Video Article

# The Inverted Heart Model for Interstitial Transudate Collection from the Isolated Rat Heart

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## Abstract

The present protocol describes a unique approach that enables the collection of cardiac transudate (CT) from the isolated, saline-perfused rat heart. After isolation and retrograde perfusion of the heart according to the Langendorff technique, the heart is inverted into an upside-down position and is mechanically stabilized by a balloon catheter inserted into the left ventricle. Then, a thin latex cap – previously cast to match the average size of the rat heart – is placed over the epicardial surface. The outlet of the latex cap is connected to silicon tubing, with the distal opening 10 cm below the base level of the heart, creating slight suction. CT continuously produced on the epicardial surface is collected in ice-cooled vials for further analysis. The rate of CT formation ranged from 17 to 147 µL/min (n = 14) in control and infarcted hearts, which represents 0.1-1% of the coronary venous effluent perfusate. Proteomic analysis and high performance liquid chromatography (HPLC) revealed that the collected CT contains a wide spectrum of proteins and purinergic metabolites.

## Video Link

The video component of this article can be found at https://www.jove.com/video/55849/

## Introduction

Heart failure (HF) is the leading cause of death in humans worldwide[1]. HF often occurs because of myocarditis, ischemic insults to the myocardium, and left ventricular remodeling, leading to the progressive deterioration of cardiac contractile function and patients' quality of life. Although advances in cardiology and cardiac surgery have remarkably lowered HF mortality, they merely serve as transient "delayers" of an inevitably progressive disease process that carries significant morbidity. Therefore, the current lack of effective treatment underscores the need to identify novel molecular targets that can prevent or even reverse HF. This includes changes in the extracellular matrix, uncontrolled cardiac immune response, and interactions between cardiac and non-cardiac cells[2].

It is important to recognize that the microenvironment that cardiac cells are exposed to directly shapes the immune and regenerative response of the injured heart. In the isolated, saline-perfused heart, CT is generated on the heart surface in the form of small droplets that are derived from the interstitial fluid space (*i.e.,* microenvironment), both under physiological and pathophysiological conditions[3,4,5]. Therefore, analysis of the CT (*i.e.,* interstitial fluid) may help to identify factors that regulate cardiac metabolism and contractile function[6] or influence immune cell functions after migration into the injured heart. Potentially, this may lead to the development of novel therapeutic strategies for the treatment of HF.

The collection of CT from murine hearts is technically challenging. In regular Langendorff-perfused hearts, the exclusive collection of CT is difficult because the mixture of the CT with coronary venous effluent perfusate unpredictably dilutes any concentration of metabolites/enzymes released from the interstitial space. One possible strategy to overcome this limitation is to exclude the venous effluent by cannulating the pulmonary and simultaneously ligating the pulmonary vein[7]. However, this method faces difficulties associated with the cannulation and ligation of the pulmonary artery and vein, causing potential leakage of venous effluent into the cardiac transudate. The concept of using a reverse heart model was first introduced by the group of Kammermeier, who inverted the isolated, perfused heart into an upside-down position and placed a thin latex cap on the epicardial surface to continuously sample CT without the contamination of venous effluent[8,9]. Using this procedure, CT was shown to provide a very sensitive measure of the metabolites released from the heart[9], the capillary transfer of fatty acids[8], and viral particles[10].

More recently, paracrine factors that may regulate the local immune response and augment cardiac angiogenesis[11] have been implicated in the beneficial effects of stem cell-based therapy for heart disease. The analysis of CT in the reversed heart may help to chemically identify these individual paracrine factors. In addition, CT may help to identify the factors involved in the *in vivo* activation of immune cells in the heart.

The detailed description of CT collection from the heart surface, provided here, is experimentally useful for researchers studying the interplay of immune cells, fibroblasts, endothelial cells, and cardiomyocytes in relation to overall cardiac function. As mentioned above, the interstitial fluid

 

carries the information for cell-to-cell communication within the heart, which can conveniently be assessed by the collection of CT. The detailed technical description, including a video protocol of how to collect CT from the reversed heart, should facilitate the future application of this unique technique.

## Protocol

All experiments were approved by the local regulatory agency (*LANUV* of Nordrhein-Westfalen, Germany) and were performed according to the guidelines of animal use. Animals were fed with a standard chow diet and received tap water *ad libitum*. All equipment and chemicals necessary to each step of the experiment are available in the **Table of Materials**.

## 1. Preparation of the Latex Cap and Intraventricular Balloon

1. Make an aluminum mold using a milling machine that matches the average size of the rat heart (bodyweight of 300-350 g). Polish the mold with superfine (10/0) emery paper.
   NOTE: The detailed metrics of the mold are shown in **Figure 1A**.
2. Vertically fix the neck of the aluminum mold to the milling machine to prepare the latex cap.
   NOTE: The milling machine causes the mold to slowly rotate. Alternatively, an electric motor can be used.
3. Pour 20 mL of liquid latex (commercially purchased, see the **Table of Materials**) into a 50 mL glass beaker.
4. Lower the mold until the entire body of the mold is immersed in the latex solution.
5. Slowly lift the mold (5 cm/min) while rotating.
6. Keep rotating the mold for an additional 15 min, until the latex on the surface of the mold is solidified.
7. Add about 1 g of talcum powder to the surface of the mold (already covered by a thin latex film) to prevent damage while detaching.
8. Gently detach with fingers the already dried latex cap from the mold surface; the latex cap is now ready for use (**Figure 1B**).
9. Connect the outlet of the latex cap to 15-cm silicon tubing (ID = 0.2 mm), used later for the collection of CT.
10. Fill the ventricular latex balloon with water and firmly fix it onto an L-shaped metal cannula connected to a 1 mL, water-filled syringe (**Figure 1C**).
    NOTE: This will be used to ensure the upright positioning of the heart (see below).
11. Make sure that the balloon is airtight by performing several deflating/inflating tests with the attached 1 mL syringe.
12. Connect the cannula, via a three-way stop, to a pressure transducer for the future measurement of intraventricular developed pressure (**Figure 1C**).

## 2. Preparation of Krebs-Henseleit Buffer (KHB) and the Langendorff Perfusion System

1. Set up a Langendorff perfusion system by using either constant-flow (driven by a roller pump) or constant-pressure (generated by static pressure in a glass column) mode.
   NOTE: Details of the Langendorff heart preparation have been described previously[12].
2. **Prepare 2 L of a modified KHB (in mM: 116.02 NaCl, 4.63 KCl, 1.10 $MgSO_4 \cdot 7H_2O$, 1.21 $K_2HPO_4$, 2.52 $CaCl_2 \cdot 2H_2O$, 24.88 $NaHCO_3$, 8.30 D-glucose, and 2.0 sodium pyruvate).**
   1. Weigh all chemicals but $CaCl_2$ and dissolve them in 1.8 L of double-distilled water in a 2-L flask.
   2. Bubble the medium with carbogen (95% $O_2$/5% $CO_2$) for at least 5 min for equilibration (pH: 7.4) under magnetic stirring.
   3. Add 0.74 g of $CaCl_2.2H_2O$ and bring up the total volume to 2 L with double-distilled water.
   4. Continue stirring and bubbling the medium with carbogen for an additional 5 min.
   5. Filter the KHB through a 0.2 µm filter to eliminate small particles that may obstruct the microcirculation of the heart.
3. **Preparation of the Langendorff perfusion system.**
   1. Place the filtered KHB into a pre-warmed water bath (38 °C); keep bubbling with carbogen to generate a pressure of 100 $cmH_2O$ inside the KHB reservoir.
   2. Connect the reservoir to the glass column to establish 100 $cmH_2O$ hydrostatic pressure for the Langendorff perfusion with KHB; continue bubbling the KHB inside the column with carbogen.
   3. Adjust the temperature of the warming system so that the temperature at the aortic cannula outlet is 37 °C.
   4. Ensure that the tubing system is bubble-free.
   5. Oxygenate the KHB with carbogen for an additional 5 min, until the $PO_2$ in the KHB reaches 500-600 mmHg (measured by a blood-gas analyzer).
4. Set up the perfusion system to run either at a constant pressure of 100 $cmH_2O$ or at a constant flow of about 10-20 mL/min using manual switching. Alternatively, use an interchangeable STH pump controller to instantly switch to the perfusion mode.

## 3. Isolation and Cannulation of the Heart

NOTE: Male Wistar rats with bodyweights of 300-350 g were used so that the sizes of hearts matched the pre-cast latex cap. Rats underwent either a ligation of the left arterial descending (LAD) for 50 min, followed by reperfusion or were sham operated. Details of the methodology for the induction of myocardial infarction (MI) were reported elsewhere[13]. The reversed-heart experiments in the infarct animals were performed 5 days after operation.

1. Anesthetize rats using an isoflurane vaporizer (2% V/V) connected to an animal holding chamber (20 L).
2. Transfer the rats to an operation table (not temperature controlled) after deep anesthesia is reached.
3. Lift the skin and muscle just below the sternum using forceps and cut around the lower margin of the ribs with heavy scissors.

4. Using fine scissors, make a small cut into the diaphragm, at the rib margin. Cut the ribs caudally to make a flap of the entire ventral chest wall.
5. Gently grab the heart with the thumb and index and middle fingers and slowly lift it upwards so that the cardiac vessels become slightly stretched.
6. Excise the heart until the aorta is fully exposed.
7. Place the heart in a 100 mL beaker containing 50 mL of ice-cold KHB (4 °C) and move it to the perfusion apparatus.
8. Immediately mount the heart via the aorta onto a dripping cannula and securely tighten it with a suture (4-0). Avoid air bubbles entering the heart.
9. Apply constant perfusion pressure (100 $cmH_2O$). Alternatively, a full flow rate (starting with 20 mL/min) can be applied.
   Note: The time from the opening of the thorax to the attachment of the heart to the perfusion cannula should take about 3 min in the hands of an experienced operator.

## 4. Reversed-heart Model

1. Gently rotate the aortic cannula until the posterior wall of the heart is in *en face* view.
2. Remove the connective tissue with scissors to expose the opening of the left atrium, making it ready for intraventricular cannulation.
3. Insert the deflated latex balloon attached to a rigid catheter via the left atrium into the left ventricle.
4. Inflate the balloon until it fills the entire ventricular cavity (the inflating volume is pre-marked on the syringe).
5. Invert the heart until it is upside-down, supporting it by the intraventricular balloon catheter.
6. As demonstrated in **Figure 1C**, mechanically stabilize the inverted heart in an upright position using the intraventricular balloon with a rigid metal catheter.
7. Adjust the position of the heart to avoid excessive twisting of the aortic root.
8. Adjust the diastolic pressure to 3-5 mmHg (measured by the intra-ventricular balloon; see **Figure 1C**).
9. Observe the epicardial surface of the heart and ensure that small droplets form.
10. Place the latex cap onto the surface of the heart by gently pushing it to cover the entire heart using the fingers.
11. Make sure that the latex cap covers most of the ventricular surface.
12. Remove the air bubbles, if any, inside the cap and the tubing by gently sucking with a 1-mL syringe.
13. Adjust the distal opening of the CT-letting tubing to 10 cm below the horizontal level of the heart.
    NOTE: This procedure ensures slight sucking by negative hydrostatic pressure.
14. Collect drops of CT in a 1.5 mL collection tube placed in ice mixed 1:1 with NaCl. Collect about 0.15-1.5 mL of CT.
    NOTE: The ice/NaCl mixture stabilizes the temperature in the collection tube to below zero (about -4 °C).
    NOTE: The sampling time depends on the experimental purpose. The flow rate of the CT is about 27 ± 20 µL/ min in sham-operated animals (n = 3) and 100 ± 47 µL/min for the coronary-ligated animals (n = 11).
15. Weigh and snap-freeze CT samples in liquid nitrogen and store them at -80 °C for later measurements.

## 5. Analysis of the CT

1. Use the CT fluid for the analysis of metabolites, depending on the scientific question.
   NOTE: The data shown in **Figure 2** and **Figure 3** were collected from a constant-pressure perfusion (100 $cmH_2O$), and about 0.15-1.5 mL of CT fluid was collected within a period of 10 min. This time and volume were sufficient for the proteomic (minimum: 50 µL; **Figure 2**)[14] and HPLC (minimum: 20 µL; **Figure 3**)[15] analysis of various purines.

## Representative Results

The reversed-heart model enables the collection of cardiac interstitial transudate in an isolated, retro-perfused rat heart (**Figure 1A-C**). When perfused at a constant pressure of 100 $cmH_2O$, the rate of interstitial fluid formation ranged between 17 and 147 µL/min, amounting to 0.1-1% of the coronary venous effluent in the isolated heart.

The protein content of the CT, as measured with the bicinchoninic acid (BCA) assay, was found to be 1.08 ± 0.40 mg/mL (n = 6). One-dimensional gel electrophoresis analysis (SDS-PAGE) revealed a wide spectrum of proteins present in the cardiac transudate (**Figure 2A**). Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) was performed on CT from the heart subjected to 50 min of ischemia/reperfusion. As shown in **Figure 2B**, several proteins were found to be upregulated in the CT of ischemic hearts. Among the proteins identified, 70.1% were extracellular matrix proteins, 4.6% were cellular membrane proteins, 17.2% were cytoplasmic proteins, and 2.3% were nuclear proteins (**Table 1**).

Purines have long been considered pivotal signaling molecules that regulate the cardiac immune response, vasomotor tone, and cardiac function, particularly after ischemic injury. The collection of CT permits the measurement of a variety of metabolites present in the cardiac interstitial fluid under pathophysiological conditions, such as MI. As shown in **Figure 3**, the concentration of AMP, GMP, NADP, adenosine, hypoxanthine, and uric acid measured by HPLC where higher in the ischemic heart, which is similar to results previously reported using other methods[16,17].

| ID | Gene Name | Location |
|---|---|---|
| Apoc1 | apolipoprotein C1 | Extracellular Space |
| ApoE | apolipoprotein E | Extracellular Space |
| Agt | angiotensinogen | Extracellular Space |
| Ces1c | carboxylesterase 1C | Extracellular Space |
| Alb | albumin | Extracellular Space |
| Klkb1 | kallikrein B1 | Extracellular Space |
| Cst3 | cystatin C | Extracellular Space |
| F13a1 | coagulation factor XIII A chain | Extracellular Space |
| Cp | ceruloplasmin (ferroxidase) | Extracellular Space |
| Adipoq | adiponectin, C1Q and collagen domain containing | Extracellular Space |
| Mfap4 | microfibrillar associated protein 4 | Extracellular Space |

**Table 1: List of upregulated proteins in the CT of ischemic hearts.** CT from ischemic hearts was analyzed by 2D-DIGE and identified by proteomics. Please click here to download this table.

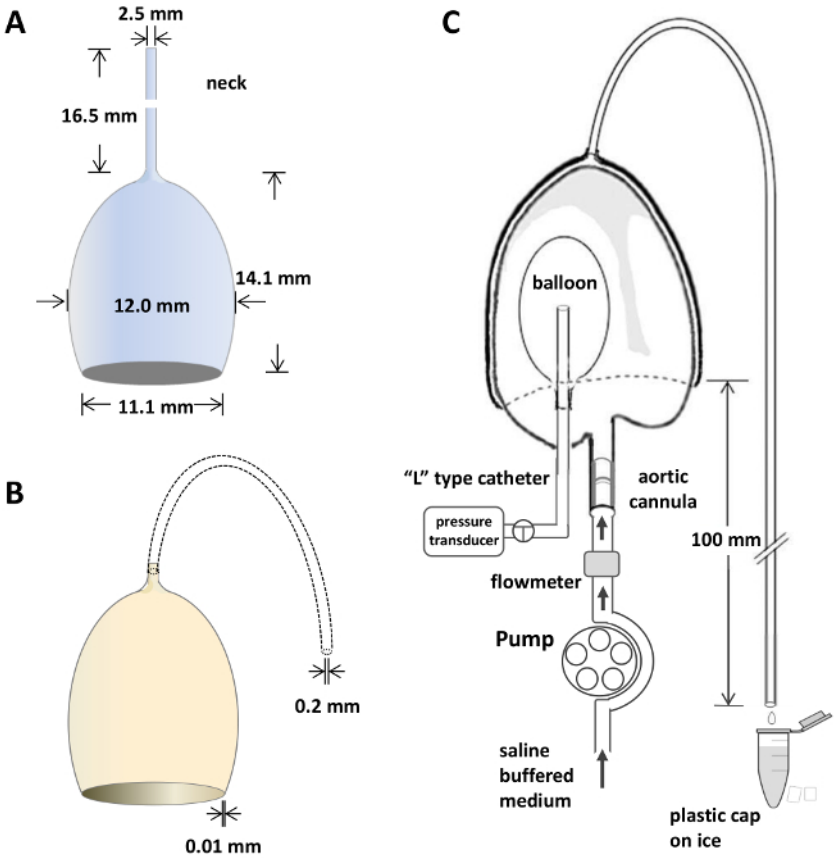


**Figure 1: Schematic drawings of the reversed-heart model.** (**A**) An aluminum mold was constructed from an appropriately shaped and sized rat heart. (**B**) After immersing the mold into latex solution, a latex cap, with a thickness of about 0.01 mm, was casted. (**C**) In a Langendorff heart apparatus, the heart was perfused via the aorta using an aortic cannula, which was later inverted into an upside-down position and was mechanically supported by an intra-ventricular balloon placed into the left ventricle. Intraventricular pressure development was monitored via a pressure transducer. The latex cap covered nearly 90% of the surface of both the right and left ventricle, and the outlet was connected to silicon tubing (ID = 0.2 mm), with the distal opening 10 cm below the base of the heart. This generated slightly negative hydrostatic pressure. The cardiac transudate is usually collected into an ice-cooled, 1.5 mL tube. Please click here to view a larger version of this figure.

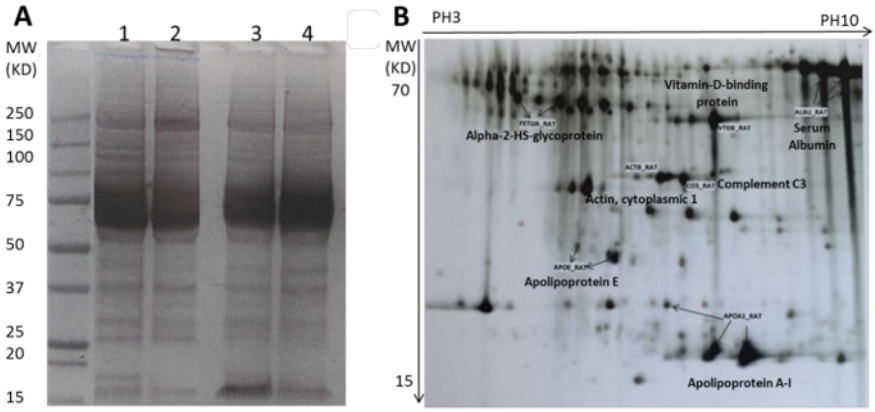


**Figure 2: Proteomic analysis of the CT.** Proteins in the CT were separated by SDS-PAGE (**A**) and identified by 2D-DIGE analysis (**B**). For (A), lanes 1-4 indicate cardiac samples from individual hearts (1 and 2 = sham; 3 and 4 = infarcted). For (B), 2D-DIGE was performed on the CT from an infarcted heart. The protein identity was confirmed by liquid chromatography (LC)-MS/MS[14,15]. Please click here to view a larger version of this figure.

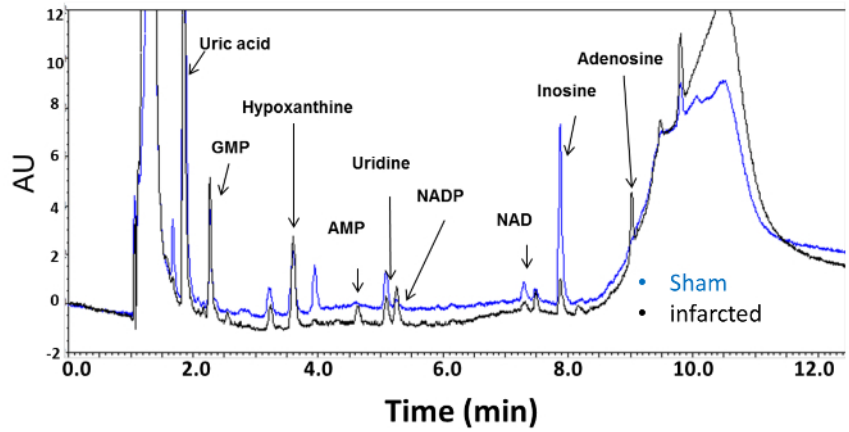


**Figure 3: Purines in the CT.** Various purines present in the CT were analyzed by HPLC. Representative HPLC runs from the sham (blue) and infarcted (black) heart show that the infarcted heart exhibits a higher interstitial concentration of AMP and adenosine, but not hypoxanthine. Please click here to view a larger version of this figure.

## Discussion

The reversed-heart model is based on the well-established Langendorff heart perfusion technique[12] and is performed by simply inverting the heart into an upside-down position and holding this position using a rigid intra-ventricular balloon catheter. In such a way, cardiac interstitial transudate can be physically separated from coronary venous effluent perfusate, dripping by gravity from the base of the heart[9]. The CT can be continuously collected by means of a thin and flexible latex cap placed on the surface of the entire heart.

The method is easy to perform, with minimal cost in addition to that of the Langendorff apparatus. Nevertheless, some steps are technically critical for obtaining reproducible and stable results. These include ensuring that the latex cap properly fits the shape of the hearts and covers about 90% of the surface of the ventricles, not the atria. The time from excising the heart from the animal to the performance of the retro-perfusion should be less than 3 min, as prolonged ischemia bears the risk of altering cardiac metabolism and the formation of cardiac transudate. The diastolic left ventricular pressure, measured by the intra-ventricular balloon, should be set to fill the ventricular cavity (3-5 mmHg). An over-inflated balloon may alter coronary flow by vascular compression. The sampling vial (1.5 mL tube) should be kept on ice to avoid any potential degradation of metabolites and proteins of interest.

Additionally, successful experiments are critically dependent upon good manual handling during the preparation, isolation, and cannulation of the heart. This requires practice. To protect hearts from ischemic damage, all preparations should be carried out with ice-cold KHB. Since the sizes of the hearts may vary between rats, in spite of similar bodyweights, it is advisable to have latex caps prepared with slightly different dimensions that suit the different sizes of the hearts.

The isolated reversed-heart method has been previously described for the isolated rat[5,6,7,8,9,10] and guinea pig heart[16] and was used for different purposes. In our present description of the methodology, we have introduced some modifications to the experimental setup and to sample processing. For example, the cannulation of the pulmonary artery, as introduced by de Deckere *et al.*[7], was not performed here, since the inverted position of the heart prevents potential contamination with venous effluent perfusate. The collecting device for CT was simplified by introducing a slight negative pressure by lowering the opening of the CT-letting tubing to 10 cm beneath the reverse heart. This makes it easier to immediately cool down the transudate samples. To ensure the rapid cooling of the sample CT, the collection cups were precooled to

a temperature of -4 °C by placing them into an equal-volume mixture of ice and NaCl (*i.e.,* a 1:1 ratio). This permitted the rapid cooling of the collected CT samples.

Generally, one should keep in mind that the isolated rat heart differs from *in vivo* physiological conditions, in that the interstitial fluid formation is most likely less than in the saline-perfused heart. The CT formed by the isolated heart may therefore not entirely mimic the true composition of the *in vivo* interstitial fluid. Additionally, the present setup does not permit the full exclusion of potential contamination with venous effluent perfusate. However, since the venous outlet is located at the cardiac base (the lowest level of the upright heart), we do not believe that contamination contributes to the collection process.

The present protocol describes a unique method to sample cardiac interstitial fluid, which contains a multitude of metabolites and proteins released into the interstitial fluid by cardiomyocytes and non-cardiac cells, such as immune cells, endothelial cells, vascular smooth muscle cells, fibroblasts, and pericytes. Cardiac interstitial transudate is formed as the result of fluid transport across the endothelial barrier[10], together with a small fraction of lymphatic fluid. It contains a mixture of cardiac metabolites[7], soluble factors in the interstitial space, and secretomes of cardiac and/or non-cardiac cells[9]. Therefore, several cell types contribute to the formation of CT. In addition, there are several factors that affect the rate of formation. First, oncotic pressure seems to be a major determinant that regulates trans-capillary fluid movement, since the increase of oncotic pressure by adding dextran or albumin to the perfusion medium significantly reduced the formation of CT[9,10]. Second, an increase in vascular permeability during hypoxia[9,16], including MI, augments the extravasation of the perfusate and thus the formation of CT. Therefore, in future studies, the increase of oncotic pressure may be a suitable means to minimize the volume of CT, thereby enriching the targeted molecules of interest.

## Disclosures

The authors declare that they have no competing financial interests.

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