

MOLECULAR BIOLOGY-RELATED PROTOCOLS

M1: Genomic DNA Isolation from Mouse Tail Samples

Currently, there are several methods available to extract DNA from mouse tails. Almost all of the protocols published are variations of the original method developed by Richard Palmiter and Ralph Brinster in 1985 (Palmiter et al. 1985). The method shown below is taken from Laird et al. (1991) and does not require a phenol:chloroform extraction, which is also commonly used. This protocol can be utilized for mouse tail genomic DNA extraction, other small tissue genomic DNA extraction, or mammalian cell genomic DNA extraction.

Materials

Lysis Buffer

- 100 mM Tris (pH 8.0)
- 5 mM EDTA (pH 8.0)
- 0.2% SDS
- 200 mM NaCl
- 100 mg/ml Proteinase K

Isopropanol

Procedure

1. Prepare lysis buffer.

The lysis buffer can be prepared ahead of time. If the proteinase K is added to the buffer during preparation, the lysis buffer should be aliquoted and stored at -20°C . The lysis buffer can be prepared minus the proteinase K and stored at room temperature, then the proteinase K can be added immediately prior to digestion.

2. Cut 1.0 cm of mouse tail and transfer to a 1.5 mL centrifuge tube.
3. Add 500 μl lysis buffer to mouse tails and incubate at 55°C with rocking or rolling overnight.

Adequate mixing during digestion is important. The tissue should no longer be visible after digestion. However, hair and bone tissue may still be present.

4. Remove from incubator and spin 10 minutes at 14,000 g to pellet hairs and debris.
5. Pour supernatant in a fresh tube and avoid transferring hairs.
6. Add 500 μl of isopropanol (or a volume equal to that used in step 3).
7. Mix by inversion. The DNA will appear as a long thread-like precipitate.
8. With a clean, sterile micropipette tip, spool out the DNA and transfer to a new tube.
9. Store the open tube on the bench until the remaining alcohol has evaporated.

10. Dissolve the genomic DNA in 200-500 ml of 10 mM TrisCl, TE (pH 8.0), or ddH₂O, by rocking gently overnight at room temperature.

The yield of DNA usually varies from 5 to 12 mg/mm of mouse tail.

Polymerase Chain Reaction and Agarose Gel Analysis to Identify the Genotype of Mouse Models

Polymerase Chain Reaction (PCR) is a technique utilized for a wide variety of applications. PCR is now one of the most widely used techniques in molecular biology; it is rapid, relatively inexpensive, and a simple means of amplifying DNA from a minute amount of template DNA. PCR is a technique used to amplify specific DNA sequences in vitro. One application for PCR is to couple it with agarose or acrylamide gel analysis to determine the genotype of mouse models.

Materials

NovaTaq DNA Polymerase Kit (Novagen cat # 71003-5)
– Taq Polymerase
– Reaction Buffer with MgCl₂
ddH₂O
dNTP
Primers
Agarose (Molecular Biology grade)
10X TBE
Ethidium Bromide (Molecular Biology grade)
DNA ladder (Promega 50bp DNA Step Ladder, cat # G4521)
Loading Buffer (Promega, Cat # G1181)

Procedure

1. Using 0.2 ml tubes, set up 20 ml reactions with the following components on ice. You can make a Master Mix of the components excluding the template gDNA.

2.0mL	10 x PCR buffer with MgCl ₂ (1X final concentration with 1.5 mM final MgCl ₂ concentration)
0.4mL	10mM dNTP (0.2 mM final concentration)
0.5mL	Taq DNA Polymerase
0.6mL	10 mM forward primer (0.3 mM final concentration)
0.6mL	10 mM reverse primer (0.3 mM final concentration)
1.0mL	genomic DNA or H ₂ O (control)
14.9mL	H ₂ O
20mL	
2. If the thermal cycler does not have a heated lid, then overlay the reaction mixtures with 1 drop (~50µl) of light mineral oil to prevent evaporation.

- Place the tubes or microtiter plate in the thermal cycler and amplify the gDNA using the following program.

95°C	2 min	X 1
95°C	45 sec	X 35
57°C	45 sec	
72°C	1 min	
72°C	7 min	X 1
4°C	hold	

The annealing temperature will vary depending on the melting temperature of the primers.

- Prepare a 4% (w/v) Agarose Gel (0.5 X TBE running buffer) with Ethidium Bromide (0.5 mg/ml final concentration).

The percentage of agarose will vary based on the size of the fragments that are to be separated.

- Load the Gel and run at ~100-150V for ~2 hours.
- Take a picture and analyze the gel.

Tissue genotyping protocols on small embryonic samples, or direct PCR on small tails

(Jensen lab method)

Tissue is isolated:

E10-E12: take the yolk sac, avoid placental tissue that may adhere.

E13-E19: take a paw/limb.

Tails: cut a size off the 0.3-0.5 cm tail piece that is app. 1mm in length. Too much tissue makes the PCR go bad.

Store the tissue in small eppendorfs at -20c until the genotyping is started.

- Make 1x PCR homogenization buffer. (This buffer has a high pH that is suitable for Taq amplification), add Proteinase K to 0.5 mg/ml from the 20 mg/ml stock.
- Add digestion mix (100 ul) to each tube. Make sure that all tissue is in the solution, and not on the side of the vial above the fluid. Use a tip to push it down, if necessary.
- incubate tubes in the racks at 55°C in the incubation oven. Do not use a heating block.
- Next morning, vortex each tube briefly – the tissue is completely dissolved.

- Incubate all tubes on a PCR machine that can hold 500ul tubes at 98°C for 10 minutes. Alternatively, boil in a water bath. This treatment denatures the protease, and opens the DNA for PCR.

Now the solution is ready for PCR. If not running this immediately, the vials can be stored at -20° indefinitely.

PCR Genotyping, direct from above

Set up reactions as above, use following thermal cycle profile.

95°C initial Denaturing

then:

95°C Denaturing, 1 minute

55°C Annealing, 30"

72°C Elongation, 30"

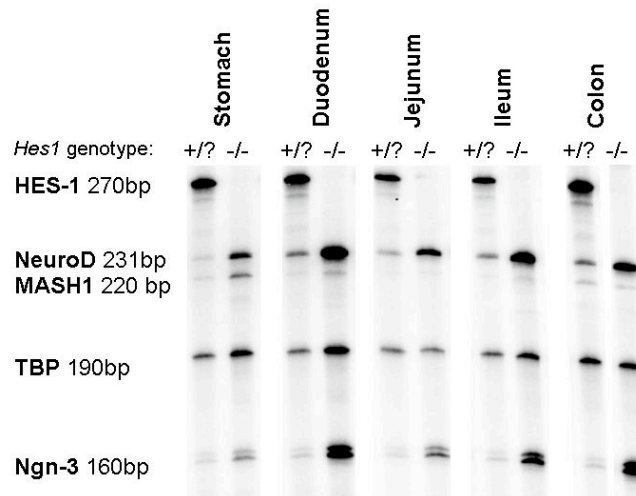
For 30 cycles.

Use the heated lid, and do not use oil.

After PCR, add 10 ul from each vial to 2 ul 5x loading buffer – use a multichannel pipette. Mix. Load samples onto a 2.5-3% agarose gel (HighRes gel) with EtBr.

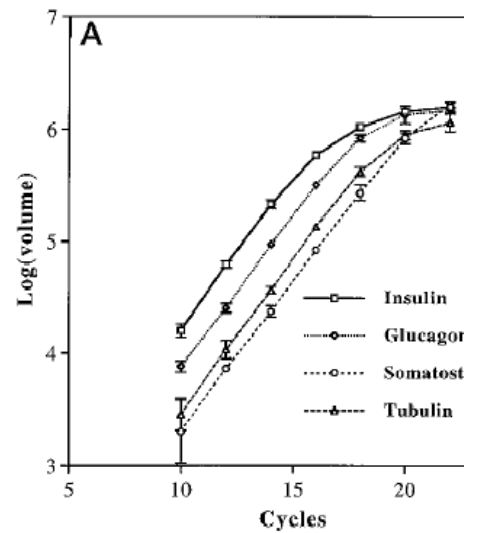
Run gel for app. 2 hours. Take picture of the Gel and interpret.

M2: Multiplex-RT-PCR



Multiplex RT-PCR is a technique that allows for screening the expression of multiple mRNA's in a single PCR reaction. It is highly sensitive, and provides semi-quantitative data where a gene of interest is measured against one, or more, internal standard genes. The technique is described in detail in [Jensen et al.](#), J. Biol. Chem, **vol.** 271, pp. 18749-58, and comments as to the quantitative aspects, controls, and detection of amplicon interference, if this should occur, can be found there.

Figure, right. Typical multiplex amplification of 4 independent cDNA's. The tissue was neonatal rat islets, and the mRNA's for the 3 predominant hormone genes, insulin, glucagon and somatostatin was amplified together with an internal standard, here tubulin. These are abundant mRNAs, and the cycle numbers are therefore very low. AS you can see, by extrapolating the insulin curve downwards, it would have been detectable after only 5 cycles of PCR. This shows the sensitivity of the current technique. The reason for this is that radioactive labeling is used, and that the amount of cold nucleotides present during PCR is lower then normal. Notice that while the most abundant amplicon plateaus, the others continue to amplify. The reason for this is that this is a non-competitive amplification, and we can conclude that it is the insulin primer/insulin product ratio that determines maximal levels – not the exhaustion of dNTP's or loss of Taq-activity. Notice also that the slopes are very similar – not to be expected, as amplicons are completely independent. However, due to the constraints in primer design, and amplicon size/GC-content, these amplicons amplify with a very similar kinetics. The above experiment was done by removing small aliquots during cycling, from 3 independent tubes. Such a control experiment is always sound to start out with before relying on the quantitative capabilities of MPX-RT-PCR.



cDNA Synthesis

Use total RNA from the selected tissues/cell lines.

Do not necessarily check for RNA degradation by agarose gel electrophoresis - you'll get this information the first time you run the PCR.

Dilute all RNA samples to 0.2 $\mu\text{g}/\mu\text{l}$ in a small eppendorf tube.

1. Make cDNA synthesis mastermix, on ice:

One reaction, 25 μl :

5.0 μl 5x RT-buffer
 1.0 μl dNTPs (25mM)
 1.0 μl Random Primers
 2.5 μl DTT (0.1 mM)
 1.0 μl RNAsin (Promega) (40 U)
 7.5 μl DEPC water
 1.0 μl M-MLV Rev. transcriptase (200U)

Mix by pipetting, do not vortex, keep on ice when you do the following:

2. Denature the diluted RNA on a PCR machine: 85°C for 10 minutes (removes secondary structures)

3. Transfer to ice, pipet 5 μl RNA (1 μg) into new small tubes, and add 20 μl RT-mix. Pipet to mix.

4. Leave 10' at room temp. IMPORTANT - very little priming at higher temperatures.

5. Transfer to PCR-machine, Plate temp. 37°C, incubate one hour. Alternatively, use a hybridization oven or incubator set at 37°C.

6. Finally, dilute the cDNA reaction with 50 µl Water (increases pipetting accuracy later).

Comments:

RNA isolation: Any method will do. We normally use the RNAzol (Cinna/Biotex) isolation solution, which works well. TriZol from Life Technologies is as good.

We prefer to use the M-MLV RT enzyme for no other reason that it works. Other enzymes (superscript, AMV) are surely also usable. However, with the above protocol we observe a very reproducible cDNA synthesis, with less than 20% variation between 10 identical reactions. (intra-assay variation).

We purchase the M-MLV RT from Gibco and the 5x buffer and DTT is supplied.

A critical point in random primed cDNA synthesis is the use of enough Random hexamers - too little gives very poor yield - therefore, do not decrease the amount of these.

Generally: random primed cDNA synthesis gives the most consistent results. In contrast: With Oligo-dT primed cDNA synthesis you'll find larger variations in expression level of the same gene, depending on the position of the PCR-primers. If these are positioned in the outmost 5' end of the mRNA, you'll often observe less signal when compared to a set amplifying the gene in the 3' end. This is caused two phenomena: 1) by the difficulty of some Rev. transcriptases to read through existing secondary structures, and 2) If you have some degradation you will not be able to prime corresponding 5' ends of mRNA's during cDNA synthesis.

PCR reactions, in standard 200 µl tubes

1/50 th of a cDNA-reaction is used for one PCR-reaction. This generally, depending on tissue-type, reflects the RNA-content of approximately 1500-2000 cells. Thaw PCR buffer, MgCl₂, dNTP's and primers on the lab bench.

1. Make the PCR-mastermix at room temperature. Gloves are not necessary.

One reaction, 25 µl:

2.5 µl 10x PCR-buffer
x µl MgCl₂

(Sometimes MgCl₂ is added to the 10x buffer, if not, add up to 1.5 mM final conc.)

0.125 µl dNTPs (8 mM dA;G;TTP's, 4 mM dCTP)

0.25 µl EACH of the Primers (20 pmol/µl)

(18 - 0.5*y)µl Water (y=the number of genes

analysed)

0.25 µl Taq-polymerase (Promega) (2.5 U)

0.125 µl γ -³²P-dCTP

Mix by vortexing.

2. Pipet 3 µl diluted cDNA into PCR-tubes.

3. Add 22 µl PCR-mastermix (do not mix).

4. Thermocycle the tubes for a set number of cycles. Use the following thermal profile:

1.1 96°C - 1'00"
2.1 96°C - 0'30"
2.2 55°C - 0'30"
2.3 73°C - 0'30"

Repeat step 2.1 - 2.3 for x cycles.

The above thermal profile have been changed from the JBC published profile. The above works consistently. Remember to use a heated lid, otherwise, add 25ul oil to the samples.

5. After thermal cycling, mix 10 µl PCR-reaction with 10 µl Gel loading buffer (95% formamide/20 mM EDTA and 0.03% Xylenecyanol/Bromophenolblue).

6. Before gel running denature this sample at 94°C for 2'00".

7. Load 4-6 µl on a preheated, denaturing sequencing gel (7M Urea/6% polyacrylamide/1x TBE).

8. Run the gel for 3 h at 65 W. Transfer gel to Whatman 3MM paper. Cover with household wrap. Cut away excessive gel/paper material. Label gel with name on back.

9. Dry gel. 15-30 minutes in geldrier at 80°C is sufficient.

10. Expose gel to phosphoimage storage screen (or radiographic film).

Next day, Scan and analyse data.

Comments:

The risk of PCR contamination is very limited in this assay, as very few cycles (15-25) of amplification is used. However, if you are routinely working with plasmids encoding the gene you are assaying from cellular material, a certain possibility of contamination is present. Therefore: never use pipettes for PCR that has been in contact with plasmid. The best way to avoid this is to dedicate a set of pipettes only for PCR-work. Use these pipettes only when you make primer dilutions and set up PCR. They should also not be used for PCR-product manipulation, as when you make your samples ready for loading.

It is not necessary to make any of the above manipulations on ice.

The make and type of PCR machine is not as critical as people think they are. If you use a PCR machine with a sensor tube, take care of the following steps. Check that the amount of oil in the sensor tube matches the volume in the PCR-tubes. And always use new oil.

Position tubes together in the PCR-machine, and locate the sensor-tube in the middle of the reaction tubes. This secures the most identical thermal profile of the different tubes.

You can get less than 10% variation in absolute product yield between tubes. For this reason, we recognize PCR is as reliable as any technique is.

PCR reactions, Microtube strip/plate setup

Below are comments as to set up PCR taking advantage of multi-channel pipetting.

Make the PCR-mastermix at room temperature. Gloves are not necessary.

One reaction, 25 μ l:	2.5 μ l 10x PCR-buffer, Dynazyme Buff. 1.5 mM MgCl ₂
	0.125 μ l dNTPs (8 mM dA;G;TTP's, 4 mM dCTP)
	0.25 μ l EACH of the Primers (20 pmol/ μ l)
	(19 - x) μ l Water (x=the number of genes analysed)
	0.25 μ l Taq-polymerase (Dynazyme) (0.5 U)
Mix by vortexing.	0.125 μ l γ - ³² P-dCTP

Pipet 3 μ l cDNA into a PCR-plate. The smart way of doing this is: Aliquot your cDNA into strips of 8, and make the proper dilution with water. This set will be your reference, and from this you can easily pipet using multichannel pipette your cDNA out into either microtitreplates or 8-strip 200 μ l sample tubes. These cDNA dilutions can be easily used for subsequent PCR setups if the reaction order is the same between experiments. I use scotch tape as lid instead of the supplied plastic lids - these are very difficult to remove without the risk of "slipping", causing cDNA to spill out all over the place.

Add 22 μ l PCR-mastermix (do not mix).

Thermocycle the tubes for a set number of cycles, use only PCR blocks suited for 200 μ l sample tubes, or plates. If you use plates, use a rubber mat. Use the heated lid. The rubber mat can be cleaned in 1N HCl between setups, so you avoid back-contamination. Remember, the mat is slightly radioactive after the run.

Use the following thermal profile:

1.1 96°C - 1'00"

2.1 96°C - 0'30"

2.2 55°C - 0'30"

2.3 73°C - 0'30"

Repeat step 2.1 - 2.3 for x cycles.

The above thermal profile has been changed from the JBC published profile. The above works consistently also for high GC-count amplicons.

The above temperature profile is very quick - generally reaction times takes less than 2 hours - Furthermore, sequencing gel running can be performed at 75W for 2 hours. This makes it possible to do PCR and gel running the same day.

After thermal cycling, mix 10 μ l PCR-reaction with 10 μ l Gel loading buffer (95% formamide/20 mM EDTA and 0.03% Xylenecyanol/Bromophenolblue). Before gel running denature this sample at 94°C for 2'00". This mix can be easily, and very quickly performed using multichannel pipettes into plastic microtitreplates. The denaturing is performed by heating up the whole plate on a PCR machine.

Load 4-6 μl on a preheated, denaturing sequencing gel (7M Urea/6% polyacrylamide/1x TBE).

NOTE: the loading procedure can be massively speeded up if a comb with proper spacing between wells is used. Make sure that your comb width fits the spacing of your multichannel pipette. This way 8 samples can be simultaneously loaded. Remember to clean wells properly of remaining acrylamide before attempting loading. The sample has to "float" by itself down into the slot. Alternatively, use special pipette strips with a flattened bottom – these allow you to reach into the wells.

Run the gel for 3 h at 65 W. Dry. Expose to phosphoimage storage screen (or radiographic film). Next day,

Scan and analyze data.

M3: Amplification of Genes for ISH Probe Generation

This protocol covers the generation of plasmids used for in-vitro translation in order to make an RNA probe for In situ hybridization. This protocol can also be used for any cloning purpose using a proof-reading heat-stable polymerase of a gene of interest. We normally design primers to amplify inside the coding region, and not less than 700bp in length for the full amplicon. Primers are designed with no modifications, and are 20-22 bp in size and contain from 11-13G/C's per primer. These are similar to the specifications of Multiplex-RT-PCR primers, and in most cases, a single primer for the MPX can be used with a new designed primer further downstream.

cDNA Synthesis

Similar as that listed under the multiplex RT PCR protocol, except for the use of OligodT primers. It can also be beneficial to use a more processive Reverse Transcriptase than the MMLV-RT listed under the cDNA synthesis. So far, we lack data on the difference between Reverse transcriptases.

PCR

Combine the following to make a mastermix:

2.5 μl	10x PCR Buffer.
2.5 μl	10x Enhancer solution.
0.5 μl	50 mM MgSO_4 = 1 mM final
0.75 μl	10 mM dNTPs = 300 μM final
17 μl	H_2O , freshly tapped
0.25 μl	Platinum Pfx Polymerase (2.5u/ μl , from InVitroGen).

Vortex briefly.

Make a template/primer tube, per reaction>

400 μM final.

1 μl	Template cDNA (10ng)
1 μl	Primer Mix of both (20mM). =

Add 23 μ l Mastermix to each template/primer. Mix by two pipettings up and down. Overlay with 25 μ l Oil.

Have the following program ready on the PCR machine:

94°C: 2 minutes	}	<u>Cycle for 30 to 35 cycles</u>
94°C: 30 seconds		
55°C: 30 seconds		
68°C: 1.00 minute.		
68°C: 20 minutes.		

If the PCR machine can cool, set it to cool after completion of the program.

Load 5 μ l on a resolving agarose gel and check for amplification. Resolution, and gelling % depending on PCR product size. Normally a 2% gel will resolve products at 700-800 bp well.

Run Gel, and analyse under UV (Molecular Core Lab).

Proceed to Cloning using the TOPO PCR Cloning kit

As per manufacturers instructions, combine the following:

	2 μ l Fresh PCR product
	1 μ l Salt solution (in kit – chemically competent)
or	1 μ l Dilute Salt (in kit – electrocompetent)
	2 μ l H ₂ O
	1 μ l pCR-TOPO vector (Either PCR4-Blunt, or PCR-Zero blunt-TOPO)
total	6 μ l.

Incubate on the bench for 5-10 minutes. (22-23°C)

Place the reaction on ice.

Transformation and Plating

Chemical competent cells: add 2 μ l of the cloning reaction into a vial of competent E.Coli. Incubate on ice for 5-30 minutes.

Heat shock the cells at 42°C, for 30 seconds.

Immediately transfer to ice, and add 250 μ l SOC medium (at RT).

Close tube and shake horizontally at 37°C for one hour.

Spread 50 μ l of the transformation on a prewarmed 50 μ g/ml Kanamycin plate, and incubate O/N at 37°C.

M4: Enzymatic manipulations of DNA, Plasmids

This protocol covers the manipulation of plasmids, such as Klenow fill-in, dephosphorylation, ligation using T4 ligase and DNA extraction.

Restriction digests for cloning purposes (for more info, see Current Protocols, section 3.1.)

In order to generate sufficient amounts of fragments, linearized vectors, we generally digest 10 ug of the desired vector.

Assume 1 unit of restriction enzyme digests 1 ug of vector in one hour. In these cases we will like to be sure that the vector is cut 100%, and for that reason over-digest. It is better to digest for longer periods, rather than increasing the amount of restriction enzyme. A typical digest of 10 ug of plasmid will require 10 units of enzyme for 3 hours (3 times overdigest). O/N is even better.

Phenol-Chloroform extractions (for more info, see Current Protocols, section 2.1.1.)

Enzymes, or other proteins, may be removed by a phenol/chloroform extraction of the reaction. The protein is extracted to the phenol phase, and the aqueous phase is subsequently precipitated again.

- Do the extraction as follows:
- Add up to a total volume of 50 ul.
- Add 50 ul Phenol/Chloroform mix (molecular grade, premade from vendor). Wear gloves, phenol is very reactive upon contact with human skin, and makes nasty burns. Do in hood.
- Vortex for 10 seconds.
- Spin at max RPM for 1 minute.
- Gently remove the upper, aqueous phase, avoid the interphase.
- Measure the volume with a pipette (most likely around 40 ul).
- Add 1 volume of Chloroform/Isoamylalcohol (CIAA, this removes traces of Phenol). Do in hood, as Chloroform is volatile, and carcinogenic.
- Vortex for 10 seconds.
- Spin at max RPM for 1 minute.
- Remove the upper aqueous phase. Adjust volume back to 50 ul with Water.
- Precipitate with 1/3 volume (17 ul) 7.5M NH₄Ac, and 2 volumes (100 ul) EtOH (100%). Put in -80c freezer for 20 minutes, or -20 for 1 hour.
- Spin the precipitate at max RPM for 10 minutes, remove liquid, wash with 70% icce-cold EtOH, spin briefly, and remove EtOH. Let air dry for 10-15 minutes.
- Resuspend in desired volume of Water.

Dephosphorylation of plasmid. (Current Prot. Section 3.10.1)

Plasmids that receive fragments, as dephosphorylated, in order to avoid closure, and self-ligation. Fragments to be inserted are never dephosphorylated.

Of the phenol/CHCl₃ extracted plasmid, resuspend in 21 ul water:

Combine the following 2.5 \square 10x Alkaline phosphatase Buffer.

2.0 ul calf intestinal alkaline phosphatase, CIAP (Promega).

Incubate for 30 minutes at 37c. in oven.

Change temperature to 75c, and incubate a further 15 minutes. (CIAP is effectively denatured at 75 c).

Klenow enzyme Fill-in of 5' overhangs (Current Prot. Section 3.5.9)

This protocol covers the filling-in of overhangs, so that restriction fragments can be inserted into blunt-ended vectors.

Klenow enzyme is quite independent of buffer specifications, so it will generally work in most restriction buffers.

1. Digest the plasmid according to your protocol. Normally a reaction runs in 25 ul.
2. You can heat denature the restriction enzyme at this point, but current protocols argues that it is not necessary.
3. add 2 ul of 0.25mM dNTP mix.
4. add 5 units of Klenow enzyme, mix by pipetting, incubate at 30c for 15 minutes.
5. Stop the reaction by heating to 75c for 10 minutes.

Ligation of inserts into plasmids (Current Prot. Section 3.14.1)

Ligation of inserts into opened plasmids are performed by incubating a mix of insert and opened vector with T4 DNA ligase. Normally, all vectors receiving inserts are dephosphorylated to avoid self-ligation. If inserts are inserted using two different restriction enzymes, this step can be by-passed, as the vector does not have the ability to self-ligate. The T4 DNA ligase comes with a 10x buffer that includes ATP, so no ATP has to be added.

Normally, ligations of DNA termini that are overlapping is done at 12-16 c, whereas ligations of blunt-ended fragments are done at 25 c. This is due to the fact that the ligase is more active at 25 c, and with blunt ends there is nothing gained from overlapping ends. Blunt end ligations also require more enzyme, up to 10-100-fold. However, never let the enzyme amount to more than 10% volume of the total mix.

Ligations will typically be performed using app. 100 ng of the target vector, and various amounts of insert, that will range from 1-5 fold molar amounts of the vector. The inserts should generally be gel-purified, if these are derived from another donor vector. If the insert is a PCR fragment, this is not necessary, unless the PCR reaction is dirty. In that case, gel-purification is essential.

Controls for ligation are generally two: 1. Make a control reaction without insert. This will test for the amount of undigested acceptor plasmid, and self-ligation. 2. Make a control reaction without ligase. This reaction checks for presence of uncut plasmid. Normally control 1 is vital, and control 2 is optional.

See example below.

	Hes1-phRL	Hes1-phRL	Hes1-phRL	control 1	ngn3-pGL2	ngn3-pGL2	ngn3-pGL2	Control 1	Control 2
H2O	16 µl	14 µl	12 µl	17 µl	16 µl	14 µl	12 µl	17 µl	12 µl
10x ligase buffer	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
insert	1 µl	3 µl	5 µl	0 µl	1 µl	3 µl	5 µl	0 µl	5 µl
Vector	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
T4 ligase	1 µl	1 µl	1 µl	1 µl	2 µl	2 µl	2 µl	2 µl	0 µl

M5: Bio-Rad Protein Assay (BRADFORD METHOD)

This Assay allows for protein determination, using a spectrophotometric method.

1. Make protein (BSA)-standards:

0,1µg/µl
0,2µg/µl
0,5µg/µl
1,0µg/µl
2,0µg/µl
3,0µg/µl
4,0µg/µl
5,0µg/µl

You can freeze these, and store at - 20°C and reuse.

2. Make double-setup of all unknowns

Dilute samples, e.g. 1: 5, 1: 10

Blind: 800µl H₂O

+200µl Bio-Rad Assay reagent

Standard/samples: 795µl H₂O

+ 5µl Std./sample

+ 200µl Bio-Rad

Transfer to 1 ml Cuvette.

Read over blind at. OD_{595nm}

Plot standard curve – read unknowns.

Above protocol MODIFIED FOR SPECTRA MAX 190 96-well plate format

1. Bio-Rad BSA Standarts set : 0.125mg/ml, 0.25mg/ml, .5mg/ml, 0.75mg/ml, 1.mg/ml, 1.5mg/ml, 2.0mg/ml, Stocks stored at –20c

2. Make Rx (96 well plate): Sample (ul?) or Standard (5ul) or Blank, bring to 150ul with dH₂O + 150 ul and add Quick Start Bradford Dye Reagent, 1X (making a total of 300ul)
Read at 595nm within 30min. Use a microplate reader.

M6: Intracellular Flow Cytometry Staining Protocol

Materials

Staining Buffer
1x PBS
1% FBS or BSA
0.1% NaN₃

Permeabilization Buffer (Make fresh each time)
Staining Buffer
0.5% saponin

GolgiPlug
Pharmingen Cat.# 51-2301KZ (use at 1:1000), or substitute 10 ug/mL Brefeldin A
(You may have to use GolgiStop [Monensin] for some cells/activations)

Procedure:

1. Add GolgiPlug (1:1000) to cells and culture for 3-5 hours longer at 37° C.
2. Harvest cells from flasks, samples, or homogenized tissues.
3. Count cells and spin down in 15 mL or conical tubes.
4. Resuspend to 10⁶ to 10⁷ per mL in staining buffer+GolgiPlug (1:1000).
5. Aliquot 100 uL/well into 96 well plate wells, as many wells as you need per sample.
6. Spin down in plate at 300 x g (rcf), 5 min, 4° C (all spins from here on are in these conditions.), flick plate out and blot on paper towel stack.
7. Stain for surface markers first (steps 7-11), then intracellular antigens next. If you do not wish to stain for any surface markers, proceed directly to step 12.
8. ****Skip step 8 if you are not staining cells with strong Fc receptor binding.****
9. Fc receptor blocking:
10. Dilute FcBlock antibody 0.25 ug/10⁶ cells, 50 uL/well in staining buffer+GolgiPlug
11. Aliquot FcBlock antibody to all wells and resuspend using a multichannel pipettor.

12. Incubate plate on ice for 5 minutes, while diluting staining antibodies
13. Dilute antibodies in 1.5 mL or 0.6 mL eppendorf tubes, enough for 50 or 100 uL per sample in staining buffer + GolgiPlug (1:1000). (keep Abs in dark until use) Follow concentrations recommended by manufacturer, usually 0.5-1 ug per 10⁶ cells.
14. Aliquot each antibody to appropriate wells, 50 or 100 uL/well. If using FcBlock antibody steps, aliquot 50 uL regular antibodies right on top of the 50 uL FcBlock antibodies already present.
15. Incubate on ice for 30 minutes in the dark.
16. Wash 3x with staining buffer+GolgiPlug (1:1000), 200 uL/well.
17. ***Intracellular Steps***
18. Resuspend in 100 uL staining buffer+Golgi Plug (1:1000).
19. Add 100 uL 4% paraformaldehyde (in fume hood) for a final concentration of 2% paraformaldehyde.
20. Incubate for 15-30 min. at room temp, in the dark to fix cells.
21. Spin down and wash once more with regular staining buffer
22. Wash once with 200 uL/well permeabilization buffer.
23. Dilute antibodies in permeabilization buffer and aliquot to well, 50 or 100 uL per well. Use identical concentrations as you would for surface staining. Also, try to use directly conjugated antibodies for intracellular staining.
24. Incubate on ice in the dark for 30 min.
25. Wash 3x in permeabilization buffer, 100 uL/well.
26. Wash 2x in regular staining buffer.
27. Arrange small cluster tubes in the 96 well-type rack or flow tubes in a test tube rack. Pre-aliquot 100 uL regular staining buffer into cluster tubes or 300 uL into flow tubes. Resuspend stained cells in 200 uL regular staining buffer and transfer to cluster tubes for flow analysis.

M7: Pancreas Digestion Protocol

1. Remove pancreas from each mouse, cut into at least 3 pieces, and drop into 15 mL conical tubes containing 1-2 mL cold Solution A. After removing all pancreata, move to step 2.

2. (Optional: Quick-spin pancreas pieces before aspirating Solution A.) Carefully aspirate Solution A from each tube, taking care not to remove any pancreas tissue.
3. Add 1.0 mL Solution B per tube and place in 37^o C water bath. Swirl often for 20-40 min. (Stop when tissue appears almost as a single-cell suspension.)
4. Add 5 mL cold Solution C to each tube.
5. Homogenize in glass homogenizer 2 times.
6. Resuspend well, flash-spin, and transfer to a new 15 mL conical tube.
7. Spin down at 300 x g for 10 min.
8. Resuspend in 1.0 mL Solution C.
9. Aliquot into 2 wells of a 24-well plate, 500 uL per well. Add 500 uL 2 x PMA/Ionomycin (stimulated) in Solution C or Solution C alone (unstimulated).
10. Culture for 3 hours at 37^o C, 10% CO₂.
11. Remove supernatant to 1.5 mL eppendorf tubes, wash once with 200 uL PBS/GolgiPlug, and add 200 uL Cell Dissociation Buffer (+GolgiPlug).
12. Incubate 5-10 min. at 37^o C.
13. Remove to eppendorf tube, wash once more with 200 uL PBS/GolgiPlug, transferring wash to eppendorf tube as well. Remove 50 uL for counting.
14. Spin eppendorf tubes at 300 x g 7-10 min.
15. Resuspend in staining buffer(+GolgiPlug) and proceed to intracellular staining.

Materials

Prepare and sterile filter:

Solution A:

1 x PBS
5% FBS
1% glucose (w:v)
1:1000 (v:v) Golgi Plug*

Solution B:

1 x PBS
15% FBS
5 mg/mL collagenase
mg/mL DNase (optional)

1:1000 (v:v) Golgi Plug*

Solution C:

CM

1:1000 (v:v) Golgi Plug*

Golgi Plug is necessary for intracellular staining only.

2x PMA/Ionomycin:

2 ug/mL PMA, 200 ng/mL Ionomycin (1 uL 10 mg/mL PMA, 10 uL 10 mg/mL Ionomycin)