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Sample Testing Recommendations

CELLnTEC media have been developed to optimize the isolation and further growth of epithelial cells from human, mouse or rat tissue. We appreciate you conducting a side-by-side test with your current medium to evaluate CELLnTEC medium's performance in your laboratory.

Medium performance is known to be affected by many factors. Please consider the following when designing and conducting your evaluation experiment:

Seeding Density

- CELLnTEC's defined media encourage the growth of progenitor cells without the need for additional components such as feeder layers, conditioned medium, plate coating or other supplements (i.e. BPE). However at low seeding densities, such factors or treatments may be beneficial – please see the Resources Page on www.cellntec.com for details.

Supplementation

- If you are currently supplementing your medium with non-defined components (e.g. BPE), a specific cell population dependent on these components may have been selected. For a complete and accurate comparison, CELLnTEC media can be tested with and without the non-defined components.

Switching Media

- Cells grown in one medium become accustomed to the medium's component profile, especially if it contains FBS or BPE. Such established cells should be weaned away from their original medium, and allowed at least 10 days in the new medium before testing.

Trypsinization

- Serum free media are not suitable for stopping the trypsinization reaction, as they do not contain the necessary protein concentration. To avoid the use of serum we recommend rProtease, whose reaction does not have to be stopped . Please refer to the Resources Page on www.cellntec.com for protocols.

Age of Cell Culture

- Over several passages, certain cell sub-populations are preferentially selected by the medium formulation. Such "selected" populations require more time to adapt to new media. For optimal comparison we recommend the use of freshly isolated or early passage cultures, or alternatively at least the adaptation period described above.

Other Variables

- Isolation efficiency and culture growth are also affected by variables such as tissue donor, age of donor, species, animal strain (especially for mice), donor age. For more information about your specific situation, please contact CELLnTEC.

Thank you and we look forward to hearing your results.

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Isolation of Primary Corneal Epithelium

A wide range of approaches exist for the isolation and growth of early passage corneal epithelium cell cultures. For optimal results, we suggest the following procedure.

In particular we recommend the seeding density listed below, as this has been found to be an important factor in isolation, as well as the use of the PCT medium CnT-20, which offers maximum isolation efficiency in a fully defined environment.

1. Cut corneal tissue sample into small pieces
2. Incubate overnight at 4°C in CnT-20 with 0.7 units/mL dispase and 2X Antibiotic/antimycotic (CnT-ABM)
3. Separate epithelium from underlying tissue using sterile forceps
4. Centrifuge 5min 1000rpm, wash once
5. Add 1x rProtease* to separate epithelial cells
6. Dissociate any cell clumps by pipetting, and wash
7. Seed cells in CnT-20 at $8x10^3$ per sq. cm
8. Change medium every 2-3 days after cells have attached

*We recommend the use of rProtease for cell separation, based on its effectivity, price, and ease of use. In particular, no stopping of the digestion is required, as it is with trypsin. Should trypsin be used, be sure to use FCS-supplemented medium to completely stop the reaction once separation has reached the desired level.
rProtease solution: TypLE Select (Cat # 12563-011 Invitrogen)

For the latest information about these products, please see www.cellntec.com

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Isolation of Primary Mammary Epithelium

A wide range of approaches exist for the isolation and growth of early passage mammary epithelium cell cultures. For optimal results, we suggest the following procedure.

In particular we recommend the seeding densities listed below, and the direct seeding of organoids (cell clumps) following dispase/collagenase treatment, thereby avoiding the additional dissociation step required for single cells (which generally lowers the isolation efficiency).

CnT-22 has been developed to provide efficient isolation efficiency and growth rates in a defined environment at moderate seeding densities. For researchers who are restricted to very small tissue samples and thus lower densities, the non-defined CnT-54 (containing BPE) may offer still better results.

1. Cut mammary tissue sample into small pieces, remove some of the fat
2. Incubate overnight at 37°C in CnT-22 or CnT-54 with 0.7 units/mL dispase + 500 ug/mL collagenase 2 + 3X antibiotic/antimycotic (CnT-ABM)
3. Vigorously pipette or vortex to break up clumps
4. Centrifuge 5min 1000rpm, wash until all the fat is removed
5. Pass through 40micron mesh filter (cell strainer), wash filter with 10 mL medium. Reverse wash organoids off filter into new tube
6. Wash epithelial organoids once or twice with medium
7. Seed organoids directly into plate (recommended), or if single cells are required, digest organoids with rProtease* before seeding cells at $4x10^3$ per sq. cm in CnT-22, or $2x10^3$ per sq. cm in CnT-54.
8. Change medium every 2-3 days after cells have attached

*We recommend the use of rProtease for cell separation, based on its effectivity, price, and ease of use. In particular, no stopping of the digestion is required, as it is with trypsin. Should trypsin be used, be sure to use FCS-supplemented medium to completely stop the reaction once separation has reached the desired level.
rProtease solution: TypLE Select (Cat # 12563-011 Invitrogen)

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Isolation of Primary Human Prostate Epithelium

A wide range of approaches exist for the isolation and growth of early passage prostate epithelium cell cultures. For optimal results, we suggest the following procedure.

In particular we recommend the seeding densities listed below, and the direct seeding of organoids (cell clumps) following dispase/collagenase treatment, thereby avoiding the additional dissociation step required for single cells (which generally lowers the isolation efficiency).

CnT-12 has been developed to provide efficient isolation efficiency and growth rates in a defined environment at moderate seeding densities. For researchers who are restricted by very small tissue samples and thus lower densities, the non-defined CnT-52 (containing BPE) may provide still better results.

1. Cut prostate tissue sample into small pieces
2. Incubate overnight at 37°C in CnT-12 or CnT-52 with 0.7 units/mL dispase + 500 ug/mL collagenase 1 + 3X antibiotic/antimycotic (CnT-ABM)
3. Vigorously pipette or vortex to break up clumps
4. Centrifuge 5min 1000rpm, wash once
5. Pass through 40micron mesh filter (cell strainer), wash filter with 10ml medium. Reverse wash organoids off filter into new tube
6. Wash epithelial organoids once or twice with medium
7. Seed organoids directly into plate (recommended), or if single cells are required, digest organoids with rProtease* before seeding cells at $4x10^3$ per sq. cm in CnT-12, or $2x10^3$ per sq. cm in CnT-52.
8. Change medium every 2-3 days after cells have attached

*We recommend the use of rProtease for cell separation, based on its effectivity, price, and ease of use. In particular, no stopping of the digestion is required, as it is with trypsin. Should trypsin be used, be sure to use FCS-supplemented medium to completely stop the reaction once separation has reached the desired level.
rProtease solution: TypLE Select (Cat # 12563-011 Invitrogen)

For the latest information about these products, please see www.cellntec.com

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Isolation of Primary Human Small Airway Epithelium

A wide range of approaches exist for the isolation and growth of early passage small airway epithelium cell cultures. For optimal results, we suggest the following procedure.

In particular we recommend the seeding densities listed below, and the direct seeding of organoids (cell clumps) following dispase/collagenase treatment, thereby avoiding the additional dissociation step required for single cells (which generally lowers the isolation efficiency).

CnT-17 has been developed to provide efficient isolation efficiency and growth rates in a defined environment at moderate seeding densities. For optimal results with this medium, we recommend a density of $4x10^3$ per sq. cm in CnT-17.

1. Cut lung tissue into small pieces, 0.5x0.5x0.5cm or smaller
2. Incubate with 10 mg/mL Dispase + 5 ug/mL collagenase 1 in CnT-17 at 37ºC for 12-18h
3. Vigorously pipette or vortex to break up clumps
4. Centrifuge 5min 1000rpm, wash with medium
5. Pass through 40micron mesh filter (cell strainer), wash filter with 10 mL medium. Reverse wash organoids off filter into new tube
6. Wash epithelial organoids once or twice with medium
7. Seed organoids directly into plate (recommended), or if single cells are required, digest organoids with rProtease* before seeding cells at $4x10^3$ per sq. cm in CnT-17.
8. Change medium every 2-3 days after cells have attached

*We recommend the use of rProtease for cell separation, based on its effectivity, price, and ease of use. In particular, no stopping of the digestion is required, as it is with trypsin. Should trypsin be used, be sure to use FCS-supplemented medium to completely stop the reaction once separation has reached the desired level. rProtease solution: TypLE Select (Cat # 12563-011 Invitrogen)

For the latest information about these products, please see www.cellntec.com

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Protocol for the cultivation of CELLnTEC human keratinocytes

Immediately upon delivery
Remove the vial from the shipping container, and check that it is still frozen.
Transfer frozen vial to liquid nitrogen until you are ready to thaw and begin culture.

Thawing cells
Thaw vial in waterbath at 37°C until just melted.
Immediately transfer the cells into 10ml CnT-07 and centrifuge 5 min. at 160g.
Resuspend pellet in 5ml CnT-07.
Seed the cells from one vial (1.2 x 10^6 cells) into a $75cm^2$ culture flask.
Change medium after 2 days, then every 3rd day.

Growth
Let the cells grow at 35°C and 5% CO_2 until they reach confluency (approximately 7-10 days).
Passage the cells at the latest 1 day after confluence (differentiation may begin when waiting longer).

Passaging
Aspirate the medium.
Add 2ml 1x rProtease and incubate until all cells are detached (ca. 10 min. at 35°C).
Add 5ml CnT-07 to the detached cells and resuspend 2-3 times vigorously.
Spin the cells at 160xg for 5 min.
Aspirate supernatant and resuspend the pellet in 5ml CnT-07 medium.
Count cells and seed at the appropriate density.

Seeding
Seed cells at a density of 0.8-1x10^4 ¢/cm^2 in culture flasks in 5ml CnT-07/25cm^2.
Until you become experienced with the cells, we recommend the following:

- Seed 1x10^4 ¢/cm^2 back into the same old flask after rinsing it with another 5ml CnT-07
- Seed 1x10^4 ¢/cm^2 into a new flask
- Seed 3x10^4 ¢/cm^2 into a new flask

Medium change
Aspirate all medium and replace with fresh CnT-07. Change medium 2 days after seeding, then every 3 days.

Freezing
Treat subconfluent monolayers with rProtease as above.
Count cells - place the cells on ice while counting.
Adjust the cell concentration to 2.4 x 10^6 cells/ml with cold CnT-07.
Add drop-wise the same amount of cold 2 x freezing medium, while gently swirling the tube (final concentration 1.2 x 10^6 cells/ml).
Immediately add 1 ml cell suspension into labeled cryotubes.
Immediately transfer tubes to a NALGENE® Cryo 1°C Freezing container (#5100-0001).
Immediately place at –80°C, leave at least overnight.
Transfer tubes to liquid nitrogen for long-term storage.

Solutions
rProtease: Tryple™ Select (12563-011, Invitrogen)
2x freezing medium: CnT-07, 40% fetal calf serum, 20% DMSO

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Protocol for the cultivation of CELLnTEC mouse keratinocytes

Immediately upon delivery
Remove the vial from the shipping container, and check that it is still frozen.
Transfer frozen vial to liquid nitrogen until you are ready to thaw and begin culture.

Thawing cells
Thaw vial in waterbath at 37°C until just melted.
Immediately transfer the cells into 10ml CnT-02 and centrifuge 5 min. at 160g.
Resuspend pellet in 5ml CnT-02.
Seed the cells from one vial (1.5 x 10^6 cells) into a 25cm^2 culture flask.
Change medium after 2 days, then every 3rd day.

Growth
Let the cells grow at 35°C and 5% CO_2 until they reach confluency (approximately 5-7 days). Passage the cells at the latest 1 day after confluence (differentiation may begin when waiting longer).

Passaging
Aspirate the medium.
Add 2ml 1x rProtease and incubate until all cells are detached (ca. 10 min. at 35°C).
Add 5ml CnT-02 to the detached cells and resuspend 2-3 times vigorously.
Spin the cells at 160xg for 5 min.
Aspirate supernatant and resuspend the pellet in 5ml CnT-02 medium.
Count cells and seed at the appropriate density.

Seeding
Seed cells at a density of 3x10^4 ¢/cm^2 in culture flasks in 5ml CnT-02/25cm^2.
Until you become experienced with the cells, we recommend the following:

- Seed 3x10^4 ¢/cm^2 back into the same old flask after rinsing it with another 5ml CnT-02
- Seed 5x10^4 ¢/cm^2 into a new flask
- Seed 3x10^4 ¢/cm^2 into a new flask

Medium change
Aspirate all medium and replace with fresh CnT-02.
Change medium 2 days after seeding, then every 3 days.

Freezing
Treat subconfluent monolayers with rProtease as above.
Count cells - place the cells on ice while counting.
Adjust the cell concentration to 3 x 10^6 cells/ml with cold CnT-02.
Add drop-wise the same amount of cold 2 x freezing medium, while gently swirling the tube (final concentration 1.5 x 10^6 cells/ml).
Immediately add 1 ml cell suspension into labeled cryotubes.
Immediately transfer tubes to a NALGENE® Cryo 1°C Freezing container (#5100-0001).
Immediately place at –80°C, leave at least overnight.
Transfer tubes to liquid nitrogen for long-term storage.

Solutions
rProtease: Tryple™ Select (#12563-011, Invitrogen)
2x freezing medium: CnT-02, 40% fetal calf serum, 20% DMSO

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Isolation of Primary Mouse Keratinocytes

The isolation of primary mouse keratinocytes can be a challenging process. A number of factors influence the success of this process, including the mouse strain, and variation between different individuals.

For maximum efficiency, we recommend using epidermis from neo-natal mice. Between embryonic day 18 and 20 provides good cell activity, before any significant hair follicle formation has occurred, enabling easier separation of epidermis and dermis.

It is also important to use a number of embryos wherever possible, as it is common for the cells from one or two individuals to establish very poorly in culture.

Despite the challenges of establishing mouse keratinocytes in vitro, with certain strains, such as C57BL/6, with experience it does become possible to be routinely successful at establishing these cells in vitro.

For establishing primary mouse keratinocyte cultures, CELLnTEC recommends the basic protocol summarized below, in which our progenitor cell targeted medium CnT-07 is used to maximize isolation efficiency and early passage growth.

1. Remove the skin from a mouse embryo at approximately ED18-20, remaining as sterile as possible.
2. Incubate the skin in CnT-07 with 5 U/mL Dispase (10 mg/mL) and 10X antibiotic/antimycotic (e.g. Pen/Step/Amphotericin) for 8 hours at 4°C.
3. Gently separate the epidermis from the dermis using sterile forceps, cut it into small pieces, and add 1x rProtease* until cells separate (generally for 10-15 mins).
4. Add 2-2.5 volumes of CnT-07 and transfer cell suspension to appropriate tube for centrifuging.
5. Seed cells at 3×10^4 per cm^2 in CnT-07 into uncoated cell culture flasks. At confluency cells may also be detached using rProtease (see separate passaging protocol).

*We recommend the use of rProtease for cell separation, based on its effectivity, price, and ease of use. In particular, no stopping of the digestion is required, as it is with trypsin. Should trypsin be used, be sure to use FCS-supplemented medium to completely stop the reaction once separation has reached the desired level. rProtease solution: TypLE Select (Cat # 12563-011 Invitrogen)

This summary is intended as a guide only. For our customers, CELLnTEC representatives are available for basic trouble shooting should problems arise.

In case of persistent problems with the establishment of mouse keratinocytes, CELLnTEC offers a service of culture establishment from your own specific mice – please see www.cellntec.com for more details.

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Suggested Seeding Densities For CELLnTEC Media

Medium	Target	Species	Seeding Density
CnT-02	Keratinocytes	Human	9×10^3 cells / cm^2
CnT-02	Keratinocytes	Mouse	2×10^4 cells / cm^2
CnT-07	Keratinocytes	Human	9×10^3 cells / cm^2
CnT-07	Keratinocytes	Mouse	2×10^4 cells / cm^2
CnT-11	Prostate	Rat	4×10^3 cells / cm^2
CnT-12	Prostate	Human	4×10^3 cells / cm^2
CnT-52	Prostate	Human	2×10^3 cells / cm^2
CnT-14	Small Airway	Rat	4×10^3 cells / cm^2
CnT-15	Large Airway	Rat	4×10^3 cells / cm^2
CnT-17	Airway	Human	4×10^3 cells / cm^2
CnT-16	Bladder	Rat	2×10^4 cells / cm^2
CnT-18	Bladder	Human	4×10^3 cells / cm^2
CnT-20	Cornea	Human	8×10^3 cells / cm^2
CnT-22	Mammary	Human	4×10^3 cells / cm^2
CnT-54	Mammary	Human	2×10^3 cells / cm^2
CnT-24	Oral Epithelium	Human	4×10^3 cells / cm^2

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Transfection of cells using Polyethylenimine (PEI)

Cells should be seeded a day prior to transfection in 6 well plates at a density of 300,000 cells per well.

To 50ul of basal medium (containing no additives ie serum, antibiotics or other proteins)

Add 2ug DNA; mix by pipetting up-down

Add 15ul PEI (from 1mg/ml solution) ie 15ug; mix by pipetting up-down

Incubate for 8 min at room temp.

Add 450ul complete medium containing 10% FCS

Apply mixture to cells. Note: cells should have been washed previously with PBS 2x

Incubate for 2 hour (in incubator); rock the plate occasionally ie. every 10min.

Wash cells 3x with PBS

Add 2mls of complete medium

Incubate overnight Note: some cell death will occur during this period.

Change medium

Transfection efficiency for mouse keratinocytes is ~15%

Preparation of PEI stock solution

(Polyethylenimine, linear,MW-25,000; cat. No.23966 Polysciences, Inc Warrington,PA 18976)

Dissolve PEI powder to a concentration of 2mg/ml in water which has been heated to 80°C.
Allow solution to cool to room temp.
Adjust pH to 7.0 with 5M HCl
Filter sterilize
Freeze aliquots at –80°C

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Important Note For Keratinocyte Differentiation using PCT media

CnT-07 is recommended for isolation and maintenance of keratinocyte progenitor cells.

However for differentiation of these cells (both 2D and 3D), we recommend the use of CnT-02, because the PCT formulation of CnT-07 is known to cause a delay in the onset of terminal differentiation.

As with all differentiation work, it is important that cells reach confluency before calcium levels are increased. Thus the protocol we recommend for differentiation of cells in CnT-07 is as follows:

1. Seed cells as usual in CnT-07
2. Allow cells to proliferate until they approach confluency
3. One day before confluency (and calcium switch), change the medium to CnT-02
4. At confluency add 1.2 mM Ca(2+), triggering the onset of terminal differentiation

Due to the similarity of the CnT-02 and CnT-07 formulations, cells can be easily switched between these media.

Following this protocol, it is possible to obtain the full differentiation of cells normally maintained in a PCT medium.