ExoLution™ Plus cfDNA + exoRNA Isolation Kit

Protocol Guide

Research Use Only. Not for use in diagnostic procedures.

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Purpose

The ExoLution™ Plus cfDNA + exoRNA Isolation Kit is an isolation kit that simultaneously extracts cell-free DNA (cfDNA) and exosomal RNA (exoRNA) from the plasma fraction of whole blood. The Kit is designed for use with Asuragen© QuantideX® exoMutation kitted products.

The ExoLution Plus cfDNA + exoRNA Isolation Kit is for research use only (RUO) and not for use in diagnostic procedures.

Limitations

- This Kit has been evaluated for use with QuantideX exoMutation kitted products as indicated in the associated kit protocol guide.
- Refer to the appropriate QuantideX exoMutation kit protocol guide for correct blood collection tube usage and pre-processing steps.
- This Kit is intended for Research Use Only. Not for use in diagnostic procedures.

Warnings and Precautions

- Use appropriate personal protective equipment when working with these materials.
- Follow Universal Precautions in compliance with OSHA 1910.1030, CLSI M29, or other applicable guidance when handling human samples.
- RNase/DNase contamination can cause degradation of the RNA samples and cDNA products. Use nuclease-free filter pipette tips and nuclease-free tubes. Clean bench surfaces before beginning work.
- RNA/DNA carryover contamination can result in false positive signals. Use appropriate precautions in sample handling, workflow, and pipetting.
- Use of alternative plastics other than those provided or recommended in the **Required but not Provided** section may increase risk of isolation failure.
- Prior to use, ensure that all equipment (e.g., centrifuges, pipettes) are maintained, calibrated, and validated according to the manufacturer's instructions.
- **WARNING!** The Exosome Elution Buffer and Nucleic Acid Prep Buffer contain guanidine salts. Do not mix with bleach or strong acids as this may liberate toxic gases.
- Use proper pipetting techniques to minimize sample cross-contamination due to splashing when adding buffers to columns.

Principle of the Procedure

The ExoLution™ Plus cfDNA + exoRNA Isolation Kit is designed to simultaneously capture cell-free DNA (cfDNA) and exosomal RNA (exoRNA) from the plasma fraction of whole blood. Whole blood can be collected in K_2EDTA collection tubes or commercially available stabilizer collection tubes designated by the downstream Asuragen© QuantideX® exoMutation Kit protocol guide. The resultant eluant contains total nucleic acid (cfDNA and exoRNA) and is ready for use in an Asuragen QuantideX exoMutation kit.

Background Information

The ExoLution™ Plus cfDNA + exoRNA Isolation Kit uses a spin column-based technology for co-isolation of cfDNA and nucleic acid content contained in exosomes from plasma. The Kit may be used for 0.5 – 5 mL of human plasma from whole blood samples. For more information on sample isolation volume requirements, consult the protocol guide of the downstream Asuragen QuantideX exoMutation kit.

The isolation consists of two sequential bind – wash – elute steps to isolate highly pure nucleic acids. First, circulating nucleic acids and exosomes are selectively bound to the ExoLution Plus Column Unit. Second, the captured components are lysed and conditioned for silica-based column nucleic acid isolation.

The ExoLution™ Plus cfDNA + exoRNA Isolation Kit is free of phenol and does not require lengthy organic phase separation to access the samples' nucleic acid content. The procedure allows for parallel processing of multiple samples.

Workflow

Plasma is combined at a 1 to 1 ratio with 2X Binding Buffer to prepare the exosomes and cell-free DNA to bind to the Column Unit, followed by the Exosome Wash Buffer to remove background materials. Exosome Elution Buffer is applied to the column to release exoRNA and cell-free DNA. The elution is combined with Conditioning Buffer to precipitate additional contaminants.

Conditioned eluate supernatant is combined with isopropanol and bound to a Spin Column. The Spin Column is washed with both Nucleic Acid Prep Buffer and Nucleic Acid Wash Buffer. The total nucleic acid is eluted using Nucleic Acid Elution Buffer and collected for downstream assay use.

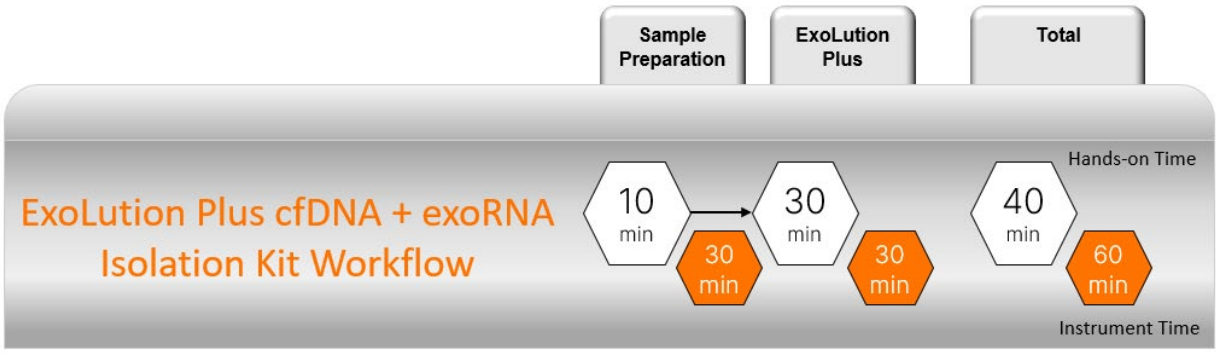


Figure 1. The workflow is streamlined from sample-to-isolate and can be performed in less than 2 hours with less than 1-hour hands-on time for 6 samples.

Reagents Supplied With the Kit

Item #	Description	Storage Temp.
A00659	2X Binding Buffer	18 to 25°C
A00660	Exosome Wash Buffer	18 to 25°C
A00661	Conditioning Buffer	18 to 25°C
A00662	Exosome Elution Buffer*	18 to 25°C
A00663	Spin Columns	18 to 25°C
A00657	Nucleic Acid Prep Buffer†	18 to 25°C
A00664	2 mL Collection Tubes	18 to 25°C
A00658	Nucleic Acid Wash BufferΦ	18 to 25°C
A00667	Nucleic Acid Elution Buffer	18 to 25°C
352070	50 mL Conical Tubes	18 to 25°C
A00666	Column Units	18 to 25°C
022431021	Elution Tubes	18 to 25°C

***** Contains a guanidine salt. Do not mix with bleach & take appropriate safety measures when handling. For additional safety information see §**Warnings and Precautions** and the item Safety Data Sheet (SDS).
† Contains a guanidine salt and ethanol. Do not mix with bleach & take appropriate safety measures when handling. For additional safety information see §**Warnings and Precautions** and item SDS.
Φ Add 68 mL of non-denatured Ethanol (96 – 100%) prior to use. Assign expiration of 1 year after addition of ethanol. See §**Pre-Isolation Steps**.

Required but not Provided

- General laboratory equipment and workspace to perform nucleic acid isolations
- Centrifuge capable of spinning 2 mL tubes at 16,000 x g at room temperature (15-25°C): Eppendorf 5425, or equivalent
- Centrifuge capable of spinning 50 mL conical tubes at 5,000 x g at room temperature (15-25°C) with swinging-bucket rotor: Allegra X-15, or equivalent
- Vortex mixer
- Pipettes with an accuracy range between 2–20 µL, 20–200 µL, and 100–1000 µL
- 1.5 or 2 mL Microcentrifuge tubes
- Non-denatured Ethanol / Ethyl alcohol (96 - 100% purity)
- Isopropanol / 2-propanol (>99% purity)
- 0.8 µm Leur Lock syringe filter: Millipore P/N SLAAR33SB, or equivalent
- 20 mL syringe: Becton Dickinson P/N 302830, or equivalent
- 15 mL sterile conical tube
- Chemical-resistant laboratory marker

Number of Reactions

- The reagents provided are sufficient for up to 50 isolations.

Reagent Stability

- The Kit reagents are stable through the labelled date when stored under the specified conditions.
- ExoLution Plus Nucleic Acid Wash Buffer (A00658) concentrate expires at the labeled date when stored under the specified conditions. Following the addition of ethanol, ExoLution Plus Nucleic Acid Wash Buffer (A00658) expiration is 1 year from the ethanol addition date or remains at the labeled expiration date, whichever occurs first.

Storage, Handling & Disposal

- Store reagents at 18 to 25°C.
- Use appropriate personal protective equipment when working with these materials.
- Do not pool components from different reagent lots.
- Do not use components from one kit lot in combination with components from a different kit lot.
- Do not use reagents after the labeled expiration date.
- Dispose of contents/containers in accordance with local, regional, national, territorial, provincial, and international regulations. Review the Safety Data Sheets (SDS) to determine the safe disposal of materials for your region. SDSs are available for download at https://asuragen.com/myasuragen. Avoid release to the environment.
- Contact Asuragen Technical Support immediately if the reagent packaging arrives damaged or opened. Asuragen Technical Support

Procedural Steps

Pre-Isolation Steps

- 68 mL of non-denatured ethanol (96 - 100%) must be added to ExoLution Plus Nucleic Acid Wash Buffer prior to use. Mix well to generate a homogenous solution. Mark the corresponding box on the bottle label to acknowledge addition of ethanol and include addition date. **NOTE**: Nucleic Acid Wash Buffer expires 1 year after addition of ethanol.
- ExoLution Plus Exosome Elution Buffer may form crystals during storage. In this case, warm buffer to 37°C with occasional swirling to re-dissolve any formed crystals.
- If selectable, set all centrifuges to rcf instead of rpm. If this is not an option, then calculate rpm needed to perform the isolation steps at the designated rcf.
- Equilibrate any sample to room temperature (15-25°C) before starting the ExoLution Plus procedure. Frozen samples may be equilibrated in a 25°C water bath.
- To ensure homogeneity, gently invert every sample approximately 5 times before starting the ExoLution Plus procedure.
- Sample tracking is critical in the ExoLution Plus workflow as the sample is passed over a large Column Unit, eluted, captured to a small Spin Column, and then eluted in the final step. It is critical to use a chemical-resistant labeling marker to label the lids and sides of tubes to minimize sample swapping or cross-contamination.

Overview of the ExoLution™ Plus cfDNA + exoRNA Isolation Kit

The provided reagents are sufficient for up to 50 isolations, with a recommended batch size of 6-12 samples. The isolation protocol involves 4 key sets of procedures:

1. Plasma collection and pre-processing
2. Isolation of cfDNA + exoRNA
3. Eluate conditioning
4. Total nucleic acid isolation

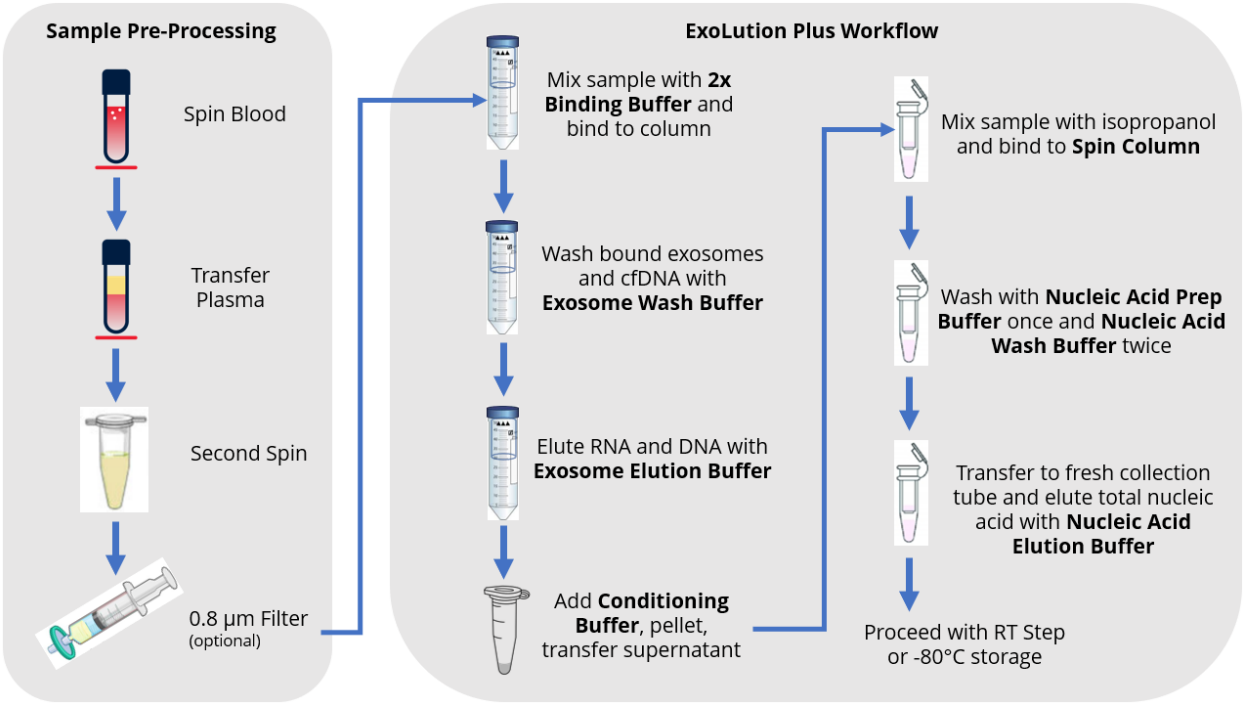


Figure 2. ExoLution™ Plus cfDNA + exoRNA Isolation Kit workflow.

Plasma collection and pre-processing

Whole blood must be processed into plasma ≤4 hours post-venipuncture for K_2EDTA collection tubes utilizing two separate centrifugation steps. In addition to using K_2EDTA collection tubes, whole blood may be collected in tubes containing stabilizers that may enable additional time between collection and processing. Refer to the protocol guide of the downstream Asuragen© QuantideX® exoMutation kit for more information on compatibility with stabilizer collection tubes.

Note: Storage of whole blood in K_2EDTA collection tubes >4 hours post-venipuncture may increase hemolysis and has not been verified for compatibility in this system.

The plasma pre-processing steps are recommended for K_2EDTA collection tubes. Refer to manufacturer recommendations for processing plasma in collection tubes containing stabilizers.

1. Centrifuge the blood collection tube at room temperature (15-25°C) for 15 minutes at 1600-2000 x g using a balanced swinging bucket centrifuge.
2. Carefully remove the plasma fraction and transfer to a second tube.
3. Centrifuge a second time at room temperature (15-25°C) for 10 minutes at 2500-3000 x g.
4. Following the second spin, plasma should be carefully removed making sure not to disturb any debris at the bottom of the tube.
5. Optional: Attach a 0.8 µm filter to a syringe and push the plasma through the filter into a 15 mL tube.

   Note: The use of a 0.8 µm syringe filter (0.8 µm Leur Lock filter: Millipore P/N SLAAR33SB, or equivalent) is not a requirement but should be incorporated if typical workflow leads to clogged Column Units during the ExoLution Plus cfDNA + exoRNA Isolation Kit. Certain collection procedures or using frozen plasma may require the syringe filter step. This filter step results in minor sample volume loss; therefore, process ample plasma to ensure correct plasma input volume according to the downstream Asuragen© QuantideX® exoMutation kit protocol guide.
6. Proceed directly to §**Isolation of cfDNA + exoRNA** or store plasma at -80°C.

Isolation of cfDNA + exoRNA

1. Mix an equal volume of **2X Binding Buffer** and filtered (optional) plasma in a 15 mL tube.

   Note: For more information on assay compatibility and sample isolation volume, consult the protocol guide of the downstream Asuragen QuantideX exoMutation kit.
2. Mix by slowly pipetting up and down 5-10 times.
3. Load the sample mixture onto a **Column Unit**. Close the lid and centrifuge for 1 min at 500 x g.

   Note: If any sample remains on the membrane, spin again for 1 min at 5,000 x g. See **Troubleshooting** for more information on a clogged Column Unit.
4. Add 10 mL of **Exosome Wash Buffer** to the **Column Unit**. Close the lid and centrifuge for 5 min at 5000 x g.
5. Transfer the **Column Unit** to a fresh 50 mL collection tube. Discard the flow through and the first collection tube.

   Note: Ensure that flow though does not contact the **Column Unit** during its removal.
6. Add 400 µL of **Exosome Elution Buffer** to the center of the column membrane.

   Note: Make sure the **Exosome Elution Buffer** is evenly distributed across the membrane surface. Do not touch the membrane with the pipet tip.
7. Centrifuge for 5 min at 5000 x g to collect the eluate containing total nucleic acid.
8. Transfer the eluate to a fresh microcentrifuge tube (not supplied).

Eluate Conditioning

1. Add 200 µL of **Conditioning Buffer** to the eluate in previous step, vortex on high for 5 sec and incubate for 1 min at room temperature (15-25°C).
2. Spin the tube for 5 min at 14,000 x g to collect the precipitate.

   Note: If the solution does not appear clear after centrifugation, repeat this step.
3. Carefully transfer the supernatant to a fresh microcentrifuge tube (not supplied). Discard the tube containing the pelleted precipitate.

   Note: Do not touch the pellet with the pipet tip when removing the supernatant.

Total Nucleic Acid Isolation

1. Add 350 µL of 100% isopropanol to the supernatant and mix by pipetting up and down 5-10 times.
2. Assemble a **Spin Column** in a provided 2 mL collection tube. Add up to 700 µL of the mixture from Step 1 onto the **Spin Column** assembly.
3. Centrifuge for 30 sec at 11,000 x g. Discard the flow through and re-assemble the column on the same collection tube.

   CRITICAL: Repeat this step until all of the sample has been run through the silica column.
4. Add 400 µL of **Nucleic Acid Prep Buffer** to the **Spin Column**, close the lid and centrifuge 30 sec at 11,000 x g. Discard the flow through and re-assemble the column on the same collection tube.
5. Add 700 µL of **Nucleic Acid Wash Buffer** to the **Spin Column**, close the lid and centrifuge 30 sec at 11,000 x g. Discard the flow through and re-assemble the column on the same collection tube.

   Note: Add 68 mL of non-denatured ethanol prior to use. Assign expiration of 1 year after ethanol addition.
6. Repeat the previous step for a total of two washes.
7. Transfer the **Spin Column** to a fresh 2 mL collection tube. Centrifuge for 3 min at 16,000 x g to dry the membrane.
8. Transfer the **Spin Column** into a 1.5 mL **Elution Tube** provided in the Kit. Add 12 µL of **Nucleic Acid Elution Buffer** directly to the membrane. Close the lid and incubate for 5 min at room temperature (15-25°C).
9. Centrifuge for 1 min at 16,000 x g.

   Note: To avoid damage to the lids, place the tubes into the centrifuge with at least one empty position between each tube. Orient the lids so that they point in the direction opposite to the rotor rotation.
10. Discard Spin Column and proceed directly to downstream applications (e.g., reverse transcription) or freeze the isolated material at -80°C.

    Note: Please refer to the protocol guide of the downstream Asuragen© QuantideX® exoMutation kit for more information on isolated nucleic acid stability.

Troubleshooting

Observation	Potential Cause(s)	Suggested Action(s)
Plasma appears cloudy or contains particulates	Incomplete separation of blood components	Centrifuge blood samples again under recommended conditions to ensure complete separation. Avoid large particulates when transferring plasma. Smaller particulates should be removed during filtration. Consider turning off centrifuge brake to avoid resuspension of the pellet.
Low plasma volume	Underfilled blood collection tube.	Carefully pipette plasma without touching or disturbing the pellet. Consider using a pipette with a narrower tip.
Clogged Column Unit	Plasma not filtered or improperly filtered	Filter plasma through the indicated syringe filter before beginning the isolation process to remove particulates and debris. See §**Plasma collection and pre-processing**.
Crystals observed in buffer	Buffer stored at low temperatures	Warm the buffer bottle to 18 to 25°C with occasional swirling until crystals dissolve. If crystals persist, gently heat to 37°C, continuously monitoring until crystals dissolve. Stop heating when crystals are dissolved.
Downstream internal control (IC) QC	Hemolysis from prolonged storage in K_2EDTA collection tube	Whole blood must be processed into plasma ≤4 hours post-venipuncture for K_2EDTA collection tubes. Prolonged whole blood storage in K_2EDTA collection tubes leads to hemolysis that can lead to downstream assay inhibition.
	Ethanol not added to nucleic acid wash buffer	Check if ethanol has been added to the Nucleic Acid Wash Buffer and mixed well prior to first use. Initial starting volume is 85 mL (68 mL ethanol added to 15 mL buffer). Subtracting known use from the bottle can help estimate correct initial addition of ethanol.
	Incorrect centrifugation settings	Ensure centrifugation is set to rcf as recommended. If only rpm is available, calculate the correct rpm to achieve the required rcf. Check calibration of centrifuge to ensure accuracy. Perform all centrifuge steps at recommended speeds to ensure complete capture of eluate from column surface. See §**Required but not Provided** for required centrifuge specifications. See §**Isolation of cfDNA + exoRNA** and §**Total Nucleic Acid Isolation** for centrifuge speed settings per procedural step.

Observation	Potential Cause(s)	Suggested Action(s)
	Samples not brought to room temperature or unevenly thawed	Ensure samples are completely thawed and at room temperature (15-25°C) before processing. Mix samples gently post-thaw to ensure uniformity.
	Inadequate whole blood collection tube mixing before plasma pre-processing	Gently invert blood collection tube approximately 5 times to ensure homogeneity before starting the extraction process.
	Inadequate plasma mixing with 2X Binding Buffer before extraction	Ensure plasma is well mixed with 2X Binding Buffer before adding the sample mixture to the Column Unit. If issues persist, consider briefly vortexing the 2X Binding Buffer/plasma mixture prior to loading on the Column Unit.
	Contamination or degradation of reagents	Check all reagents for signs of contamination or expiration. If contamination is suspected, use a new Kit if possible.
Inconsistent downstream internal control (IC) QC	Variable sample quality or handling	Standardize sample handling procedures and check the quality of each sample before processing. If variability remains, assess pre-analytical factors such as collection and storage conditions. Use serological pipet most appropriate for each step. Use smallest pipet available based on each prescribed volume. When handling plasma and plasma/binding buffer mixture, pipet slowly to ensure accurate volume.
	Equipment malfunction or improper settings	Check and calibrate equipment to ensure it is operating within specified parameters. If issues persist, consider servicing the equipment or using an equivalent device.
Insufficient eluate volume from Column Unit or Spin Column	Incorrect (too slow) centrifugation settings	Perform all centrifuge steps at recommended speeds to ensure complete capture of eluate from column surface. See §**Required but not Provided** for required centrifuge specifications. See §**Isolation of cfDNA + exoRNA** and §**Total Nucleic Acid Isolation** for centrifuge speed settings per procedural step. Repeat centrifugation step to capture more eluate volume from Column Unit or Spin Column.
Extreme overage of eluate volume from Column Unit or Spin Column	Incorrect (too slow) centrifugation settings; wash buffer carry over into eluate	Perform all centrifuge steps at recommended speeds to ensure removal of wash buffers from column surface. See §**Required but not Provided** for required centrifuge specifications. See §**Isolation of cfDNA + exoRNA** and §**Total Nucleic Acid Isolation** for centrifuge speed settings per procedural step. Repeat centrifugation steps to remove wash buffer from Column Unit or Spin Column.

Disclaimers

1. This product is intended for Research Use Only. Not for use in diagnostic procedures.
2. This product may not be resold, modified for resale, or used to manufacture commercial products without the written approval of Asuragen®.
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