Miltenyi Biotec

Embryoid Body Dissociation Kit
human and mouse

Order no. 130-096-348

Contents

1. Description

Components 6 vials, containing

2.5 mL Enzyme P

2×50 mL Buffer X

1.5 mL Buffer Y

1 vial Enzyme A (lyophilized powder)

1 mL Buffer A

Size For 50 digestions.

The specified number of digestions is valid when embryoid bodies at several stages generated from a starting cell number of 1×10^6/10 mL (mouse) and 4×10^6/10 mL (human) are used.

Storage Upon arrival immediately store Enzyme P in aliquots at –20 °C. Store all other components at 2–8 °C upon arrival. Reconstitute Enzyme A before the date indicated on the box label. For information about reconstitution and storage after reconstitution of the lyophilized component refer to chapter 2.1.

1.1 Background information

Embryoid body (EB) formation is a crucial step in many ES or iPS cell differentiation protocols. For subsequent cell analysis or isolation of specific cell populations of the EB, single-cell suspensions are a prerequisite, for example, to achieve the highest possible purity and recovery during cell separations with MACS® Technology. The gentleMACS™ Dissociator provides optimized programs to attain single-cell suspensions from *in vitro* generated EBs.

EBs are cellular aggregates generated from differentiating pluripotent stem cells grown in suspension in defined cell numbers and media. Cell aggregation is initiated by suspension culture in hanging drops or as mass suspension culture in non-tissue culture plates or spinner flasks. During the three dimensional differentiation process, *in vivo* embryonic development is recapitulated to a limited extent and differentiated cell types of all three germ layers are generated. Embryoid bodies are a well studied model system for investigating early stages of development and deriving distinct cell types from pluripotent stem cells.

1.2 Applications

- Dissociation of EBs into single-cell suspensions for subsequent cell separations using MACS Technology.
- Cultivation of EB-derived cells.
- Phenotyping or enumeration of individual EB-derived cell populations by flow cytometry.
- Dissociation of pluripotent stem cell-derived neurospheres.

  ▲ **Note:** The Embryoid Body Dissociation Kit is not suitable for the dissociation of tissue-derived neurospheres. In this case use the Neurosphere Dissociation Kit (P) (# 130-095-943) or the Neurosphere Dissociation Kit (T) (# 130-095-944) depending on the antigen epitope of interest.

1.3 Reagent and instrument requirements

- Buffer: Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} and Mg^{2+}
- EB culture medium
- Pre-Separation Filters, 70 µm (# 130-095-823)
- (Optional) gentleMACS Dissociator (# 130-093-235)
- (Optional) gentleMACS M Tubes (# 130-093-236, # 130-096-335)
- (Optional) ART® 1000 REACH™ pipet tips (Molecular BioProducts, Inc.) for removal of dissociated material from the closed M Tubes.

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2. Protocol

▲ The dissociation protocols have been optimized for embryoid bodies generated from a starting cell number of 1×10^6/10 mL (mouse) and 4×10^6/10 mL (human).

▲ For cell culture experiments subsequent to embryoid body dissociation, all steps should be performed under sterile conditions.

▲ Partially dissociated EBs may adhere unspecifically to plastic surfaces such as the tube walls. Thus, avoid unnecessary rotation of tubes during dissociation and keep the EBs covered with enzyme solution at all times.

2.1 Reagent preparation

1. Prepare Enzyme A by reconstitution of the lyophilized powder in its vial with 1 mL of Buffer A. Do not vortex. Prepare aliquots of appropriate volume and avoid repeated freeze-thaw-cycles. Store aliquots at –20 °C. This solution is stable for 6 months after reconstitution. For cell culture experiments subsequent to tissue dissociation, Enzyme A should be sterile filtered.
2. Prepare 1950 µL enzyme mix 1 by adding 50 µL of Enzyme P to 1900 µL of Buffer X and vortex. Pre-heat the mixture to 37 °C for 10–15 minutes before use.

   ▲ **Note:** For enumeration or magnetic cell separation of $CD309^+$ (KDR^+, $Flk\text{-}1^+$, VEGFR2) cells add 10 µL of Enzyme P to 1940 µL of Buffer X.
3. Prepare 30 µL enzyme mix 2 by adding 20 µL of Buffer Y to 10 µL of Enzyme A.
4. Add 30 µL enzyme mix 2 to 1950 µL enzyme mix 1.

Enzyme mix 1		Enzyme mix 2	
Enzyme P 50 µL	Buffer X 1900 µL	Buffer Y 20 µL	Enzyme A 10 µL

2.2 Automated dissociation using the gentleMACS™ Dissociator

▲ For details on the use of the gentleMACS™ Dissociator, refer to the gentleMACS Dissociator user manual.
The protocol is not compatible with the gentleMACS Octo Dissociator or the gentleMACS Octo Dissociator with Heaters.

2.2.1 Automated dissociation of up to 7 days old EBs

1. Transfer up to 20 mL of EB-containing cell culture medium into the gentleMACS M Tube. Centrifuge at 300×g for 2 minutes. Discard supernatant.
2. Add 10 mL of DPBS to the gentleMACS M Tube. Resuspend EBs carefully. Centrifuge at 300×g for 2 minutes. Discard supernatant.
3. Add 1980 µL of the combined pre-heated enzyme mix 1 and 2 to the gentleMACS M Tube and tightly close the lid.
4. Incubate sample for 10 minutes at 37 °C without agitation and ensure EBs are covered by the enzyme solution.
5. Invert the M Tube in one movement and attach it upside down onto the sleeve of the gentleMACS Dissociator. Keep the tube upside down during the following incubation and dissociation steps 6–9.
6. Run the gentleMACS Program **EB_01**.
7. Once the program is finished incubate sample for 5 minutes at 37 °C without agitation.
8. Attach M Tube upside down onto the sleeve of the gentleMACS Dissociator.
9. Run the gentleMACS Program **EB_02**.
10. Once the program is finished add 8 mL of cell culture medium or DPBS to the M Tube.
11. Resuspend sample and apply the cell suspension to a Pre-Separation Filter, 70 µm, placed on a suitable (13–15 mL) tube.

    ▲ **Note:** Dissociated EBs can be removed from the closed M Tube by pipetting through the septum-sealed opening in the center of the cap of the M Tube. Use ART 1000 REACH 1000 µL pipette tips.
12. Wash Pre-Separation Filter, 70 µm, with 3 mL of EB culture medium or DPBS.
13. Discard filter and centrifuge cell suspension at 300×g for 5 minutes. Aspirate supernatant completely.
14. Resuspend cells with appropriate buffer or medium to the required volume for further applications.

2.2.2 Automated dissociation of 8–20 days old EBs

1. Transfer up to 20 mL of EB-containing cell culture medium into the gentleMACS M Tube. Centrifuge at 300×g for 2 minutes. Discard supernatant.
2. Add 10 mL of DPBS to the gentleMACS M Tube. Resuspend EBs carefully. Centrifuge at 300×g for 2 minutes. Discard supernatant.
3. Add 1980 µL of the combined pre-heated enzyme mix 1 and 2 to the gentleMACS M Tube and tightly close the lid.
4. Incubate sample for 10 minutes at 37 °C without agitation and ensure EBs are covered by the enzyme solution.
5. Invert the M Tube in one movement and attach it upside down onto the sleeve of the gentleMACS Dissociator. Keep the tube upside down during the following incubation and dissociation steps 6–12.
6. Run the gentleMACS Program **EB_01**.
7. Once the program is finished incubate sample for 10 minutes at 37 °C without agitation.
8. Attach M Tube upside down onto the sleeve of the gentleMACS Dissociator.
9. Run the gentleMACS Program **EB_02**.
10. Once the program is finished incubate sample for 5 minutes at 37 °C without agitation.
11. Attach M Tube upside down onto the sleeve of the gentleMACS Dissociator.
12. Run the gentleMACS Program **EB_02**.
13. Once the program is finished add 8 mL of cell culture medium or DPBS to the M Tube.
14. Resuspend sample and apply the cell suspension to a Pre-Separation Filter, 70 µm, placed on a suitable (13–15 mL) tube.

    ▲ **Note:** Dissociated EBs can be removed from the closed M Tube by pipetting through the septum-sealed opening in the center of the cap of the M Tube. Use ART 1000 REACH 1000 µL pipette tips.

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15. Wash Pre-Separation Filter, 70 µm, with 3 mL of cultivation medium or DPBS.
16. Discard filter and centrifuge cell suspension at 300×g for 5 minutes. Aspirate supernatant completely.
17. Resuspend cells with appropriate buffer or medium to the required volume for further applications.

2.3 Manual dissociation

▲ The protocol below is compatible with a wide range of EB-based differentiation protocols. However, depending on cell type of interest and the protocol used for EB generation, pipetting and incubation times may have to be optimized.

▲ Dissociation by pipetting may give variable results, depending on the user. For optimal standardization, the use of the gentleMACS Dissociator is recommended (refer to section 2.2)

2.3.1 Manual dissociation of up to 7 days old EBs

1. Transfer up to 20 mL of EB-containing cell culture medium into a 50 mL tube. Centrifuge at 300×g for 2 minutes. Discard supernatant.
2. Add 10 mL of DPBS and resuspend EBs carefully. Centrifuge at 300×g for 2 minutes. Discard supernatant.
3. Add 1980 µL of the combined pre-heated enzyme mix 1 and 2.
4. Incubate for 10 minutes at 37 °C without agitation and ensure EBs are covered by the enzyme solution.
5. Start mechanical dissociation by carefully pipetting up and down using a 1000 µL pipette for 1 minute.
6. Incubate for 5 minutes at 37 °C without agitation.
7. Pipette up and down for 1 minute using a 1000 µL pipette.
8. Add 8 mL of cell culture medium or DPBS.
9. Pass the cell suspension through a Pre-Separation Filter, 70 µm, placed on a suitable (13–15 mL) tube.
10. Wash the Pre-Separation Filter with 3 mL of EB culture medium or DPBS.
11. Discard filter and centrifuge cell suspension at 300×g for 5 minutes. Aspirate supernatant completely.
12. Resuspend in an appropriate buffer or medium required for your downstream application.

2.3.2 Manual dissociation of 8–20 days old EBs

1. Transfer up to 20 mL of EB-containing cell culture medium into a 50 mL tube. Centrifuge at 300×g for 2 minutes. Discard supernatant.
2. Add 10 mL of DPBS and resuspend EBs carefully. Centrifuge at 300×g for 2 minutes. Discard supernatant.
3. Add 1980 µL of the combined pre-heated enzyme mix 1 and 2.
4. Incubate sample for 10 minutes at 37 °C without agitation and ensure EBs are covered by the enzyme solution.
5. Start mechanical dissociation by carefully pipetting up and down using a 1000 µL pipette for 1 minute.
6. Incubate sample for 10 minutes at 37 °C without agitation.
7. Pipette up and down for 1 minute using a 1000 µL pipette.
8. Incubate for 5 minutes at 37 °C without agitation.
9. Pipette up and down for 1 minute using a 1000 µL pipette.
10. Add 8 mL of cell culture medium or DPBS.
11. Pass the cell suspension through a Pre-Separation Filter, 70 µm, placed on a suitable (13–15 mL) tube.
12. Wash the Pre-Separation Filter with 3 mL of EB culture medium or DPBS.
13. Discard filter and centrifuge cell suspension at 300×g for 5 minutes. Aspirate supernatant completely.
14. Resuspend in an appropriate buffer or medium required for your downstream application.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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