Steps for preparing the TOXCAT DNA

I. Primer design

Example:

GTTGATTAGCGCGGATTCGTTACTCGAGGTCGAGGACTATGCCAAATCTATG
GTTTCTATTGTTCTTGGGACTAGTTATGATGCTGGTCGTGGAGGTGCTGCTGG
TGTTGGTGCTGGTGATGACGATGTACTGGTAGCATTTTGAGTGCTCCTGTACA
GGTCTGCCCTTTTAATGCCTTTACAGGATCCGGC

Complete 5-1 sequence
Sequence selected for TOXCAT
MPNLWFLLFLGLVMMLVVEVLLVLVLVMTMYW

In the 5' there will be a restriction site for NheI (forward primer):
Recognition Site:

5′...G▼CTAGC...3′
3′...CGATC▲G...5′

5′ GCCAAATCTATGGCTAGCTTGTTC 3′
…TCGAGGACTATGCCAAATCTATGGTTTCTATTGTTCTTGGGACTAGTTA…

In the 3' it will be a restriction site for BamHI (reverse primer):
Recognition Site:

5′...G▼GATCC...3′
3′...CCTAG▲G...5′

T M Y W
…ACGATGTACTGGCATTTTGAGTGCTCCTGTACAGGTCTGCCCTTTTA
3′ CATGACCGCCTAGGCTCACGAGGAC 5′
(↑I put here this C as insertion)

Thus this will be the primers ordered (from 5′ to 3′):

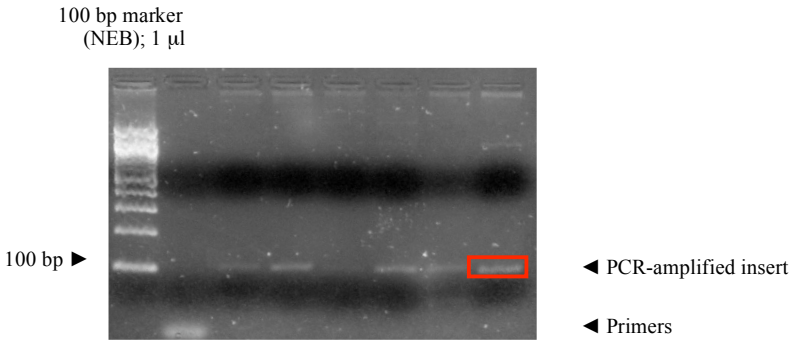
PDG2 FORWA Tm= 56.4
5′ GCCAAATCTATGGCTAGCTTGTTC **3′**

PDG3 REVER Tm= 56.4
5′ CAGGAGCACTGGGATCCGCCAGTAC **3′**

Note: The additional C nucleotide inserted at the BamHI restriction site must be included to respect the reading frame.

II. PCR

The aim is to amplify the TM sequence of interest and include the BamHI and NheI restriction sites. Prepare 100 µl reactions. Typical results are shown below. The intensity of the PCR result (red square) should not be lower than that showed in the 2% gel below (5 µl of sample loaded):



III. Gel-purify the band

Load all the sample into a 1.5 % agarose gel with big wells, cut the band using a razor blade, and purify the ADN with, for example, Qiagen QIAEX II or QIAquick gel extraction kits.

IV. NheI and BamHI digestion of the PCR-amplified and vector

Perform preferentially a sequential digestion. Here is detailed the protocol for the NEB restriction enzymes for cutting high amounts of DNA (like pcckan vector). For PCRs half of the restriction enzymes or less would be sufficient. Cut separately vector and insert.

NheI reaction (37 ºC, 2.5 h):

Sample	Volume (µl)
Gel-purified DNA	14
BSA 50x	0.5
Buffer 2	2.5
NheI (10.000 units/ml)	5
Water	3

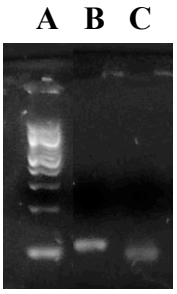
BamHI reaction (37 ºC, 1.2 h):

Sample	Volume (µl)
NheI reaction	25
BSA 50x	0.2
Buffer 2	1
Buffer TNa (see below)	2.5
BamHI (20.000 units/ml)	4
Water	2.3

Buffer TNa: Tris 400 mM, NaCl 504 mM, pH 7.9

This is an example of what to expect for the digestion of the:

i) PCR-amplified insert in 3.5% Supra Sieve agarose gel:

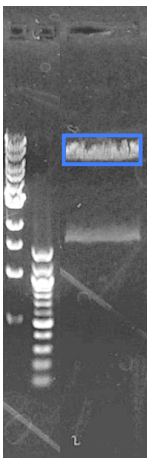


A= 1 µl NEB 100 bp ladder

B= 1 µl PCR-amplified insert before digestion

C= 4 µl PCR-amplified insert after digestion

ii) pcckan vector (ask for it to Miriam) in 0.8% agarose gel:



A= 1 µl NEB 1000 bp ladder

B= 1 µl NEB 100 bp ladder

C= Digested pcckan vector (now called pcc !!).

Repeat step III for the two lanes C of i) and ii). For pcc vector, cut the blue-boxed band. Load 4 μl of each sample into an appropriate agarose gel, and quantify using as a reference NEB ladders.

V. Ligation

Now we will ligate the two digested fragments of DNA (insert+vector). For NEB ligase, react in a volume of 20 μl, with 0.5 μl of ligase and 2 μl of 10x buffer. The ideal is to work with three different vector:insert ratios (1:5, 1:10 and 1:20), and 100 ng of vector. For calculating the ratio:

$$100 \text{ ng}/7326 \text{ bp} = x /90 \text{ bp}$$

7326 is the length of the digested vector (pcc). Adjust insert length according to sample. Ligate ON at 16 C.

VI. Transformation in DH5α

Transform in DH5α cells following manufacturer protocol. Add 5 μl of ligation reaction. Centrifuge at 3.000 rpm the whole volume of the transformation, resuspend in 100 μl of medium, and plate in LB+Amp plates. Make a Miniprep out of the colonies to get the DNA.

VII. Transformation in NT326 cells

Using the DNA of the Miniprep, transform by electroporation in NT326 cells. These cells will be used for the different TOXCAT assays.

Electroporation conditions (machine in Steitz lab): 25 mFD, 200 W, and 1.8 kV. The time constant (tau value) should be 3-4 msec

Transformation efficiency usually is very high. So, plating 20 μl of the culture yields a high number of colonies.

Steps for performing the TOXCAT assay

I. Disk diffusion assay

-Prepare 2 or 3 LB+Amp plates per sample (including controls of GpA wt and G83I).

-Add 60 µl of cloramphenicol 90 mgr/ml to a piece of filter paper (Whatman 1030024, 3 mm, 2.4 cm diameter). Add one filter paper disk to the center of each plate. Incubate plates up (not upside down) either 6 hours 37 ºC, or ON at 4 ºC. Put smooth side of the filter paper down.

- Remove the paper disks, and add 100 µl of Abs=0.6 cultures of each of the NT326 transformed with the different DNAs to each plate, and plate using a glass bead.

II. Western Blot

Now we must check that all the proteins are expressed to the same level. We will then grow cell cultures up to a D.O. of 0.6. We will make stocks of these cells, in 100 or 200 µl stocks. We will centrifuge one tube per sample (1 min, 14.000 rpm), and resuspend the sample in 30 µl concentrated sample buffer. We heat 10 min at 95 ºC, and resuspend thoroughly with the pipette tip. We load 5 µl into a SDS-PAGE gel, and make a western blot:
-Run a gel. For NuPage, run at 200 V until necessary.

-Prepare the PVDF membrane. Cut into the desired size, wash 30 s with ethanol, wash briefly with water, and incubate several minutes in transfer buffer (prepared from the 10x stock, with 20% methanol).

-Transfer at 30 V for 1 hour.

-Block 30 min. with 5% milk in TBST (all steps at RT unless otherwise indicated).

-Incubate with the primary antibody (Mouse anti-MBP, Zymed laboratories). Add 15 µl of antibody to 8 ml of TBST with 5% milk. 1 h at RT of ON at 4 °C. This antibody can be re-used once.

-Wash thrice with 5% milk in TBST. 10 min each wash. -Reblock with 10% milk in TBST (10 min).

-Incubate 30 min with the secondary antibody (anti-Mouse IgG, Promega). Add 1.5 µl to 10 ml of TBST with 2.5% milk.

-Wash thrice with in TBST (no milk). 5 min each wash.

-Stain with the BCIP/NBT kit. Bands should appear in 3-30 min.

-Stop the reaction with PBS+ 2 mM EDTA.

III. CAT assay

-Carefully prepare stocks of cultures of NT326 cells transformed with the different TOXCAT plasmids (store at -80 ºC). Vol=100 µl. Abs 600nm=0.65

-Centrifuge cultures for 5 min at 14.000 r.p.m. (table centrifuge) and remove carefully the supernatant.

-Add to the pellets 0.5 ml of Tris 0.1 M pH 8 (vortex), 20 µl of lysis buffer freshly prepared or thawed (0.1 M EDTA, 0.1 M DTT, Tris 0.1 M pH 8) and one drop of toluene (vortex). Incubate 30 min at 30 ºC.

-Prepare Standard Curve:

Use CAT stock solution (0.002U/µl), stored in the fridge of hot room.

CAT units	µl CAT stock	µl Tris 0.1 M, pH 8
0	0	40
0.005	2.5	37.5
0.01	5	35
0.02	10	30
0.03	15	25

The standard curve results must be linear, indicating proportionality between CAT concentration and signal recorded.

- Prepare cold CAM solution. Cold CAM is prepared by mixing 6.5 µl of CAM 6.5 mgr/ml (freezer) and 43.5 µl of Tris 0.1 M.

- Prepare Master Mix. For each sample: (prepare according to the number of samples)

1.0 µl	cold CAM
1.0 µl	^{3}H-CAM (400 mCi/mmol)
1.0 µl	n-Butyryl CoA (5 mg/ml)
17.0 µl	0.1 M Tris pH 8

-Add 20 µl of Master Mix to 40 µl of CAT solution (Standard Curve) or cell lysates.

-Reaction protocol:

1) Incubate premixed samples for 90 minutes at 37 ºC in heat block of hot room
2) Terminate the reaction adding 300 µl of xylene. Vortex 30 seconds.
3) Spin for 3 minutes (14.000 r.p.m.) and carefully collect 220 µl of upper phase.
4) Add 300 µl of Tris 0.1 M pH 8, vortex 30 sec and spin.
5) Collect 160 µl of the upper phase.
6) Repeat step 4.
7) Collect 100 µl of upper phase, and add 1 ml of scintillation liquid
8) Measure 3H in coulter (Program 13 in Room 420 Scintillation Analyzer)

REMOVE ALL RADIOACTIVE MATERIAL TO THE HOT ROOM AND COMPLETE WIPE TEST.