volumes of start buffer or until no material appears in the effluent.	وأوصلنا العامود بالإبرة بالرابط المناسب، وبحذر ولتجنب دخول الهواء على العامود وضعنا نقطة نقطة من الإبرة على العامود إلى أن يلصق به. 2. أزلنا النهاية المضافة عند أسفل العامود. 3.قمنا بغسل المواد في العامود ب 5 مرات من حجم العامود بمنظم البدء، على 1مل/بالدقيقة لكل 1مل من عامود "هاي ترب". 4. قمنا بغسل ب 5 مرات من حجم العامود بمنظم الإستخراج. 5. أخيراً قمنا بتعديل العامود ب 5 مرات من حجم العامود بمنظم البدء. 6. وضعنا العينة من البروتين المراد نتقيته (الهلا0201)على 1مل/بالدقيقة لكل 1مل من عامود "هاي ترب" بإستخدام الإبرة الموصلة الرابط المناسب. 7. قمنا بغسل العامود ب 5 مرات من حجم العامود بمنظم البدء. 8. ثم إستخرجنا العينة ب5 مرات من حجم العامود بمنظم الإستخراج. 9. بعد إكمال عملية الإستخراج، قم بغسل العامود ب 5 مرات من حجم العامود بمنظم الإستخراج ثم أيضاً ب 5 مرات من حجم العامود بمنظم البدء. والآن أصبح العامود جاهزاً لتنقية عينة جديدة. 10. أضفنا العينة الثانية المراد تنقيتها (البيتا2-آم) على 1مل/بالدقيقة لكل 1مل من "هاي تراب" بإستخدام الإبرة

12. Eluted with 5-10 column volumes of elution buffer. 13. When we finished the purification of the entire sample we rinse the column with water then washed with 5 column volumes 20 % ethanol at 1ml/min for the HiTrap 1ml to prevent microbial growth. Sealed the column with the supplied stoppers. And stored at 4°C to 30°C.	الموصولة بالرابط المناسب. 11. قمنا بغسل العامود ب5 مرات من حجمه بمنظم البدء. 12. قمنا بعملية الإستخراج بإضافة 5 مرات من حجم العامود من منظم الإستخراج. 13. عند الإنتهاء من نتقية العينات قمنا بغسل العامود بالماء ومن ثم بالأيثانول 20% على 1مل /بالدقيقة لكل 1مل من "هاي تراب" لمنع نمو الجراثيم. وأخيراً قمنا بختم العامود بالسدادات المزودة والمناسبة. ووضعنا العامود على درجة حرارة بين 4م° و 30م°.

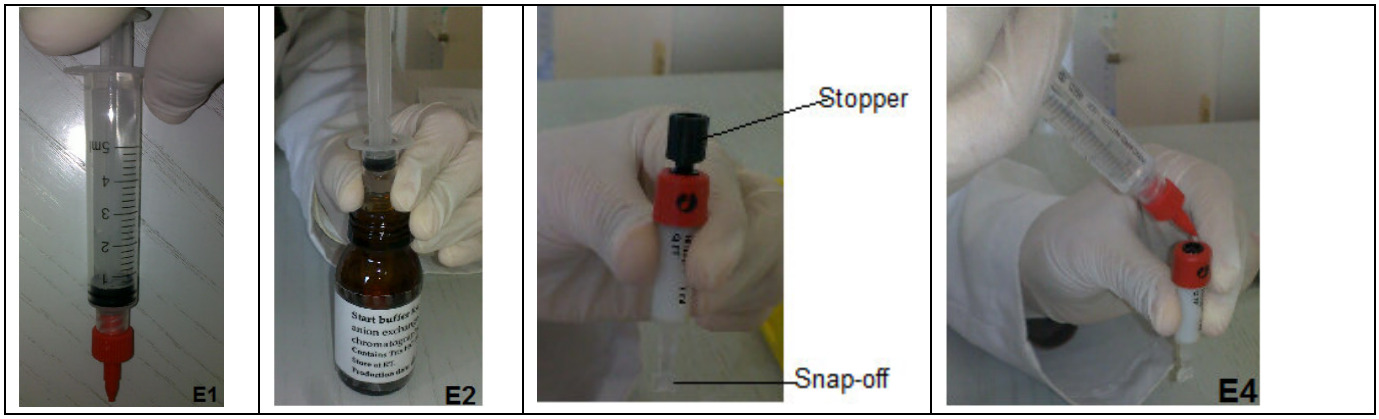


Figure 25: E1) prepare the syringe with the provider connector and fill it with the start buffer as E2), remove the stopper from the column and connect it with the syringe by drop drop as E4) to avoid introducing air to the column.

الصورة 25: ه1) جهزّ الإبرة بالرابط المناسب ومن ثم قم بملئها بمنظم البدء كما في ه2)، أزل السدادة من أعلى العامود وقبل بإيصال الإبرة بالعامود قم بوضع بعض النقاط على العامود لمنع دخول الهواء كما في ه4).

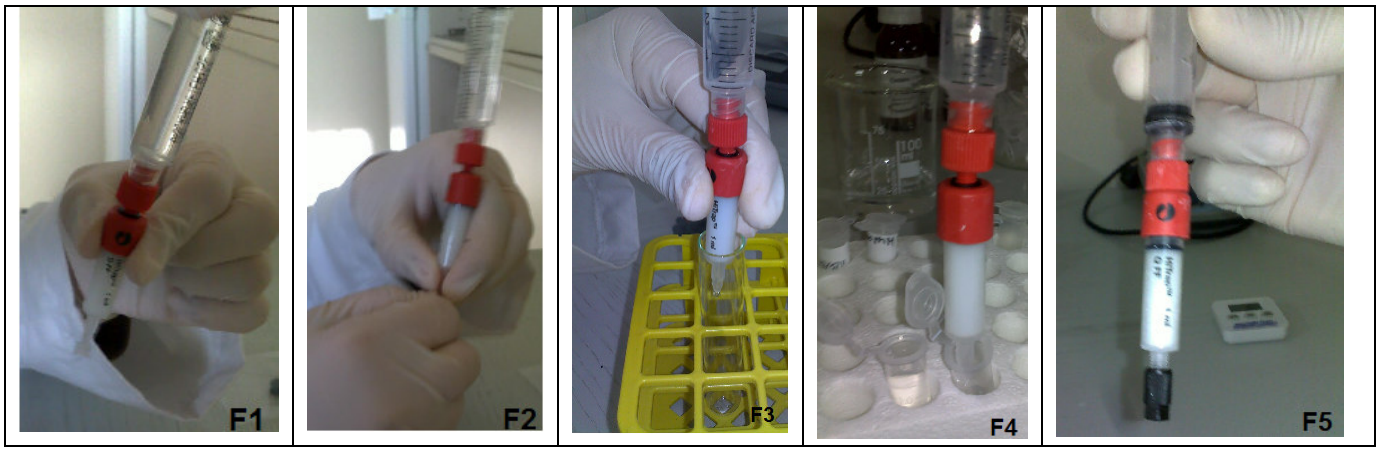


Figure 26: after the connection of syringe with the column remove the snap-off end the column as F2) wash the column with the start buffer and the elution buffer as F3) then elute the sample in a sterile eppendorf tube as F4) then when it finish the rinsing of column seal it with the supplied stopper.

الصورة 26: بعد ربط الإبرة بالعامود قم بإزالة النهاية المضافة للعامود كما في و2) ثم قم بغسل العامود بمنظم البدء ثم بمنظم الإستخراج كما في و3) ثم قم بإستخراج العينة وضعها في أنبوب نابذة كما في و4) وعند الإنتهاء من إستخراج العينة قم بغسل العامود وتنظيفه ثم أغلق الفتحات بالسدادات المناسبة.

Finally we began in the ELISA test as follows:

In the first day we dilute 5 µl of pan specific mouse anti HLA class I antibody, W6/32 in 1ml of carbonate buffer (0.1M) PH 9.6, and we putted 50 µl/well in A1, A2, A3, A4, A5, C1, C2, C3, C4 and we let it at 4°C for overnight.

In the second day we added 350 µl/well 10% w/v protein block to block the residual bind, then we washed twice with 600 µl/well of 0.05% tween in PBS at room temperature to remove the unbound W6/32 and blocking reagent, then we diluted the purified recombinant HLA-A0201 molecule 100 fold into 100µl 0.3 mM tris maleat buffer, PH 6.6, containing human β2-m

وأخيراً بدأنا في فحص إيليزا كما يلي:

في اليوم الأول قمنا بتخفيف 5 ميكروليتر من pan specific mouse anti HLA class I antibody, W6/32 في 1مل من منظم الكاربونات (0.1M) درجة الحموضة 9.6 ، ووضعنا 50 ميكروليتر/في كل من الفتحات التالية: أ1، أ2، أ3، أ4، أ5، ج1، ج2، ج3، ج4 وتركناهم على 4م° طيلة الليل.

وفي اليوم الثاني أضفنا 350 ميكروليتر/بالفتحة من 10% من البروتيين المانع لمنع أي ربط آخر، ومن بعدها قمنا بغسل الفتحات مرتين ب600 ميكروليتر/بالفتحة من 0.05% من التويين في PBS على درجة حرارة الغرفة لإزالة الW6/32 الغير مربتط بالفتحة وإيقاف ما تب

قي من الكواشف، ثم قمنا بتخفيف جزيئات الهلا المؤتلف المنقى

(100nM), peptide(optimal 1nM to 1µM) and lutrol F-68 (1g/L) and we incubated them at 18°C for 48 hours. In the fourth day of ELISA test we diluted 2 µl fo the sample reaction in 10 µl of protein block with 40 µl PBS 0.05% Tween then we added 50 µl of the sample in each this well C1, C2, C3, C4 and we added on A1,A2, A3, A4, A5, the same sample but without dilution and we let the plate for 2h at 4°C, then we washed 6×times 300µl/well with PBS 0.05% Tween at room temperature, and for detecting the binding complex, we incubated the plate for 1h at 4°C, with 49 µl of post primary block + 1 µl of protein block, then we washed 6×times 300µl/well with PBS 0.05% Tween, we added one drop of Novolink polymer /well and incubated them for 30min at room temperature to enhance the detection, then we washed 6×times 300µl/well with PBS 0.05% Tween, we added 3 µl of 3,3′5, 5′-tetramethylbenzidine hydrogenperoxide in A1, A2, A3, C1, C2, and we added 3 µl of DAB Chromogen in A4, A5, C3, C4, and we observed directly a change of color in A1, A2, A3, C1, C2 from incolor to blue color and after 30 min we observed that color was change to yellow and that was meaning there is a complex and AlHamdullillah we successful in the protocol and in our	في 100 ميكروليتر من منظم tris maleat buffer (0.3mM) درجة الحموضة 6.6، ويحتوي على البيتا2-آم (100nM)، الببتيد (1nM to 1µM) واللوترول ف-68 (1g/l) وتركناهم لمدة ساعة على 18م° لمدة 48 ساعة. وفي اليوم الرابع من فحص إيليزا قمنا بتخفيف 2 ميكروليتر من تفاعل العينة في 10 ميكروليتر من البروتيين المانع مع 40 ميكروليتر من PBS مع0.05% من التويين، ثم أضفنا 50 ميكروليتر من العينة في كل من الفتحات التالية ج1، ج2، ج3، ج4 وأضفنا في الفتحات أ1، أ2، أ3، أ4، أ5 نفس محتويات العينة ولكن من غير تخفيفها ثم تركناهم على 4م° لمدة ساعتين، وبعدها قمنا بغسل الفتحات 6 مرات ب300ميكروليتر من PBS مع التووين على درجة حرارة الغرفة، ولإستبان المركب المؤلف من الهلا والببتيد أضفنا 49 ميكروليتر من بوست برايميري بلوك مع 1 ميكروليتر من البروتيين بلوك وتركناهم لمدة ساعة على 4م°، ومن ثم قمنا بغسل الفتحات 6 مرات ب300ميكروليتر من PBS مع التووين، ثم أضفنا نقطة من النوفولينك بوليمر على كل فتحة وتركناهم لمدة 30 دقيقة على درجة حرارة الغرفة لتحفيز الإستبيان، ومن ثم قمنا بغسل 6 مرات ب300ميكروليتر من PBS مع التووين، ومن بها أضفنا 3 ميكروليتر من 3,3′5, 5′-tetramethylbenzidine hydrogenperoxide في الفتحات التالية أ1، أ2، أ3، ج1،ج2، وأضفنا 3 ميكروليتر من DAB Chromogen في الفتحات أ4، أ5، ج3، ج4 و لاحظنا أن اللون في الفتحات أ1،

أ2، أ3، ج1، ج2 قد تغير من اللون الشفاف ألى اللون الأزرق وبعد 30 دقيقة لاحظنا أن اللون الأزرق تحول إلى اللون الأصفر وهذا يعني أنه يوجد مركب وأننا الحمدلله نجحنا في البروبوكول وفي تجربتنا.	experiment.

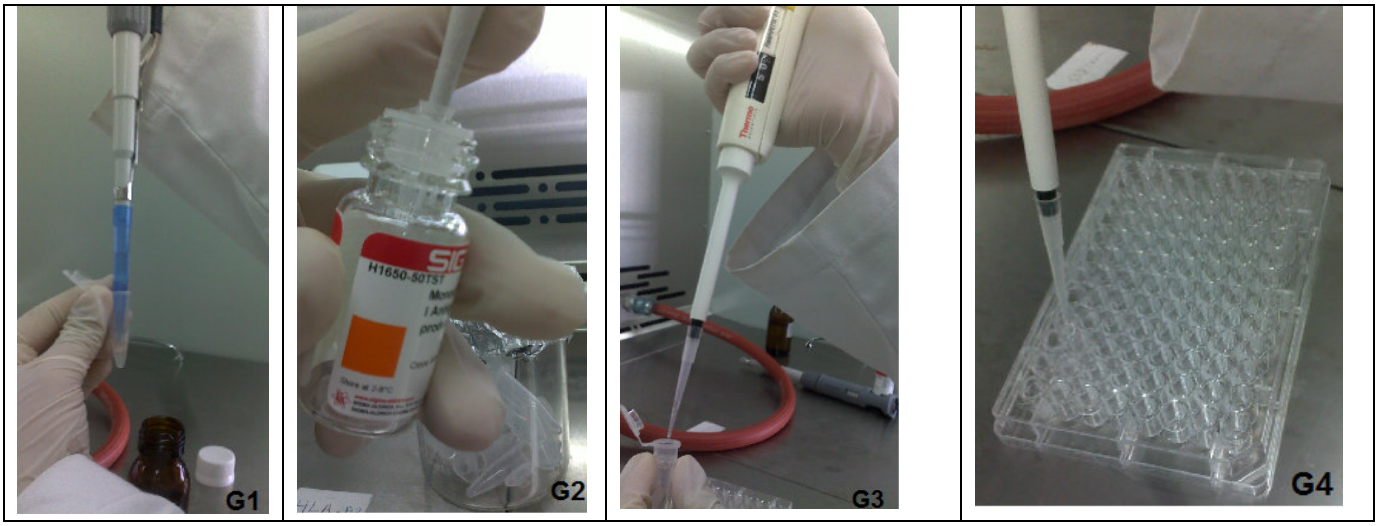


Figure 27: G3) dilute the w6/32 (G2) with the carbonate buffer (G1) and put it the wells as G4.

الصورة 27: ز**3**) خفف **w6/32** (ز**2**) مع منظم الكاربونات (ز**1**) ضعهم في الفتحات كما في ز**4**).

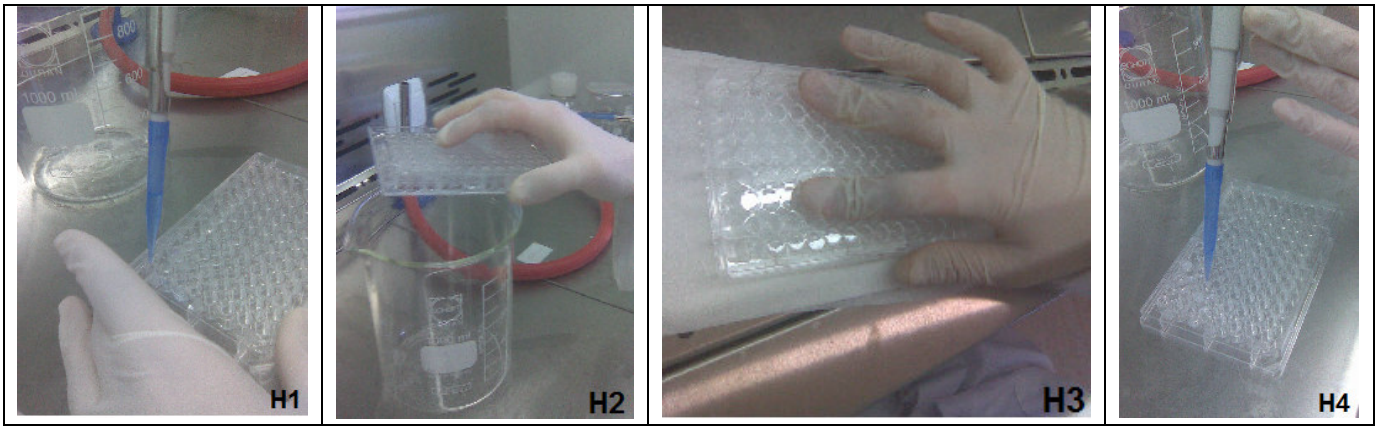


Figure 28: H1) put PBS with tween for the washing and discard them the waste as H2) then dry it by a towel as H3) then repeat the washing as the same method.

الصورة 28: ح**1**) ضع **PBS** مع التوين للغسل ثم إرميه في المهملات كما في ح**2**) ثم جففه على المنشفة كما في ح**3**) ثم أعد عملية الغسل على نفس الطريقة.

Figure 29: add the protein bock then the post primary block then the Novolink polymer but after adding each reagent should be wash by PBS with tween.

الصورة 29: أضف البروتين المانع ومن ثم البوست برايميري بلوك ومن ثم النوفولينك بوليمير ولكن بعد إضافة كل عامل يجب الغسل بال**PBS** مع التوين.

Figure30: add drop of the Novolink polymer in each well as J1) then add in part of wells TMB (J2) and in the second part DAB (J3), J4) observe the wells that have TMB change it color to blue after many seconds.

الصورة 30: أضف قطرة من النوفولينك بوليمير في كل فتحة كما في ي**1**) ثم أضف في قسم من الفتحات ال**TMB** (ي**2**) وفي القسم الثاني **DAB** (ي**3**)، ي**4**) لاحظ أن الفتحات التي تملك **TMB** تغير لونها إلى الأزرق بعد ثواني

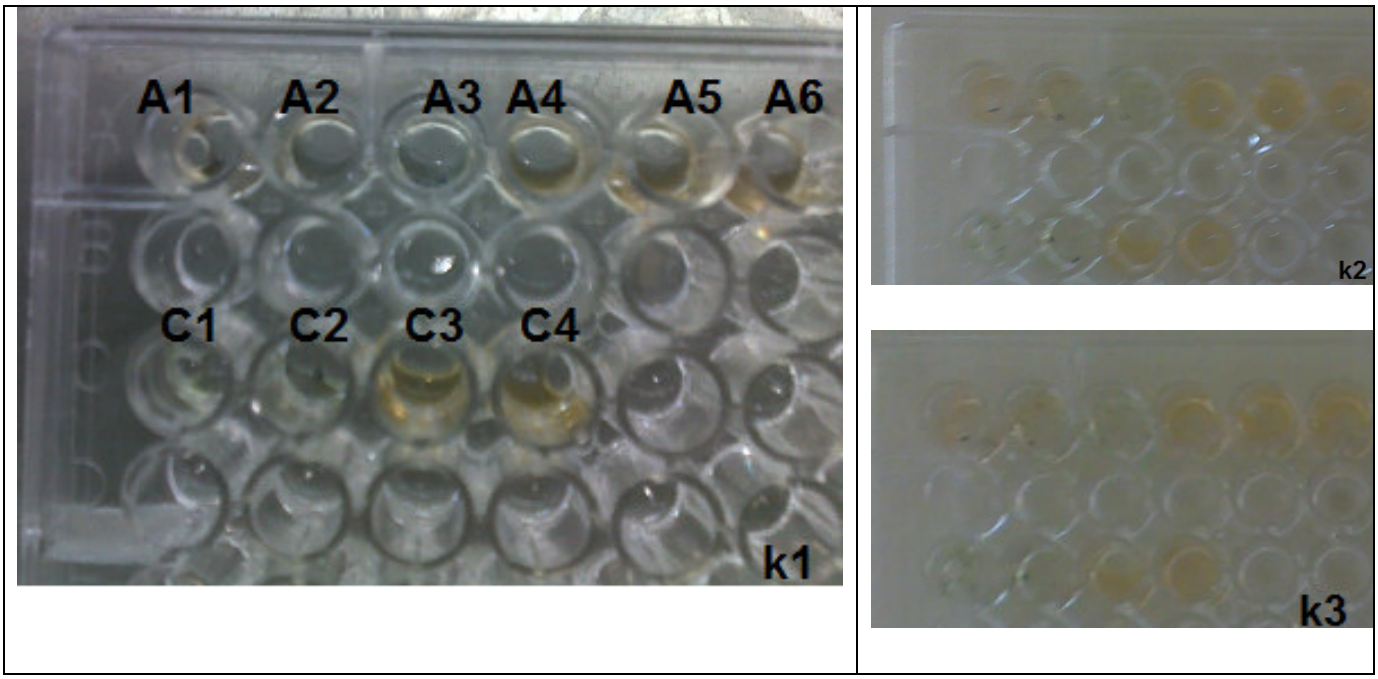


Figure 31: observe the result after 30 min the color blue change to yellow in the wells A1, A2, A3, C1, C2, that have the TMB.

الصورة 31: لاحظ النتيجة بعد 30 دقيقة اللون الأزرق تغير إلى الأصفر في الفتحات التي تملك TMB :أ1، أ2، أ3، ج1، ج2.

5 Appendix:

1-April-2011 Extraction of white blood cell from the whole blood	10:00 ⟶ 11:40
Purification of the white blood cells by kit And retrotranscription the RNA to cDNA	12:30 ⟶ 5:00
2-April-2011 Amplification of the DNA by PCR for the HLA-A0201 and β2-m and the nested PCR for HLA-A0201	9:42 ⟶ 2:30
4-April-2011 Preparation of the solutions (TAE Buffer, Tris base)	9:00 ⟶ 12:30
5-Apirl-2011 Purification on Agarose gel	8:00 ⟶ 12:30
Gel purification by Qiaquick gel extraction kit	2:00 ⟶ 3:30
7-April-2011 Preparation of vector	9:50 ⟶ 6:30
8-April-2011 Ligation	**8:00 ⟶ 1:00** **With the over night**
Growing the E.coli in the LB.medium	**1:30 ⟶ 2:30** **With the over night**
29-April-2011 Transformation with e.coli top10 Plating on LB agar Incubation the e.coli that from hospital in lb medium	11:40 ⟶ 4:30
30-April-2011 Transformation pET into e.coli top 10 and plating onto LB plate.	10:00 ⟶ 2:00
Putting the e.coli that is form the hospital in 5ml LB medium	9:00 ⟶ 11:00
1-May-2011 Competent cell the strain that is from the	10:00 ⟶ 12:00

hospital	
Transformation the pET and the HLA-A*0201 into this strain	10:00⟶12:00
Plating on LB plates	11:00⟶ 12:00
2-May-2011 Screening the pET.	9:00 ⟶9:30 Incubation from 12-16 h
Preparation of 5ml of LB medium and putting the e.coli top 10 in it.	9:30 ⟶10:15 Incubation over night.
3-May-2011 Purification the pET by Qia prep kit.	11:30⟶12:30
Competent cells for the e.coli top 10	1:00 ⟶3:00
5-May-11 Competent cells for top 10 Transformation Plating on lb agar	9:00 ⟶2:00
6-May-11 Preparation of PMSF Screening the colonies in 2.5ml with 1.25μl	9:00 ⟶3:30
7-May-11 Preparation of PMSF	9:00 ⟶ 2:00
9-May-11 Screening the colonies in 2.5ml LB medium with 1.25μl ampicillin. Preparation of PMSF	9:00 ⟶ 2:00
10-May-11 Preparation of PMSF Plate the bacteria that did for it the screening	9:00 ⟶2:00
11-May-11 Screening the colonies	9:00⟶ 10:50
12-May-11 Lysis of the bacteria Dissolve the protein Purification with chromatography QFF	9:00 ⟶7:40
13-May-11 The first day in ELISA test: preparation the coated buffer with the carbonate buffer and	10:00 ⟶11:50

we putted it into the wells plate	
14-May-11 The second day of ELISA test: preparation of PBS, tris maleat buffer and we make the complex HLA-A0201 and the peptide	9:00 ⟶ 1:00 Overnight 48hours
15-May-11 Incubation and washing in the well plate of ELISA test until arriving to the result.	12:40 ⟶ 6:10

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