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Effective isolation of functional islets from neonatal mouse pancreas

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Corresponding Author:	Guoqiang Gu Vanderbilt University School of Medicine Nashville, TN UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	Guoqiang.gu@vanderbilt.edu
Corresponding Author's Institution:	Vanderbilt University School of Medicine
Corresponding Author's Secondary Institution:	
First Author:	Guoqiang Gu
First Author Secondary Information:	
Other Authors:	Chen Huang
Order of Authors Secondary Information:	
Abstract:	Perfusion-based islet-isolation protocols from large mammalian pancreata are well established. Such protocols are readily conducted in many laboratories due to the large size of the pancreatic duct that allows for ready collagenase injection and subsequent tissue perfusion. In contrast, islet isolation from small pancreata, like that of neonatal mice, is challenging because perfusion is not readily achievable in the small pancreata. Here we describe a detailed simple procedure that recovers substantial numbers of islets from newly born mice with visual assistance. Freshly dissected whole pancreata were digested with 0.5 mg/mL collagenase IV dissolved in Hanks' Balanced Salt Solution (HBSS) at 37 °C, in microcentrifuge tubes. Tubes were tapped regularly to aid tissue dispersal. When most of the tissue was dispersed to small clusters around 1 mm, lysates were washed three to four times with culture media with 10% fetal bovine serum (FBS). Islet clusters, devoid of recognizable acinar tissues, can then be recovered under dissecting stereoscope. This method recovers 20-80 small- to large-sized islets per pancreas of newly born mouse. These islets are suitable for most conceivable downstream assays, including insulin secretion, gene expression, and culture. An example of insulin secretion assay is presented to validate the isolation process. The genetic background and degree of digestion are the largest factors determining the yield. Freshly made collagenase solution with high activity is preferred, as it aids in endocrine-exocrine isolation. The presence of cations [calcium (Ca^{2+}) and magnesium (Mg^{2+})] in all solutions and fetal bovine serum in the wash/picking media are necessary for good yield of islets with proper integrity. A dissecting scope with good contrast and magnification will also help.
Author Comments:	Dear Editor, Thanks for your help with revising the attached manuscript. In the new version, we have detailed the islet isolation process from neonatal pancreata from mouse, with added functional validations of the method. This isolation process is critical for studying how immature pancreatic beta cells become mature functional beta cells, which bears

	significant implication for deriving functional beta cells in culture for curing type I diabetes. So far, there is not a standardized detailed isolation method for newly born islets. The goal of this manuscript is to provide these details for new laboratories to successfully isolate islets from neonates. We will list several factors that affect the success of this isolation method. We envision that this protocol to be of great value for any laboratory that utilized islets form newly-born animals. Thanks for your help.
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TITLE:
Effective isolation of functional islets from neonatal mouse pancreas

AUTHORS:
Huang Chen
Department of Cell and Development Biology
Vanderbilt University Medical School
Nashville, TN, USA
Chen.huang@vanderbilt.edu

Guoqiang Gu
Department of Cell and Development Biology
Vanderbilt University Medical School
Nashville, TN, USA
Guoqiang.gu@vanderbilt.edu

CORRESPONDING AUTHOR:
Guoqiang Gu
Department of Cell and Development Biology
Vanderbilt University Medical School
Nashville, TN, USA
615-936-3634
Guoqiang.gu@vanderbilt.edu

KEYWORDS:
transplantation, collagenase, diabetes, maturation, Islets, insulin, beta cells, secretion, neonatal, hand picking.

SHORT ABSTRACT:
We describe a protocol herein for isolating intact islets from neonatal mice. Pancreata were partially digested with collagenase, followed by washing and hand picking. 20-80 islet clusters can be obtained per pancreas from newly born mice, which are suitable for several islet studies.

LONG ABSTRACT:
Perfusion-based islet-isolation protocols from large mammalian pancreata are well established. Such protocols are readily conducted in many laboratories due to the large size of the pancreatic duct that allows for ready collagenase injection and subsequent tissue perfusion. In contrast, islet isolation from small pancreata, like that of neonatal mice, is challenging because perfusion is not readily achievable in the small pancreata. Here we describe a detailed simple procedure that recovers substantial numbers of islets from newly born mice with visual assistance. Freshly dissected whole pancreata were digested with 0.5 mg/mL collagenase IV dissolved in Hanks' Balanced Salt Solution (HBSS) at 37 °C, in microcentrifuge tubes. Tubes were tapped regularly to aid tissue dispersal. When most of the tissue was dispersed to small clusters around 1 mm, lysates were washed three to four times with culture media with 10% fetal bovine serum

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INTRODUCTION:

Isolating pure pancreatic islets is essential for assaying glucose stimulated insulin secretion (GSIS) of beta cells and for islet transplantation from cadaveric donors [1-3]. It is also necessary to establish endocrine gene expression in islet cells [4, 5]. For this purpose, detailed protocols have been established to allow for isolation of pancreatic islets from large pancreata ([6] and references therein). These methods are based on enzymatic perfusion to dissociate acinar from islet tissues, coupled with gradient separation and hand picking. Thus, islet isolation from large pancreas can be performed readily in most laboratories. On the other hand, no detailed step-by-step protocol exists to allow for the isolation of islets from pancreata that are too small to perfuse.

Studying gene expression and function of neonatal islets is important. Neonate islets have different properties from adults in insulin secretion and proliferation capability [7, 8]. However, isolating islets from newly born animals, especially mice is challenging due to the small size of the newly born pancreas. The size prevents the usual perfusion process when collagenase is injected though the pancreatic duct. Indeed, several papers have presented studies along these lines, with enzyme or non-enzyme aided isolation procedures [7, 9, 10]. However, detailed description of the islet isolation process with visual aid is lacking [7, 9], making it a challenge for most researchers to perform similar studies.

We have explored several different conditions that yield high quality islets from neonatal mice. Here we present a protocol that is expected to help researchers learn the key details in the islet isolation process. This protocol is applicable to mouse pancreas up to two weeks of age, after which perfusion can be performed for routine islet isolation. Islets can be directly used for insulin secretion and gene expression assays.

PROTOCOL:

Animal usage follows the procedures specified in protocol M/11/181 approved by the Vanderbilt Institutional Animal Care and Use Committee for Gu. CD1 or CBA/Bl6 mice were purchased from commercial vendors and crossed in the Vanderbilt animal facility to obtain neonatal mice.

1. Preparation of mice, stock solutions, and equipment

1.1 For the mouse cross: set up a mouse cross and record the plugging dates to aid experimental planning. CD1 mice usually give birth around day 19 after mating. Use CD1 or CBA/Bl6 crosses to obtain CD1 or CBA/Bl6 neonates, respectively. Intercrosses between the two lines utilize CD1 females and CBA/Bl6 males.

1.2 For the collagenase stock: weigh 200 mg collagenase Type IV with a high precision balance. Transfer to a 50 mL centrifuge tube. Dissolve in 40 mL HBSS with Ca^{2+}/Mg^{2+} (1.26 and 0.5 mM, respectively) to make a 5 mg/mL stock solution.

1.2.1 Allow collagenase to dissolve for ~30 minutes with gentle shaking. Aliquot and keep the solution frozen in -20 °C as stock, which stays active for at least one year.

1.3 Autoclave to sterilize several pairs of tweezers, scissors, and P20 tips for dissection and islet picking.

1.4 For the 1 M glucose stock: weigh 9 g glucose in a 50 mL centrifuge tube, add 50 mL RPMI 1640 media to dissolve the glucose. Keep in 4 °C until use, but no more than 6 months.

2. Working solutions

Note: The day of islet isolation, prepare the following reagents.

2.1 For the collagenase type IV working solution: thaw and dilute collagenase stock on ice. Dispense 0.15 mL stock into each 1.7 mL microfuge tube. Add 1.35 mL HBSS with Ca^{2+}/Mg^{2+}. Invert the tube 5 times. Leave on ice for 15 minutes.

2.1.1 Spin at high speed for 3 minutes in a microcentrifuge. Transfer supernatant to a new tube. Leave on ice until use. Discard the tube with insoluble debris.

2.2 For complete RPMI 1640 media: add 2.5 mL 1 M glucose, 50 mL heat inactivated FBS, and 5 mL 100X Pen-strep stock into 500 mL RPMI 1640 media. Leave on ice until use.

3. Pancreas isolation and digestion

3.1 Euthanize neonates with isoflurane, followed by decapitation according to approved protocols.

3.2 Lay the mouse with its belly facing up. Spray with 70% ethanol for sterilization.

3.3 Lift the belly skin up with tweezers and cut the skin and muscle layers longitudinally along the midline with a pair of scissors, from the genital area to the rib cage. This opens the abdominal cavity to expose all internal organs.

3.4 Transfer all the internal organs to a 100 cm dish with a pair of tweezers. Locate the pancreas, which is a scattered-organ with a dorsal portion that clings to the stomach and spleen and a ventral portion that clings to the duodenum[11].

3.5 Add HBSS into the dish to submerge the organs. In solution, the pancreatic tissues no longer cling to the duodenum, stomach, or spleen. It is readily recognizable under a dissecting scope because of its typical white color. Use a pair of tweezers to peel the pancreatic tissues away from surrounding tissue and transfer to a new 60 mm dish with HBSS.

3.6 Cut each pancreas with a scissor into pieces smaller than 5 mm across any dimensions. Transfer to a 1.5 mL microcentrifuge tube. Up to five pancreata (younger than P7) can be digested in one tube. For P8-P16 mice, use one tube per pancreas.

3.7 Add 0.5-1 mL collagenase working solution to each tube (0.2 mL for each P1-P7 pancreas. Use 0.5 mL for each P8-P16 pancreas). Leave the tube in a 37 °C incubator for up to 15 minutes. Invert the tube two times every minute.

3.8 After ~5 minutes, tap the tube to monitor the digestion status of the pancreatic tissue. Continue the digestion process until most of the pancreatic tissue appears as fragmented cell clusters, with diameter <2 mm. The lysate will appear cloudy, which is due to normal acinar cell autolysis.

4. Lysate washing

4.1 Spin the lysate at 500 x g for 10 s in a microcentrifuge. Remove the supernatant layer with a P1000 pipetman. The supernatant should appear turbid but devoid of cell clusters. Do not use aspiration, which may quickly aspirate away the tissue fragments at the bottom due to the presence of some sticky DNA.

4.2 Add 1 mL RPMI 1640 complete media. Tap the tube gently to resuspend the fragmented lysate. Invert up and down 10 times. Repeat step 4.1.

4.3 Repeat step 4.2 two more times or until the supernatant appears clear. Re-suspend washed pancreatic fragments in 1 mL complete RPMI 1640 media.

5. Islet isolation

Note: For small numbers of pancreata, direct hand-picking as below (5.1) can be used for islet isolation. For large numbers of pancreata (>6), the method outlined in 5.2 is preferred.

5.1 Direct hand-picking:

5.1.1 Transfer lysate from step 4.3 to a 60 mm dish. Add 5 mL complete RPMI 1640 media. Bring under a dissecting microscope.

5.1.2 Set up the bright field illumination on the microscope. Adjust the light intensity to ~50% of the maximum power. Under this condition, islets appear as slightly pink

clusters, whereas acinar cells as dark irregular-shaped clusters (Figure 1A). A magnification of ~50X (combining the power of the objective and the eye piece) is recommended to offer the best way of visualizing islets and the pipet tip opening simultaneously for picking.

5.1.3 Pick up islets with a P20 pipette tip while avoiding acinar clusters. Dispense islets-enriched fractions to a new 60 mm dish with RPMI complete media (Figure 1B).

5.1.4 Repeat the hand-picking process twice more. Leave pure islets in a new 60 mm dish with 4 mL complete RPMI media (Figure 1C).

5.2 Gradient centrifugation:

5.2.1 Prepare a 15 mL centrifuge tube that is preloaded with 2 mL polysucrose and sodium diatrizoate at a density of 1.077 g/mL. Leave the tube on ice until use.

5.2.2 Transfer the suspension from step 4.3 onto the top of the polysucrose and sodium diatrizoate solution with a Pasteur pipette. Do not disturb the bottom layer. Tissues from up to 10 newly born or 2 P17 pancreata can be loaded into each 15 mL tube.

5.2.3 Spin the tube at ~600 x g in a swing rotor for 15 minutes at 4 °C. Use brake-off settings.

5.2.4 After centrifugation, transfer the media and interphase layers (note that under ideal operation conditions, the islets will be located in the interphase between the polysucrose and culture media after centrifugation) into a new 15 mL tube. Add 10 mL media, *mix* gently but thoroughly.

5.2.5 Spin down the islet-enriched fraction at 300 x g for 5 minutes. Remove the wash media. Repeat the wash one more time with complete RPMI media. Resuspend pellets in to 4 mL media, which contains mostly islets and ducts (Figure 1D).

5.2.6 Hand-pick the islets as outlined in section 5.1.

6. GSIS assays in isolated islets

6.1 Incubate the islets from step 5.1.4 in complete RPMI media for two hours in a 37 °C tissue culture incubator.

6.2 Use a pipette to remove RPMI under a dissecting microscope. Add 3 mL KRB solution with 2.8 mM glucose to the plate[3]. Swirl 10 times to wash the islets. Remove KRB solutions.

6.2.1 Repeat the washing process once. Incubate the islets with 3 mL KRB solution at 37 °C for one hour in an incubator.

6.3 Aliquot 1 mL KRB into each well of a 12-well plate. Pre-warm the KRB stock and plate to 37 °C in an incubator.

6.4 Wash the islets twice with pre-warmed KRB as in 6.2. Transfer 10 islets to each well of the pre-warmed plates. Incubate in a 37 °C incubator.

6.5 After 45 minutes, withdraw 50 µL of supernatant. This contains the insulin solution secreted under basal glucose.

6.6 Add 32 µL 500 mM glucose. Swirl the plate 20 times to mix the solution. Incubate in a 37 °C incubator. Swirl the plate 5 times once every 5 minutes.

6.7 After 45 minutes, withdraw 50 µL supernatant. This is the insulin secreted under basal and high glucose.

6.8 Transfer all islets to a 1.5 mL tube. Freeze in -20 °C for 30 minutes. Thaw at room temperature for 5 minutes. Repeat the freezing and thawing process.

6.9 Add 0.5 mL 70% ethanol with 20 mM HCl. Leave overnight. This is the insulin left in islets.

6.10 Use conventional ELISA to assay insulin levels, described previously[3].

REPRESENTATIVE RESULTS:

Under optimal conditions, the presented method can yield 20-80 islets from each small mouse pancreas. This number depends on the genetic background, age of mice, and the size of islets to be recovered. Among the commonly used, CD1 out-bred and C57BL/6J pure-bred mice produce less islets with smaller size than hybrids between CD1 and C57BL/6 or commercial B6CBAF1/J mice do. Direct hand picking generally gave a smaller number of islets, likely due to the exclusion of small islets that could be hard to recognized when mixed with exocrine tissues (Figure 1A-C). Gradient centrifugation can yield more islets, including smaller islets in the mix (Figure 1D). Older mice also produce more and larger islets, as expected from continued islet proliferation after birth. Interestingly, the islet number recovered is not directly correlated with pancreas size: CD1 mice usually have bigger pancreas than F1 progenies of CD1 and C57BL/6J mice crossing. Yet CD1 mice usually produce less islets than the F1 progenies.

Either insufficient- or over-digestion with collagenase results in suboptimal islet isolation. In the former case, large islets can be readily visualized, yet some islets cannot be completely separated from acinar tissues (Figure 2A). This will reduce the yield of islets, but larger islets are usually produced. In the latter case, acinar tissues can be completely dissociated from islets. Yet this compromises the islet structure, resulting in many islets with rough surfaces (Figure 2B).

Neonatal islets isolated by this method have expected insulin secretion profiles. For example, both P1 and P7 islets displayed high basal insulin secretion (Figure 3, compare the levels of insulin secretion at 2.8 mM glucose between P1-P7 and mature P24 islets), typical GSIS profiles of immature islet beta cells[7, 9]. This suggests that our islet isolation process largely conserves the functional properties of neonatal islets. We therefore expect that these islets are likely fit for other *in vitro*-based studies, including gene expression, metabolic analyses, survival assays, and stress responses.

Figure 1. Appearance of islets during the isolation process.
(A) P1 pancreata after collagenase digestion. Arrow points to a relatively large islet. Arrowheads point to two relatively small islets. (B) P1 islets after the first round hand-picking. (C) Islets after the 3rd round handpicking. (D) Islet fractions after gradient centrifugation. Arrow, a large islet. Arrowhead, a small islet.

Figure 2. Islets after insufficient- or over- digestion with collagenase.
(A) Hand picked islets after under-digestion, note the association between some islets (pink clusters, arrows) and acinar cells (dark clusters, arrowheads). (B) Islets after over-digestion, note the islets with jagged surfaces (arrows).

Figure 3. GSIS results from isolated islets.
Presented data are mean ± SEM. They represent the percentage of insulin release (amount of insulin released over the total amount of insulin contained in starting islets) within a 45 minutes assay window with indicated glucose concentration. Islets from ICR mice, via direct hand picking, were utilized for these assays. Note that P1 and P7 islets were considered immature, whereas P24 islets are mature[7, 9]. The P values are calculated between groups using t-test.

DISCUSSION

Here, we provide a step-by-step protocol on islet isolation from pancreata that are too small for conventional perfusion. It is expected to yield islets ready for all islet-based studies such as beta-cell purification, gene expression analysis, islet beta-cell maturation, proliferation, cell stress responses, cell survival, metabolism, and functional GSIS maintenance etc. This will be, to the best of our knowledge, the first detailed visual protocol that guides new researchers to perform islet isolation from neonatal mice. Without these visual aides, extensive trial and error is expected for most researchers to achieve successful islet isolation from small pancreata.

The most critical factor for a successful islet isolation is the proper degree of pancreatic digestion as outlined in steps 3.6-3.8. In general, both under- and over-digestion reduce islet yield. Over-digestion further compromises islet architecture, which makes them unsuited for functional assays. To achieve best digestion results, the reaction has to be constantly monitored to visualize the tissue fragmentation process. Counting on the duration of digestion to judge the acinar-islet separation is the least dependable method, because each batch of collagenase is different and the age/size of the starting pancreas profoundly impacts the digestion process. In case of under-digestion, large endocrine-exocrine clusters can be washed in HBSS and digested again with collagenase. The

digestion can then be monitored directly under a dissecting microscope to determine the time of continued digestion. Gentle pipetting can also be utilized to aide tissue dissociation. In this case, a P1000 tip with wide opening needs be utilized, which lessens the shearing force that could destroy the islet architecture. Over-digested islets are not recommended for most subsequent studies, but can be used for some assays, such as gene expression analysis with careful controls.

Beside the above caution, paying attention to several procedural factors can further improve the final islet yield and quality. First, Ca^{2+} and Mg^{2+} should be included in all solutions, if non-commercial sources were utilized. These cations are necessary to maintain islet integrity. Second, fresh collagenase solution with high activity is essential. Collagenase solution with low activity results in a longer time for tissue dissociation. This causes substantial exocrine cell autolysis and islet destruction. To this end, utilizing collagenase stocks with more than three-rounds of freezing-thawing is not recommended. Third, low speed centrifugation during tissue washing is recommended. The rule of thumb is to use a g force (<500 x g) that is sufficient to sediment the cell clusters while maintain cell debris in the supernatant in step 4.1 of the protocol. This speed not only avoids destroying islets, but also helps to remove cell debris to enable easier islet visualization during hand picking. Lastly, collagenase cannot be inactivated by serum [12]. Thus, its removal by washing is essential to ensure islet integrity in later studies.

Finally, it should be noted that the presented protocol should be limited to mouse pancreata from P1 to P17. It does not work well for pancreata older than P18. Conventional perfusion for these older mice is recommended for better yields and healthier islets. Moreover, the genetic background and age of mice profoundly affect the islet yield [13,14]. Therefore, even optimal conditions will yield different number of islets per pancreas, which is normal and expected.

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DISCLOSURE
The authors declare no competing financial interests for the described work.

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Figure 1

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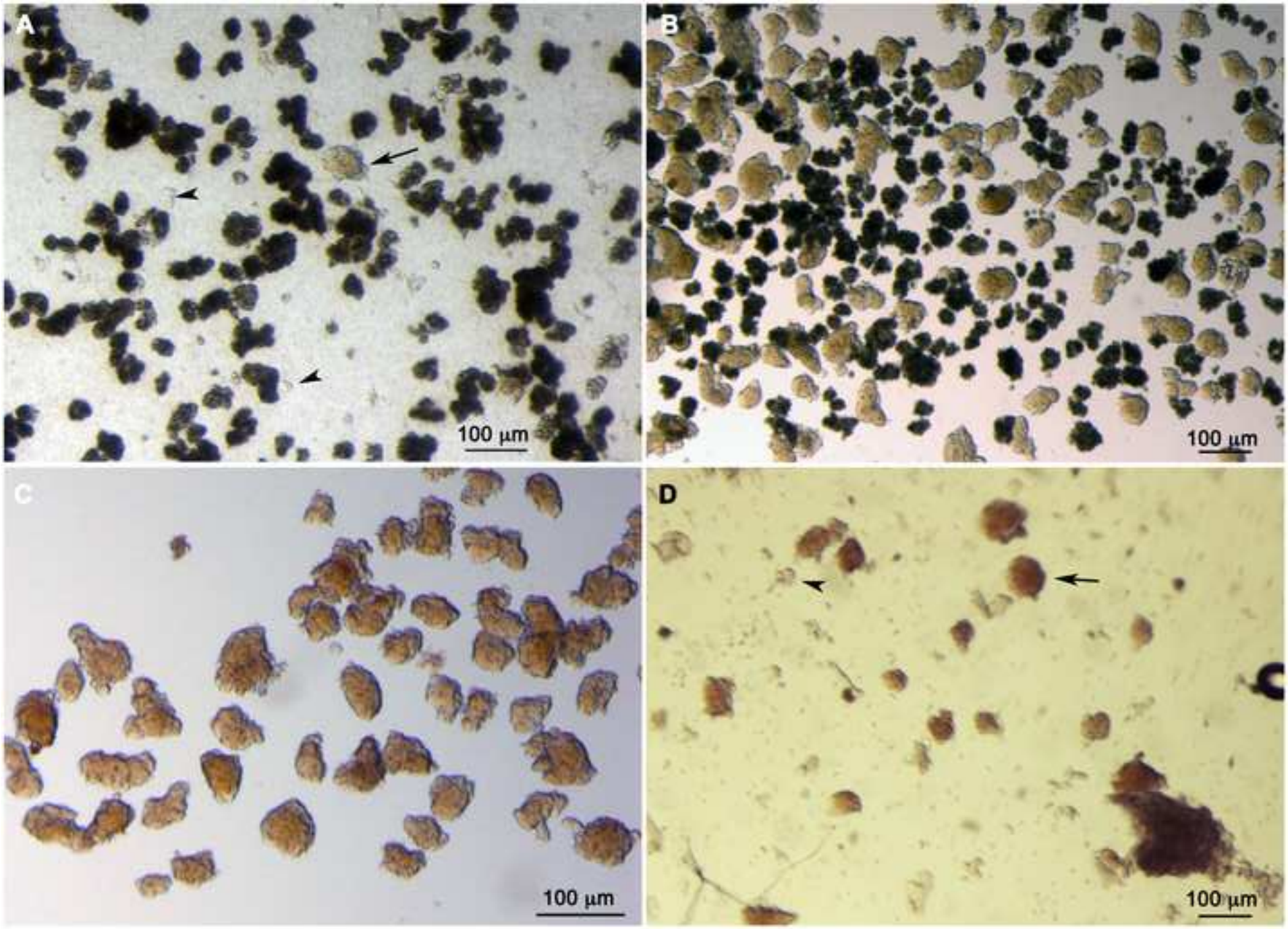

Figure 2

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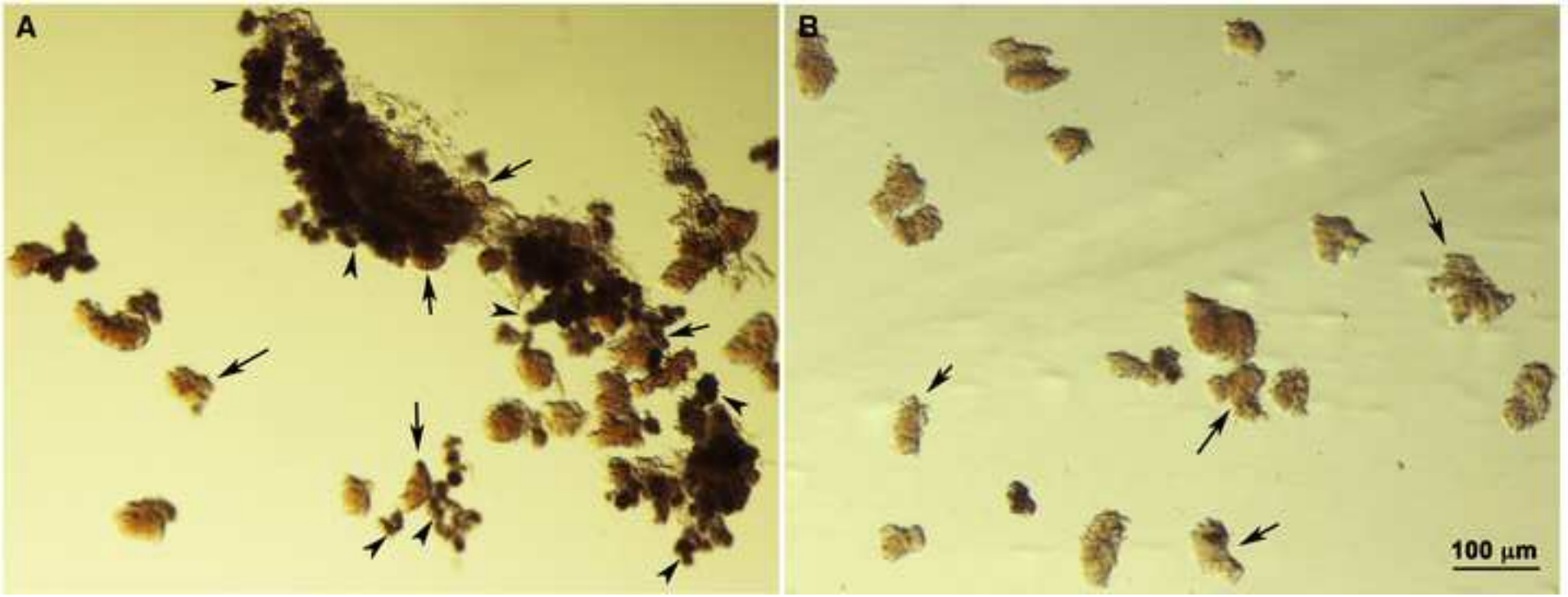

Figure 3

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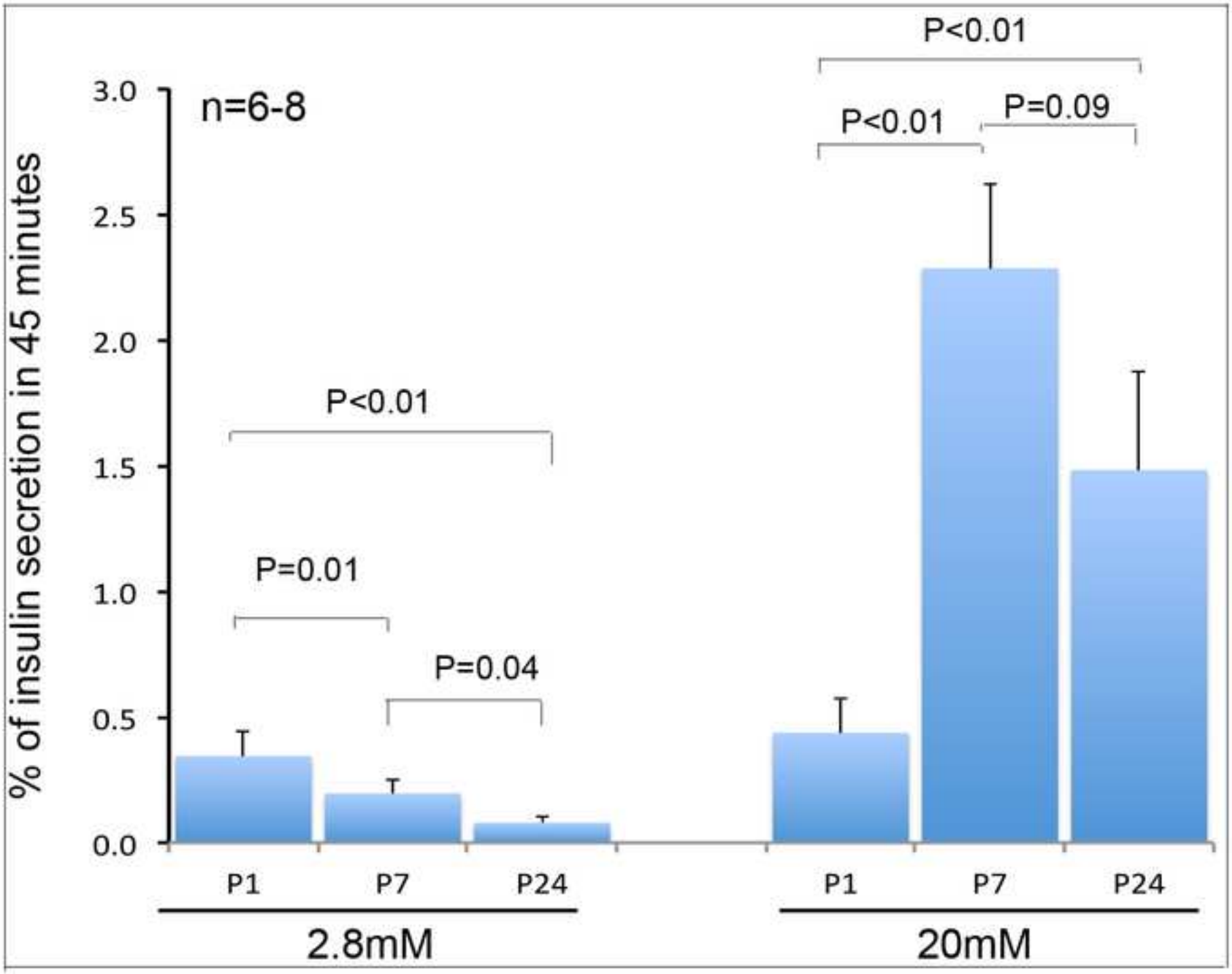

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Collagenase Type IV	Sigma Aldrich, St Louis, MO	C5138	
1X HBSS with Ca2+/Mg2+	Mediatech/Cellgro, Manassas, VA	MT21020CV	If HBSS wihout Ca2+/Mg2+ is obtained, CaCl2 and MgSO4 can be added to 1.26 and 0.5 mM,
RPMI 1640 w/o glucose	Thermo Fisher Scientific/Life Technologies, Waltham, MA	11879-020	Glucose needs to be added to specific levels to not interfere with seusequent islet usage.
Glucose	Sigma Aldrich, St Louis, MO	G-7021	
polysucrose and sodium diatrizoate solution	Sigma Aldrich, St Louis, MO	Histopaque-10771	
Stereoscope	Carl-Zeiss, Oberkochen, Germany	Stemi2000	
Stereoscope	Leica, Wetzlar, Germany	Leica M165	
Microcentrifuge	Eppendorf, Hauppauge, NY	Centrifuge 5417C	
Centrfuge	Eppendorf, Hauppauge, NY	Centrifuge 5810R	
15-ml centrfuge tubes	VWR, Radnor, PA	89039-666	
50-ml centrfuge tubes	VWR, Radnor, PA	89039-658	
Precision balance	VWR, Radnor, PA	VWR-225AC	
Microfuge tubes	VWR, Radnor, PA	87003-294	
Pipetman P1000	Fisher Scientific, Waltham, MA	F123602	
Pipetman P20	Fisher Scientific, Waltham, MA	F123600	
100X15 millimeter dish	VWR, Radnor, PA	25384-088	
60X15 millimeter dish	VWR, Radnor, PA	25384-168	
12-well plates	VWR, Radnor, PA	665-180	
Scissor	Fine Scientific Tools, Foster City, CA	14080-11	
Tweezers	Fine Scientific Tools, Foster City, CA	5708-5	
CD1 mice	Charles River Laboratories, Wilminton, MA	CD-1	
C57BL/6J	The Jackson laboratory, Farmington, CT	C57BL/6J	
B6CBAF1/J	The Jackson laboratory, Farmington, CT	B6CBAF1/J	

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CORRESPONDING AUTHOR:

Name: Guoqiang Gu

Department: Cell & Dev. Biology

Institution: Vanderbilt University Medical school

Article Title: Effective isolation of functional islets from neonatal mouse pancreas

Signature:  Date: 6/22/16

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Dear Editor Nguyen,

Thank you very much for helping with this manuscript. We also thank the reviewers for their precious input about improving the paper. Below is a detailed changes we have made to address the critiques.

From Editor:
Critique 1. Formatting: Acknowledgements – Who is Dr. XXX? We assume this is a placeholder.
Reply: Names of two colleagues helping with the manuscript were added.

Critique 2. Grammar:
-Line 44 – "Tubes will be regularly" – wrong verb tense
Reply: Changed to "were tapped regularly"

1.3 – "several pair" -
Reply: Changed to "several pairs".

1.4 – "Dissolved in"
Reply: The sentence is changed to "add 50 ml RPMI 1640 media to dissolve the glucose."

Critique 3. Additional detail is required: 6.1 note – Please describe the kind of methods used, at minimum.
Reply: A brief description of the method is added (6.2-6.10).

Critique 4. Branding: Charles River should be removed from the ethics statement and used in the materials table instead.
Reply: The information was moved to materials as suggested.

Reviewer #1: *Manuscript Summary:* Accept.

Reply: Thanks for the positive opinion.

Reviewer #2: *Manuscript Summary:* The authors describe an optimized protocol for islet isolation from neonatal mouse pancreata. This method should be useful for the developmental biology of pancreatic islets in mice.

Reply: Thanks for the positive opinion.

Major Concerns: Possible effects of the genetic background should be clarified for the application of this method. As the authors describe that "In general, ICR and CBA/Bl6 pure-bred mice produce less islets than hybrids between ICR and CBA/Bl6 mice do", is it

the size of their pancreata that results in different yields? Were these the only two strains of mice tested? What about C57BL/6 mice that are most commonly used?

Reply: Thanks for reading into these details. Now we have included more details to describe results of the regularly used CD1 and C57BL/6 lines and the pronuclear injection recipient line (B6CBAF1/J) of our studies.

To directly answer the reviewer's question: the size of the pancreas is not directly related with islet number. We have tested more than three strains with different intercrosses. Unfortunately, due to the method-nature of the journal, providing all the details in islet size, number, and function in relation with genetic background, age, and pancreatic size is out of the scope.

Minor Concerns:

1. p.1. line 44: Change "will be" to "were".
Changed as suggested.

2. p.2. line 81: Change "laymen" to "researchers". Done
Changed as suggested.

3. p.4. line 139: Change "into" to "to". Done
Changed as suggested.

4. p.6. line 237 and 256: Change "jagged" to "rough". Done
Changed as suggested.

5. p.6. line 259: Change "+" to "±". Done
Changed as suggested.

6. p.7. line 314: Fill "Dr. XXX". The names were now added.
Changed as suggested.