
DIAGNOSTIC-RELATED PROTOCOLS

D1: HLA Typing

The HLA class II molecules of the human Major Histocompatibility Complex (MHC) are encoded in the short arm of chromosome 6 in the HLA region. These glycoproteins consist of an alpha and a beta chain associated as heterodimers on the surface of antigen presenting cells such as B cells and macrophages.

The HLA-D region contains several class II genes and has three sub regions: HLA-DR, -DQ and -DP. The DQ and DP regions contain one functioning gene each for the alpha and beta chains. The DR region has only one functioning alpha chain and varying numbers of beta chains, usually one or two depending on the class II haplotype.

With the exception of the DRA molecule, the genes encoding the functional class II proteins are highly polymorphic with most of the variability localized in the second exon. This exon encodes the extracellular domain which functions as the antigen binding site for processed peptides. The variability is concentrated as distinct clusters within a relatively conserved framework region.

The HLA DQA kit is part of a Perkin Elmer human identification and forensic kit. The Polymerase Chain Reaction (PCR) is used to amplify minute amounts of DNA. The primers for amplification are biotinylated to aid in detection. The HLA DQ A alleles are detected by a reverse dot blot hybridization, where the sequence specific oligonucleotides for the polymorphisms are immobilized on hybridization strips. Hybridization is detected by color reaction of the biotinylated primers with horse radish peroxidase streptavidin and chromogen (TMB 3,3',5,5'-tetramethylbenzidine).

The HLA DQB kit is similarly structured with variations in the primers, hybridization conditions. Also the immobilized oligonucleotides are arrayed in lines instead of dots, since there are many more alleles. The pattern of lines is more complex and a computer program is used to assist in allele calling.

HLA-DQA

Materials

Amplitype –PM kit from Perkin Elmer (N808-0094) manufactured by Roche Molecular Systems, Branchburg N.J. USA. (*Available from Applied Biosystems. 1-800-327-3002*)

Contents:

Reaction Mix: *Amplitaq DNA polymerase, dNTPs MgCl₂*

Amplitype primers (*Primers include those for HLA DQ and 5 other loci used for human identification.*)

Amplitype probe strips (*50 strips for DQA and 50 for PM*)

Enzyme conjugate HRP/SA

Chromogen TMB

Control DNA

100% ethanol

Citric acid monohydrate ($C_6H_5O_7Na_3 \cdot 2H_2O$)
30% Hydrogen peroxide
Concentrated HCl
10N NaOH
Sodium citrate dihydrate ($C_6H_8O_7 \cdot H_2O$)
Sodium Chloride
Sodium dodecyl sulphate SDS
Sodium EDTA ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$)
Sodium phosphatate monophosphate, monohydrate ($NaH_2PO_4 \cdot H_2O$)

Equipment

Amplitype typing trays
Aspirator apparatus
Dispensing repipet
Thermacycler
Water bath, shaking with cover
Water bath, stationary

Other Lab Supplies

0.2ml PCR tubes and caps
Pipette tips 1-200 plugged and regular
10ml and 25 ml pipettes
Timer
Filter forceps
Balance
Stir plate and bars
pH meter
Deionized water or glass distilled water

Preparation of reagents

Chromagen solution

1. Carefully open the chromagen bottle. Slowly add 30 ml 100% ethanol to the bottle and mix until the chromagen is dissolved.
2. Store in the refrigerator, good for 6 months

1L Citrate buffer (0.1 M sodium citrate pH 5.0)

- 18.4 g trisodium citrate dihydrate
- 6g of citric acid monohydrate

Dissolve 18.4 g trisodium citrate dihydrate in 800 ml of DI water. Adjust pH to 5.0 by addition of approximately 6g of citric acid monohydrate. Adjust volume to 1L and autoclave.

20XSSPE

- 7.4 g Disodium EDTA

- 10N NaOH
- 210 g NaCl
- 27.6 g sodium phosphate, monobasic monohydrate

Dissolve 7.4 g Disodium EDTA in 800 ml of DI water. Adjust pH to 6.0 with 10N NaOH. Add 210 g NaCl and 27.6 g sodium phosphate. Adjust the pH 7.4 with 10N NaOH. Adjust volume to 1L.

20% SDS (1L)

- 200g sodium dodecyl sulphate SDS

Slowly dissolve 200g SDS in DI water. (Warming in 37 C water bath may be necessary). Adjust volume to 1L.

Hybridization solution (5XSSPE, 0.5%SDS 1L)

Add 250 mL of 20XSSPE and 25 mL of 20% SDS to 725 mL of DI water and mix thoroughly. (Warming in 37C to 55C water bath may be necessary).

Wash Solution (2.5XSSPE, 0.1%SDS 2L)

Add 250 mL of 20XSSPE and 10 ml of 20% SDS to 1740 mL of DI water and mix thoroughly. (Warming in 37C to 55C water bath may be necessary).

0.5M EDTA

- 186.1 NA₂EDTA
- 10N NaOH

Add 186.1 g NA₂EDTA to 800 ml of DI water. Dissolve by adding 10N NaOH to adjust pH to 8.0. Adjust volume to 1L and autoclave.

200 mM EDTA (10 mL)

Add 4.0 mL of 0.5M EDTA to 6 mL of DI water.

Procedure

PCR amplification

1. DNA concentration should be in the range of 0.1 to 0.5 ng/μl, so that 2 to 10 ng will be added to the PCR reaction in a volume of 20 μl. Use plugged tips.
2. Add in 40 μl of Amplitype PM PCR reaction mixture to each tube 0.2mL.
3. Pipette 40 μl of Amplitype PM primer set into each tube. Include control tubes, one tube for the positive control and another one for adding 20 μl distill water instead of DNA as negative control.

4. Add 20µl of DNA samples, and control DNA1 in the kit to the prepared tubes. Use plugged tips.
5. Place the tubes in the PCR machine. The PCR conditions are as follows:

PCR 9600 /9700

Denature at 95°C 5 min

Denature 94 C 30sec.

Anneal 60 C 30sec

Extend 72 C 30sec

Cycles 32

Incubate 72 C 10min

Hold 4C

Hybridization

6. Prior to hybridization, add 5µl of 200 mM sodium EDTA into the tubes (use a new tip for each tube).
7. Pre-warm hybridization and wash solutions in 55C water bath.
8. Remove the DNA probe strips using filter forceps take the required number of strips from of the tube. Label each strip with a pencil and place one strip in each well of the DNA typing trays. Add 3 ml of pre-warmed hybridization solution (55C) into each well at the labeled end of the strips, tilting the tray.
9. Denature the amplified DNA sample by incubation at 95 C for 3 to 10 min. in the PCR machine.
10. Add 20µl of PCR DNA sample into the well of the corresponding DNA probe strip below the surface of hybridization solution. Put the lid on the tray and mix by carefully rocking the tray. Once hybridization has begun, the strips should remain wet until the conclusion of color development.
11. Put the tray in shaking water bath at 55 C. Place 1 Kg weight on the covered tray. Rotate water bath at 50 to 70 RPM. for 15min. The temperature is very important, it should be checked by immersing a thermometer in the water bath.
12. Approximately 5 min before the end of the hybridization, prepare the enzyme conjugate solution according to the number of strips:

of strips x 3.3ml = volume of hybridization solution

of strips x 27µl = volume of enzyme conjugated HRP-SA

Mix these two solutions thoroughly and leave at room temperature.

13. After hybridization, open the tray and aspirate the content of each well. Dispense 5ml of pre-warmed wash solution into each well and rinse by gently rocking the tray for several seconds. Aspirate the solution.
14. Dispense 3ml of enzyme conjugate solution into each well and put the lid on. Place the tray in water bath at 55C for 5min.
15. Aspirate the content of each well. Dispense 5ml pre-warmed wash solution into each well. Rinse by rocking the tray for several seconds and aspirate the solution out.
16. For stringent washing, dispense 5ml of wash solution into each well. Place the tray in water bath at 55C and rotate at 50 to 70 RPM for 12 min. Aspirate out the content of each well
17. Dispense 5ml of wash solution into each well. Aspirate out the content of each well.

Color development

18. Dispense 5ml of citrate buffer into each well and place the tray on an orbital shaker for 5 min at room temperature.
19. During this step, prepare the color development solution according to the number of strips and add these reagents in order as listed.

of strips x 5ml=volume of citrate buffer
 # of strips x 0.5µl of 30%Hydrogen peroxide=volume of peroxide
 # of strips x 0.25ml of chromogen, TMB=volume of chromogen,

20. Aspirate or pour off the content of each well and add 5ml of freshly prepared color development solution.
21. Place the tray on an orbital shaker for 20 to 30 min to develop the color. Develop until the "S" or "C" dot is visible.
22. Stop the color development by removing the solution from the well and dispensing 5ml of DI water into the well. Shake for 5-10 mins. Repeat this wash step at least 3 times to prevent background color development.
23. Record the results.

Interpretation of results

1.	○	2.	○	3.	○	4.	○	C	○	1.1	○	1.2	○	1.3	○	1.3	○	All	but	○	4.1	○	4.2	○	DQA
											4					1.3				4.3					

The "1" dot is positive in the presence of HLA DQA1 0101,0102 and 0103 alleles

The "2" dot is positive in the presence of HLA DQA2 0201 allele

The “3” dot is positive in the presence of HLA DQA3 0301 allele

The “4” dot is positive in the presence of HLA DQA1 0401, 0501 and 0601 alleles

The “C” dot should be positive.

The “1.1” dot is positive in the presence of HLA DQA* 0101 only.

The “1.3” dot is positive in the presence of HLA DQA 0103 but is also positive with HLA DQA* 0102 and “4”.

There is no probe that detects only the HLA DQA* 0102 allele

The “4.1” dot is positive in the presence of HLA DQA1*0501 allele.

The “4.2” dot is positive in the presence of HLA DQA1 0401 alleles.

HLA DQB

Materials

HLA DQB manufactured by Dynal

Kit contents:

Master mix DNA polymerase, dNTPs, and biotinylated primers to amplify second exon

6.0mM MgCl₂

Hybridization strips

Strip detection kit (802.01) for color development.

Kit contents:

Substrate A

Substrate B

Denaturation Concentration

SDS concentrate

Streptavidin HRP conjugate

SSPE concentrate

Citrate Buffer concentrate

Other reagents

100% ethanol

30% Hydrogen peroxide

Equipment

Dynal typing trays

Aspirator apparatus

Dispensing repipet

Thermacycler

Water bath, rotating with cover

Water bath, stationary

Preparation of Reagents

Working Hybridization Buffer

Add 55 mL of SSPE concentrate and 213 mL of DI water and 6.9mL of SDS concentrate and mix thoroughly. (Warming in 37C to 55C water bath may be necessary).

Working Wash Buffer

Add 65 mL of SSPE concentrate and 1228.5 mL of DI water and 6.5 ml of SDS concentrate. and mix thoroughly. (Warming in 37C to 55C water bath may be necessary).

Stringent Wash Buffer pre-warm to 55C
Use 275 ml

Ambient Wash buffer (Room temperature)
Use 1025 mL

Working Citrate Buffer

Dilute 30 ml of Citrate concentrate with 570 ml DI water.

PCR amplification

1. DNA concentration should deliver 200ng in a volume of 15 μ l (at least 13ng/ μ l)
2. Add in 30 μ l of Master mix to each PCR tube.
3. Pipette 15 μ l of 6.0 mM MgCl₂ into each tube
4. Add 15 μ l of DNA samples, and control DNA in the kit to the prepared tubes.
5. Cap the tubes and place them in the PCR machine. The PCR conditions are as follows:

PCR 9600 /9700
Denature at 95 C 5 min

Denature	95 C 15sec.
Anneal	60 C 45sec
Extend	72 C 15sec
Cycles	35
Incubate	72 C 5min
Hold	15 C

Hybridization

6. Prior to hybridization, add 60ul of denaturation solution into the tubes (use a new tip for each tube Incubate at room temperature for 10 mins.

7. Warm the SSPE concentrate and SDS concentrate in 50 C water bath.
8. Remove the DNA probe strips using filter forceps take the required number of strips from the tube. Label each strip with a pencil and place one strip in each well of the DNA typing trays. Add 3 ml of pre-warmed hybridization solution (55C) into each well at the labeled end of the strips, tilting the tray.
9. Add 70ul of denatured PCR DNA sample into the well of the corresponding DNA probe strip below the surface of hybridization solution. Place the lid on the tray and mix by carefully rocking the tray.
10. Place the tray in shaking water bath at 50 C. Rotate water bath at 50 to 70 RPM. for **30min**. Aspirate the solution
11. Dispense 5ml of Ambient Wash Buffer into each well and rinse by gently rocking the tray for several seconds. Aspirate the solution.
12. Dispense 5ml of pre-warmed Stringent Wash Buffer into each well Return tray to 50C water bath and incubate for 15 mins. Aspirate the solution.

Color development

13. Prepare the Working conjugate solution while the stringent washing is going on.

of strips x 5.3ml = volume of hybridization solution

of strips x 16ul = volume of Streptavidin HRP conjugate

Mix these two solutions thoroughly.

14. Dispense 5ml working conjugate solution into each well and place the tray on an orbital shaker at room temperature for 15 mins. Aspirate the solution
15. Dispense 5ml ambient wash solution into each well. Shake the tray at room temperature for 5 mins. Aspirate the solution out. Repeat this step once.
16. Dispense 5ml of Citrate Buffer into each well. Shake the tray at room temperature for 5 mins.
17. Prepare Working Substrate (Mixture of Substrate A and Substrate B)

of strips x 4.4ml = SubstrateA

of strips x 1.1ml = Substrate B

Mix these two solutions thoroughly.
18. Aspirate out the content of each well. Dispense 5ml of working substrate into each well. Shake the tray at room temperature for 10 mins. Aspirate out the contents.
19. Dispense 5ml of DI water into each well and place the tray on an shaker for 5min. Aspirate out the contents

20. Dispense 5ml of Citrate Buffer into each well Strips may be stored in Citrate buffer for 3 days.
21. Record the results.
22. The "C" line intensity is designed to be less than the other lines. The "C" line should be lighter than the other lines. The presence of lines lighter than the "C" line indicates contamination.

Interpretation of the results:

Using the transparent overlay, record the positive lines.

Use the computer program to interpret the pattern of lines.

D2: Insulin Autoantibody Immunoprecipitation Assay

Principles

- Incubation of serum with labeled antigen with and without cold insulin overnight
- Precipitation of antibody-bound labeled antigens with protein-A/G Sepharose in a 96-well plate format, with each serum tested in duplicate
- Washing of the 96-well plates to remove unbound labeled antigens
- Counting of each well with a 96-well plate scintillation counter.
- Results expressed as an index that adjusts the delta cpm of the test serum for the delta cpm of positive and negative control sera in a particular assay.

Plan for performing the assay

Assay sera:

- | | | |
|--------|-----------|--|
| Day 1: | morning: | retrieve and thaw sera to be tested |
| | mid-day: | (1) set up incubation of sera in buffer 1
(2) prepare protein-A/G Sepharose in buffer 1
(3) Coating the 96-well plates |
| Day 2: | morning: | (1) add incubate to protein-A/G Sepharose in plates
(2) wash plates
(3) dry plates
(4) add scintillation liquid |
| | afternoon | (4) count
(5) analyze data (Part VI) |

Quality Control

All assays are run in duplicate, along with a standard positive and a negative control serum samples. If the positive control shows as < 1000 delta cpm or > 2000 delta cpm, results are regarded as invalid, and the test is re-run until the positive control result falls within this

range. One internal low positive control is used for monitoring the sensitivity and variability of assay.

Materials

Trizma Base
NaCl
Sodium Azide
Tween 20
Bovine Serum Albumin (BSA)
Protein A-Sepharose
Protein G-Sepharose
125-I human insulin
48-well plate
MultiScreen-DV filtration plates
TopSeal
TopCount
Millipore vacuum-operated 96-well plate washer
96-well Plate Shaker
Water Bath Incubater
Scintillation Liquid
Bottle-Top 500 ml-Filter Units

Procedure

Incubation of serum samples with ¹²⁵I-insulin

Each 96-well plate is sufficient for testing 24 samples in duplicate (24 duplicate with cold insulin and 24 duplicate without cold insulin. Usually, four plates can easily be run at one time (total of 96 samples).

1. Spin down sera to remove fibrin clots.
(otherwise these may partially block membrane in bottom of wells)
2. Prepare the stock solution of ¹²⁵I-insulin.
Use 1 ml of 5%BSA in PBS, dissolve the powder of 10 uCi of ¹²⁵I-insulin.
3. Calculate how much ¹²⁵I-insulin and cold insulin are required.
6.4 ml of Washing Buffer for two plates:
48 x 4.2 x 30 = 6 ml
(48 samples, with 30 ml/well; in duplicate for both with and without cold insulin wells but multiply by 4.2 rather than 4 to allow for some extra)

20,000 cpm is used for each well.

3040 ml buffer 1
160 ml ¹²⁵I-insulin
3.2 ml

2784 ml buffer 1
160 ml ¹²⁵I-insulin
256 ml humulin (or Novolin)
3.2 ml

Keep the Buffer-labeled antigen mixture on ice.

4. Mix each serum sample with buffer-antigen mixture in a PCR tube (or similar tube).

Serum: 6 ml
Buffer: 30 ml

Each sample for 2 wells.

Use the same control samples for every assay.

5. Vortex and incubate 2 hours at room temperature and overnight at 4°C.

Preparation of MultiScreen Filtration Plates and Protein A/G-Sepharose

1. Coat the plate with BSA by adding 150 µl of Buffer 1 to each well.

Incubate overnight at room temperature, after placing the plate on aluminium foil.

2. Remove the washing buffer.

3. The plates are now ready for running the assay, but can be stored at 4°C if necessary.

4. Prepare Protein-A/G Sepharose:

a) Prepare Protein A-Sepharose

- Use only plastic tubes because Protein-A sticks to glass
- For each plate, suspend ~0.75 gm Protein-A Sepharose in distilled water in a 50 ml tube. Spin down and remove the fluid phase. Repeat once with water and a second time with buffer 1.
- Finally add buffer 1 to give 62.5% concentration of Protein-A Sepharose by volume.

b) Prepare Protein G-Sepharose

- Use only plastic tubes because Protein-G sticks to glass
- For each plate, re-suspend ~0.2 gm Protein-G Sepharose in buffer 1 in 50 ml tube. Spin down and remove the fluid phase. Repeat once with buffer 1
- Finally add buffer 1 to give 40% concentration of Protein-A Sepharose by volume.

- c) Mix Protein A/G Sepharose in a 4:1 ratio.

Immunoprecipitation with Protein A-Sepharose

1. Add 50 ml of Protein A/G-Sepharose mixture to each well. Use Eppendorf multipipettor and re-suspend the Protein-A/G Sepharose after each row of the plate is done. (Will need 5 ml of Protein-A/G Sepharose per plate.)
2. Add 30 ml of overnight incubate to each well (i.e., each serum will be tested in duplicate).
3. Shake the plate on a Plate Shaker for 45 minutes at 4°C. Accurate timing important.
4. Place the plate on Millipore plate washer device (with vacuum set low).
5. Wash the plate three times in this way with 200 ml of Washing Buffer per well.
6. Add 130 ml of Washing Buffer to each well. Shake for at least 5 minutes at 4°C.
7. Wash the plate four times with 200 ml of washing buffer per well (change the plate direction after two times of washing at this stage).
8. Place the plate under a lamp for approximately 10 minutes to dry. Rotate the plate several times to ensure even drying and check its appearance. Drying is complete when deep fissures appear in the Sepharose visible in the bottom of the wells. Do not over-dry and be careful not to melt the plastic parts of the plate.
9. Add 50 ml of scintillation cocktail (Microscint-20) to each well.
10. Count on Top Count 96-well plate b counter.

Data Analysis

1. Calculate delta cpm =
mean cpm of duplicate without cold insulin - mean cpm of duplicate with cold insulin
2. CPM Index for each sample:

$$\frac{\text{Sample delta cpm} - \text{NC delta cpm}}{\text{PC delta cpm} - \text{NC delta cpm}}$$

3. Coefficient of Variation

For Duplicates:

$$\frac{(\text{High CPM} - \text{Low CPM})/1.128}{\text{-----}} \times 100$$

Mean CPM

For Triplicates:

$$\frac{(\text{High CPM} - \text{Low CPM})/1.693}{\text{Mean CPM}} \times 100$$

Buffers

Buffer 1 (150 mM NaCl, 20 mM Tris-HCl, 1%BSA, 0.1%Sodium Azide pH 7.4)

30 ml 5M NaCl
10 ml 2M Tris-HCl pH 7.4
10 gm BSA
1 gm Sodium Azide (essential to prevent bacterial contamination)
1.5 ml Tween-20
up to 1000 ml

Buffer 2 The same as buffer 1 except for 0.1%BSA

Important Points

- Buffer should be filtered (0.45 micron filter) to prevent any particles blocking the membrane in bottom of the wells of the 96 well plate (which would decrease washing efficiency and increase the assay background)
- Store buffers at 4°C in a sterile bottle for up to 3 months

D3: Combined GAD-65/ICA-512BDC In Vitro Translation & Immunoprecipitation Assay

Principles

- In vitro transcription and translation (in one step, using rabbit reticulocyte lysate) of labeled antigen (³H-Leucine-GAD65 and ³⁵S-Methionine -ICA512BDC)
- Incubation of serum with both labeled antigens together overnight
- Precipitation of antibody-bound labeled antigens with protein-A Sepharose in a 96-well plate format, with each serum tested in duplicate
- Washing of the 96-well plates to remove unbound labeled antigens
- Counting of each well with a 96-well plate scintillation counter. Emission spectra of ³⁵S and ³H partially overlap and can be corrected, allowing simultaneous measurement of antibodies against the two antigens.
- Results expressed as an index that adjusts the cpm of the test serum for the cpm of positive and negative control sera in a particular assay.

Plan for performing the assay

In vitro transcription/translation of antigens (see Part III of this protocol): Labeled antigens can then be stored at -70°C for at least one month and used in multiple assays.

Assay sera:

Day 1:	mid-day:	retrieve and thaw sera to be tested
	afternoon:	(1) set up incubation of sera in washing buffer (2) prepare 96-well plates (3) prepare protein-A Sepharose
Day 2:	morning:	(1) add incubate to protein-A Sepharose in plates (2) wash plates (3) dry plates
	afternoon	(4) count (5) analyze data (Part VII)

Quality Control

All assays are run in duplicate, along with a standard positive and a negative control serum samples. If the positive control shows as < 0.75 or > 1.25 for both GAD65Ab and ICA512Ab, results are regarded as invalid, and the test is re-run until the positive control result falls within this range. The negative control must be < 0.032 (GAD65Ab) or < 0.05 (ICA512) for results to be valid.

Proficiency is monitored through the International Diabetes Workshop Islet Cell Antibody Test Program. The following proficiency results have been determined to be unacceptable and necessitate trouble shooting of the assay:

Sensitivity	Specificity
$< 70\%$	$< 80\%$

Materials

Trizma Base
NaCl
Sodium Azide
Tween 20
Bovine Serum Albumin (BSA)
TCA
Protein A-Sepharose (Amersham/Pharmacia)
TNT Reticulocyte In Vitro Transcription/Translation Kit
RNasin 40,000 u/ml
 ^{35}S -Methionine
 ^3H -Leucine
Sizing Column
48-well plate
MultiScreen-DV filtration plates
TopSeal
TopCount
Millipore vacuum-operated 96-well plate washer
96-well Plate Shaker

Water Bath Incubater
 Filtering Funnel
 GF/C Whatman Glass Fiber Filter 2.4 cm diameter
 Scintillation Liquid
 Bottle-Top 500 ml-Filter Units

Procedure

In Vitro Transcription/Translation

Reaction

In a given reaction either GAD65 is labeled with ³H-Leucine or ICA512bdc is labeled with ³⁵S-Methionine. All reagents and tubes must be sterile, otherwise RNase may destroy RNA. Keep all reagents and tubes on ice while on the bench.

1. Set up a reaction tube (the reaction volume depending on the amount of ³⁵S or ³H available) that will contain DNA, plus one control tube that will contain no DNA and should theoretically not precipitate any detectable radioactivity)

Store reagents at -20°C, except for the Reticulocyte Lysate, which is stored at -70°C.

Important: the Reticulocyte Lysate must be thawed rapidly just before use. To do this, roll the tube between hands. Do not use a hot water bath. Each tube can only be thawed twice, after which there will be a significant decrease in the amount of product. Reticulocytes have ribosomes but no nucleus (normoblasts have nucleus).

The TNT kit gives high incorporation (~50%) and consequently there is need to purify the labeled product to remove free radiolabeled methionine or leucine with a sizing column.

2. Add in following order:

	Reaction tubes with DNA	Control tube without DNA
Water (double distilled, sterile)	14-15 ml	6.5 ml
*"TNT" Reaction Buffer	2 ml	1.0 ml
Rnasin (inhibits RNase)	1 ml	1.0 ml
*Amino Acid Mixture (with no Leucine for GAD65, or no Methionine for ICA512BDC.)	1-2 ml	1.0 ml
DNA plasmid (GAD65 or ICA-512BDC)	1-2 mg	-----
³⁵ S-Methionine or ³ H-Leucine (1,000Ci/mmol)	4 ml (5ml for ³ H)	2.0 ml
*"TNT" RNA Polymerase	1 ml	1.0 ml
"TNT" Rabbit Reticulocyte Lysate	25 ml	12.5 ml

Items marked with an asterisk (*) are included in the TNT kit.

3. Mix the reagents in each reaction tube by pipetting up and down. Do not vortex as this will create bubbles that interfere with the reaction.

4. Incubate the reaction at 30°C for 90 minutes.

Purification

1. Open the top cover of the column and then bottom cover and let it dry.
2. Add 1 ml of washing buffer to the column and let it go through.
3. Add reaction mix onto the column, let it go into the column, add small amount of washing buffer to wash the wall of column and then add more washing buffer.
4. Collect the whole red part from the column (labeled protein product will come out together with hemoglobin present in reticulocyte lysate).

Analysis by Trichloroacetic Acid (TCA) Precipitation

1. Remove 2 ml from each reaction tube for analysis and store the remainder at -70°C.
2. Place 25% wt./vol TCA on ice
3. Add 2 ml of reaction to 98 ml of 1M NaOH/2% H₂O₂. (Hydrolyses aminoacyl tRNAs and bleached hemoglobin)
4. Vortex briefly and incubate at 37°C for 10 minutes.
5. Add 900 ml of ice-cold 25% TCA. Incubate on ice for at least 30 minutes. (Precipitation of the protein)
6. Place Whatman glass fiber filters on aluminium foil (one per tube, plus an additional filter for determining "total counts" as described below).
7. Collect the precipitate from each tube on a filter by vacuum (see diagram). First, place the filter on the fenestrated platform of the funnel. Add 1 ml of cold 5% TCA to wet the filter, followed by the contents of one of the reaction tubes.
8. Rinse the filter 5 times with 1 ml of cold 5% TCA (wash away any free ³⁵S-Methionine or ³H-Leucine).
9. Determine the "total counts": Take another 2 ml from the control tube with no DNA. Place this on a filter. Omit the washing step for this filter (do not place on funnel).
10. Dry each filter under a lamp for at least 10 minutes.
11. Place dried filter in a glass scintillation vial. Add 5 ml of scintillation liquid, shake laterally (don't invert) and count with b counter.
12. Calculate percentage incorporation for each reaction tube. This will be needed to determine how much volume to use in the assay:
% incorporation = $\frac{\text{cpm}(\text{with DNA}) - \text{cpm}(\text{without DNA})}{\text{cpm}(\text{total})}$
13. Labeled GAD65 or ICA512bdc can be stored at -70°C for at least one month.

Incubation of Serum Samples with ^3H -GAD65 and ^{35}S -ICA512BDC

Each 96-well plate is sufficient for testing 48 samples in duplicate (42 test samples sera plus two positive, two negative control serum, and two internal controls). Usually, four plates can easily be run at one time (total of 192 samples).

1. Spin down sera to remove fibrin clots (otherwise these may partially block membrane in bottom of wells)
2. Calculate how much ^3H -GAD65 and ^{35}S -ICA512BDC is required.

12 ml of Washing Buffer for two plates:

$$96 \times 2.5 \times 50 = 12 \text{ ml}$$

(96 samples, with 50 ml/well; in duplicate but multiply by 2.5 rather than 2 to allow for some extra)

20,000 cpm of TCA precipitate is used for each antigen for each well.

$$96 \times 2.5 \times 20,000 = 4.8 \times 10^6 \text{ cpm of both GAD65 and ICA512BDC for two plates.}$$

In this example, say the GAD65 reaction tube being used contains 1.2×10^6 cpm/2ml of TCA precipitate (or 0.6×10^6 cpm/ml), as determined by the calculation at the end of Part III, then:

$$4.8 \times 10^6 \text{ cpm required} / 0.6 \times 10^6 \text{ cpm per ml in the reaction tube} = 8 \text{ ml required from the tube}$$

Therefore, add 8 ml from the GAD65 reaction tube to 12 ml washing Buffer for two plates. Using a similar method calculate the volume required from the ICA512BDC reaction tube. Keep the Buffer-labeled antigen mixture on ice.

3. Mix each serum sample with Buffer-antigen mixture in a PCR tube (or similar tube).

Total volume: $50 \text{ ml/well} \times 2.5 = 125 \text{ ml}$ (to test each serum in duplicate; the factor of 2.5 allows for some extra)

$$\text{Serum: } 2 \text{ ml/well} \times 2.5 = 5 \text{ ml}$$

$$\text{Buffer-antigen mixture: } 125 \text{ ml} - 5 \text{ ml} = 120 \text{ ml}$$

Therefore mix 5 ml of serum with 120 ml of buffer-antigen mixture

4. Using the same control samples for every plate. One positive control (PC1) and one negative control (NC1) are used for ICA512bdc in which only ^{35}S antigen will be added and PC2 and NC2 for GAD65 in which only ^3H antigen will be added.
5. Vortex and incubate overnight at 4°C .

Preparation of MultiScreen Filtration Plates and Protein A-Sepharose

1. Coat the plate with BSA by adding 200 ml of Washing Buffer to each well.

Incubate overnight at room temperature, after placing the plate on aluminium foil.

2. Remove the washing buffer.
3. The plates are now ready for running the assay, but can be stored at 4°C if necessary.
4. Prepare Protein-A Sepharose:
 - Use only plastic tubes because Protein-A sticks to glass
 - For each plate, suspend ~0.75 gm Protein-A Sepharose in distilled water in a 50 ml tube. Spin down and remove the fluid phase. Repeat once with water and a second time with washing buffer.
 - Finally add washing buffer to give 50% concentration of Protein-A Sepharose by volume.

Immunoprecipitation with Protein A-Sepharose

1. Add 25 ml of 50% Protein A-Sepharose to each well.

Use Eppendorf multipipettor and resuspend the Protein-A Sepharose after each row of the plate is done. (Will need 2.5 ml of 50% Protein-A Sepharose per plate.)
2. Add 50 ml of overnight incubate to each of 2 wells (i.e., each serum will be tested in duplicate).
3. Shake the plate on a Plate Shaker for 45 minutes at 4°C.

Accurate timing is important.
4. Place the plate on Millipore plate washer device (with vacuum set low).
5. Wash the plate three times in this way with 200 ml of Washing Buffer per well.
6. Add 120 ml of Washing Buffer to each well. Shake for at least 5 minutes at 4°C.
7. Wash the plate four times with 200 ml of washing buffer per well (change the plate direction after two times of washing at this stage).
8. Place the plate under a lamp for approximately 10 minutes to dry. Rotate the plate several times to ensure even drying and check it's appearance. Drying is complete when deep fissures appear in the Sepharose visible in the bottom of the wells.

Do not over-dry and be careful not to melt the plastic parts of the plate.
9. Add 30 ml of scintillation cocktail (Microscint-20) to each well.
10. Count on Top Count 96-well plate b counter.

Set two windows, window A 2.9-10 for 3-H and window B 10-256 for 35-S

Data Analysis

1. Data correction

Spill rate of 35-S between two windows

$$r1(PC1) = \text{windowB}/(\text{windowA} + \text{windowB})$$

$$r1.1 = (1 - r1)$$

Spill rate of 3-H

$$r2(PC2) = \text{windowA}/(\text{windowA} + \text{windowB})$$

$$r2.1 = (1 - r2)$$

$$\text{Total PC1} = \text{PC1_windowA} + \text{PC1_windowB}$$

$$\text{Total PC2} = \text{PC2_windowA} + \text{PC2_windowB}$$

$$\text{NC1} = \text{NC1_windowB}/r1$$

$$\text{NC2} = \text{NC2_windowA}/r2$$

$$\text{Sample cpm of 3-H} = \text{windowA} - r1.1(\text{windowA} + \text{windowB})/(r2 - r1.1)$$

$$\text{Sample cpm of 35-S} = \text{windowB} - r2.1(\text{windowA} + \text{windowA})/(r2 - r1.1)$$

2. CPM Index for each sample

Index for ICA512BDC:

$$\frac{\text{Sample CPM of 35-S} - \text{NC1}}{\text{Total PC1} - \text{NC1}}$$

Index for GAD65:

$$\frac{\text{Sample CPM of 3-H} - \text{NC2}}{\text{Total PC2} - \text{NC2}}$$

3. Coefficient of Variation

For Duplicates:

$$\frac{(\text{High CPM} - \text{Low CPM})/1.128}{\text{Mean CPM}} \quad \times 100$$

For Triplicates:

$$\frac{(\text{High CPM} - \text{Low CPM})/1.693}{\text{Mean CPM}} \quad \times 100$$

Reagents

TBS/azide buffer (150 mM NaCl, 20 mM Tris-HCl, 0.1% Sodium Azide pH 7.4)

30 ml 5M NaCl
10 ml 2M Tris-HCl pH 7.4
1 gm Sodium Azide (essential, to prevent bacterial contamination)
up to 1000 ml

Washing buffer (0.15% Tween-20, 0.1% BSA in TBS/azide buffer)

1.5 ml Tween-20
1 gm BSA
plain buffer to 1000 ml

Important Points

- Buffer should be filtered (0.45 micron filter) to prevent any particles blocking the membrane in bottom of the wells of the 96 well plate (which would decrease washing efficiency and increase the assay background)
- Store buffers at 4°C in a sterile bottle for up to 3 months

D4: Mouse ELISPOT Assay

Materials

Kit: BD ELISPOT Mouse IFN-g Set #551083, store at 4C

Contents

Capture Antibody (purified anti-mouse IFN-g, 1mg/ml)
Detection Antibody (biotinylated anti-mouse IFN-g, 0.5mg/ml)
Streptavidin-HRP
BD ELISPOT Plates

AEC Substrate
AEC Chromogen

PBS sterile
PBS + Tween (250ul in 500ml PBS)
PBS + FBS 10%

Procedure

Day 1

In hood: Capture antibody 50ul + sterile PBS 10ml and coat 1 plate with 100ul per well

Store 4C overnight

Day 2

- 1) In hood: Wash plate x 3 with PBS (200ul/well)
- 2) Block with culture medium 200ul/well x 2 hours room temperature
- 3) Then dump medium
- 4) PEPTIDES AND CON A:

After blocking, take peptides 1mg/ml and Con A 100ug/ml

For 10ug/ml concentration peptide

Add 20ul peptide to 1ml medium

For 50ug/ml concentration peptide

Add 50ul to 1ml medium

For 0.5ug/ml Con A

Add 5ul Con A to 1ml medium

For 1ug/ml Con A

10ul Con A to 1 ml medium

Add 100ul of peptide/medium to corresponding well and let sit in incubator

- 5) CELLS

Harvest spleen, RBC lyse and wash x 2 in medium (make sure medium is warmed)
Resuspend cells in medium. If you want 400,000 cells/well, resuspend 4 million cells/ml

If you want 200,000 cells/well, resuspend 2 million cells/ml

Add 100ul cells to each well, incubate x 48 hours

Day 3 Holiday

Day 4

- 1) Dump cells and wash with PBS x 3 (allow wells to soak 3-4 minutes at each wash step)
- 2) Wash x 3 PBS + Tween
- 3) Blot dry well
- 4) Add Detection antibody 40ul + PBS 10%FBS 10ml, 100ul per well and incubate room temperature x 2 hours
- 5) Wash with PBS + Tween x 4 (allow to soak 1-2 minutes at each wash step), dry well
- 6) Add Streptavidin-HRP 100ul + PBS 10%FBS 10ml, 100ul/well and incubate in dark at room temperature x 1 hour
- 7) Wash PBS + Tween x 3 (allow to soak 1-2 minutes at each wash step)
- 8) Wash PBS x 3, dry well
- 9) AEC Substrate 10ml + AEC Chromogen 200ul and add 100ul per well. Monitor 5-60 minutes for spot formation
- 10) Stop substrate reaction by washing well with dH₂O. Air dry in dark for 2 hours or overnight. Read plate.

D5: Insulin Release and Immunoassay

Plating the cells

Materials

24-well tissue culture plate

6-well tissue culture plate

Complete medium (for INS-1 cells RPMI-1640/10%FBS/10mM Hepes/1mM Na-Pyruvate/50 μ M 2-mercaptoethanol/100 U/ml penicillin/100 μ g/ml streptomycin)

Low glucose media (as complete media but 5.6mM glucose)

0.25% Trypsin-EDTA

Hemacytometer

Trypan Blue

10 x SAB (1.14M NaCl/ 47mM KCl/ 12mM KH₂PO₄/11.6mM MgSO₄)

10xKAB (0.64M NaCl/ 0.55M KCl/ 12mM KH₂PO₄/11.6mM MgSO₄)

1M Hepes pH 7.2

0.25M CaCl₂

NaHCO₃

Glucose

BSA

PBS

Trypsinize cells and resuspend in complete media to get a single cell suspension. Take a sample, stain with Trypan Blue to determine viability and count in hemacytometer.

Plate appropriate number of cells in 24-well plate to achieve 90-100% confluency and an additional well in 6-well plate well for Western Blot control sample.

We plate 1.5×10^5 INS-1 cells/well/ml complete media in 24-well plate and let it grow for 6 days and add 0.5 ml fresh complete media on day 3 or 4.

Day before the Insulin Release assay aspirate the media and replace with 1 ml of low glucose media.

Insulin Release

Day 7: Prepare 200mls of 1 x SAB/ 0.2%BSA/2.8mM glucose and let the buffer sit in an open container inside CO₂ incubator for a minimum 30 of minutes before use. Aspirate low glucose medium from 24-well plate. Wash twice (very gently) with 1 ml of the buffer and replace with 1 ml of buffer for 2 hour preincubation.

Prepare desired buffers for the Insulin Release assay and let sit in open containers inside CO₂-incubator for a minimum of 1 hour before use. After 2 hour preincubation aspirate buffer and replace with 1 ml desired condition buffer. We use 3-4 replicates per condition.

We generally use the following conditions:

- 1xSAB/0.2% BSA/2.8 mM glucose

- 1xSAB/0.2% BSA/8.3 mM glucose
- 1xSAB/0.2% BSA/11.1 mM glucose
- 1xSAB/0.2% BSA/10 mM leucine
- 1xSAB/0.2% BSA/2.8 mM glucose/10 μ M forskolin
- 1xSAB/0.2% BSA/11.1 mM glucose/10 μ M forskolin
- 1xSAB/0.2% BSA/10 mM leucine/10 μ M forskolin
- 1xKAB/0.2% BSA/2.8 mM glucose/10 μ M forskolin

1xSAB buffer (200ml)

10xSAB	20ml
1M Hepes pH 7.2	4ml
0.25M CaCl ₂	2ml
NaHCO ₃	.428g
H ₂ O	to 200ml
BSA	.4g

Add appropriate amount of glucose/ leucine/ forskolin.

Prepare 1X KAB using same ratio of ingredients.

Harvest the samples

After 2-hour incubation place the plate on ice. Transfer 500 μ l of buffer into a 1.5ml centrifuge tube carefully avoiding transferring any cells, centrifuge 14K, 5 minutes +4°C. Transfer into 2ml 96-well storage plate. Add 500 μ l of PBS/1%BSA/0.02% Na-Azide and mix. These are the secreted samples.

Add 5 μ l of 10% Triton X-100 to remaining buffer in 24-well plate, sonicate 10 seconds, transfer into 2ml 96-well storage plate. Add 500 μ l of PBS/1%BSA/0.02% Na-Azide and mix. These are the insulin content samples.

Store plates in +4°C until assayed.

Harvest the cells from 6-well plate for western blot analysis; rinse twice with PBS and lyse the cells in Laemmli Sample Buffer.

Determine DNA concentration

Use Picogreen dsDNA Quantitation Kit , Molecular Probe, cat#P7589 to determine DNA concentration of the content samples. You will need these numbers to determine the suitable dilution for the Insulin Elisa and to normalize the secreted insulin results.

INSULIN ELISA IMMUNOASSAY

We use Ultra Sensitive Rat Insulin Elisa Kit from Crystal Chem Inc, cat# 90060.

Dilute content and secreted samples in PBS/0.2%BSA.

Based on buffer condition in Insulin release use 2, 10 or 20 μ l of secreted samples in the Elisa, 5 μ l of content samples and 5 μ l of the standard provided with the Elisa kit.

First reaction: Pipets Samples or standards in 100 μ l of Sample Diluent into antibody coated wells. Incubate on a plate shaker in +4°C overnight.

Discard the samples by shaking the plate, wash 5 times with 300 μ l of Washing Buffer.

Second Reaction: Add 100 μ l of Rat Insulin Enzyme Conjugate. Incubate on plate shaker 30 minutes in RT.

Discard the conjugate, was 7 times times with 300 μ l of Washing Buffer.

Enzyme reaction: For a kinetic assay add 100 μ l of Enzyme Substrate Solution and let the reaction proceed about 40 minutes in RT. Stop the reaction with 100 μ l Stopping Solution when the numbers seem appropriate. Measure the end point assay numbers at 450 nm and calculate the Insulin concentration.