



Standard Operating Procedure

Cell-Free Benchtop Pause Assay (PURExpress)

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SOP: Cell-Free Benchtop Pause Assay

1. Purpose and Scope

This procedure expresses reverse transcriptase / ART enzyme domains using a reconstituted cell-free protein synthesis system and runs a bulk primer-extension assay to test for motif-specific arrest at the 12-nucleotide AT tract. Oligonucleotide sequences are specified in Document 3.

2. Reagents and Equipment

- PURExpress In Vitro Protein Synthesis Kit (New England Biolabs, #E6800L)
- Linearised expression constructs encoding phage ART or human ORF2p RT variants
- PAGE-purified DNA template oligonucleotides and 6-FAM labelled primer (Document 3)
- 15% urea-PAGE denaturing gel apparatus, 1× TBE
- Thermocycler or incubator capable of 40 °C and 95 °C
- Fluorescence gel scanner with 488 nm excitation (Typhoon or equivalent)

SAFETY

Unpolymerised acrylamide is a neurotoxin and is readily absorbed through skin. Prepare and pour gels in a fume hood with nitrile gloves. Urea-formamide loading buffer is a reproductive hazard. Dispose of acrylamide waste per institutional policy.

3. Procedure

1 Cell-free protein expression

Thaw PURExpress Solution A, Solution B and the enzyme mix on ice. Assemble 25 μ L translation reactions per the NEB protocol, with 250 ng linearised template DNA per reaction. Incubate at 30 °C for 2 hours.

2 Primer annealing

Anneal the 6-FAM primer to each template strand at a 1:1.2 primer-to-template molar ratio in 10 mM Tris-HCl pH 8.0, 50 mM NaCl. Heat to 95 °C for 2 minutes, then cool to 20 °C at approximately 1 °C per minute. Prepare consensus and control templates in parallel from the same primer stock.

3 Reaction assembly

Combine the cell-free expression mixture with the annealed template-primer complex in reverse transcriptase buffer: 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 5 mM DTT.

4 Initiation and time course

Initiate extension by adding dNTPs to 500 μ M each, at 30 °C. Remove 5 μ L aliquots at 15 s, 30 s, 1 min, 2 min, 5 min and 10 min. Quench each immediately into an equal volume of 2 $\times$ urea-PAGE loading buffer containing 95% formamide and 20 mM EDTA.

5 Denaturing PAGE readout

Denature samples at 95 °C for 5 minutes. Resolve on a 15% urea-polyacrylamide gel at 200 V in 1 $\times$ TBE. Scan for 6-FAM fluorescence and quantify band intensities.

4. Required Controls

Each of the following distinguishes a result from an artefact. A pause observed without them is not interpretable.

CONTROL	COMPOSITION	DISTINGUISHES
No enzyme	Annealed template-primer, buffer, dNTPs	Extension from contaminating polymerase activity
No dNTP	Full reaction, dNTPs omitted	Primer degradation and gel artefacts from unextended primer
No template	Primer alone with enzyme and dNTPs	Primer self-extension or dimer formation
Iso-thermal control template	Control template, otherwise identical	Sequence-specific arrest from composition or T_m effects

WHY LABEL INTERFERENCE IS NOT ON THIS LIST

The consensus and control templates are run with the same labelled primer, so the fluorophore cannot account for a difference between them. Label interference is a prerequisite for the single-molecule stage rather than a condition for interpreting this gel. It is specified in section 5.

5. Prerequisites for Single-Molecule Measurement

The bulk assay above establishes whether arrest occurs and how much of the population arrests. It cannot measure the force required to clear an arrest or the dwell time of a single arrest event. Those quantities require single-molecule methods, and those methods impose three requirements that the bulk assay does not.

REQUIREMENT	HOW IT IS MET	WHY THE MEASUREMENT NEEDS IT
Fluorescently labelled enzyme	Conjugate the purified reverse transcriptase or ART variant to a fluorophore suitable for single-molecule detection, and purify the conjugate away from free dye	Single-molecule fluorescence follows the protein, not the nucleic acid. An unlabelled enzyme produces no signal to track.
Activity match between labelled and unlabelled protein	Run the bulk assay in section 3 with the labelled conjugate and with the unlabelled protein in parallel, and compare arrest fraction and time course	Fluorophore conjugation can alter enzyme kinetics. Without a demonstrated match, a single-molecule trace describes the conjugate rather than the enzyme.
Activity match between labelled and unlabelled primer	Run the same reaction with the 6-FAM primer and with the unlabelled primer of identical sequence, supplied in Document 3	Separates a label effect on the nucleic acid from a label effect on the protein, so that a mismatch can be attributed to one or the other.

All three use the gel readout described in section 3 and require no additional instrumentation. None needs to be complete before the bulk comparison in section 4 is run. All three need to be complete before a single-molecule result is interpretable.

6. Interpreting the Gel

Expected band positions for the sequences in Document 3:

BAND	LENGTH	MEANING
Unextended primer	18 nt	No extension occurred
Arrest products	19–30 nt	Polymerase entered the motif and stopped within it
Full-length product	49 nt	Read-through to the template 5' end

- A **positive result** is accumulation in the 19–30 nt window on the consensus template, with the control template showing a higher full-length fraction at the same timepoints.

- A **negative result** is indistinguishable band distributions between consensus and control.
- An **uninterpretable result** is arrest on both templates, which indicates the pause is composition-driven rather than sequence-specific, or a reaction condition is limiting.

TEMPLATE GEOMETRY

In the current design the primer 3' terminus abuts the motif directly, with zero nucleotides of template between them. Extension therefore enters the motif on its first catalytic step. This is sufficient to detect arrest but gives no processive run-in, so a pause cannot be distinguished from a failure to initiate. Adding 15–20 nt of neutral sequence between the primer site and the motif, in a second-generation template, would produce a run-in band and make the two cases separable on the same gel.

7. Handling and Storage

- Resuspend lyophilised oligonucleotides in TE pH 8.0 to 100 μ M. Store at -20°C .
- Keep the 6-FAM primer in amber tubes or foil-wrapped. Fluorescein photobleaches and is pH sensitive below pH 7.
- Limit freeze-thaw cycles on the labelled primer; aliquot the working stock on first use.
- PURExpress components are single-use once thawed. Do not refreeze.