CULTREX® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

Cultrex® Embryoid Body Formation Kit

Reagent kit for formation of embryoid bodies by hES cells and iPS cells

96 samples

Catalog # 3550-096-K

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I. Quick Reference Procedure for Cultrex® Embryoid Body Formation Kit:

Read through the complete Instructions For Use prior to using this kit. Determine the optimal seeding density for each cell line used. In general, 3,000 cells per well is a good starting point.

1. Culture undifferentiated human ES or iPS cells under feeder cell free conditions using Cultrex® Stem Cell Qualified RGF BME (catalog# 3434-005-02).
2. Thaw 10X EB Formation ECM for two hours or overnight in a 4°C refrigerator and keep it on ice.
3. Thaw 500X ROCK Inhibitor Y-27632 at room temperature and then keep it on ice.
4. Harvest cells (section VII.B) and prepare single cell suspension in DMEM containing 10% FBS, 1X EB Formation Matrix and 1X ROCK inhibitor Y-27632 (section VI).
5. Aliquot 100 µl of cell suspension per well of the 96-well EB Formation Plate.
6. Centrifuge the plate at 200 x *g* for 3 minutes at room temperature in a swinging bucket rotor.
7. Incubate the plate at 37 °C, 5% CO2 in a tissue culture incubator for 24-48 hours to compete formation of embryoid bodies (EBs).
8. Collect EBs and/or continue with differentiation protocol of your choice.

II. Background

Cultrex® Embryoid Body Formation Kit offers a useful tool for generating consistent, reproducible and identical in size embryoid bodies (EBs). The kit has been qualified to form EBs from human pluripotent stem cells (hESCs and iPSCs).

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have the unique ability to differentiate into cells of three embryonic germ layers (endoderm, ectoderm and mesoderm)[1, 2]. The majority of differentiation protocols start with the generation of embryoid bodies (EBs) – three-dimensional multicellular aggregates of pluripotent stem cells. Although pluripotent stem cells placed in suspension culture will form EBs spontaneously[3], the efficiency of this process varies and is not reproducible. Most current methods avoid preparing single cell suspension, due to low survival of pluripotent stem cells in single cell culture[1, 4, 5], and require keeping cells in large clumps, thus generating EBs of different size and shapes. Size of EBs is one of the key factors effecting differentiation of PSCs and needs to be controlled[6, 7]. To solve these problems and help to improve reproducibility of

differentiation procedures, Trevigen developed the Cultrex® Embryoid Body Formation Kit. It contains ROCK Inhibitor Y-27632, which significantly increases survival of PSCs in single cell culture[8]; specialized EB Formation Matrix to stimulate cell aggregation and development of EBs[9]; and a 96 Well EB Formation Plate that allows to control the size of EBs and create a single EB in each well.

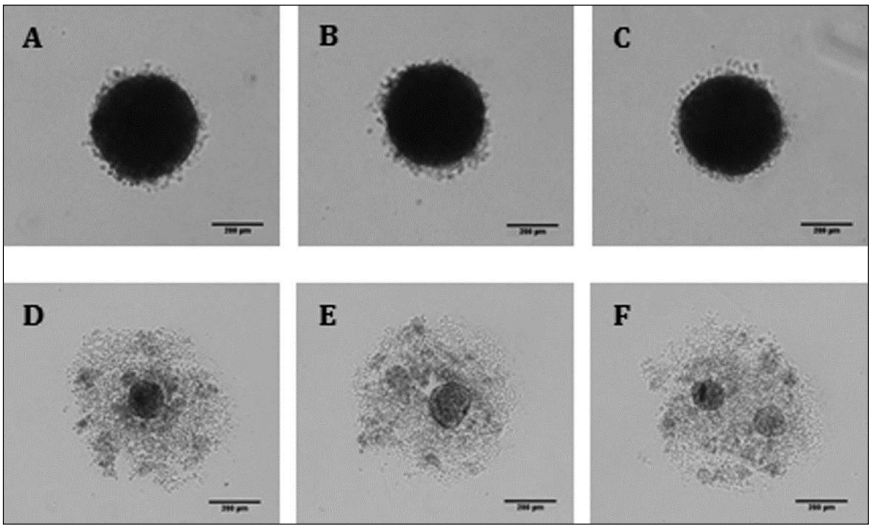

Figure 1. Cultrex® Embryoid Body Formation Kit helps to generate identical in size EBs.
A, B, C - EBs formed from human iPSCs using Cultrex® Embryoid Body Formation Kit; shown EBs generated with 3,000 cells per well after 48 hours.
D, E, F - EBs formed from human iPSCs using conventional method; 3,000 cells per well after 48 hours.
Each image (A-F) represents individual well of 96-well plate.

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of these products may not yet have been fully investigated; therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.
3. The **CULTREX® Embryoid Body Formation Kit** contains reagents that may be harmful if swallowed, or come in contact with skin or eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Material safety data sheets are available on request.

IV. Materials Supplied

Component	Quantity	Storage	Catalog #
10X EB Formation Matrix	1.1 ml	-80˚C	3550-096-01
500X ROCK Inhibitor Y-27632	35 µl	-80˚C	3550-096-02
96 Well EB Formation Plate	Each	RT	3550-096-03

V. Materials/Equipment Required But Not Supplied

Equipment

1. Laminar flow hood.
2. Cell culture incubator to maintain 5%CO_2 and 37˚C.
3. Low speed centrifuge (e.g. Eppendorf 5810R) with swinging bucket rotor and plate holders.
4. Pipette aid.
5. Multichannel micropipettor.
6. Micropipettors
7. Hemocytometer or automated cells counter (e.g. Bio-Rad TC10).
8. Microscope (e.g. ZEISS Invertoskop 40C) equipped with 4X and 10X objectives.

Reagents

1. Human pluripotent stem cells (hESCs or iPSCs).
2. DMEM/F12 (e.g. Life Technologies, catalog#10565018)
3. ACCUTASE® (e.g. STEMCELL Technologies, catalog# 07920).
4. DMEM (Dulbecco's Modified Eagle Medium) (e.g. Life Technologies, catalog#11965092).
5. FBS (Fetal bovine serum) (e.g. Atlas Biologicals, catalog# FP-0500-A).
6. Trypan Blue (e.g. Bio-Rad, catalog# 145-0013) or equivalent viability stain.

Disposables

1. 1 - 200 µl wide bore pipet tips (e.g. Genesee Scientific, catalog# 22-611).
2. 1 - 200 µl and 200 – 1000 µl pipet tips.
3. Sterile 25 ml reservoirs for multichannel micropipettor.
4. 15 ml and 50 ml conical tubes.
5. 1, 5, and 10 ml serological pipettes.

VI. Reagent Preparation

1. **10X EB Formation Matrix.**
   10X EB Formation Matrix should be thawed at 4 °C, kept on ice prior to use and diluted with ice-cold medium.
2. **500X ROCK Inhibitor Y-27632.**
   500X ROCK Inhibitor Y-27632 should be thawed at room temperature and then kept on ice prior to use.

VII. Assay Protocol

These procedures should be performed in a laminar flow hood utilizing aseptic technique to prevent contamination.

A. Maintenance of undifferentiated human ES cells or iPS cells in feeder-free culture.

Cultrex® Embryoid Body Formation Kit can be used to form EBs from undifferentiated human ES cells or iPS cells grown in feeder-free culture.

1. Pluripotent stem cells must be healthy and in an active proliferating phase prior to use in the assay. Do not use cells for EB formation, if there are more than 15% differentiated cells in the culture.
2. Maintain undifferentiated cells in StemPro Medium (Lite Technologies, catalog# A1000701), mTeSR™1 or mTeSR™2 (STEMCELL Technologies, catalog## 05850 and 05860, respectively), or other medium of your choice according to manufacturer's instructions.
3. Trevigen recommends using Cultrex® Stem Cell Qualified RGF BME (catalog# 3434-005-02) as a coating substrate for feeder-free stem cell culture.
4. Prior to starting the assay calculate the required number of cells to form EBs of preferred size as follows:
   Total number of cells = number of cells per EB X number of wells.
5. One 60 mm dish yields about 2 x 10^6 cells.

B. Harvesting undifferentiated cells and preparation of a single cell suspension.

6. Equilibrate DMEM/F12 and ACCUTASE® at room temperature for 30 min.
7. Aspirate the growth medium from cell culture dish and rinse the cells twice with 3 ml DMEM/F12 per 60 mm dish. Aspirate and discard DMEM/F12.
8. Add 1.5 ml ACCUTASE® per 60 mm dish and incubate at 37˚C, 5% CO_2 for 5 to 10 minutes until cells have dissociated from the plate.
9. Gently pipette the cell suspension a few times with 5 ml serological pipette to break up cell aggregates.
10. Transfer cells to a 15 ml conical tube and add 1.5 ml of DMEM-10%FBS.
11. Centrifuge cells at 200 x *g* for 3 minutes at room temperature.
12. Aspirate supernatant and gently resuspend cells in 1 ml of DMEM-10%FBS.
13. Count cells and evaluate cell viability by Trypan Blue exclusion or equivalent test. Do not use cells if their viability is less than 90%.
14. Dilute cells, if necessary with DMEM-10% FBS to prepare a single cell suspension at concentration 1 x 10^6 cells per 1 ml.

C. Formation of EBs.

15. Thaw 10X EB Formation Matrix at 4 °C and keep on ice prior to use.
16. Thaw 500X ROCK Inhibitor Y-27632 and keep on ice prior to use.

17. Add necessary amount of DMEM-10%FBS (see Table 1) into 15 ml conical tube and incubate it on ice for 5 – 10 min.
18. Add 10X EB Formation Matrix to ice cold DMEM-10% FBS and mix well.

NOTE: 10X EB Formation Matrix should be kept on ice and added to the ice-cold medium.

19. Warm up DMEM-10% FBS with 1X EB Formation Matrix at 37ºC and then add ROCK Inhibitor Y-27632 and cells (see Table 1).

Table 1: Amount of reagents required to generate EBs of 3,000 cell size.

Reagent	1 Well	96 Wells
DMEM-10%FBS	86.8 µl	9.55 ml
10X EB Formation Matrix (4°C)	10 µl	1.1 ml
500X ROCK Inhibitor Y-27632 (4°C)	0.2 µl	22 µl
Cell suspension at 1 x 10^6 cells/ml (step 14)	3 µl	330 µl
Total	**100 µl**	**11 ml***

***Extra 15% added to compensate for possible loss with the use of multichannel pipettor and sterile reservoirs.**

20. Cap the tube and gently invert it a few times to mix.
21. Using wide bore pipet tips, dispense 100 µl of the resulting single cell suspension per well of 96 Well EB Formation Plate.

NOTE: Use multichannel micropipettor, sterile 25 ml reservoirs and wide bore pipet tips to dispense single cell suspension in multiple wells or all 96 wells.

22. Centrifuge the plate at 200 x *g* for 3 minutes at room temperature in a swinging bucket rotor.
23. Incubate the plate at 37 °C, 5% CO_2 in a tissue culture incubator for 24-48 hours to promote EB formation.
24. EBs can be collected and transferred to gelatin-coated or tissue culture treated 96-well plate (not provided) using multichannel micropipettor and wide bore pipet tips.
25. Continue with differentiation protocol of your choice.

D. Differentiation of EBs in 96 Well EB Formation Plate.

EBs do not attach to EB Formation Plate, therefore care should be taken when changing medium in the wells with EBs.

26. After EBs are formed (see step 23), add 100 µl DMEM-10%FBS per well to the EB Formation Plate, so that the volume in each well will be 200 µl.
27. Incubate the plate at 37 °C, 5% CO_2 in a tissue culture incubator until next medium change.
28. Replace half of the medium in the wells with the fresh DMEM-10%FBS.

Carefully aspirate 100 µl of medium from each well and add 100 µl of fresh medium.

29. Repeat step 28 every 3 - 4 days for the period of 14 - 21 days, or until differentiation protocol is complete.

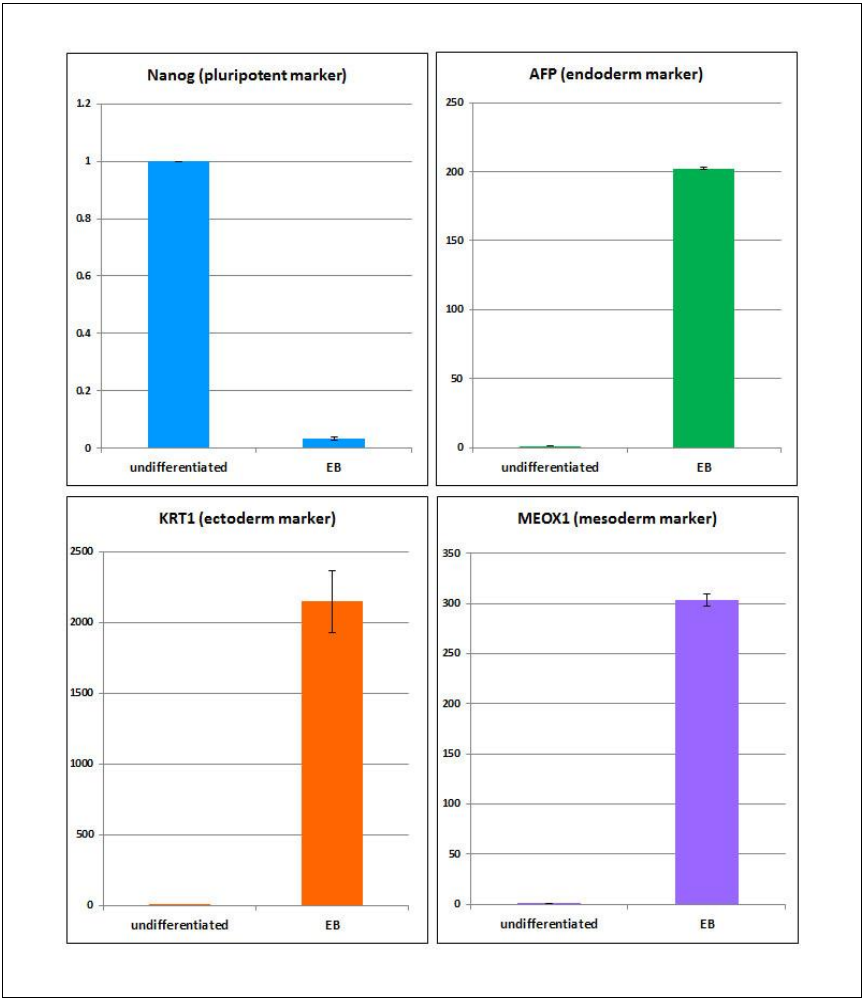

Figure 2. EBs generated from human iPS cells using Cultrex® Embryoid Body Formation Kit can be differentiated in three germ lineages.
EBs were formed from iPSCs using Cultrex® Embryoid Body Formation Kit and incubated for 21 days in DMEM-10%FBS in 96-well EB Formation Plate followed by quantitative RT-PCR analysis. Undifferentiated iPS cells were cultured in mTeSR1 medium on RGF BME-coated plate.

VIII. Troubleshooting

Troubleshooting Guide		
Problem	**Cause**	**Solution**
Cells did not form EBs.	Cells were not healthy.	Cells should be in an active proliferation phase.
		There should be less than 15% differentiated cells in cell culture.
		Cells viability should be more than 90%.
	Cells were stressed during harvesting and preparation of a single cell suspension (section VII B).	Do not incubate cells in ACCUTASE® longer than necessary to detach cells (monitor under microscope).
		Do not pipette cell suspension with excess force using narrow pipet tip or 2 ml serological pipette. Use 5 ml serological pipette.
		Add ROCK Inhibitor (Y-27632) to DMEM-10% FBS at 1X working concentration; it will increase survival of single cells. Use this medium in steps 10-14 (section VII B).

IX. References

1. Thomson, J.A. *et al*. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145-47.
2. Itskocitz-Eldor, J. *et al*. 2000. Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. *Mol. Med* 6: 88-95.
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4. Reubinoff, B.E. *et al.* 2000. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotechnol*. 18:399–404.
5. Pyle, A.D., Lock, L.F., and Donovan, P.J. 2006. Neurotrophins mediate human embryonic stem cell survival. *Nat. Biotechnol*. 24:344–350.
6. Peerani R., *et al*. 2007. Niche-mediated control of human embryonic stem cell self-renewal and differentiation. *EMBO J*. 26:4744 – 55.
7. Bauwens C.L., *et al*. 2008. Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. *Stem Cells*. 26(9):2300-10.
8. Watanabe K., *et al*. 2007. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol*. 25(6):681-6.
9. Goh S.K., Olsen P and Banerjee I. 2013. Extracellular matrix aggregates from differentiating embryoid bodies as a scaffold to support ESC proliferation and differentiation. *PLoS One*. 2013. 8(4):e61856.

X. Reagent and Buffer Composition.

1. 10X EB Formation Matrix.

Proprietary mixture of extracellular matrix proteins derived from murine EHS sarcoma cells optimized for embryoid bodies formation. It is provided in DMEM with 10 µg/ml gentamycin.
For optimal stability store at -80ºC. For short-term storage up to 3 months can be kept at -20ºC. Avoid multiple freeze-thaws.

2. 500X ROCK Inhibitor (Y-27632).

(R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide Dihydrochloride.
Molecular Formula: $C_{14}H_{21}N_3O \cdot 2HCl \cdot H_2O$.
500X ROCK Inhibitor provided as 5 mM stock solution in sterile H_2O.
Recommended working concentration is 10 µM.
Store at -80ºC.

3. 96 Well EB Formation Plate.

Clear, round bottom 96-well plate with low adhesion surface to promote embryoid body formation.

XI. Related Products:

Catalog#	Description	Size
3434-005-02	Cultrex® Stem Cell Qualified RGF BME, PathClear®	5 ml
3415-001-03	Cultrex® Stem Cell Qualified Human BME, PathClear®	1 mg
3400-010-03	Cultrex® Stem Cell Qualified Laminin I, PathClear®	1 mg
3420-001-03	Cultrex® Stem Cell Qualified Human Fibronectin, PathClear®	1 mg
3420-001-03	Cultrex® Stem Cell Qualified Human Vitronectin, PathClear®	200 µg
3432-005-01	Cultrex® Basement Membrane Extract, PathClear®	5 ml
3433-005-01	Cultrex® Reduced Growth Factor BME, PathClear®	5 ml
3532-005-02	Cultrex® Basement Membrane Extract, Type 2, PathClear®	5 ml
3533-005-02	Cultrex® RGF BME, Type 2, PathClear®	5 ml
3632-005-02	Cultrex® Basement Membrane Extract, Type 3, PathClear®	5 ml
3410-010-01	Cultrex® Mouse Collagen IV	1 mg
3440-100-01	Cultrex® Rat Collagen I	100 mg
3442-050-01	Cultrex® Bovine Collagen I	50 mg
3445-005-01	Cultrex® 3-D Culture Matrix™ BME, PathClear®	5 ml
3446-005-01	Cultrex® 3-D Culture Matrix™ Laminin I	5 ml
3447-020-01	Cultrex® 3-D Culture Matrix™ Collagen I	100 mg
3438-100-01	Cultrex® Poly-L-Lysine	100 ml
3439-100-01	Cultrex® Ploy-D-Lysine	100 ml

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

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