Whole Embryo Culture, E6-E9
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Components required for media:

A. Raw materials

Rat serum (Valley Biomedical "Rat Serum Special Collection", must be ordered as product "AS3061-SC" and specify 500mL quantity, also indicate in notes to dispense in 125mL aliquots; it is an off-menu item so need to use special order form in Gladstone Market, enter price estimate as $1100 for the 500mL quantity) – once it arrives, keep at -80C
Fetal bovine serum, cell culture grade
DMEM/F-12 (ThermoFisher 11039021)
GlutaMAX (ThermoFisher 35050061)
ITS-X (ThermoFisher 51500056)
Penicillin/Streptomycin (ThermoFisher 15070063)
DMSO, cell culture grade
b-estradiol (Sigma E8875) – stored at RT
Progesterone (Sigma P3972) – stored at 4C
N-acetyl cysteine (Sigma A7250) – stored at 4C
4-hydroxytamoxifen (Biogems 6833585, 10mg) – stored at -20C (if using Cre/Dre-ERT2)
PBS

B. APE preparation at 2000X:

APE simulates the in-utero hormonal environment, and provides protection from oxidative damage when embryos are grown in atmospheric oxygen.

1. First, make three individual reagent stocks in cell-culture grade DMSO:
    NAC 4348X: dilute 26.61mg in 1.5mL DMSO
    progesterone 4000X: dilute 3mg in 2.4mL DMSO
    estradiol 50000X: dilute 3.27mg in 30mL DMSO
2 Next, make 3mL APE 2000X mix by adding the components together:
    NAC 4348X: 1380uL
    progesterone 4000X: 1500uL
    estradiol 50000X: 120uL
3. If possible, sterile filter (with PTFE or Nylon; do NOT use PVDF or cellulose acetate), aliquot to 30uL and store at -80C for up to 1-2 years

C. FBS Preparation:

Divide 1L FBS into 50mL aliquots and freeze at -20C.
When ready to use a new 50mL aliquot, thaw first at 37C, then heat-inactivate at 56C for 30 minutes. Divide heat-inactivated FBS into 5mL aliquots, and freeze at -20C.

D. Rat serum preparation:

When ready to use a 125mL aliquot, thaw first at 37C, then heat-inactivate at 56C for 30 minutes. Cool to RT and sterile filter the rat serum with 220nm filter (to prevent clogging may have to pass through 450nm filter first, or use multiple 220nm filters in parallel). Divide filtered, heat-inactivated rat serum into 15mL aliquots in 50mL conical vials, and freeze at -20C.

E. 4OH-Tamoxifen preparation (for Cre/Dre-ERT2 induction):
Dilute 10mg 4OHT in 2mL 100% etOH to make 5mg/mL stock (12.9mM), and store at -20C. From that stock, remove 30uL to fresh sterile tube and add to 357uL DMSO to make a 1mM stock, then aliquot into PCR tube strips, 5uL per tube. These aliquots are also stored at -20C.

Media Preparation:

A. Dissection Medium (makes 50mL initially):

DMEM/F12 (+/-HEPES, no phenol red)	43.5mL
heat-inactivated FBS (see above)	5mL
pen/strep at 100X	500uL
ITS-X at 100X	500uL
GlutaMAX at 100X	500uL
APE preparation at 2000X (see above)	25uL

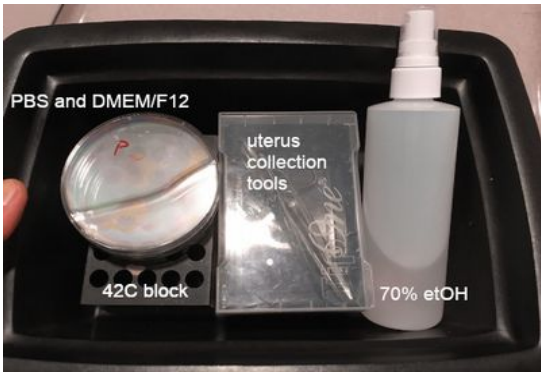

Figure 1

B-1. Culture Medium E7.5+ (makes 50mL):

Dissection Medium (see above)	35mL
heat-inactivated rat serum (see above)	15mL
--OR--	
DMEM/F12 (+/-HEPES, no phenol red)	30.5mL
heat-inactivated rat serum (see above)	15mL
heat-inactivated FBS (see above)	3.5mL
pen/strep at 100X	350uL
ITS-X at 100X	350uL
GlutaMAX at 100X	350uL
APE preparation at 2000X (see above)	18uL

B-2. Culture Medium E6.5+ (makes 36mL):

DMEM/F12 (+/-HEPES, no phenol red)	15mL
heat-inactivated FBS (see above)	5mL
heat-inactivated rat serum (see above)	15mL
pen/strep at 100X	350uL
ITS-X at 100X	350uL
GlutaMAX at 100X	350uL
APE preparation at 2000X (see above)	15uL

Embryo Dissection and Culture Initiation:

1. warm culture medium, dissection medium, DMEM/F-12, and PBS in 37C dry bath
2. clean / sterilize laboratory bench with ethanol
3. load a P20 (E6.5-E7.5) or P200 (E7.5+) with a low-retention wide-orifice tip, or can sterilize a razor blade and use it to cut off a pipette tip for transferring embryos
4. set one benchtop removable thermal block to 42C (two if doing a live imaging session)
5. set up dissection dishes:
    a. add 12mL DMEM/F12 with HEPES (no phenol red) to 10cm petri dish; warm in incubator
    b. add 12mL PBS to another 10cm petri dish; warm in incubator

c. fill 6cm round bottom petri dishes (2 per pregnant dam expected) for dissection with 6mL dissection medium each, place in incubator
d. fill 6cm center well dishes (1 per expected dam) with 1.5mL culture medium, place in incubator
e. fill the outer torus "moat" of additional 6cm center well dishes (1-2 per expected dam) with 3mL culture medium, place in incubator – this is for long-term culture

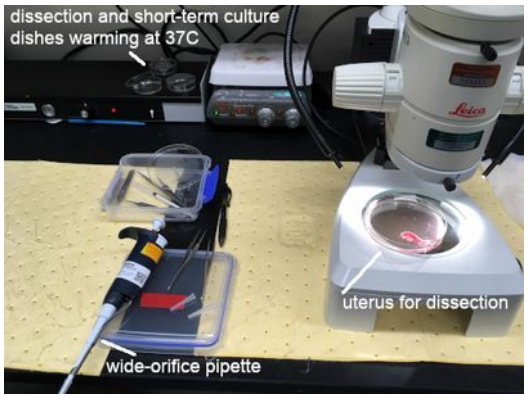

Figure 2

6. turn on fluorescent screening light source on screening microscope
7. turn on slide warmers to 37-40C
8. go to animal facility with clean tub containing 42C heat block, PBS and DMEM/F12 dishes from incubator (place on heat block), ethanol spraybottle, and scissors/forceps for dissection (Figure 1)
9. euthanize and dissect out uterus from dam; transfer uterus to PBS first and shake gently to wash, then transfer it to DMEM/F-12 on 42C pre-heated block; return to lab with uterus
10. dissect embryos:
    a. bring 6cm dissection dishes and one center well dish per dam to dissection room, and place on slide warmer at 37C
    b. place 10cm dish with uterus on slide warmer also
    c. remove gestational sacs from uterus one at a time, and use the forceps to quickly transfer each one to the two 6cm dishes (half of litter into each) with dissection medium (Figure 2)
    d. microdissect embryos (including removing Reichert's membrane) in 6cm dish, while maintaining 37C as best as possible i.e. quickly (Figure 3)

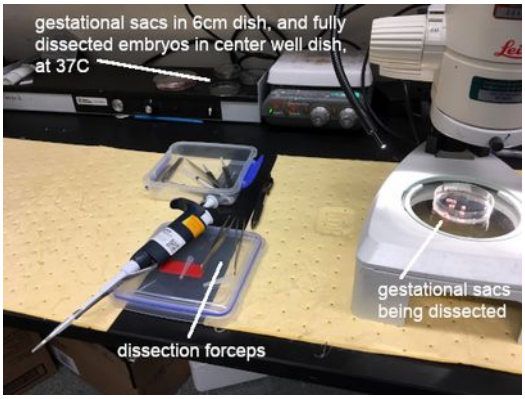

Figure 3

    e. transfer dissected embryos into center well dish on slide warmer (use P20 or P200 with low-retention wide-orifice tips) to maintain at 37C
11. screen embryos with fluorescence microscope if desired, and transfer positive embryos to the "moat" portion of fresh 37C center well dishes prepared for long term culture
12. if desired, before placing in long term culture, can incubate with 4OHT for Cre/Dre recombination; typical use at 2uM (1:500 of 1mM stock) for 2hr, then washes once with culture medium and return to culture with no 4OHT
13. place in incubator at 37C and 5% CO2, on an orbital shaker platform at 50-70rpm (Figure 4) – embryos will go around the moat like a lazy river
14. change culture medium every day or every other day, can use a 25mL serological pipette to transfer embryos as needed

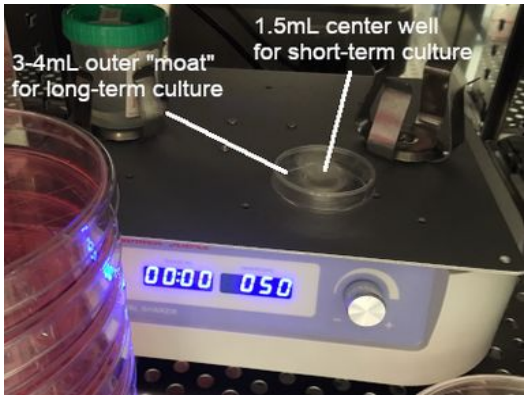

Figure 4