STANDARD OPERATING PROCEDURE

Purification and Kinetic Enzymatic Assay of Recombinant Human Kinase V-321

SOP-BIO-2026-042

Revision: 3.1 | **Supersedes:** Rev 3.0

Document ID	SOP-BIO-2026-042	**Effective Date**	July 15, 2026
Department	Molecular Biochemistry & Bioassays	**Review Cycle**	Bi-annual (Next: Jan 2027)
Target Molecule	Human Kinase V-321 (His_6-tagged)	**Expression Host**	*E. coli* BL21(DE3) pET-28a(+)
Primary Author	Dr. Elena Rostova, Senior Biochemist	**Technical Approver**	Dr. Marcus Vance, VP Operations
Quality Assurance	Dr. Sarah Jenkins, QA Compliance Manager	**Classification**	Controlled Operating Protocol

1. PURPOSE & SCOPE

This Standard Operating Procedure (SOP) outlines the validated workflow for cell lysis, Immobilized Metal Affinity Chromatography (IMAC) purification, size-exclusion buffer exchange, and continuous spectrophotometric NADH-coupled kinase activity assay of human recombinant Kinase V-321.

- **Scope:** Applies to all research personnel within Molecular Biochemistry at Meridian Biosciences Inc. executing lot release, stability testing, or kinetic profiling for Project Kinase-V321.
- **Quality Goal:** Maintain lot consistency with protein purity $\geq 95\%$, specific activity ≥ 280 U/mg, and assay coefficient of variation (CV) $< 5\%$.

2. SAFETY PRECAUTIONS & HAZARD CONTROL

SAFETY WARNING & HAZARD CONTROL Strict adherence to Biosafety Level 2 (BSL-2) practices is mandatory. All operations involving chemical lysis reagents and inhibitors must be conducted with appropriate Personal Protective Equipment (PPE).

- **PMSF (Phenylmethylsulfonyl fluoride):** Highly toxic serine protease inhibitor ($LD_{50} = 200$ mg/kg). Causes severe skin burns. Prepare fresh solutions in anhydrous Isopropanol inside a certified chemical fume hood.
- **Acrylamide & SDS (SDS-PAGE):** Neurotoxin and suspected carcinogen. Handle monomer gels in the fume hood.
- **Imidazole & Dithiothreitol (DTT):** Corrosive skin irritants. Avoid inhalation of dust or contact with eyes.
- **Required PPE:** Lab coat, safety goggles with side shields, double nitrile gloves, and closed-toe non-porous footwear.

3. REAGENTS, MATERIALS & EQUIPMENT

3.1 Chemical Reagents & Solutions

Reagent / Chemical	Grade / Specification	CAS No.	Storage	Safety / Prep Note
Tris-HCl	Mol. Biology Grade ($\geq 99.8\%$)	77-86-1	Room Temp	Buffer base; adjust pH with HCl
Sodium Chloride (NaCl)	ACS Reagent ($\geq 99.0\%$)	7647-14-5	Room Temp	High purity ionic strength agent
Imidazole	ReagentPlus ($\geq 99.0\%$)	288-32-4	Room Temp	Corrosive; light sensitive
Ni-NTA Sepharose High Perf.	5 mL Prepacked Column	N/A	4–8°C	Do not freeze; store in 20% EtOH
Adenosine 5'-triphosphate (ATP)	Disodium Salt ($\geq 98.0\%$)	34369-07-8	-20°C	Prepare 100 mM stock in dH_2O
NADH (Reduced NAD^+)	Disodium Salt ($\geq 98\%$)	606-68-8	-20°C (Desic.)	Prepare fresh daily; light sensitive
Phosphoenolpyruvate (PEP)	Monopotassium Salt	4265-07-2	-20°C	Prepare 50 mM stock in buffer
PK/LDH Enzyme Suspension	Rabbit Muscle Mix	N/A	2–8°C	Do not freeze or vortex vigorously
Substrate Peptide STK-1	Synthetic ($\geq 98.0\%$ HPLC)	Custom-STK1	-80°C	Resuspend in ultrapure dH_2O

3.2 Equipment Specifications

Equipment Type	Model / Manufacturer	Calibration Standard	Operational Criteria
FPLC System	ÄKTA pure 25 (Cytiva)	Annual IQ/OQ (CAL-014)	Flow rate range: 0.1–25 mL/min
UV-Vis Microplate Reader	SpectraMax M5 (Molecular Dev.)	NIST Traceable Filters	Temp stability: 30.0 ± 0.2°C
Ultrasonic Disruptor	Sonifier SFX-550 (Branson)	Microtip Calibration	Output: 40% amplitude, pulsed
Refrigerated Centrifuge	Avanti J-26S XP (Beckman)	Rotor Cert. (JA-25.50)	Max RCF: $50,000 \times g$ at 4°C

4. REAGENT & BUFFER PREPARATION

Prepare all buffers using Milli-Q ultrapure water (18.2 M$\Omega \cdot$cm resistivity at 25°C). Filter chromatography buffers through a 0.22 μm PES membrane prior to use.

- **Lysis Buffer L-1 (500 mL):** 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM Imidazole, 10% (v/v) Glycerol, 0.1% (v/v) Triton X-100. Store at 4°C. Add fresh 1.0 mM PMSF and 0.5 mg/mL Lysozyme prior to use.
- **Wash Buffer W-1 (500 mL):** 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 40 mM Imidazole, 5% (v/v) Glycerol. Store at 4°C.
- **Elution Buffer E-1 (250 mL):** 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 300 mM Imidazole, 10% (v/v) Glycerol. Store at 4°C.
- **Kinase Assay Buffer A-1 (5x Stock, 100 mL):** 250 mM HEPES-NaOH (pH 7.5), 50 mM $MgCl_2$, 5 mM EGTA, 0.05% (v/v) Tween-20. Aliquot and store at -20°C. Supplement with 1 mM DTT on day of assay.

5. STEP-BY-STEP EXPERIMENTAL PROTOCOL

Phase I: Cell Lysis & Clarification

1. Retrieve frozen *E. coli* BL21(DE3) cell pellet (approx. 10.0 g wet weight) from -80°C freezer and thaw on ice for 25 min.
2. Resuspend pellet in 50 mL ice-cold Lysis Buffer L-1 (5 mL/g cell paste) using a magnetic stirrer at 4°C for 15 min.
3. Add 50 μL of 1000x PMSF stock (100 mM in Isopropanol) for a final concentration of 1.0 mM.
4. Perform ultrasonic lysis on ice: 10 cycles of 30 s ON, 30 s OFF at 40% output amplitude. Maintain temperature below 8°C.
5. Centrifuge crude lysate at $24,000 \times g$ for 35 min at 4°C. Collect clarified supernatant and filter through a 0.45 μm syringe filter.

Phase II: IMAC Chromatography Purification (Ni-NTA)

1. Mount a 5 mL Ni-NTA Sepharose column onto ÄKTA pure FPLC. Flush with 5 Column Volumes (CV) Milli-Q water at 2.5 mL/min.
2. Equilibrate column with 8 CV of Lysis Buffer L-1 at 2.0 mL/min.
3. Load filtered supernatant onto column at 1.0 mL/min to maximize target binding. Collect flow-through (FT).
4. Wash column with 10 CV of Lysis Buffer L-1, followed by 8 CV of Wash Buffer W-1 at 2.0 mL/min.
5. Elute bound Kinase V-321 using 100% Elution Buffer E-1 over 5 CV at 1.5 mL/min. Collect 1.5 mL fractions. Monitor A_{280} UV.

Phase III: Buffer Exchange & Storage

1. Pool peak fractions ($A_{280} > 0.6$ AU). Load onto PD-10 desalting column pre-equilibrated with Storage Buffer S-1 (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT, 20% Glycerol).
2. Concentrate protein using Amicon Ultra-15 10 kDa MWCO centrifugal unit at $4,000 \times g$ (4°C) to ~ 2.5 mg/mL.
3. Measure protein concentration by UV spectrophotometry at 280 nm ($\epsilon_{280} = 54,490$ $M^{-1}cm^{-1}$).
4. Aliquot 50 μL into cryovials, snap-freeze in liquid nitrogen, and store at -80°C.

> **CRITICAL HOLDING POINT:** Purified Kinase V-321 aliquots may be stored at -80°C for up to 6 months. Avoid repeated freeze-thaw cycles (> 2 cycles results in $> 15\%$ loss of activity).

6. KINETIC ENZYMATIC ASSAY PROTOCOL

Enzymatic activity of Kinase V-321 is determined via a continuous NADH-coupled spectrophotometric assay at 340 nm ($\epsilon_{NADH} = 6,220$ $M^{-1}cm^{-1}$).

6.1 Assay Reaction Setup (96-Well Microplate)

Reaction Component	Stock Conc.	Vol / Well (μL)	Final Conc. (200 μL Total)
Kinase Assay Buffer A-1	5x	40.0	1x (50 mM HEPES pH 7.5)
Substrate Peptide STK-1	1.0 mM	20.0	100 μM
NADH Solution	10.0 mM	4.0	0.20 mM
Phosphoenolpyruvate (PEP)	50.0 mM	4.0	1.0 mM
PK/LDH Enzyme Suspension	Commercial	5.0	10 U/mL PK / 15 U/mL LDH
Kinase V-321 (Enzyme Sample)	1.0 μM	10.0	50 nM (Target concentration)
Milli-Q H_2O	N/A	97.0	Fill to final volume
ATP (Initiator - Add last)	10.0 mM	20.0	1.0 mM
TOTAL REACTION VOLUME		**200.0 μL**	**Assay Temp: 30.0°C**

6.2 Spectrophotometric Measurement Procedure

1. Preheat SpectraMax M5 microplate reader chamber to 30.0°C.
2. Pipette buffer, substrate, coupling enzymes, and Kinase V-321 into duplicate wells (Table 6.1).
3. Incubate microplate inside reader for 5 min to reach thermal equilibrium (30°C).
4. Initiate reaction by adding 20.0 μL of 10.0 mM ATP solution with a multi-channel pipette.
5. Record absorbance at $\lambda = 340$ nm every 15 s for 20 min (81 data points total).

7. CALCULATIONS & MATHEMATICAL FORMULAS

Calculate the linear reaction rate (ΔA_{340}/min) from the initial velocity region (minutes 2 to 7).

Enzyme Volumetric Activity Formula:

$$\text{Volumetric Activity (U/mL)} = \left(\frac{\Delta A_{340}/\text{min}}{\epsilon_{\text{NADH}} \times d} \right) \times \left(\frac{V_{\text{total}}}{V_{\text{enzyme}}} \right) \times D$$

- ΔA_{340}/min: Linear rate of absorbance decrease at 340 nm (min^{-1})
- ϵ_{NADH}: Molar extinction coefficient of NADH (6.22 mM^{-1}cm^{-1})
- d: Optical pathlength for 200 μL in 96-well plate (0.588 cm)
- V_{total}: Total assay volume (0.200 mL) | V_{enzyme}: Enzyme volume added (0.010 mL)
- D: Sample dilution factor ($D = 20$ for 1.0 μM stock dilution)

Specific Activity Calculation:

$$\text{Specific Activity (U/mg)} = \frac{\text{Volumetric Activity (U/mL)}}{\text{Protein Concentration (mg/mL)}}$$

One Unit (U) catalyzes phosphorylation of 1.0 μmol STK-1 peptide per minute at 30°C under standard assay conditions.

8. QUALITY CONTROL ACCEPTANCE CRITERIA

Purified Kinase V-321 batches must meet all specifications in Table 8.1 prior to lot release.

QC Parameter	Acceptance Specification	Lot V321-08	Status
Protein Purity (SDS-PAGE)	Single main band at 48.5 kDa $\geq 95.0\%$	98.2%	✔ PASS
Purification Yield	≥ 5.0 mg protein per liter cell culture	8.4 mg/L	✔ PASS
Specific Activity	≥ 280 U/mg at 30.0°C	345 ± 12 U/mg	✔ PASS
Absorbance Ratio (A_{280}/A_{260})	$1.65 \leq$ Ratio ≤ 1.85 (Low nucleic acid contamination)	1.76	✔ PASS
Enzymatic Precision (CV)	Intra-assay replicate CV $\leq 4.0\%$	1.8%	✔ PASS
Bacterial Endotoxin	< 0.10 EU/μg protein (LAL Assay)	0.025 EU/μg	✔ PASS

9. TROUBLESHOOTING GUIDE

Observed Issue	Probable Cause	Corrective Action
Low Protein Yield (< 2 mg/L)	Incomplete cell lysis or poor induction in host cells.	Increase sonication cycles; verify IPTG concentration (0.5 mM) and induction temp (18°C overnight).
Protein Precipitates in Elution	Rapid imidazole shift or high protein density (> 5 mg/mL).	Perform step-wise desalting; add 10% Glycerol and 1 mM DTT to Elution Buffer E-1. Keep on ice.
High Background Rate in Blank	Spontaneous NADH oxidation or ATPase contamination.	Prepare fresh NADH daily; filter PEP solution; run control wells lacking STK-1 substrate.
Non-linear Rate ($R^2 < 0.98$)	Substrate exhaustion or rapid thermal denaturation.	Lower enzyme concentration to 25 nM; ensure reader temperature is stable at 30.0°C.

10. REVISION HISTORY & APPROVALS

END OF STANDARD OPERATING PROCEDURE — MERIDIAN BIOSCIENCES INC. — CONFIDENTIAL

Rev #	Date	Author	Description of Revision
1.0	10-JAN-2024	**Dr. Elena Rostova**	Initial release for pilot-scale Kinase V-321 assay.
2.0	14-FEB-2025	**Dr. Elena Rostova**	Updated IMAC wash buffer imidazole conc. from 20 mM to 40 mM.
3.0	10-NOV-2025	**Dr. Marcus Vance**	Added continuous NADH spectrophotometric kinetic protocol.
3.1	15-JUL-2026	**Dr. Elena Rostova**	Current Version: Optimized assay buffer pH and added STK-1 specs.

Role	Printed Name & Title	Signature & Approval Date
Primary Author	Dr. Elena Rostova Senior Biochemist	*Elena Rostova* (15-JUL-2026)
Technical Reviewer	Dr. Marcus Vance VP Scientific Operations	*Marcus Vance* (16-JUL-2026)
Quality Assurance	Dr. Sarah Jenkins QA Compliance Manager	*Sarah Jenkins* (17-JUL-2026)