Standard Operating Procedure
for
Hazardous Chemicals

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Building and rooms: Davison Life Sciences Building, Lab B310

Chemical(s)	Isopropanol, chloroform, ethanol, liquid nitrogen (LN), SDS, ethylenediamine tetraacetic acid (EDTA), isoamyl alcohol (IAA), ltris(hydroxymethyl)aminomethane (Tris-base), sodium hydroxide (NaOH), potassium acetate.
Process	**TA cloning**
Specific Hazards *referred to MSDSs for more detailed information*	Chloroform is a probable human carcinogen, reproductive hazard and skin/eye irritant. IAA is flammable, and may be harmful upon skin/eye contact, ingestion or inhalation. LN is a frostbite hazard. NaOH is a corrosive base, may cause burns to skin and eyes upon contact and to mucous membranes if inhaled or ingested.
Personal protective equipment	Must wear 3-5 mil nitrile gloves. Chemical safety goggles and lab coat should be worn when splash potential exist
Engineering/ventilation controls	All operations involving chloroform, and IAA must be done in a chemical fume hood.
Special handling procedures and storage requirements	Store chloroform, IAA, and alcohol in the flammable cabinets under the hood. Store all the reagents on bench top, 4°C or -20°C accordingly.
Spill and accident procedures *for hazardous chemicals only*	Skin exposure: Rinse affected skin with plenty of water while removing contaminated clothing/shoes. Rinse for > 15 minutes. Eye exposure: Wash eyes for > 15 minutes. For both cases, seek medical attention immediately.
	Small (< 2L): Absorb with vermiculite or spill pads and transfer absorbed material to a closed container. Label and date as hazardous waste for disposal. Notify PIs.
	Large (> 2L): Evacuate the room, notify PIs and call 2-5801 to request emergency spill assistance from the Environmental Safety Division.
Waste disposal	Chloroform, IAA waste must be collected and labeled as hazardous waste according to the SOP for Hazardous Waste Disposal.
Special approval	No special authorization needed after SOP training & reading MSDSs.
Prepared by	Name/date: Kate Tay, 9/8/2010
Reviewed by	Name/date: C-J Tsai, 9/9/2010

TA Cloning

TA ligation (Invitrogen 461370, Dual Promoter TA Cloning Kit) or home-made pCX T-vectors
Ligation should be done **right after** PCR reaction. The A-overhangs on the PCR products are unstable overtime.

Half-reaction	needed	final	
10x Ligation buffer[1]	1 µl	1 x	Mix, short spin
10mM ATP	0.5µl	0.5 mM	14°C, O/N
pCRII or pCX vector	0.5µl	~10 ng	Stored at -20°C
Insert (fresh PCR product)[2]	1 µl	see below	(can be re-used if needed,
Ligase	0.5µl	2 units	label clearly w/ vector + insert and date)
ddH_2O	6.5µl		
	10.0µl		OK to use NEB ligase as well

[1] The 10X ligase buffer precipitates after thawing on ice. Vortex vigorously and spin down before use. NEVER thaw the ligase buffer at room temp.

[2] Shoot for a molar ratio of insert : vector > 3:1 ([insert length bp]/[vector length bp]x10 ng x3). For good PCR products, 1 µl is usually sufficient. Too much DNA will reduce transformation efficiency.

Prepared LB+Kan plate

	Final	1L
LB (low salt) Powder	--	20 g
Agar	15%	15 g
[100mg/ml] Kan	100 mg/L	1 ml
[100mg/ml] Amp	100 mg/L	1 ml

Autoclave & cool to touch
\+ Antibiotic & pour

25 ml per plate;
40 plates per liter

Transformation

1. Turn on water bath to 42°C (or warm a beaker of water to 42°C by microwave)

2. Ligation mix 2µl
   Top10 C.C. (purple)* 15µl
   Tap only once to mix GENTLY!
   Do Not pipette up & down!
   On ice, 30 min

3. Heat Shock: 42°C, 30sec (Do Not mix or shake!) & chill on ice
4. + 500 µl SOC/LB (in laminar flow)
5. Shake, 37°C, 1 hr, 225 rpm (Warm up LB + Kan plate at 37°C, 10min)
6. Take 200 µl of the transformant mix
   → pCRII spread 200 µl is more than enough
   → pCXSN series need to spread more, ~300 µl

   and + 20 µl [40 mg/ml] X-gal (light sensitive)
   + 20 µl [10 mM] IPTG

   Double check!
   TOP 10 (general use) requires only X-gal
   TOP 10F' (methylated/toxic genes) requires both X-gal & IPTG
   pCX series does not require any

5. Spread all on plate & dry
6. Incubate @ 37°C, O/N

* Compentent cells are expensive. They should be kept cold at all time and thaw only on ice.
* Each vial has 50 µl and can be used for up to 3 reactions (at least 15 µl each).
* For multiple transformations, add 2 µl ligation reaction to a fresh tube on ice. Transfer 15 µl of cells to each tube, then add the 3rd ligation to the remaining cells in the original CC tube.
* If you have only 1 transformation to perform, use the whole vial or wait until you have more.
* Re-frozen competent cells have drastically reduced transformation efficiency.

Colony PCR

For blue/white selection, incubate the plate at 4°C for > 1 hr

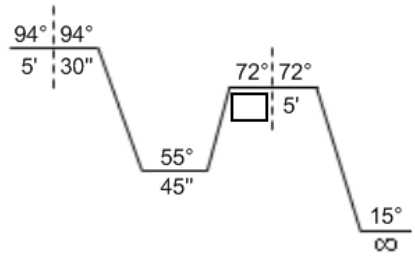
		20 colonies
Colony Lysis buffer	**stock**	10ml
1% Triton X-100	100%	100 µl
20 mM Tris, pH8.5	500 mM	400 µl
2 mM EDTA	100 mM	200 µl
s/w		9.3 ml

1. Aliquot 50 µl of lysis buffer into each tube.
2. Number the (white) colonies. Pick a part of the colony with a pipet tip and mix with lysis buffer by pipetting up and down several times. Re-incubate the plate at 37°C for a few hrs.
3. 95°C, 10 min
4. Centrifuge: 2 min, RT, max. speed
5. Transfer supernatant to new tube (optional)
6. Take 1 µl lysate for colony PCR. Stored the rest at -20°C

7. Prepare **master mix** [# of reactions plus ~10% (or one reaction) extra) for PCR:

	Lysate	1 µl
10x	REDTaq buffer	1 µl
10mM	dNTP	0.25 µl
5µM	forward primer*	0.5 µl
5µM	reverse primer *	0.5 µl
0.5 U	REDTaq	0.5 µl
1.5mM	$MgCl_2$	3 µl
	s/w	3.25 µl
		10 µl

35x



* Use both gene-specific F + vector F AND gene-specific F + vector R to determine the insert orientation in the vector (expect one positive and one negative reaction).

8. Run 5µl on a gel.

For PCR-positive colonies:

Pick colonies (choose 4)

1. Prepare 5 ml LB liq + 5 µl [100 mg/ml] Kan in culture tubes (final [Kan] = 100 mg/L)
2. Wipe the pipet shaft with 70% EtOH. Pick the desired colony with a pipet tip and eject the tip into the LB media
3. Shake, 37°C, O/N, 225 rpm (8-16 hrs, the shorter the better, no more than 16 hrs!)
4. Use immediately or store at 4°C overnight for miniprep (or up to 2 weeks max for subculturing).

Glycerol Stock preparation:

1. Take 0.5 ml cultures for glycerol stock in a 2-ml cryo-tube
   + 0.5 ml LB (no antibiotic) w/ 80% glycerol (1:1 ratio)
2. Snap-freeze in LN before storing at -80°C.

Miniprep by alkaline lysis

GTE buffer (50 mM glucose, 25 mM Tris-HCl pH8, 10 mM EDTA pH8)	Sol. 1 → on ice from 4°C
0.2M NaOH/1%SDS (make **fresh weekly** w/ 10M NaOH and 20% SDS)	Sol. 2 → RT
5 M potassium acetate (pH4.8)	Sol. 3 → on ice from 4°C
RNase A (DNase-free), 10 mg/ml	
Chloroform:IAA = 24:1	
Isopropanol	

1. Transfer 1.5 ml cultures to a new 1.7 ml tube, centrifuge at RT, max. speed, 45 sec.
   Discard supernatant directly. Repeat for up to 2 more times (in the same tube). Unused bacterial waste needs to be autoclaved.

2. Resuspend the pellet in 200µl of **cold** GTE (1) by pipetting up/down.
   Disturb the pellet to ensure **complete suspension**! (Turns turbid).

3. Denature with 400µl of 0.2N NaOH/1% SDS (2), **gently** invert 5 times (become clear). NO incubation.

4. Neutralize with 300µl of **cold** 5M KOAc (3), **gently** invert (white protein precipitate form). NO incubation.

5. Centrifuge, RT, max, 10 min. Transfer supernatant (900µl clear, only DNA/RNA) to new 1.5ml.

6. Add 1.8 µl RNase A (invert gently once) to 20 µg/ml. Incubate at 37°C, 20-40 min.

7. Extract with 400 µl chloroform:IAA, Vortex 5 sec. Centrifuge: RT, max, 1 min.
   Transfer top phase to new 1.5 ml (4x175 µl, tilt the tube to form a ball).
   DO NOT touch the interphase or the bottom layer! Leave behind some top phase (quality is more important – you will get more than you need)

8. (Repeat once more #7). Chloroform waste goes into a specifically labeled waste jar.

9. Precipitate DNA with 1 Vol (700 µl) isopropanol, invert 5 times, and **immediately** centrifuge, RT, max, 10 min. Discard supernatant. EXTREMELY CAREFUL!

10. Wash the white pellet with 500 µl 70% EtOH; Centrifuge: 5 min, max, RT
    Discard supernatant. EXTREMELY CAREFUL!

11. Vacuum dry: 7-10 min, 35°C, 400-500 mmbar. Make sure no residual EtOH remains. Over-dried pellet may be difficult to resuspend (and difficult to "see").

12. Samples on ice! Completely dissolve the pellet in 30 µl ddH_2O, vortex 2 sec, short spin 2 sec.

13. Nanodrop and check 200 ng on a gel. Over-loading will affect QC assessment.

14. For sequencing purpose, dilute a small aliqout to 200 or 500 ng/µl (depending on plasmid size, refer to sequencing SOP). Store at -20°C (concentrated form is desired for long-term storage).

* Stored at -20°C
* Definitely No PEG! Minor RNA contamination is ok, or go back to #6 (Repeat RNase A)

For 2 samples/cultures:

50 µl	20% SDS
20 µl	10M NaOH
930 µl	s/w